# Toward understanding of the positive regulation of ATP-sensitive $K^+$ channels with regard to physiology and pathophysiology of the pancreatic β-cell

**Dissertation**

der Mathematisch-Naturwissenschaftlichen Fakultät
der Eberhard Karls Universität Tübingen
zur Erlangung des Grades eines
Doktors der Naturwissenschaften
(Dr. rer. nat.)

vorgelegt von
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Tübingen
2020

Gedruckt mit Genehmigung der Mathematisch-Naturwissenschaftlichen Fakultät der Eberhard Karls Universität Tübingen

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| Tag der mündlichen Qualifikation: | 28.05.2020. |
| Dekan: | Prof. Dr. Wolfgang Rosenstiel |
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*“Nothing in life is to be feared, it is only to be understood. Now is the time to understand more, so that we may fear less.”*

Marie Curie

For Danijel Soldo

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# Abbreviations

| | |
|---|---|
| $[Ca^{2+}]_c$ | cytosolic $Ca^{2+}$ concentration |
| a.u. | arbitrary units |
| $[^3H]$GBM | radioligand-bound glibenclamide |
| ABC | ATP binding cassette |
| ABCC8 | gene encoding sulfonylurea receptors (SUR) |
| ADP | adenosine diphosphate |
| AK | adenylate kinase |
| ANP | atrial natriuretic peptide |
| ATP | adenosine triphosphate |
| BK | $Ca^{2+}$-activated $K^+$ channel of large conductance |
| $BK^{-/-}$ | BK channel-knockout |
| BPip2a | nucleotide- and $Mg^{2+}$-free control solution |
| BPip2b | nucleotide-free control solution |
| $Ca^{2+}$ | calcium ion |
| cAMP | 3',5'-cyclic adenosine monophosphate |
| CDKN1C | gene encoding protein p57 |
| CFTR | cystic fibrosis transmembrane conductance regulator |
| CHI | congenital hyperinsulinism |
| CK | creatine kinase |
| Cryo-EM | Cryogenic electron microscopy |

| | |
|---|---|
| DCEBIO | 5,6-Dichloro-1-ethyl-1,3-dihydro-2H-benzimidazole-2-one |
| $FADH_2$ | reduced form of flavin adenine dinucleotide |
| FCCP | carbonylcyanide-p-trifluoromethoxyphanylhidrazone |
| FRET | Förster resonance energy transfer |
| Fig. | figure |
| GALR | galanin receptor |
| GIRK | G protein-activated inwardly rectifying potassium channel |
| GLUT1 | glucose transporter type 1 |
| GLUT2 | glucose transporter type 2 |
| GBM | glibenclamide |
| GLP-1 | glucagon-like peptide-1 |
| GTP | guanosin triphosphate |
| HEK-293 | human embryonic kidney cells |
| IGF-II | gene encoding a growth factor |
| $K^+$ | potassium ion |
| $K_{ATP}$ channel | ATP-sensitive potassium channel |
| $K_{ATP}$ current | ATP-sensitive potassium current |
| Kir6.2 | inward rectifier potassium channel 6.2 |
| KCNJ11 | gene encoding Kir6.2 |
| $K_{Ca}$ | calcium-activated potassium channel |
| Kv | voltage-dependent potassium channel |

| | |
|---|---|
| L0 | linker |
| LXR | liver X receptor |
| MDR | multidrug |
| mTOR | mammalian target of rapamycin |
| myr-PKI | myristoylated protein kinase A inhibitory peptide |
| $Na^+$ | sodium ion |
| NADH | reduced form of nicotinamide adenine dinucleotide |
| Nav | voltage-dependent sodium channel |
| NBD | nucleotide binding domain |
| ND | neonatal diabetes |
| NN414 | 6-Chloro-3-[[1-methylcyclopropyl] amino]-4H-thieno[3,2-e]-1,2,4-thiadiazine 1,1-dioxide |
| $NP_o$ | open probability |
| PCr | phosphocreatine |
| $P_i$ | inorganic phosphate, $PO_4H_3$ |
| PKA | protein kinase A |
| SERCA | sarco/endoplasmic reticulum $Ca^{2+}$-ATPase |
| SK4 | $Ca^{2+}$-dependent $K^+$ channel of intermediate conductance |
| $SK4^{-/-}$ | SK4 channel-knockout |
| SSC | stimulus-secretion coupling |
| SUR1 | sulfonylurea receptor 1 |
| $SUR1^{-/-}$ | SUR1-knockout |

| | |
|---|---|
| SUR2A | sulfonylurea receptor 2A |
| SUR2B | sulfonylurea receptor 2B |
| SSTR | somatostatin receptor |
| SSTR2 | somatostatin receptor 2 |
| SSTR5 | somatostatin receptor 5 |
| TMD | transmembrane domain |
| VDCC | voltage-dependent $Ca^{2+}$ channel |
| Vm | membrane potential |
| VU0071063 | 7-[[4-(1,1-Dimethylethyl)phenyl]methyl]-3,7-dihydro-1,3-dimethyl-1H-purine-2,6-dione |
| WT | wild type |

## Summary

Pancreatic β-cells secrete the hormone insulin in response to increased postprandial glucose via a mechanism called stimulus-secretion coupling (SSC). The underlying molecular events in SSC involve different ion channels, whereby the crucial role in the regulation of membrane potential of the β-cell and triggering insulin release is played by neuroendocrine type ATP-sensitive $K^+$ ($K_{ATP}$) channels. Their activity is affected by interaction between nucleotides (ATP and ADP) and channel forming subunits (SUR1 and Kir6.2). Channel activity is regulated positively by binding of ATP or ADP to the SUR1 subunit and negatively by nucleotide binding to the Kir6.2. Furthermore, mutations in genes encoding the $K_{ATP}$ channel produce insulin secretion disorders such as neonatal diabetes (ND) and congenital hyperinsulinism (CHI). Accordingly, $K_{ATP}$ channels are pharmacological targets for drugs (sulfonylureas and $K_{ATP}$ channel openers) that bind directly and regulate insulin release independent of glucose concentrations. The present work offers further insight regarding the mechanism underlying the positive regulation of $K_{ATP}$ channels. Additionally, it also examines new pharmacological approaches in the treatment of CHI.

Contrary to the widely accepted hypothesis that activation of $K_{ATP}$ channels requires MgATP binding and MgATP hydrolysis by SUR1, this thesis clearly demonstrates that ATP-binding, without hydrolysis, stimulates neuroendocrine type $K_{ATP}$ channel openings. Binding of ATP, under non-hydrolytic conditions, opens channels comprising SUR1 mutants with increased ATP affinity and Kir6.2 pores with abolished nucleotide antagonism. Hence, ATP exerts purely agonistic action on SUR1. Furthermore, the thesis demonstrates that the $K_{ATP}$ channel opener diazoxide does not need ATP hydrolysis for its action and that its action is further supported by ATP.

Current treatment options for CHI are limited to severe cases due to the lack of efficacy and undesirable effects of medications. The first-line drug used for treatment of CHI is diazoxide. Despite advances in alternatives to diazoxide therapy, there is still need for better medication. This thesis examines new potential approaches for treating CHI. First, two new $K_{ATP}$ channel openers, NN414 and VU0071063 were

tested. The opener NN414 suppressed oscillations of the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) in human β-cells isolated from CHI patients and dose-dependently inhibited glucose-induced insulin secretion in murine β-cells. Furthermore, NN414 activated $K_{ATP}$ channels with mutated SUR1 at a 50-fold lower concentration than diazoxide. Although VU0071063 is able to suppress the oscillations of $[Ca^{2+}]_c$ in human CHI β-cells, it is not appropriate for therapy due to its detrimental effects on ATP production. When $K_{ATP}$ channels are functionally impaired as a result of mutations in the *ABCC8* or *KCNJ11* gene, the effect of diazoxide is disabled. Therefore, it is of great importance to focus on targeting other steps of SSC in order to reduce insulin secretion. This study proposes openers of $Ca^{2+}$-activated $K^+$ channels of intermediate conductance (SK4 channels) as an alternative approach.

## Zusammenfassung

Pankreatische β-Zellen sezernieren das Hormon Insulin als Reaktion auf eine erhöhte postprandiale Glukosekonzentration über einem Mechanismus, der als Stimulus-Sekretion-Kopplung (SSK) bezeichnet wird. Die zugrunde liegenden molekularen Ereignisse in der SSK umfassen verschiedene Ionenkanäle, wobei die entscheidende Rolle in der Regulation des Membranpotentials der β-Zelle liegt sowie in der durch neuroendokrine ATP-sensitive $K^+$-Kanäle ($K_{ATP}$-Kanäle) ausgelösten Insulinfreisetzung. Ihre Aktivität wird durch die Interaktion zwischen Nukleotiden (ATP und ADP) und den zwei Kanaluntereinheiten (SUR1 und Kir6.2) beeinflusst. Während die Kanalaktivität durch die ATP- oder ADP-Bindung an die SUR1-Untereinheit positiv reguliert wird, reguliert die Bindung von Nukleotiden an die Kir6.2-Untereinheit die Kanäle negativ. Mutationen in Genen, die den $K_{ATP}$-Kanal kodieren, verursachen zudem Insulinsekretionsstörungen wie neonatalen Diabetes (ND) und kongenitalen Hyperinsulinismus (CHI). Dementsprechend sind $K_{ATP}$-Kanäle pharmakologische Angriffspunkte für die Medikamente (wie z.B. Sulfonylharnstoffe und $K_{ATP}$-Kanalöffner), die durch direkte Bindung die Insulinausschüttung unabhängig von der Glukosekonzentration regulieren. Die vorliegende Arbeit bietet weitere Erkenntnisse über den Mechanismus der positiven Regulation von $K_{ATP}$-Kanälen. Darüber hinaus werden neue pharmakologische Ansätze zur Behandlung von CHI untersucht.

Im Gegensatz zur allgemein akzeptierten Hypothese, dass die Aktivierung von $K_{ATP}$-Kanälen eine MgATP-Bindung und eine MgATP-Hydrolyse durch SUR1 erfordert, zeigt diese Arbeit deutlich, dass die Stimulation der neuroendokrinen $K_{ATP}$-Kanalöffnungen ohne Nukleotid-Hydrolyse erfolgt. Die Bindung von ATP unter nicht-hydrolytischen Bedingungen öffnet Kanäle, welche SUR1-Mutanten mit erhöhten ATP-Affinitäten und Kir6.2-Poren, die nicht durch ATP gehemmt werden, enthalten. Außerdem zeigt diese Arbeit, dass der $K_{ATP}$-Kanalöffner Diazoxid für seine Wirkung keine ATP-Hydrolyse benötigt, seine Wirkung aber durch ATP unterstützt wird.

Die aktuellen Therapiemöglichkeiten für schwere Fälle von CHI sind einerseits durch die mangelnde Wirksamkeit der Medikamente und anderseits durch die unerwünschten Arzneimittelwirkungen eingeschränkt. Medikament der ersten Wahl

ist momentan noch Diazoxid. Trotz der Entwicklung von Alternativen zu diesem Wirkstoff besteht noch immer ein großer Bedarf an besseren Medikamenten. Diese Arbeit untersucht neue Konzepte für die Behandlung von CHI. Es wurden zwei neue $K_{ATP}$-Kanalöffner, NN414 und VU0071063, getestet. Der Öffner NN414 unterdrückte die Oszillationen der zytosolischen $Ca^{2+}$-Konzentration ($[Ca^{2+}]_c$) in humanen β-Zellen, die aus CHI-Patienten isoliert wurden. Zusätzlich zeigte er eine dosisabhängige Hemmung der glukoseinduzierten Insulinsekretion in murinen β-Zellen. Im Vergleich zu Diazoxid aktivierte NN414 in einer 50-fach niedrigeren Konzentration die $K_{ATP}$-Kanäle mit mutierter SUR1-Untereinheit. Obwohl die Substanz VU0071063 in der Lage ist, die Oszillationen der $[Ca^{2+}]_c$ in menschlichen CHI β-Zellen zu hemmen, ist sie aufgrund der schädlichen Auswirkungen auf die ATP-Produktion nicht für die therapeutische Anwendung geeignet. Wenn $K_{ATP}$-Kanäle funktionell beeinträchtigt sind, wird die Wirkung von Diazoxid durch einige Mutationen im *ABCC8*- oder *KCNJ11*-Gen deaktiviert. Aus diesem Grund wäre es von großer Bedeutung, sich auf andere Schritte der SSK zu konzentrieren, um die Insulinsekretion zu reduzieren. Diese Studie schlägt als alternativen Ansatz Öffner von $Ca^{2+}$-aktivierten $K^+$-Kanälen der intermediären Leitfähigkeit (SK4-Kanäle) vor.

## List of the publications of the thesis

The present work is a cumulative dissertation. Reviews and research are already published in the following journals:

**Sikimic J, McMillen T.S, Bleile C, Dastvan F, Quast U, Krippeit-Drews P, Drews G, Bryan J (2019)**

ATP binding without hydrolysis switches sulfonylurea receptor 1 (SUR1) to outward-facing conformations that activate $K_{ATP}$ channels. J Biol Chem 294(10):3707-3719.

**Bauer C, Kaiser J, Sikimic J, Krippeit-Drews P, Düfer M, Drews G (2018)**

ATP mediates a negative autocrine signal on stimulus-secretion coupling in mouse pancreatic β-cells. Endocrine 63(2):270-283.

**Undank S, Kaiser J, Sikimic J, Düfer M, Krippeit-Drews P, Drews G (2017)**

Atrial natriuretic peptide affects stimulus-secretion coupling of pancreatic β-cells. Diabetes 66(11):2840-2848.

**Maczewsky J, Sikimic J, Bauer C, Krippeit-Drews P, Wolke C, Lendeckel U, Barthlen W, Drews G (2017).**

The LXR ligand T0901317 acutely inhibits insulin secretion by affecting mitochondrial metabolism. Endocrinology 158(7):2145-2145.

In addition, the following unpublished manuscript is included:

**Sikimic J, Hoffmeister T, Gresch A, Barthlen W, Wolke C, Lendeckel U, Krippeit-Drews P, Düfer M, Drews G.**

Possible new strategies for the treatment of congenital hyperinsulinism

## Declaration of contribution of author and co-authors

**Jelena Sikimic, Tim McMillen, Cita Bleile, Frank Dastvan, Uli Quast, Peter Krippeit-Drews, Gisela Drews and Joseph Bryan (2019).**

**ATP binding without hydrolysis switches sulfonylurea receptor 1 (SUR1) to outward-facing conformations that activate $K_{ATP}$ channels. J Biol Chem 294(10):3707-3719.**

The whole study was conducted under supervision of Joseph Bryan and Gisela Drews. Human embryonic kidney cells (HEK-293) were provided from Joseph Bryan. The SUR1 and Kir6.2 plasmids and stable transfections in HEK-293 cells were done by Frank Dastvan. Cita Bleile investigated the effect of $GTP^{4-}$ on $SUR1_{Q1179R}$/$Kir6.2_{G334D}$ channels and did some of the patch clamp measurements with $ATP^{4-}$ on $SUR1_{Q1179R}$/$Kir6.2_{G334D}$ and $SUR1_{E1507Q}$/$Kir6.2_{G334D}$ channels. Tim McMillen carried out all [$^3$H]GBM-binding experiments. Transient transfections and all other patch clamp measurements were carried out and evaluated by me. My work contributed clarifying the gating of pancreatic $K_{ATP}$ channels. The manuscript was written by Joseph Bryan and me. Uli Quast, Gisela Drews and Peter Krippeit-Drews contributed to the scientific discussion and revision of the manuscript.

**Bauer C, Kaiser J, Sikimic J, Krippeit-Drews P, Düfer M, Drews G (2018).**

**ATP mediates a negative autocrine signal on stimulus-secretion coupling in mouse pancreatic β-cells. Endocrine 63(2):270-283.**

The whole study was conducted under supervision of Gisela Drews. All series of measurements and evaluations which are not described in detail below were carried out by Cita Bauer. Cita Bauer and Julia Kaiser conducted and evaluated measurements of $[Ca^{2+}]_c$ with $P2X_1$ antagonist suramin in β-cells from C57BL/6N mice. The measurements of insulin secretion with islets from C57BL/6N mice preincubated with ATP were done by Julia Kaiser. Julia Kaiser and I carried out and evaluated measurements of membrane potential in the perforated patch-clamp mode with $P2X_1$ antagonist NF-279 in C57BL/6N β-cells. I investigated the effect of ATP on $[Ca^{2+}]_c$ in islets from C57BL/6N mice. This study provided further insight into the effect of extracellular ATP on stimulus secretion coupling in mouse pancreatic β-

cells. The manuscript was written by Gisela Drews and Cita Bauer. Peter Krippeit-Drews and Martina Düfer contributed to the scientific discussion and revision of the manuscript. Julia Kaiser and I were involved in the revision of the manuscript and thus contributed to the development and finalization of the study.

**Undank S, Kaiser J, Sikimic J, Düfer M, Krippeit-Drews P, Drews G (2017).**

**Atrial natriuretic peptide affects stimulus-secretion coupling of pancreatic β-cells. Diabetes 66(11):2840-2848.**

The whole study was conducted under supervision of Gisela Drews. All series of measurements and evaluations which are not described in detail below were carried out by Sabrina Undank. Julia Kaiser investigated the effect of ANP in the presence of myr-PKI or GLP-1 on insulin secretion. Julia Kaiser and I carried out and evaluated the effect of ANP on the membrane potential in the perforated patch-clamp mode in $SUR1^{-/-}$ β-cells. I conducted and evaluated measurements that investigate the effect of ANP in the presence of PKA inhibitor myr-PKI on the $NP_o$ of single $K_{ATP}$ channels by using the patch-clamp technique in the cell attached mode in C57BL/6N β-cells. This work evaluated the effects of ANP on β-cell function. The manuscript was written by Gisela Drews and Sabrina Undank. Peter Krippeit-Drews and Martina Düfer contributed to the scientific discussion and revision of the manuscript. Julia Kaiser and I were involved in the revision of the manuscript.

**Maczewsky J, Sikimic J, Bauer C, Krippeit-Drews P, Wolke C, Lendeckel U, Barthlen W, Drews G (2017).**

**The LXR ligand T0901317 acutely inhibits insulin secretion by affecting mitochondrial metabolism. Endocrinology 158(7):2145-2145.**

The whole study was conducted under supervision of Gisela Drews. Human Langerhans islets were isolated and provided by Winfried Barthlen, Carmen Wolke and Uwe Lendeckel. Jonas Maczewsky conducted and evaluated all measurements of $[Ca^{2+}]_c$ with T0901317 and GW3965, patch clamp measurements, measurements of membrane potential and microelectrode-array experiments. Furthermore, the effects of T0901317 on insulin secretion and the concentration of ROS species were also investigated by Jonas Maczewsky. The effect of T0901317 on human β-cells

was investigated by Cita Bauer and me. I carried out and evaluated the measurements of ATP concentration and measurements of $[Ca^{2+}]_c$ with 25-Hydroxycholesterol. *In vivo* experiments were planned and carried out by Gisela Drews, Peter Krippeit-Drews and Jonas Maczewsky. This work identified new mechanisms of action of the LXR-agonist T0901317. The manuscript was written by Gisela Drews and Jonas Maczewsky. Peter Krippeit-Drews contributed to the scientific discussion and revision of the manuscript. I was involved in the discussion and revision of the manuscript.

**Sikimic J, Hoffmeister T, Gresch A, Barthlen W, Wolke C, Lendeckel U, Krippeit-Drews P, Düfer M, Drews G.**

**Possible new strategies for the treatment of congenital hyperinsulinism**

The whole study was conducted under supervision of Gisela Drews. Human Langerhans islets were isolated and provided by Winfried Barthlen, Carmen Wolke and Uwe Lendeckel. Theresa Hoffmeister conducted and evaluated measurements of $[Ca^{2+}]_c$ with simvastatin in human β-cells isolated from pancreatic tissue of CHI patients. Anne Gresch carried out and evaluated measurements of $[Ca^{2+}]_c$ with dextromethorphan in human β-cells isolated from pancreatic tissue of CHI patients. I conducted and evaluated all other measurements of $[Ca^{2+}]_c$ with diazoxide, NN414, VU0071063, DCEBIO and galanin in human β-cells isolated from pancreatic tissue of CHI patients. I also carried out and evaluated the measurements of $[Ca^{2+}]_c$, and measurements of ΔΨ with VU0071063 and DCEBIO in β-cells isolated from C57BL/6N and $SUR1^{-/-}$ mice. My work contributed to exploring better strategies in the treatment of CHI. The manuscript was written by Gisela Drews and me. Martina Düfer and Peter Krippeit-Drews contributed to the scientific discussion of the manuscript.

# 1 Introduction

Accurate regulation of blood sugar concentration is essential for an organism, as glucose is the most important energy supplier for all organs. Some organs, especially the brain, are critically affected by disorders affecting glucose metabolism. Various hormones are responsible for regulation of glucose homeostasis. The most important, and the only hormone that directly lowers blood glucose concentration, is insulin, which is secreted from β-cells of the islets of Langerhans. Healthy β-cells act as a glucose sensor and adjust the release of insulin in response to the concentration of postprandial circulating glucose. Metabolically induced changes in electrical activity are responsible for adequate insulin secretion.

Reduced secretion of insulin, or insufficient action of existing insulin on its target organs, leads to diabetes mellitus. By contrast, the increase of insulin secretion, as occurs in congenital hyperinsulinism (CHI), dramatically reduces blood glucose levels, which in turn leads to severe brain damage. Insulin secretion occurs in a pulsatile fashion. This property of β-cells is essential in avoiding the development of insulin resistance (Goodner *et al.* 1977; Peiris *et al.* 1992; Porksen *et al.* 2002; Satin *et al.* 2015). The pulsatile release of insulin is a result of the unique ability of the β-cell to generate oscillations in electrical activity and cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$), thereby linking glucose metabolism to insulin secretion via stimulus-secretion coupling (SSC).

## 1.1 Stimulus-secretion coupling and regulation of insulin secretion

Insulin secretion occurs in a biphasic manner as a result of enhanced blood glucose concentration (above 6 mM), which mirrors changes in the membrane potential and $[Ca^{2+}]_c$ of the pancreatic β-cell. Appropriately, the pancreatic β-cells contain different voltage-dependent and ligand-activated ion channels. With stimulating glucose concentrations, the opening and closure of these channels lead to oscillations in the membrane potential (Santos *et al.* 1991), which are parallel with $[Ca^{2+}]_c$ oscillations (Gilon *et al.* 1993). Oscillations in $[Ca^{2+}]_c$ are required for the pulsatile secretion of insulin (Kjems *et al.* 2002; Gilon *et al.* 1993). This oscillatory pattern of the electrical activity of the murine pancreatic β-cells is characterized by a bursting pattern. The bursting is characterized by active phases (i.e., bursts) in which action potentials are

elicited, separated by inactive or silent phases (the interbursts) with no electrical activity (Manning Fox *et al.* 2006; Bertram *et al.* 2010).

### 1.1.1 Stimulus secretion coupling in murine β-cells

In SSC of pancreatic β-cells, glucose enters the β-cell via an insulin-independent glucose transporter (predominantly GLUT2 for murine β-cells) in response to elevated blood glucose concentrations (Thorens 2001; McCulloch *et al.* 2011); the glucose is then metabolized by glycolysis. As a result of glycolysis, pyruvate and the reducing equivalent NADH are produced. Pyruvate is used in the tricarboxylic acid cycle in mitochondria, which in turn produces mNADH and $FADH_2$. NADH produced in glycolysis is transferred into mitochondria via shuttle systems, such as malate-aspartate and glycerol phosphate (MacDonald 1982; Eto *et al.* 1999). Consequently, concentrations of NADH and $FADH_2$ in mitochondria are elevated. These reducing equivalents are able to generate the proton gradient across the inner mitochondrial membrane by transferring their electrons across the respiratory chain. $F_1F_0$-ATPase uses this gradient to produce ATP. Furthermore, the proton transfer leads to hyperpolarization of the mitochondrial membrane potential ($\Delta\Psi$) (Fujimoto *et al.* 2007; Drews *et al.* 2015b). There is evidence that strongly support the hypothesis that only ATP, synthesized from reducing equivalents produced in glycolysis, regulates $K_{ATP}$ channels (Dukes *et al.* 1994; Gerbitz *et al.* 1996; Krippeit-Drews *et al.* 2003). Conversely, the ATP generated from the metabolism of pyruvate and the reduction equivalents generated in the citric acid cycle is used to produce the bulk cytosolic ATP ($[ATP]_c$) (3-5 mM) which is needed to meet the energy demands of the cell (Detimary *et al.* 1998; Drews *et al.* 2015b). One hypothesis proposes that the ATP produced from a glycolytic reducing equivalents is first converted to phosphocreatine (PCr) by a mitochondrial creatine kinase (CK), which is then transported to the plasma membrane into the direct vicinity of the $K_{ATP}$ channels (Gerbitz *et al.* 1996; Krippeit-Drews *et al.* 2003; Dzeja and Terzic 2003). There, the ATP concentration is determined by membrane-bound enzymes like CK and adenylate kinase (AK), forming a “metabolic barrier” that shields the $K_{ATP}$ channels from the high cellular bulk $[ATP]_c$ (Dzeja and Terzic 2003; Krippeit-Drews *et al.* 2003; Schulze *et al.* 2007; Stanojevic *et al.* 2008). Whereas membrane-associated AK produces ADP from ATP and AMP and positively regulates the $K_{ATP}$ channels, membrane-associated CK produces ATP from ADP and PCr, leading to an increase in the ATP:ADP ratio,

which causes the closure of $K_{ATP}$ channels (Schulze *et al.* 2007). When a certain number of $K_{ATP}$ channels are closed, a still unidentified depolarizing current depolarizes the membrane to the threshold for opening of L-type voltage-dependent $Ca^{2+}$ channels (VDCCs) (Braun *et al.* 2008; Santos *et al.* 1991; Gilon *et al.* 1993; Taylor *et al.* 2005). The channels open, enabling $Ca^{2+}$ to enter the β-cell and trigger insulin secretion (Ashcroft and Rorsman 1989). This influx of $Ca^{2+}$ and the increase in $Ca^{2+}$ concentration are essential for the release of insulin. These events characterize the first phase of insulin secretion (Straub and Sharp 2002; Henquin 2009).

As long as glucose concentrations are at stimulatory levels, insulin secretion is continual and the bursting process regenerates (Tarasov *et al.* 2012). During the second phase, a rapid increase of $[Ca^{2+}]_c$ leads to $Ca^{2+}$ entering mitochondria through $Ca^{2+}$ uniporters and the elevation of mitochondrial concentration of $Ca^{2+}$ ($[Ca^{2+}]_m$), which results in a depolarization of ΔΨ. The concomitantly decreased proton gradient attenuates the driving force of ATP synthesis by $F_1F_0$-ATPase and yields reduced ATP production (Krippeit-Drews *et al.* 2000; Drews *et al.* 2015a; Kindmark *et al.* 2001; Bertram *et al.* 2010; Kennedy *et al.* 2002; Detimary *et al.* 1998). In turn, a distinct number of $K_{ATP}$ channels is activated. Additionally, elevated $[Ca^{2+}]_c$, during the active phase, stimulates $Ca^{2+}$-activated $K^+$ channels ($K_{Ca}$) (Göpel *et al.* 1999; Goforth *et al.* 2002; Bertram *et al.* 2010). The outcome of these events is hyperpolarization of the membrane potential, which subsequently decreases $[Ca^{2+}]_c$ (Tarasov *et al.* 2012). Decreased $[Ca^{2+}]_c$ then reduces the activity of $K_{Ca}$ channels and hyperpolarizes ΔΨ, which increases ATP production again and accordingly turns off $K_{ATP}$ channels. The reduction of both $K_{ATP}$ and $K_{Ca}$ currents, allows elevation of $[Ca^{2+}]_c$ and the initiation of a new cycle of bursting (Drews *et al.* 2015b). Thus, depolarization of ΔΨ reflects decreased ATP synthesis, while the hyperpolarization of ΔΨ mirrors increased production of ATP.

### 1.1.2 Stimulus secretion coupling in human β-cells

SSC in human β-cells largely resembles that in murine β-cells. Still, there are some differences, which are presumably the consequence of the less organized cytoarchitecture of human islets and alterations in the expression of specific ion channels and membrane transporters (Braun *et al.* 2008; Rorsman and Braun 2013; Rorsman and Ashcroft 2018; Cabrera *et al.* 2006). The electrical activity in human β-

cells is already elicited already at approximately 5 mM glucose (Rorsman and Braun 2013). Compared with mouse β-cells, human β-cells express predominantly GLUT1 and GLUT3 glucose transporters (McCulloch *et al.* 2011; Rorsman and Braun 2013). As described for murine β-cells (section 1.1.1), glucose uptake via a glucose transporter accelerates glucose metabolism leading to increased ATP production, which further leads to the closure of the $K_{ATP}$ channels. In addition to L-type and P/Q-type VDCCs, human β-cells comprise T-type VDCCs, which play a role in pacemaking, i.e. they facilitate the initiation of action potential firing (Braun *et al.* 2008; Rorsman *et al.* 2012; Rorsman and Ashcroft 2018; Misler *et al.* 1992b). Furthermore, T-type VDCCs are also known as low-voltage-activated $Ca^{2+}$ channels, because they are activated at more negative membrane potentials than other types of VDCCs (above -60 mV) (Rorsman and Braun 2013; Braun *et al.* 2008). The closure of $K_{ATP}$ channels increases membrane resistance and allows occasional spontaneous opening of T-type VDCCs (Rorsman *et al.* 2012; Rorsman and Braun 2013). Further depolarization of the membrane allows opening more T-type $Ca^{2+}$ channels and when the threshold is achieved, L-Type VDCCs open (above -50 mV) followed by voltage-dependent $Na^+$ ($Na_v$) channels (above -40 mV) (Braun *et al.* 2008; Rorsman and Braun 2013). Although $Na_v$ channels exist in the murine pancreatic β-cells, it has been shown that they are fully inactivated at the resting potential and probably physiologically insignificant (Plant 1988; Drews *et al.* 2015b). However, in human β-cells, they play an important role in action potential generation (Braun *et al.* 2008; Pressel and Misler 1990; Barnett *et al.* 1995). The opening of $Na_v$ channels and associated $Na^+$ influx lead to a depolarization of the cell membrane that is sufficient for the activation of P/Q-type VDCCs (Braun *et al.* 2008; Rorsman and Braun 2013; Rorsman *et al.* 2012). Concomitant $Ca^{2+}$-influx results in increased $[Ca^{2+}]_c$, which triggers exocytosis of insulin granules. Braun *et al.* showed that in human β-cells P/Q-type VDCCs are more tightly coupled to exocytosis than L-type VDCCs (Braun *et al.* 2008). Opening of $K_{Ca}$ channels and to a lesser extent voltage-dependent $K^+$ channels ($K_V$) channels repolarizes action potential (Rorsman and Braun 2013) Different patterns of electrical activity have also been noticed between species. Unlike in mice, in human β-cells bursting electrical activity is seen only occasionally and action potential firings are more frequent (Misler *et al.* 1992a; Riz *et al.* 2014; Skelin Klemen *et al.* 2017).

### 1.2 Involvement of different ion channels in oscillations of β-cells

As already noted, metabolic processes and the electrical activity of various ion channels are responsible for the bursting process. Of special interest are $K_{ATP}$ channels, which couple cellular metabolism with the electrical activity in β-cells, and VDCCs, which are crucial for the influx of $Ca^{2+}$ and thus the triggering of insulin secretion (Meissner and Schmelz 1974; Ashcroft and Rorsman 1989; Drews *et al.* 2015b). However, other ion channels contribute to the electrical activity pattern of the β-cell as well. Of particular interest are $K_v$ channels, $Na_v$ channels in human β-cells (see Section 1.1.2), and $K_{Ca}$ channels. The $K_v$ channels primarily contribute in action potential repolarization and modulate insulin secretion only in the presence of membrane depolarization (Trube *et al.* 1986; Jacobson and Philipson 2007; Jacobson *et al.* 2007; MacDonald and Wheeler 2003). The involvement of $K_{Ca}$ channels in the regulation of the membrane potential oscillations of β-cells has been discussed. Investigating the effect of an increased $Ca^{2+}$ influx on membrane potential, Rosario *et al.* demonstrated that the activation of $K_{Ca}$ current could modify the interval of the interbursts (Rosario *et al.* 1993; Drews *et al.* 2015b). The existence of $K_{Ca}$ channels has been detected in pancreatic β-cells, and depending on their single-channel conductance, there are three groups of $K_{Ca}$ channels: BK channels (large-conductance or $K_{Ca}1.1$), SK4 channels (intermediate-conductance or $K_{Ca}3.1$), and SK1, SK2, and SK3 channels (small-conductance) (Tamarina *et al.* 2003; Düfer *et al.* 2009; Jacobson *et al.* 2010; Düfer *et al.* 2011; Kukuljan *et al.* 1991; Braun *et al.* 2008). However, their role in β-cells is not yet fully understood. Activation of BK channels is dependent on both $[Ca^{2+}]_c$ and membrane depolarization (Drews *et al.* 2015b). It leads to a hyperpolarizing efflux of $K^+$ that can affect different parameters of SSC. However, different groups have reported contradictory results regarding the role of BK channels in action potentials. Kukuljan *et al.* failed to detect an effect of BK channels on electrical activity in the β-cells of rodents (Kukuljan *et al.* 1991). By contrast, Düfer *et al.* demonstrated that the genetic ablation of BK channels or its pharmacologic inhibition with iberiotoxin decreased glucose-stimulated insulin secretion, broadened the action potentials, and abolished the after hyperpolarization but did not affect the action potential frequency, the plateau potential at which action potential starts, nor the $[Ca^{2+}]_c$ (Düfer *et al.* 2011). Furthermore, mice with genetically ablated BK channels ($BK^{-/-}$ mice) are normoglycemic with impaired glucose tolerance

(Düfer *et al.* 2011). One reason could be that the ablation of BK channels mirrors a reduction in β-cell mass (Rorsman and Ashcroft 2018). This is also supported by the fact that BK channels are important for the ability of the β-cell to handle oxidative stress, which is indicated by an increased rate of apoptosis in $BK^{-/-}$ mice (Düfer *et al.* 2011; Rorsman and Ashcroft 2018). In human β-cells, Braun *et al.* showed that pharmacologic inhibition of BK channels using iberiotoxin leads to increased amplitude of action potentials and increased insulin secretion by 70% (Braun *et al.* 2008). Accordingly, it was assumed that BK channels are involved in the repolarization of individual action potentials to plateau potential of murine and human β-cells (Braun *et al.* 2008; Düfer *et al.* 2011; Drews *et al.* 2015b). Due to the fact that the blockage of BK channels does not influence membrane potential oscillations (Atwater *et al.* 1979; Henquin 1990; Kukuljan *et al.* 1991; Houamed *et al.* 2010), these channels are not considered to play a role in the regulation of the burst pattern (Drews *et al.* 2015b).

Unlike BK channels, the activation of SK4 channels is independent of membrane depolarization, but is strictly regulated by $[Ca^{2+}]_c$ (Jensen *et al.* 1998; Vogalis *et al.* 1998). *In vitro* experiments on murine β-cells from mice lacking functional SK4 channels ($SK4^{-/-}$) demonstrated increased frequency and broadening of action potentials, as well as increased $[Ca^{2+}]_c$ (Düfer *et al.* 2009). The same effects have been observed in experiments on β-cells from C57BL/6N mice where the SK4 current was pharmacologically inhibited with the SK4-specific inhibitor TRAM-34 (Düfer *et al.* 2009). According to the above-described model of murine β-cell oscillations, the role in termination of the active (burst) phase could be ascribed partially to the current that comprises both $K_{ATP}$ and SK4 currents (Drews *et al.* 2015b). This current was initially described by Göpel *et al.*, who documented a $Ca^{2+}$-dependent $K^+$ current that was activated during oscillatory electrical activity, which they named $K_{slow}$, due to its slow activation (Göpel *et al.* 1999). Further studies by Kanno *et al.* showed that 48% of $K_{slow}$ could be attributed to $K_{ATP}$ and that the rest belongs to sulfonylurea-independent $K^+$-current (Kanno *et al.* 2002). Regarding murine β-cells it has been demonstrated that knockout or pharmacological inhibition of SK4 channels significantly reduces $K_{slow}$ (Düfer *et al.* 2009), leading to the assumption that SK4 channels make up the other component of $K_{slow}$.

### 1.3 Modulation of insulin secretion

As already explained, the main stimulus for insulin secretion from the β-cells is glucose. In addition to glucose, a number of various other substances modulate insulin secretion in different ways. Some of them are able to initiate insulin secretion independently, without the presence of another secretagogue (e.g., glucose). These substances include the amino acid leucin and certain drugs like sulfonylureas (Yang *et al.* 2010; Hellman *et al.* 1971; Rorsman and Ashcroft 2018). In contrast, neurotransmitters (released by nerve endings within the islet) and various circulating hormones are effective only in the presence of glucose (Rorsman and Ashcroft 2018). Some of the substances that enhance the glucose-stimulated insulin secretion include glucagon, acetylcholine, glucagon like peptide 1 (GLP-1), glucose-dependent insulin-releasing peptide (GIP), oxytocin, vasoactive intestinal peptide (VIP), and opioids (Irwin et al, 2013). The hormone somatostatin, the neuropeptide galanin, and catecholamines have inhibitory modulatory effects on glucose-stimulated electrical activity by inhibiting action potential firing and hence insulin secretion (Nilsson *et al.* 1989; Drews *et al.* 2015b). All three agents have similar action mechanisms. It has been suggested that they hyperpolarize membrane potential ($V_m$) after binding to specific membrane receptors which are coupled to Gi/o-proteins (Ullrich and Wollheim 1988; Drews *et al.* 1990; Nilsson *et al.* 1989; Dunne *et al.* 1989; Braun 2014), but the underlying mechanisms are still not fully understood. The effect of somatostatin on β-cells is mediated via somatostatin receptors (SSTRs) and is a result of inhibiting the adenylate cyclase-cAMP pathway, activating a G protein-gated inwardly rectifying $K^+$ channel (GIRK), and reducing P/Q type $Ca^{2+}$ currents (Patel *et al.* 1994; Kailey *et al.* 2012; Braun 2014). Additionally, somatostatin also directly inhibits exocytosis by activating the protein phosphatase calcineurin (Renström *et al.* 1996). Galanin via galanin receptors (GALRs) and catecholamines via $\alpha_2$ adrenoreceptors inhibit the insulin secretion by a mechanism resembling that of somatostatin (Drews *et al.* 1990; Ullrich and Wollheim 1989; Jones *et al.* 1987). Studies from Tang *et al.* have demonstrated that galanin mediates its inhibition by $G_{o2}$ and not by other $G_i/G_o$ proteins in pancreatic β-cells (Tang *et al.* 2012). They showed that the effect of galanin on $K_{ATP}$ and $Ca^{2+}$ currents were completely abolished in β-cells from mice lacking $G_{o2}$ (Tang *et al.* 2012).

It has been proposed that SSTRs, GALRs and $\alpha_2$ adrenoreceptors in pancreatic β-cell are coupled to the $K_{ATP}$ channels, (Ribalet and Eddlestone 1995; Smith *et al.* 2001; Dunne *et al.* 2004). Even though the experiments showed that this coupling is present in insulin-secreting cell lines (Ribalet and Eddlestone 1995), it has not been established in murine or human β-cells (Doyle and Egan 2003). Rorsman *et al.* have postulated that in mouse β-cells, the inhibition of electrical activity is mediated by the activation of low-conductance $K^+$ channels, which differ from $K_{ATP}$ channels (Rorsman *et al.* 1991). Findings from Düfer *et al.* and Sieg *et al.* (Sieg *et al.* 2004; Düfer *et al.* 2004) support this theory, as noradrenalin and galanin showed their effects in the β-cells of mice lacking functional $K_{ATP}$ channels. Moreover, adrenaline and somatostatin were able to reduce insulin secretion in the presence of sulfonylureas in INS-1 cells (Abel *et al.* 1996). Additionally, recent experiments with human β-cells showed that somatostatin hyperpolarized the membrane potential in the presence of tolbutamide (Kailey *et al.* 2012) arguing against coupling between SSTRs and $K_{ATP}$ channels.

### 1.4 ATP-sensitive potassium channels

Functional $K_{ATP}$ channels are comprised of two subunits, a sulfonylurea receptor (SURx) (Aguilar-Bryan *et al.*, 1995) and a $K^+$-selective inward rectifier (Kir6.x) (Inagaki *et al.* 1995), assembled as hetero-octamers $(SURx/Kir6x)_4$ (Shyng and Nichols 1997). SURx is a member of the ATP-binding cassette transporter subfamily (ABCC family) (Aguilar-Bryan *et al.* 1995), which also includes the cystic fibrosis transmembrane conductance regulator (CFTR) and the multidrug resistance associated protein (MDR) (Wilkens 2015). Unlike other ABC proteins, the SURx receptor has no identified transport function. Instead, it associates with Kir6.x to form the $K_{ATP}$ channel and plays a role in the regulation and fine-tuning of the channel gating.

The expression of various combinations of SURx (SUR1, SUR2A or SUR2B) and Kir6x (Kir6.1 or Kir6.2) leads to the formation of $K_{ATP}$ channels that differ not only in their localization, but also in their electrophysiological properties and their sensitivity to nucleotides and drugs (Tinker *et al.* 2018). Accordingly, SUR1/Kir6.2 channels are found in the pancreas and brain (Aguilar-Bryan *et al.* 1995; Doyle *et al.* 1998; Inagaki *et al.* 1997; Aguilar-Bryan and Bryan 1999), SUR2A/Kir6.2 channels are found in the

heart and skeletal muscle (Inagaki *et al.* 1996), whereas SUR2B/Kir6.1 and SUR2B/Kir6.2 channels are identified in vascular and non-vascular smooth muscle (Isomoto *et al.* 1996; Yamada *et al.* 1997). Moreover, two types of $K_{ATP}$ channel complexes (SUR1/Kir6.2 and SUR2B/Kir6.1) are also identified in the hair follicle, where they probably regulate the follicle growth cycle (Shorter *et al.* 2008). Two variants of the same gene (*ABCC9*), SUR2A and SUR2B differ by only 42 amino acids in the C-terminus, due to alternative splicing (Isomoto *et al.* 1996).

Neuroendocrine type $K_{ATP}$ channels are comprised of four Kir6.2 subunits that form the pore (Aguilar-Bryan and Bryan 1999; Aguilar-Bryan *et al.* 1995; Inagaki *et al.* 1997; Doyle *et al.* 1998), which is surrounded by four regulatory SUR1 subunits. Subunits SUR1 and Kir6.2 are encoded by *ABCC8* and *KCNJ11* genes, respectively, located on chromosome band 11p15.1 (Aguilar-Bryan *et al.* 1995). Recent structural studies (Martin *et al.* 2017a; Lee *et al.* 2017), have confirmed earlier findings regarding the assembly of pancreatic $K_{ATP}$ channels. The Kir6.2 subunit consists of two transmembrane helices and N- and C-terminal cytoplasmic domains (Hibino *et al.* 2010). As a member of the ABC family of proteins, SUR1 possesses a characteristic ABC core comprised of two asymmetric cytosolic nucleotide binding domains (NBD1 and NBD2) and two bundles of six transmembrane domain helices (TMD1 and TMD2) (Fig. 1) (Tusnady *et al.* 2006; Martin *et al.* 2017a; Lee *et al.* 2017; Puljung 2018). In addition, SUR1 has an N-terminal bundle of five transmembrane helices (TMD0), that serves to couple conformational changes in the SUR1 to the pore, followed by the linker termed L0, which plays a critical role in channel activation (Babenko and Bryan 2003; Tusnady *et al.* 2006; Martin *et al.* 2017a). Each NBD has Walker A and B motifs, which are involved in nucleotide binding and in the modulation of channel activity (Gribble *et al.* 1997; Matsuo *et al.* 2005). For other ABC proteins, it has been reported that glutamate of the Walker B motifs plays a role in the catalytic activity of NBDs (Linton and Higgins 2007). However, SURs have been described to have a conserved catalytic glutamate residue (E1506 or E1507 that is based on the human L78208 *ABCC8* isoform) in their NBD2 Walker B, whereas the Walker B of NBD1 has an aspartate (D855 in SUR1) (Aittoniemi *et al.* 2009; Männikkö *et al.* 2011). This difference is consistent with findings that the NBDs of SUR are asymmetrical and have different ATPase activity. Additionally,

measurements of ATPase activity have shown that the activity of NBD2 is higher than the activity measured for NBD1 (Matsuo *et al.* 2005; de Wet *et al.* 2007).

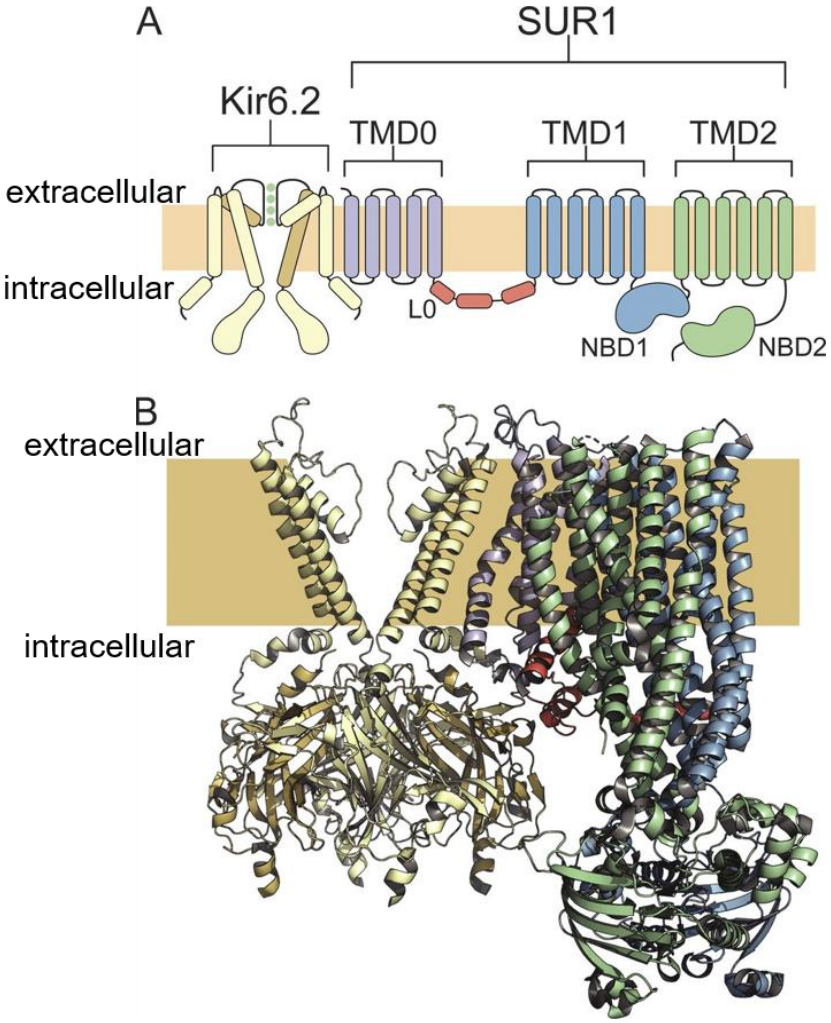


**Fig. 1: Structure of pancreatic $K_{ATP}$ channel.** A) Transmembrane topology of the pancreatic β cell $K_{ATP}$ complex. Only two (of four) Kir6.2 subunits and one (of four) SUR1 subunits are shown. B) Side view of the structure of $K_{ATP}$ channel complex. For clarity, the pore domains of two of the Kir6.2 subunits have been removed, and only one SUR1 subunit is shown.
Kir6.2 subunits: yellow and brown, SUR1 is color-coded as follows: TMD0: lavender, L0: orange, TMD1-NBD1: blue and TMD2-NBD2: green. Modified after (Puljung 2018).

Functional $K_{ATP}$ channels are incorporated into the plasma membrane only in the case of the coexpression of both subunits (Inagaki *et al.*, 1995, 1996). Assembly of the subunits happens in the endoplasmic reticulum (ER) and is under the control of an ER retention signal (RKR) (Zerangue *et al.* 1999). This retention signal is a tripeptide (Arg-Lys-Arg) and is present in both subunits, preventing them from leaving the ER before assembly of the full channel (Zerangue *et al.* 1999). The appropriate

association of SUR1 and Kir6.2 results in the mutual shielding of retention signals and allows the channel to traffic to the cell membrane (Yan *et al.* 2007; Quan *et al.* 2011). The COOH terminus of the SUR1 subunit has an additional trafficking signal. The deletion of certain amino acids in this region reduces the expression of $K_{ATP}$ channels (Sharma *et al.* 1999).

### 1.4.1 Regulation of pancreatic $K_{ATP}$ channels

In pancreatic β-cells, $K_{ATP}$ channels are essential for linking cellular metabolism and membrane electrical activity and thus are regulators of insulin secretion. The activity of $K_{ATP}$ channels is subject to complex regulation, which is determined by a variety of factors. The channels are regulated positively, by ATP and ADP binding to the SUR1 subunit, and negatively, by nucleotide binding to Kir6.2 (Cook and Hales 1984; Hopkins *et al.* 1992; Dunne and Petersen 1986; Kakei *et al.* 1986; Tucker *et al.* 1997; Gribble *et al.* 1998). Additionally, metabolites such as phosphoinositides (Baukrowitz *et al.* 1998; Shyng and Nichols 1998; Baukrowitz and Fakler 2000), long-chain acyl-Co-A esters (Schulze *et al.* 2003), and phosphorylation (Light *et al.* 2002; Beguin *et al.* 1999; Lin and Chai 2008) play an important role in the positive modulation of channel activity (Tinker *et al.* 2018)

The pharmacologic modulation of the channels by interaction with SUR1 is of clinical importance. Sulfonylureas (like glibenclamide (GBM)) and glinides (like repaglinide) exert an inhibitory effect on channel activity (Sturgess *et al.* 1988; Panten *et al.* 1990; Gribble and Reimann 2003; Dabrowski *et al.* 2001). By contrast, diazoxide, which is a benzothiadiazine derivate, is an agonist of $K_{ATP}$ channels, which effectively hyperpolarizes the β-cell membrane potential and reduces insulin secretion (Sturgess *et al.* 1988; Bryan *et al.* 2005). The regions where diazoxide binds include transmembrane domains (TMDs) 6-11 and NBD1 (Babenko *et al.* 2000). The literature suggests that diazoxide requires $Mg^{2+}$ and hydrolyzable ATP for its action (Larsson *et al.* 1993; Kozlowski *et al.* 1989).

The proper explanation of how nucleotides regulate the $K_{ATP}$ channels is still a matter of debate. Early electrophysiological studies showed that ATP and ADP independent of $Mg^{2+}$, inhibit the channels by interacting with the pore (Cook and Hales 1984; Ashcroft and Kakei 1989). On the other hand, numerous electrophysiological studies have demonstrated that co-application of MgADP antagonizes the inhibitory effect of

ATP on the $K_{ATP}$ channels through interaction with the SUR1 and enhances their activity (Dunne and Petersen 1986; Ueda *et al.* 1997; Gribble *et al.* 1997; Nichols *et al.* 1996). Moreover, Ueda *et al.* have shown that the binding of the 8-azido analog of ATP to NBD1 does not require $Mg^{2+}$, but that the affinity for MgATP at NBD2 is significantly greater than for $ATP^{4-}$ (Ueda *et al.* 1997). In experiments from Nichols *et al.* (Nichols *et al.* 1996), $K_{ATP}$ channels with a mutation in SUR1, G1479R, known to cause CHI, failed to respond to MgADP co-application. Located in the cytoplasmic region between Walker A and Walker B within NBD2, the G1479 residue is very close to a region that is proposed to play an important role in nucleotide hydrolysis in several ABC proteins (Smit *et al.* 1993; Carson and Welsh 1995). Accordingly, Nichols *et al.* (Nichols *et al.* 1996) proposed that MgADP is needed for the channel stimulation and that NBD2 could be a critical site for it.

This proposal raises a question of where does MgADP come from. It is generally accepted that ABC proteins are ATPases and have a catalytic cycle. Current models of regulation of ABC proteins propose that ATP binding and ATP hydrolysis provide energy or the so-called “power stroke” for the conformational switch of the two transmembrane helical domains, from an inward- to an outward-facing conformation needed to translocate the substrate (Linton and Higgins 2007; Wilkens 2015). Due to structural similarities with the other members of the ABC protein family, it has been assumed that ATP hydrolysis, rather than ATP binding alone, is required to switch the SUR into the conformation that is responsible for channel openings (Fig. 2). Thus, the posthydrolytic MgADP-bound conformation of SUR1 (Fig. 2D) has been expected to be responsible for positive regulation of the channels (Zingman *et al.* 2001; Zingman *et al.* 2002; Bienengraeber *et al.* 2000). This idea has been supported by experiments with non-hydrolyzable analogs (e.g. AMPPNP, AMPPCP and ATPγS) and by studies suggesting that the action of $K^+$ channel agonists (e.g., diazoxide for SUR1- and pinacidil for SUR2-based channels) requires ATP hydrolysis (Larsson *et al.* 1993; Hambrock *et al.* 1998; Schwanstecher *et al.* 1998). The reports that SUR1 has low levels of ATPase activity (de Wet *et al.* 2007; Mikhailov *et al.* 2005) further support the explanation of how MgADP increases channel open probability in spite of strong inhibition by ATP binding to the Kir6.2 pore (Dunne and Petersen 1986). Additionally, the $K_{ATP}$ channel opener diazoxide has been proposed to open $K_{ATP}$

channels by stabilizing this stimulatory, posthydrolytic MgADP-bound state of SUR1 (Nichols *et al.* 1996; Shyng *et al.* 1997; Moreau *et al.* 2005).

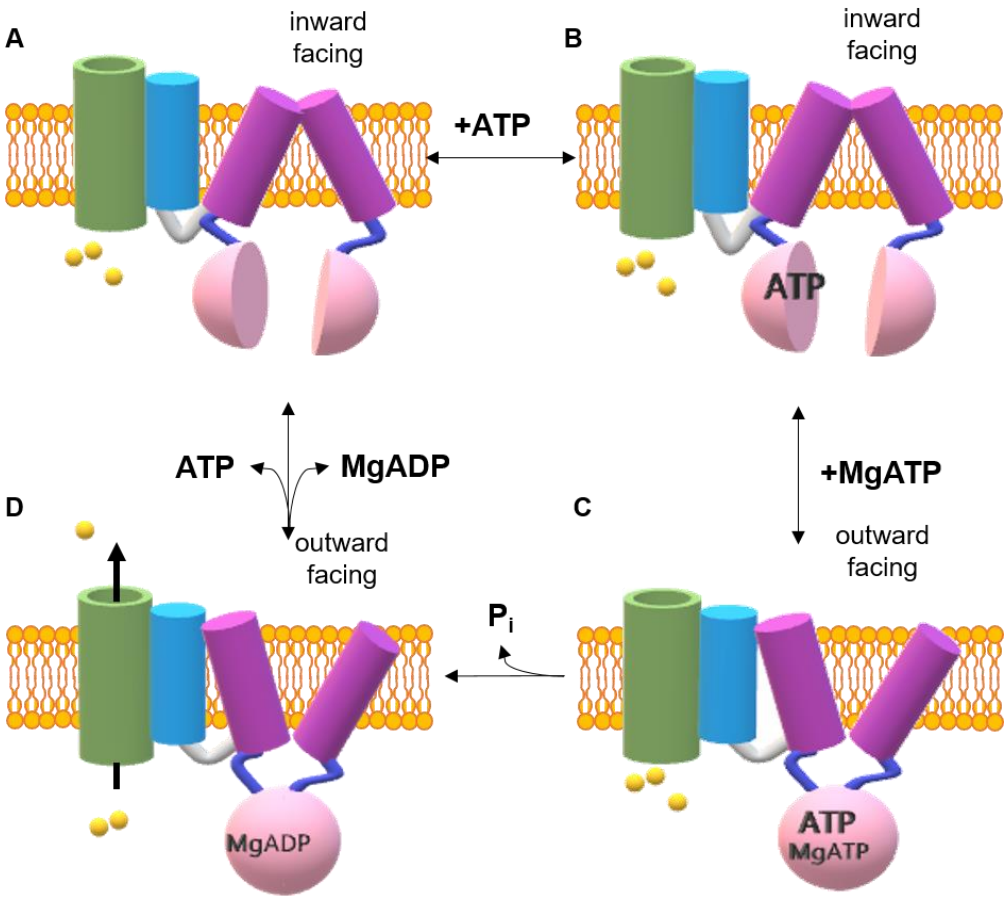


**Fig. 2: Schematic of the current model for $K_{ATP}$ channel gating.** A) The state of the Kir6.2/SUR1 complex having the NBDs in an open dimer configuration and TMDs in a closed or inward-facing conformation. B) ATP binding to the NBD1 enhances binding of MgATP to the NBD2. C) Binding of MgATP at NBD2 induces dimerization of NBDs which results in the conformational change of TMDs from an inward- to an outward-facing state. MgATP has been hydrolyzed to MgADP. D) The posthydrolytic MgADP-bound state of SUR1 stimulates the channel activity by stabilizing the outward-facing state of SUR1. ATP hydrolysis initiates dissociation of the dimer. Zingman *et al.* argued that conversion of ADP to ATP by CK probably facilitates the ATPase cycle (Zingman *et al.* 2001) and resets the SUR1 into the initial, inward-facing conformation. Phosphate (Pi) and ADP are released to restore the basal state (Linton and Higgins 2007; Wilkens 2015).
$K^+$ ions: yellow; Kir6.2 pore: green; SUR1-TMD0: blue; L0 linker: grey; SUR1-TMD1 and -TMD2: magenta; SUR1-NBDs: rose. Modified after (Ortiz *et al.* 2012).

By contrast, Choi *et al.* failed to identify the violations of microscopic reversibility and detailed balance predicted by strong coupling between ATP hydrolysis and channel gating (Choi *et al.* 2008). In addition, in the study on SUR1 monomers Ortiz *et al.*

demonstrated that ATP binding under nonhydrolytic conditions was enough to switch SUR1 allosterically between two different conformations (Ortiz *et al.* 2013; Ortiz *et al.* 2012). They used [$^3$H] labeled GBM as a probe to assess two different conformations of SUR1. The ATP binding reduced the binding of [$^3$H] GBM, which led to the suggestion that the ATP-bound state of SUR1 with the lowest affinity for [$^3$H] GBM is an outward-facing state, responsible for channel openings (Ortiz *et al.* 2013). Hence, as in other ABC proteins, ATP binding to the NBDs of SUR1 results in their dimerization and a reconfiguration of TMD1 and TMD2 from a non-stimulatory (inward-facing) to a stimulatory (outward-facing) state for the pore. Two recent cryoEM studies of SUR1/Kir6.2 channels, stabilized by the addition of GBM, showed that SUR1 with GBM bound is in the inward-facing conformation (Li *et al.* 2017; Martin *et al.* 2017a).

Consequently, it is important to further investigate the effect of ATP on the $K_{ATP}$ channel complex and to explore whether ATP affects the channel activation when hydrolysis is disabled.

### 1.5 Pathophysiological role of pancreatic $K_{ATP}$ channels

Defects in ion channels (channelopathies) can be causes of many diseases. In the case of $K_{ATP}$ channels, they are a result of a mutation in a gene encoding an ion channel or a subunit. Dysfunctions mediated by mutations in $K_{ATP}$ channels are associated with a variety of insulin secretion disorders. A gain of channel function is the cause of neonatal diabetes mellitus (NDM) (Aguilar-Bryan and Bryan 2008). The disease manifests in different spectrums, from transient to permanent and severe forms, including neurological manifestation present in developmental delay-epilepsy-neonatal diabetes (DEND) syndrome (Gloyn *et al.* 2004; Flanagan *et al.* 2007; Rubio-Cabezas and Ellard 2013). Aside from neonatal diabetes, DEND syndrome includes developmental delay, autism, and epilepsy (Gloyn *et al.* 2004). The loss of function mutations in genes encoding $K_{ATP}$ channels cause CHI (more in section 1.5.1) (James *et al.* 2009).

#### 1.5.1 Congenital hyperinsulinism

CHI is a rare heterogeneous genetic disorder and still the most frequent cause of severe, persistent hypoglycemia in neonates, infants, and children. The main

reasons for developing CHI are defects in important genes regulating pancreatic β-cell function. In the last two decades, progress has been made in detecting the genes responsible for CHI. To date, there have been reported mutations in 14 essential genes regulating insulin secretion: *ABCC8, KCNJ11, GLUD1, GCK, HADH, SLC16A1, UCP2, HNF4A, HNF1A, HK1, PGM1, PMM2, CACNA1D, and FOXA2* (Galcheva *et al.* 2019; Demirbilek *et al.* 2017; Banerjee *et al.* 2019; Vajravelu *et al.* 2018).

Defects in the above-mentioned genes are responsible for the failure of β-cells to respond to normal regulatory mechanisms, leading to inappropriate and excessive insulin release despite low blood glucose levels, resulting in frequent episodes of hypoglycemia (Hussain 2007; Banerjee *et al.* 2019). Some excellent reviews offer more details regarding molecular mechanisms underlying the pathophysiology of CHI e.g. (Banerjee *et al.* 2019; Galcheva *et al.* 2019). The most prevalent and most severe cases of CHI are due to mutations in the genes that encode SUR1 and Kir6.2 subunits (*ABCC8* and *KCNJ11*, respectively) (James *et al.* 2009). Almost 60% of all detectable mutations are due to one of these two mutations (Vajravelu and De León 2018). According to the Human Gene Mutation Database (HGMD), 448 *ABCC8* and 66 *KCNJ11* mutations have been reported (Galcheva *et al.* 2019). Autosomal recessive and dominant mutations in $K_{ATP}$ channel genes (*ABCC8* and *KCNJ11*) either lead to defects in the channel assembly and transport to the cell membrane or disable the activation of the expressed channels by MgADP (Dunne *et al.* 2004). Thus, the affected β-cells will be continuously depolarized and unable to regulate insulin secretion properly.

Three histologically distinct forms of CHI have been described: focal, diffuse, and atypical. In the focal form, affected β-cells are localized in only one specific part of the pancreas, surrounded by a tissue of normal morphology. Conversely, in diffuse CHI all pancreatic β-cells are affected (Rahier *et al.* 2011). The genetic etiologies of focal and diffuse CHI are distinct. Focal CHI is in most cases caused by two events: paternally inherited autosomal recessive *ABCC8* or *KCNJ11* mutation and somatic loss of the maternal segment in the 11p15 region (extending from 11p15.1 to 11p15.5) resulting in paternal isodisomy (Rahier *et al.* 2011; Damaj *et al.* 2008; de Lonlay *et al.* 1997; Verkarre *et al.* 1998). As a result, the β-cells in the focal lesion have two copies of the mutated SUR1 gene inherited from the father. The 11p15

region contains several imprinted genes involved in cell proliferation, including *CDKN1C*, *H19*, and *IGF2* (Damaj *et al.* 2008; Shuman *et al.* 2006). The *CDKN1C* and *H19* genes are known to inhibit cell proliferation and to be maternally expressed (Damaj *et al.* 2008). They are absent in the focal CHI due to loss of the maternal imprinting of chromosome 11p15 allele (de Lonlay *et al.* 1997; Fournet *et al.* 2001). On the other hand, paternally expressed *IGF2*, which promotes cell growth, is still present. This unbalanced expression of imprinted genes allows the growth of the focal lesion and explains the hyperplasia in the focal CHI (Damaj *et al.* 2008; James *et al.* 2009; Verkarre *et al.* 1998; de Lonlay *et al.* 1997; Fournet *et al.* 2001). In contrast to focal CHI, diffuse CHI varies in inheritance mechanisms and involved genes (Arnoux *et al.* 2010). Morphologically, it is characterized by large β-cells with abnormally large nuclei (Rahier *et al.* 2011). If the histology of the tissue does not match one of the forms described, it is considered an atypical form of CHI. The atypical form is characterized by a mosaic pattern of hyper-functional islets spread over the pancreas (Sempoux *et al.* 2011).

Continuous hypoglycemia is responsible for seizures and, eventually, for severe brain damage (Kapoor *et al.* 2009). Thus, it is crucial to diagnose CHI rapidly and to begin an effective and suitable treatment as early as possible. Treatment options include medical therapy (with a pharmacological approach) and surgical intervention. The first-line drug for treating CHI is the $K_{ATP}$ channel agonist diazoxide (Aynsley-Green *et al.* 2000). However, numerous side effects of diazoxide limit its use. Some of the most common undesired effects are $Na^+$ and fluid retention, hypertrichosis, and loss of appetite. Certain life-threatening side effects, such as cardiac failure, pulmonary hypertension, hyperuricemia, bone marrow suppression, and anemia are reasons for its cessation (Shah *et al.* 2017; Welters *et al.* 2015; Arya, Mohammed*, et al.* 2014; Nebesio *et al.* 2007; Yildizdas *et al.* 2008; Demirel *et al.* 2011; Timlin *et al.* 2017). Notably, diazoxide is effective only when $K_{ATP}$ channels are functional (Aynsley-Green *et al.* 2000). When the therapy with diazoxide fails alternatives include glucagon, somatostatin analogues, nifedipine and novel medications like GLP1-receptor antagonist (Exendin-(9-39)), and sirolimus (Modan-Moses *et al.* 2011; Le Quan Sang *et al.* 2012; Mohnike *et al.* 2008; Neylon *et al.* 2013; Eichmann *et al.* 1999; Yorifuji *et al.* 2013; Arya, Güemes*, et al.* 2014). These drugs also have numerous undesirable effects, which may be reasons to reconsider their therapeutic

usefulness: gastrointestinal symptoms, the formation of gall stones, the suppression of pituitary hormones, necrotizing enterocolitis, hypotension, immune suppression, thrombocytosis, impaired immune response, and many more (Hawkes *et al.* 2016; Hussain 2008; McMahon *et al.* 2017; Demirbilek *et al.* 2014; Durmaz *et al.* 2014; Szymanowski *et al.* 2016; Banerjee *et al.* 2017; van der Steen *et al.* 2018). Recently, a new full human monoclonal antibody to the insulin receptor XmetD (also known as XOMA 358 or RZ358) has been proposed as a novel therapeutic strategy (Corbin *et al.* 2014a; Corbin *et al.* 2014b; Issafras *et al.* 2014; Johnson *et al.* 2017a). It has advanced into phase 2b of a clinical trial. After phase 2a, it was reported that it improves glycemic control in patients with persistent hypoglycemia (Johnson *et al.* 2017b).

For patients in whom CHI cannot be managed medically, surgical treatment is indicated. While partial pancreatectomy is beneficial for patients with focal CHI, in the case of diffuse and drug-unresponsive CHI, a near-total pancreatectomy is required (Adzick *et al.* 2004; Barthlen 2011; Adzick *et al.* 2019; Barthlen *et al.* 2016). Due to different post-operative complications such as recurrent hypoglycemia, pancreatic exocrine insufficiency, and diabetes, diffuse CHI patients are far from being cured after surgery (Meissner *et al.* 2003; Arnoux *et al.* 2010; Lovvorn *et al.* 1999). In order to hinder the development of diabetes postsurgically, 70–90% resection of the pancreas may be considered; however, the outcome is still very unpredictable (Barthlen 2011; Barthlen *et al.* 2010).

In the light of the various aspects of the foregoing discussion, it is of great importance to explore new pharmacological options for CHI therapy to maintain euglycemia and reduce the severe side effects that result from current medical and surgical treatment.

# 2 The objective of the present work

The present study consists of two parts. The focus of the first part is set on the regulation of the pancreatic $K_{ATP}$ channels, and elucidation of the channel activation by nucleotides. The second part investigates new potential approaches for treating CHI.

The mechanism by which nucleotides bring the SUR1 into the conformation that is stimulatory for the pore remains controversial. Previous biochemical studies on SUR1 monomers have demonstrated that ATP hydrolysis is not necessary for conformational change of SUR1. Instead, such change is done solely by ATP binding (Ortiz *et al.* 2013; Ortiz *et al.* 2012). Accordingly, this work aims to discern whether these findings concerning SUR1 monomers are applicable when tested on full $K_{ATP}$ channels. More directly stated, the goal is to investigate whether the energy of ATP binding is sufficient to induce channel openings. Additionally, it has been shown that mutations in SUR1, which cause ND, have a higher affinity for ATP. Hence, those channels spend more time in the open state that results in ND (Ortiz *et al.* 2013; Ortiz *et al.* 2012). Accordingly, this work investigates the response of full $K_{ATP}$ channels with mutated SUR1 to the addition of ATP under non-hydrolytic conditions. The work also investigates whether ATP hydrolysis is needed for the effect of the $K_{ATP}$ channel agonist diazoxide (Nichols *et al.* 1996).

CHI has been characterized as a rare heterogeneous genetic disorder in neonates, infants, and children (see section 1.5.1). Despite the fact that the $K_{ATP}$ channel opener diazoxide causes numerous undesirable effects, it remains the main drug in therapy for CHI due to either poor effectiveness or more severe side effects of the alternatives. For its action, diazoxide needs functional $K_{ATP}$ channels, which are not present in some CHI patients. Therefore, new potential approaches that can silence β-cells were investigated in the scope of the present work. The research involved new drugs that interact with $K_{ATP}$ channels as well as with $K_{ATP}$ channel-independent targets. These were tested on β-cells isolated from mice lacking functional $K_{ATP}$ channels and on human β-cells obtained from the tissue of CHI patients after pancreatectomies.

# 3 ATP hydrolysis is not needed for $K_{ATP}$ channel activation

As described in section 1.4, the pancreatic $K_{ATP}$ channels comprise two subunits, namely SUR1 and Kir6.2, and are dually regulated by nucleotides, depending on which subunit of the channel they bind (Ashcroft 2005). Channel activity is inhibited by ATP or ADP interaction with the pore (Cook and Hales 1984; Tucker *et al.* 1997), which is independent of $Mg^{2+}$ ions (Ashcroft and Kakei 1989). By contrast, the binding of nucleotides to the regulatory SUR1 subunit, which possesses the potential for enzymatic activity, stimulates channel openings. The explanation of how the channels are stimulated is more complex.

Based on the findings of Ortiz *et al.* (Ortiz *et al.* 2013; Ortiz *et al.* 2012) obtained from experiments on SUR1 monomers, the focus of the work presented here, was on further investigating the need for ATP hydrolysis for $K_{ATP}$ channel gating. In order to test this need, wild type (WT) or mutant SUR1 subunits and WT or mutant Kir6.2 were co-expressed in HEK-293 cells. The channel activity of these $K_{ATP}$ channels was measured in the inside/out configuration by using the patch clamp technique. The $K_{ATP}$ currents were measured at a membrane potential of −50 mV, and inward currents are shown as downward deflections (Fig. 3.1.1, Fig. 3.1.2, and Fig. 3.1.3). Analyses to estimate the open probability of the channels ($NP_O$) were done in IgorPro7 with user-written software (adapted by Prof. Dr. Joseph Bryan and Prof. Dr. Peter Krippeit-Drews).

## 3.1 Results

### 3.1.1 Effect of ATP on SUR1/Kir6.2 and SUR1/Kir6.2$_{G334D}$ $K_{ATP}$ channels

Measurements conducted on WT SUR1/Kir6.2 channels confirm that application of 1 mM MgATP or 10 mM $ATP^{4-}$ reduces the activity of the channels (Fig. 3.1.1A and B) (Sikimic *et al.* 2019). According to findings reported by Ortiz *et al.* (Ortiz *et al.* 2013; Ortiz *et al.* 2012), WT SUR1 has a significantly lower affinity for $ATP^{4-}$ than it has for MgATP. Therefore, different concentrations of nucleotides were used. To test how ATP interaction with a regulatory subunit affects channel activity, we used $K_{ATP}$ channels that lack the inhibitory effect at the pore. The mutant pore subunit Kir6.2$_{G334D}$ has an aspartate (D) substituted for glycine (G) at position 334. Previous studies have reported that this substitution, identified in cases of ND (Masia *et al.*

2007), greatly reduces the affinity of Kir6.2 for ATP and in that manner diminishes ATP antagonism at the pore, but it does not affect the properties of the channel (Li *et al.* 2000; Proks *et al.* 2014, 2013). In contrast to measurements on WT SUR1/Kir6.2 channels, 1 mM MgATP acted as an agonist when applied on WT SUR1/Kir6.2$_{G334D}$ channels and increased the open probability ($P_o$) and number (N) of open channels ($NP_o$) (Fig. 3.1.1C) (Sikimic *et al.* 2019). Without $Mg^{2+}$ that is a cofactor needed for hydrolysis, 10 mM $ATP^{4-}$ had no significant effect on channel open probability (Fig. 3.1.1C) (Sikimic *et al.* 2019). The data are consistent with the experiments on SUR1 monomers showing significantly higher affinity for MgATP *vs.* $ATP^{4-}$ (Ortiz *et al.* 2013; Ortiz *et al.* 2012).

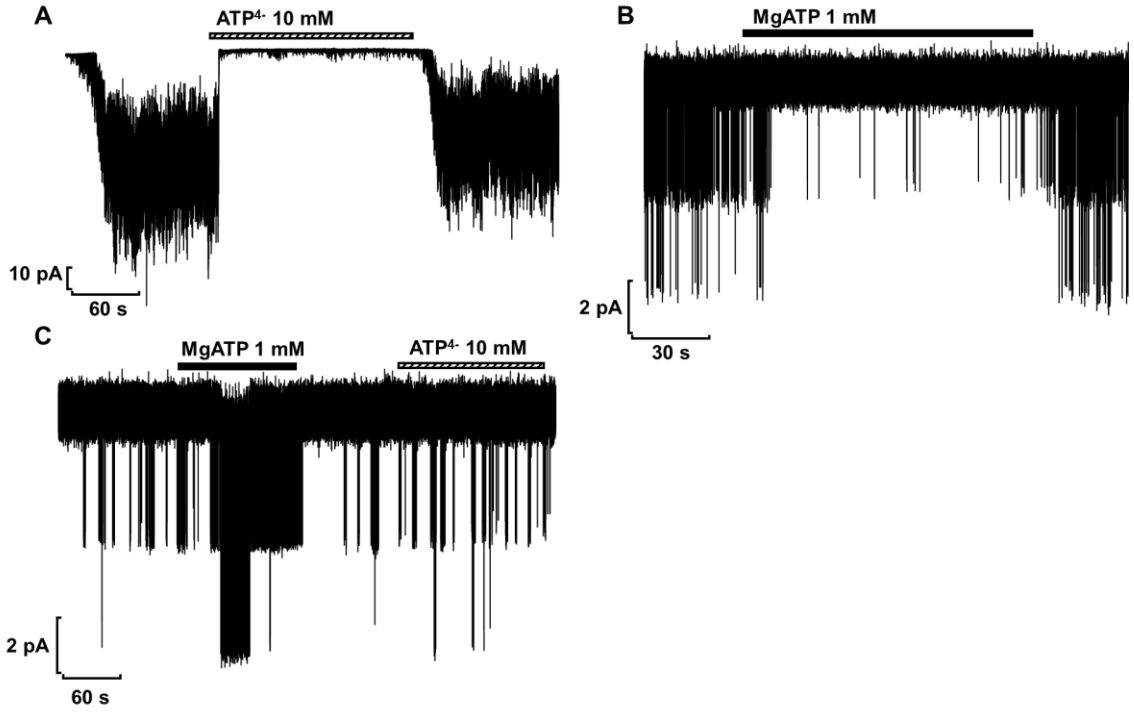


**Fig. 3.1.1: Effect of nucleotides (MgATP and $ATP^{4-}$) on $K_{ATP}$ channel activity.** A) Representative recording showing inhibition of WT SUR1/WT Kir6.2 channels by 10 mM $ATP^{4-}$. B) Representative recording showing inhibition of WT SUR1/WT Kir6.2 channels by 1 mM MgATP. C) Representative recording showing suppression of WT SUR1/Kir6.2$_{G334D}$ channel activity by 1 mM MgATP and 10 mM $ATP^{4-}$ (Sikimic *et al.* 2019).

### 3.1.2 Effect of ATP on $SUR1_{Q1179R}$/$Kir6.2_{G334D}$ and $SUR1_{E1507Q}$/$Kir6.2_{G334D}$ $K_{ATP}$ channels

In order to further investigate whether ATP binding is enough to stimulate channel openings, we used $K_{ATP}$ channels in which the SUR1 had higher affinity for ATP ($\pm Mg^{2+}$) and ATP antagonism at the Kir6.2 pore was abolished. Based on the allosteric effects of ATP on [$^3$H]GBM binding it has been reported that several SUR1 receptors carrying ND mutations (i.e. $SUR1_{Q1179R}$ (Christesen *et al.* 2005; Babenko 2008) and $SUR1_{E1507Q}$ (Can Thi Bich *et al.* 2014)) have increased affinities for nucleotides (Ortiz and Bryan 2015; Ortiz *et al.* 2013; Ortiz *et al.* 2012). Accordingly, SUR1 subunits with these mutations were co-expressed with a mutated Kir6.2 pore, $Kir6.2_{G334D}$. Fig. 3.1.2A shows that $ATP^{4-}$ rapidly enhanced the spontaneous activity of the $SUR1_{Q1179R}$/$Kir6.2_{G334D}$ channels. The $NP_o$ was significantly increased from 0.22 ± 0.14 to 0.99 ± 0.53 with $ATP^{4-}$ at a concentration of 10 mM (Fig. 3.1.2B) (Sikimic *et al.* 2019).

Next, the response of $SUR1_{E1507Q}$/$Kir6.2_{G334D}$ channels to $ATP^{4-}$ was tested. In other ABC proteins, the substitution of a glutamine (Q) for the catalytic glutamate (E) in position 1507 of NBD2 strongly reduces ATPase and transport activities (Orelle *et al.* 2003; Tombline *et al.* 2004). In structural studies, this substitution has been used to eliminate ATP hydrolysis and to trap ATP-bound conformations (Oldham *et al.* 2007; Smith *et al.* 2002; Moody *et al.* 2002). The addition of $ATP^{4-}$ without $Mg^{2+}$ as a cofactor further stimulated $SUR1_{E1507Q}$/$Kir6.2_{G334D}$ channel openings (Fig. 3.1.2C) (Sikimic *et al.* 2019). The open probability of $SUR1_{E1507Q}$/$Kir6.2_{G334D}$ channels increased significantly from 0.08 ± 0.03 under control conditions (nucleotide- and $Mg^{2+}$-free solution (Bpip2a)) to 0.99 ± 0.53 after application of 10 mM $ATP^{4-}$ (Fig. 3.1.2D). Thus, the findings presented here indicate that ATP acts as a $K_{ATP}$ channel agonist when the inhibitory effect of ATP at the Kir6.2 pore is absent, and hydrolysis is disabled.

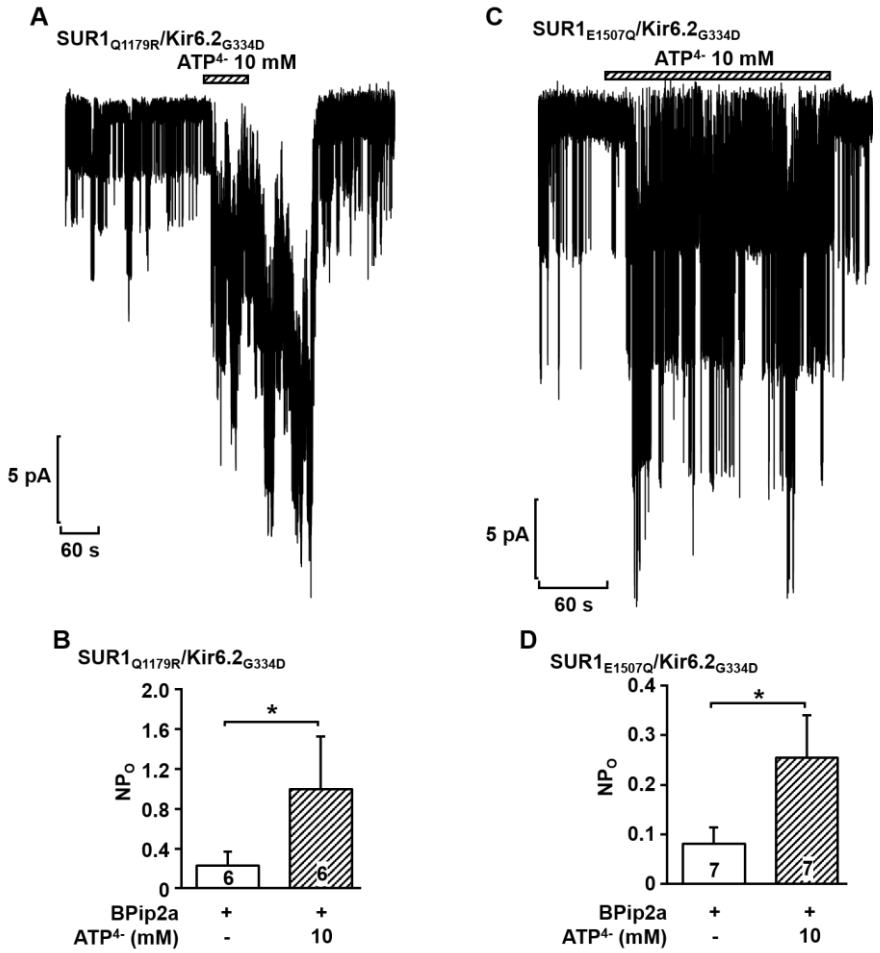


**Fig. 3.1.2: ATP in the absence of $Mg^{2+}$, needed for hydrolysis, activates $K_{ATP}$ channels with ND mutations in SUR1 that have higher affinity for ATP.** A) Representative recording showing activation of $SUR1_{Q1179R}/Kir6.2_{G334D}$ channels by 10 mM $ATP^{4-}$. B) Summary of results from 6 experiments on $SUR1_{Q1179R}/Kir6.2_{G334D}$ channels. C) Representative recording showing activation of $SUR1_{E1507Q}/Kir6.2_{G334D}$ channels by 10 mM $ATP^{4-}$. D) Summary of results from 7 experiments on $SUR1_{E1507Q}/Kir6.2_{G334D}$ channels. Bpip2a is the nucleotide- and $Mg^{2+}$-free control solution. Significance was determined using the non-parametric Wilcoxon signed rank test. $p < 0.05$. (Sikimic *et al.* 2019).

### 3.1.3 $ATP^{4-}$ stabilizes the action of $K_{ATP}$ channel agonists

In order to test whether ATP binding is enough to support the action of channel openers, the ability of diazoxide to activate channels expressing mutant $SUR1_{E1507K}$ and WT Kir6.2 was tested. The mutation E1507K (NM_001287174.2 (ABCC8):c.4519G>A (p.Glu1507Lys)) in exon 37 of SUR1, where lysine (K) has been substituted for the catalytic glutamate (E) at position 1507 in NBD2, is associated with CHI (Huopio *et al.* 2000). Noteworthy, amino acid numbering E1507 refers to the L78208 human *ABCC8* isoform that contains an additional residue in NBD1 and it is equivalent to E1506 described in the study of Huopio *et al.* (Huopio *et*

*al.* 2000; Männikkö *et al.* 2011). This mutation was used because: 1) CHI patients who carry this mutation respond well to diazoxide therapy (Huopio *et al.* 2000; Pinney *et al.* 2008) and 2) without the catalytic glutamate, $SUR1_{E1507K}$/WT Kir6.2 channels are expected to have impaired ATP hydrolysis. When patches from cells expressing $SUR1_{E1507K}$/WT Kir6.2 channels were pulled into a nucleotide-free solution, channels were spontaneously activated (Fig. 3.1.3A) (Sikimic *et al.* 2019). Application of 1 mM MgATP rapidly closed the channels, and co-application of 340 µM diazoxide reopened them. This verifies that MgATP inhibits channel activity and supports the action of diazoxide, as it is expected from patient response. Fig. 3.1.3B demonstrates that application of $ATP^{4-}$, without $Mg^{2+}$ needed for hydrolysis, inhibits channel activity by interaction with the WT Kir6.2 pore (Sikimic *et al.* 2019). Further administration of diazoxide reopened the channels showing that this $K_{ATP}$ channel opener does not need hydrolysis for its agonistic action.

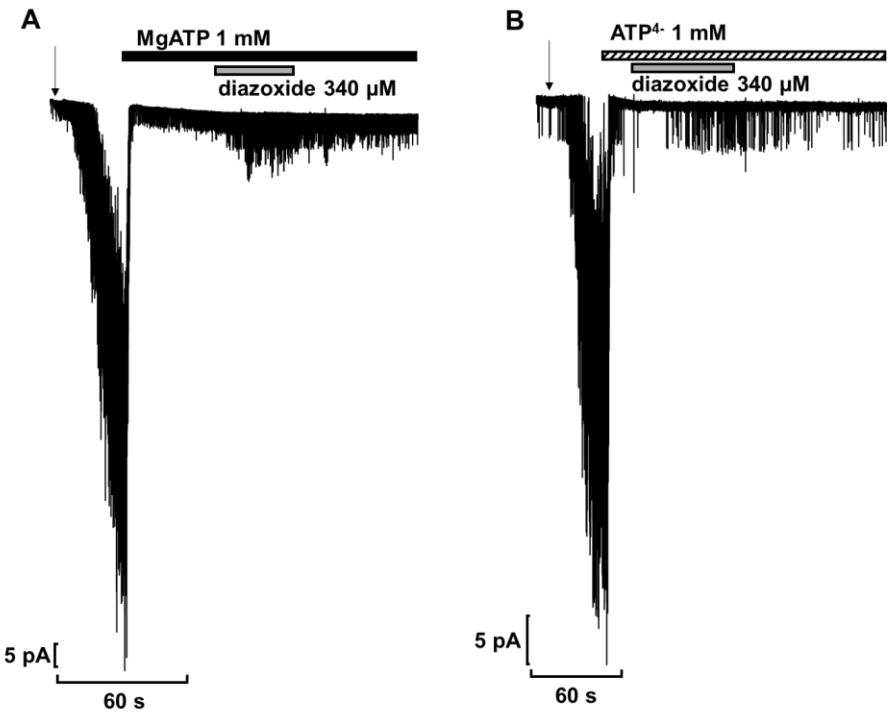


**Fig. 3.1.3: Activation of $K_{ATP}$ channels associated with CHI ($SUR1_{E1507K}$/WT Kir6.2) by the $K_{ATP}$ channel opener diazoxide.** At the arrows, patches were pulled into nucleotide-free media which activates a large number of channels as inhibitory nucleotides leave the pore. A) Representative recording showing the inhibitory effect of 1 mM MgATP. Concomitant application of diazoxide (340 µM) increases channel activity. B) Representative recording showing the inhibitory effect of 1 mM $ATP^{4-}$. Concomitant application of diazoxide (340 µM) rapidly increases the channel activity. Activation was observed in five-out-of-five experiments for both MgATP and $ATP^{4-}$ (Sikimic *et al.* 2019).

The response to diazoxide is consistent with the positive allosteric coupling between channel opener and nucleotide-binding sites seen with SUR1 alone, where diazoxide stabilizes nucleotide-bound conformations (Ortiz *et al.* 2013).

## 3.2 Discussion

Pancreatic $K_{ATP}$ channels couple cellular metabolism to electrical activity in β-cells and thus play an essential role in insulin secretion. It is generally accepted that nucleotide binding to the Kir6.2 pore inhibits $K_{ATP}$ channels, whereas nucleotide interactions with SUR1 activate channel openings (Nichols *et al.* 1996; Shyng *et al.* 1997; Proks *et al.* 2010; Tinker *et al.* 2018). The underlying processes for nucleotide activation still need to be clarified. As noted, early electrophysiological studies on $K_{ATP}$ channels clearly demonstrated that MgADP counteracts the inhibitory effect at the pore (Dunne and Petersen 1986; Kakei *et al.* 1986). Furthermore, the NBD2 of SUR1 has been proposed to be important for the positive regulation of channels (Nichols *et al.* 1996; Shyng *et al.* 1997; Ueda *et al.* 1997; Gribble *et al.* 1998). Due to structural similarities with other ABC protein family members and according to the electrophysiological studies, the current consensus argues that MgATP after binding to SUR1 must be hydrolyzed to MgADP to stimulate channel openings (Zingman *et al.* 2001; Proks *et al.* 2010). However, our results together with findings from other groups (Ortiz *et al.* 2013; Ortiz *et al.* 2012; Puljung *et al.* 2019; Choi *et al.* 2008) indicate that strong coupling between channel gating and ATP hydrolysis is not required.

### 3.2.1 Binding of ATP is sufficient for the activation of pancreatic $K_{ATP}$ channels

Direct ATP hydrolysis by SUR1 and full-channel complex (SUR1/Kir6.2) has been described in a few studies, which have found that assembly with Kir6.2 increases the rate of ATP hydrolysis of SUR1 ($V_{max}$ for SUR1 was 9 ± 1.7 nmol Pi/min/mg *vs.* 110 ± 7.6 nmol Pi/min/mg for the full channel complex) (de Wet *et al.* 2007; Mikhailov *et al.* 2005). Mikhailov *et al.* argued that this finding could be due to the assumption that the SUR1/Kir6.2 complex uses this energy from ATP hydrolysis for dimerization of NBD1 and NBD2 to bind nucleotides, as it is suggested by the enzymatic cycle of other ABC proteins (Linton and Higgins 2007; Mikhailov *et al.* 2005). However, this rate of ATP hydrolysis is still lower than the average rate reported for some other members of the ABCC subfamily, such as CFTR (~125 nmol Pi/min/mg), MRP1

(~235 nmol Pi/min/mg) or P-glycoprotein (~ 825 nmol Pi/min/mg) (Chang *et al.* 1997; Sharom *et al.* 1995; Shapiro and Ling 1994; Mao *et al.* 1999). The hypothesis that MgADP-bound posthydrolytic conformation of SUR, generated as a part of the enzymatic cycle, could activate channel opening was most concisely described by Zingman *et al*. and Bienengraber *et al.* (Zingman *et al.* 2001; Bienengraeber *et al.* 2000). Numerous studies have shown that the NBD2 of SUR1 and SUR2 subunits exhibits $Mg^{2+}$-dependent ATPase activity (Zingman *et al.* 2001; de Wet *et al.* 2007; Mikhailov *et al.* 2005; Bienengraeber *et al.* 2000; Masia *et al.* 2005). To prove the theory that SURs also follow the enzymatic cycle of ABC proteins, Zingman *et al.* used beryllium fluoride (BeF) and orthovanadate in electrophysiological studies to simulate pre- and post-hydrolytic states, respectively, in the NBDs of SUR2A (Zingman *et al.* 2001). Their results were interpreted to mean that the prehydrolytic state trapped by BeF does not promote SUR2A/Kir6.2 channel openings and mimics MgATP-bound conformation. In view of these results, they suggested that binding of MgATP to NBD2 is unable to open the channels. Conversely, the state trapped by orthovanadate imitates an MgADP-bound posthydrolytic state. This state was able to antagonize ATP-induced inhibition and favored the activation of SUR2A/Kir6.2 channels (Zingman *et al.* 2001). One of the drawbacks of the theory of Zingman *et al.* is the fact that their measurements were performed on NBDs from SUR2A subunits. However, it has been shown that SUR1 and SUR2A differ both structurally and in the binding affinities for MgATP (Vedovato *et al.* 2018; de Wet *et al.* 2010). According to de Wet *et al.*, SUR2A has approximately four times higher Km for MgATP than SUR1 (400 µM and 100 µM, respectively), which implies that SUR1 binds MgATP more tightly (de Wet *et al.* 2010; de Wet *et al.* 2007). To estimate the potency of different $Mg^{2+}$-nucleotides on activation of $K_{ATP}$ channels via SUR1, Proks *et al.* used $K_{ATP}$ channels (SUR1/Kir6.2$_{G334D}$) with a mutation on Kir6.2 that abolishes the inhibitory effect of nucleotides at the pore (Masia *et al.* 2007; Proks *et al.* 2010). They demonstrated that both MgATP and MgADP were able to activate the channels (Proks *et al.* 2010). Nevertheless, MgADP exhibited more than tenfold higher potency than MgATP. Noteworthy, the poorly hydrolyzable ATP analogue MgATPγS activated SUR1/Kir6.2$_{G334D}$ channels to the same extent as MgATP. The effect of the non-hydrolyzable analogue MgAMP-PNP was insignificant, which according to Proks *et al.* further supports the idea that MgATP has to be first hydrolyzed to MgADP to activate channels (Proks *et al.* 2010). Conversely, binding studies on SUR1

monomers have found that the non-hydrolyzable analogue MgAMP-PNP failed to switch the conformation of SUR1 due to its binding to asymmetric NBD1 (Ortiz *et al.* 2013). In this manner, dimerization of NBDs is blocked and the conformational switch of TMDs is disabled (Ortiz *et al.* 2013). Accordingly, the MgAMP-PNP binding to the asymmetric NBD1 stabilizes SUR1 in an inward-facing conformation which results in reduced channel activity (Ortiz *et al.* 2013). The hypothesis that NBD1 is asymmetric is in accordance with recent structural studies (Martin *et al.* 2017a; Lee *et al.* 2017) and, together with data from Ortiz *et al.,* strongly opposes the argument of Proks *et al.* (Proks *et al.* 2010).

By using $K_{ATP}$ channels that lack the inhibitory effect at the pore (SUR1/Kir6.2$_{G334D}$), we were able to test the direct effect of MgATP on channels through SUR1 as well. We determined that MgATP is effective in stimulating the channel openings, where it increased the open probability by approximately seven to eightfold (Fig.3.1.1C) (Sikimic *et al.* 2019). However, in the absence of $Mg^{2+}$, the activation of these channels by ATP ($ATP^{4-}$) was very modest (Fig. 3.1.1C) (Sikimic *et al.* 2019). Similarly, the recent publication of Puljung *et al.* (Puljung *et al.* 2019), which used FRET with fluorescent nucleotides, reported that ATP and ADP do not need $Mg^{2+}$ to bind to NBD2. However, they argued that NBDs can dimerize only in the presence of $Mg^{2+}$ and induce the conformational change of SUR1, as it is required for channel activation (Puljung *et al.* 2019). These results are also consistent with the data of Ortiz *et al.* who found, using a radioligand binding assay, that WT SUR1 monomers have a higher affinity for ATP in the presence of $Mg^{2+}$ (Ortiz *et al.* 2013; Ortiz *et al.* 2012). According to Ortiz *et al.*, the $IC_{50}$ value was increased approximately 100-fold when $Mg^{2+}$ was excluded from experiments (Ortiz *et al.* 2013; Ortiz *et al.* 2012). However, Ortiz *et al.* also demonstrated that multiple mutations in SUR1 that cause ND (including Q1179R and E1507Q) have an ability to bind $ATP^{4-}$ (or MgATP) at least tenfold more tightly than WT SUR1 (Ortiz *et al.* 2013; Ortiz *et al.* 2012). Thus, we used those two ND-related mutations in SUR1 to further elucidate the channel opening mechanism. In this case, $ATP^{4-}$ (in the absence of $Mg^{2+}$, which is needed for hydrolysis) showed clear agonist action on the SUR1$_{Q1179R}$/Kir6.2$_{G334D}$ channels (Fig. 3.1.2A, B) (Sikimic *et al.* 2019). Furthermore, the channels where hydrolysis was disabled due to the mutation in the SUR1 subunit (SUR1$_{E1507Q}$/Kir6.2$_{G334D}$) were significantly activated by $ATP^{4-}$ (Fig. 3.1.2C, D) (Sikimic *et al.* 2019). The results

presented in Fig. 3.1.1C and Fig. 3.1.2 together with previous reports from Ortiz et al. (Ortiz *et al.* 2013; Ortiz *et al.* 2012) indicate that nucleotide-induced conformational switch depends on the affinity of SUR1 to $ATP^{4-}$ or MgATP. The data further supported the hypothesis that ATP binding, a key part of any ABC protein enzymatic cycle, is enough to induce the conformational change of SUR1 that is responsible for the positive regulation of $K_{ATP}$ channels. Even though the NBD2 of SUR1 possesses ATPase activity, it is not necessary for the activation of the channels. Accordingly, we assume that NBD1, with a higher affinity for ATP than NBD2, is occupied and that ATP binding at NBD2 leads to dimerization and repositioning of TMD1 and TMD2 from an inward- to outward-facing conformation (Sikimic *et al.* 2019). According to recent structural studies, this outward-facing conformation transmits a signal to the Kir6.2 pore through TMD0 and L0 and in that manner it promotes channel openings (Wu *et al.* 2018).

Choi and colleagues (Choi *et al.* 2008) documented additional support for our hypothesis. They used $K_{ATP}$ single channel recordings to track conformational changes of $K_{ATP}$ channels. As a result, their findings clearly demonstrated that the gating of SUR1/Kir6.2 channel obeys microscopic reversibility (Choi *et al.* 2008). If the gating of $K_{ATP}$ channels is tightly coupled and dependent on an irreversible process such as ATP hydrolysis, then the thermodynamic equilibrium does not occur, and microscopic reversibility is violated (Rothberg and Magleby 2001). The principle of equilibrium suggests that single channel recordings of ion channels are time reversible. As such, their characteristics are the same in both directions, forward or reverse (Rothberg and Magleby 2001; Csanády 2017; Choi *et al.* 2008). Additionally, an exponential decline of the dwell-time distributions of the recordings, with the maximum at $t = 0$, indicated gating at thermodynamic equilibrium (Colquhoun and Hawkes 1995). A peaked distribution illustrates a nonequilibrium process, such as that seen in CFTR gating, where dwell-time distributions had maxima at times greater than zero (Kijima and Kijima 1987; Csanády *et al.* 2010). As demonstrated in the study of Choi et al., dwell-time distributions of open (burst) and closed (interburst) states of the $K_{ATP}$ channels exhibited a clear exponential decline, demonstrating an ability for microscopic reversibility (Choi *et al.* 2008). Thus, channel gating and positive regulation are not strictly coupled to ATP hydrolysis at SUR1.

### 3.2.2 Hydrolysis is not necessary for the action of a $K_{ATP}$ channel agonist

The SUR1 mutation E1507K, which is associated with CHI, causes a reduction but not complete loss of $K_{ATP}$ channels in pancreatic β-cells (Huopio *et al.* 2000; Huopio *et al.* 2003). The conserved catalytic glutamate residue (E1507) follows Walker B motif within NBD2 of SUR1 (Männikkö *et al.* 2011). It has been reported that equivalent residue in other ABC proteins is involved in the binding of $Mg^{2+}$ from Mg-nucleotides and in ATP hydrolysis (Payen *et al.* 2003). This may explain the resistance of channels with the mutation E1507K to metabolic activation by MgADP (Männikkö *et al.* 2011). Furthermore, de Wet *et al.* showed that ATPase activity is reduced when the catalytic residues of NBD2 of SUR1 are mutated (de Wet *et al.* 2007; Vedovato *et al.* 2015). Under the assumption that the $K_{ATP}$ channel agonist, diazoxide, activates $K_{ATP}$ channels only in the presence of Mg-nucleotides (Kozlowski *et al.* 1989; Larsson *et al.* 1993; Shyng *et al.* 1997), addition of diazoxide to the channels that have mutations in NBD1 or NBD2 (suppressing the stimulatory effect of MgADP) should result in a lack of activation. However, CHI patients who carry the mutation E1507K respond well to diazoxide treatment (Pinney *et al.* 2008; Huopio *et al.* 2000). When ATP hydrolysisis is impaired by the mutation E1507K, it can be assumed that channels with this mutation should be resistant to activation by MgATP, but not by MgADP (Vedovato *et al.* 2015). By contrast, the literature shows that activation by both nucleotides is impaired (Gribble *et al.* 1997; Shyng *et al.* 1997; Proks *et al.* 2014; Vedovato *et al.* 2015). Furthermore, in a prior study on ND and WT SUR1 monomers, Ortiz and Bryan revealed that diazoxide prefers to bind to the ATP-bound state of SUR1 and supports ATP-driven receptor switching (Ortiz and Bryan 2015). Our results on $SUR1_{E1507K}$/Kir6.2 channels with $ATP^{4-}$ further indicate that diazoxide does not need hydrolysis for its action (Fig. 3.1.3B) (Sikimic *et al.* 2019). Instead, diazoxide stabilizes ATP binding, thereby further supporting the outward-facing conformation of SUR1, which results in channel openings.

### 3.2.3 Clinical significance of $K_{ATP}$ channel regulation

Ortiz *et al.*, estimating the affinity of radiolabeled GBM, assumed that inward-facing conformation of SUR1 describes a non-stimulatory state, where affinity for GBM is the highest, while the outward-facing conformation is a stimulatory state, with the lowest affinity for GBM and the highest for channel agonists (Ortiz *et al.* 2013; Ortiz

*et al.* 2012). This assumption has been confirmed by recent cryo-EM studies (Martin *et al.* 2017a; Wu *et al.* 2018; Lee *et al.* 2017; Martin *et al.* 2017b) that suggested that the pharmacological approach can be used to assess the structure and relate it to activity. As binding studies have already revealed and we have presented here, SUR1 has a weaker affinity to $ATP^{4-}$ than to MgATP and therefore $ATP^{4-}$ is less potent in activating channels (Fig. 3.1.1C) (Sikimic *et al.* 2019). This dependence has clinical significance. It has been shown that mutations of SUR1 with higher affinity for ATP ($\pm Mg^{2+}$) cause ND (Ortiz and Bryan 2015; Ortiz *et al.* 2013; Ortiz *et al.* 2012). According to the findings that ATP-induced conformational changes in SUR1 have negative allosteric effects on GBM binding (Martin *et al.* 2017b; Lee *et al.* 2017), it is thought that treatment with sulfonylureas would reduce ATP agonistic action on ND-related mutant channels and reduce the channel openings. Conversely, it was found that mutations in SUR1 with attenuated affinity cause CHI (Ortiz and Bryan 2015). $K_{ATP}$ channels that comprise these mutated SUR1 subunits spend more time in the non-stimulatory conformation, which is a state that can possibly be amended through treatment with $K_{ATP}$ channel openers (e.g. diazoxide) that stabilizes ATP binding.

# 4 Toward finding new approaches for treating CHI

Mutations of genes that encode $K_{ATP}$ channel subunits can lead to various metabolic diseases (Quan *et al.* 2011). Thus, $K_{ATP}$ channels are important drug targets. Clinically relevant $K_{ATP}$ channel openers are used for treatment of CHI. In spite of its numerous side effects, some of which are life-threatening, diazoxide is still the first-line drug in a medical management of CHI. Current treatment options are limited due to either lack of efficacy or strong adverse effects. Therefore, it is of great importance to find new approaches which could silence β-cells and be therapeutically beneficial.

In this work several drugs were investigated that silence β-cells either by interacting with the $K_{ATP}$ channel or with $K_{ATP}$ channel-independent targets. To test their effects, measurements of $[Ca^{2+}]_c$ were conducted on β-cells isolated from C57BL/6N and $SUR1^{-/-}$ mice and on human β-cells. The β-cells were obtained by dispersing islets of Langerhans by trypsin treatment. Human islets of Langerhans were obtained from different biopsies of children undergoing pancreatic surgery according to the approvals of the Ethics Commission of the Universitätsmedizin Greifswald (BB 050/13). The islets were taken from biopsies of six CHI patients. Genetic studies showed that five patients had mutations in the *ABCC8* gene that encodes the SUR1 subunit of $K_{ATP}$ channels. Three of them had a mutation typical among the Ashkenazi Jewish population (c.3989_9G>A) (Glaser *et al.* 2011; Nestorowicz *et al.* 1996). In one biopsy no mutation was identified. According to a postsurgical evaluation of the biopsies by the Department of Pathology at the University Hospital Greifswald, the tissue was identified as healthy or pathological. Islets from areas affected (termed pathological) and not affected (termed healthy) by the disease were isolated. Furthermore, measurements of $\Delta\Psi$, patch-clamp measurements and measurement of insulin secretion were conducted on β-cells obtained from C57BL/6N and $SUR1^{-/-}$ mice.

## 4.1 Results

### 4.1.1 $K_{ATP}$ channel openers in the treatment of CHI

#### 4.1.1.1 Effects of diazoxide on $[Ca^{2+}]_c$ of human β-cells

The $K_{ATP}$ channels (SUR1/Kir6.2) in pancreatic β-cells play a crucial role in coupling cellular metabolism with electrical activity. In electrically inactive or hyperpolarized β-cells, $K_{ATP}$ channels are open. Accordingly, this is one of the strategies used in the treatment of CHI. Diazoxide is an already established opener of $K_{ATP}$ channels and its effect on $[Ca^{2+}]_c$ was tested as a control. In β-cells, isolated from the healthy pancreatic tissue of CHI patients, 250 µM diazoxide significantly abolished mean $[Ca^{2+}]_c$, elicited in the presence of 10 mM glucose, from 0.70 ± 0.06 to 0.53 ± 0.01 (Fig. 4.1.1.1A, B).

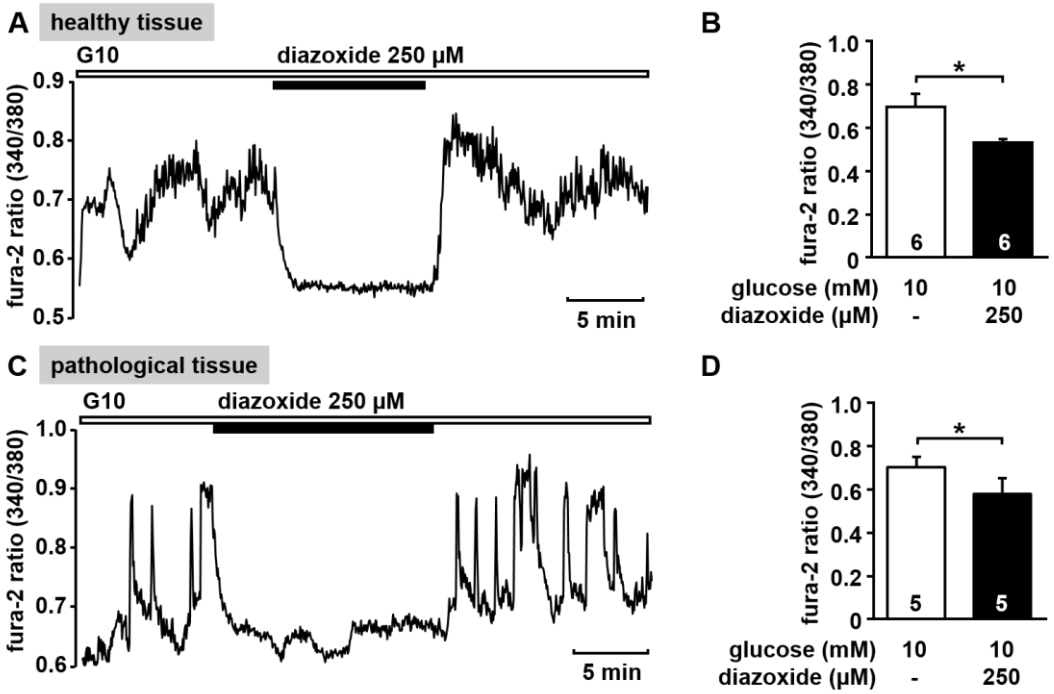


**Fig. 4.1.1.1: Diazoxide suppresses the oscillations of $[Ca^{2+}]_c$ in humane CHI β-cells in the presence of 10 mM glucose (G10).** A) Representative recording showing the effect of 250 µM diazoxide on the oscillations of $[Ca^{2+}]_c$ in a human β-cell isolated from healthy pancreatic tissue of CHI patients. B) Summary of all experiments. Diazoxide (250 µM) decreases the mean fluorescence ratio in healthy human CHI β-cells. n = 6 from 3 different pancreatectomies. C) Representative recording showing the effect of 250 µM diazoxide in a human β-cell obtained from pathological pancreatic tissue of CHI patients. D) Summary of all experiments. Diazoxide (250 µM) decreases the mean fluorescence ratio in human CHI β-cells. n = 5 from 2 different pancreatectomies of focal and mosaic CHI. n represents the number of recordings. * $p \leq 0.05$.

Similar results were obtained in experiments with diazoxide application on human β-cells isolated from the focal and mosaic lesions of two different biopsies (0.70 ± 0.05 under control conditions *vs.* 0.58 ± 0.07 after application of 250 µM diazoxide) (Fig. 4.1.1.1C, D).

### 4.1.1.2 Effects of NN414 on $[Ca^{2+}]_c$ of human β-cells

The diazoxide analog, NN414, has been reported to be a selective agonist of pancreatic β-cell $K_{ATP}$ channels (SUR1/Kir6.2), and to have a higher potency than diazoxide (>100-fold) (Dabrowski *et al.* 2003). Therefore, NN414 could be useful as a therapeutic agent in diseases in which excessive insulin release must be attenuated (Dabrowski *et al.* 2003; Carr *et al.* 2003). At a concentration of 5 µM, NN414 completely abolished oscillations of $[Ca^{2+}]_c$ induced by 10 mM glucose and reduced $[Ca^{2+}]_c$ to basal levels (Fig. 4.1.1.2A, B). The mean fluorescence ratio was reduced from 0.75 ± 0.03 under control conditions (in 10 mM glucose) to 0.64 ± 0.03 in the presence of NN414 in β-cells obtained from healthy human pancreatic tissue. The application of 5 µM NN414 to β-cells taken from three different biopsies of pathological pancreatic tissue (focal, diffuse and mosaic) lowered the mean ratio from 0.72 ± 0.03 in 10 mM glucose to 0.62 ± 0.03 in the presence of NN414 (Fig. 4.1.1.2C, D).

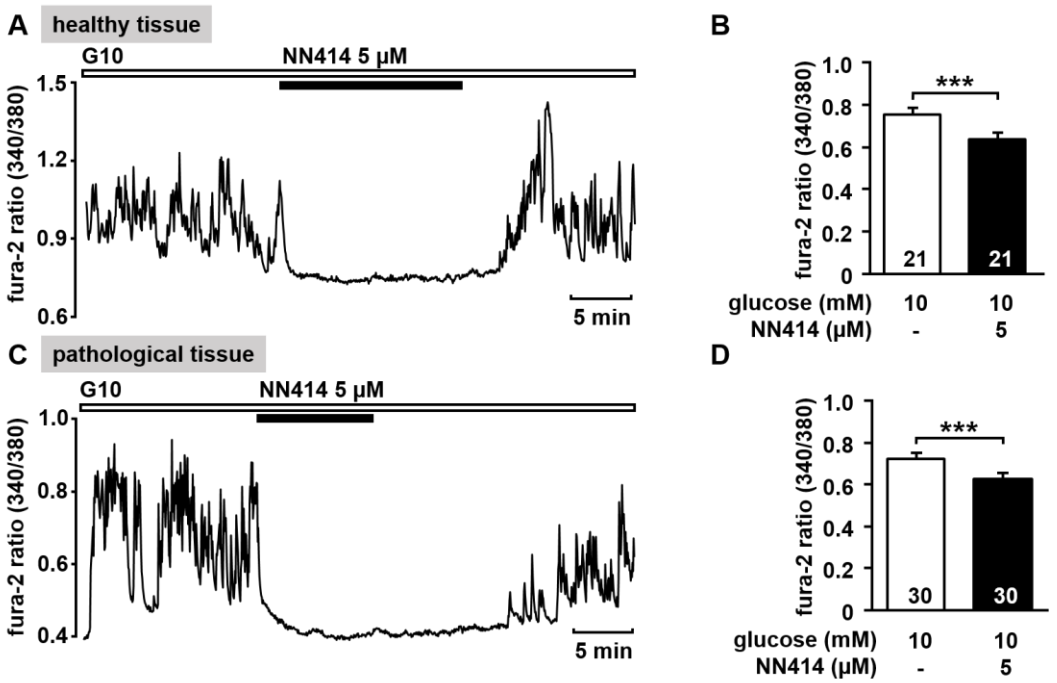


**Fig. 4.1.1.2: NN414 suppresses the oscillations of $[Ca^{2+}]_c$ in human CHI β-cells in the presence of 10 mM glucose (G10).** A) Representative recording showing the effect of 5 µM NN414 on the oscillations of $[Ca^{2+}]_c$ in a human β-cell isolated from healthy pancreatic tissue of CHI patients. B) Summary of all experiments. NN414 (5 µM) decreases the mean fluorescence ratio in healthy human CHI β-cells. n = 21, obtained from 3 different pancreatectomies. C) Representative recording showing the effect of 5 µM NN414 in a human β-cell obtained from pathological pancreatic tissues of CHI patients. D) Summary of all experiments. NN414 (5 µM) decreases the mean fluorescence ratio. n = 30, obtained from 3 different pancreatectomies of focal, diffuse and mosaic CHI. n represents the number of recordings. *** $p \leq 0.001$.

### 4.1.1.3 Effects of VU0071063 on $[Ca^{2+}]_c$ of human β-cells

In a recent study, Raphemot *et al.* discovered a novel xanthine derivative VU0071063 that directly and selectively activates $K_{ATP}$ channels (Raphemot *et al.* 2014). They found that VU0071063 is more potent and activates $K_{ATP}$ channels with a faster kinetic than diazoxide. These findings encouraged us to test the effect of VU0071063 on changes in $[Ca^{2+}]_c$ on human CHI islet cells. In β-cells isolated from the healthy part of pancreatic tissue, 30 µM VU0071063 immediately abolished glucose-induced $Ca^{2+}$ oscillations. The mean fluorescence ratio decreased from 0.57 ± 0.03 in 10 mM glucose to 0.48 ± 0.01 in the presence of VU0071063 (Fig. 4.1.1.3A, B). The administration of VU0071063 (30 µM) on β-cells from pathological CHI pancreatic tissue (focal and difuse lesion) resulted in a prompt reduction of $[Ca^{2+}]_c$ in all four measurements. Fig. 4.1.1.3C shows a typical recording. The mean

fluorescence ratio changed from 0.59 ± 0.04 under control conditions to 0.50 ± 0.02 in the presence of VU0071063 (Fig. 4.1.1.3D). On account of limited pathological material, which explains the low number of experiments, statistical significance between the groups was not reached.

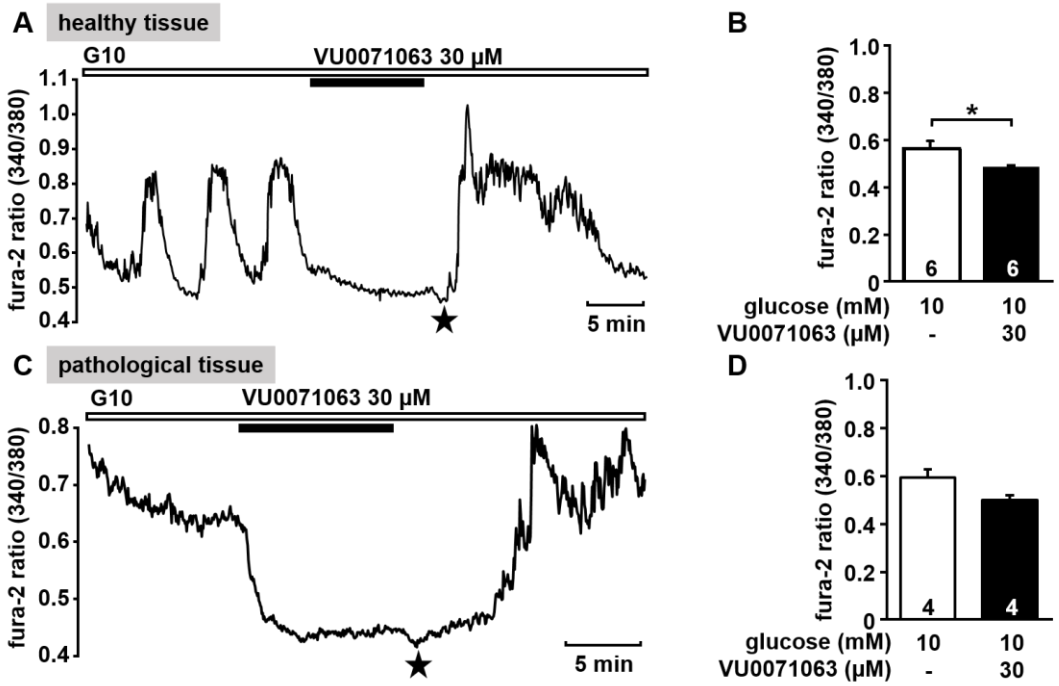


**Fig. 4.1.1.3: VU0071063 suppresses the oscillations of $[Ca^{2+}]_c$ in human CHI β-cells in the presence of 10 mM glucose (G10).** A) Effect of 30 µM VU0071063 on the oscillations of $[Ca^{2+}]_c$ in a human β-cell obtained from healthy pancreatic tissue of CHI patients. Note the drop in $[Ca^{2+}]_c$ after removal of VU0071063 (asterisk). B) Summary of all experiments. VU0071063 (30 µM) decreases the mean fluorescence ratio in healthy human CHI β-cells. n = 6, obtained from 2 different pancreatectomies. C) Representative recording showing the effect of 30 µM VU0071063 in a human β-cell obtained from pathological pancreatic tissue of CHI patients. Note the drop in $[Ca^{2+}]_c$ after removal of VU0071063 (asterisk). D) Summary of all experiments. VU0071063 (30 µM) decreases the mean fluorescence ratio, but due to low number of experiments, statistical significance is not reached. n = 4, obtained from 2 different pancreatectomies of focal and diffuse CHI. n represents the number of recordings. * $p \leq 0.05$.

### 4.1.1.4 VU0071063 silences β-cells in a $K_{ATP}$ channel-independent manner

In both healthy and pathological CHI β-cells, a drop of $[Ca^{2+}]_c$ was noticed directly after withdrawal of VU0071063 (Fig. 4.1.1.3A, B asterisks). This drop is assumed to occur due to ATP-dependent sequestration of $Ca^{2+}$ into the ER (Maczewsky *et al.* 2017). Thus, this observation implies that VU0071063 may affect additional targets

besides $K_{ATP}$ channels. To evaluate this assumption, the $[Ca^{2+}]_c$ of β-cells from SUR1$^{-/-}$ mice, lacking functional $K_{ATP}$ channels, was measured and compared to that of C57BL/6N mice. It was found that VU0071063 (30 µM) suppressed $Ca^{2+}$ oscillations and lowered $[Ca^{2+}]_c$ in β-cells of both genotypes (Fig. 4.1.1.4A, B). The mean fluorescence ratio decreased from 0.67 ± 0.01 to 0.56 ± 0.01 in C57BL/6N β-cells and from 0.69 ± 0.01 to 0.66 ± 0.01 in SUR1$^{-/-}$ β-cells (i.e. the effect was weaker in the cells obtained from knockout mice). The data obtained with SUR1$^{-/-}$ β-cells make evident that VU0071063 affects other SSC parameters beside $K_{ATP}$ channels in order to reduce $[Ca^{2+}]_c$.

As mentioned in section 1.2, a part of the $Ca^{2+}$-dependent hyperpolarizing current, $K_{slow}$, belongs to $K_{Ca}$ channels. Thus, it was investigated whether SK4 or BK channels are involved in the effect of VU0071063. To test their potential involvement, the β-cells from SK4$^{-/-}$ and BK$^{-/-}$ mice were used and the maximal fluorescence ratio was taken to compare the effect of VU0071063 under different experimental conditions. The effect on $K_{ATP}$ channels was excluded by pharmacological inhibition with 1 mM tolbutamide. Fig. 4.1.1.4C shows a representative recording from the series with SK4$^{-/-}$ mice. The addition of VU0071063 (30 µM) abolished the influx of $Ca^{2+}$. The maximal fluorescence ratio decreased from 0.99 ± 0.02 under control conditions (10 mM glucose in the presence of 1 mM tolbutamide) to 0.73 ± 0.02 in the presence of VU0071063 (Fig. 4.1.1.4D). VU0071063 had a similar effect on $[Ca^{2+}]_c$ of β-cells from BK$^{-/-}$ mice (Fig. 4.1.1.4E). It reduced the maximal fluorescence ratio from 1.08 ± 0.02 to 0.70 ± 0.02 (Fig. 4.1.1.4F).

Similar to human CHI β-cells (mentioned in section 4.1.1.3), $[Ca^{2+}]_c$ was further reduced after the removal of the compound in both C57BL/6N and SUR1$^{-/-}$ murine β-cells (0.050 ± 0.02 and 0.054 ± 0.02 respectively) (Fig. 4.1.1.4A, asterisks). This points to alterations in mitochondrial metabolism, which can cause changes in $K_{ATP}$ channel activity (Drews *et al.* 2015b) as the mitochondrial membrane potential is directly linked to ATP production. For this reason, to evaluate mitochondrial ATP production (Krippeit-Drews *et al.* 2000), the effects of VU0071063 on the mitochondrial membrane potential (ΔΨ) were measured. As shown in Fig. 4.1.1.4G, a rise in the glucose concentration, which causes an immediate increase in ATP production, is accompanied by a hyperpolarization of ΔΨ, which is indicated by a

decrease of the fluorescence signal. The fluorescence signal in C57BL/6N β-cells was increased from 1629 ± 58 a.u. in the presence of 10 mM glucose to 2473 ± 96 a.u. in the presence of 30 µM VU0071063 (Fig. 4.1.1.4H). Similarly, VU0071063 strongly and reversibly depolarized mitochondrial membrane potential in $SUR1^{-/-}$ β-cells from 1516 ± 55 a.u. to 2021 ± 87 a.u. (Fig. 4H). As a control at the end of each experiment carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was added to initiate the maximal depolarization of ΔΨ. This substance is a potent protonophore that reduces the proton gradient across the inner mitochondrial membrane.

Due to its detrimental effects on ATP production, the substance VU0071063 may not be suitable for use in humans. Therefore, it was excluded from the following experiments.

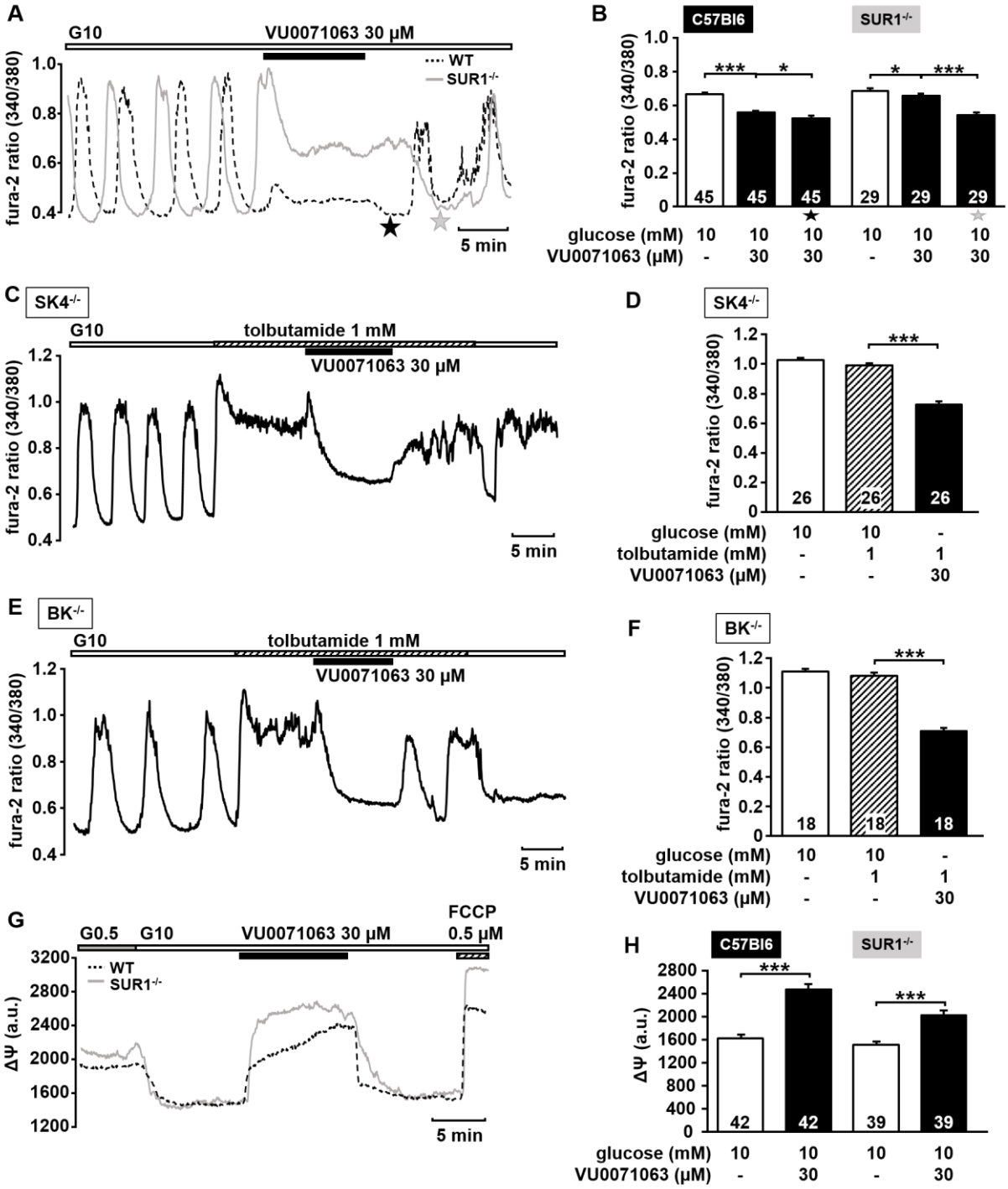


**Fig. 4.1.1.4: VU0071063 affects the ATP production in murine β-cells in the presence of 10 mM glucose (G10).** A) Comparing effects of 30 µM VU0071063 on $[Ca^{2+}]_c$ in β-cells obtained from C57BL/6N and SUR1$^{-/-}$ mice. Note the drop in $[Ca^{2+}]_c$ after removal of VU0071063 (black asterisk: C57BL/6N, grey asterisk: SUR1$^{-/-}$). B) Summary of all experiments. VU0071063 (30 µM) decreases the mean fluorescence ratio in both C57BL/6N and SUR1$^{-/-}$ β-cells. n = 45 for C57BL/6N and n = 29 for SUR1$^{-/-}$. C) Representative recording showing the reduction of $[Ca^{2+}]_c$ by 30 µM VU0071063 in a β-cell from SK4$^{-/-}$ mice when the $K_{ATP}$ channels are inhibited by 1mM tolbutamide. D) Summary of all experiments. VU0071063 (30 µM) decreases the maximal fluorescence ratio in murine SK4$^{-/-}$ β-cells in the presence of 10 mM glucose and 1 mM tolbutamide. n = 26.

E) Representative recording showing the reduction of $[Ca^{2+}]_c$ by 30 µM VU0071063 in a β-cell from $BK^{-/-}$ mice when the $K_{ATP}$ channels are inhibited by 1mM tolbutamide. F) Summary of all experiments. VU0071063 (30 µM) decreases the maximal fluorescence ratio in murine $BK^{-/-}$ β-cells in the presence of 10 mM glucose and 1 mM tolbutamide. n = 18. G) Comparing effects of 30 µM VU0071063 on ΔΨ in β-cells obtained from C57BL/6N and $SUR1^{-/-}$ mice. FCCP leads to maximal depolarization of ΔΨ. H) Summary of all experiments. VU0071063 (30 µM) depolarizes ΔΨ in both C57BL/6N and $SUR1^{-/-}$ β-cells. n = 42 for C57BL/6N and n = 39 for $SUR1^{-/-}$. n represents the number of recordings obtained from 3 different preparations. * $p \leq 0.05$, *** $p \leq 0.001$.

#### 4.1.1.5 NN414 stimulates $SUR1_{E1507K}$/WT Kir6.2 channels activity at a much lower concentration than diazoxide

As mentioned in section 3.1.3, the $SUR1_{E1507K}$ mutation is associated with a loss of $K_{ATP}$ channel activity and is a cause of dominantly inherited CHI (Huopio *et al.* 2000). It has been reported that patients, who carry dominant CHI, have a mild form of the disease and respond well to the treatment with diazoxide (Huopio *et al.* 2000). However, diazoxide is known to cause numerous side effects and it would be beneficial to have drugs that are more specific for pancreatic $K_{ATP}$ channels and have a higher potency. This study tested and compared the effects of 5 µM NN414 and 340 µM diazoxide on a $SUR1_{E1507K}$/WT Kir6.2 channel. In the experiment, $SUR1_{E1507K}$ and WT Kir6.2 were co-expressed in HEK-293 cells, and channel activity was measured as mean current in the inside-out configuration of the patch clamp technique in the presence of 1 mM MgATP. The concentration of 340 µM diazoxide was used to fully activate the channels (Huopio *et al.* 2000). After pulling a membrane patch, in the control bath solution without adenine nucleotides (Bpip2b), the channels open spontaneously due to the absence of nucleotides at the pore. The activity of $SUR1_{E1507K}$/WT Kir6.2 channels was rapidly and significantly reduced by application of MgATP due to its interaction with the Kir6.2 pore (Fig. 4.1.1.5A). The mean current of $SUR1_{E1507K}$/WT Kir6.2 channels under control conditions was 494.95 ± 157.88 pA and fell to 10.14 ± 2.78 pA after the addition of 1 mM MgATP (Fig. 4.1.1.5B). The concomitant application of 340 µM diazoxide augmented the channel activity (the mean current amounted to 59.25 ± 17.37 pA in the presence of diazoxide). The stimulatory effect of diazoxide was not significant in the analysis of variance (ANOVA) posttest, but significance was reached when it was evaluated with a nonparametric statistical test. By contrast, NN414 (5 µM) at a more than 60-fold lower concentration than diazoxide produced a significant activation of $K_{ATP}$ channels

in the presence of 1 mM MgATP (32.92 ± 9.88 pA in the presence of MgATP *vs.* 153.67 ± 58.08 pA after addition of 5 µM NN414) (Fig. 4.1.1.5A, B).

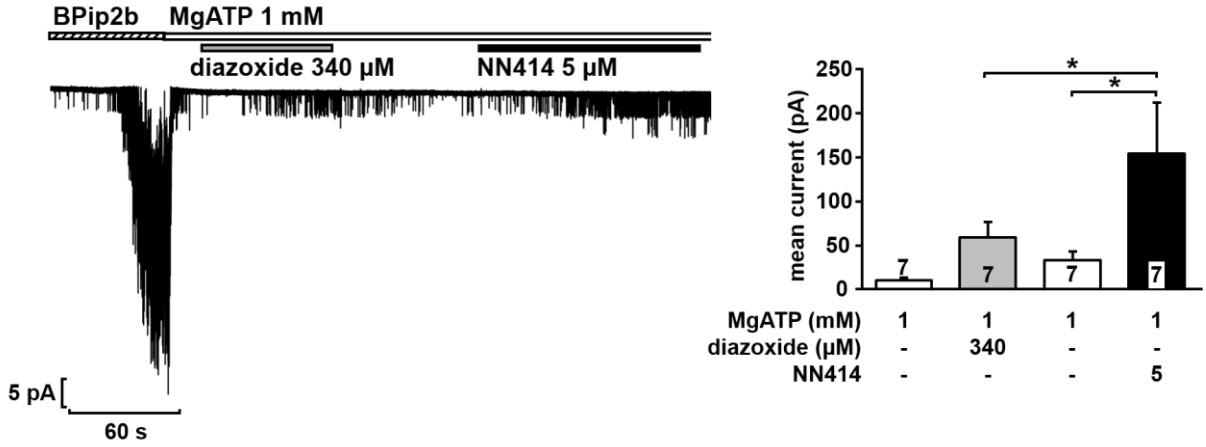


**Fig. 4.1.1.5: NN414 opens SUR1$_{E1507K}$/WT Kir6.2 channels in lower concentration than diazoxide.** A) Representative recording comparing the effect of 340 µM diazoxide and 5 µM NN414 to counteract the inhibitory effect of 1 mM MgATP and reopen the $K_{ATP}$ channels associated with CHI (SUR1$_{E1507K}$/WT Kir6.2). B) Summary of all experiments. NN414 (5 µM) increases the mean current to a greater extent than it does diazoxide (340 µM) in HEK-293 cells transfected with mutated SUR1 (SUR1$_{E1507K}$) and WT Kir6.2. n = 7. n represents the number of different experiments. * p ≤ 0.05.

### 4.1.1.6 Comparison of the effects of diazoxide and NN414 on insulin secretion in murine β-cells

As noted in section 1.1, the fine-tuned regulation of SSC in the pancreatic β-cells controls the release of insulin from the storage vesicles. The experiments in this work have indicated an inhibitory effect of diazoxide and NN414 on the parameters of SSC. Accordingly, they appear to cause an interruption in the oscillatory pattern of $[Ca^{2+}]_c$ and decreased $Ca^{2+}$ influx. Notably, $Ca^{2+}$ influx is the trigger for insulin secretion. One of the advantages of NN414 is that it activates pancreatic β-cell $K_{ATP}$ channels and decreases $[Ca^{2+}]_c$ at a much lower concentration than diazoxide ((Dabrowski *et al.* 2003) and own observations). To further assess its potency in comparison to diazoxide, different concentrations of both drugs were tested on insulin release in pancreatic β-cells from C57BL/6N mice. Insulin secretion was measured after one hour of incubation of islets of Langerhans with diazoxide at low (50 µM) and high (250 µM) concentrations, or with NN414 at concentrations ranging from 0.1 to 5 µM, in the presence of 10 mM glucose (Fig. 4.1.1.6). Control of the metabolic integrity of the islets of Langerhans, used for insulin secretion experiments,

was based on the increase of insulin secretion from substimulatory (3 mM) to stimulatory (10 mM) concentrations of glucose. Fig. 4.1.1.6 illustrates that the islets of Langerhans reacted adequately to glucose, insofar as a significant increase from 3 mM to 10 mM glucose was observed in each case (white bars). As seen in Fig. 4.1.1.6, diazoxide concentrations of 50 µM and 250 µM reduced glucose-induced insulin secretion by 75% and 95%, respectively. The high concentration of diazoxide lowered insulin release almost to the same level as the substimulatory glucose concentration. Similarly, results obtained with NN414 demonstrated a decrease of insulin release in a concentration-dependent manner. Compared with diazoxide, NN414 showed the same effect but at much lower concentrations. NN414 at a concentration of 0.5 µM already decreased the glucose-induced insulin secretion by the same percentage as diazoxide at a 100-fold higher concentration. Furthermore, NN414 at a concentration of 5 µM suppressed the glucose-induced insulin secretion by 97% (i.e. to the same level as it was in the presence of substimulatory glucose concentration). Insulin secretion was 0.05 ± 0.01 ng insulin/(islet*h) in the presence of 3 mM glucose *vs.* 0.04 ± 0.01 in the presence of 5 µM NN414.

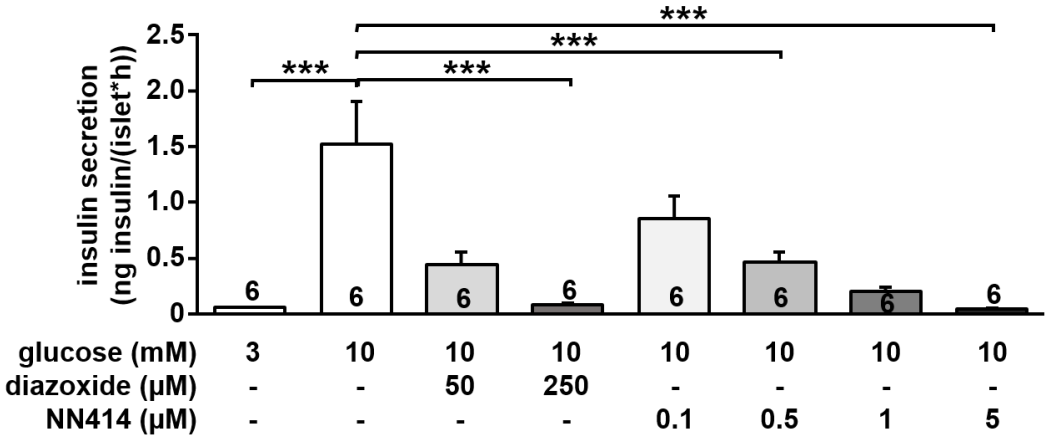


**Fig. 4.1.1.6: NN414 is more potent in decreasing glucose-induced insulin secretion than diazoxide.** Comparative inhibitory effects of diazoxide (50 and 250 µM) and NN414 (0.1, 0.5, 1 and 5 µM) on glucose-induced (G3/G10) insulin secretion in the islets of Langerhans obtained from C57BL/6N mice. n = 6 from 5 different preparations. n represents the number of different experiments. * $p \leq 0.05$, *** $p \leq 0.001$.

### 4.1.2 $K_{ATP}$ channel-independent drugs

The next two sections present approaches that could be effective in CHI cases when $K_{ATP}$ channels are not expressed or when they do not respond to metabolic regulation.

#### 4.1.2.1 SK4 channel openers as potential therapeutics

As already mentioned in section 1.1, in addition to $K_{ATP}$ and voltage-gated $K^+$ channels, pancreatic β-cells express $K^+$ channels regulated by the cytosolic $Ca^{2+}$ concentration ($K_{Ca}$) (Drews *et al.* 2015b). It has been demonstrated that the $K_{Ca}$ channels of intermediate conductance (SK4) play an important role in the $K^+$ current ($K_{slow}$) that contributes to β-cell hyperpolarization at the end of a burst phase with electrical activity (Düfer *et al.* 2009; Goforth *et al.* 2002; Krippeit-Drews *et al.* 2000; Göpel *et al.* 1999). Previous results from Düfer *et al.* demonstrated that activation of SK4 channels hyperpolarized the membrane potential of pancreatic β-cells from C57BL/6N mice (Düfer *et al.* 2009). Because it is known that approximately 50% of $K_{slow}$ belongs to the $K_{ATP}$ current (Kanno *et al.* 2002), the sulfonylurea-insensitive $K_{Ca}$ component could be even more significant in β-cells that lack functional $K_{ATP}$ channels, resembling the situation in CHI channelopathies.

To verify this assumption, the effect of the potent SK4 opener DCEBIO (Singh *et al.* 2001) was evaluated on β-cells from $SUR1^{-/-}$ mice. DCEBIO effectively abolished the glucose-induced oscillations of $[Ca^{2+}]_c$ (Fig. 4.1.2.1A). In a concentration of 100 µM, DCEBIO reduced the mean fluorescence ratio from 0.39 ± 0.02 to 0.31 ± 0.02 (Fig. 4.1.2.1B). Because $[Ca^{2+}]_c$ is determined by the cell membrane potential, the $V_m$ was measured in the perforated-patch configuration. Fig. 4.1.2.1C shows the action potential firing in the presence of 15 mM glucose. The addition of DCEBIO (100 µM) hyperpolarized the plateau potential and decreased the membrane potential from −31.2 ± 2.1 mV under control conditions to −46.7 ± 4.8 mV (Fig. 4.1.2.1D). Next, it was tested whether DCEBIO exhibits similar effects on human β-cells isolated from healthy and pathological CHI pancreatic biopsies. As expected, DCEBIO (100 µM) significantly reduced mean $[Ca^{2+}]_c$ in the presence of 10 mM glucose (0.75 ± 0.04 under control conditions *vs.* 0.59 ± 0.03 after the addition of DCEBIO) (Fig. 4.1.2.1E, F). Furthermore, DCEBIO was tested on β-cells isolated from tissues obtained from patients with mosaic and diffuse forms of CHI, and it suppressed the oscillations of

$[Ca^{2+}]_c$ (Fig. 4.1.2.1G). In the presence of 10 mM glucose, the mean fluorescence ratio was 0.70 ± 0.03 before the addition of 100 µM DCEBIO compared with 0.58 ± 0.03 after (Fig. 4.1.2.1H).

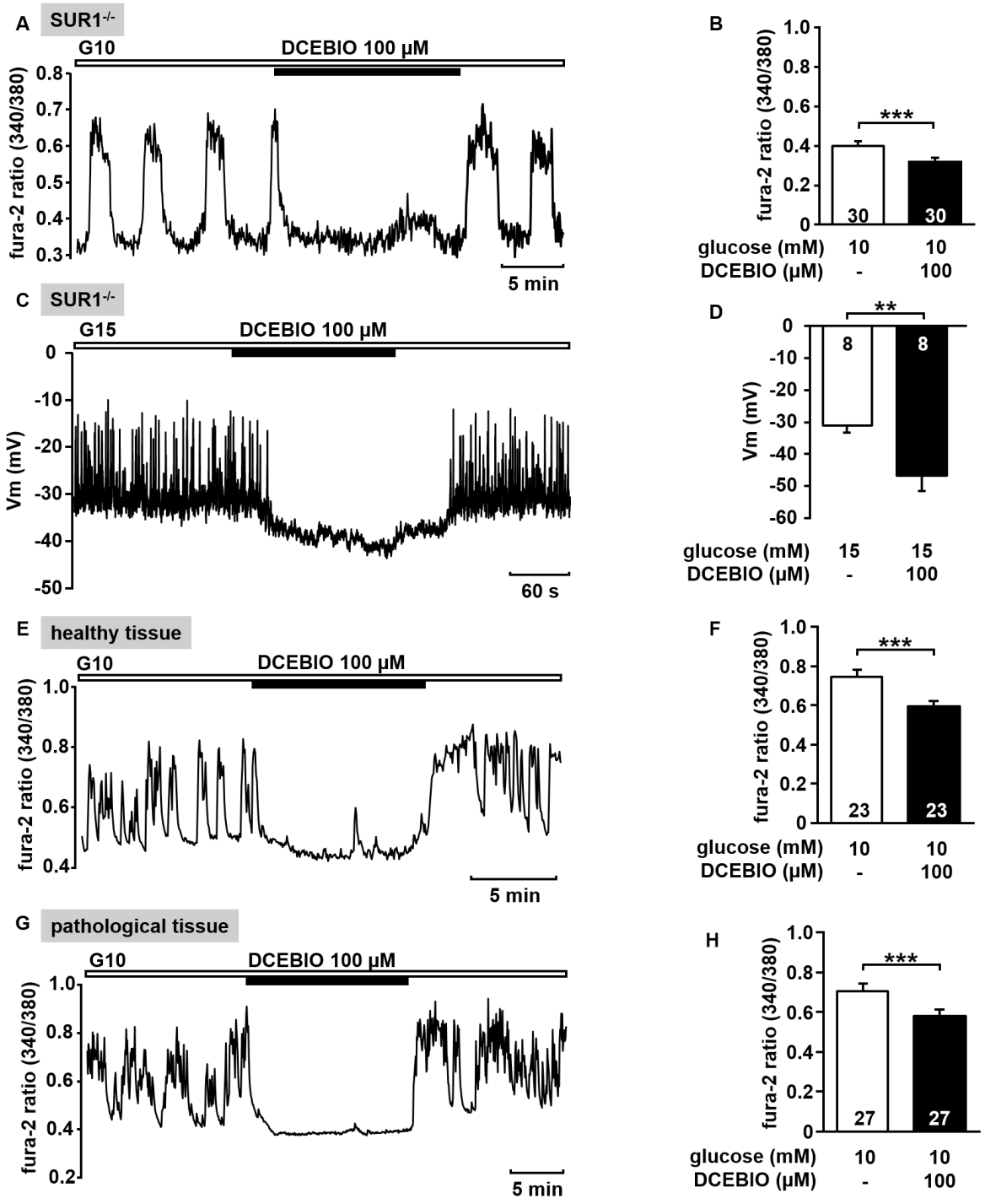


**Fig. 4.1.2.1: DCEBIO hyperpolarizes β-cells from SUR1$^{-/-}$ mice in the presence of 15 mM glucose (G15) and abolishes the oscillations of $[Ca^{2+}]_c$ in both β-cells from SUR1$^{-/-}$ mice and human CHI β-cells in the presence of 10 mM glucose (G10).** A) Representative recording showing the effect of 100 µM DCEBIO on the oscillations of $[Ca^{2+}]_c$ in a β-cell obtained from a SUR1$^{-/-}$ mouse.

B) Summary of all experiments. DCEBIO (100 µM) decreases the mean fluorescence ratio in SUR1$^{-/-}$ β-cells. n = 30 obtained from 3 different preparations. C) Representative recording showing the effect of 100 µM DCEBIO on $V_m$ in a β-cell obtained from a SUR1$^{-/-}$ mouse. D) Summary of all experiments. DCEBIO (100 µM) hyperpolarizes the Vm in SUR1$^{-/-}$ β-cells. n = 8 obtained from 4 different preparations. E) Representative recording showing the effect of 100 µM DCEBIO in a human β-cell obtained from healthy pancreatic tissue of CHI patients. F) Summary of all experiments. DCEBIO (100 µM) decreases the mean fluorescence ratio in healthy human CHI β-cells. n = 23 obtained from 2 different pancreatectomies. G) Representative recording showing the effect of 100 µM DCEBIO in a human β-cell obtained from pathological pancreatic tissue of CHI patients. H) Summary of all experiments. DCEBIO (100 µM) decreases the mean fluorescence ratio in pathological human CHI β-cells. n = 27 obtained from 2 different pancreatectomies of mosaic and diffuse CHI. n represents the number of experiments. ** $p \leq 0.01$, *** $p \leq 0.001$.

#### 4.1.2.2 Galanin does not affect the $[Ca^{2+}]_c$ of human β-cells

Galanin is a neuropeptide that is distributed in the central and peripheral nervous system and the intestinal neuroendocrine system. It acts mostly as a hyperpolarizing neuromodulator and possesses an inhibiting effect on insulin secretion in murine β-cells (Drews *et al.* 1990), probably via a Gi protein-coupled receptor. However, its precise mechanism of action remains uncertain. Düfer *et al.* found that galanin can hyperpolarize mouse β-cells that lack functional $K_{ATP}$ channels (Düfer *et al.* 2004). Hence, application of galanin could be a rational approach for the pharmacological treatment of CHI.

For this reason, the effect of galanin was studied on healthy and pathological human CHI β-cells. Contrary to expectations, galanin at a concentration of 100 nM was without effect (Fig. 4.1.2.2A, C). In healthy β-cells obtained from tissue surrounding a focal CHI lesion, the mean fluorescence ratio amounted to 0.83 ± 0.03 before and 0.82 ± 0.04 after the addition of galanin, respectively (p = 0.79) (Fig. 4.1.2.2B). Moreover, galanin was tested on two pathological CHI β-cells isolated from diffuse pancreatic tissue (genetic mutation not identified). However, in both cases, galanin did not affect glucose-stimulated $Ca^{2+}$ oscillations (the mean fluorescence ratio amounted to 0.52 *vs.* 0.56 tested in the first cell and 0.69 *vs.* 0.66 in the second β-cell) (Fig. 4.1.2.2D).

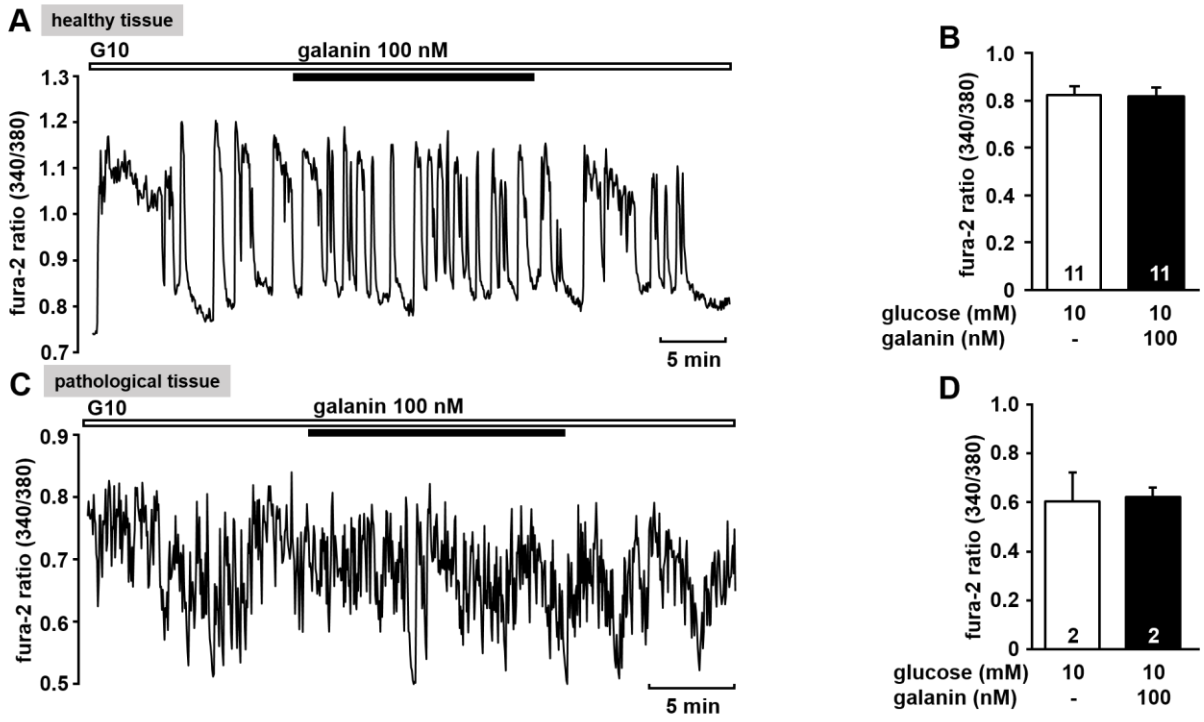


**Fig. 4.1.2.2: Galanin has no effect on the oscillations of $[Ca^{2+}]_c$ in human CHI β-cells in the presence of 10 mM glucose (G10).** A) Representative recording showing the effect of 100 nM galanin on the oscillations of $[Ca^{2+}]_c$ in a human β-cell obtained from healthy pancreatic tissue of CHI patients. B) Summary of all experiments. Galanin (100 nM) does not change the mean fluorescence ratio in healthy human CHI β-cells. n = 11 obtained from 1 pancreatectomy. C) Representative recording showing the effect of 100 nM galanin in a human β-cell obtained from pathological pancreatic tissue of CHI patients. D) Summary of all experiments. Galanin (100 nM) does not change the mean fluorescence ratio in pathological human CHI β-cells. n = 2 obtained from 2 different pancreatectomies. n represents the number of experiments.

## 4.2. Discussion

CHI is a heterogeneous disease in terms of its clinical features and morphology. However, excessive and inappropriate insulin secretion from pancreatic β-cells are common (Arnoux *et al.* 2010; Shah *et al.* 2017; Banerjee *et al.* 2019). The strategy for long-term management of CHI is to bring the β-cell in quiescence, decrease cytosolic $Ca^{2+}$ influx, and to appropriately reduce insulin secretion. Due to the challenge of managing CHI patients, new drug therapies are needed. The present study explored new potential targets and approaches for future drug development for the treatment of CHI.

### 4.2.1 Possible $K_{ATP}$ channel-dependent strategies to treat CHI

Diazoxide is the first line drug for the treatment, because it is usually effective in all forms of CHI except severe cases caused by mutations in the genes that encode $K_{ATP}$ channel (*ABCC8* and *KCNJ11*). Oral administration of this drug is an advantage in the treatment of CHI. However, numerous side effects complicate its usage. Its severe side effects are a consequence of the non-selectivity of diazoxide for pancreatic $K_{ATP}$ channels (SUR1/Kir6.2) (Coetzee 2013). In addition to the pancreatic $K_{ATP}$ channels, it activates those of smooth muscles (SUR2B/Kir6.2 and SUR2B/Kir6.1) and exerts a weak stimulatory effect on the $K_{ATP}$ channels of the cardiac muscle (SUR2A/Kir6.2) (D'Hahan *et al.* 1999). One of the most common adverse effects of diazoxide, hypertrichosis, can be explained by the activation of both SUR1/Kir6.2 and SUR2B/Kir6.1 channels in hair follicles (Burton *et al.* 1975; Davies *et al.* 2005; Shorter *et al.* 2008). Another common and also the most clinically concerning side effect of diazoxide is fluid and sodium retention, which causes edema or fluid overload that might be the indirect cause of cardiac failure (Welters *et al.* 2015; Demirbilek *et al.* 2017; Herrera *et al.* 2018). Additional side effects related to treatment with diazoxide include neutropenia, thrombocytopenia, hyperuricemia and life threatening pulmonary hypertension (Welters *et al.* 2015; Demirbilek *et al.* 2017; Herrera *et al.* 2018; Timlin *et al.* 2017). Because diazoxide is still the first line drug and there are CHI patients who respond well to diazoxide therapy, identifying more selective diazoxide analogues with less side effects seems to be a reasonable step towards better treatment options.

#### 4.2.1.1 NN414

As one of the approaches to improve CHI therapy, we tested more specific β-cell $K_{ATP}$ channel openers. Compared with diazoxide, NN414 is reported to be a SUR1 selective agonist, 100-fold more potent, suggesting that the drug is effective at a much lower concentration (Dabrowski *et al.* 2003). This characteristic of NN414 was confirmed by the data in the present work, where NN414 at a concentration of 0.5 µM decreased glucose-induced insulin secretion to the same extent as 50 µM diazoxide did (section 4.1.1.6). Furthermore, NN414 was more effective in activating $K_{ATP}$ channels related to CHI ($SUR1_{E1507K}$/WT Kir6.2) than diazoxide was (section 4.1.1.5). This dominantly inherited mutation in SUR1 has been connected to the development

of diabetes later in life (Huopio *et al.* 2000). The early, prediabetic stages of type 2 diabetes mellitus (T2DM) are known to be normally characterized by a compensatory hypersecretion of insulin and associated with elevated intracellular $[Ca^{2+}]_c$. Elevated $[Ca^{2+}]_c$ could cause a high rate of β-cell apoptosis, which has also been reported for the focal form of CHI (Glaser *et al.* 1999). Accordingly, therapy with $K_{ATP}$ channel openers might be helpful in CHI to delay the initiation of apoptosis and its progression to diabetes by counteracting excess hormone release (Huopio *et al.* 2000; Hansen *et al.* 2004).

The $K_{ATP}$ channel agonist NN414 has been used in numerous *in vitro* and *in vivo* studies to induce β-cell rest, thereby preserving β-cell function and preventing apoptosis (Ritzel *et al.* 2004; Nielsen *et al.* 2002). An animal *in vivo* study revealed significant potential for NN414 in the treatment of disorders that result from excessive insulin release (Alemzadeh *et al.* 2004). Alemzadeh *et al*. showed in a six-week study that NN414 reduced hyperinsulinemia and improved glucose responsiveness in Zucker obese rats in a dose-dependent manner, and thus NN414 entered human clinical trials for the treatment of T2DM. In healthy subjects it inhibited insulin release, was well tolerated, and did not cause clinically relevant changes in safety parameters, except side effects on the gastrointestinal system (Zdravkovic *et al.* 2005). NN414 was advanced into phase 2 of clinical trials, where it showed a tendency to improve the β-cell secretory function in diabetic patients (Choi 2003; Sarabu and Tilley 2005). The clinical trial was stopped due to elevated liver enzymes in treated patients (Adis R&D ; Zdravkovic *et al.* 2007).

Based on the literature and the results presented here, NN414 is a good candidate for therapy of CHI. It is SUR1 selective, acts at low doses and shows the reproducible $Ca^{2+}$-lowering effect in human β-cells obtained from pancreatic tissue of CHI patients. The elevation of liver enzymes by NN414 should not be seen as a criterion for excluding the drug, although it would be desirable to develop NN414 analogs without this adverse effect. Noteworthy, an increase in liver enzymes is one of the most reported side effects for two currently proposed diazoxide alternatives for the long-term management and treatment of CHI, octreotide and sirolimus (Demirbilek *et al.* 2017; Koren *et al.* 2013; Ben-Ari *et al.* 2013; Demirbilek *et al.* 2014).

#### 4.2.1.2 VU0071063

Recently, a novel $K_{ATP}$ channel activator, VU0071063, was discovered (Raphemot *et al.* 2014). The VU0071063 is reportedly more selective for SUR1/Kir6.2 channels than for SUR2A/Kir6.2 and SUR2A/Kir6.1 channels. Furthermore it has been demonstrated to directly activate SUR1/Kir6.2 channels expressed in HEK-293 cells, with higher potency than diazoxide (Raphemot *et al.* 2014). Data from experiments with human CHI β-cells and murine β-cells presented in this study are in agreement with the findings that VU0071063 reduces glucose-induced $[Ca^{2+}]_c$ in murine β-cells (Fig. 4.1.1.3A-D and Fig. 4.1.1.4A). However, the observation that the removal of VU0071063 from the solution initiated a transient drop in $[Ca^{2+}]_c$ indicates that the compound has additional effects in pancreatic β-cells, apart from the direct activation of the channels. Further measurements on β-cells from $SUR1^{-/-}$ mice (Fig. 4.1.1.4A, B), lacking functional $K_{ATP}$ channels, suggested that the mechanism of action of VU0071063 is not solely the activation of $K_{ATP}$ channels, but it can affect other parameters of SSC as well. This assumption is supported by the following observations: 1) The removal of VU0071063 caused a transient drop in $[Ca^{2+}]_c$ in both C57BL/6N and $SUR1^{-/-}$ β-cells, presumably due to ATP-dependent SERCA activation. 2) VU0071063 strongly and reversibly depolarized ΔΨ in C57BL/6N and $SUR1^{-/-}$ β-cells, implying inhibition of ATP formation. 3) VU0071063 rapidly and significantly decreased $[Ca^{2+}]_c$ in $SUR1^{-/-}$ β-cells, which may be a result of the release of $Ca^{2+}$ from $Ca^{2+}$ stores due to the lack in ATP and secondary $Ca^{2+}$-dependent inactivation of L-type VDCCs. Taken together, the data suggest that the $Ca^{2+}$-lowering effect of VU0071063 is caused by a dual mechanism: 1) activation of the pancreatic $K_{ATP}$ channels either directly through the binding of the drug to the channels or indirectly by ATP depletion; 2) interference with other parameters of SSC, independent of the $K_{ATP}$ channel.

Due to the fact that ATP depletion can have detrimental effects on ATP-dependent processes, the existing molecular structure of VU0071063 is unsuitable for use in humans. Thus, molecular modification of the compound should be considered.

### 4.2.2 Possible $K_{ATP}$ channel-independent strategies to treat CHI

When $K_{ATP}$ channels are impaired or not properly expressed because of certain mutations in *ABCC8* or *KCNJ11* genes, the effect of an opener (e.g., diazoxide) might be disabled (Glaser *et al.* 2000; Saint-Martin *et al.* 2011; Vajravelu and De León 2018). As a result, patients are unresponsive to therapy with diazoxide (Arya, Güemes*, et al.* 2014). In this case, it could be useful to focus on targeting mechanisms (i.e., different steps of SSC) that can induce β-cell rest independently of the $K_{ATP}$ channel and attenuate insulin release. Currently available alternatives to diazoxide therapy are somatostatin analogues (octreotide, octreotide-LAR and lanreotide), sirolimus and exendin-(9-39) (Demirbilek *et al.* 2017). Octreotide, a short-acting somatostatin analogue, suppresses insulin secretion by binding with a high affinity to SSTR subtype 2 and subtype 5 (SSTR2 and SSTR5) (Katz and Erstad 1989; Lamberts *et al.* 1996; van der Steen *et al.* 2018). It has been shown that human pancreatic β-cells dominantly express the SSTR2 receptor, but the other isoforms also contribute to the somatostatin effect (Kailey *et al.* 2012; Braun 2014). As already mentioned in Section 1.3, somatostatin exerts its effect after binding to SSTRs that are coupled to Gi/o proteins. Activation of SSTRs exhibits multifactorial modulation of β-cells, involving the inhibition of the adenylate cyclase-cAMP pathway, activation of GIRK channels, decrease of $Ca^{2+}$ influx via P/Q-type VDCCs and direct inhibition of exocytosis (Patel 1999; Doyle and Egan 2003; Braun 2014). Despite good response to initial doses, it is nevertheless unsuitable for long-term treatment because of tachyphylaxis after initiation of treatment (Demirbilek *et al.* 2017; Welters *et al.* 2015). Long-acting somatostatin analogues (octreotide long-acting release (sandostatin-LAR) and lanreotide) have similar mechanism of action as octreotide and show the same side effects (Le Quan Sang *et al.* 2012; Shah *et al.* 2015; Dastamani *et al.* 2019; van der Steen *et al.* 2018). However, their long dosing interval (once every four weeks) is beneficial for patients (Demirbilek *et al.* 2017). Sirolimus, a mammalian target of the rapamycin (mTOR) inhibitor is an immunosuppressive and anti-proliferative agent that has been used in patients with diffuse CHI, unresponsive to diazoxide and octreotide therapy (Güemes *et al.* 2019; Arya, Güemes*, et al.* 2014). It suppresses insulin release by various mechanisms, which have not all been fully elucidated (Szymanowski *et al.* 2016). It has been proposed that downregulation of the mTOR pathway decreases insulin production in

pancreatic β-cells and β-cell growth and may restore ketogenesis (Güemes *et al.* 2019; Wullschleger *et al.* 2006). However, severe and life threatening side effects have been reported for this drug, which restricts its use (Houde *et al.* 2010; Demirbilek *et al.* 2017; Güemes *et al.* 2019).

#### 4.2.2.1 Opening of SK4 channels in the treatment of CHI

As described in section 1.1, apart from $K_{ATP}$ channels, SK4 channels have been proposed to contribute to $K_{slow}$, the hyperpolarizing current that terminates bursts of action potentials in β-cells (Düfer *et al.* 2009; Drews *et al.* 2015b). Accordingly, SK4 channels may play a dominant role in inducing hyperpolarization in β-cells without functional $K_{ATP}$ channels, which would be of great importance in the treatment of CHI. Data in the present study demonstrate that an opener of the SK4 channel, DCEBIO, was able to abolish $[Ca^{2+}]_c$ and to hyperpolarize the membrane potential in $SUR1^{-/-}$ β-cells (lacking functional $K_{ATP}$ channels) (section 4.1.2.1). Furthermore, DCEBIO was highly effective in silencing human β-cells from healthy and pathological pancreatic tissue of CHI patients. Experiments on human CHI β-cells additionally support the idea of targeting SK4 channels in the treatment of CHI. However, SK4 channels are expressed in various organs such as the salivary gland and lungs, and in the cells of the hematopoietic system (Hoffman *et al.* 2003). Additionally, they are present in the endothelium where they are involved in the regulation of vascular tone and blood pressure (Ledoux *et al.* 2006; Sheng *et al.* 2009; Nam *et al.* 2017). Openers of SK4 channels increase agonist-evoked endothelial nitric oxide synthesis and arteriolar vasodilation (Sheng *et al.* 2009). Thus, targeting SK4 channels as a strategy in the treatment of CHI patients would require the search for new agonists with high selectivity for β-cells, because unspecific SK4 channel openers are expected to exert severe side effects in numerous other organs (e.g. erythrocytes, lung, endothelium and immune cells) (Jensen *et al.* 1998; Hoffman *et al.* 2003; Begenisich *et al.* 2004; Ledoux *et al.* 2006; Sheng *et al.* 2009; Nam *et al.* 2017).

#### 4.2.2.2 The neuropeptide galanin

*In vitro* studies with rodents have suggested the neuropeptide galanin has a role in suppressing insulin secretion as an adaptive effect of the autonomic nervous system by a $K_{ATP}$ channel-independent mechanism, which is still not completely understood (Drews *et al.* 1990; Tang *et al.* 2012). Düfer *et al.* demonstrated that galanin suppresses electrical activity of β-cells isolated from $SUR1^{-/-}$ mice (Düfer *et al.* 2004). Accordingly, galanin may affect insulin secretion in CHI β-cells through a mechanism similar to that of somatostatin analogs. However, different *in vitro* and *in vivo* studies with galanin in humans described controversial effects ranging from inhibition to no effect (Ahren *et al.* 1991; Holst *et al.* 1993; Lang *et al.* 2015; Tang *et al.* 2012; Hussain *et al.* 2016). In this study, galanin did not reduce the $[Ca^{2+}]_c$ of human β-cells isolated from CHI patients (Fig. 4.1.2.2). These results are in agreement with recent findings from Rorsman and Ashcroft, who demonstrated the expression of galanin receptors in mouse β-cells, but not in human β-cells (Rorsman and Ashcroft 2018).

# 5 Literature

Abel, K. B., Lehr, S., and Ullrich, S. 1996. 'Adrenaline-, not somatostatin-induced hyperpolarization is accompanied by a sustained inhibition of insulin secretion in INS-1 cells. Activation of sulphonylurea $K^+$ATP channels is not involved', *Pflugers Arch*, 432(1): 89-96.

Adis R&D, Insight Database. 'Investigational Drugs Database', Springer, Accessed May 2019.

Adzick, N. S., De León, D. D., States, L. J., Lord, K., Bhatti, T. R., Becker, S. A., and Stanley, C. A. 2019. 'Surgical treatment of congenital hyperinsulinism: Results from 500 pancreatectomies in neonates and children', *J Pediatr Surg*, 54(1): 27-32.

Adzick, N. S., Thornton, P. S., Stanley, C. A., Kaye, R. D., and Ruchelli, E. 2004. 'A multidisciplinary approach to the focal form of congenital hyperinsulinism leads to successful treatment by partial pancreatectomy', *J Pediatr Surg*, 39(3): 270-5.

Aguilar-Bryan, L., and Bryan, J. 1999. 'Molecular biology of adenosine triphosphate-sensitive potassium channels', *Endocr Rev*, 20(2): 101-35.

Aguilar-Bryan, L., and Bryan, J. 2008. 'Neonatal diabetes mellitus', *Endocr Rev*, 29(3): 265-91.

Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., Clement, J. P. th, Boyd, A. E., 3rd, Gonzalez, G., Herrera-Sosa, H., Nguy, K., Bryan, J., and Nelson, D. A. 1995. 'Cloning of the beta cell high-affinity sulfonylurea receptor: a regulator of insulin secretion', *Science*, 268(5209): 423-6.

Ahren, B., Ar'Rajab, A., Bottcher, G., Sundler, F., and Dunning, B. E. 1991. 'Presence of galanin in human pancreatic nerves and inhibition of insulin secretion from isolated human islets', *Cell Tissue Res*, 264(2): 263-7.

Aittoniemi, J., Fotinou, C., Craig, T. J., de Wet, H., Proks, P., and Ashcroft, F. M. 2009. 'Review. SUR1: a unique ATP-binding cassette protein that functions as an ion channel regulator', *Philos Trans R Soc Lond B Biol Sci*, 364(1514): 257-67.

Alemzadeh, Ramin, Fledelius, Christian, Bodvarsdottir, Thora, and Sturis, Jeppe. 2004. 'Attenuation of hyperinsulinemia by NN414, a SUR1/Kir6.2 selective $K^+$-adenosine triphosphate channel opener, improves glucose tolerance and lipid profile in obese Zucker rats', *Metabolism*, 53(4): 441-47.

Arnoux, J. B., de Lonlay, P., Ribeiro, M. J., Hussain, K., Blankenstein, O., Mohnike, K., Valayannopoulos, V., Robert, J. J., Rahier, J., Sempoux, C., Bellanne, C., Verkarre, V., Aigrain, Y., Jaubert, F., Brunelle, F., and Nihoul-Fékété, C. 2010. 'Congenital hyperinsulinism', *Early Hum Dev*, 86(5): 287-94.

Arya, V. B., Güemes, M., Nessa, A., Alam, S., Shah, P., Gilbert, C., Senniappan, S., Flanagan, S. E., Ellard, S., and Hussain, K. 2014. 'Clinical and histological heterogeneity of congenital hyperinsulinism due to paternally inherited heterozygous ABCC8/KCNJ11 mutations', *Eur J Endocrinol*, 171(6): 685-95.

Arya, V. B., Mohammed, Z., Blankenstein, O., De Lonlay, P., and Hussain, K. 2014. 'Hyperinsulinaemic hypoglycaemia', *Horm Metab Res*, 46(3): 157-70.

Ashcroft, F. M. 2005. 'ATP-sensitive potassium channelopathies: focus on insulin secretion', *J Clin Invest*, 115(8): 2047-58.

Ashcroft, F. M., and Kakei, M. 1989. 'ATP-sensitive $K^+$ channels in rat pancreatic beta-cells: modulation by ATP and $Mg^{2+}$ ions', *J Physiol*, 416: 349-67.

Ashcroft, F. M., and Rorsman, P. 1989. 'Electrophysiology of the pancreatic beta-cell', *Prog Biophys Mol Biol*, 54(2): 87-143.

Atwater, I., Ribalet, B., and Rojas, E. 1979. 'Mouse pancreatic beta-cells: tetraethylammonium blockage of the potassium permeability increase induced by depolarization', *J Physiol*, 288: 561-74.

Aynsley-Green, A., Hussain, K., Hall, J., Saudubray, J. M., Nihoul-Fékété, C., De Lonlay-Debeney, P., Brunelle, F., Otonkoski, T., Thornton, P., and Lindley, K. J. 2000. 'Practical management of hyperinsulinism in infancy', *Arch Dis Child Fetal Neonatal Ed*, 82(2): F98-F107.

Babenko, A. P. 2008. 'A novel ABCC8 (SUR1)-dependent mechanism of metabolism-excitation uncoupling', *J Biol Chem*, 283(14): 8778-82.

Babenko, A. P., and Bryan, J. 2003. 'Sur domains that associate with and gate $K_{ATP}$ pores define a novel gatekeeper', *J Biol Chem*, 278(43): 41577-80.

Babenko, A. P., Gonzalez, G., and Bryan, J. 2000. 'Pharmaco-topology of sulfonylurea receptors. Separate domains of the regulatory subunits of $K_{ATP}$ channel isoforms are required for selective interaction with $K^+$ channel openers', *J Biol Chem*, 275(2): 717-20.

Banerjee, I., De León, D., and Dunne, M. J. 2017. 'Extreme caution on the use of sirolimus for the congenital hyperinsulinism in infancy patient', *Orphanet J Rare Dis*, 12(1): 70.

Banerjee, I., Salomon-Estebanez, M., Shah, P., Nicholson, J., Cosgrove, K. E., and Dunne, M. J. 2019. 'Therapies and outcomes of congenital hyperinsulinism-induced hypoglycaemia', *Diabet Med*, 36(1): 9-21.

Barnett, D. W., Pressel, D. M., and Misler, S. 1995. 'Voltage-dependent $Na^+$ and $Ca^{2+}$ currents in human pancreatic islet beta-cells: evidence for roles in the generation of action potentials and insulin secretion', *Pflugers Arch*, 431(2): 272-82.

Barthlen, W. 2011. 'Surgery in congenital hyperinsulinism-tips and tricks not only for surgeons. A practical guide', *Semin Pediatr Surg*, 20(1): 56-9.

Barthlen, W., Mohnike, W., and Mohnike, K. 2010. 'Techniques in pediatric surgery: congenital hyperinsulinism', *Horm Res Paediatr*, 74(6): 438-43.

Barthlen, W., Varol, E., Empting, S., Wieland, I., Zenker, M., Mohnike, W., Vogelgesang, S., and Mohnike, K. 2016. 'Surgery in Focal Congenital

Hyperinsulinism (CHI) - The "Hyperinsulinism Germany International" Experience in 30 Children', *Pediatr Endocrinol Rev*, 14(2): 129-37.

Baukrowitz, T., and Fakler, B. 2000. '$K_{ATP}$ channels: linker between phospholipid metabolism and excitability', *Biochem Pharmacol*, 60(6): 735-40.

Baukrowitz, T., Schulte, U., Oliver, D., Herlitze, S., Krauter, T., Tucker, S. J., Ruppersberg, J. P., and Fakler, B. 1998. 'PIP2 and PIP as determinants for ATP inhibition of KATP channels', *Science*, 282(5391): 1141-4.

Begenisich, T., Nakamoto, T., Ovitt, C. E., Nehrke, K., Brugnara, C., Alper, S. L., and Melvin, J. E. 2004. 'Physiological roles of the intermediate conductance, $Ca^{2+}$-activated potassium channel Kcnn4', *J Biol Chem*, 279(46): 47681-7.

Beguin, P., Nagashima, K., Nishimura, M., Gonoi, T., and Seino, S. 1999. 'PKA-mediated phosphorylation of the human $K_{ATP}$ channel: separate roles of Kir6.2 and SUR1 subunit phosphorylation', *EMBO J*, 18(17): 4722-32.

Ben-Ari, J., Greenberg, M., Nemet, D., Edelstein, E., and Eliakim, A. 2013. 'Octreotide-induced hepatitis in a child with persistent hyperinsulinemia hypoglycemia of infancy', *J Pediatr Endocrinol Metab*, 26(1-2): 179-82.

Bertram, R., Sherman, A., and Satin, L. S. 2010. 'Electrical bursting, calcium oscillations, and synchronization of pancreatic islets', *Adv Exp Med Biol*, 654: 261-79.

Bienengraeber, M., Alekseev, A. E., Abraham, M. R., Carrasco, A. J., Moreau, C., Vivaudou, M., Dzeja, P. P., and Terzic, A. 2000. 'ATPase activity of the sulfonylurea receptor: a catalytic function for the $K_{ATP}$ channel complex', *FASEB J*, 14(13): 1943-52.

Braun, M. 2014. 'The somatostatin receptor in human pancreatic beta-cells', *Vitam Horm*, 95: 165-93.

Braun, M., Ramracheya, R., Bengtsson, M., Zhang, Q., Karanauskaite, J., Partridge, C., Johnson, P. R., and Rorsman, P. 2008. 'Voltage-gated ion channels in human pancreatic beta-cells: electrophysiological characterization and role in insulin secretion', *Diabetes*, 57(6): 1618-28.

Bryan, J., Crane, A., Vila-Carriles, W. H., Babenko, A. P., and Aguilar-Bryan, L. 2005. 'Insulin secretagogues, sulfonylurea receptors and $K_{ATP}$ channels', *Curr Pharm Des*, 11(21): 2699-716.

Burton, J. L., Schutt, W. H., and Caldwell, I. W. 1975. 'Hypertrichosis due to diazoxide', *Br J Dermatol*, 93(6): 707-11.

Cabrera, O., Berman, D. M., Kenyon, N. S., Ricordi, C., Berggren, P. O., and Caicedo, A. 2006. 'The unique cytoarchitecture of human pancreatic islets has implications for islet cell function', *Proc Natl Acad Sci U S A*, 103(7): 2334-9.

Can Thi Bich, N., Vu Chi, D., Bui Phuong, T., Nguyen Ngoc, K., Nguyen Phu, D., Ellard, S., Craig, M., and Nguyen Thi, H. . 2014. 'The Result of Sulphonylureas

Treatment in Patients with Neonatal Diabetes Mellitus due to *KCNJ1111/ABCC8* Gene Mutations in Vietnam', *ESPE*, 82.

Carr, R. D., Brand, C. L., Bodvarsdottir, T. B., Hansen, J. B., and Sturis, J. 2003. 'NN414, a SUR1/Kir6.2-selective potassium channel opener, reduces blood glucose and improves glucose tolerance in the VDF Zucker rat', *Diabetes*, 52(10): 2513-8.

Carson, M. R., and Welsh, M. J. 1995. 'Structural and functional similarities between the nucleotide-binding domains of CFTR and GTP-binding proteins', *Biophys J*, 69(6): 2443-8.

Chang, X. B., Hou, Y. X., and Riordan, J. R. 1997. 'ATPase activity of purified multidrug resistance-associated protein', *J Biol Chem*, 272(49): 30962-8.

Choi, J. K. 2003. 'NN-414. Novo Nordisk', *Curr Opin Investig Drugs*, 4(4): 455-8.

Choi, K. H., Tantama, M., and Licht, S. 2008. 'Testing for violations of microscopic reversibility in ATP-sensitive potassium channel gating', *J Phys Chem B*, 112(33): 10314-21.

Christesen, H. T., Sjobald, S., Brusgaard, K., Papadopolou, D., and Jaobson, B.B. 2005. 'Permanent neonatal diabetes in a child with an ABCC8 gene mutation.', *Horm Res*(64): 135.

Coetzee, W. A. 2013. 'Multiplicity of effectors of the cardioprotective agent, diazoxide', *Pharmacol Ther*, 140(2): 167-75.

Colquhoun, D., and Hawkes, A. G. 1995. 'Desensitization of N-methyl-D-aspartate receptors: a problem of interpretation', *Proc Natl Acad Sci U S A*, 92(22): 10327-9.

Cook, D. L., and Hales, C. N. 1984. 'Intracellular ATP directly blocks $K^+$ channels in pancreatic B-cells', *Nature*, 311(5983): 271-3.

Corbin, J. A., Bhaskar, V., Goldfine, I. D., Bedinger, D. H., Lau, A., Michelson, K., Gross, L. M., Maddux, B. A., Kuan, H. F., Tran, C., Lao, L., Handa, M., Watson, S. R., Narasimha, A. J., Zhu, S., Levy, R., Webster, L., Wijesuriya, S. D., Liu, N., Wu, X., Chemla-Vogel, D., Lee, S. R., Wong, S., Wilcock, D., and White, M. L. 2014a. 'Improved glucose metabolism in vitro and in vivo by an allosteric monoclonal antibody that increases insulin receptor binding affinity', *PLoS One*, 9(2): e88684.

Corbin, J. A., Bhaskar, V., Goldfine, I. D., Issafras, H., Bedinger, D. H., Lau, A., Michelson, K., Gross, L. M., Maddux, B. A., Kuan, H. F., Tran, C., Lao, L., Handa, M., Watson, S. R., Narasimha, A. J., Zhu, S., Levy, R., Webster, L., Wijesuriya, S. D., Liu, N., Wu, X., Chemla-Vogel, D., Lee, S. R., Wong, S., Wilcock, D., Rubin, P., and White, M. L. 2014b. 'Inhibition of insulin receptor function by a human, allosteric monoclonal antibody: a potential new approach for the treatment of hyperinsulinemic hypoglycemia', *MAbs*, 6(1): 262-72.

Csanády, L. 2017. 'CFTR gating: Invisible transitions made visible', *J Gen Physiol*, 149(4): 413-16.

Csanády, L., Vergani, P., and Gadsby, D. C. 2010. 'Strict coupling between CFTR's catalytic cycle and gating of its Cl- ion pore revealed by distributions of open channel burst durations', *Proc Natl Acad Sci U S A*, 107(3): 1241-6.

D'Hahan, N., Moreau, C., Prost, A. L., Jacquet, H., Alekseev, A. E., Terzic, A., and Vivaudou, M. 1999. 'Pharmacological plasticity of cardiac ATP-sensitive potassium channels toward diazoxide revealed by ADP', *Proc Natl Acad Sci U S A*, 96(21): 12162-7.

Dabrowski, M., Larsen, T., Ashcroft, F. M., Bondo Hansen, J., and Wahl, P. 2003. 'Potent and selective activation of the pancreatic beta-cell type $K_{ATP}$ channel by two novel diazoxide analogues', *Diabetologia*, 46(10): 1375-82.

Dabrowski, M., Wahl, P., Holmes, W. E., and Ashcroft, F. M. 2001. 'Effect of repaglinide on cloned beta cell, cardiac and smooth muscle types of ATP-sensitive potassium channels', *Diabetologia*, 44(6): 747-56.

Damaj, L., le Lorch, M., Verkarre, V., Werl, C., Hubert, L., Nihoul-Fékété, C., Aigrain, Y., de Keyzer, Y., Romana, S. P., Bellanne-Chantelot, C., de Lonlay, P., and Jaubert, F. 2008. 'Chromosome 11p15 paternal isodisomy in focal forms of neonatal hyperinsulinism', *J Clin Endocrinol Metab*, 93(12): 4941-7.

Dastamani, A., Güemes, M., Pitfield, C., Morgan, K., Rajab, M., Rottenburger, C., Bomanji, J., De Coppi, P., Dattani, M., and Shah, P. 2019. 'The Use of a Long-Acting Somatostatin Analogue (Lanreotide) in Three Children with Focal Forms of Congenital Hyperinsulinaemic Hypoglycaemia', *Horm Res Paediatr*, 91(1): 56-61.

Davies, G. C., Thornton, M. J., Jenner, T. J., Chen, Y. J., Hansen, J. B., Carr, R. D., and Randall, V. A. 2005. 'Novel and established potassium channel openers stimulate hair growth in vitro: implications for their modes of action in hair follicles', *J Invest Dermatol*, 124(4): 686-94.

de Lonlay, P., Fournet, J. C., Rahier, J., Gross-Morand, M. S., Poggi-Travert, F., Foussier, V., Bonnefont, J. P., Brusset, M. C., Brunelle, F., Robert, J. J., Nihoul-Fékété, C., Saudubray, J. M., and Junien, C. 1997. 'Somatic deletion of the imprinted 11p15 region in sporadic persistent hyperinsulinemic hypoglycemia of infancy is specific of focal adenomatous hyperplasia and endorses partial pancreatectomy', *J Clin Invest*, 100(4): 802-7.

de Wet, H., Fotinou, C., Amad, N., Dreger, M., and Ashcroft, F. M. 2010. 'The ATPase activities of sulfonylurea receptor 2A and sulfonylurea receptor 2B are influenced by the C-terminal 42 amino acids', *FEBS J*, 277(12): 2654-62.

de Wet, H., Mikhailov, M. V., Fotinou, C., Dreger, M., Craig, T. J., Venien-Bryan, C., and Ashcroft, F. M. 2007. 'Studies of the ATPase activity of the ABC protein SUR1', *FEBS J*, 274(14): 3532-44.

Demirbilek, H., Rahman, S. A., Buyukyilmaz, G. G., and Hussain, K. 2017. 'Diagnosis and treatment of hyperinsulinaemic hypoglycaemia and its implications for paediatric endocrinology', *Int J Pediatr Endocrinol*, 2017: 9.

Demirbilek, H., Shah, P., Arya, V. B., Hinchey, L., Flanagan, S. E., Ellard, S., and Hussain, K. 2014. 'Long-term follow-up of children with congenital hyperinsulinism on octreotide therapy', *J Clin Endocrinol Metab*, 99(10): 3660-7.

Demirel, F., Unal, S., Cetin, II, Esen, I., and Arasli, A. 2011. 'Pulmonary hypertension and reopening of the ductus arteriosus in an infant treated with diazoxide', *J Pediatr Endocrinol Metab*, 24(7-8): 603-5.

Detimary, P., Gilon, P., and Henquin, J. C. 1998. 'Interplay between cytoplasmic $Ca^{2+}$ and the ATP/ADP ratio: a feedback control mechanism in mouse pancreatic islets', *Biochem J*, 333 ( Pt 2): 269-74.

Doyle, D. A., Morais Cabral, J., Pfuetzner, R. A., Kuo, A., Gulbis, J. M., Cohen, S. L., Chait, B. T., and MacKinnon, R. 1998. 'The structure of the potassium channel: molecular basis of K+ conduction and selectivity', *Science*, 280(5360): 69-77.

Doyle, M. E., and Egan, J. M. 2003. 'Pharmacological agents that directly modulate insulin secretion', *Pharmacol Rev*, 55(1): 105-31.

Drews, G. , Krippeit-Drews, P. , and Düfer, M. 2015b. 'Electrophysiology of Islet Cells.' in, *Islets of Langerhans* (Springer Netherlands).

Drews, G., Bauer, C., Edalat, A., Düfer, M., and Krippeit-Drews, P. 2015a. 'Evidence against a $Ca^{2+}$-induced potentiation of dehydrogenase activity in pancreatic beta-cells', *Pflugers Arch*, 467(11): 2389-97.

Drews, G., Debuyser, A., Nenquin, M., and Henquin, J. C. 1990. 'Galanin and epinephrine act on distinct receptors to inhibit insulin release by the same mechanisms including an increase in K+ permeability of the B-cell membrane', *Endocrinology*, 126(3): 1646-53.

Düfer, M., Gier, B., Wolpers, D., Krippeit-Drews, P., Ruth, P., and Drews, G. 2009. 'Enhanced glucose tolerance by SK4 channel inhibition in pancreatic beta-cells', *Diabetes*, 58(8): 1835-43.

Düfer, M., Haspel, D., Krippeit-Drews, P., Aguilar-Bryan, L., Bryan, J., and Drews, G. 2004. 'Oscillations of membrane potential and cytosolic $Ca^{2+}$ concentration in $SUR1^{-/-}$ beta cells', *Diabetologia*, 47(3): 488-98.

Düfer, M., Neye, Y., Horth, K., Krippeit-Drews, P., Hennige, A., Widmer, H., McClafferty, H., Shipston, M. J., Haring, H. U., Ruth, P., and Drews, G. 2011. 'BK channels affect glucose homeostasis and cell viability of murine pancreatic beta cells', *Diabetologia*, 54(2): 423-32.

Dukes, I. D., McIntyre, M. S., Mertz, R. J., Philipson, L. H., Roe, M. W., Spencer, B., and Worley, J. F., 3rd. 1994. 'Dependence on NADH produced during glycolysis for beta-cell glucose signaling', *J Biol Chem*, 269(15): 10979-82.

Dunne, M. J., Bullett, M. J., Li, G. D., Wollheim, C. B., and Petersen, O. H. 1989. 'Galanin activates nucleotide-dependent $K^+$ channels in insulin-secreting cells via a pertussis toxin-sensitive G-protein', *EMBO J*, 8(2): 413-20.

Dunne, M. J., Cosgrove, K. E., Shepherd, R. M., Aynsley-Green, A., and Lindley, K. J. 2004. 'Hyperinsulinism in infancy: from basic science to clinical disease', *Physiol Rev*, 84(1): 239-75.

Dunne, M. J., and Petersen, O. H. 1986. 'Intracellular ADP activates $K^+$ channels that are inhibited by ATP in an insulin-secreting cell line', *FEBS Lett*, 208(1): 59-62.

Durmaz, E., Flanagan, S. E., Parlak, M., Ellard, S., Akcurin, S., and Bircan, I. 2014. 'A combination of nifedipine and octreotide treatment in an hyperinsulinemic hypoglycemic infant', *J Clin Res Pediatr Endocrinol*, 6(2): 119-21.

Dzeja, P. P., and Terzic, A. 2003. 'Phosphotransfer networks and cellular energetics', *J Exp Biol*, 206(Pt 12): 2039-47.

Eichmann, D., Hufnagel, M., Quick, P., and Santer, R. 1999. 'Treatment of hyperinsulinaemic hypoglycaemia with nifedipine', *Eur J Pediatr*, 158(3): 204-6.

Eto, K., Tsubamoto, Y., Terauchi, Y., Sugiyama, T., Kishimoto, T., Takahashi, N., Yamauchi, N., Kubota, N., Murayama, S., Aizawa, T., Akanuma, Y., Aizawa, S., Kasai, H., Yazaki, Y., and Kadowaki, T. 1999. 'Role of NADH shuttle system in glucose-induced activation of mitochondrial metabolism and insulin secretion', *Science*, 283(5404): 981-5.

Flanagan, S. E., Patch, A. M., Mackay, D. J., Edghill, E. L., Gloyn, A. L., Robinson, D., Shield, J. P., Temple, K., Ellard, S., and Hattersley, A. T. 2007. 'Mutations in ATP-sensitive $K^+$ channel genes cause transient neonatal diabetes and permanent diabetes in childhood or adulthood', *Diabetes*, 56(7): 1930-7.

Fournet, J. C., Mayaud, C., de Lonlay, P., Gross-Morand, M. S., Verkarre, V., Castanet, M., Devillers, M., Rahier, J., Brunelle, F., Robert, J. J., Nihoul-Fékété, C., Saudubray, J. M., and Junien, C. 2001. 'Unbalanced expression of 11p15 imprinted genes in focal forms of congenital hyperinsulinism: association with a reduction to homozygosity of a mutation in ABCC8 or KCNJ11', *Am J Pathol*, 158(6): 2177-84.

Fujimoto, S., Nabe, K., Takehiro, M., Shimodahira, M., Kajikawa, M., Takeda, T., Mukai, E., Inagaki, N., and Seino, Y. 2007. 'Impaired metabolism-secretion coupling in pancreatic beta-cells: role of determinants of mitochondrial ATP production', *Diabetes Res Clin Pract*, 77 Suppl 1: S2-10.

Galcheva, S., Demirbilek, H., Al-Khawaga, S., and Hussain, K. 2019. 'The Genetic and Molecular Mechanisms of Congenital Hyperinsulinism', *Front Endocrinol (Lausanne)*, 10: 111.

Gerbitz, K. D., Gempel, K., and Brdiczka, D. 1996. 'Mitochondria and diabetes. Genetic, biochemical, and clinical implications of the cellular energy circuit', *Diabetes*, 45(2): 113-26.

Gilon, P., Shepherd, R. M., and Henquin, J. C. 1993. 'Oscillations of secretion driven by oscillations of cytoplasmic $Ca^{2+}$ as evidences in single pancreatic islets', *J Biol Chem*, 268(30): 22265-8.

Glaser, B., Blech, I., Krakinovsky, Y., Ekstein, J., Gillis, D., Mazor-Aronovitch, K., Landau, H., and Abeliovich, D. 2011. 'ABCC8 mutation allele frequency in the Ashkenazi Jewish population and risk of focal hyperinsulinemic hypoglycemia', *Genet Med*, 13(10): 891-4.

Glaser, B., Thornton, P., Otonkoski, T., and Junien, C. 2000. 'Genetics of neonatal hyperinsulinism', *Arch Dis Child Fetal Neonatal Ed*, 82(2): F79-86.

Gloyn, A. L., Pearson, E. R., Antcliff, J. F., Proks, P., Bruining, G. J., Slingerland, A. S., Howard, N., Srinivasan, S., Silva, J. M., Molnes, J., Edghill, E. L., Frayling, T. M., Temple, I. K., Mackay, D., Shield, J. P., Sumnik, Z., van Rhijn, A., Wales, J. K., Clark, P., Gorman, S., Aisenberg, J., Ellard, S., Njolstad, P. R., Ashcroft, F. M., and Hattersley, A. T. 2004. 'Activating mutations in the gene encoding the ATP-sensitive potassium-channel subunit Kir6.2 and permanent neonatal diabetes', *N Engl J Med*, 350(18): 1838-49.

Goforth, P. B., Bertram, R., Khan, F. A., Zhang, M., Sherman, A., and Satin, L. S. 2002. 'Calcium-activated $K^+$Channels of Mouse β-cells are Controlled by Both Store and Cytoplasmic $Ca^{2+}$', *The Journal of General Physiology*, 120(3): 307-22.

Goodner, C. J., Walike, B. C., Koerker, D. J., Ensinck, J. W., Brown, A. C., Chideckel, E. W., Palmer, J., and Kalnasy, L. 1977. 'Insulin, glucagon, and glucose exhibit synchronous, sustained oscillations in fasting monkeys', *Science*, 195(4274): 177-9.

Göpel, S. O., Kanno, T., Barg, S., Eliasson, L., Galvanovskis, J., Renstrom, E., and Rorsman, P. 1999. 'Activation of $Ca^{2+}$-dependent $K^+$ channels contributes to rhythmic firing of action potentials in mouse pancreatic beta cells', *J Gen Physiol*, 114(6): 759-70.

Gribble, F. M., and Reimann, F. 2003. 'Differential selectivity of insulin secretagogues: mechanisms, clinical implications, and drug interactions', *J Diabetes Complications*, 17(2 Suppl): 11-5.

Gribble, F. M., Tucker, S. J., and Ashcroft, F. M. 1997. 'The essential role of the Walker A motifs of SUR1 in $K_{ATP}$ channel activation by Mg-ADP and diazoxide', *EMBO J*, 16(6): 1145-52.

Gribble, F. M., Tucker, S. J., Haug, T., and Ashcroft, F. M. 1998. 'MgATP activates the beta cell $K_{ATP}$ channel by interaction with its SUR1 subunit', *Proc Natl Acad Sci U S A*, 95(12): 7185-90.

Güemes, M., Dastamani, A., Ashworth, M., Morgan, K., Ellard, S., Flanagan, S. E., Dattani, M., and Shah, P. 2019. 'Sirolimus: Efficacy and Complications in Children With Hyperinsulinemic Hypoglycemia: A 5-Year Follow-Up Study', *J Endocr Soc*, 3(4): 699-713.

Hambrock, A., Loffler-Walz, C., Kurachi, Y., and Quast, U. 1998. '$Mg^{2+}$ and ATP dependence of $K_{ATP}$ channel modulator binding to the recombinant sulphonylurea receptor, SUR2B', *Br J Pharmacol*, 125(3): 577-83.

Hansen, J. B., Arkhammar, P. O., Bodvarsdottir, T. B., and Wahl, P. 2004. 'Inhibition of insulin secretion as a new drug target in the treatment of metabolic disorders', *Curr Med Chem*, 11(12): 1595-615.

Hawkes, C. P., Adzick, N. S., Palladino, A. A., and De León, D. D. 2016. 'Late Presentation of Fulminant Necrotizing Enterocolitis in a Child with Hyperinsulinism on Octreotide Therapy', *Horm Res Paediatr*, 86(2): 131-36.

Hellman, B., Sehlin, J., and Taljedal, I. 1971. 'Uptake of alanine, arginine and leucine by mammalian pancreatic beta-cells', *Endocrinology*, 89(6): 1432-9.

Henquin, J. C. 1990. 'Role of voltage- and $Ca^{2+}$-dependent $K^+$ channels in the control of glucose-induced electrical activity in pancreatic B-cells', *Pflugers Arch*, 416(5): 568-72.

Henquin, J. C. 2009. 'Regulation of insulin secretion: a matter of phase control and amplitude modulation', *Diabetologia*, 52(5): 739-51.

Herrera, A., Vajravelu, M. E., Givler, S., Mitteer, L., Avitabile, C. M., Lord, K., and De Leon, D. D. 2018. 'Prevalence of Adverse Events in Children With Congenital Hyperinsulinism Treated With Diazoxide', *J Clin Endocrinol Metab*, 103(12): 4365-72.

Hibino, H., Inanobe, A., Furutani, K., Murakami, S., Findlay, I., and Kurachi, Y. 2010. 'Inwardly rectifying potassium channels: their structure, function, and physiological roles', *Physiol Rev*, 90(1): 291-366.

Hoffman, J. F., Joiner, W., Nehrke, K., Potapova, O., Foye, K., and Wickrema, A. 2003. 'The hSK4 (KCNN4) isoform is the $Ca^{2+}$-activated $K^+$ channel (Gardos channel) in human red blood cells', *Proc Natl Acad Sci U S A*, 100(12): 7366-71.

Holst, J. J., Bersani, M., Hvidberg, A., Knigge, U., Christiansen, E., Madsbad, S., Harling, H., and Kofod, H. 1993. 'On the effects of human galanin in man', *Diabetologia*, 36(7): 653-7.

Hopkins, W. F., Fatherazi, S., Peter-Riesch, B., Corkey, B. E., and Cook, D. L. 1992. 'Two sites for adenine-nucleotide regulation of ATP-sensitive potassium channels in mouse pancreatic beta-cells and HIT cells', *J Membr Biol*, 129(3): 287-95.

Houamed, K. M., Sweet, I. R., and Satin, L. S. 2010. 'BK channels mediate a novel ionic mechanism that regulates glucose-dependent electrical activity and insulin secretion in mouse pancreatic beta-cells', *J Physiol*, 588(Pt 18): 3511-23.

Houde, V. P., Brule, S., Festuccia, W. T., Blanchard, P. G., Bellmann, K., Deshaies, Y., and Marette, A. 2010. 'Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue', *Diabetes*, 59(6): 1338-48.

Huopio, H., Otonkoski, T., Vauhkonen, I., Reimann, F., Ashcroft, F. M., and Laakso, M. 2003. 'A new subtype of autosomal dominant diabetes attributable to a mutation in the gene for sulfonylurea receptor 1', *Lancet*, 361(9354): 301-7.

Huopio, H., Reimann, F., Ashfield, R., Komulainen, J., Lenko, H. L., Rahier, J., Vauhkonen, I., Kere, J., Laakso, M., Ashcroft, F., and Otonkoski, T. 2000. 'Dominantly inherited hyperinsulinism caused by a mutation in the sulfonylurea receptor type 1', *J Clin Invest*, 106(7): 897-906.

Hussain, K. 2007. 'Insights in congenital hyperinsulinism', *Endocr Dev*, 11: 106-21.

Hussain, K. 2008. 'Diagnosis and management of hyperinsulinaemic hypoglycaemia of infancy', *Horm Res*, 69(1): 2-13.

Hussain, M. A., Akalestou, E., and Song, W. J. 2016. 'Inter-organ communication and regulation of beta cell function', *Diabetologia*, 59(4): 659-67.

Inagaki, N., Gonoi, T., Clement, J. P. th, Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S., and Bryan, J. 1995. 'Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor', *Science*, 270(5239): 1166-70.

Inagaki, N., Gonoi, T., Clement, J. P., Wang, C. Z., Aguilar-Bryan, L., Bryan, J., and Seino, S. 1996. 'A family of sulfonylurea receptors determines the pharmacological properties of ATP-sensitive $K^+$ channels', *Neuron*, 16(5): 1011-7.

Inagaki, N., Gonoi, T., and Seino, S. 1997. 'Subunit stoichiometry of the pancreatic beta-cell ATP-sensitive $K^+$ channel', *FEBS Lett*, 409(2): 232-6.

Isomoto, S., Kondo, C., Yamada, M., Matsumoto, S., Higashiguchi, O., Horio, Y., Matsuzawa, Y., and Kurachi, Y. 1996. 'A novel sulfonylurea receptor forms with BIR (Kir6.2) a smooth muscle type ATP-sensitive $K^+$ channel', *J Biol Chem*, 271(40): 24321-4.

Issafras, H., Bedinger, D. H., Corbin, J. A., Goldfine, I. D., Bhaskar, V., White, M. L., Rubin, P., and Scannon, P. J. 2014. 'Selective allosteric antibodies to the insulin receptor for the treatment of hyperglycemic and hypoglycemic disorders', *J Diabetes Sci Technol*, 8(4): 865-73.

Jacobson, D. A., Kuznetsov, A., Lopez, J. P., Kash, S., Ammala, C. E., and Philipson, L. H. 2007. 'Kv2.1 ablation alters glucose-induced islet electrical activity, enhancing insulin secretion', *Cell Metab*, 6(3): 229-35.

Jacobson, D. A., Mendez, F., Thompson, M., Torres, J., Cochet, O., and Philipson, L. H. 2010. 'Calcium-activated and voltage-gated potassium channels of the pancreatic islet impart distinct and complementary roles during secretagogue induced electrical responses', *J Physiol*, 588(Pt 18): 3525-37.

Jacobson, D. A., and Philipson, L. H. 2007. 'Action potentials and insulin secretion: new insights into the role of Kv channels', *Diabetes Obes Metab*, 9 Suppl 2: 89-98.

James, C., Kapoor, R. R., Ismail, D., and Hussain, K. 2009. 'The genetic basis of congenital hyperinsulinism', *J Med Genet*, 46(5): 289-99.

Jensen, B. S., Strøbaek, D., Christophersen, P., Jørgensen, T. D., Hansen, C., Silahtaroglu, A., Olesen, S. P., and Ahring, P. K. 1998. 'Characterization of the

cloned human intermediate-conductance $Ca^{2+}$-activated $K^+$ channel', *Am J Physiol*, 275(3): C848-56.

Johnson, K. W., Neale, A., Gordon, A., Roessig, J., Bezwada, P., Vukelich, S., Goldfine, I., and Rubin, P. 2017a. 'Attenuation of Insulin Action by an Allosteric Insulin Receptor Antibody in Healthy Volunteers', *J Clin Endocrinol Metab*, 102(8): 3021-28.

Johnson, K.W, Neale, A.C, Gordon, A, De León-Crutchlow, D.D.C, Hussain, K, Mohnike, K.L., Vukelich, S, Roessig, J.M, and Rubin, P.D. 2017b. "Activity of Xoma 358, an Inhibitor of insulin Action Following Short-Term Administration to Congenital Hyperinsulinism Patients." In, 38. Endocrine Reviews.

Jones, P. M., Fyles, J. M., Persaud, S. J., and Howell, S. L. 1987. 'Catecholamine inhibition of $Ca^{2+}$-induced insulin secretion from electrically permeabilised islets of Langerhans', *FEBS Lett*, 219(1): 139-44.

Kailey, B., van de Bunt, M., Cheley, S., Johnson, P. R., MacDonald, P. E., Gloyn, A. L., Rorsman, P., and Braun, M. 2012. 'SSTR2 is the functionally dominant somatostatin receptor in human pancreatic beta- and alpha-cells', *Am J Physiol Endocrinol Metab*, 303(9): E1107-16.

Kakei, M., Kelly, R. P., Ashcroft, S. J., and Ashcroft, F. M. 1986. 'The ATP-sensitivity of $K^+$ channels in rat pancreatic B-cells is modulated by ADP', *FEBS Lett*, 208(1): 63-6.

Kanno, T., Rorsman, P., and Göpel, S. O. 2002. 'Glucose-dependent regulation of rhythmic action potential firing in pancreatic β-cells by kATP-channel modulation', *The Journal of Physiology*, 545(2): 501-07.

Kapoor, R. R., Flanagan, S. E., James, C., Shield, J., Ellard, S., and Hussain, K. 2009. 'Hyperinsulinaemic hypoglycaemia', *Arch Dis Child*, 94(6): 450-7.

Katz, M. D., and Erstad, B. L. 1989. 'Octreotide, a new somatostatin analogue', *Clin Pharm*, 8(4): 255-73.

Kennedy, R. T., Kauri, L. M., Dahlgren, G. M., and Jung, S. K. 2002. 'Metabolic oscillations in beta-cells', *Diabetes*, 51 Suppl 1: S152-61.

Kijima, S., and Kijima, H. 1987. 'Statistical analysis of channel current from a membrane patch. I. Some stochastic properties of ion channels or molecular systems in equilibrium', *J Theor Biol*, 128(4): 423-34.

Kindmark, H., Kohler, M., Brown, G., Branstrom, R., Larsson, O., and Berggren, P. O. 2001. 'Glucose-induced oscillations in cytoplasmic free $Ca^{2+}$ concentration precede oscillations in mitochondrial membrane potential in the pancreatic beta-cell', *J Biol Chem*, 276(37): 34530-6.

Kjems, L. L., Ravier, M. A., Jonas, J. C., and Henquin, J. C. 2002. 'Do oscillations of insulin secretion occur in the absence of cytoplasmic $Ca^{2+}$ oscillations in beta-cells?', *Diabetes*, 51 Suppl 1: S177-82.

Koren, I., Riskin, A., Barthlen, W., and Gillis, D. 2013. 'Hepatitis in an infant treated with octreotide for congenital hyperinsulinism', *J Pediatr Endocrinol Metab*, 26(1-2): 183-5.

Kozlowski, R. Z., Hales, C. N., and Ashford, M. L. 1989. 'Dual effects of diazoxide on ATP-$K^+$ currents recorded from an insulin-secreting cell line', *Br J Pharmacol*, 97(4): 1039-50.

Krippeit-Drews, P., Backer, M., Düfer, M., and Drews, G. 2003. 'Phosphocreatine as a determinant of K(ATP) channel activity in pancreatic beta-cells', *Pflugers Arch*, 445(5): 556-62.

Krippeit-Drews, P., Düfer, M., and Drews, G. 2000. 'Parallel oscillations of intracellular calcium activity and mitochondrial membrane potential in mouse pancreatic B-cells', *Biochem Biophys Res Commun*, 267(1): 179-83.

Kukuljan, M., Goncalves, A. A., and Atwater, I. 1991. 'Charybdotoxin-sensitive K(Ca) channel is not involved in glucose-induced electrical activity in pancreatic beta-cells', *J Membr Biol*, 119(2): 187-95.

Lamberts, S. W., van der Lely, A. J., de Herder, W. W., and Hofland, L. J. 1996. 'Octreotide', *N Engl J Med*, 334(4): 246-54.

Lang, R., Gundlach, A. L., Holmes, F. E., Hobson, S. A., Wynick, D., Hokfelt, T., and Kofler, B. 2015. 'Physiology, signaling, and pharmacology of galanin peptides and receptors: three decades of emerging diversity', *Pharmacol Rev*, 67(1): 118-75.

Larsson, O., Ämmälä, C., Bokvist, K., Fredholm, B., and Rorsman, P. 1993. 'Stimulation of the KATP channel by ADP and diazoxide requires nucleotide hydrolysis in mouse pancreatic beta-cells', *J Physiol*, 463: 349-65.

Le Quan Sang, K. H., Arnoux, J. B., Mamoune, A., Saint-Martin, C., Bellanné-Chantelot, C., Valayannopoulos, V., Brassier, A., Kayirangwa, H., Barbier, V., Broissand, C., Fabreguettes, J. R., Charron, B., Thalabard, J. C., and de Lonlay, P. 2012. 'Successful treatment of congenital hyperinsulinism with long-acting release octreotide', *Eur J Endocrinol*, 166(2): 333-9.

Ledoux, J., Werner, M. E., Brayden, J. E., and Nelson, M. T. 2006. 'Calcium-activated potassium channels and the regulation of vascular tone', *Physiology (Bethesda)*, 21: 69-78.

Lee, K. P. K., Chen, J., and MacKinnon, R. 2017. 'Molecular structure of human $K_{ATP}$ in complex with ATP and ADP', *Elife*, 6.

Li, L., Wang, J., and Drain, P. 2000. 'The I182 region of k(ir)6.2 is closely associated with ligand binding in K(ATP) channel inhibition by ATP', *Biophys J*, 79(2): 841-52.

Li, N., Wu, J. X., Ding, D., Cheng, J., Gao, N., and Chen, L. 2017. 'Structure of a Pancreatic ATP-Sensitive Potassium Channel', *Cell*, 168(1-2): 101-10 e10.

Light, P. E., Manning Fox, J. E., Riedel, M. J., and Wheeler, M. B. 2002. 'Glucagon-like peptide-1 inhibits pancreatic ATP-sensitive potassium channels via a protein kinase A- and ADP-dependent mechanism', *Mol Endocrinol*, 16(9): 2135-44.

Lin, Y. F., and Chai, Y. 2008. 'Functional modulation of the ATP-sensitive potassium channel by extracellular signal-regulated kinase-mediated phosphorylation', *Neuroscience*, 152(2): 371-80.

Linton, K. J., and Higgins, C. F. 2007. 'Structure and function of ABC transporters: the ATP switch provides flexible control', *Pflugers Arch*, 453(5): 555-67.

Lovvorn, H. N., 3rd, Nance, M. L., Ferry, R. J., Jr., Stolte, L., Baker, L., O'Neill, J. A., Jr., Schnaufer, L., Stanley, C. A., and Adzick, N. S. 1999. 'Congenital hyperinsulinism and the surgeon: lessons learned over 35 years', *J Pediatr Surg*, 34(5): 786-92; discussion 92-3.

MacDonald, M. J. 1982. 'Evidence for the malate aspartate shuttle in pancreatic islets', *Arch Biochem Biophys*, 213(2): 643-9.

MacDonald, P. E., and Wheeler, M. B. 2003. 'Voltage-dependent $K^+$ channels in pancreatic beta cells: role, regulation and potential as therapeutic targets', *Diabetologia*, 46(8): 1046-62.

Maczewsky, J., Sikimic, J., Bauer, C., Krippeit-Drews, P., Wolke, C., Lendeckel, U., Barthlen, W., and Drews, G. 2017. 'The LXR Ligand T0901317 Acutely Inhibits Insulin Secretion by Affecting Mitochondrial Metabolism', *Endocrinology*, 158(7): 2145-54.

Männikkö, R., Flanagan, S. E., Sim, X., Segal, D., Hussain, K., Ellard, S., Hattersley, A. T., and Ashcroft, F. M. 2011. 'Mutations of the same conserved glutamate residue in NBD2 of the sulfonylurea receptor 1 subunit of the KATP channel can result in either hyperinsulinism or neonatal diabetes', *Diabetes*, 60(6): 1813-22.

Manning Fox, J. E., Gyulkhandanyan, A. V., Satin, L. S., and Wheeler, M. B. 2006. 'Oscillatory membrane potential response to glucose in islet beta-cells: a comparison of islet-cell electrical activity in mouse and rat', *Endocrinology*, 147(10): 4655-63.

Mao, Q., Leslie, E. M., Deeley, R. G., and Cole, S. P. 1999. 'ATPase activity of purified and reconstituted multidrug resistance protein MRP1 from drug-selected H69AR cells', *Biochim Biophys Acta*, 1461(1): 69-82.

Martin, G. M., Kandasamy, B., DiMaio, F., Yoshioka, C., and Shyng, S. L. 2017b. 'Anti-diabetic drug binding site in a mammalian KATP channel revealed by Cryo-EM', *Elife*, 6.

Martin, G. M., Yoshioka, C., Rex, E. A., Fay, J. F., Xie, Q., Whorton, M. R., Chen, J. Z., and Shyng, S. L. 2017a. 'Cryo-EM structure of the ATP-sensitive potassium channel illuminates mechanisms of assembly and gating', *Elife*, 6.

Masia, R., Enkvetchakul, D., and Nichols, C. G. 2005. 'Differential nucleotide regulation of $K_{ATP}$ channels by SUR1 and SUR2A', *J Mol Cell Cardiol*, 39(3): 491-501.

Masia, R., Koster, J. C., Tumini, S., Chiarelli, F., Colombo, C., Nichols, C. G., and Barbetti, F. 2007. 'An ATP-binding mutation (G334D) in KCNJ11 is associated with a sulfonylurea-insensitive form of developmental delay, epilepsy, and neonatal diabetes', *Diabetes*, 56(2): 328-36.

Matsuo, M., Kimura, Y., and Ueda, K. 2005. '$K_{ATP}$ channel interaction with adenine nucleotides', *J Mol Cell Cardiol*, 38(6): 907-16.

McCulloch, L. J., van de Bunt, M., Braun, M., Frayn, K. N., Clark, A., and Gloyn, A. L. 2011. 'GLUT2 (SLC2A2) is not the principal glucose transporter in human pancreatic beta cells: implications for understanding genetic association signals at this locus', *Mol Genet Metab*, 104(4): 648-53.

McMahon, A. W., Wharton, G. T., Thornton, P., and De León, D. D. 2017. 'Octreotide use and safety in infants with hyperinsulinism', *Pharmacoepidemiol Drug Saf*, 26(1): 26-31.

Meissner, H. P., and Schmelz, H. 1974. 'Membrane potential of beta-cells in pancreatic islets', *Pflugers Arch*, 351(3): 195-206.

Meissner, T., Wendel, U., Burgard, P., Schaetzle, S., and Mayatepek, E. 2003. 'Long-term follow-up of 114 patients with congenital hyperinsulinism', *Eur J Endocrinol*, 149(1): 43-51.

Mikhailov, M. V., Campbell, J. D., de Wet, H., Shimomura, K., Zadek, B., Collins, R. F., Sansom, M. S., Ford, R. C., and Ashcroft, F. M. 2005. '3-D structural and functional characterization of the purified $K_{ATP}$ channel complex Kir6.2-SUR1', *EMBO J*, 24(23): 4166-75.

Misler, S., Barnett, D. W., Gillis, K. D., and Pressel, D. M. 1992a. 'Electrophysiology of stimulus-secretion coupling in human beta-cells', *Diabetes*, 41(10): 1221-8.

Misler, S., Barnett, D. W., Pressel, D. M., Gillis, K. D., Scharp, D. W., and Falke, L. C. 1992b. 'Stimulus-secretion coupling in beta-cells of transplantable human islets of Langerhans. Evidence for a critical role for $Ca^{2+}$ entry', *Diabetes*, 41(6): 662-70.

Modan-Moses, D., Koren, I., Mazor-Aronovitch, K., Pinhas-Hamiel, O., and Landau, H. 2011. 'Treatment of congenital hyperinsulinism with lanreotide acetate (Somatuline Autogel)', *J Clin Endocrinol Metab*, 96(8): 2312-7.

Mohnike, K., Blankenstein, O., Pfuetzner, A., Pötzsch, S., Schober, E., Steiner, S., Hardy, O. T., Grimberg, A., and van Waarde, W. M. 2008. 'Long-term non-surgical therapy of severe persistent congenital hyperinsulinism with glucagon', *Horm Res*, 70(1): 59-64.

Moody, J. E., Millen, L., Binns, D., Hunt, J. F., and Thomas, P. J. 2002. 'Cooperative, ATP-dependent association of the nucleotide binding cassettes during the catalytic cycle of ATP-binding cassette transporters', *J Biol Chem*, 277(24): 21111-4.

Moreau, C., Gally, F., Jacquet-Bouix, H., and Vivaudou, M. 2005. 'The size of a single residue of the sulfonylurea receptor dictates the effectiveness of $K_{ATP}$ channel openers', *Mol Pharmacol*, 67(4): 1026-33.

Nam, Y. W., Orfali, R., Liu, T., Yu, K., Cui, M., Wulff, H., and Zhang, M. 2017. 'Structural insights into the potency of SK channel positive modulators', *Sci Rep*, 7(1): 17178.

Nebesio, T. D., Hoover, W. C., Caldwell, R. L., Nitu, M. E., and Eugster, E. A. 2007. 'Development of pulmonary hypertension in an infant treated with diazoxide', *J Pediatr Endocrinol Metab*, 20(8): 939-44.

Nestorowicz, A., Wilson, B. A., Schoor, K. P., Inoue, H., Glaser, B., Landau, H., Stanley, C. A., Thornton, P. S., Clement, J. P. th, Bryan, J., Aguilar-Bryan, L., and Permutt, M. A. 1996. 'Mutations in the sulonylurea receptor gene are associated with familial hyperinsulinism in Ashkenazi Jews', *Hum Mol Genet*, 5(11): 1813-22.

Neylon, O. M., Moran, M. M., Pellicano, A., Nightingale, M., and O'Connell, M. A. 2013. 'Successful subcutaneous glucagon use for persistent hypoglycaemia in congenital hyperinsulinism', *J Pediatr Endocrinol Metab*, 26(11-12): 1157-61.

Nichols, C. G., Shyng, S. L., Nestorowicz, A., Glaser, B., Clement, J. P. th, Gonzalez, G., Aguilar-Bryan, L., Permutt, M. A., and Bryan, J. 1996. 'Adenosine diphosphate as an intracellular regulator of insulin secretion', *Science*, 272(5269): 1785-7.

Nielsen, F. E., Bodvarsdottir, T. B., Worsaae, A., MacKay, P., Stidsen, C. E., Boonen, H. C., Pridal, L., Arkhammar, P. O., Wahl, P., Ynddal, L., Junager, F., Dragsted, N., Tagmose, T. M., Mogensen, J. P., Koch, A., Treppendahl, S. P., and Hansen, J. B. 2002. '6-Chloro-3-alkylamino-4H-thieno[3,2-e]-1,2,4-thiadiazine 1,1-dioxide derivatives potently and selectively activate ATP sensitive potassium channels of pancreatic beta-cells', *J Med Chem*, 45(19): 4171-87.

Nilsson, T., Arkhammar, P., Rorsman, P., and Berggren, P. O. 1989. 'Suppression of insulin release by galanin and somatostatin is mediated by a G-protein. An effect involving repolarization and reduction in cytoplasmic free $Ca^{2+}$ concentration', *J Biol Chem*, 264(2): 973-80.

Oldham, M. L., Khare, D., Quiocho, F. A., Davidson, A. L., and Chen, J. 2007. 'Crystal structure of a catalytic intermediate of the maltose transporter', *Nature*, 450(7169): 515-21.

Orelle, C., Dalmas, O., Gros, P., Di Pietro, A., and Jault, J. M. 2003. 'The conserved glutamate residue adjacent to the Walker-B motif is the catalytic base for ATP hydrolysis in the ATP-binding cassette transporter BmrA', *J Biol Chem*, 278(47): 47002-8.

Ortiz, D., and Bryan, J. 2015. 'Neonatal Diabetes and Congenital Hyperinsulinism Caused by Mutations in ABCC8/SUR1 are Associated with Altered and Opposite Affinities for ATP and ADP', *Front Endocrinol (Lausanne)*, 6: 48.

Ortiz, D., Gossack, L., Quast, U., and Bryan, J. 2013. 'Reinterpreting the action of ATP analogs on $K_{ATP}$ channels', *J Biol Chem*, 288(26): 18894-902.

Ortiz, D., Voyvodic, P., Gossack, L., Quast, U., and Bryan, J. 2012. 'Two neonatal diabetes mutations on transmembrane helix 15 of SUR1 increase affinity for ATP and ADP at nucleotide binding domain 2', *J Biol Chem*, 287(22): 17985-95.

Panten, U., Heipel, C., Rosenberger, F., Scheffer, K., Zunkler, B. J., and Schwanstecher, C. 1990. 'Tolbutamide-sensitivity of the adenosine 5'-triphosphate-dependent $K^+$ channel in mouse pancreatic B-cells', *Naunyn Schmiedebergs Arch Pharmacol*, 342(5): 566-74.

Patel, Y. C. 1999. 'Somatostatin and its receptor family', *Front Neuroendocrinol*, 20(3): 157-98.

Patel, Y. C., Greenwood, M. T., Warszynska, A., Panetta, R., and Srikant, C. B. 1994. 'All five cloned human somatostatin receptors (hSSTR1-5) are functionally coupled to adenylyl cyclase', *Biochem Biophys Res Commun*, 198(2): 605-12.

Payen, L. F., Gao, M., Westlake, C. J., Cole, S. P., and Deeley, R. G. 2003. 'Role of carboxylate residues adjacent to the conserved core Walker B motifs in the catalytic cycle of multidrug resistance protein 1 (ABCC1)', *J Biol Chem*, 278(40): 38537-47.

Peiris, A. N., Stagner, J. I., Vogel, R. L., Nakagawa, A., and Samols, E. 1992. 'Body fat distribution and peripheral insulin sensitivity in healthy men: role of insulin pulsatility', *J Clin Endocrinol Metab*, 75(1): 290-4.

Pinney, S. E., MacMullen, C., Becker, S., Lin, Y. W., Hanna, C., Thornton, P., Ganguly, A., Shyng, S. L., and Stanley, C. A. 2008. 'Clinical characteristics and biochemical mechanisms of congenital hyperinsulinism associated with dominant $K_{ATP}$ channel mutations', *J Clin Invest*, 118(8): 2877-86.

Plant, T. D. 1988. 'Na$^+$ currents in cultured mouse pancreatic B-cells', *Pflugers Arch*, 411(4): 429-35.

Porksen, N., Hollingdal, M., Juhl, C., Butler, P., Veldhuis, J. D., and Schmitz, O. 2002. 'Pulsatile insulin secretion: detection, regulation, and role in diabetes', *Diabetes*, 51 Suppl 1: S245-54.

Pressel, D. M., and Misler, S. 1990. 'Sodium channels contribute to action potential generation in canine and human pancreatic islet B cells', *J Membr Biol*, 116(3): 273-80.

Proks, P., de Wet, H., and Ashcroft, F. M. 2010. 'Activation of the $K_{ATP}$ channel by Mg-nucleotide interaction with SUR1', *J Gen Physiol*, 136(4): 389-405.

Proks, P., de Wet, H., and Ashcroft, F. M. 2013. 'Molecular mechanism of sulphonylurea block of $K_{ATP}$ channels carrying mutations that impair ATP inhibition and cause neonatal diabetes', *Diabetes*, 62(11): 3909-19.

Proks, P., de Wet, H., and Ashcroft, F. M. 2014. 'Sulfonylureas suppress the stimulatory action of Mg-nucleotides on Kir6.2/SUR1 but not Kir6.2/SUR2A $K_{ATP}$ channels: a mechanistic study', *J Gen Physiol*, 144(5): 469-86.

Puljung, M. C. 2018. 'Cryo-electron microscopy structures and progress toward a dynamic understanding of $K_{ATP}$ channels', *J Gen Physiol*, 150(5): 653-69.

Puljung, M., Vedovato, N., Usher, S., and Ashcroft, F. 2019. 'Activation mechanism of ATP-sensitive $K^+$ channels explored with real-time nucleotide binding', *Elife*, 8.

Quan, Y., Barszczyk, A., Feng, Z. P., and Sun, H. S. 2011. 'Current understanding of K ATP channels in neonatal diseases: focus on insulin secretion disorders', *Acta Pharmacol Sin*, 32(6): 765-80.

Rahier, J., Guiot, Y., and Sempoux, C. 2011. 'Morphologic analysis of focal and diffuse forms of congenital hyperinsulinism', *Semin Pediatr Surg*, 20(1): 3-12.

Raphemot, R., Swale, D. R., Dadi, P. K., Jacobson, D. A., Cooper, P., Wojtovich, A. P., Banerjee, S., Nichols, C. G., and Denton, J. S. 2014. 'Direct activation of beta-cell $K_{ATP}$ channels with a novel xanthine derivative', *Mol Pharmacol*, 85(6): 858-65.

Renström, E., Ding, W. G., Bokvist, K., and Rorsman, P. 1996. 'Neurotransmitter-induced inhibition of exocytosis in insulin-secreting beta cells by activation of calcineurin', *Neuron*, 17(3): 513-22.

Ribalet, B., and Eddlestone, G. T. 1995. 'Characterization of the G protein coupling of a somatostatin receptor to the $K^+$ATP channel in insulin-secreting mammalian HIT and RIN cell lines', *J Physiol*, 485 ( Pt 1): 73-86.

Ritzel, R. A., Hansen, J. B., Veldhuis, J. D., and Butler, P. C. 2004. 'Induction of beta-cell rest by a Kir6.2/SUR1-selective $K_{ATP}$-channel opener preserves beta-cell insulin stores and insulin secretion in human islets cultured at high (11 mM) glucose', *J Clin Endocrinol Metab*, 89(2): 795-805.

Riz, M., Braun, M., and Pedersen, M. G. 2014. 'Mathematical modeling of heterogeneous electrophysiological responses in human beta-cells', *PLoS Comput Biol*, 10(1): e1003389.

Rorsman, P., and Ashcroft, F. M. 2018. 'Pancreatic beta-Cell Electrical Activity and Insulin Secretion: Of Mice and Men', *Physiol Rev*, 98(1): 117-214.

Rorsman, P., Bokvist, K., Ämmälä, C., Arkhammar, P., Berggren, P. O., Larsson, O., and Wahlander, K. 1991. 'Activation by adrenaline of a low-conductance G protein-dependent $K^+$ channel in mouse pancreatic B cells', *Nature*, 349(6304): 77-9.

Rorsman, P., and Braun, M. 2013. 'Regulation of insulin secretion in human pancreatic islets', *Annu Rev Physiol*, 75: 155-79.

Rorsman, P., Braun, M., and Zhang, Q. 2012. 'Regulation of calcium in pancreatic alpha- and beta-cells in health and disease', *Cell Calcium*, 51(3-4): 300-8.

Rosario, L. M., Barbosa, R. M., Antunes, C. M., Silva, A. M., Abrunhosa, A. J., and Santos, R. M. 1993. 'Bursting electrical activity in pancreatic beta-cells: evidence that the channel underlying the burst is sensitive to $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels', *Pflugers Arch*, 424(5-6): 439-47.

Rothberg, B. S., and Magleby, K. L. 2001. 'Testing for detailed balance (microscopic reversibility in ion channel gating', *Biophys J*, 80(6): 3025-6.

Rubio-Cabezas, O., and Ellard, S. 2013. 'Diabetes mellitus in neonates and infants: genetic heterogeneity, clinical approach to diagnosis, and therapeutic options', *Horm Res Paediatr*, 80(3): 137-46.

Saint-Martin, C., Arnoux, J. B., de Lonlay, P., and Bellanné-Chantelot, C. 2011. '$K_{ATP}$ channel mutations in congenital hyperinsulinism', *Semin Pediatr Surg*, 20(1): 18-22.

Santos, R. M., Rosario, L. M., Nadal, A., Garcia-Sancho, J., Soria, B., and Valdeolmillos, M. 1991. 'Widespread synchronous $[Ca^{2+}]_i$ oscillations due to bursting electrical activity in single pancreatic islets', *Pflugers Arch*, 418(4): 417-22.

Sarabu, R., and Tilley, J. 2005. 'Recent advances in therapeutic approaches to type 2 diabetes.' in A. M. Doherty (ed.), *Annual Reports in Medicinal Chemistry* (Elsevier).

Satin, L. S., Butler, P. C., Ha, J., and Sherman, A. S. 2015. 'Pulsatile insulin secretion, impaired glucose tolerance and type 2 diabetes', *Mol Aspects Med*, 42: 61-77.

Schulze, D., Rapedius, M., Krauter, T., and Baukrowitz, T. 2003. 'Long-chain acyl-CoA esters and phosphatidylinositol phosphates modulate ATP inhibition of $K_{ATP}$ channels by the same mechanism', *J Physiol*, 552(Pt 2): 357-67.

Schulze, D. U., Düfer, M., Wieringa, B., Krippeit-Drews, P., and Drews, G. 2007. 'An adenylate kinase is involved in $K_{ATP}$ channel regulation of mouse pancreatic beta cells', *Diabetologia*, 50(10): 2126-34.

Schwanstecher, M., Sieverding, C., Dorschner, H., Gross, I., Aguilar-Bryan, L., Schwanstecher, C., and Bryan, J. 1998. 'Potassium channel openers require ATP to bind to and act through sulfonylurea receptors', *EMBO J*, 17(19): 5529-35.

Sempoux, C., Capito, C., Bellanne-Chantelot, C., Verkarre, V., de Lonlay, P., Aigrain, Y., Fekete, C., Guiot, Y., and Rahier, J. 2011. 'Morphological mosaicism of the pancreatic islets: a novel anatomopathological form of persistent hyperinsulinemic hypoglycemia of infancy', *J Clin Endocrinol Metab*, 96(12): 3785-93.

Shah, P., Rahman, S. A., Demirbilek, H., Güemes, M., and Hussain, K. 2017. 'Hyperinsulinaemic hypoglycaemia in children and adults', *Lancet Diabetes Endocrinol*, 5(9): 729-42.

Shah, P., Rahman, S. A., McElroy, S., Gilbert, C., Morgan, K., Hinchey, L., Senniappan, S., Levy, H., Amin, R., and Hussain, K. 2015. 'Use of Long-Acting Somatostatin Analogue (Lanreotide) in an Adolescent with Diazoxide-Responsive Congenital Hyperinsulinism and Its Psychological Impact', *Horm Res Paediatr*, 84(5): 355-60.

Shapiro, A. B., and Ling, V. 1994. 'ATPase activity of purified and reconstituted P-glycoprotein from Chinese hamster ovary cells', *J Biol Chem*, 269(5): 3745-54.

Sharma, N., Crane, A., Clement, J. P. th, Gonzalez, G., Babenko, A. P., Bryan, J., and Aguilar-Bryan, L. 1999. 'The C terminus of SUR1 is required for trafficking of $K_{ATP}$ channels', *J Biol Chem*, 274(29): 20628-32.

Sharom, F. J., Yu, X., Chu, J. W., and Doige, C. A. 1995. 'Characterization of the ATPase activity of P-glycoprotein from multidrug-resistant Chinese hamster ovary cells', *Biochem J*, 308 ( Pt 2): 381-90.

Sheng, J. Z., Ella, S., Davis, M. J., Hill, M. A., and Braun, A. P. 2009. 'Openers of SKCa and IKCa channels enhance agonist-evoked endothelial nitric oxide synthesis and arteriolar vasodilation', *FASEB J*, 23(4): 1138-45.

Shorter, K., Farjo, N. P., Picksley, S. M., and Randall, V. A. 2008. 'Human hair follicles contain two forms of ATP-sensitive potassium channels, only one of which is sensitive to minoxidil', *FASEB J*, 22(6): 1725-36.

Shuman, C., Smith, A. C., Steele, L., Ray, P. N., Clericuzio, C., Zackai, E., Parisi, M. A., Meadows, A. T., Kelly, T., Tichauer, D., Squire, J. A., Sadowski, P., and Weksberg, R. 2006. 'Constitutional UPD for chromosome 11p15 in individuals with isolated hemihyperplasia is associated with high tumor risk and occurs following assisted reproductive technologies', *Am J Med Genet A*, 140(14): 1497-503.

Shyng, S., Ferrigni, T., and Nichols, C. G. 1997. 'Regulation of $K_{ATP}$ channel activity by diazoxide and MgADP. Distinct functions of the two nucleotide binding folds of the sulfonylurea receptor', *J Gen Physiol*, 110(6): 643-54.

Shyng, S. L., and Nichols, C. G. 1998. 'Membrane phospholipid control of nucleotide sensitivity of $K_{ATP}$ channels', *Science*, 282(5391): 1138-41.

Shyng, S., and Nichols, C. G. 1997. 'Octameric stoichiometry of the $K_{ATP}$ channel complex', *J Gen Physiol*, 110(6): 655-64.

Sieg, A., Su, J., Munoz, A., Buchenau, M., Nakazaki, M., Aguilar-Bryan, L., Bryan, J., and Ullrich, S. 2004. 'Epinephrine-induced hyperpolarization of islet cells without KATP channels', *Am J Physiol Endocrinol Metab*, 286(3): E463-71.

Sikimic, J., McMillen, T. S., Bleile, C., Dastvan, F., Quast, U., Krippeit-Drews, P., Drews, G., and Bryan, J. 2019. 'ATP binding without hydrolysis switches sulfonylurea receptor 1 (SUR1) to outward-facing conformations that activate $K_{ATP}$ channels', *J Biol Chem*, 294(10): 3707-19.

Singh, S., Syme, C. A., Singh, A. K., Devor, D. C., and Bridges, R. J. 2001. 'Benzimidazolone activators of chloride secretion: potential therapeutics for cystic fibrosis and chronic obstructive pulmonary disease', *J Pharmacol Exp Ther*, 296(2): 600-11.

Skelin Klemen, M., Dolensek, J., Slak Rupnik, M., and Stozer, A. 2017. 'The triggering pathway to insulin secretion: Functional similarities and differences between the human and the mouse beta cells and their translational relevance', *Islets*, 9(6): 109-39.

Smit, L. S., Wilkinson, D. J., Mansoura, M. K., Collins, F. S., and Dawson, D. C. 1993. 'Functional roles of the nucleotide-binding folds in the activation of the cystic fibrosis transmembrane conductance regulator', *Proc Natl Acad Sci U S A*, 90(21): 9963-7.

Smith, P. A., Sellers, L. A., and Humphrey, P. P. 2001. 'Somatostatin activates two types of inwardly rectifying $K^+$ channels in MIN-6 cells', *J Physiol*, 532(Pt 1): 127-42.

Smith, P. C., Karpowich, N., Millen, L., Moody, J. E., Rosen, J., Thomas, P. J., and Hunt, J. F. 2002. 'ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer', *Mol Cell*, 10(1): 139-49.

Stanojevic, V., Habener, J. F., Holz, G. G., and Leech, C. A. 2008. 'Cytosolic adenylate kinases regulate $K_{ATP}$ channel activity in human beta-cells', *Biochem Biophys Res Commun*, 368(3): 614-9.

Straub, S. G., and Sharp, G. W. 2002. 'Glucose-stimulated signaling pathways in biphasic insulin secretion', *Diabetes Metab Res Rev*, 18(6): 451-63.

Sturgess, N. C., Kozlowski, R. Z., Carrington, C. A., Hales, C. N., and Ashford, M. L. 1988. 'Effects of sulphonylureas and diazoxide on insulin secretion and nucleotide-sensitive channels in an insulin-secreting cell line', *Br J Pharmacol*, 95(1): 83-94.

Szymanowski, M., Estebanez, M. S., Padidela, R., Han, B., Mosinska, K., Stevens, A., Damaj, L., Pihan-Le Bars, F., Lascouts, E., Reynaud, R., Ferreira, C., Bansept, C., de Lonlay, P., Saint-Martin, C., Dunne, M. J., Banerjee, I., and Arnoux, J. B. 2016. 'mTOR Inhibitors for the Treatment of Severe Congenital Hyperinsulinism: Perspectives on Limited Therapeutic Success', *J Clin Endocrinol Metab*, 101(12): 4719-29.

Tamarina, N. A., Wang, Y., Mariotto, L., Kuznetsov, A., Bond, C., Adelman, J., and Philipson, L. H. 2003. 'Small-conductance calcium-activated $K^+$ channels are expressed in pancreatic islets and regulate glucose responses', *Diabetes*, 52(8): 2000-6.

Tang, G., Wang, Y., Park, S., Bajpayee, N. S., Vi, D., Nagaoka, Y., Birnbaumer, L., and Jiang, M. 2012. 'Go2 G protein mediates galanin inhibitory effects on insulin release from pancreatic beta cells', *Proc Natl Acad Sci U S A*, 109(7): 2636-41.

Tarasov, A. I., Griffiths, E. J., and Rutter, G. A. 2012. 'Regulation of ATP production by mitochondrial $Ca^{2+}$', *Cell Calcium*, 52(1): 28-35.

Taylor, J. T., Huang, L., Keyser, B. M., Zhuang, H., Clarkson, C. W., and Li, M. 2005. 'Role of high-voltage-activated calcium channels in glucose-regulated beta-cell calcium homeostasis and insulin release', *Am J Physiol Endocrinol Metab*, 289(5): E900-8.

Thorens, B. 2001. 'GLUT2 in pancreatic and extra-pancreatic gluco-detection (review)', *Mol Membr Biol*, 18(4): 265-73.

Timlin, M. R., Black, A. B., Delaney, H. M., Matos, R. I., and Percival, C. S. 2017. 'Development of Pulmonary Hypertension During Treatment with Diazoxide: A Case Series and Literature Review', *Pediatr Cardiol*, 38(6): 1247-50.

Tinker, A., Aziz, Q., Li, Y., and Specterman, M. 2018. 'ATP-Sensitive Potassium Channels and Their Physiological and Pathophysiological Roles', *Compr Physiol*, 8(4): 1463-511.

Tombline, G., Bartholomew, L. A., Tyndall, G. A., Gimi, K., Urbatsch, I. L., and Senior, A. E. 2004. 'Properties of P-glycoprotein with mutations in the "catalytic carboxylate" glutamate residues', *J Biol Chem*, 279(45): 46518-26.

Trube, G., Rorsman, P., and Ohno-Shosaku, T. 1986. 'Opposite effects of tolbutamide and diazoxide on the ATP-dependent $K^+$ channel in mouse pancreatic beta-cells', *Pflugers Arch*, 407(5): 493-9.

Tucker, S. J., Gribble, F. M., Zhao, C., Trapp, S., and Ashcroft, F. M. 1997. 'Truncation of Kir6.2 produces ATP-sensitive $K^+$ channels in the absence of the sulphonylurea receptor', *Nature*, 387(6629): 179-83.

Tusnady, G. E., Sarkadi, B., Simon, I., and Varadi, A. 2006. 'Membrane topology of human ABC proteins', *FEBS Lett*, 580(4): 1017-22.

Ueda, K., Inagaki, N., and Seino, S. 1997. 'MgADP antagonism to $Mg^{2+}$-independent ATP binding of the sulfonylurea receptor SUR1', *J Biol Chem*, 272(37): 22983-6.

Ullrich, S., and Wollheim, C. B. 1988. 'GTP-dependent inhibition of insulin secretion by epinephrine in permeabilized RINm5F cells. Lack of correlation between insulin secretion and cyclic AMP levels', *J Biol Chem*, 263(18): 8615-20.

Ullrich, S., and Wollheim, C. B. 1989. 'Galanin inhibits insulin secretion by direct interference with exocytosis', *FEBS Lett*, 247(2): 401-4.

Vajravelu, M. E., Chai, J., Krock, B., Baker, S., Langdon, D., Alter, C., and De Leon, D. D. 2018. 'Congenital Hyperinsulinism and Hypopituitarism Attributable to a Mutation in FOXA2', *J Clin Endocrinol Metab*, 103(3): 1042-47.

Vajravelu, M. E., and De León, D. D. 2018. 'Genetic characteristics of patients with congenital hyperinsulinism', *Curr Opin Pediatr*, 30(4): 568-75.

van der Steen, I., van Albada, M. E., Mohnike, K., Christesen, H. T., Empting, S., Salomon-Estebanez, M., Greve Rasmussen, A., Verrijn Stuart, A., van der Linde, A. A. A., Banerjee, I., and Boot, A. M. 2018. 'A Multicenter Experience with Long-Acting Somatostatin Analogues in Patients with Congenital Hyperinsulinism', *Horm Res Paediatr*, 89(2): 82-89.

Vedovato, N., Ashcroft, F. M., and Puljung, M. C. 2015. 'The Nucleotide-Binding Sites of SUR1: A Mechanistic Model', *Biophys J*, 109(12): 2452-60.

Vedovato, N., Rorsman, O., Hennis, K., Ashcroft, F. M., and Proks, P. 2018. 'Role of the C-terminus of SUR in the differential regulation of beta-cell and cardiac KATP channels by MgADP and metabolism', *J Physiol*, 596(24): 6205-17.

Verkarre, V., Fournet, J. C., de Lonlay, P., Gross-Morand, M. S., Devillers, M., Rahier, J., Brunelle, F., Robert, J. J., Nihoul-Fékété, C., Saudubray, J. M., and Junien, C. 1998. 'Paternal mutation of the sulfonylurea receptor (SUR1) gene and maternal loss of 11p15 imprinted genes lead to persistent hyperinsulinism in focal adenomatous hyperplasia', *J Clin Invest*, 102(7): 1286-91.

Vogalis, F., Zhang, Y., and Goyal, R. K. 1998. 'An intermediate conductance $K^+$ channel in the cell membrane of mouse intestinal smooth muscle', *Biochim Biophys Acta*, 1371(2): 309-16.

Welters, A., Lerch, C., Kummer, S., Marquard, J., Salgin, B., Mayatepek, E., and Meissner, T. 2015. 'Long-term medical treatment in congenital hyperinsulinism: a descriptive analysis in a large cohort of patients from different clinical centers', *Orphanet J Rare Dis*, 10: 150.

Wilkens, S. 2015. 'Structure and mechanism of ABC transporters', *F1000Prime Rep*, 7: 14.

Wu, J. X., Ding, D., Wang, M., Kang, Y., Zeng, X., and Chen, L. 2018. 'Ligand binding and conformational changes of SUR1 subunit in pancreatic ATP-sensitive potassium channels', *Protein Cell*, 9(6): 553-67.

Wullschleger, S., Loewith, R., and Hall, M. N. 2006. 'TOR signaling in growth and metabolism', *Cell*, 124(3): 471-84.

Yamada, M., Isomoto, S., Matsumoto, S., Kondo, C., Shindo, T., Horio, Y., and Kurachi, Y. 1997. 'Sulphonylurea receptor 2B and Kir6.1 form a sulphonylurea-sensitive but ATP-insensitive K+ channel', *J Physiol*, 499 ( Pt 3): 715-20.

Yan, F. F., Lin, Y. W., MacMullen, C., Ganguly, A., Stanley, C. A., and Shyng, S. L. 2007. 'Congenital hyperinsulinism associated ABCC8 mutations that cause defective trafficking of ATP-sensitive $K^+$ channels: identification and rescue', *Diabetes*, 56(9): 2339-48.

Yang, J., Chi, Y., Burkhardt, B. R., Guan, Y., and Wolf, B. A. 2010. 'Leucine metabolism in regulation of insulin secretion from pancreatic beta cells', *Nutr Rev*, 68(5): 270-9.

Yildizdas, D., Erdem, S., Küçükosmanoğlu, O., Yilmaz, M., and Yüksel, B. 2008. 'Pulmonary hypertension, heart failure and neutropenia due to diazoxide therapy', *Adv Ther*, 25(5): 515-9.

Yorifuji, T., Kawakita, R., Hosokawa, Y., Fujimaru, R., Matsubara, K., Aizu, K., Suzuki, S., Nagasaka, H., Nishibori, H., and Masue, M. 2013. 'Efficacy and safety of long-term, continuous subcutaneous octreotide infusion for patients with different subtypes of KATP-channel hyperinsulinism', *Clin Endocrinol (Oxf)*, 78(6): 891-7.

Zdravkovic, M., Kruse, M., Rost, K. L., Moss, J., and Kecskes, A. 2007. 'The effects of NN414, a SUR1/Kir6.2 selective potassium channel opener in subjects with type 2 diabetes', *Exp Clin Endocrinol Diabetes*, 115(6): 405-6.

Zdravkovic, M., Kruse, M., Rost, K. L., Moss, J., Kecskes, A., and Dyrberg, T. 2005. 'The effects of NN414, a SUR1/Kir6.2 selective potassium channel opener, in healthy male subjects', *J Clin Pharmacol*, 45(7): 763-72.

Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. 1999. 'A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane $K_{ATP}$ channels', *Neuron*, 22(3): 537-48.

Zingman, L. V., Alekseev, A. E., Bienengraeber, M., Hodgson, D., Karger, A. B., Dzeja, P. P., and Terzic, A. 2001. 'Signaling in channel/enzyme multimers: ATPase transitions in SUR module gate ATP-sensitive $K^+$ conductance', *Neuron*, 31(2): 233-45.

Zingman, L. V., Hodgson, D. M., Bienengraeber, M., Karger, A. B., Kathmann, E. C., Alekseev, A. E., and Terzic, A. 2002. 'Tandem function of nucleotide binding domains confers competence to sulfonylurea receptor in gating ATP-sensitive $K^+$ channels', *J Biol Chem*, 277(16): 14206-10.

# 6 Acknowledgment

The present work was carried out from February 2016 to July 2019 under the scientific supervision of Prof. Dr. Gisela Drews at the Pharmaceutical Institute of the Eberhard Karls University of Tübingen.

First, my special thanks goes to my supervisor Prof. Dr. Drews for giving me the opportunity to work on this exciting research topic, for the trust she has placed in me, and for the motivation and support she has given me during the preparation of this work. She has allowed me to be a part of her working group, to learn and progress. I had the opportunity to attend interesting congresses, to work independently on the scientific questions with my own ideas and suggested solutions, and I could always rely on an open ear and support when problems arose.

My sincere thanks also go to Prof. Dr. Susanne Ullrich for accepting to be my co-supervisor and for the comments and encouragement and her availability whenever necessary.

My sincere gratitude for Prof. Dr. Joseph Bryan and his wife Lydia Aguilar Bryan for priceless advice throughout my PhD. I am very gratefull for allowing me to work together on the $K_{ATP}$ project, for the opportunity to present our work in conferences and warm hospitality during my stay in Seattle.

I would also like to express appreciation to Prof. Dr. Peter Krippeit-Drews for his helpfulness, scientific discussions, and technical solutions.

I would also like to thank Prof. Dr. Bertolt Gust and Prof. Dr. Leonard Kaysser for their willingness to act as further examiners.

I would like to show sincere gratitude to Prof. Dr. Winfried Barthlen and Prof. Dr. Carmen Wolke from Greifswald for their friendly cooperation and providing us valuable material that was essential for my research. Further thanks go to Prof. Dr. Martina Düfer and her work group for the scientific exchange during this time and to all former and present members of the work group Drews.

Colleagues from the work group of Prof. Dr. Joseph Bryan, Dr. Mc Mullen and Frank Dastvan, I am grateful for their warm welcome in the PNRI laboratory in Seattle, for

teaching me new techniques and providing us with necessary HEK-293 cells and mutations that were essential for the completion of this work.

My thanks also go to technical assistant Isolde Breuning for her tireless work in measuring countless RIA samples, to Ilona Böhler and Clement Kabagema-Bilan for taking care of the mice, and Mrs. Weber and Mrs. Leitermann for their help with all administrative issues and paperwork.

I would also like to thank all my friends and family members for providing me with emotional support as well as with continuous encouragement throughout my entire PhD.

Special thanks go to my parents, who have accompanied and supported me on all my journeys. Thank you from my heart for your financial and emotional support on my long way through my studies and doctorate. Above all, I would like to thank my dear fiancée Danijel for his patience, his support, and the constructive words in difficult times and the security he gave me. This journey would be much duller without you by my side.

# 7 Publications

## 7.1 Scientific publications

**2018**

**Sikimic J, McMillen TS, Bleile C, Dastvan F, Quast U, Krippeit-Drews P, Drews G, Bryan J.**, 2018. ATP-binding without hydrolysis switches sulfonylurea receptor 1 (SUR1) to outward-facing conformations that activate $K_{ATP}$ channels. J Biol Chem 294(10):3707-3719.

**Bauer C, Kaiser J, Sikimic J, Krippeit-Drews P, Düfer M, Drews G.**, 2018. ATP mediates a negative autocrine signal on stimulus-secretion coupling in mouse pancreatic β-cells. Endocrine 63(2):270-283.

**2017**

**Undank S, Kaiser J, Sikimic J, Düfer M, Krippeit-Drews P, Drews G.**, 2017. Atrial Natriuretic Peptide Affects Stimulus-Secretion Coupling of Pancreatic β-Cells. Diabetes 66(11):2840-2848.

**Maczewsky J, Sikimic J, Bauer C, Krippeit-Drews P, Wolke C, Lendeckel U, Barthlen W, Drews G.**, 2017. The LXR Ligand T0901317 Acutely Inhibits Insulin Secretion by Affecting Mitochondrial Metabolism. Endocrinology 158(7):2145-2145.

**2015**

**Srdic-Rajic T, Konic-Ristic A, Tišma-Miletic N, Kardum N, Galun D, Sikimic J, Glibetic M, Milicevic M.,** 2015. Efekti Ekstrakta Bele Imele na Markere Aktivacije i Agregacije Trombocita. Acta Chirurgica Iugoslavica 62(2):5-13.

## 7.2 Conference and meeting contributions

**Mai 29 - June 1, 2019**

Diabetes Kongress 2019 in Berlin

Poster: “ATP triggers $K_{ATP}$ channel opening without hydrolysis”

**Sikimic J**, Krippeit-Drews P, Drews G, Bryan J.

**March 27 - 29, 2019**

DPhG-Doktorandentagung in Darmstadt

Short talk: "ATP binding without hydrolysis switches SUR1 to outward-facing conformation that activate $K_{ATP}$ channels".

**Sikimic J**, McMillen TS, Bleile C, Dastvan F, Quast U, Krippeit-Drews P, Drews G, Bryan J.

**September 18 - 21, 2018**

Inaugural Conference of the Transmembrane Transporter Society: "The Transporter Transition" in Vienna, Austria

Poster: "Association of SUR1/ABCC8 with the $K_{ATP}$ channel pore subunit Kir6.2/KCNJ11 alters the receptor's allosteric properties".

**Sikimic J**, McMillen TS, Bleile C, Dastvan F, Krippeit-Drews P, Drews G, Bryan J.

**September 29 - October 1, 2018**

6th DZD Diabetes Research School des Deutschen Zentrums für Diabetesforschung (DZD) , Berlin

**March 6 - 12, 2018**

7th FEBS-Special Meeting ATP-Binding Cassette Proteins: From Multidrug Resistance to Genetic Diseas - ABC2018, Inssbruck, Austria

Dr. Josef Steiner Fellowship; short talk and poster: "Association of SUR1/ABCC8 with the $K_{ATP}$ channel pore subunit Kir6.2/KCNJ11 alters the receptor's allosteric properties".

**Sikimic J**, McMillen TS, Bleile C, Dastvan F, Quast U, Krippeit-Drews P, Drews G, Bryan J.

# 8 Curriculum Vitae

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| 02/2016 - 08/2019 | PhD student and research associate at Eberhard Karls University of Tübingen, Faculty of Science, Institute of Pharmacy, Department of Pharmacology, Toxicology and Clinical Pharmacy, Prof. Dr. Gisela Drews |
| 05/2015 - 01/2016 | Research assistant at Institute for Oncology and Radiology, Department of Experimental Pharmacology |
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| 01/2013 – 11/2013 | Internship for Clinical Chemist in Clinical Center “Bezanijska Kosa” in Belgrade, Republic of Serbia |

**Education**

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| 02/2014 | Stateexam for Clinical Chemist, Belgrade, Republic of Serbia |
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| 09/2002 – 06/2006 | High school, Pozarevacka gimnazija in Pozarevac, Republic of Serbia |

# 9 Appendix - Publications

1. Sikimic J, McMillen T.S, Bleile C, Dastvan F, Quast U, Krippeit-Drews P, Drews G, Bryan J (2019)

   **ATP binding without hydrolysis switches sulfonylurea receptor 1 (SUR1) to outward-facing conformations that activate $K_{ATP}$ channels.** J Biol Chem 294(10):3707-3719.

2. Sikimic J, Hoffmeister T, Gresch A, Barthlen W, Wolke C, Lendeckel U, Krippeit-Drews P, Düfer M, Drews G. (Paper in preparation)

   **Possible new strategies for the treatment of congenital hyperinsulinism**

3. Maczewsky J, Sikimic J, Bauer C, Krippeit-Drews P, Wolke C, Lendeckel U, Barthlen W, Drews G (2017).

   **The LXR ligand T0901317 acutely inhibits insulin secretion by affecting mitochondrial metabolism.** Endocrinology 158(7):2145-2145.

4. Undank S, Kaiser J, Sikimic J, Düfer M, Krippeit-Drews P, Drews G (2017)

   **Atrial natriuretic peptide affects stimulus-secretion coupling of pancreatic β-cells.** Diabetes 66(11):2840-2848.

5. Bauer C, Kaiser J, Sikimic J, Krippeit-Drews P, Düfer M, Drews G (2018)

   **ATP mediates a negative autocrine signal on stimulus-secretion coupling in mouse pancreatic β-cells.** Endocrine 63(2):270-283.

**ATP binding without hydrolysis switches sulfonylurea receptor 1 (SUR1) to outward-facing conformations that activate $K_{ATP}$ channels**

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*Published: The Journal of Biological Chemistry.* 2019 March 8; 294, 3707-3719. Epub 2018 Dec.

**Abstract**

Neuroendocrine-type ATP-sensitive $K^+$ ($K_{ATP}$) channels are metabolite sensors coupling membrane potential with metabolism, thereby linking insulin secretion to plasma glucose levels. They are octameric complexes, $(SUR1/Kir6.2)_4$, comprising sulfonylurea receptor 1 (SUR1 or ABCC8) and a $K^+$-selective inward rectifier (Kir6.2 or KCNJ11). Interactions between nucleotide-, agonist-, and antagonist-binding sites affect channel activity allosterically. Although it is hypothesized that opening these channels requires SUR1-mediated MgATP hydrolysis, we show here that ATP binding to SUR1, without hydrolysis, opens channels when nucleotide antagonism on Kir6.2 is minimized and SUR1 mutants with increased ATP affinities are used. We found that ATP binding is sufficient to switch SUR1 alone between inward- or outward-facing conformations with low or high dissociation constant, KD, values for the conformation-sensitive channel antagonist [$^3$H]glibenclamide ([$^3$H]GBM), indicating that ATP can act as a pure agonist. Assembly with Kir6.2 reduced SUR1's $K_D$ for [$^3$H]GBM. This reduction required the Kir N terminus (KNtp), consistent with KNtp occupying a “transport cavity,” thus positioning it to link ATP-induced SUR1 conformational changes to channel gating. Moreover, ATP/GBM site coupling was constrained in WT SUR1/WT Kir6.2 channels; ATP-bound channels had a lower KD for [$^3$H]GBM than ATP-bound SUR1. This constraint was largely eliminated by the Q1179R neonatal diabetes-associated mutation in helix 15, suggesting that a

“swapped” helix pair, 15 and 16, is part of a structural pathway connecting the ATP/GBM sites. Our results suggest that ATP binding to SUR1 biases KATP channels toward open states, consistent with SUR1 variants with lower KD values causing neonatal diabetes, whereas increased KD values cause congenital hyperinsulinism.

**Introduction**

Neuroendocrine-type ATP-sensitive $K^+$ $(K_{ATP})2$ channels comprise an ATP-binding cassette (ABC) protein (1), ABCC8/SUR1, and a $K^+$-selective inward rectifier (2), KCNJ11/Kir6.2, assembled as heterooctamers (3–5), $(SUR1/Kir6.2)_4$. In pancreatic β-cells, these channels are metabolite sensors that couple cellular metabolism with membrane electrical activity to link insulin secretion with blood glucose levels. This coupling is critical for normal physiology; loss of channel function is a cause of congenital hyperinsulinism (Ref. 6; for reviews, see Refs. 7 and 8), whereas gain-of-function mutations in Kir6.2 (9) and SUR1 (10) cause neonatal diabetes (ND) (for reviews, see Refs. 11 and 12). Gain of function is one cause of mature-onset diabetes of the young (13), whereas a ABCC8/SUR1 polymorphism, the Ala amino acid allele at p.A1369S, is associated with an increased risk for type 2 diabetes (14).

Channel activity is regulated positively by ATP and ADP binding to SUR1 and negatively by nucleotide binding to Kir6.2 (15–20). Additionally, multiple metabolites, including phosphoinositides (21–23) and long-chain acyl-CoA esters (24–26), and phosphorylation (27–29) positively modulate channel activity. Pharmacologic modulation of SUR1 by channel antagonists, sulfonylureas like glibenclamide (GBM) and glinides like repaglinide (30–36), and by agonists like diazoxide (Refs. 31 and 37–39; for a review, see Ref. 40) is clinically important. These physiologic and pharmacologic modulators all affect channel gating via allosteric interactions in the sense that their binding sites on SUR1 are coupled to and known to be physically distinct from one another based on structural studies (41, 42). The available data are consistent with the idea that SUR1 exists in multiple conformations differing in affinity for ligands and ability to activate channel openings.

How nucleotides regulate $K_{ATP}$ channels remains an open question. Early electrophysiological studies (15) showed that although ATP inhibited activity,

MgADP, acting through SUR1, activated channel openings (16–18, 20). The finding that SUR1 was in the ABC protein family (1), whose members are ATPases that use the energy of ATP binding and hydrolysis to transport substrates across membranes (43–45), suggested that ATP hydrolysis on SUR1 might generate a conformation that activated channel openings. The idea that a "posthydrolytic," MgADP-bound conformation of SUR1, generated as part of an ABC enzymatic cycle, uniquely activates channel openings was most concisely described by Zingman et al. (46, 47) and Bienengraeber et al. (48) and reviewed in Zingman et al. (49). The idea was supported by experiments with nonhydrolyzable analogs, e.g. AMPPNP, AMPPCP, and ATPγS, and by reports that the action of potassium channel agonists, e.g. diazoxide for SUR1-based channels and a pinacidil analog, P1075, for related SUR2-based channels, required ATP hydrolysis to reach an activating state (50–52).

The need for hydrolysis was questioned by Choi et al. (53), who failed to find the changes in microscopic reversibility and detailed balance expected for strong coupling, and by Ortiz et al. (54, 55), who used [$^3$H]GBM to show that ATP binding was enough to switch SUR1, in the absence of Kir6.2, between conformations with high and low affinities for the ligand. In other ABC proteins, ATP binding to the nucleotide-binding domains (NBDs) results in NBD dimerization and a reconfiguration of the transmembrane helical domains (TMDs) from "inward- to outward-facing" states. Thus, Ortiz et al. (54) proposed that outward-facing, ATP-bound conformations of SUR1 with lowest affinity for [$^3$H]GBM and highest affinity for the agonist diazoxide would activate channel openings without the need for hydrolysis. We tested this proposal here using $K_{ATP}$ channels whose Kir6.2 pores are not inhibited by ATP.

In other ABC proteins, hydrolysis of ATP is essential to "reset" the transporter conformation and allow cyclic substrate transport, but the function of the low rate of hydrolysis reported for SUR1 is not yet clear (56, 57). Despite its structural relation to other ABC transporters, SUR1 is usually assumed to be a "regulatory protein," not a transporter. However, the localization of GBM bound in the central cavity of SUR1 by cryo-EM (41, 42) together with earlier studies aimed at defining the GBM-binding site and defining the role(s) of the N terminus, KNtp, of Kir6.2 in GBM binding and channel gating (for a review, see Ref. 40) suggests that the transport idea deserves revisiting. The implication is that SUR1 is a "cryptic," albeit frustrated, peptide

transporter whose “substrate” is KNtp. This idea suggests that evolution has linked two fundamental transport mechanisms, ATP-driven substrate transport and ion transport, to couple cellular metabolism with membrane potential, thus effectively marrying the transport functionality of an ATP-binding cassette protein, *ABCC8*/SUR1, with the regulation of gating of an ion channel, Kir6.2/*KCNJ11*. Clearly, SUR1 cannot fully “transport” Kir6.2, but the idea implies a mechanism by which ATP-driven changes in SUR1 can couple directly to the pore by moving KNtp.

We have focused on ATP-driven conformational changes in SUR1, including testing the need for ATP hydrolysis to activate channel openings and defining the properties of different SUR1 conformations. We show that when the inhibitory action of ATP on the Kir6.2 pore is eliminated, $ATP^{4-}$ acts as a classic channel agonist even when potential nucleotide hydrolysis is minimized by limiting $Mg^{2+}$ and/or by substitution of glutamine for the catalytic glutamate, $SUR1_{E1507Q}$, needed for hydrolysis. We confirm that outward-facing conformations of SUR1 alone, i.e. without Kir6.2, have reduced affinity for [$^3$H]GBM and show that assembly with full-length Kir6.2 increases the affinity and attenuates the negative allosteric effect of ATP on GBM interactions (58). One ND mutation, $SUR1_{Q1179R}$, in transmembrane helix 15 near the GBM-binding site markedly increases coupling between the GBM and ATP sites. This suggests that helix 15 and helix 16, which cross over from TMD2 to contact NBD1, are part of a structural pathway or network connecting these sites. MgADP, like MgATP, efficiently switches the conformation of SUR1 alone but has no significant effect on [$^3$H]GBM binding to wildtype (WT) SUR1 in channels. The results support the hypothesis that ATP acts as an agonist by switching SUR1 from inactive inward-facing to active outward-facing conformations that bias channels toward open states.

**Results**

*ATP activation of SUR1/Kir6.2$_{G334D}$ $K_{ATP}$ channels*

To test whether ATP can act directly as a channel agonist, the inhibitory action of ATP binding to the Kir6.2 pore was minimized by coexpressing SUR1 with a mutant pore subunit, $Kir6.2_{G334D}$. This substitution, identified in cases of neonatal diabetes (59), reduces the affinity of Kir6.2 for inhibitory ATP, thus minimizing ATP antagonism (60–62). Fig. 1, A and B, confirm that $ATP^{4-}$ (10 mm) or MgATP (1 mm) reduce the

activity of WT SUR1/WT Kir6.2 channels. In contrast, MgATP acts as an agonist on WT SUR1/Kir6.2$_{G334D}$ channels, increasing the product of the open probability (Po) and number (N) of open channels, NPo, 7–8-fold (Fig. 1C). The different nucleotide concentrations were chosen because SUR1 has a significantly lower KD for MgATP versus $ATP^{4-}$ as determined by their respective abilities to stabilize conformations that bind GBM more weakly (54, 55). WT Kir6.2 has estimated $K_D$ values of ~5–20 μm (19, 63, 64) for inhibitory ATP ($\pm Mg^{2+}$) and should be nearly saturated in either case. The agonist action is reversible: after washout of MgATP, channel activity rapidly returns to basal levels. The application of $ATP^{4-}$ (10 mm) had no significant effect on channel open probability.

*$ATP^{4-}$ activation of SUR1$_{Q1179R}$/Kir6.2$_{G334D}$ $K_{ATP}$ channels*

Several ND SUR1 mutant receptors, including SUR1$_{Q1179R}$, have lower KD values for nucleotides based on the allosteric effect(s) of $ATP^{4-}$ or MgATP on [$^3$H]GBM binding (54, 55, 65). SUR1$_{Q1179R}$/Kir6.2$_{G334}$D channels were used to determine whether nucleotide binding, without the $Mg^{2+}$ cofactor needed for ATP hydrolysis (56), was enough to activate channel openings. Fig. 2, A and D, show that the NPo of spontaneously active SUR1$_{Q1179R}$/Kir6.2$_{G334D}$ channels is increased significantly by $ATP^{4-}$ (10 mm), consistent with nucleotide binding being enough to stabilize conformations of SUR1 that activate channel openings. Together with Fig. 1C, the results show that, in the absence of antagonism at Kir6.2, MgATP and more importantly $ATP^{4-}$ are channel agonists, albeit agonists with markedly different KD values.

*$GTP^{4-}$ activation of SUR1$_{Q1179R}$/Kir6.2$_{G334D}$ $K_{ATP}$ channels*

We tested whether $GTP^{4-}$, without $Mg^{2+}$, was enough to activate SUR1$_{Q1179R}$/Kir6.2$_{G334D}$ channels. Fig. 2, B and E, show that $GTP^{4-}$ (10 mm), like $ATP^{4-}$, significantly increases channel activity. The results suggest that a lower KD for nucleotides may underlie the increased activation of the SUR1$_{A1369}$ variant associated with type 2 diabetes risk (14) rather than the proposed altered rate of ATP hydrolysis (66, 67).

*$ATP^{4-}$ activation of $SUR1_{E1507Q}$/$Kir6.2_{G334D}$ $K_{ATP}$ channels*

To test further whether nucleotide binding is sufficient to select and stabilize SUR1-activating conformers, a glutamine was substituted for the “catalytic” glutamate in NBD2. This substitution strongly reduces ATPase and transport activity in related proteins (68, 69), and Glu → Gln substitutions have been used to trap ATP-bound conformations for structural studies (70–72). Fig. 2, C and F, show that $SUR1_{E1507Q}$/$Kir6.2_{G334D}$ channels, lacking the catalytic glutamate and $Mg^{2+}$, are significantly activated by $ATP^{4-}$. The results imply that $ATP^{4-}$ and $GTP^{4-}$ are channel agonists whose binding energies are enough to shift the population of SUR1 conformers toward those that increase channel openings.

*$ATP^{4-}$ supports the action of $K_{ATP}$ channel agonists*

To assess whether ATP binding is sufficient to support the action of channel openers, the ability of diazoxide to activate $SUR1_{E1507K}$/WT Kir6.2 channels was tested. The $SUR1_{E1507K}$ mutation was used because congenital hyperinsulinism patients with this Glu → Lys mutation respond to diazoxide (73), and, without the catalytic glutamate, $SUR1_{E1507K}$/WT Kir6.2 channels are expected to have impaired ATP hydrolysis. Fig. 3A shows that when patches from cells expressing $SUR1_{E1507K}$/WT Kir6.2 channels are pulled into nucleotide-free medium, channels are activated as nucleotide antagonism on their WT pores is reduced. Application of $ATP^{4-}$ rapidly inhibits channel activity as expected, but coapplication of diazoxide shows that ATP4− does support its agonist action. Fig. 3B confirms that MgATP inhibits channel activity and supports the action of diazoxide as expected from patient responses. The response to diazoxide is consistent with the positive allosteric coupling between channel opener and nucleotide-binding sites seen with SUR1 alone where diazoxide stabilizes nucleotide-bound conformations (55).

*Assembly with Kir6.2 lowers the dissociation constant, $K_G$, of SUR1 for GBM*

We asked how assembly with Kir6.2 affects the GBM-binding site and the intramolecular linkage between the ATP- and GBM-binding sites within the receptor. The dissociation constant, $K_G$, values for [$^3$H]GBM binding to receptors and channels are needed to assess the impact of adding nucleotides; thus, $K_G$ values were determined from homologous competition experiments where [$^3$H]GBM binding is

competed by increasing concentrations of unlabeled GBM. KG values were obtained by simultaneously fitting a homologous competition model (74) using data at two concentrations of [$^3$H]GBM (see “Experimental procedures”). The approach is illustrated in Fig. 4, A–C, for the WT receptor alone or coexpressed with Kir6.2 or Kir6.2$_{G334D}$. The results extend earlier studies in isolated membranes (75) and live cells (76) showing that $K_{ATP}$ channels bind [$^3$H]GBM more tightly than SUR1 alone. Our binding assays were done without $Mg^{2+}$ and ATP, conditions where cryo-EM studies show SUR1 in inward-facing conformations with GBM bound and a closed Kir6.2 pore (77, 78). Under these conditions, WT channels have approximately a 7.2-fold lower $K_G$ for GBM. Table 1 summarizes the data for four receptors and corresponding channels, including WT, one congenital hyperinsulinism (SUR1$_{E1507K}$), and two neonatal diabetes ABCC8/SUR1 mutations (SUR1$_{E1507Q}$ and SUR1$_{Q1179R}$). In all four cases, the $K_G$ values for channels are significantly lower than for SUR1 alone. There was no significant difference in KG values between channels assembled with WT Kir6.2 versus Kir6.2$_{G334D}$. An average value for the increased stability of GBM–WT SUR1/Kir6.2 versus GBM–WT SUR1 complexes was estimated from the difference in free energies, $\Delta\Delta G = -1.2 \pm 0.1$ kcal/mol (mean ± S.E., n = 9), derived from the respective dissociation constants. The average ΔΔG value, −0.8 kcal/mol, for all the WT and mutant channels is somewhat lower. The structural changes underlying the increased stability of GBM–channel complexes are not well defined, but previous studies implicate the N terminus of Kir6.2, KNtp, as a major factor in the higher affinity of SUR1/Kir6.2 channels versus SUR1 for GBM (Refs. 75 and 76 and “Discussion”).

Consistent with our earlier results (54, 55) and cryo-EM structures (41, 42, 77, 78), the high affinity of GBM binding strongly selects and stabilizes inward-facing conformations of SUR1 with a ΔG0 value of approximately −12.4 or −13.6 kcal/mol for receptors or channels, respectively. The difference in binding energies of SUR1 versus SUR1/Kir6.2 reflects an increased stability of GBM-bound versus unliganded channels. We used the thermodynamic cycle shown in Fig. 4D to estimate the increased stability. We assumed channels and subunits are in equilibrium and that GBM interacts with different energies with SUR1 alone versus SUR1 in channels (data are summarized in Table 1). The products of the dissociation constants along the two branches forming GBM–channel complexes must be equal. Kchan, the

dissociation constant for channels into subunits, is not known but must be low because channels are stable and complexes can be isolated (41, 42, 57, 77–79). The $K_G$ values for WT receptors versus channels gave an estimated value for α of ~0.14, indicating channels complexed with GBM are ~7 times more stable than unliganded $K_{ATP}$ channels. The average stabilization, for all channels versus receptors, is ~4-fold based on the average ΔΔG value.

*Assembly with Kir6.2 alters the allosteric coupling between the GBM and ATP-binding sites in WT SUR1*

Table 1 shows that assembly with Kir6.2 alters the SUR1 GBM-binding site, increasing its affinity for GBM. The $K_G$ values were used to assess how channel assembly alters the negative allosteric coupling between GBM- and ATP-binding sites. The effects of increasing concentrations of MgATP on the binding of fixed concentrations of [$^3$H]GBM were compared for SUR1 alone or SUR1/Kir6.2 channels. The GBM- and ATP-binding sites are physically distinct; thus, a reduction in bound [$^3$H]GBM indicates negative allosteric coupling between these two binding sites. Fig. 5 shows stronger negative coupling for SUR1 alone than for SUR1/Kir6.2 channels. In both cases, a significant fraction of bound [$^3$H]GBM remains at the highest, saturating concentrations of MgATP, but the fraction remaining is significantly lower for SUR1 alone; assembly with Kir6.2 markedly attenuates the ability of nucleotides to reduce [$^3$H]GBM binding to SUR1/Kir6.2 channels versus SUR1 alone (58). Our previously described ternary complex model (54), shown in Fig. 5, inset, was used to interpret the data. In this model, the receptor (R) is presumed to have coupled GBM- and ATP-binding sites. The coupling factor, β, is a measure of the interaction between sites; β = 1 equates to no coupling, whereas β > 1 reflects an increase in the $K_D$ for the binding of one ligand when the opposite ligand-binding site is occupied. In this model, the fraction of bound [$^3$H]GBM remaining at saturating concentrations of nucleotide reflects the higher $K_D$ (=β$K_G$), i.e. a reduced affinity, of MgATP-bound SUR1 for [$^3$H]GBM.

The parameters $K_T$, the dissociation constant for ATP, and β, the allosteric constant, were estimated in two ways using Equation 1 given under “Experimental procedures.” In both cases, the individual $K_G$ values were fixed (parameters in Table 2). In one case, the two data sets were fit simultaneously (globally). This assumes

the KT for ATP-binding is the same for both SUR1 and channels and yields a single KT value and best-fit estimates of β for SUR1 alone versus channels. In the alternative case, $K_T$ and β were estimated independently for each data set. The two estimates are nearly identical (Table 2). The curves in Fig. 5 were generated using the global parameters, but curves generated using the independent parameters are visually the same. In this four-state model, the fraction of bound [$^3$H]GBM for SUR1 alone reflects the ~5-fold lower affinity of SUR1-MgATP–bound complexes for [$^3$H]GBM. The data in Table 1 show that channels have ~7-fold higher affinity for [$^3$H]GBM, which is reflected in the larger fraction of bound [$^3$H]GBM seen in ATP-bound SUR1/Kir6.2 channels. It is worth noting that this negative allosteric effect is reciprocal; GBM–SUR1, alone or in channels, binds MgATP more weakly (compare $K_T$ versus $\beta K_T$ values in Table 2).

*$SUR1_{Q1179R}$, an ABCC8 neonatal diabetes mutation, affects allosteric coupling*

Assembly with Kir6.2 markedly affects the coupling between the ATP and GBM sites in WT SUR1 (Fig. 5), but there are no informative data available on the structural pathway(s) or amino acid network(s) linking these sites. Analyses of coupling in *ABCC8*/SUR1 mutants provide one approach to identify amino acids in potential pathways. We tested whether the Gln → Arg substitution, $SUR1_{Q1179R}$, might affect coupling. Position 1179 is near the top of helix 15, within 20 nm of the GBM-binding site (41). Helices 15 and 16 are the pair of helices from TMD2 that cross over to interact with NBD1. Intuitively, this “swapped” pair of helices is a potential pathway or link between the ATP- and GBM-binding sites. Fig. 6 compares the reduction of bound [$^3$H]GBM by MgATP from WT SUR1/WT Kir6.2 versus ND mutant $SUR1_{Q1179R}$/WT Kir6.2 channels. The Gln → Arg substitution largely reverses the constraint imposed by assembly with Kir6.2 (Fig. 5). In this experiment, the WT control data set includes the channel data shown in Fig. 5. The curves are based on fits to the ternary complex model (Fig. 5, inset) with values for WT channels of KG = 0.25 (0.19–0.32) nm, β = 2.2 (2.1–2.3), and $K_T$ = 38 (27.1–53.6) μm and for the $SUR1_{Q1179R}$/wtKir6.2 channels of $K_G$ = 1.1 (3.2–4.8) nm, β = 7.3 (6.3–8.6), and $K_T$ = 18 (14.9–21.9) μm. Values are given as means with 95% confidence intervals (lower-upper values).

*Effects of MgADP on SUR1 versus SUR1/Kir6.2*

MgADP increases the open probability of WT $K_{ATP}$ channels and stabilizes more outward-facing receptor conformations (42); thus, the effect of MgADP on GBM binding was determined for WT SUR1 versus WT SUR1/WT Kir6.2 channels. Ortiz et al. (54) used an excess of MgAMP to minimize conversion of added ADP to ATP by adenylate kinase and reported that MgADP stabilized SUR1 conformations with lower affinity for GBM somewhat better than MgATP. We added hexokinase plus glucose to reduce low levels of contaminant ATP and to convert any generated ATP to ADP. Under these conditions, MgADP was nearly as effective at switching the conformation of WT SUR1 as MgATP (dissociation constant for ADP, $K_{ADP}$, ~55 (38–107) versus $K_T$ ~38 (24–52) μm, respectively) with a β value of 1.8 (1.6–1.9) but had no significant effect on the GBM interaction with channels (Fig. 7).

**Discussion**

ATP has dual effects on (SUR1/Kir6.2)4 neuroendocrine $K_{ATP}$ channels. Nucleotide interactions with the Kir6.2 pore inhibit channel openings, whereas binding to SUR1, including potential enzymatic activities, activate openings. This study focused on the ATP-binding cassette subunit *ABCC8*/SUR1, whether enzymatic hydrolysis of ATP by SUR1 is needed for channel activation, identifying which receptor conformation(s) activates channel openings, and the allosteric pathways coupling the SUR1 ATP- and GBM-binding sites.

*ATP binding to SUR1 is sufficient to activate $K_{ATP}$ channels*

Prior results (53–55) and the data in Figs. 2 and 3 show that strong coupling between ATP hydrolysis and channel gating is not required. ATP binding, a key part of any ABC protein enzymatic cycle, is enough to switch SUR1 to activating conformations. Like other ABC proteins. SUR1 interacts with two ATPs with differing affinities (80). We assume that NBD1, with a higher affinity for ATP ($\pm Mg^{2+}$), is occupied and that ATP binding at NBD2 results in NBD dimerization and a repositioning of TMDs 1 and 2 from inward- to more outward-facing conformations as confirmed by cryo-EM structures of $K_{ATP}$ channels (42, 57). The agonist action of $ATP^{4-}$, without $Mg^{2+}$, on SUR1 mutant channels with impaired ATPase activity is clearly demonstrable when the inhibitory effect of ATP binding on the Kir6.2 pore is minimized (Fig. 2). This

demonstrates that ATP is a $K_{ATP}$ channel agonist and identifies ATP-bound, outward-facing states of SUR1 as activating conformations. This agonist action and our earlier reports (54, 55) on nucleotide-induced switching of SUR1 conformations show the dependence of switching, and thus agonist action, on the affinity of SUR1 for $ATP^{4-}$ or MgATP. SUR1 has a weaker affinity for $ATP^{4-}$ than MgATP and is less potent at switching conformations and activating channel openings (Fig. 1C). This dependence is physiologically and clinically important; *ABCC8*/SUR1 mutations, identified in cases of neonatal diabetes, have higher affinities for ATP, whereas mutations identified in cases of congenital hyperinsulinism have reduced affinities (65).

The idea that ATP is a $K_{ATP}$ channel agonist is counterintuitive as it is widely appreciated that eliminating nucleotides, for example by pulling insulin-secreting β-cell membrane patches into nucleotide-free medium, a maneuver that favors inward-facing conformations, produces a large increase in $K_{ATP}$ channel openings (see Fig. 3 for example). Despite this apparent contradiction, the results are readily rationalized. Removing nucleotides eliminates their inhibitory action on the Kir6.2 pore and shifts the SUR1 population in favor of inward-facing, nonactivating states, but the remaining equilibrium fraction of outward-facing, activating conformers supports channel openings. If this is correct, added sulfonylureas, e.g. tolbutamide or GBM, should act as “inverse agonists” by selecting and stabilizing inward-facing, nonactivating conformers. This idea is supported by early studies (37, 81–84), including the first report showing that the sulfonylurea receptor was part of $K_{ATP}$ channels and that active, open channels, without added $Mg^{2+}$ or ATP, were inhibited by tolbutamide or GBM (30). The idea that GBM binding will stabilize inward-facing conformers is a consequence of the high affinity of SUR1 for GBM and is directly supported by cryo-EM studies (77, 78).

*Assembly with Kir6.2 alters ATP-induced switching of SUR1 between conformations with high or low affinity for GBM*

ATP binding switches SUR1 to outward-facing conformations that activate channel openings (Figs. 2 and 3). This ATP-induced conformational change noncompetitively reduces [$^3$H]GBM binding to SUR1 alone or in $K_{ATP}$ channels (Figs. 5⇑–7). The reduction of bound [$^3$H]GBM by ATP is incomplete; at high, saturating concentrations of ATP, the remaining bound fraction reflects a new, lower-affinity equilibrium

between [$^3$H]GBM and ATP-bound SUR1 either alone or in channels. We used a four-state equilibrium model (Fig. 5, inset, and Equation 1) to quantify the allosteric reduction of bound [$^3$H]GBM by ATP and to evaluate the effect(s) of pairing SUR1 with Kir6.2 on this allosteric coupling. Equation 1 relates bound [$^3$H]GBM to the concentrations of GBM and ATP, their respective dissociation constants ($K_G$ for [$^3$H]GBM and $K_T$ for nucleotides), and an allosteric coupling constant, β. The products, $\beta K_G$ and $\beta K_T$, reflect the dissociation constants for ATP-bound and GBM-bound complexes, respectively. As described above, we assume ATP binding at NBD2 drives the conformational change (54, 55). In structural terms, this reflects switching SUR1 from inward-facing, nucleotide-free “apo” or singly liganded states with the greatest affinity for GBM to outward-facing, ATP-bound states with lower affinity.

To apply the four-state model, the dissociation constants for [$^3$H]GBM, $K_G$, are determined independently (Fig. 4 and Table 1) and assumed constant when fitting the ATP inhibition data. Determinations of $K_T$ and β for WT SUR1 alone or in channels (Fig. 5 and Table 2) indicate that the affinities of ATP-bound SUR1 alone or in channels for [$^3$H]GBM are ~5- or ~2.2-fold weaker than the unliganded molecules, respectively (compare $K_G$ versus $\beta K_G$ values in Table 2). Thus, the greater reduction in bound [$^3$H]GBM seen for SUR1 alone reflects the ~5-fold weaker affinity of ATP-bound SUR1 for [$^3$H]GBM. The lesser ATP effect observed for channels reflects the smaller change in affinities. The negative allosteric effects are reciprocal; GBM-bound SUR1 alone or in channels has a corresponding lower affinity for ATP (compare $K_T$ versus $\beta K_T$ values in Table 2). The data have two implications. First, the results imply that the attenuation of the agonist action of ATP underlies the inhibitory effect of GBM on WT $K_{ATP}$ channels. Basically, ATP binding to SUR1 biases channels toward open states; reducing the affinity of SUR1 for ATP reduces channel activity. Second, the negative allosteric linkage is clinically important; glibenclamide is now a common therapy for the treatment of cases of neonatal diabetes (85), and GBM therapy will attenuate the excess agonist action of ATP on *ABCC8*/SUR1 mutations with higher affinities for ATP. Thus, GBM therapy attenuates the consequences of neonatal diabetes ABCC8 mutations with higher affinities for ATP by reducing their affinities for nucleotides.

We applied the four-state model to assess how MgADP affects ATP/GBM site coupling with somewhat surprising results. In agreement with earlier studies (54), MgADP did stabilize lower-affinity conformers of SUR1, but the effect on full channels was negligible (Fig. 7). The estimated $K_{ADP}$ for MgADP binding to SUR1 alone was like the $K_T$ for MgATP, but the allosteric factor, β = 1.8, was low, and the reduction in bound [$^3$H]GBM was less than for MgATP (Fig. 7). The result suggests that the energy of MgADP binding at both NBD1 and NBD2 may not be enough to stabilize SUR1 conformers in full channels that have a lower $K_G$ for [$^3$H]GBM; ATP binding to NBD1 may be needed. Structural studies on channels with both GBM and ADP present and/or bound are needed to determine whether GBM prevents dimerization of the NBDs or affects reconfiguration of the TMDs to activating conformations.

*A potential structural pathway coupling the SUR1 ATP- and GBM-binding sites*

Our results (Fig. 5 and Refs. 54, 55, and 65) and early studies on the effects of nucleotides on [$^3$H]GBM binding clearly demonstrate negative allosteric effects (51, 58, 86–88). However, essentially nothing is known about possible amino acid networks or structural pathways coupling the ATP-binding sites (NBDs) with the GBM-binding site in SUR1. One obvious candidate is the pair of “crossover” helices (15 and 16) that link NBD1 with TMD2 in SUR1 (57, 77, 78) and other ABC proteins. We tested this possibility using $SUR1_{Q1179R}$, a well-studied neonatal diabetes mutation (54, 55, 65, 89, 90). The Gln → Arg substitution at position 1179, near the top of helix 15 within 20 nm of the GBM-binding site (Fig. 8 and Ref. 41), effectively eliminates the constraint on coupling imposed by assembly with Kir6.2 (Fig. 7) This substitution places a positive charge adjacent to Lys-1180 and approximately one helical turn from Arg-1174. The introduced charge has two effects, increasing the KG of the “Arg” variant for [$^3$H]GBM and increasing the allosteric factor β significantly. The results are consistent with the swapped transmembrane helices 15 and 16 coupling ATP binding and NBD dimerization with the sulfonylurea-binding site.

*Why does assembly with Kir6.2 increase the affinity of SUR1 for [$^3$H]GBM?*

As noted above, to apply the four-state model, the dissociation constants for [$^3$H]GBM were determined separately (Fig. 4 and Table 1). The results, although consistent with earlier studies reporting that SUR1 in channels binds [$^3$H]GBM 5–10-

fold more tightly than SUR1 alone (75, 91), are not well understood. The increased affinity is not unique to GBM; repaglinide, a related channel antagonist, has a 100–150-fold higher affinity for SUR1 in channels versus the receptor alone (75, 76). Early mechanistic attempts to account for these differences proposed that KNtp, the N terminus of Kir6.2, contributed in a direct fashion to GBM binding (for reviews, see Refs. 40, 92, and 93). A variety of data support a direct interaction, including the finding that truncations of KNtp eliminate the difference in affinity between channels and receptors (75, 76) but increase channel open probability and reduce the inhibitory action of the sulfonylurea antagonist tolbutamide (76, 94–96). Photoaffinity labeling with an azido derivative of GBM labels Kir6.2 but not truncated subunits missing KNtp, which implies that KNtp is near GBM (97). Restricting the positioning of KNtp by fusing the Kir and SUR N and C termini, SUR1–Kir6.2 (3–5), or by constructing “triple fusions,” SUR1–(Kir6.2)2, markedly reduces the affinities for MgATP and GBM (3). Additional studies tested the effects of a synthetic 32-amino-acid “KNtp,” showing it reduces the open probability of ΔN32Kir6.2/SUR1 channels but increases channel activity when applied to full-length channels (96). This suggested that SUR1 has a binding site for KNtp, whose occupation in truncated channels partially restores function, but competing with the endogenous KNtp mimics the effects of truncation (96). If KNtp is near GBM, as implied by these early results, the cryo-EM localization of bound GBM (41, 42) suggests that KNtp can access the central cavity and that SUR1 is a cryptic or frustrated peptide transporter. A cryo-EM study by Wu et al. (42) provides some support for this idea, reporting that electron densities near the GBM site may be due to KNtp, although the densities are not strong enough to allow modeling of the Kir N terminus. How this cryptic transport activity and potential movement(s) of KNtp during ATP-driven SUR1 conformational changes are coupled to channel gating remains an intriguing open question.

**Summary**

We have shown that ATP acts as a $K_{ATP}$ channel agonist, switching SUR1 from inward- to outward-facing conformations that can activate channel openings. This is easily demonstrated when the inhibitory effects of ATP on the pore are minimized and ND mutants of SUR1 with higher affinity for ATP are tested. ATP binding is sufficient; strong coupling to ATP hydrolysis is not required. ATP-induced switching has a negative allosteric effect on GBM binding; channel activating, outward-facing

conformations have the lowest affinity for GBM as expected for a channel antagonist. The allosteric effects are reciprocal; bound GBM reduces the affinity of SUR1 for ATP; thus, treatment with sulfonylureas will reduce the agonist action of ATP on WT and ND mutant channels and reduce channel openings. The structural links between the NBDs and GBM-binding site are not defined, but results with one ND SUR1 mutant, $SUR1_{Q1179R}$, suggest involvement of helices 15 and 16 in TMD2 that contact NBD1. The biochemical prediction of ternary complexes, i.e. SUR1 liganded with both GBM and ATP, requires confirmation by cryo-EM studies.

**Experimental procedures**

*Molecular biology and cell culture.* WT or mutant hamster Abcc8/SUR1 cDNAs (National Institutes of Health NCBI sequence L40623.1) were cloned into the multiple cloning site of the Tet-On® 3G bicistronic inducible expression vector, pTRE3G-ZsGreen1 (TaKaRa Bio USA, Inc.). The ZsGreen1 marker was replaced by either WT human KCNJ11/Kir6.2 (National Institutes of Health reference sequence NG_012446) or $KCNJ11/Kir6.2_{G334D}$ in which aspartic acid was substituted for glycine at position 334. A puromycin resistance cassette was either engineered into the resulting plasmid or cotransfected into HEK 293 Tet-On cells carrying the Tet-On 3G transactivator (TaKaRa, Inc.). Amino acid numbering is based on the hamster sequence (NIH NCBI sequence L40623.1) to facilitate comparison with the numbering in the cryo-EM publications (41, 42, 77, 78). The exception is $SUR1_{A1369S}$ where the numbering follows the human reference sequence (National Institutes of Health NCBI accession number AH003589) without incorporation of exon 17. The equivalent numbering in the hamster sequence includes exon 17. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and glutamine. Stable cell lines were selected and subcloned using puromycin (600 μm). Expression was induced by addition of doxycycline (300 μm) and tested for characterization of channel activity from 24 to 72 h. In some experiments, transient transfections were done and induced with doxycycline after 24 h.

*Membrane isolation.* For membrane preparation, doxycycline-induced cells were centrifuged for 10 min at 500 × g at 37 °C and lysed by incubation in ice-cold hypotonic buffer containing 10 mm HEPES, 1 mm EGTA, and a protease inhibitor, HALT (Thermo Fisher), at pH 7.4. After 30 min on ice, the swollen cells were broken

by 50 strokes in a tight-fitting Dounce homogenizer. Unbroken cells and nuclei were removed by centrifugation at 1,200 × g for 10 min at 4 °C. The supernatant was centrifuged at 105 × g at 4 °C for 60 min, and the resulting membrane pellet was resuspended in a buffer containing 5 mm HEPES, 5 mm KCl, and 139 mm NaCl at pH 7.4 (4 °C). Protein concentration was determined by the BCA method using BSA as the standard. The protein concentration was adjusted to 2.0 mg ml−1, and suspensions were frozen at −80 °C.

*[$^3$H]GBM homologous displacement experiments without nucleotides.* The dissociation constant, $K_G$, of SUR1 for [$^3$H]GBM, in the absence of $Mg^{2+}$ and nucleotides, was measured using isolated membranes from cells expressing WT or mutant SUR1 ± Kir6.2. Membranes were suspended in a buffered solution (139 mm NaCl, 5 mm KCl, 1 mm EDTA, and 50 mm HEPES, pH 7.4) with 0.3 or 1 nm [$^3$H]GBM plus increasing concentrations of unlabeled GBM for 30 min at 37 °C and then analyzed by rapid filtration as described previously (58) to determine total binding. The estimated values for free $Mg^{2+}$, <10 nm, were calculated using MAXC (98) assuming contaminating $Ca^{2+}$ and $Mg^{2+}$ levels as high as 5 μm. Triplicate determinations were made for each GBM concentration per experiment; the data from three or more experiments were analyzed following the procedure described by Swillens (74) for homologous displacement curves. Analysis and global fitting were done with user-written functions in Origin2018 (OriginLab Corp., Northampton, MA) or in Prism 7 (GraphPad Software, Inc., La Jolla, CA). The data were weighted using a factor of (1/cpm)2.

*[$^3$H]GBM binding in nucleotide-containing solutions.* [$^3$H]GBM binding to isolated membranes was analyzed by rapid filtration as described previously (58). The [$^3$H]GBM concentration, ∼1 nm, was determined for each experiment. MgATP-containing solutions (concentrations ranging from 10 nm to 10 mm MgATP) with a calculated free $Mg^{2+}$ concentration of 1 mm were prepared using the BioEqCalc program from Akers and Goldberg (99). Solutions contained 50 mm HEPES, pH 7.4, 5 mm KCl, and varying amounts of $Na_2ATP$, $MgCl_2$, and NaCl to give the appropriate MgATP concentration at an ionic strength of 0.2 m.

In the experiments using MgADP, hexokinase (1 unit) and glucose (10 mm) were added to scavenge contaminant ATP and ATP generated by adenylate kinase. ATP

determinations using luciferase indicate that the ATP levels were <0.1% of added ADP. The solution conditions, pH, ionic strength, and free $Mg^{2+}$ concentrations, for the MgADP experiments were equivalent to those for MgATP described above.

*Model fitting.* The four-state equilibrium model (Fig. 5, inset) described previously (54) was used to estimate the dissociation constant, $K_T$, for binding of ATP (or $K_{ADP}$ for ADP) at NBD2 of SUR1 and the allosteric constant β. The dissociation constants ($K_G$) for [$^3$H]GBM were estimated independently as described above and held constant. Following Alper and Gelb (100) and Christopoulos (101), a transformed binding equation was used with nonlinear least-squares methods to estimate approximately normally distributed values for $pK_T$ and pβ and to estimate confidence intervals. Formula (Eq. 1) Here, $K_T = 10^{pK_T}$ and $\beta = 10^{p\beta}$.

*Electrophysiology.* Patch-clamp recordings were done in the inside-out configuration. $K_{ATP}$ currents were measured at a membrane potential of −50 mV (pipette voltage, +50 mV); inward currents are shown as downward deflections. Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, March-Hugstetten, Germany) and had a resistance of 6–8 megaohms. Currents were recorded with an EPC-9 patch-clamp amplifier using Patchmaster software (HEKA, Lambrecht, Germany). Single-channel $K_{ATP}$ channel currents were about −4 pA at a holding potential of −50 mV, corresponding to a single-channel conductance of 80 picosiemens.

The pipette solution contained 130 mmol/liter KCl, 1.2 mmol/liter $MgCl_2$, 2 mmol/liter $CaCl_2$, 10 mmol/liter EGTA, and 10 mmol/liter HEPES; pH was adjusted to 7.4 with KOH at 25 °C. The magnesium-free bath solution contained 130 mmol/liter KCl, 4.6 mmol/liter $CaCl_2$, 10 mmol/liter EDTA, and 20 mmol/liter HEPES with pH adjusted to 7.2 with KOH at 25 °C. The MgATP bath solution contained 130 mmol/liter KCl, 2 mmol/liter $CaCl_2$, 10 mmol/liter EGTA, 1 mmol/liter $Na_2ATP$, 1.7 mmol/liter $MgCl_2$, and 20 mmol/liter HEPES with pH adjusted to 7.2 with KOH at 25 °C. Analyses to estimate NPo were done offline in IgorPro 7 (Wavemetrics, Inc., Lake Oswego, OR) with user-written software.

*Statistics.* Data values are given as means ± S.E. (n = number of replications). Estimated parameter values are reported as means ± (lower–higher) 95% confidence

intervals. In Fig. 2, the number of channels in patches from randomly selected cells and randomly selected locations varied widely, presumably due to differences in expression levels and/or cell-surface distribution. To determine the significance of the action of nucleotides on SUR1/Kir6.2$_{G334D}$ $K_{ATP}$ channels, in the absence of $Mg^{2+}$, a nonparametric statistic, W, in the Wilcoxon signed-rank test was applied (102). This is a paired-difference test. Patches are chosen randomly, and the NPo values are determined for a fixed time, typically 30–60 s before and 30–60 s at the end of an application of nucleotide. The absolute differences in NPo values, plus versus minus nucleotide, were calculated and ranked smallest to largest. In all cases, the signed differences were positive; i.e. application of nucleotides always increased the NPo. The rank times the difference values were summed to give W. Comparison of W with calculated values for small sample numbers or using online Wilcoxon tests (e.g. http://www.vassarstats.net/wilcoxon.html)3 gave estimates of statistical significance. p values <0.05 were considered significant.

**Author contributions:** J. S., T. S. M., C. B., F. D., U. Q., P. K.-D., and J. B. data curation; J. S., T. S. M., C. B., U. Q., P. K.-D., G. D., and J. B. formal analysis; J. S., T. S. M., C. B., F. D., U. Q., P. K.-D., G. D., and J. B. validation; J. S., T. S. M., C. B., F. D., U. Q., P. K.-D., G. D., and J. B. investigation; J. S., T. S. M., C. B., F. D., U. Q., P. K.-D., G. D., and J. B. methodology; J. S., U. Q., and J. B. writing-original draft; J. S., T. S. M., C. B., F. D., U. Q., P. K.-D., G. D., and J. B. writing-review and editing; T. S. M., U. Q., and J. B. visualization; U. Q. and J. B. conceptualization; P. K.-D., G. D., and J. B. supervision; J. B. resources; J. B. software; J. B. funding acquisition.

**Acknowledgment:** We thank Dr. Robert Goldberg for providing an updated version of BioCalcEq running under Mathematica v11.

This work was supported by National Institutes of Health Grant DK098647 (to J. B.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

**References**

1. Aguilar-Bryan, L., Nichols, C. G., Wechsler, S. W., Clement, J. P., 4th, Boyd, A. E., 3rd, González, G., Herrera-Sosa, H., Nguy, K., Bryan, J., and Nelson, D. A. (1995) Cloning of the β cell high-affinity sulfonylurea receptor: a regulator of insulin secretion. Science 268, 423–426

2. Inagaki, N., Gonoi, T., Clement, J. P., 4th, Namba, N., Inazawa, J., Gonzalez, G., Aguilar-Bryan, L., Seino, S., and Bryan, J. (1995) Reconstitution of IKATP: an inward rectifier subunit plus the sulfonylurea receptor. Science 270, 1166–1170

3. Clement, J. P., 4th, Kunjilwar, K., Gonzalez, G., Schwanstecher, M., Panten, U., Aguilar-Bryan, L., and Bryan, J. (1997) Association and stoichiometry of KATP channel subunits. Neuron 18, 827–838

4. Inagaki, N., Gonoi, T., and Seino, S. (1997) Subunit stoichiometry of the pancreatic β-cell ATP-sensitive K+ channel. FEBS Lett. 409, 232–236

5. Shyng, S., and Nichols, C. G. (1997) Octameric stoichiometry of the KATP channel complex. J. Gen. Physiol. 110, 655–664

6. Thomas, P. M., Cote, G. J., Wohllk, N., Haddad, B., Mathew, P. M., Rabl, W., Aguilar-Bryan, L., Gagel, R. F., and Bryan, J. (1995) Mutations in the sulfonylurea receptor gene in familial persistent hyperinsulinemic hypoglycemia of infancy. Science 268, 426–429

7. Aguilar-Bryan, L., and Bryan, J. (1999) Molecular biology of adenosine triphosphate-sensitive potassium channels. Endocr. Rev. 20, 101–135

8. Bryan, J., Muñoz, A., Zhang, X., Düfer, M., Drews, G., Krippeit-Drews, P., and Aguilar-Bryan, L. (2007) ABCC8 and ABCC9: ABC transporters that regulate K+ channels. Pflugers Arch 453, 703–718

9. Hattersley, A. T., and Ashcroft, F. M. (2005) Activating mutations in Kir6.2 and neonatal diabetes: new clinical syndromes, new scientific insights, and new therapy. Diabetes 54, 2503–2513

10. Babenko, A. P., Polak, M., Cavé, H., Busiah, K., Czernichow, P., Scharfmann, R., Bryan, J., Aguilar-Bryan, L., Vaxillaire, M., and Froguel, P. (2006) Activating mutations in the ABCC8 gene in neonatal diabetes mellitus. N. Engl. J. Med. 355, 456–466

11. Aguilar-Bryan, L., and Bryan, J. (2008) Neonatal diabetes mellitus. Endocr. Rev. 29, 265–291

12. Hattersley, A. T., and Patel, K. A. (2017) Precision diabetes: learning from monogenic diabetes. Diabetologia 60, 769–777

13. Bowman, P., Flanagan, S. E., Edghill, E. L., Damhuis, A., Shepherd, M. H., Paisey, R., Hattersley, A. T., and Ellard, S. (2012) Heterozygous ABCC8 mutations are a cause of MODY. Diabetologia 55, 123–127

14. Emdin, C. A., Klarin, D., Natarajan, P., CARDIOGRAM Exome Consortium, Florez, J. C., Kathiresan, S., and Khera, A. V. (2017) Genetic variation at the sulfonylurea receptor, type 2 diabetes, and coronary heart disease. Diabetes 66, 2310–2315

15. Cook, D. L., and Hales, C. N. (1984) Intracellular ATP directly blocks K+ channels in pancreatic β-cells. Nature 311, 271–273

16. Dunne, M. J., and Petersen, O. H. (1986) Intracellular ADP activates K+ channels that are inhibited by ATP in an insulin-secreting cell line. FEBS Lett. 208, 59–62

17. Kakei, M., Kelly, R. P., Ashcroft, S. J., and Ashcroft, F. M. (1986) The ATP-sensitivity of K+ channels in rat pancreatic β-cells is modulated by ADP. FEBS Lett. 208, 63–66

18. Hopkins, W. F., Fatherazi, S., Peter-Riesch, B., Corkey, B. E., and Cook, D. L. (1992) Two sites for adenine-nucleotide regulation of ATP-sensitive potassium channels in mouse pancreatic β-cells and HIT cells. J. Membr. Biol. 129, 287–295

19. Tucker, S. J., Gribble, F. M., Zhao, C., Trapp, S., and Ashcroft, F. M. (1997) Truncation of Kir6.2 produces ATP-sensitive K+ channels in the absence of the sulphonylurea receptor. Nature 387, 179–183

20. Gribble, F. M., Tucker, S. J., Haug, T., and Ashcroft, F. M. (1998) MgATP activates the β cell KATP channel by interaction with its SUR1 subunit. Proc. Natl. Acad. Sci. U.S.A. 95, 7185–7190

21. Baukrowitz, T., Schulte, U., Oliver, D., Herlitze, S., Krauter, T., Tucker, S. J., Ruppersberg, J. P., and Fakler, B. (1998) PIP2 and PIP as determinants for ATP inhibition of $K_{ATP}$ channels. Science 282, 1141–1144

22. Shyng, S. L., and Nichols, C. G. (1998) Membrane phospholipid control of nucleotide sensitivity of KATP channels. Science 282, 1138–1141

23. Baukrowitz, T., and Fakler, B. (2000) KATP channels: linker between phospholipid metabolism and excitability. Biochem. Pharmacol. 60, 735–740

24. Bränström, R., Leibiger, I. B., Leibiger, B., Corkey, B. E., Berggren, P. O., and Larsson, O. (1998) Long chain coenzyme A esters activate the pore-forming subunit (Kir6.2) of the ATP-regulated potassium channel. J. Biol. Chem. 273, 31395–31400

25. Rohács, T., Lopes, C. M., Jin, T., Ramdya, P. P., Molnár, Z., and Logothetis, D. E. (2003) Specificity of activation by phosphoinositides determines lipid regulation of Kir channels. Proc. Natl. Acad. Sci. U.S.A. 100, 745–750

26. Schulze, D., Rapedius, M., Krauter, T., and Baukrowitz, T. (2003) Long-chain acyl-CoA esters and phosphatidylinositol phosphates modulate ATP inhibition of KATP channels by the same mechanism. J. Physiol. 552, 357–367

27. Béguin, P., Nagashima, K., Nishimura, M., Gonoi, T., and Seino, S. (1999) PKA-mediated phosphorylation of the human KATP channel: separate roles of Kir6.2 and SUR1 subunit phosphorylation. EMBO J. 18, 4722–4732

28. Light, P. E., Manning Fox, J. E., Riedel, M. J., and Wheeler, M. B. (2002) Glucagon-like peptide-1 inhibits pancreatic ATP-sensitive potassium channels via a protein kinase A- and ADP-dependent mechanism. Mol. Endocrinol. 16, 2135–2144

29. Lin, Y. F., and Chai, Y. (2008) Functional modulation of the ATP-sensitive potassium channel by extracellular signal-regulated kinase-mediated phosphorylation. Neuroscience 152, 371–380

30. Sturgess, N. C., Ashford, M. L., Cook, D. L., and Hales, C. N. (1985) The sulphonylurea receptor may be an ATP-sensitive potassium channel. Lancet 2, 474–475

31. Sturgess, N. C., Kozlowski, R. Z., Carrington, C. A., Hales, C. N., and Ashford, M. L. (1988) Effects of sulphonylureas and diazoxide on insulin secretion and nucleotide-sensitive channels in an insulin-secreting cell line. Br. J. Pharmacol. 95, 83–94

32. Ashcroft, F. M., Kakei, M., Gibson, J. S., Gray, D. W., and Sutton, R. (1989) The ATP- and tolbutamide-sensitivity of the ATP-sensitive K-channel from human pancreatic β cells. Diabetologia 32, 591–598

33. Panten, U., Heipel, C., Rosenberger, F., Scheffer, K., Zünkler, B. J., and Schwanstecher, C. (1990) Tolbutamide-sensitivity of the adenosine 5′-triphosphate-dependent K+ channel in mouse pancreatic β-cells. Naunyn Schmiedebergs Arch. Pharmacol. 342, 566–574

34. Hu, S., Wang, S., Fanelli, B., Bell, P. A., Dunning, B. E., Geisse, S., Schmitz, R., and Boettcher, B. R. (2000) Pancreatic β-cell K(ATP) channel activity and membrane-binding studies with nateglinide: A comparison with sulfonylureas and repaglinide. J. Pharmacol. Exp. Ther. 293, 444–452

35. Dabrowski, M., Wahl, P., Holmes, W. E., and Ashcroft, F. M. (2001) Effect of repaglinide on cloned β cell, cardiac and smooth muscle types of ATP-sensitive potassium channels. Diabetologia 44, 747–756

36. Quast, U., Stephan, D., Bieger, S., and Russ, U. (2004) The impact of ATP-sensitive K+ channel subtype selectivity of insulin secretagogues for the coronary vasculature and the myocardium. Diabetes 53, Suppl. 3, S156–S164

37. Dunne, M. J., Illot, M. C., and Peterson, O. H. (1987) Interaction of diazoxide, tolbutamide and ATP4− on nucleotide-dependent K+ channels in an insulin-secreting cell line. J. Membr. Biol. 99, 215–224

38. Lebrun, P., Devreux, V., Hermann, M., and Herchuelz, A. (1989) Similarities between the effects of pinacidil and diazoxide on ionic and secretory events in rat pancreatic islets. J. Pharmacol. Exp. Ther. 250, 1011–1018

39. Niki, I., and Ashcroft, S. J. (1991) Possible involvement of protein phosphorylation in the regulation of the sulphonylurea receptor of a pancreatic β-cell line, HIT T15. Biochim. Biophys. Acta 1133, 95–101

40. Bryan, J., Crane, A., Vila-Carriles, W. H., Babenko, A. P., and Aguilar-Bryan, L. (2005) Insulin secretagogues, sulfonylurea receptors and KATP channels. Curr. Pharm. Des. 11, 2699–2716

41. Martin, G. M., Kandasamy, B., DiMaio, F., Yoshioka, C., and Shyng, S. L. (2017) Anti-diabetic drug binding site in a mammalian KATP channel revealed by cryo-EM. Elife 6, e31054

42. Wu, J. X., Ding, D., Wang, M., Kang, Y., Zeng, X., and Chen, L. (2018) Ligand binding and conformational changes of SUR1 subunit in pancreatic ATP-sensitive potassium channels. Protein Cell 9, 553–567

43. Linton, K. J. (2007) Structure and function of ABC transporters. Physiology 22, 122–130

44. Oldham, M. L., Davidson, A. L., and Chen, J. (2008) Structural insights into ABC transporter mechanism. Curr. Opin. Struct. Biol. 18, 726–733

45. Rees, D. C., Johnson, E., and Lewinson, O. (2009) ABC transporters: the power to change. Nat. Rev. Mol. Cell Biol. 10, 218–227

46. Zingman, L. V., Alekseev, A. E., Bienengraeber, M., Hodgson, D., Karger, A. B., Dzeja, P. P., and Terzic, A. (2001) Signaling in channel/enzyme multimers: ATPase transitions in SUR module gate ATP-sensitive K+ conductance. Neuron 31, 233–245

47. Zingman, L. V., Hodgson, D. M., Bienengraeber, M., Karger, A. B., Kathmann, E. C., Alekseev, A. E., and Terzic, A. (2002) Tandem function of nucleotide binding domains confers competence to sulfonylurea receptor in gating ATP-sensitive K+ channels. J. Biol. Chem. 277, 14206–14210

48. Bienengraeber, M., Alekseev, A. E., Abraham, M. R., Carrasco, A. J., Moreau, C., Vivaudou, M., Dzeja, P. P., and Terzic, A. (2000) ATPase activity of the sulfonylurea receptor: a catalytic function for the KATP channel complex. FASEB J. 14, 1943–1952

49. Zingman, L. V., Alekseev, A. E., Hodgson-Zingman, D. M., and Terzic, A. (2007) ATP-sensitive potassium channels: metabolic sensing and cardioprotection. J. Appl. Physiol. 103, 1888–1893

50. Dickinson, K. E., Bryson, C. C., Cohen, R. B., Rogers, L., Green, D. W., and Atwal, K. S. (1997) Nucleotide regulation and characteristics of potassium channel opener binding to skeletal muscle membranes. Mol. Pharmacol. 52, 473–481

51. Hambrock, A., Löffler-Walz, C., Kurachi, Y., and Quast, U. (1998) Mg2+ and ATP dependence of KATP channel modulator binding to the recombinant sulphonylurea receptor, SUR2B. Br. J. Pharmacol. 125, 577–583

52. Schwanstecher, M., Sieverding, C., Dörschner, H., Gross, I., Aguilar-Bryan, L., Schwanstecher, C., and Bryan, J. (1998) Potassium channel openers require ATP to bind to and act through sulfonylurea receptors. EMBO J. 17, 5529–5535

53. Choi, K. H., Tantama, M., and Licht, S. (2008) Testing for violations of microscopic reversibility in ATP-sensitive potassium channel gating. J. Phys. Chem. B 112, 10314–10321

54. Ortiz, D., Voyvodic, P., Gossack, L., Quast, U., and Bryan, J. (2012) Two neonatal diabetes mutations on transmembrane helix 15 of SUR1 increase affinity for ATP and ADP at nucleotide binding domain 2. J. Biol. Chem. 287, 17985–17995

55. Ortiz, D., Gossack, L., Quast, U., and Bryan, J. (2013) Reinterpreting the action of ATP analogs on KATP channels. J. Biol. Chem. 288, 18894–18902

56. de Wet, H., Mikhailov, M. V., Fotinou, C., Dreger, M., Craig, T. J., Vénien-Bryan, C., and Ashcroft, F. M. (2007) Studies of the ATPase activity of the ABC protein SUR1. FEBS J 274, 3532–3544

57. Lee, K. P. K., Chen, J., and MacKinnon, R. (2017) Molecular structure of human KATP in complex with ATP and ADP. Elife 6, e32481

58. Hambrock, A., Löffler-Walz, C., and Quast, U. (2002) Glibenclamide binding to sulphonylurea receptor subtypes: dependence on adenine nucleotides. Br. J. Pharmacol. 136, 995–1004

59. Masia, R., Koster, J. C., Tumini, S., Chiarelli, F., Colombo, C., Nichols, C. G., and Barbetti, F. (2007) An ATP-binding mutation (G334D) in KCNJ11 is associated with a sulfonylurea-insensitive form of developmental delay, epilepsy, and neonatal diabetes. Diabetes 56, 328–336

60. Li, L., Wang, J., and Drain, P. (2000) The I182 region of KIR6.2 is closely associated with ligand binding in KATP channel inhibition by ATP. Biophys. J. 79, 841–852

61. Proks, P., de Wet, H., and Ashcroft, F. M. (2013) Molecular mechanism of sulphonylurea block of KATP channels carrying mutations that impair ATP inhibition and cause neonatal diabetes. Diabetes 62, 3909–3919

62. Proks, P., de Wet, H., and Ashcroft, F. M. (2014) Sulfonylureas suppress the stimulatory action of Mg-nucleotides on Kir6.2/SUR1 but not Kir6.2/SUR2A KATP channels: a mechanistic study. J. Gen. Physiol. 144, 469–486

63. Babenko, A. P., Gonzalez, G., and Bryan, J. (1999) Two regions of sulfonylurea receptor specify the spontaneous bursting and ATP inhibition of KATP channel isoforms. J. Biol. Chem. 274, 11587–11592

64. Babenko, A. P., and Bryan, J. (2001) A conserved inhibitory and differential stimulatory action of nucleotides on KIR6.0/SUR complexes is essential for excitation-metabolism coupling by KATP channels. J. Biol. Chem. 276, 49083–49092

65. Ortiz, D., and Bryan, J. (2015) Neonatal diabetes and congenital hyperinsulinism caused by mutations in ABCC8/SUR1 are associated with altered and opposite affinities for ATP and ADP. Front. Endocrinol. 6, 48

66. Fatehi, M., Raja, M., Carter, C., Soliman, D., Holt, A., and Light, P. E. (2012) The ATP-sensitive K+ channel ABCC8 S1369A type 2 diabetes risk variant increases MgATPase activity. Diabetes 61, 241–249

67. Fatehi, M., Carter, C. R., Youssef, N., Hunter, B. E., Holt, A., and Light, P. E. (2015) Molecular determinants of ATP-sensitive potassium channel MgATPase activity: diabetes risk variants and diazoxide sensitivity. Biosci. Rep. 35, e00238

68. Orelle, C., Dalmas, O., Gros, P., Di Pietro, A., and Jault, J. M. (2003) The conserved glutamate residue adjacent to the Walker-B motif is the catalytic base for ATP hydrolysis in the ATP-binding cassette transporter BmrA. J. Biol. Chem. 278, 47002–47008

69. Tombline, G., Bartholomew, L. A., Tyndall, G. A., Gimi, K., Urbatsch, I. L., and Senior, A. E. (2004) Properties of P-glycoprotein with mutations in the “catalytic carboxylate” glutamate residues. J. Biol. Chem. 279, 46518–46526

70. Moody, J. E., Millen, L., Binns, D., Hunt, J. F., and Thomas, P. J. (2002) Cooperative, ATP-dependent association of the nucleotide binding cassettes during the catalytic cycle of ATP-binding cassette transporters. J. Biol. Chem. 277, 21111–21114

71. Smith, P. C., Karpowich, N., Millen, L., Moody, J. E., Rosen, J., Thomas, P. J., and Hunt, J. F. (2002) ATP binding to the motor domain from an ABC transporter drives formation of a nucleotide sandwich dimer. Mol. Cell 10, 139–149

72. Oldham, M. L., Khare, D., Quiocho, F. A., Davidson, A. L., and Chen, J. (2007) Crystal structure of a catalytic intermediate of the maltose transporter. Nature 450, 515–521

73. Pinney, S. E., MacMullen, C., Becker, S., Lin, Y. W., Hanna, C., Thornton, P., Ganguly, A., Shyng, S. L., and Stanley, C. A. (2008) Clinical characteristics and biochemical mechanisms of congenital hyperinsulinism associated with dominant KATP channel mutations. J. Clin. Investig. 118, 2877–2886

74. Swillens, S. (1995) Interpretation of binding curves obtained with high receptor concentrations: practical aid for computer analysis. Mol. Pharmacol. 47, 1197–1203

75. Hansen, A. M., Hansen, J. B., Carr, R. D., Ashcroft, F. M., and Wahl, P. (2005) Kir6.2-dependent high-affinity repaglinide binding to β-cell KATP channels. Br. J. Pharmacol. 144, 551–557

76. Kühner, P., Prager, R., Stephan, D., Russ, U., Winkler, M., Ortiz, D., Bryan, J., and Quast, U. (2012) Importance of the Kir6.2 N-terminus for the interaction of glibenclamide and repaglinide with the pancreatic KATP channel. Naunyn Schmiedebergs Arch. Pharmacol. 385, 299–311

77. Li, N., Wu, J. X., Ding, D., Cheng, J., Gao, N., and Chen, L. (2017) Structure of a pancreatic ATP-sensitive potassium channel. Cell 168, 101–110.e10

78. Martin, G. M., Yoshioka, C., Rex, E. A., Fay, J. F., Xie, Q., Whorton, M. R., Chen, J. Z., and Shyng, S. L. (2017) Cryo-EM structure of the ATP-sensitive potassium channel illuminates mechanisms of assembly and gating. Elife 6, e24149

79. Mikhailov, M. V., Campbell, J. D., de Wet, H., Shimomura, K., Zadek, B., Collins, R. F., Sansom, M. S., Ford, R. C., and Ashcroft, F. M. (2005) 3-D structural and functional characterization of the purified KATP channel complex Kir6.2-SUR1. EMBO J. 24, 4166–4175

80. Matsuo, M., Kioka, N., Amachi, T., and Ueda, K. (1999) ATP binding properties of the nucleotide-binding folds of SUR1. J. Biol. Chem. 274, 37479–37482

81. Trube, G., Rorsman, P., and Ohno-Shosaku, T. (1986) Opposite effects of tolbutamide and diazoxide on the ATP-dependent K+ channel in mouse pancreatic β-cells. Pflugers Arch. 407, 493–499

82. Ashcroft, F. M., Kakei, M., Kelly, R. P., and Sutton, R. (1987) ATP-sensitive K+ channels in human isolated pancreatic β-cells. FEBS Lett. 215, 9–12

83. Schmid-Antomarchi, H., De Weille, J., Fosset, M., and Lazdunski, M. (1987) The receptor for antidiabetic sulfonylureas controls the activity of the ATP-modulated K+ channel in insulin-secreting cells. J. Biol. Chem. 262, 15840–15844

84. Zünkler, B. J., Lenzen, S., Männer, K., Panten, U., and Trube, G. (1988) Concentration-dependent effects of tolbutamide, meglitinide, glipizide, glibenclamide and diazoxide on ATP-regulated K+ currents in pancreatic β-cells. Naunyn Schmiedebergs Arch. Pharmacol. 337, 225–230

85. Lemelman, M. B., Letourneau, L., and Greeley, S. A. W. (2018) Neonatal diabetes mellitus: an update on diagnosis and management. Clin. Perinatol. 45, 41–59

86. Schwanstecher, M., Löser, S., Rietze, I., and Panten, U. (1991) Phosphate and thiophosphate group donating adenine and guanine nucleotides inhibit glibenclamide binding to membranes from pancreatic islets. Naunyn Schmiedebergs Arch. Pharmacol. 343, 83–89

87. Schwanstecher, M., Löser, S., Brandt, C., Scheffer, K., Rosenberger, F., and Panten, U. (1992) Adenine nucleotide-induced inhibition of binding of sulphonylureas to their receptor in pancreatic islets. Br. J. Pharmacol. 105, 531–534

88. Schwanstecher, M., Löser, S., Chudziak, F., and Panten, U. (1994) Identification of a 38-kDa high affinity sulfonylurea-binding peptide in insulin-secreting cells and cerebral cortex. J. Biol. Chem. 269, 17768–17771

89. Christesen, H. B. T., Sjoblad, S., Brusgaard, K., Papadopoulou, D., and Jacobsen, B. B. (2005) Permanent neonatal diabetes in a child with an ABBC8 gene mutation. Horm. Res. 64, 135

90. Babenko, A. P. (2008) A novel ABCC8 (SUR1)-dependent mechanism of metabolism-excitation uncoupling. J. Biol. Chem. 283, 8778–8782

91. Stephan, D., Winkler, M., Kühner, P., Russ, U., and Quast, U. (2006) Selectivity of repaglinide and glibenclamide for the pancreatic over the cardiovascular KATP channels. Diabetologia 49, 2039–2048

92. Bryan, J., Vila-Carriles, W. H., Zhao, G., Babenko, A. P., and Aguilar-Bryan, L. (2004) Toward linking structure with function in ATP-sensitive K+ channels. Diabetes 53, Suppl. 3, S104–S112

93. Babenko, A. P. (2005) KATP channels “vingt ans après”: ATG to PDB to Mechanism. J. Mol. Cell. Cardiol. 39, 79–98

94. Koster, J. C., Sha, Q., and Nichols, C. G. (1999) Sulfonylurea and K+-channel opener sensitivity of KATP channels.Functional coupling of Kir6.2 and SUR1 subunits. J. Gen. Physiol. 114, 203–213

95. Reimann, F., Tucker, S. J., Proks, P., and Ashcroft, F. M. (1999) Involvement of the N-terminus of Kir6.2 in coupling to the sulphonylurea receptor. J. Physiol. 518, 325–336

96. Babenko, A. P., and Bryan, J. (2002) SUR-dependent modulation of KATP channels by an N-terminal KIR6.2 peptide. Defining intersubunit gating interactions. J. Biol. Chem. 277, 43997–44004

97. Vila-Carriles, W. H., Zhao, G., and Bryan, J. (2007) Defining a binding pocket for sulfonylureas in ATP-sensitive potassium channels. FASEB J. 21, 18–25

98. Bers, D. M., Patton, C. W., and Nuccitelli, R. (2010) A practical guide to the preparation of Ca2+ buffers. Methods Cell Biol. 99, 1–26

99. Akers, D. L., and Goldberg, R. N. (2001) BioEqCalc: a package for performing equilibrium calculations on biochemical reactions. Math. J. 8, 27

100. Alper, J. S., and Gelb, R. I. (1993) Application of nonparametric statistics to the estimation of the accuracy of Monte Carlo confidence intervals in regression analysis. Talanta 40, 355–361

101. Christopoulos, A. (1998) Assessing the distribution of parameters in models of ligand-receptor interaction: to log or not to log. Trends Pharmacol. Sci. 19, 351–357

102. Siegel, S. (1956) Non-parametric Statistics for the Behavioral Sciences, pp. 75–85, McGraw-Hill, New York

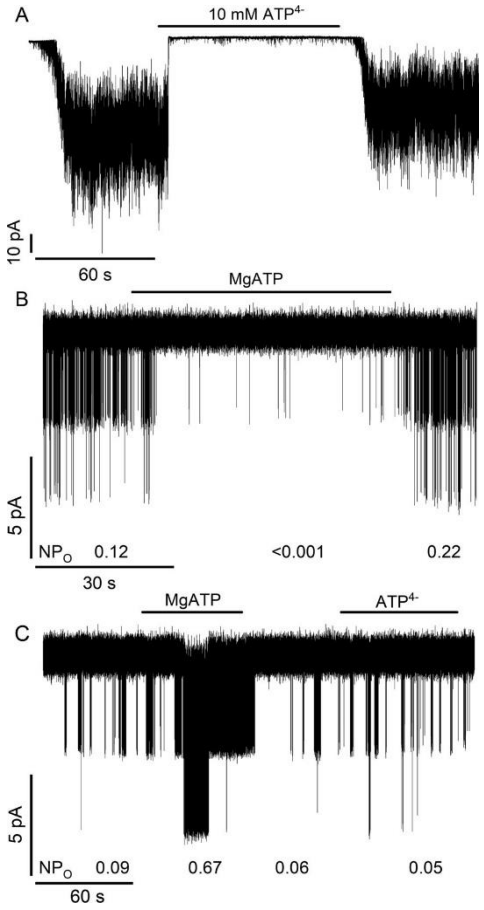


Figure 1. Assay for the action of nucleotides on $K_{ATP}$ channels. (A) $ATP^{4-}$ (10 mM), without $Mg^{2+}$, or (B), MgATP (1 mM) inhibits wtSUR1/wtKir6.2 channels. (C) wtSUR1/Kir6.2$_{G334D}$ channels, where the G334D mutant Kir6.2 subunit has a low affinity for adenine nucleotides, are activated by MgATP (1 mM), but not significantly affected by $ATP^{4-}$ (10 mM). In (B and C) the $NP_O$ values (current = number of open channels times channel open probability) were estimated over 20-to-45 second intervals before, during or after the application of nucleotides.

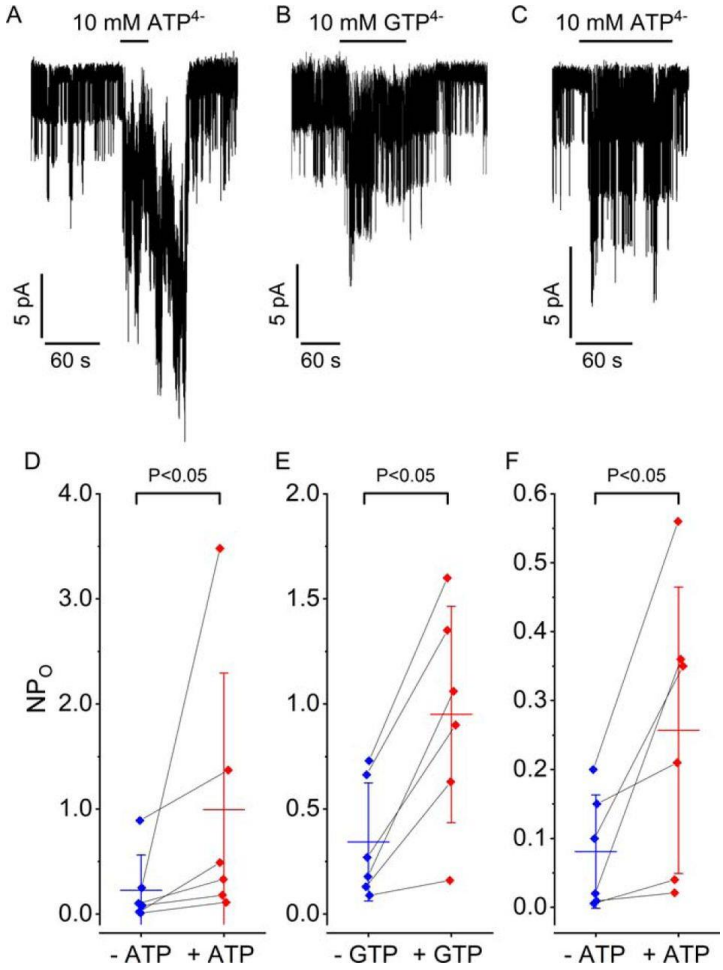


Fig. 2: $ATP^{4-}$ (10 mm) and $GTP^{4-}$ (10 mm) activate $K_{ATP}$ channels with ND mutations in SUR1 that have a lower $K_D$ for ATP. (A and B) Activation of $SUR1_{Q1179R}/Kir6.2_{G334D}$ channels by $ATP^{4-}$ or $GTP^{4-}$. (D and E) Summary of the results from twelve experiments on $SUR1_{Q1179}/Kir6.2_{G334D}$ channels. (C) Activation of $SUR1_{E1507Q}/Kir6.2_{G334D}$ channels by $ATP^{4-}$ and (F) summary of results from 7 experiments on $SUR1_{E1507Q}/Kir6.2_{G334D}$ channels. Red and blue lines are the means ± SEM. Significance was determined using the non-parametric Wilcoxon signed-rank test.

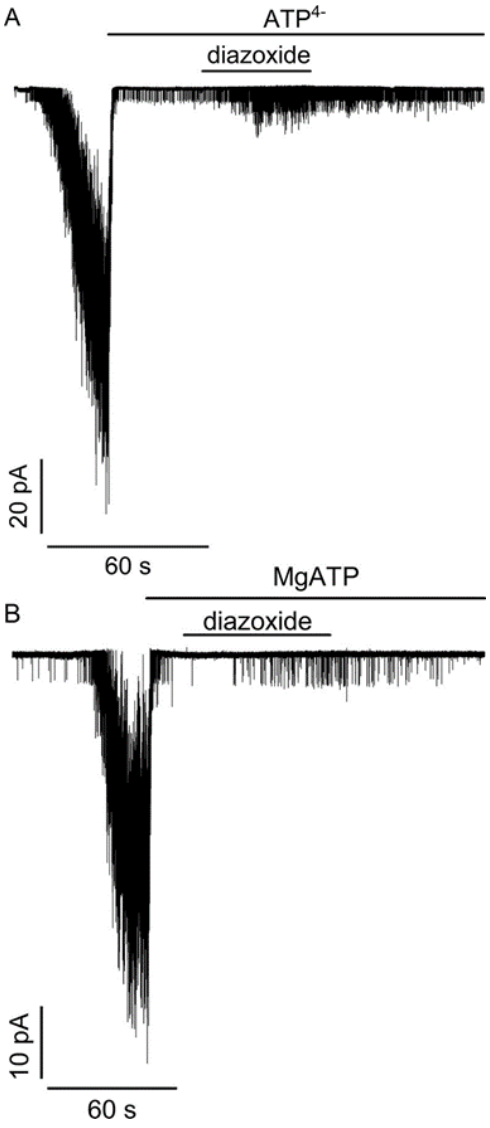


Fig. 3: Activation of $SUR1_{E1507K}$/WT Kir6.2 channels associated with congenital hyperinsulinism by the agonist diazoxide. At the arrows, patches were pulled into nucleotide-free media which activates a large number of channels as inhibitory nucleotides leave the pore. (A) Application of $ATP^{4-}$ (10 mM) or (B) MgATP (1 mM) rapidly inhibits channel activity. Concurrent application of diazoxide (340 µM) increases channel activity in both cases. Activation was observed in five out-of-five trials for $ATP^{4-}$ and for MgATP.

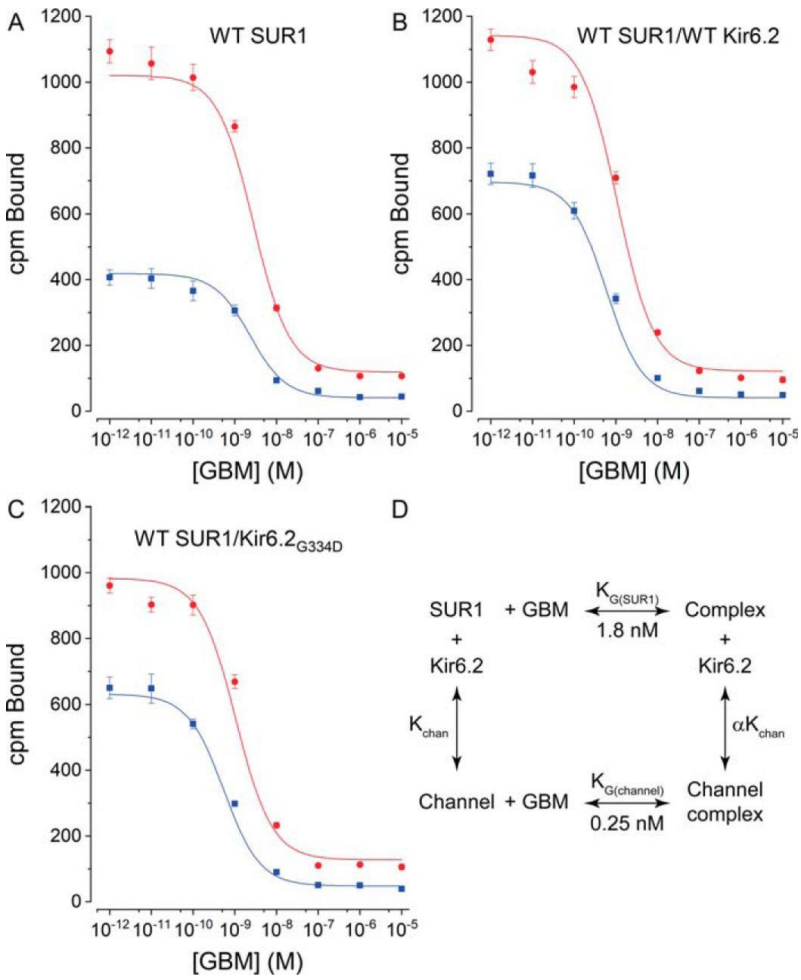


Fig. 4: Assembly with Kir6.2 increases the affinity of SUR1 for [$^3$H]GBM. (A-C) Dissociation constants, $K_G$'s, for [$^3$H]GBM were determined by displacement of [$^3$H]GBM, 0.3 (blue) or 1 (red) nM, by unlabeled GBM. Plots are for wtSUR1 alone, wtSUR1/wtKir6.2 and wtSUR1/Kir6.2$_{G334D}$, respectively. The data are presented as the mean values (±SEM, n = 9) of the total bound radioactivity. The radioactivity remaining at the foot of the binding curves shows non-specific binding which increases with higher [$^3$H]GBM concentrations. The curves are global best fits to a homologous displacement model with radioligand depletion (74). Table 1 gives a summary of $K_G$ values for wildtype (wt) and mutant SUR1s and corresponding channels. (D) The thermodynamic cycle used to estimate GBM stabilization of $K_{ATP}$ channels. Binding assays were done in the absence of ATP and $Mg^{2+}$ with 1 mM EDTA added.

Table 1: Summary of dissociation constants ($K_G$)

| Sample | Mean | CI lower– upper | *p* value |
|---|---|---|---|
| | *nM* | | |
| WT SUR1 alone | 1.8 | 1.4–2.2 | |
| WT SUR1/WT Kir6.2 | 0.25 | 0.2–0.3 | <0.001 |
| WT SUR1/Kir6.2$_{G334D}$ | 0.4 | 0.3–0.5 | <0.001 |
| SUR1$_{Q1179R}$ | 3.9 | 3.2–4.8 | |
| SUR1$_{Q1179R}$/WT Kir6.2 | 1.1 | 0.7–1.6 | <0.001 |
| SUR1$_{Q1179R}$/WT Kir6.2$_{G334D}$ | 1.1 | 0.8–1.5 | <0.001 |
| SUR1$_{E1507K}$ | 1.7 | 1.4–2.0 | |
| SUR1$_{E1507K}$/WT Kir6.2 | 0.6 | 0.5–0.9 | <0.001 |
| SUR1$_{E1507Q}$ | 0.8 | 0.4–1.5 | |
| SUR1$_{E1507Q}$/Kir6.2 | 0.3 | 0.2–0.5 | <0.01 |

CI values are lower and upper 95% confidence intervals. *p* values were determined using one-way ANOVA with Bonferroni's test for multiple comparisons.

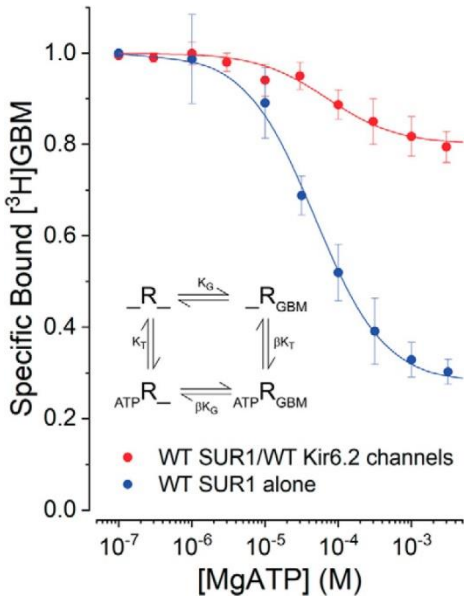


Fig. 5: Association with Kir6.2 affects the allosteric properties of WT SUR1. Increasing MgATP reduces the binding of [$^3$H]GBM (1 nM). The data are given as Specific Bound [$^3$H]GBM defined as [$^3$H]GBM bound in the presence of MgATP divided by the radioactivity bound without nucleotide. The inset shows the four-state ternary complex model. R is defined as SUR1 with ATP bound to NBD1, the non-canonical nucleotide-binding site. ATP can induce NBD dimerization by binding to NBD2 with or without bound GBM. The $K_G$ values from Fig. 4, estimated in the absence of ATP, were used to estimate β and $K_T$. The curves were generated using the global-fit parameters in Table 2; data are means ± SEM, n = 6.

Table 2: Summary of binding parameters from Fig. 5.

| Species | $K_G$ | β | $K_T$ | $\beta K_G$ | $\beta K_T$ |
|---|---|---|---|---|---|
| | *nM* | | µM | *nM* | µM |
| **Global fits** | | | | | |
| SUR1 | 1.8 (1.5–2.2) | 5.0 (4.5–5.6) | 34.1 (29.6–40.4) | ~9 | ~170 |
| SUR1/Kir6.2 | 0.25 (0.19–0.32) | 2.2 (2.0–2.4) | 34.1 (29.6–40.4) | ~0.6 | ~75 |
| **Independent fits** | | | | | |
| SUR1 | 1.8 (1.5–2.2) | 5.0 (4.5–5.6) | 34.4 (27.7–42.6) | ~9 | ~170 |
| SUR1/Kir6.2 | 0.25 (0.19–0.32) | 2.2 (2.0–2.4) | 39.4 (22.3–69.7) | ~0.6 | ~86 |

Data are given as mean values and 95% Confidence Intervals (lower-upper values).

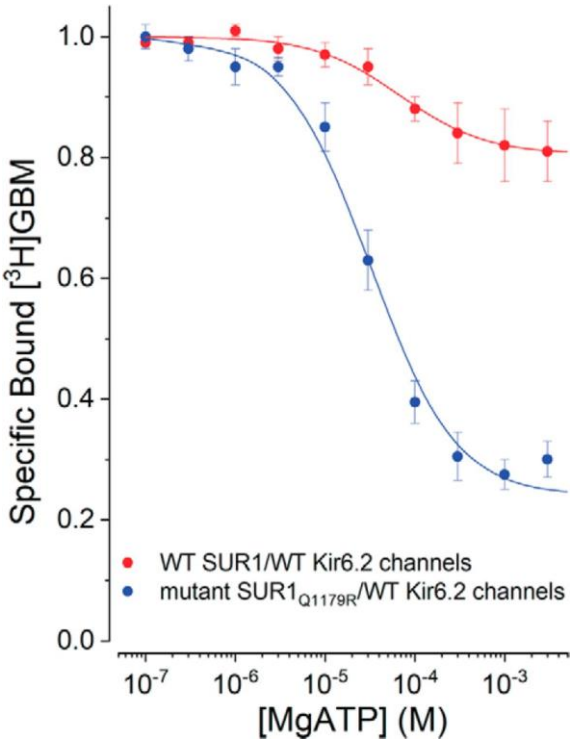


Fig. 6: Effect of the $SUR1_{Q1179R}$ ND mutation on allosteric coupling in $K_{ATP}$ channels. Reduction of bound [$^3$H]GBM (1 nM) by increasing MgATP. Curves are the best fits of the ternary complex model (Fig. 5; inset) to the data. The $K_G$ values from Table 1 were used to estimate β and KT. The best-fit parameters are given in the text. Points are means ± SEM, n = 6. The wildtype $SUR_{1Q1179}$/Kir6.2 channel values include those shown in Fig. 5.

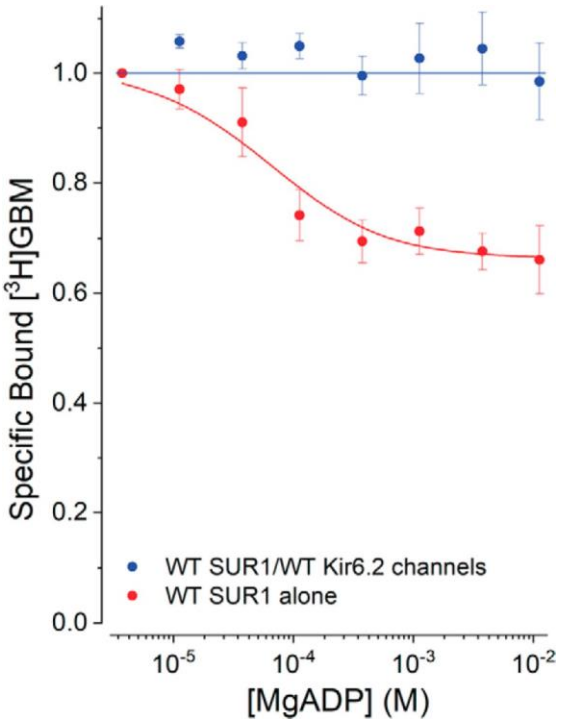


Fig. 7: Effect of MgADP on allosteric coupling in SUR1 alone versus SUR1 in $K_{ATP}$ channels. Reduction of bound [$^3$H]GBM (1 nM) by increasing MgADP. The $K_G$ values from Fig. 4 were used to estimate β and $K_D$. The SUR1 curve was drawn using best-fit parameters, mean (lower CI-upper CI): $K_G$ = 0.25 nM (0.19-0.32), KD = 55 (28-107) µM and β = 1.8 (1.6-1.9)). Points are means ± SEM, n = 6.

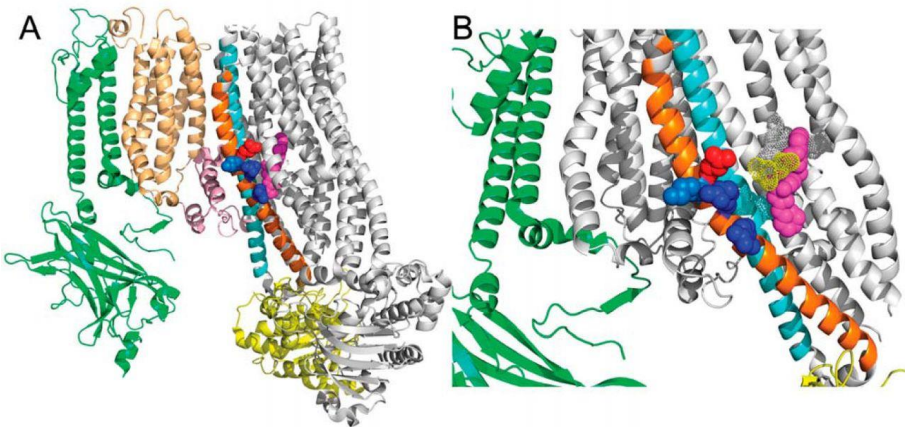


Fig. 8: Positioning of amino acid Gln-1179 in helix 15 relative to the GBM-binding site. Panel (A) shows one Kir6.2 subunit (green) interacting with SUR1 core (grey) through TMD0 (tan) and L0 (pink). Helices 15 and 16 are orange and cyan respectively. Residues Q1179 (red), K1180 (light blue), R1182 (dark blue), R1187 (dark blue) and GBM (magenta) are shown. NBD1 is shown in yellow. Panel (B) is an enlarged view of the GBM site with the helices in the forefront hidden. The dotted surfaces show the amino acids identified in Martin *et a.l* (41) as part of the GBM binding site.

**Possible new strategies for the treatment of congenital hyperinsulinism**

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Paper in preparation.

**Abstract**

Objective: Congenital hyperinsulinism (CHI) is a rare disease characterized by persistent hypoglycemia as a result of inappropriate insulin secretion which can lead to irreversible neurological defects in infants. Poor efficacy and strong adverse effects of the current medications impede successful treatment. The aim of the study was to investigate new approaches to silence β-cells and thus attenuate insulin secretion.

Research design and methods: In the scope of our research, we tested substances more selective and more potent than the gold standard diazoxide that also interact with neuroendocrine ATP-sensitive $K^+$ ($K_{ATP}$) channels. Additionally, $K_{ATP}$ channel-independent targets as $Ca^{2+}$-activated $K^+$ channels of intermediate conductance (SK4) and L-type $Ca^{2+}$ channels were also investigated. Experiments were performed using human β-cells isolated from tissue of CHI patients and β-cells obtained from C57BL/6N or SUR1 knockout ($SUR1^{-/-}$) mice. The cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) was used as a parameter for insulin secretion and was determined by fura-2 fluorescence. The mitochondrial membrane potential (ΔΨ) was determined by rhodamine 123 fluorescence.

Results: The selective $K_{ATP}$ channel opener NN414 (5 µM) diminished $[Ca^{2+}]_c$ in isolated human β-cells stimulated with 10 mM glucose. VU0071063 (30 µM), another $K_{ATP}$ channel opener considered to be selective, lowered $[Ca^{2+}]_c$ in human CHI β-cells and in β-cells lacking functional $K_{ATP}$ channels ($SUR1^{-/-}$ β-cells), showing that $[Ca^{2+}]_c$ is influenced by additional effects besides KATP channels. The drug affected ΔΨ in both C57BL/6N and $SUR1^{-/-}$ β-cells. An opener of SK4 channels DCEBIO (100 µM) significantly decreased $[Ca^{2+}]_c$ in $SUR1^{-/-}$ and human CHI β-cells. To target L-type $Ca^{2+}$ channels we tested two already approved drugs, dextromethorphan (DXM) and simvastatin. DXM (100 µM) efficiently diminished $[Ca^{2+}]_c$ in stimulated $SUR1^{-/-}$ β-cells as well as in stimulated human CHI β-cells. Similar effects on $[Ca^{2+}]_c$ were seen in experiments with simvastatin (7.2 µM). The neuropeptide galanin was without effect in human β-cells.

Conclusions: NN414 seems to provide a good alternative to the currently used $K_{ATP}$ channel opener diazoxide. Targeting SK4 channels by channel openers or L-type $Ca^{2+}$ channels with DXM or simvastatin might be a valuable approach for treatment of CHI caused by mutations of $K_{ATP}$ channels not treatable by $K_{ATP}$ channel openers.

## 1 Introduction

Congenital hyperinsulinism (CHI) is a rare heterogeneous genetic disorder, but the most frequent cause of severe, persistent hypoglycemia in neonates, infants and children. The main reasons for developing CHI are defects in important genes regulating pancreatic β-cell function. To date, mutations in 14 essential genes controlling insulin secretion have been reported including *ABCC8* and *KCNJ11. ABCC8* and *KCNJ11* genes encode the $K_{ATP}$ channel subunits SUR1 and Kir6.2, respectively, and mutations in these genes represent the most prevalent cause of CHI. Defects in these genes are responsible for the failure of β-cells to respond to normal regulatory mechanisms, leading to inappropriate and excessive insulin release despite low blood glucose concentrations resulting in frequent episodes of hypoglycemia (1, 2). There are some excellent reviews giving detailed information about molecular mechanisms underlying the pathophysiology of CHI (1-5).

Based on histopathological observations, three distinct forms of CHI are described: focal, diffuse and atypical. In focal CHI affected β-cells are localized only in small

specific parts of the pancreas. Conversely, in diffuse CHI all pancreatic β-cells seem to be affected (6). If the histology of the tissue does not fit in one of the forms, it is regarded as an atypical form of CHI. It is characterized by a mosaic-like assembly of hyper-functional islets spread over the pancreas (7).

Persistent hypoglycemia is responsible for seizures and finally for severe brain damage (8). Thus, it is necessary to diagnose CHI rapidly and to start as early as possible with a suitable treatment. Treatment options include medical therapy and surgical intervention (9). First-line drug for treating CHI is the $K_{ATP}$ channel agonist diazoxide (10). However, numerous side effects of diazoxide limit its use. Some of the most common undesired effects are $Na^+$ and fluid retention, hypertrichosis and loss of appetite. Life threatening side effects also occur including cardiac failure, pulmonary hypertension, hyperuricemia, bone marrow suppression, and anemia (11-16). Additionally, diazoxide is only effective when $K_{ATP}$ channels are functional (10). Alternatives to the therapy with diazoxide and novel medications include glucagon, somatostatin analogues, nifedipine, GLP1-receptor antagonists (exendin-(9-39)), and sirolimus ((17-22), reviewed in (3)). Many of these drugs act by lowering the $Ca^{2+}$ influx into β-cell (23-25). The aforementioned drugs also have numerous undesirable effects, which may be a reason for reconsidering their therapeutic usefulness: gastrointestinal symptoms, formation of gall stones, suppression of pituitary hormones, necrotizing enterocolitis, hypotension, immune suppression, thrombocytosis, impaired immune response and many more (26-31). Recently, a new full human monoclonal antibody to the insulin receptor XMetD (also known as XOMA 358 or RZ358) has been proposed as a novel therapeutic strategy (32-35). First results in a Phase 2a clinical trial exhibited an improved glycemic control in patients with persistent hypoglycemia (36).

In patients that cannot be treated sufficiently with drugs, surgical treatment is indicated. While partial pancreatectomy is beneficial for patients with focal CHI (37, 38), in case of diffuse and drug-unresponsive CHI, near-total pancreatectomy is usually required (39, 40). Due to different post-operative complications like recurrent hypoglycemia, pancreatic exocrine insufficiency and diabetes, patients with diffuse CHI are far from being cured after surgery (41, 42). In order to reduce the development of diabetes postsurgically, a 70 to 90% resection of pancreas has been considered, however, the outcome is still unpredictable (39, 43).

Taken together, it is of great importance to explore new pharmacological options for CHI therapy in order to maintain euglycemia and reduce severe side effects from current medical and surgical treatment. Aim of this study was to find new strategies which are able to silence β-cell by inhibiting extensive $Ca^{2+}$ influx into the cell. For this purpose, new and approved drugs interacting with $K_{ATP}$ channels as well as with $K_{ATP}$ channel-independent targets have been tested on β-cells obtained from biopsies of CHI patients and β-cells from C57BL/6N and $SUR1^{-/-}$ mice.

**2 Materials and Methods**

*2.1 Cell and islet preparation.* Human islets of Langerhans were obtained from different biopsies of children undergoing pancreatic surgery according to the approvals of the ethic commission of the Universitätsmedizin Greifswald (BB 050/13). The islets were taken from biopsies of eleven CHI patients. Ten patients had mutations in the *ABCC8* gene encoding the SUR1 subunit of $K_{ATP}$ channels. Three of them had mutation typical among the Ashkenazi Jewish population (c.3989_9G>A) (44, 45). For one biopsy of a patient no mutation was found (Table 1). According to postsurgical evaluation of the biopsies by the Department of Pathology at the University Hospital Greifswald, the tissue was identified as healthy or pathological. Islets from areas affected (termed pathological) and not affected (termed healthy) by the disease were isolated. They were isolated by injecting collagenase (2 mg/ml) into the biopsy material and by handpicking islets after digestion at 37 °C. Afterwards islets were cultured in a CMRL 1066 medium with 5.5 mM glucose supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES and 2 mM L-glutamine.

Mouse islets of Langerhans were isolated from adult C57BL/6N mice or SUR1 knockout ($SUR1^{-/-}$) mice on a C57BL/6N background. The mice were bred in the animal facility of the Department of Pharmacology at the University of Tübingen. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and German laws were followed. Islets were isolated and cultured as previously described (46). For experiments single islet cells or cell clusters were used, obtained by dispersing islets by trypsin treatment.

*2.2 Solutions and chemicals.* Measurements of $[Ca^{2+}]_c$ were performed with a bath solution which contained (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 10 HEPES and glucose as indicated, pH 7.4 adjusted with NaOH. The same bath solution was used for the determination of the mitochondrial membrane potential (ΔΨ).

NN414, diazoxide, simvastatin and galanin were obtained from Sigma-Aldrich (Schnelldorf, Germany). DCEBIO was either purchased from Tocris Bioscience (Bristol, United Kingdom) or Santa Cruz (Heidelberg, Germany), fura-2-AM from Biotrend (Köln, Germany) and dextromethorphan (DXM) from Alfa Aeser (Thermo Fisher Scientific). Rhodamine 123 (Rh123), RPMI 1640 medium, CMRL 1066 medium, fetal calf serum (FCS), penicillin/streptomycin, glutamine and trypsin were from Invitrogen (Karlsruhe, Germany). Collagenase used for human biopsy material was obtained from Serva (Heidelberg, Germany). All other chemicals were obtained from Sigma-Aldrich or Carl Roth (Karlsruhe, Germany) in the purest form available.

*2.3 Measurements of $[Ca^{2+}]_c$.* Details are described in (46) In brief, islet cells were loaded with 5 µM fura-2-AM for 30-35 min at 370C. The cells were perifused with bath solution with the indicated test substances. Fluorescence was excited at 340 and 380 nm, emission was filtered (LP515) and measured by a digital camera. Fluorescence ratios of emitted fluorescence intensities (F340/F380) were determined every 3 seconds with background subtraction and at the end of each interval the mean ratio was evaluated for a period of 5 to 8 min to compare $[Ca^{2+}]_c$ under different experimental conditions.

*2.4 Measurements of the mitochondrial membrane potential (ΔΨ).* ΔΨ was measured by Rh123 fluorescence at 480 nm excitation wave length as described in (47). The effects were evaluated by averaging the values of the last 60 s of each interval before solution change.

*2.5 Statistics.* Each series of experiments with islets cells from mice was performed with at least 3 independent mouse preparations. Number of preparations for recordings with human islet cells varied and is indicated for every series. Means ± SEM are given for the indicated number of experiments. Statistical significance of differences was assessed by Student's t test. Multiple comparisons were made by

ANOVA followed by Student-Newman-Keuls test. P values ≤ 0.05 were considered significant.

## 3 Results

### *3.1 Effects of nifedipine and low glucose on the cytosolic $Ca^{2+}$ concentration in human β-cells*

Oscillations of the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) are driven by fluctuations of the membrane potential and $[Ca^{2+}]_c$ is the trigger signal for insulin secretion. $[Ca^{2+}]_c$ is the best surrogate parameter for insulin secretion. It can be determined easily and online with few cell material which is an enormous advantage when working with β-cells from human tissue. Glucose-stimulated insulin secretion in human pancreatic β-cells is completely suppressed by pharmacologic blockage of L-type $Ca^{2+}$ channels (48). As a control for proper response we tested the effect of the L-type $Ca^{2+}$ channel blocker nifedipine on $[Ca^{2+}]_c$ in human β-cells isolated from healthy pancreatic tissue of CHI patients. Fig. 1A presents a typical recording with periodic oscillations of $[Ca^{2+}]_c$ in the presence of a stimulating glucose concentration of 15 mM glucose. The addition of nifedipine at a concentration of 5 µM diminished $[Ca^{2+}]_c$ significantly. The mean fluorescence ratio was reduced from 0.84 ± 0.05 under control conditions to 0.65 ± 0.04 after application of nifedipine (Fig. 1B).

To test the glucose responsiveness of healthy human β-cells isolated from patients with focal CHI, we lowered the glucose concentration from 10 mM to 0.5 mM (Fig. 1C). Out of the 50 β-cells tested, only 17 responded to low glucose with discontinuation of the oscillations and a drop in $[Ca^{2+}]_c$ (the mean fluorescence ratio was reduced from 0.77 ± 0.05 in the presence of 10 mM glucose to 0.65 ± 0.04 at a glucose concentration of 0.5 mM glucose) (Fig. 1D). One possible explanation for the lack of a $Ca^{2+}$-lowering effect in many cells obtained from pancreatic tissue classified as “healthy” by the pathologists could be stress, which can affect metabolic integrity. The stress may be a result of transport of the biopsy material within Greifswald and shipment of the isolated islets from Greifswald to the research laboratories in Tübingen or Münster where the measurements of $[Ca^{2+}]_c$ were performed.

### *3.2 $K_{ATP}$ channel openers*

#### *3.2.1 Effects of diazoxide on $[Ca^{2+}]_c$ of human β-cells*

$K_{ATP}$ channels (SUR1/Kir6.2) of pancreatic β-cells play a crucial role as they couple cellular metabolism to electrical activity. In electrically inactive β-cells, $K_{ATP}$ channels are open. CHI is characterized by permanently active β-cells and thus opening of these channels is one strategy to treat it. Diazoxide is an opener of $K_{ATP}$ channels that is already established in CHI therapy. We tested the effect of diazoxide on $[Ca^{2+}]_c$ as a control. In human β-cells isolated from healthy pancreatic tissue of CHI patients, 250 µM diazoxide significantly decreased $[Ca^{2+}]_c$ in the presence of 10 mM glucose from 0.70 ± 0.06 to 0.53 ± 0.01 (Fig. 2A, B). Similar results were obtained with diazoxide applied to human β-cells isolated from pathological pancreatic tissue of two patients with focal CHI (0.70 ± 0.05 under control conditions vs. 0.58 ± 0.07 after application of 250 µM diazoxide) (Fig. 2C, D).

#### *3.2.2 Effects of NN414 on $[Ca^{2+}]_c$ of human β-cells*

The diazoxide analogue NN414 is suggested to be a selective agonist of pancreatic β-cell $K_{ATP}$ channels which is 100-fold more potent than diazoxide (49). Therefore, it has been proposed as useful drug for the treatment of diseases with excessive insulin secretion (49). NN414, in a concentration of 5 µM, completely abolished oscillations of $[Ca^{2+}]_c$ and reduced $[Ca^{2+}]_c$ to basal levels (Fig. 3A, B). The mean fluorescence ratio was reduced from 0.75 ± 0.03 under control conditions to 0.64 ± 0.03 in the presence of NN414 in β-cells obtained from healthy human pancreatic tissue of CHI patients. Application of 5 µM NN414 to human β-cells taken from three different forms (focal, diffuse and atypical) of pathological pancreatic tissue lowered the mean fluorescence ratio from 0.72 ± 0.03 in 10 mM glucose to 0.62 ± 0.03 in the presence of NN414 (Fig. 3C, D).

#### *3.2.3 VU0071063 silences β-cells in a $K_{ATP}$ channel-dependent and -independent manner*

Recently, Raphemot et al. discovered a novel xanthine derivative, VU0071063 that directly and selectively activates $K_{ATP}$ channels (50). They found that VU0071063 is more potent and activates $K_{ATP}$ channels with a faster kinetic than diazoxide. These

findings encouraged us to test its effect on changes in $[Ca^{2+}]_c$ on human β-cells from CHI patients. In human β-cells isolated from healthy pancreatic tissue of CHI patients, 30 µM VU0071063 immediately abolished glucose-induced $Ca^{2+}$ oscillations. Mean fluorescence ratio decreased from 0.57 ± 0.03 under control conditions with 10 mM glucose to 0.48 ± 0.01 in the presence of VU0071063 (Fig. 4A, B). Administration of VU0071063 (30 µM) on β-cells from pathological CHI pancreatic tissue (focal lesion) induced a prompt reduction of $[Ca^{2+}]_c$ in all four measurements. Fig. 4C shows a typical example. The mean fluorescence ratio changed from 0.59 ± 0.04 under control conditions to 0.50 ± 0.02 in the presence of VU0071063. Due to the limited pathological material, which explains the low number of experiments, the difference between the groups did not reach statistical significance.

In human β-cells obtained from both healthy and pathological pancreatic tissue of CHI patients, a drop of $[Ca^{2+}]_c$ was noticed directly after withdrawal of VU0071063 (Fig 4 A, C asterisks). Presumably, this drop is due to ATP-dependent sequestration of $Ca^{2+}$ into the ER (51). This suggests that VU0071063 affects additional targets besides $K_{ATP}$ channels. To evaluate this assumption, $[Ca^{2+}]_c$ of β-cells from C57BL/6N mice and $SUR1^{-/-}$ mice was measured. VU0071063 (30 µM) suppressed $Ca^{2+}$ oscillations and lowered $[Ca^{2+}]_c$ in both genotypes (Fig. 4 E, F). The mean fluorescence ratio decreased from 0.67 ± 0.01 to 0.56 ± 0.01 in C57BL/6N β-cells and from 0.69 ± 0.01 to 0.66 ± 0.01 in $SUR1^{-/-}$ β-cells, i.e. the effect was weaker in the cells of the knock-out mice. Similar to human β-cells the drug further reduced $[Ca^{2+}]_c$ after its removal in both, C57BL/6N and $SUR1^{-/-}$ mouse β-cells (reduction of $[Ca^{2+}]_c$ was 0.050 ± 0.02 and 0.054 ± 0.02, respectively (Fig. 4F, asterisks)). This points to alterations in mitochondrial metabolism which can cause changes in KATP channel activity (52). As the mitochondrial membrane potential is almost at all times directly linked to ATP production (53), we evaluated mitochondrial ATP production (54) by measuring the effects of VU0071063 on the mitochondrial potential (ΔΨ). A rise in the glucose concentration causes an immediate increase in ATP production. This increase is accompanied by a hyperpolarization of ΔΨ which is indicated by a decrease of the fluorescence signal (Fig. 4G). In β-cells of C57BL/6N mice the fluorescence signal was increased from 1629 ± 58 a.u. in the presence of 10 mM glucose to 2473 ± 96 a.u. after addition of 30 µM VU0071063 (Fig 4H). Likewise,

VU0071063 applied to SUR1$^{-/-}$ β-cells, strongly and reversibly depolarized mitochondrial membrane potential from 1516 ± 55 a.u. to 2021 ± 87 a.u. (Fig. 4G, H).

*3.3 $K_{ATP}$ channel-independent drugs*

In the following part we present drugs and potential strategies which could be effective in CHI β-cells lacking functional $K_{ATP}$ channels.

*3.3.1 SK4 channel openers as a potential approach*

In addition to $K_{ATP}$ and voltage-gated $K^+$ channels, pancreatic β-cells express $K^+$ channels regulated by the cytosolic $Ca^{2+}$ concentration ($K_{Ca}$) (52). Depending on their single channel conductance, there are three groups whose existence has been detected in pancreatic β-cells (55-58). It has been demonstrated that the $K_{Ca}$ channels of intermediate conductance (SK4, KCa3.1) play an important role in the $K^+$ current ($K_{slow}$) that contributes to β-cell hyperpolarization at the end of a burst phase with electrical activity (54, 56, 59, 60). Previous results from Düfer et al. (56) demonstrated that activation of SK4 channels hyperpolarized the membrane potential of pancreatic β-cells from C57BL/6N mice. Since about 50% of Kslow is $K_{ATP}$ current (61), the sulfonylurea-insensitive $K_{Ca}$ component could be even more significant in β-cells lacking functional $K_{ATP}$ channels which resembles the situation in CHI channelopathies.

To verify this assumption, we evaluated the effect of the SK4 opener DCEBIO on β-cells isolated from SUR1$^{-/-}$ mice. DCEBIO effectively abolished the glucose-induced oscillations of $[Ca^{2+}]_c$ (Fig. 5A). DCEBIO (100 µM) reduced the mean fluorescence ratio from 0.39 ± 0.02 to 0.31 ± 0.02 (Fig. 5B). Next we tested the effect of DCEBIO on human β-cells from healthy and pathological tissue of pancreatectomies. As expected, DCEBIO (100 µM) significantly reduced $[Ca^{2+}]_c$ in the presence of 10 mM glucose (0.75 ± 0.04 under control conditions vs. 0.59 ± 0.03 after addition of DCEBIO) (Fig. 5 C, D). Furthermore, DCEBIO was tested on human β-cells isolated from pancreatic tissues with mosaic and diffuse forms of CHI. It suppressed the oscillations of $[Ca^{2+}]_c$ (Fig. 5E). In the presence of 10 mM glucose, the mean fluorescence ratio was 0.70 ± 0.03 in comparison to 0.58 ± 0.03 after application of 100 µM DCEBIO (Fig. 5F).

### *3.3.2 Galanin does not affect* $[Ca^{2+}]_c$ *of human β-cells*

Galanin is a neuropeptide found in the central and peripheral nervous system and the intestinal neuroendocrine system. It mostly acts as a hyperpolarizing neuromodulator. It possesses an inhibiting effect on insulin secretion in murine β-cells (62), probably via $G_i$ protein-coupled receptors. However, the exact mechanism of action is still a matter of debate. Düfer and colleagues showed that galanin is able to hyperpolarize mouse β-cells lacking functional $K_{ATP}$ channels (63). Thus, application of the neuropeptide could be a reasonable approach in the therapy of CHI.

For this reason the effect of galanin was studied on β-cells from healthy and pathological human pancreatic tissue of CHI patients. Contrary to the expectation, galanin in a concentration of 100 nM was without effect (Fig. 6A, C). In human β-cells obtained from healthy pancreatic tissue of CHI patients, mean fluorescence ratio amounted to 0.83 ± 0.03 before and 0.82 ± 0.04 after addition of galanin, respectively (Fig. 6B). We were able to test galanin on two pathological CHI β-cells isolated from pancreatic tissue of a patient with the diffuse form of CHI. In both experiments galanin did not affect the glucose-stimulated $Ca^{2+}$ oscillations. The mean fluorescence ratio amounted to 0.52 *vs.* 0.56 tested in the first cell and 0.69 *vs.* 0.66 in the second islet cell (Fig. 6D).

### *3.3.3 Effect of dextromethorphan on* $[Ca^{2+}]_c$ *of human β-cells*

Dextromethorphan (DXM) is a known antagonist of NMDA receptors. Active NMDA receptors can activate other ion channels, like $Ca^{2+}$-activated $K^+$ channels or KATP channels and thus potentiate $K^+$ outflow (64). A block of NMDA receptors leads to prolonged depolarization and increases insulin secretion (65). Lesser-known is its ability to directly inhibit L-type $Ca^{2+}$ channels. Carpenter et al. found that DXM moderately inhibits L-type $Ca^{2+}$ channels thereby lowering $[Ca^{2+}]_c$. This effect was observed with permanently depolarized cells under stimulating glucose concentrations (66). Since permanent depolarization is a characteristic of CHI β-cells, DXM may offer a possibility to rescue, i.e. silence the overstimulated cells.

The measurement in Fig. 7A shows a recording of $[Ca^{2+}]_c$ of a permanently depolarized β-cell from a $SUR1^{-/-}$ mouse in the presence of 10 mM glucose.

Application of DXM in a concentration of 100 µM rapidly reduced $[Ca^{2+}]_c$, i.e. the drug significantly lowered the mean fluorescence ratio from 0.82 ± 0.01 to 0.67 ± 0.02 (Fig. 7B). Likewise, 100 µM DXM markedly decreased $[Ca^{2+}]_c$ in human β-cells obtained from healthy pancreatic tissue of CHI patients as well as in β-cells obtained from pathological tissue of patients with diffuse and focal CHI (Fig. 7C, E). The mean fluorescence ratio was lowered in healthy cells from 0.86 ± 0.06 to 0.73 ± 0.04 and in pathological cells from 1.04 ± 0.09 to 0.82 ± 0.04, respectively (Fig. 7 D, F).

*3.3.4 Statins as a potential strategy to silence human β-cells*

Lipid-lowering statins are inhibitors of the enzyme HMG-CoA-reductase which plays a significant role in cholesterol synthesis by converting HMG-CoA to mevalonate. For these drugs it has been reported that they increase the risk of type 2 diabetes (67). Different studies have been conducted in order to enlighten the mechanism how the statins impair insulin secretion. In the study using β-cells isolated from rats, Yada et al. (68) showed that simvastatin in a concentration of 3 µg/ml acutely blocked L-type $Ca^{2+}$ channels, thus lowering insulin secretion. Furthermore, Yaluri et al. demonstrated that simvastatin diminished glucose-stimulated insulin secretion and $[Ca^{2+}]_c$ in MIN6 β-cells via multiple mechanisms (69).

Hence, we considered simvastatin as a potential therapeutic strategy to treat CHI. In order to confirm that simvastatin shows its effect when functional $K_{ATP}$ channels are lacking, we measured $[Ca^{2+}]_c$ in β-cells from $SUR1^{-/-}$ mice (Fig. 8A). Simvastatin in a concentration of 7.2 µM (according to the concentration of 3 µg/ml that was used in the study of Yada et al. (68)) rapidly decreased the glucose-stimulated $Ca^{2+}$ oscillations. The mean fluorescence ratio changed from 0.46 ± 0.02 to 0.35 ± 0.02 (Fig. 8B). Further, we tested simvastatin on human β-cells isolated from healthy pancreatic tissue of CHI patients (Fig. 8C). The reduction in $[Ca^{2+}]_c$ was significant as well (mean fluorescence ratio: 0.84 ± 0.05 under stimulating glucose concentrations vs. 0.64 ± 0.03 after administration of 7.2 µM simvastatin) (Fig. 8D). Fig. 8E shows a measurement of $[Ca^{2+}]_c$ in a β-cell isolated from pathological pancreatic tissue of a patient with focal CHI. Similar to the previously shown findings, $[Ca^{2+}]_c$ declined (mean fluorescence ratio: 0.85 ± 0.06 vs. 0.63 ± 0.02) (Fig. 8F).

## 4 Discussion

### *4.1 Possible $K_{ATP}$ channel-dependent strategies to treat CHI*

In the present study β-cells isolated from biopsies of CHI patients were used to search for new strategies to treat the disease. Noteworthy, the aim of the study was not to suggest novel drugs for CHI treatment but to search for novel targets and concepts for future drug development. β-cells from healthy tissue were sensitive to the L-type $Ca^{2+}$ channel blocker nifedipine. This maneuver resulted in a decrease of $[Ca^{2+}]_c$ as expected from numerous observations with murine β-cells and insulin-secreting tumor cell lines as well as the restricted number of studies with human β-cells. This shows that sufficient cells of the biopsy material resisted to stress induced by preparation, culture or transport and preserved metabolic integrity to receive reliable and reproducible results. This is also confirmed by the results observed with diazoxide used as gold standard in CHI treatment. Noteworthy, nifedipine has been used for the treatment of diazoxide-unresponsive CHI (19, 29), but due to reported hypotension in patients with mutations in the *ABCC8* gene, it is not commonly used in the treatment of CHI (29, 70).

Diazoxide is usually effective in all forms of CHI including severe cases caused by mutations in the genes encoding $K_{ATP}$ channels (*ABCC8* and *KCNJ11*). The severe side effects are a consequence of the non-selectivity of diazoxide for pancreatic $K_{ATP}$ (SUR1/Kir6.2) channels (71). Besides pancreatic $K_{ATP}$ channels, the drug activates those of smooth muscles (SUR2B/Kir6.2 and SUR2B/Kir6.1) and exerts weak stimulatory effects on $K_{ATP}$ channels of the cardiac muscle (SUR2A/Kir6.2) (72). One of the most common adverse effects of diazoxide, hirsutism could be explained by activating both SUR1/Kir6.2 and SUR2B/Kir6.2 channels in hair follicles (73, 74).

#### *4.1.1 NN414*

As one strategy to improve CHI therapy we tested more β-cell specific $K_{ATP}$ channel openers. In comparison to diazoxide, NN414 is reported to be a selective SUR1 agonist, 100-fold more potent than diazoxide suggesting that the drug is effective at much lower concentrations (49). Early, prediabetic stages of type 2 diabetes mellitus (T2DM) are normally characterized by compensatory hypersecretion of insulin. $K_{ATP}$ channel openers have been suggested as beneficial medication to counteract

excessive hormone release in prediabetic patients as insulin hypersecretion may cause or contribute to the development of glucose intolerance and β-cell degeneration in T2DM (75). NN414 has been used in numerous in vitro and in vivo studies to achieve β-cell rest, thereby preserving β-cell function and preventing apoptosis (76, 77). An animal in vivo study revealed a significant potential of NN414 in the treatment of disorders resulting from excessive insulin release (78). Alemzadeh et al. showed in a six-week study that NN414 reduced hyperinsulinemia and improved glucose responsiveness in Zucker obese rats in a dose-dependent manner. NN414 entered human clinical trials for the treatment of T2DM. In healthy subjects it inhibited insulin release, was well tolerated and did not induce clinically relevant changes in safety parameters besides side effects on the gastrointestinal tract (79). NN414 was advanced in phase 2 of clinical trials where it showed a tendency to improve β-cell secretory function in diabetic patients (80, 81). The clinical trial was stopped because of elevated liver enzymes in treated patients (81, 82). The SUR1 selectivity, the low doses and the reproducible $Ca^{2+}$-lowering effect observed in our study in healthy and pathological β-cells suggest to consider NN414 as a potential drug for the treatment of CHI. Liver enzymes have to be monitored during therapy with NN414 but moderate elevation of their plasma concentration is no criterion to exclude the drug although it would be desirable to develop NN414 analogues without this drug effect. Notably, increased concentrations of circulating liver enzymes is one of the most reported side effects for octreotide that is used off-label as second-line therapeutic in the long-term management of CHI and sirolimus that is proposed for patients resistant to diazoxide and octreotide (28, 83, 84), reviewed in (3).

### *4.1.2 VU0071063*

Recently, a novel $K_{ATP}$ channel activator, VU0071063 was discovered (50). VU0071063 is reported to be more selective for SUR1/Kir6.2 channels than for SUR2A/Kir6.2 and SUR2A/Kir6.1 channels. It has been demonstrated that it opens SUR1/Kir6.2 channels with a higher potency than diazoxide (50). Our data with human β-cells isolated from CHI patients demonstrate a beneficial characteristic of VU0071063 which supports the findings that it activates $K_{ATP}$ channels expressed in HEK-293 cells (50) and reduces glucose-stimulated $Ca^{2+}$ influx in murine β-cells (Fig. 4A-D). However, the observation that removal of VU0071063 from the solution initiated a transient drop in [Ca2+]c suggests that the drug does not selectively

interfere with KATP channels but also with ATP production (51). This assumption is supported by the following observations: 1) VU0071063 caused a transient drop in $[Ca^{2+}]_c$ in response to drug removal in C57BL/6N and SUR1$^{-/-}$ β-cells which is presumably due to ATP-dependent SERCA activation. 2) VU0071063 strongly and reversibly depolarized ΔΨ in both C57BL/6N and SUR1$^{-/-}$ β-cells which points to inhibition of ATP formation. Additionally, VU0071063 rapidly and significantly decreased $[Ca^{2+}]_c$ in SUR1$^{-/-}$ β-cells. This may be owing to $Ca^{2+}$-release from $Ca^{2+}$-stores because of the lack in ATP and secondary $Ca^{2+}$-dependent inactivation of L-type $Ca^{2+}$-channels. Our data suggest that the $Ca^{2+}$-lowering effect of VU0071063 is caused by a dual mechanism: 1) direct opening of $K_{ATP}$ channels and indirect opening of $K_{ATP}$ channels by ATP depletion; 2) interference with SERCA function and $Ca^{2+}$-release. In conclusion, since VU0071063 let expect detrimental effects on mitochondria, thereby impairing all ATP-dependent processes, this compound seems not suitable for use in humans without structural modifications avoiding this side effect.

### *4.2 Possible $K_{ATP}$ channel-independent strategies to treat CHI*

#### *4.2.1 Current second and third line therapy regimen targeting $K_{ATP}$ channel independent pathways*

There are mutations in *ABCC8* or *KCNJ11* genes known to disrupt the cell surface expression of $K_{ATP}$ channels (4, 85, 86). In this case openers e.g. diazoxide are ineffective in the treatment of CHI (87, 88). For these patients it is indispensable to find drugs targeting mechanisms which can induce β-cell rest and inhibit insulin release independent of $K_{ATP}$ channels. Currently available alternatives to diazoxide therapy are somatostatin analogues (octreotide, octreotide-LAR and lanreotide), sirolimus and exendin-(9-39) (3). Octreotide, a short-acting synthetic somatostatin analogue, inhibits insulin secretion by binding to and activating somatostatin receptors 2 and 5 (SSTR2 and SSTR5) (89). Activation of SSTRs shows multifactorial modulation of β-cells which involves inhibition of adenylate cyclase/cAMP pathway, activation of G protein-activated inwardly rectifying $K^+$ (GIRK) channels, decrease of $Ca^{2+}$ influx via P/Q-type $Ca^{2+}$ channels and inhibition of exocytosis (24, 90, 91). Infants respond well to initial doses of octreotide, but tachyphylaxis after a few doses makes it not suitable for the long-term treatment.

Long-acting somatostatin analogues (octreotide-LAR and lanreotide) have similar effects as octreotide, but have the advantage that they are given once every four weeks which improves therapy compliance and quality of life (21, 92, 93). However, due to a similar mechanism of action as octreotide, long-acting somatostatin analogues show similar side effects (94). Sirolimus, a mammalian target of rapamycin (mTOR) inhibitor, is an immunosuppressive and anti-proliferative agent that has been used in patients with diffuse CHI, unresponsive to diazoxide and octreotide therapy (22, 95). It suppresses insulin release by different mechanisms which have not been fully elucidated (30). It has been proposed that downregulation of mTOR pathway decreases insulin production in pancreatic β-cells, β-cell growth and may restore ketogenesis (95, 96). Furthermore, upregulation of liver gluconeogenesis by sirolimus contributes to insulin resistance (97). However, severe and life threatening side effects reported for the above mentioned drugs restrict their use.

### *4.2.2 Opening of SK4 channels*

Beside $K_{ATP}$ channels, $Ca^{2+}$-activated $K^+$ channels of intermediate conductance (SK4, KCa3.1) contribute to $K_{slow}$, the hyperpolarizing current that terminates bursts of action potentials in β-cells (56, 60). Accordingly, SK4 channels may become predominant regulators of membrane potential and insulin secretion when functional $K_{ATP}$ channels are absent which makes these channels seem ideal as drug targets in CHI. Our data show that an opener of SK4 channels, DCEBIO, was able to strongly reduce $[Ca^{2+}]c$ in $SUR1^{-/-}$ β-cells. Furthermore, DCEBIO was highly effective in silencing human β-cells obtained from healthy and pathological pancreatic tissue of CHI patients. These experiments provide valuable support for the idea of targeting SK4 channels in the treatment of CHI. To follow this strategy in the treatment of CHI patients would of course require the search for new SK4 channels openers with high selectivity for β-cells since unspecific SK4 channel openers are expected to exert severe side effects in numerous organs (e.g. lung, cells of the hematopoietic system and salivary glands) (98-100).

*4.2.3 The neuropeptide galanin*

In vitro studies with rodents suggest that the neuropeptide galanin suppresses insulin secretion by a $K_{ATP}$ channel-independent mechanism which is not fully understood so far (62). Düfer et al. demonstrated that galanin suppresses electrical activity of β-cells from SUR1$^{-/-}$ mice (63). This suggests that galanin possibly affects insulin secretion in CHI β-cells by a similar mechanism as somatostatin analogues. However, different in vitro and in vivo studies with galanin on insulin secretion in humans describe controversial effects (101-105), i.e. inhibition or no effect. In our study, galanin did not reduce $[Ca^{2+}]_c$ of human CHI β-cells. This is in agreement with recent findings of Rorsman and Ashcroft who demonstrated that galanin receptors are expressed in mouse β-cells but not in human β-cells (106). These observations show that galanin is not suitable for the treatment of CHI.

*4.2.4 DXM as L-type $Ca^{2+}$ channel antagonist*

The block of NMDA receptors by DXM is expected to increase insulin secretion (65). However, DXM has a higher affinity for L-type $Ca^{2+}$ channels than to its known target, the NMDA receptor (107). As mentioned before, DXM moderately inhibits L-type $Ca^{2+}$ channels, thereby lowering $[Ca^{2+}]_c$ in permanently depolarized β-cells under stimulating glucose concentrations (66). This is exactly what we observe in our experiments with depolarized β-cells isolated from pancreatic tissue of CHI patients and with depolarized β-cells from SUR1$^{-/-}$ mice (Fig. 7). Thus, DXM could be an alternative strategy in the treatment of CHI especially in the diffuse form of CHI. In patients with focal lesions the drug could cause and undesired increase in $[Ca^{2+}]_c$ and insulin secretion in healthy islets. The benefits of this drug are that it is already available as a pharmacon and that inhibition of L-type $Ca^{2+}$ channels by e.g. nifedipine is already a proved treatment of CHI (19, 29). Considering side effects of nifedipine, like hypotension, the moderate effect of DXM on L-type $Ca^{2+}$ channels could be of advantage (66).

*4.2.5 Simvastatin*

It was shown that simvastatin lowers insulin secretion by blocking L-type $Ca^{2+}$ channels (68, 69). The effect of simvastatin on $[Ca^{2+}]_c$ was comparable with that of nifedipine (69). Accordingly, the risk to develop diabetes mellitus is increased under

the therapy with statins (67). With respect to CHI patients, this side effect could constitute a suitable therapeutic approach. Our results obtained from experiments with β-cells isolated from SUR1$^{-/-}$ mice and human β-cells isolated from patients with CHI point toward a possible beneficial effect of simvastatin in the treatment of CHI (Fig.8). Noteworthy, statins are widely used and well tolerated in the long-term therapy. In contrast to nifedipine, which affects the cardiovascular system, statins have no effect on blood pressure or heart rate (108). Additionally, it has been proposed that statins induce hepatic gluconeogenesis in human liver cells by activation of the pregnane X receptor (PXR) (109, 110), which could also counteract hypoglycemic conditions in CHI patients.

*Conclusions:* There is a clear need to develop novel approaches to prevent hypoglycaemia in CHI patients and better therapies with less side effects for the different forms of CHI. In this study we had access to biopsy material of CHI patients and could give suggestions which drugs or targets should be studied in future. Promising results were obtained with NN414, DCEBIO, DXM and simvastatin.

**Disclosure Summary:** The authors have nothing to disclose

**Contribution of the authors**: J.S. researched data, wrote and edited the manuscript; T.H. and A.G. researched data and contributed to discussion and edited the manuscript; W.B., C.W. and U.L. provided human β-cells and contributed to discussion; M.D. and P.K. D. contributed to discussion and study design and edited the manuscript; G.D. designed the study, wrote and edited the manuscript, contributed to discussion, and is the guarantor of the whole study.

**Acknowledgement:** This work was supported by a grant from the DFG (G.D.).

**References**

1. Dunne MJ, Cosgrove KE, Shepherd RM, Aynsley-Green A, Lindley KJ. Hyperinsulinism in infancy: from basic science to clinical disease. Physiol Rev. 2004;84(1):239-75.

2. Banerjee I, Salomon-Estebanez M, Shah P, Nicholson J, Cosgrove KE, Dunne MJ. Therapies and outcomes of congenital hyperinsulinism-induced hypoglycaemia. Diabet Med. 2019;36(1):9-21.

3. Demirbilek H, Rahman SA, Buyukyilmaz GG, Hussain K. Diagnosis and treatment of hyperinsulinaemic hypoglycaemia and its implications for paediatric endocrinology. Int J Pediatr Endocrinol. 2017;2017:9.

4. Vajravelu ME, De Leon DD. Genetic characteristics of patients with congenital hyperinsulinism. Curr Opin Pediatr. 2018;30(4):568-75.

5. Galcheva S, Demirbilek H, Al-Khawaga S, Hussain K. The Genetic and Molecular Mechanisms of Congenital Hyperinsulinism. Front Endocrinol (Lausanne). 2019;10:111.

6. Rahier J, Guiot Y, Sempoux C. Morphologic analysis of focal and diffuse forms of congenital hyperinsulinism. Semin Pediatr Surg. 2011;20(1):3-12.

7. Sempoux C, Capito C, Bellanne-Chantelot C, Verkarre V, de Lonlay P, Aigrain Y, et al. Morphological mosaicism of the pancreatic islets: a novel anatomopathological form of persistent hyperinsulinemic hypoglycemia of infancy. J Clin Endocrinol Metab. 2011;96(12):3785-93.

8. Kapoor RR, Flanagan SE, James C, Shield J, Ellard S, Hussain K. Hyperinsulinaemic hypoglycaemia. Arch Dis Child. 2009;94(6):450-7.

9. Yorifuji T, Horikawa R, Hasegawa T, Adachi M, Soneda S, Minagawa M, et al. Clinical practice guidelines for congenital hyperinsulinism. Clin Pediatr Endocrinol. 2017;26(3):127-52.

10. Aynsley-Green A, Hussain K, Hall J, Saudubray JM, Nihoul-Fékété C, De Lonlay-Debeney P, et al. Practical management of hyperinsulinism in infancy. Arch Dis Child Fetal Neonatal Ed. 2000;82(2):F98-F107.

11. Shah P, Rahman SA, Demirbilek H, Guemes M, Hussain K. Hyperinsulinaemic hypoglycaemia in children and adults. Lancet Diabetes Endocrinol. 2017;5(9):729-42.

12. Welters A, Lerch C, Kummer S, Marquard J, Salgin B, Mayatepek E, et al. Long-term medical treatment in congenital hyperinsulinism: a descriptive analysis in a large cohort of patients from different clinical centers. Orphanet J Rare Dis. 2015;10:150.

13. Nebesio TD, Hoover WC, Caldwell RL, Nitu ME, Eugster EA. Development of pulmonary hypertension in an infant treated with diazoxide. J Pediatr Endocrinol Metab. 2007;20(8):939-44.

14. Yildizdas D, Erdem S, Kucukosmanoglu O, Yilmaz M, Yuksel B. Pulmonary hypertension, heart failure and neutropenia due to diazoxide therapy. Adv Ther. 2008;25(5):515-9.

15. Demirel F, Unal S, Cetin, II, Esen I, Arasli A. Pulmonary hypertension and reopening of the ductus arteriosus in an infant treated with diazoxide. J Pediatr Endocrinol Metab. 2011;24(7-8):603-5.

16. Timlin MR, Black AB, Delaney HM, Matos RI, Percival CS. Development of Pulmonary Hypertension During Treatment with Diazoxide: A Case Series and Literature Review. Pediatr Cardiol. 2017;38(6):1247-50.

17. Mohnike K, Blankenstein O, Pfuetzner A, Potzsch S, Schober E, Steiner S, et al. Long-term non-surgical therapy of severe persistent congenital hyperinsulinism with glucagon. Horm Res. 2008;70(1):59-64.

18. Neylon OM, Moran MM, Pellicano A, Nightingale M, O'Connell MA. Successful subcutaneous glucagon use for persistent hypoglycaemia in congenital hyperinsulinism. J Pediatr Endocrinol Metab. 2013;26(11-12):1157-61.

19. Eichmann D, Hufnagel M, Quick P, Santer R. Treatment of hyperinsulinaemic hypoglycaemia with nifedipine. Eur J Pediatr. 1999;158(3):204-6.

20. Modan-Moses D, Koren I, Mazor-Aronovitch K, Pinhas-Hamiel O, Landau H. Treatment of congenital hyperinsulinism with lanreotide acetate (Somatuline Autogel). J Clin Endocrinol Metab. 2011;96(8):2312-7.

21. Le Quan Sang KH, Arnoux JB, Mamoune A, Saint-Martin C, Bellanne-Chantelot C, Valayannopoulos V, et al. Successful treatment of congenital hyperinsulinism with long-acting release octreotide. Eur J Endocrinol. 2012;166(2):333-9.

22. Senniappan S, Brown RE, Hussain K. Sirolimus in severe hyperinsulinemic hypoglycemia. N Engl J Med. 2014a;370(25):2448-9.

23. Baş F, Darendeliler F, Demirkol D, Bundak R, Saka N, Günöz H. Successful therapy with calcium channel blocker (nifedipine) in persistent neonatal hyperinsulinemic hypoglycemia of infancy. J Pediatr Endocrinol Metab. 1999;12(6):873-8.

24. Kailey B, van de Bunt M, Cheley S, Johnson PR, MacDonald PE, Gloyn AL, et al. SSTR2 is the functionally dominant somatostatin receptor in human pancreatic beta- and alpha-cells. Am J Physiol Endocrinol Metab. 2012;303(9):E1107-16.

25. Gromada J, Brock B, Schmitz O, Rorsman P. Glucagon-like peptide-1: regulation of insulin secretion and therapeutic potential. Basic Clin Pharmacol Toxicol. 2004;95(6):252-62.

26. Hawkes CP, Adzick NS, Palladino AA, De Leon DD. Late Presentation of Fulminant Necrotizing Enterocolitis in a Child with Hyperinsulinism on Octreotide Therapy. Horm Res Paediatr. 2016;86(2):131-6.

27. McMahon AW, Wharton GT, Thornton P, De Leon DD. Octreotide use and safety in infants with hyperinsulinism. Pharmacoepidemiol Drug Saf. 2017;26(1):26-31.

28. Demirbilek H, Shah P, Arya VB, Hinchey L, Flanagan SE, Ellard S, et al. Long-term follow-up of children with congenital hyperinsulinism on octreotide therapy. J Clin Endocrinol Metab. 2014;99(10):3660-7.

29. Durmaz E, Flanagan SE, Parlak M, Ellard S, Akcurin S, Bircan I. A combination of nifedipine and octreotide treatment in an hyperinsulinemic hypoglycemic infant. J Clin Res Pediatr Endocrinol. 2014;6(2):119-21.

30. Szymanowski M, Estebanez MS, Padidela R, Han B, Mosinska K, Stevens A, et al. mTOR Inhibitors for the Treatment of Severe Congenital Hyperinsulinism: Perspectives on Limited Therapeutic Success. J Clin Endocrinol Metab. 2016;101(12):4719-29.

31. Banerjee I, De Leon D, Dunne MJ. Extreme caution on the use of sirolimus for the congenital hyperinsulinism in infancy patient. Orphanet J Rare Dis. 2017;12(1):70.

32. Corbin JA, Bhaskar V, Goldfine ID, Bedinger DH, Lau A, Michelson K, et al. Improved glucose metabolism in vitro and in vivo by an allosteric monoclonal antibody that increases insulin receptor binding affinity. PLoS One. 2014a;9(2):e88684.

33. Corbin JA, Bhaskar V, Goldfine ID, Issafras H, Bedinger DH, Lau A, et al. Inhibition of insulin receptor function by a human, allosteric monoclonal antibody: a potential new approach for the treatment of hyperinsulinemic hypoglycemia. MAbs. 2014b;6(1):262-72.

34. Issafras H, Bedinger DH, Corbin JA, Goldfine ID, Bhaskar V, White ML, et al. Selective allosteric antibodies to the insulin receptor for the treatment of hyperglycemic and hypoglycemic disorders. J Diabetes Sci Technol. 2014;8(4):865-73.

35. Patel P, Charles L, Corbin J, Goldfine ID, Johnson K, Rubin P, et al. A unique allosteric insulin receptor monoclonal antibody that prevents hypoglycemia in the SUR-1(-/-) mouse model of KATP hyperinsulinism. MAbs. 2018;10(5):796-802.

36. Kirk W. Johnson ACN, Allan Gordon, Diva Del Carmen De Leon-Crutchlow, Khalid Hussain, Klaus Ludwig Mohnike, Sabine Vukelich, Julie M. Roessig and Paul D. Rubin. Activity of Xoma 358, an Inhibitor of Insulin Action Following Short-Term Administration to Congenital Hyperinsulinism Patients. Endocrine Reviews. June 2017;38(3).

37. Barthlen W, Varol E, Empting S, Wieland I, Zenker M, Mohnike W, et al. Surgery in Focal Congenital Hyperinsulinism (CHI) - The "Hyperinsulinism Germany International" Experience in 30 Children. Pediatr Endocrinol Rev. 2016;14(2):129-37.

38. Adzick NS, Thornton PS, Stanley CA, Kaye RD, Ruchelli E. A multidisciplinary approach to the focal form of congenital hyperinsulinism leads to successful treatment by partial pancreatectomy. J Pediatr Surg. 2004;39(3):270-5.

39. Barthlen W, Mohnike W, Mohnike K. Techniques in pediatric surgery: congenital hyperinsulinism. Horm Res Paediatr. 2010;74(6):438-43.

40.Adzick NS, De Leon DD, States LJ, Lord K, Bhatti TR, Becker SA, et al. Surgical treatment of congenital hyperinsulinism: Results from 500 pancreatectomies in neonates and children. J Pediatr Surg. 2019;54(1):27-32.

41. Lovvorn HN, 3rd, Nance ML, Ferry RJ, Jr., Stolte L, Baker L, O'Neill JA, Jr., et al. Congenital hyperinsulinism and the surgeon: lessons learned over 35 years. J Pediatr Surg. 1999;34(5):786-92; discussion 92-3.

42.Meissner T, Wendel U, Burgard P, Schaetzle S, Mayatepek E. Long-term follow-up of 114 patients with congenital hyperinsulinism. Eur J Endocrinol. 2003;149(1):43-51.

43. Barthlen W. Surgery in congenital hyperinsulinism-tips and tricks not only for surgeons. A practical guide. Semin Pediatr Surg. 2011;20(1):56-9.

44.Glaser B, Blech I, Krakinovsky Y, Ekstein J, Gillis D, Mazor-Aronovitch K, et al. ABCC8 mutation allele frequency in the Ashkenazi Jewish population and risk of focal hyperinsulinemic hypoglycemia. Genet Med. 2011;13(10):891-4.

45. Nestorowicz A, Wilson BA, Schoor KP, Inoue H, Glaser B, Landau H, et al. Mutations in the sulonylurea receptor gene are associated with familial hyperinsulinism in Ashkenazi Jews. Hum Mol Genet. 1996;5(11):1813-22.

46.Gier B, Krippeit-Drews P, Sheiko T, Aguilar-Bryan L, Bryan J, Dufer M, et al. Suppression of KATP channel activity protects murine pancreatic beta cells against oxidative stress. J Clin Invest. 2009;119(11):3246-56.

47.Edalat A, Schulte-Mecklenbeck P, Bauer C, Undank S, Krippeit-Drews P, Drews G, et al. Mitochondrial succinate dehydrogenase is involved in stimulus-secretion coupling and endogenous ROS formation in murine beta cells. Diabetologia. 2015;58(7):1532-41.

48. Braun M, Ramracheya R, Bengtsson M, Zhang Q, Karanauskaite J, Partridge C, et al. Voltage-gated ion channels in human pancreatic beta-cells: electrophysiological characterization and role in insulin secretion. Diabetes. 2008;57(6):1618-28.

49. Dabrowski M, Larsen T, Ashcroft FM, Bondo Hansen J, Wahl P. Potent and selective activation of the pancreatic beta-cell type K(ATP) channel by two novel diazoxide analogues. Diabetologia. 2003;46(10):1375-82.

50. Raphemot R, Swale DR, Dadi PK, Jacobson DA, Cooper P, Wojtovich AP, et al. Direct activation of beta-cell KATP channels with a novel xanthine derivative. Mol Pharmacol. 2014;85(6):858-65.

51.Maczewsky J, Sikimic J, Bauer C, Krippeit-Drews P, Wolke C, Lendeckel U, et al. The LXR Ligand T0901317 Acutely Inhibits Insulin Secretion by Affecting Mitochondrial Metabolism. Endocrinology. 2017;158(7):2145-54.

52. Drews G, Krippeit-Drews P, Düfer M. Electrophysiology of islet cells. Adv Exp Med Biol. 2010;654:115-63.

53.Drews G, Bauer C, Edalat A, Dufer M, Krippeit-Drews P. Evidence against a Ca(2+)-induced potentiation of dehydrogenase activity in pancreatic beta-cells. Pflugers Arch. 2015;467(11):2389-97.

54. Krippeit-Drews P, Düfer M, Drews G. Parallel oscillations of intracellular calcium activity and mitochondrial membrane potential in mouse pancreatic B-cells. Biochem Biophys Res Commun. 2000;267(1):179-83.

55. Tamarina NA, Wang Y, Mariotto L, Kuznetsov A, Bond C, Adelman J, et al. Small-conductance calcium-activated K+ channels are expressed in pancreatic islets and regulate glucose responses. Diabetes. 2003;52(8):2000-6.

56. Düfer M, Gier B, Wolpers D, Krippeit-Drews P, Ruth P, Drews G. Enhanced glucose tolerance by SK4 channel inhibition in pancreatic beta-cells. Diabetes. 2009;58(8):1835-43.

57. Jacobson DA, Mendez F, Thompson M, Torres J, Cochet O, Philipson LH. Calcium-activated and voltage-gated potassium channels of the pancreatic islet impart distinct and complementary roles during secretagogue induced electrical responses. J Physiol. 2010;588(Pt 18):3525-37.

58. Düfer M, Neye Y, Horth K, Krippeit-Drews P, Hennige A, Widmer H, et al. BK channels affect glucose homeostasis and cell viability of murine pancreatic beta cells. Diabetologia. 2011;54(2):423-32.

59. Goforth PB, Bertram R, Khan FA, Zhang M, Sherman A, Satin LS. Calcium-activated K+Channels of Mouse β-cells are Controlled by Both Store and Cytoplasmic Ca2+. The Journal of General Physiology. 2002;120(3):307-22.

60. Göpel SO, Kanno T, Barg S, Eliasson L, Galvanovskis J, Renstrom E, et al. Activation of Ca(2+)-dependent K(+) channels contributes to rhythmic firing of action potentials in mouse pancreatic beta cells. J Gen Physiol. 1999;114(6):759-70.

61. Kanno T, Rorsman P, Göpel SO. Glucose-dependent regulation of rhythmic action potential firing in pancreatic β-cells by kATP-channel modulation. The Journal of Physiology. 2002;545(2):501-7.

62. Drews G, Debuyser A, Nenquin M, Henquin JC. Galanin and epinephrine act on distinct receptors to inhibit insulin release by the same mechanisms including an increase in K+ permeability of the B-cell membrane. Endocrinology. 1990;126(3):1646-53.

63. Düfer M, Haspel D, Krippeit-Drews P, Aguilar-Bryan L, Bryan J, Drews G. Oscillations of membrane potential and cytosolic Ca(2+) concentration in SUR1(-/-) beta cells. Diabetologia. 2004;47(3):488-98.

64. Ngo-Anh TJ, Bloodgood BL, Lin M, Sabatini BL, Maylie J, Adelman JP. SK channels and NMDA receptors form a Ca2+-mediated feedback loop in dendritic spines. Nat Neurosci. 2005;8(5):642-9.

65. Marquard J, Otter S, Welters A, Stirban A, Fischer A, Eglinger J, et al. Characterization of pancreatic NMDA receptors as possible drug targets for diabetes treatment. Nat Med. 2015;21(4):363-72.

66. Carpenter CL, Marks SS, Watson DL, Greenberg DA. Dextromethorphan and dextrorphan as calcium channel antagonists. Brain Res. 1988;439(1-2):372-5.

67. Sattar N, Preiss D, Murray HM, Welsh P, Buckley BM, de Craen AJ, et al. Statins and risk of incident diabetes: a collaborative meta-analysis of randomised statin trials. Lancet. 2010;375(9716):735-42.

68.Yada T, Nakata M, Shiraishi T, Kakei M. Inhibition by simvastatin, but not pravastatin, of glucose-induced cytosolic Ca2+ signalling and insulin secretion due to blockade of L-type Ca2+ channels in rat islet beta-cells. Br J Pharmacol. 1999;126(5):1205-13.

69. Yaluri N, Modi S, Lopez Rodriguez M, Stancakova A, Kuusisto J, Kokkola T, et al. Simvastatin Impairs Insulin Secretion by Multiple Mechanisms in MIN6 Cells. PLoS One. 2015;10(11):e0142902.

70. Güemes M, Shah P, Silvera S, Morgan K, Gilbert C, Hinchey L, et al. Assessment of Nifedipine Therapy in Hyperinsulinemic Hypoglycemia due to Mutations in the ABCC8 Gene. J Clin Endocrinol Metab. 2017;102(3):822-30.

71. Coetzee WA. Multiplicity of effectors of the cardioprotective agent, diazoxide. Pharmacol Ther. 2013;140(2):167-75.

72. D'Hahan N, Moreau C, Prost AL, Jacquet H, Alekseev AE, Terzic A, et al. Pharmacological plasticity of cardiac ATP-sensitive potassium channels toward diazoxide revealed by ADP. Proc Natl Acad Sci U S A. 1999;96(21):12162-7.

73. Davies GC, Thornton MJ, Jenner TJ, Chen YJ, Hansen JB, Carr RD, et al. Novel and established potassium channel openers stimulate hair growth in vitro: implications for their modes of action in hair follicles. J Invest Dermatol. 2005;124(4):686-94.

74. Burton JL, Schutt WH, Caldwell IW. Hypertrichosis due to diazoxide. Br J Dermatol. 1975;93(6):707-11.

75. Hansen JB, Arkhammar PO, Bodvarsdottir TB, Wahl P. Inhibition of insulin secretion as a new drug target in the treatment of metabolic disorders. Curr Med Chem. 2004;11(12):1595-615.

76. Ritzel RA, Hansen JB, Veldhuis JD, Butler PC. Induction of beta-cell rest by a Kir6.2/SUR1-selective K(ATP)-channel opener preserves beta-cell insulin stores and insulin secretion in human islets cultured at high (11 mM) glucose. J Clin Endocrinol Metab. 2004;89(2):795-805.

77. Nielsen FE, Bodvarsdottir TB, Worsaae A, MacKay P, Stidsen CE, Boonen HC, et al. 6-Chloro-3-alkylamino-4H-thieno[3,2-e]-1,2,4-thiadiazine 1,1-dioxide derivatives potently and selectively activate ATP sensitive potassium channels of pancreatic beta-cells. J Med Chem. 2002;45(19):4171-87.

78. Alemzadeh R, Fledelius C, Bodvarsdottir T, Sturis J. Attenuation of hyperinsulinemia by NN414, a SUR1/Kir6.2 selective K+-adenosine triphosphate channel opener, improves glucose tolerance and lipid profile in obese Zucker rats. Metabolism. 2004;53(4):441-7.

79. Zdravkovic M, Kruse M, Rost KL, Moss J, Kecskes A, Dyrberg T. The effects of NN414, a SUR1/Kir6.2 selective potassium channel opener, in healthy male subjects. J Clin Pharmacol. 2005;45(7):763-72.

80. Choi JK. NN-414. Novo Nordisk. Curr Opin Investig Drugs. 2003;4(4):455-8.

81. Sarabu R, Tilley J. Recent advances in therapeutic approaches to type 2 diabetes. In: Doherty AM, editor. Annual Reports in Medicinal Chemistry. 40: Elsevier; 2005. p. 167-81.

82. Zdravkovic M, Kruse M, Rost KL, Moss J, Kecskes A. The effects of NN414, a SUR1/Kir6.2 selective potassium channel opener in subjects with type 2 diabetes. Exp Clin Endocrinol Diabetes. 2007;115(6):405-6.

83. Koren I, Riskin A, Barthlen W, Gillis D. Hepatitis in an infant treated with octreotide for congenital hyperinsulinism. J Pediatr Endocrinol Metab. 2013;26(1-2):183-5.

84. Ben-Ari J, Greenberg M, Nemet D, Edelstein E, Eliakim A. Octreotide-induced hepatitis in a child with persistent hyperinsulinemia hypoglycemia of infancy. J Pediatr Endocrinol Metab. 2013;26(1-2):179-82.

85. Glaser B, Thornton P, Otonkoski T, Junien C. Genetics of neonatal hyperinsulinism. Arch Dis Child Fetal Neonatal Ed. 2000;82(2):F79-86.

86. Saint-Martin C, Arnoux JB, de Lonlay P, Bellanne-Chantelot C. KATP channel mutations in congenital hyperinsulinism. Semin Pediatr Surg. 2011;20(1):18-22.

87. Arya VB, Guemes M, Nessa A, Alam S, Shah P, Gilbert C, et al. Clinical and histological heterogeneity of congenital hyperinsulinism due to paternally inherited heterozygous ABCC8/KCNJ11 mutations. Eur J Endocrinol. 2014;171(6):685-95.

88. Macmullen CM, Zhou Q, Snider KE, Tewson PH, Becker SA, Aziz AR, et al. Diazoxide-unresponsive congenital hyperinsulinism in children with dominant mutations of the beta-cell sulfonylurea receptor SUR1. Diabetes. 2011;60(6):1797-804.

89. Katz MD, Erstad BL. Octreotide, a new somatostatin analogue. Clin Pharm. 1989;8(4):255-73.

90. Doyle ME, Egan JM. Pharmacological agents that directly modulate insulin secretion. Pharmacol Rev. 2003;55(1):105-31.

91. Braun M. The somatostatin receptor in human pancreatic beta-cells. Vitam Horm. 2014;95:165-93.

92. Shah P, Rahman SA, McElroy S, Gilbert C, Morgan K, Hinchey L, et al. Use of Long-Acting Somatostatin Analogue (Lanreotide) in an Adolescent with Diazoxide-Responsive Congenital Hyperinsulinism and Its Psychological Impact. Horm Res Paediatr. 2015;84(5):355-60.

93. Dastamani A, Guemes M, Pitfield C, Morgan K, Rajab M, Rottenburger C, et al. The Use of a Long-Acting Somatostatin Analogue (Lanreotide) in Three Children with Focal Forms of Congenital Hyperinsulinaemic Hypoglycaemia. Horm Res Paediatr. 2019;91(1):56-61.

94. van der Steen I, van Albada ME, Mohnike K, Christesen HT, Empting S, Salomon-Estebanez M, et al. A Multicenter Experience with Long-Acting Somatostatin Analogues in Patients with Congenital Hyperinsulinism. Horm Res Paediatr. 2018;89(2):82-9.

95. Maria G, Antonia D, Michael A, Kate M, Sian E, Sarah FE, et al. Sirolimus: Efficacy and Complications in Children With Hyperinsulinemic Hypoglycemia: A 5-Year Follow-Up Study. J Endocr Soc. 2019;3(4):699-713.

96. Wullschleger S, Loewith R, Hall MN. TOR signaling in growth and metabolism. Cell. 2006;124(3):471-84.

97. Houde VP, Brule S, Festuccia WT, Blanchard PG, Bellmann K, Deshaies Y, et al. Chronic rapamycin treatment causes glucose intolerance and hyperlipidemia by upregulating hepatic gluconeogenesis and impairing lipid deposition in adipose tissue. Diabetes. 2010;59(6):1338-48.

98. Hoffman JF, Joiner W, Nehrke K, Potapova O, Foye K, Wickrema A. The hSK4 (KCNN4) isoform is the Ca2+-activated K+ channel (Gardos channel) in human red blood cells. Proc Natl Acad Sci U S A. 2003;100(12):7366-71.

99. Begenisich T, Nakamoto T, Ovitt CE, Nehrke K, Brugnara C, Alper SL, et al. Physiological roles of the intermediate conductance, Ca2+-activated potassium channel Kcnn4. J Biol Chem. 2004;279(46):47681-7.

100. Jensen BS, Strobaek D, Christophersen P, Jorgensen TD, Hansen C, Silahtaroglu A, et al. Characterization of the cloned human intermediate-conductance Ca2+-activated K+ channel. Am J Physiol. 1998;275(3):C848-56.

101. Ahren B, Ar'Rajab A, Bottcher G, Sundler F, Dunning BE. Presence of galanin in human pancreatic nerves and inhibition of insulin secretion from isolated human islets. Cell Tissue Res. 1991;264(2):263-7.

102. Holst JJ, Bersani M, Hvidberg A, Knigge U, Christiansen E, Madsbad S, et al. On the effects of human galanin in man. Diabetologia. 1993;36(7):653-7.

103. Lang R, Gundlach AL, Holmes FE, Hobson SA, Wynick D, Hokfelt T, et al. Physiology, signaling, and pharmacology of galanin peptides and receptors: three decades of emerging diversity. Pharmacol Rev. 2015;67(1):118-75.

104. Tang G, Wang Y, Park S, Bajpayee NS, Vi D, Nagaoka Y, et al. Go2 G protein mediates galanin inhibitory effects on insulin release from pancreatic beta cells. Proc Natl Acad Sci U S A. 2012;109(7):2636-41.

105. Hussain MA, Akalestou E, Song WJ. Inter-organ communication and regulation of beta cell function. Diabetologia. 2016;59(4):659-67.

106. Rorsman P, Ashcroft FM. Pancreatic beta-Cell Electrical Activity and Insulin Secretion: Of Mice and Men. Physiol Rev. 2018;98(1):117-214.

107. Jaffe DB, Marks SS, Greenberg DA. Antagonist drug selectivity for radioligand binding sites on voltage-gated and N-methyl-D-aspartate receptor-gated Ca2+ channels. Neurosci Lett. 1989;105(1-2):227-32.

108. Thompson PD, Panza G, Zaleski A, Taylor B. Statin-Associated Side Effects. J Am Coll Cardiol. 2016;67(20):2395-410.

109. Gotoh S, Negishi M. Statin-activated nuclear receptor PXR promotes SGK2 dephosphorylation by scaffolding PP2C to induce hepatic gluconeogenesis. Sci Rep. 2015;5:14076.

110. van Stee MF, de Graaf AA, Groen AK. Actions of metformin and statins on lipid and glucose metabolism and possible benefit of combination therapy. Cardiovasc Diabetol. 2018;17(1):94.

Table 1: Genetic characteristics of patients

| Patient No. | Age at surgery (months) | Genetics | Form |
|---|---|---|---|
| **1** | 4 | ABCC8: c.3989-9G>A | focal |
| **2** | 6 | ABCC8 p.Gly1479Arg | mosaic |
| **3** | 3 | ABCC8: c.2509C>T; p.(Arg837*) | focal |
| **4** | 24 | no mutation was found | diffuse |
| **5** | 10 | ABCC8: c.3992-9G>A (also known as c.3989-9G>A ) | focal |
| **6** | 8 | ABCC8: c.3970G>T;p.(Glu1324*) in Exon 32 paternal heterozygot | focal |
| **7** | 10 | ABCC8: c.(3989-9G>A);(=) Exon 32-33 | focal |
| **8** | 7,5 | ABCC8: c.2509C>T, p.(Arg837*) | focal |
| **9** | 33 | ABCC8, details unknown | diffuse |
| **10** | 13,5 | ABCC8 : c1183A>T (p.Ile 395 Phe) c4146T>G (pSer 1382 Arg) | diffuse |
| **11** | 6 | ABCC8, details unknown | focal |

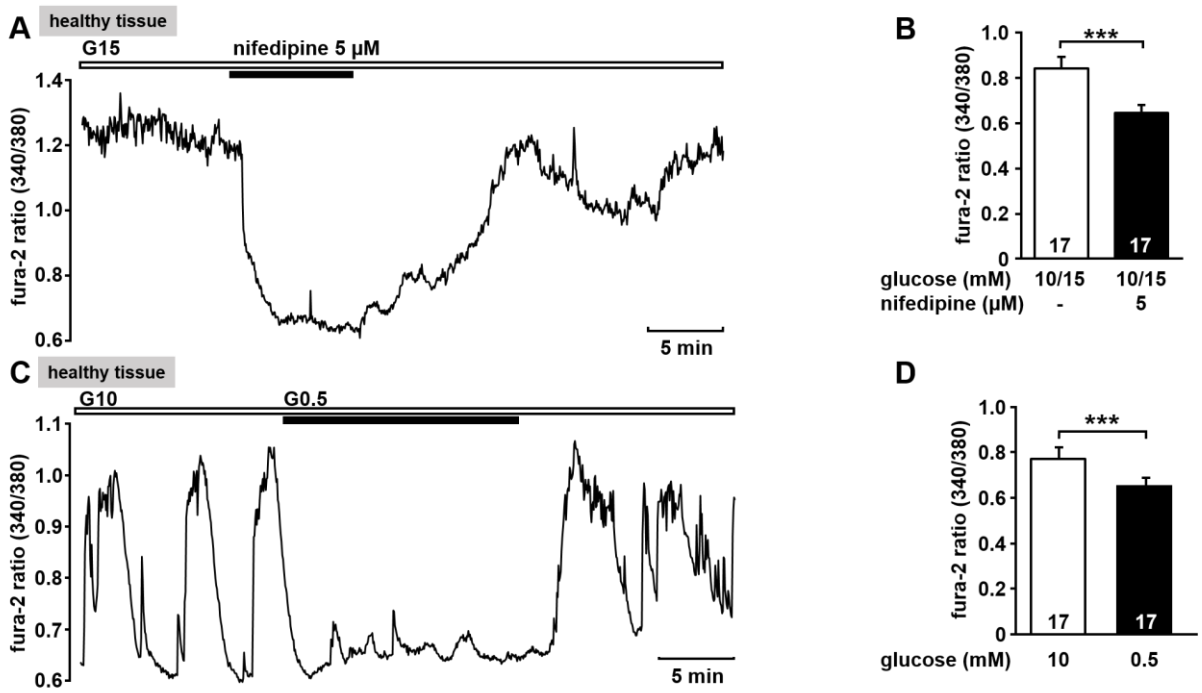


Fig. 1: Nifedipine and low glucose concentration reduce $[Ca^{2+}]_c$ in human β-cells. (A) Representative recording showing inhibition of glucose-induced oscillations of $[Ca^{2+}]_c$ by nifedipine (5 µM) in the presence of 15 mM glucose in a human β-cell isolated from healthy pancreatic tissue of a CHI patient. (B) Summary of all experiments recorded in the presence of 10 or 15 mM glucose. (C) Representative recording presenting the effect of 0.5 mM glucose on glucose-induced oscillations of $[Ca^{2+}]_c$ in a human β-cell isolated from healthy pancreatic tissue of a CHI patient. (D) Summary of all respective experiments. The numbers in the columns indicate the number of experiments with different cell clusters isolated from healthy pancreatic tissue of 2 CHI patients for series with nifedipine and 6 CHI patients for low glucose series. ***p≤0.001

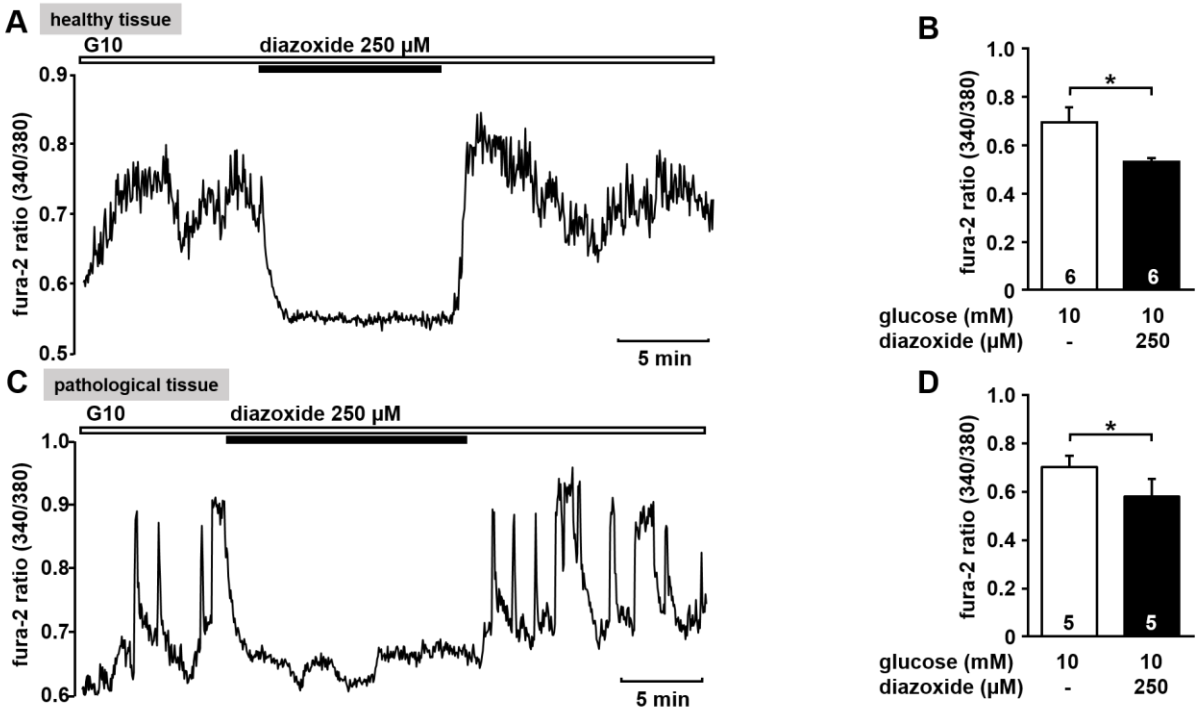


Fig. 2: Diazoxide affects $[Ca^{2+}]_c$ in human β-cells. Representative recordings showing the influence of diazoxide (250 µM) on glucose-induced oscillations of $[Ca^{2+}]_c$ in the presence of 10 mM glucose in human β-cells isolated from healthy (A) and pathological (C) pancreatic tissue of CHI patients. (B, D) Summary of all respective experiments. The numbers in columns indicate the number of different experiments with different cell clusters isolated from healthy pancreatic tissue of 1 CHI patient and pathological pancreatic tissue of 2 CHI patients. *p≤0.05

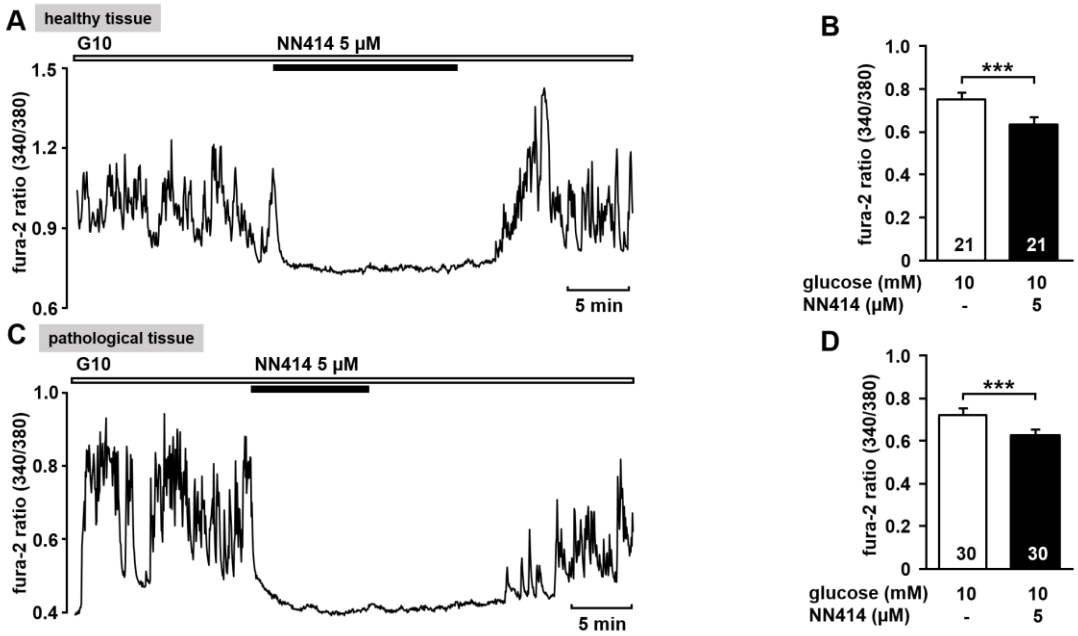


Fig. 3: NN414 is more effective than diazoxide. Representative recordings showing the reduction of glucose-induced oscillations of $[Ca^{2+}]_c$ by NN414 (5 µM) in the presence of 10 mM glucose in human β-cells isolated from healthy (A) and pathological (C) pancreatic tissue of CHI patients. (B, D) Summary of all respective experiments. The numbers in columns indicate the number of different experiments with different cell clusters isolated from healthy pancreatic tissue of 3 CHI patients and pathological pancreatic tissue of 3 CHI patients. ***p≤0.001

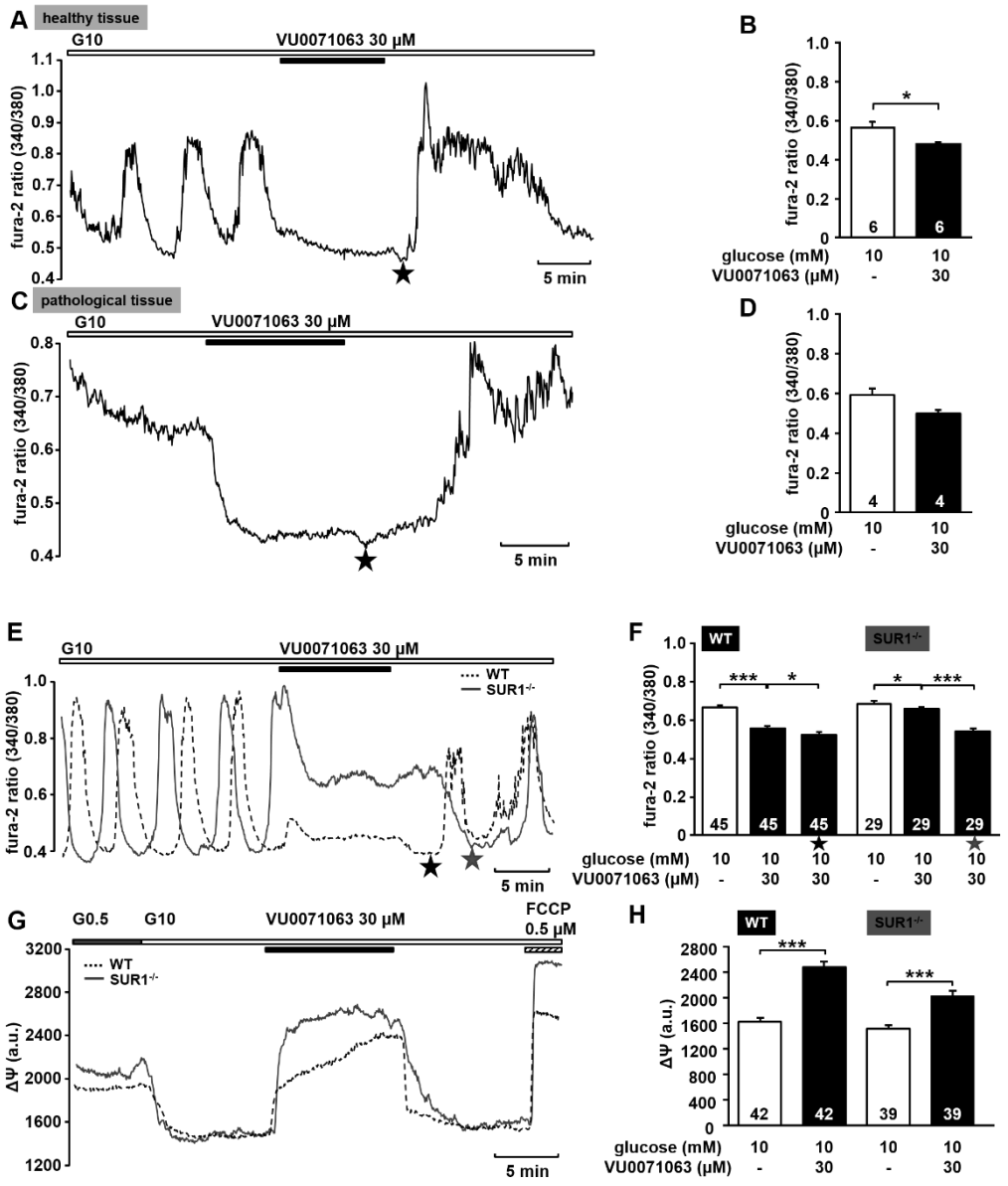


Fig. 4: $K_{ATP}$ channel-dependent and -independent effects of VU0071063. Representative recordings showing reduction of glucose-induced oscillations of $[Ca^{2+}]_c$ by VU0071063 (30 µM) in the presence of 10 mM glucose in human β-cells isolated from healthy (A) and pathological (C) pancreatic tissue of CHI patients. (B, D) Summary of all respective experiments. The numbers in columns indicate the number of different experiments with different cell clusters isolated from healthy biopsies of 2 CHI patients as well as from pathological biopsies of 2 CHI patients. *p≤0.05. In 4 out of 4 human β-cells isolated from pathological pancreatic tissue of CHI patients, VU0071063 (30 µM) rapidly reduced $[Ca^{2+}]_c$, but due to the low number of experiments, the effect is not significant. (E) Representative recordings showing the effect of VU0071063 (30 µM) on glucose-induced oscillations of $[Ca^{2+}]_c$ induced by 10 mM glucose in β-cells from C57BL/6N (dashed curve) and $SUR1^{-/-}$ (grey curve) mice. VU0071063 significantly reduced $[Ca^{2+}]_c$ in β-cells from $SUR1^{-/-}$ mice, revealing $K_{ATP}$

channel-independent effects of the compound. Possibly, the drop in ATP production causes a release of $Ca^{2+}$ from stores which in turn induces a $Ca^{2+}$-dependent inactivation of L-type $Ca^{2+}$ channels. Note the drop in $[Ca^{2+}]_c$ after removal of VU0071063 (black asterisk: C57BL/6N, grey asterisk: $SUR1^{-/-}$) (F) Summary of all respective experiments. (G) Representative recordings showing the effect of VU0071063 (30 µM) on mitochondrial membrane potential (ΔΨ) in β-cells obtained from C57BL/6N (dashed curve) and $SUR1^{-/-}$ (grey curve) mice. (H) Summary of all respective experiments. The numbers in columns indicate the number of different experiments with different cell clusters isolated from 3 C57BL/6N and 3 $SUR1^{-/-}$ mice. *p≤0.05, ***p≤0.001

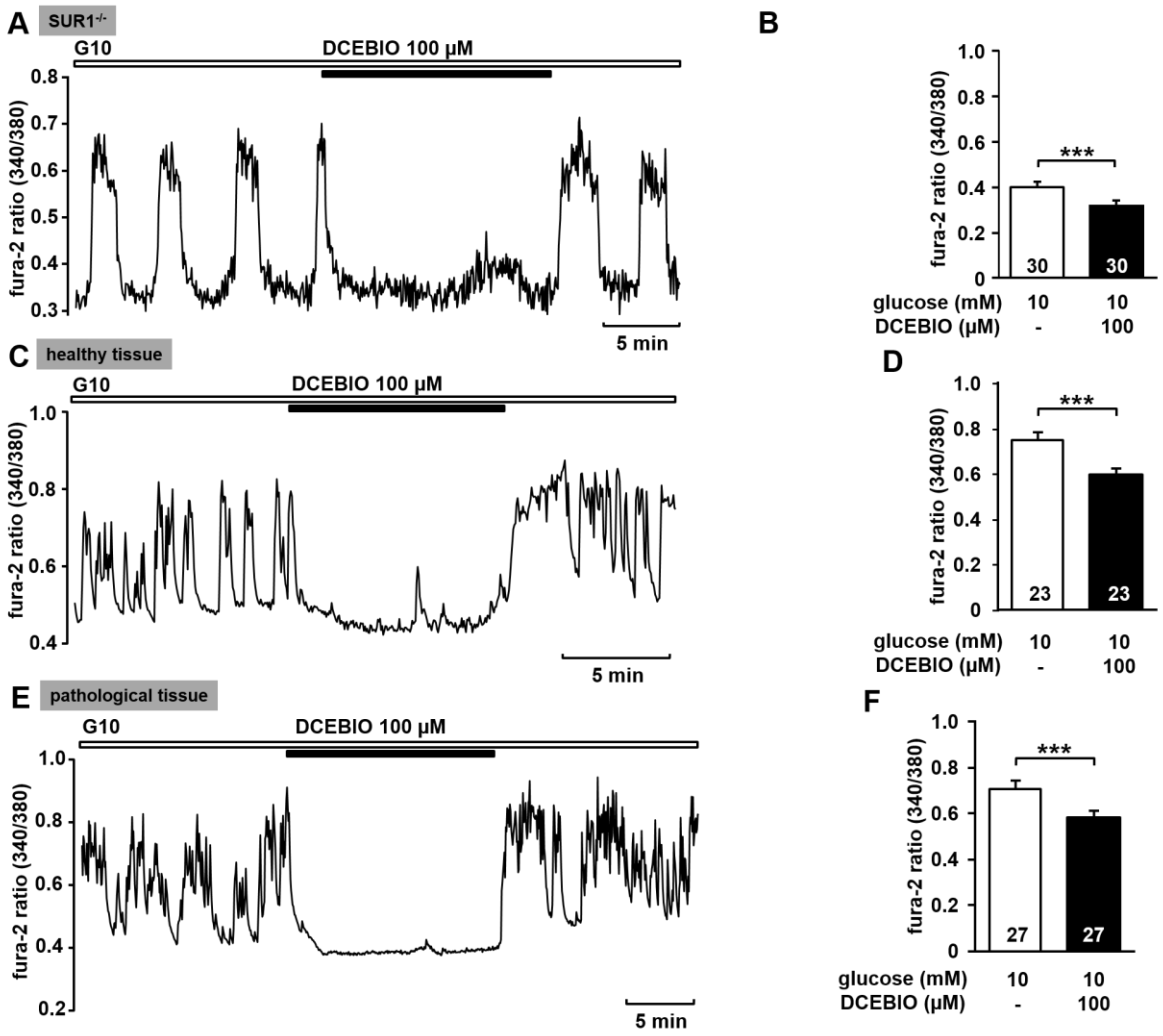


Fig. 5: The SK4 channel opener DCEBIO reduces $[Ca^{2+}]_c$ in β-cells isolated from SUR1$^{-/-}$ mice and in human β-cells. (A) Representative recording showing rapid inhibition of glucose-induced oscillations of $[Ca^{2+}]_c$ by DCEBIO (100 µM) in the presence of 10 mM glucose in β-cells from SUR1$^{-/-}$ mice. (B) Summary of all respective experiments. The numbers in columns indicate the number of different experiments with different cell clusters from 3 SUR$^{1-/-}$ mice. ***p≤0.001. Representative recordings showing the reduction of glucose-induced oscillations of $[Ca^{2+}]_c$ by DCEBIO (100 µM) in the presence of 10 mM glucose in human β-cells isolated from healthy pancreatic tissue (C) and pancreatic tissue affected by CHI (E). (D, F) Summary of all respective experiments. The numbers in columns indicate the number of different experiments with different cell clusters isolated from healthy pancreatic tissue of 2 CHI patients and pathological pancreatic tissue of 2 CHI patients. ***p≤0.001.

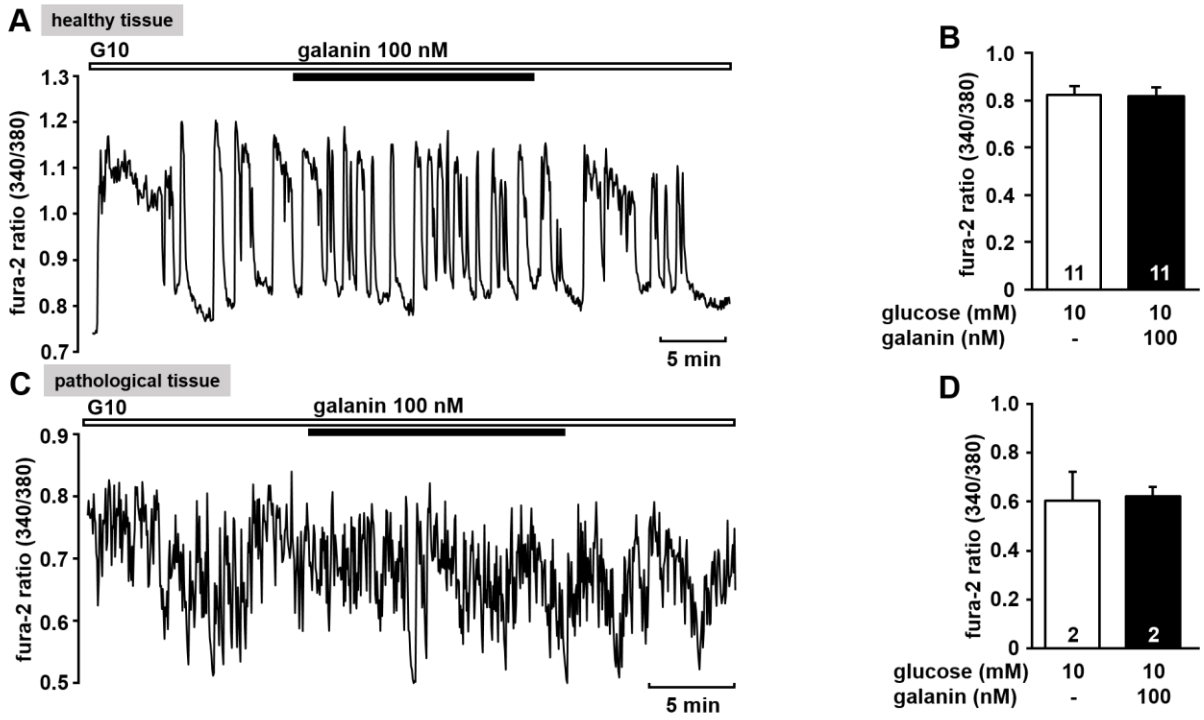


Fig. 6: Galanin does not affect $[Ca^{2+}]_c$ in human β-cells. Representative recordings showing the lack of effect of galanin (100 nM) on glucose-induced oscillations of $[Ca^{2+}]_c$ in the presence of 10 mM glucose in a human β-cell isolated from healthy (A) and pathological (C) pancreatic tissue of CHI patients. (B, D) Summary of all respective experiments. The number in the columns indicate the number of experiments with different cell clusters obtained from healthy pancreatic tissue of 1 CHI patient and pathological pancreatic tissue of 1 CHI patient.

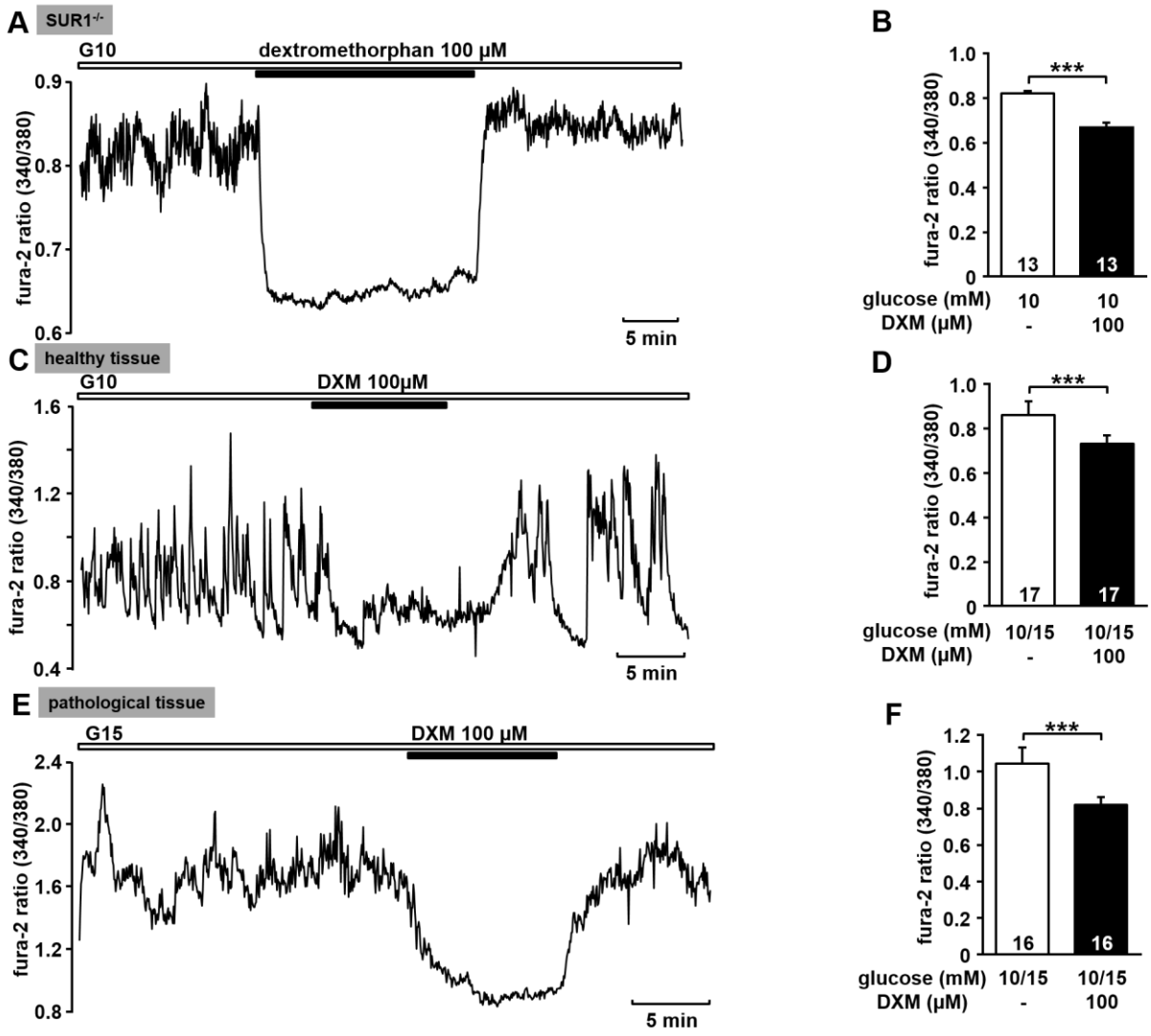


Fig. 7: DXM lowers $[Ca^{2+}]_c$ in β-cells lacking functional $K_{ATP}$ channel (A) Representative recording showing rapid inhibition of glucose-induced oscillations of $[Ca^{2+}]_c$ by DXM (100 µM) in the presence of 10 mM glucose in β-cells from SUR1$^{-/-}$ mice. (B) Summary of all respective experiments. The numbers in columns indicate the number of different experiments with different cell clusters from 3 SUR1$^{-/-}$ mice. ***p≤0.001. Representative recordings showing reduction of glucose-induced oscillations of $[Ca^{2+}]_c$ by DXM (100 µM) in the presence of 10 or 15 mM glucose in human β-cells isolated from healthy (C) pancreatic tissue and pancreatic tissue affected by CHI (E). (D, F) Summary of all respective experiments. The numbers in columns indicate the number of different experiments with different cell clusters from healthy biopsies of 3 CHI patients and pathological biopsies of 4 CHI patients. ***p≤0.001.

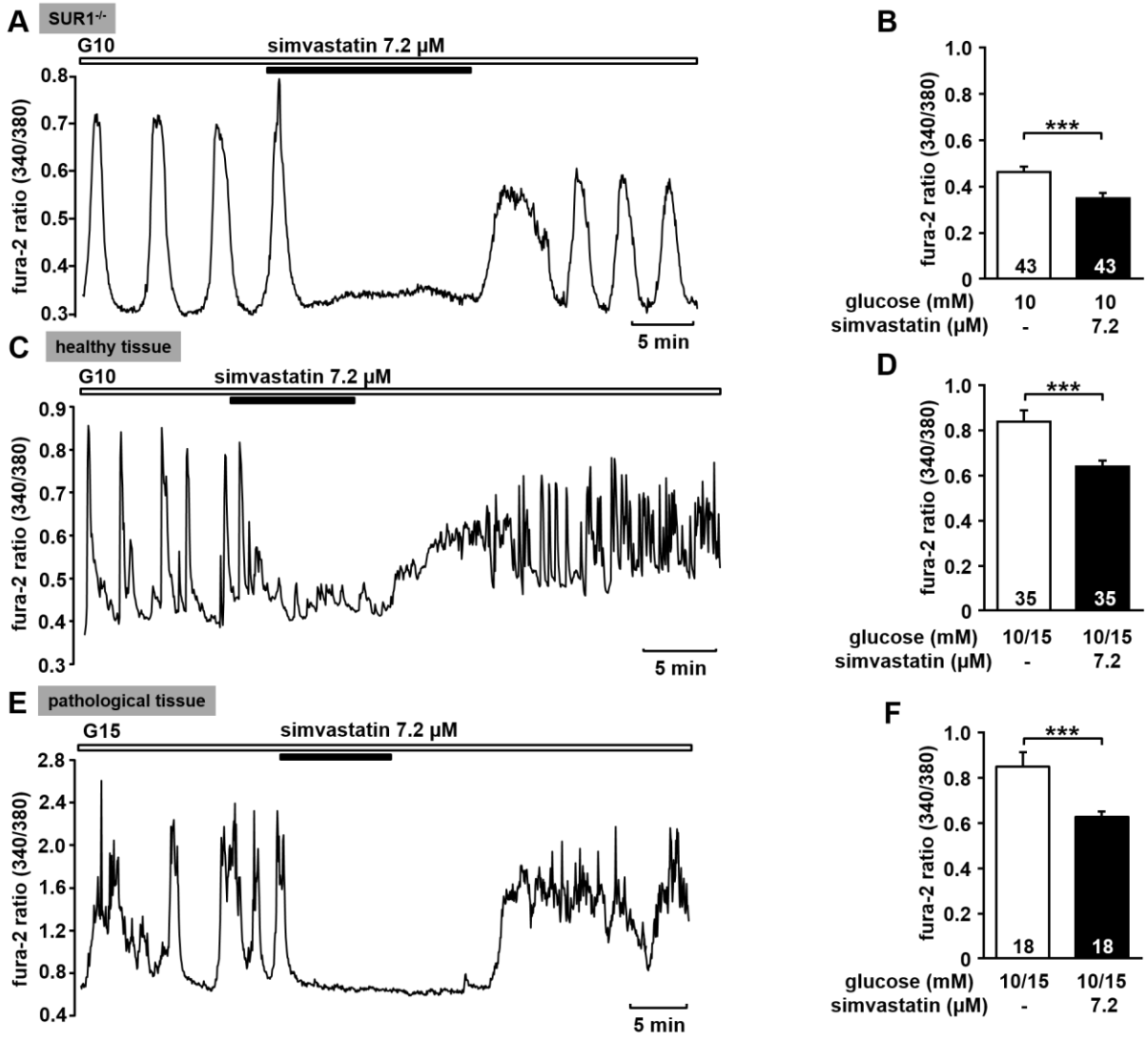


Fig. 8: Simvastatin as a potential strategy to silence β-cells affected by CHI. (A) Representative recording showing rapid inhibition of glucose-induced oscillations of $[Ca^{2+}]_c$ by simvastatin (7.2 µM) in the presence of 10 mM glucose in β-cells from SUR1$^{-/-}$. (B) Summary of all respective experiments. The numbers in columns indicate the number of different experiments with cell clusters obtained from 3 SUR1$^{-/-}$ mice. ***p≤0.001. Representative recordings showing reduction of glucose-induced oscillations of $[Ca^{2+}]_c$ by simvastatin (7.2 µM) in the presence of 10 or 15 mM glucose in human β-cells isolated from healthy (C) pancreatic tissue and pancreatic tissue affected by CHI (E). (D, F) Summary of all respective experiments. The numbers in columns indicate the number of different experiments with different cell clusters isolated from healthy pancreatic tissue of 3 CHI patients and pathological pancreatic tissue of 4 CHI patients. ***p≤0.001.

**The LXR Ligand T0901317 Acutely Inhibits Insulin Secretion by Affecting Mitochondrial Metabolism**

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*Published:* Endocrinology, Volume 158, Issue 7, 1 July 2017, Pages 2145–2154,

**Abstract**

The role of liver X receptor (LXR) in pancreatic β-cell physiology and pathophysiology is still unclear. It has been postulated that chronic LXR activation in β-cells induces lipotoxicity, a key step in the development of β-cell dysfunction, which accompanies type 2 diabetes mellitus. In most of these studies, the LXR ligand T0901317 has been administered chronically in the micromolar range to study the significance of LXR activation. In the current study, we have evaluated acute effects of T0901317 on stimulus-secretion coupling of β-cells. We found that 10 µM T0901317 completely suppressed oscillations of the cytosolic $Ca^{2+}$ concentration induced by 15 mM glucose. Obviously, this effect was due to inhibition of mitochondrial metabolism. T0901317 markedly depolarized the mitochondrial membrane potential, thus inhibiting adenosine triphosphate (ATP) production and reducing the cytosolic ATP concentration. This led in turn to a huge increase in $K_{ATP}$ current and hyperpolarization of the cell membrane potential. Eventually, T0901317 inhibited glucose-induced insulin secretion. These effects were rapid in on-set and not compatible with the activation of a nuclear receptor. In vivo, T0901317 acutely increased the blood glucose concentration after intraperitoneal application. In summary, these data clearly demonstrate that T0901317 exerts acute effects on stimulus-secretion coupling. This observation questions the chronic use of T0901317 and limits the interpretation of results obtained under these experimental conditions.

**Introduction**

The liver X receptor (LXR) is a member of the family of transcription factors and is closely related to nuclear receptors, such as the peroxisome proliferator-activated receptors, the farnesoid X receptor (FXR), and the retinoid X receptor. These receptors are important regulators of lipid and glucose homeostasis (1–3). Interestingly, in liver LXR acts as a glucose sensor (i.e., physiological glucose concentrations induce expression of LXR target genes with an efficacy similar to that of oxysterols, the known endogenous LXR ligands) (4). During the last years, it became evident that these receptors not only affect insulin sensitivity and glucose handling in peripheral organs like liver, but also affect β-cell function. LXRα and LXRβ are present in pancreatic islets and insulin-secreting MIN6 cells (5). As LXR belongs to a group of nuclear receptors, it is commonly expected that stimulation of LXR induces changes in gene expression. However, the consequences of LXR activation for β-cell function are still a matter of debate. This may be owing to the fact that in most studies chronic administration of the LXR ligand T0901317 in the micromolar range was chosen to activate the LXR. Efanov et al. (5) showed that 1 µM T0901317 enhanced glucose-induced insulin secretion of rat islets. However, an important effect of the compound was first seen at day 3 of administration. The stimulatory effect on insulin secretion was completely blocked by the pyruvate carboxylase inhibitor phenylacetic acid.

In MIN6 cells inhibition of glucose-induced insulin secretion was observed after 48 hours treatment with 2, 5, and 10 µM T0901317, respectively (6). Under these conditions, the authors found a small reduction in adenosine triphosphate (ATP) content of MIN6 cells and an increase in $K_{ATP}$ current. Inhibitory effects on glucose-induced insulin secretion by chronic T0901317 administration were also reported for HIT cells and mouse islets (7). Accordingly, the authors report that glucose tolerance is deteriorated in mice treated with T0901317 for 1 week. This contrasts with the finding that LXRβ-knockout (KO) mice show impaired glucose tolerance (8). Islets from these mice have reduced glucose-induced insulin secretion. Noteworthy, LXRα-KO mice and mice that lack both receptor subtypes are not glucose-intolerant. Another study reveals that chronic activation of LXR by 10 µM T0901317 in β-cells and INS-1 cells induces reactive oxygen species (ROS) and lipid accumulation leading to lipotoxicity and apoptosis (9). Liang et al. (10) showed that 10 µM

T0901317 enhanced palmitate-induced apoptosis in INS-1 cells. LXR is also present in human islets (11). In this study, it was shown that preincubation with 1 µM T0901317 increased glucose-induced insulin secretion of isolated human islets. The importance of LXR for human islets is also demonstrated by the fact that in subjects of European ancestry at increased risk for type 2 diabetes, a common variation within the *NR1H2* gene (encoding LXRβ) impaired insulin secretion, which may facilitate the development of type 2 diabetes (12). Taken together, the studies indicate a physiological significance of LXR in β-cells although a proof is missing that the effects observed with T0901317 are mediated by LXR activation. In this study, we tested T0901317 for acute alterations in β-cell function. According to our data it cannot be excluded that changes in gene expression in response to T0901317 treatment (6, 9, 10) are due to rapid ATP depletion (13, 14) and not primarily and/or exclusively caused by LXR activation.

**Materials and Methods**

*Cell and islet preparation.* The details are described in Gier et al. (15). Male and female C57Bl/6 mice were used in equal shares. The FXR-KO mice on a C57Bl/6 background were housed under same conditions. The mice were bred in the animal facility of the Department of Pharmacology at the University of Tübingen. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985; 16) and German laws were followed. Mouse islets were dispersed to single cells and cell clusters, respectively, by trypsin treatment. Human islets were obtained from two biopsies from children aged 6 and 11 months with focal congenital hyperinsulinism during pancreatic surgery according to the approvals of the ethic commission of the Universitätsmedizin Greifswald (BB 050/13). The children are carriers of mutations in the *ABCC8* gene, which encodes for the SUR1 subunit of the $K_{ATP}$ channel. The islets were taken from areas not affected by the mutations. They were isolated by injecting collagenase (2 to 4 mg/mL) into the biopsy material and by handpicking after digestion at 37°C. Human islets were cultured in a medium with 5.5 mM glucose.

*Solutions and chemicals.* Recordings of the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) were performed with a bath solution (bath 1), which contained (in millimolars) the following: 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, glucose as indicated, 10 HEPES, and pH 7.4 adjusted with sodium hydroxide solution (NaOH). The same bath solution was used for the determination of the mitochondrial membrane potential ($\Delta\Psi$), for the measurements on a microelectrode array (MEA), ROS measurements, and the cell-attached $K_{ATP}$ current recordings. For membrane potential (Vm) measurements in the perforated patch configuration [$Ca^{2+}$] in bath 1 was raised to 10 mM. Bath 1 was modified by adding 0.1% bovine serum albumin and used as incubation medium for ATP measurement. Krebs-Ringer-HEPES (KRH) solution for insulin secretion was composed of (in millimolars) the following: 120 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 2.5 $CaCl_2$, glucose as indicated, 10 HEPES, 0.5% bovine serum albumin, and pH 7.4 adjusted with NaOH. The control solution to register KATP channel activity in the inside-out configuration was composed of (in millimolars) the following: 130 KCl, 4.6 $CaCl_2$, 10 EDTA, 20 HEPES, and pH adjusted to 7.2 with potassium hydroxide solution (KOH). The pipette solution for cell-attached and inside-out recordings consisted of (in millimolars) the following: 130 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 20 HEPES, and pH adjusted to 7.4 with KOH. For membrane potential measurements with the perforated patch technique, the pipette solution was composed of (in millimolars) the following: 10 KCl, 10 NaCl, 70 $K_2SO4$, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 20 HEPES, 0.27 amphotericin B, and pH adjusted to 7.15 with KOH.

T0901317 and GW3965 were obtained from Biomol (Hamburg, Germany). Fura-2-AM was purchased from Biotrend (Köln, Germany) and 25-OH cholesterol from Santa Cruz (Heidelberg, Germany). Rhodamine 123 (Rh123), CMRL medium, RPMI 1640 medium, fetal calf serum, 2',7'-dichlorodihydrofluorescein-diacetate (DCDHF-DA), dihydroethidium (DHE), ATP determination kit, and penicillin/streptomycin were from Invitrogen (Karlsruhe, Germany). Collagenase used for human biopsy material was obtained from Serva (Heidelberg, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany) in the purest form available.

*Measurement of $[Ca^{2+}]_c$.* Details are described in Gier et al. (15). In brief, cells were loaded with 5 µM Fura-2-AM for 35 minutes at 37°C. Fluorescence was excited at 340 and 380 nm, and emission was filtered (LP515) and measured by a digital

camera. $[Ca^{2+}]_c$ was calculated according to an in vitro calibration. The maximum amplitude of $Ca^{2+}$ oscillations (max. $[Ca^{2+}]_c$) was taken to compare $[Ca^{2+}]_c$ under different experimental conditions.

*Measurements of ΔΨ.* ΔΨ was measured as Rh123 fluorescence at 480-nm excitation wavelength as described in Edalat et al. (17). To evaluate the effects, the values were averaged over 50 seconds at the end of each interval.

*ATP measurement.* After preparation, 20 islets were incubated for 30 minutes at 37°C in incubation medium containing either 15 mM glucose or 15 mM glucose and 30 µM T0901317. The incubation medium was removed, and islets were lysed in a solution containing 200 mM NaOH and 0.5 mM EDTA. Enzymes were inhibited by heat (60°C). The ATP concentration of the samples was determined on the same day luminometrically with a Luminometer 1253 (Bioorbit, Turku, Finland). Bioluminescence assays were performed in triplicate by adding 10 µL of test solution to 490 µL standard reaction solution, which was prepared according to the instructions of the manufacturer.

*Patch-clamp measurements.* Patch pipettes were pulled from borosilicate glass capillaries (Harvard Apparatus, March-Hugstetten, Germany). Membrane currents and potentials were recorded with an EPC-9 patch-clamp amplifier using "Patchmaster" software (HEKA, Lambrecht, Germany). As with T0901317, too many channels were open to resolve single channel open probability, so $K_{ATP}$ channel activity was determined by averaging the current over baseline for the last 30 seconds. For Vm measurements, the plateau potential in the control and wash-out phases as well as the most negative potential during the administration of T0901317 was measured. For inside-out measurements, $K_{ATP}$ currents were determined at a membrane potential of −50 mV. Single channel open probability was analyzed for the last 15 seconds.

*Measurements of electrical activity by the MEA technique.* Extracellular potential recordings were obtained with the MEA technique as described previously (18) using a MEA 2100 amplifier system and MC-Rack software (Multi Channel System, Reutlingen, Germany). An islet was placed on an electrode by means of a glass holding pipette (Reproline, Rheinbach, Germany) and a micromanipulator

(Scientifica, Uckfield, UK). For analysis, the fraction of plateau phase (FOPP ~ percentage of time with burst activity) was calculated by Jupyter Notebook using an IPython in house-made protocol, which analyzes the typical pattern of the extracellular potential. The FOPP was determined over 15 minutes at the end of each experimental maneuver.

*Insulin secretion.* Details for steady-state incubations are described in Gier et al. (15). Briefly, after preparation, islets were kept overnight in RPMI 1640 culture medium with 11.1 mM glucose. Before starting secretion experiments, islets were kept for 1 hour in KRH solution with 5.6 mM glucose and for further 30 minutes in a KRH solution with 3 mM glucose to silence metabolic activity. After this procedure, insulin in the supernatant was collected for 1 hour at 37°C under conditions as indicated. Levels of insulin were determined by radioimmunoassay (Merck Millipore, Darmstadt, Germany).

*In vivo application of T0901317.* For in vivo experiments, male C57Bl/6 mice at an age of 5 months were used. T0901317 dissolved in dimethyl sulfoxide was injected intraperitoneally (IP) at a concentration of 50 mg/kg body weight. Control mice received dimethyl sulfoxide. Blood glucose concentration (BGC) was determined directly before the injection and 5, 10, 20, 40, and 60 minutes thereafter. During the experiment, mice had free access to water and food.

*Measurement of ROS.* ROS production was measured by using the fluorescent dyes DCDHF-DA and DHE. In the cells, DCDHF-DA is oxidized to the fluorescent 2',7'-dichlorofluorescein (DCF), which detects H2O2 and other ROS species. DHE is oxidized to 2-hydroxyethidium (2-OH-$E^+$), which mainly indicates $O^{2-}$ formation. After 1-hour incubation under conditions as indicated, the clusters were loaded for 15 minutes with 20 µM DCDHF-DA and 10 µM DHE, respectively. Fluorescence was excited at 480 nm, and the intensity of the emitted light [arbitrary units (a.u.)] was measured.

*Statistics.* Each series of experiments was performed with islets or islet cells from at least three independent preparations. Means ± standard error of the mean are given for the indicated number of experiments (cells or islets). Statistical significance of differences was assessed by using Student t test. Multiple comparisons were made

by analysis of variance followed by Student-Newman-Keuls test. P values ≤ 0.05 were considered significant.

**Results**

*Effects of T0901317 on the cytosolic $Ca^{2+}$ concentration of β-cells*

T0901317 in the concentration of 10 µM completely suppressed oscillations of $[Ca^{2+}]_c$ elicited in the presence of 15 mM glucose [Fig. 1(a)]. Maximum $[Ca^{2+}]_c$ was reduced from 438 ± 66 nM under control conditions to 87 ± 18 nM in the presence of T0901317. Directly after removal of T0901317, $[Ca^{2+}]_c$ dropped to 53 ± 14 nM (asterisk). During the continued wash-out phase, it augmented to 427 ± 64 nM (arrowhead). Because FXR (*NR1H4*) and LXR (α *NR1H3* and β *NR1H2*) belong to a group of nuclear receptors with structural similarities and act on metabolism in a coordinated manner (19), we wanted to exclude that T0901317 affects the FXR. T0901317 had identical effects in β-cells of FXR-KO mice (Supplemental Fig. 1). Thus, the drug seems not to affect the FXR. Preincubation of wild-type islet cells with 1 µM of the sarco/endoplasmic reticulum $Ca^{2+}$-ATPase inhibitor thapsigargin suppressed the drop in $[Ca^{2+}]_c$ at the beginning of the wash-out phase [Fig. 1(c) and 1(d), asterisk] showing that the drop in $[Ca^{2+}]_c$ seen in Fig. 1(a) (asterisk) was due to ATP-dependent $Ca^{2+}$ sequestration into the endoplasmic reticulum (20). As mentioned earlier, LXR was found in human islets (11). Interestingly, T0901317 evoked identical effects on $[Ca^{2+}]_c$ of dispersed human β-cells with rapid suppression of $Ca^{2+}$ oscillations and a biphasic wash-out [Fig. 1(e) and (f)].

The reduction in $[Ca^{2+}]_c$ was not unique for the LXR ligand T0901317. Another synthetic LXR agonist, GW3965, and the endogenous LXR ligand 25-OH cholesterol yielded similar results leading to immediate decrease in maximal $[Ca^{2+}]_c$ (Supplemental Fig. 2).

*Effect of T0901317 on the ΔΨ of β-cells and ATP concentration of islets*

The ΔΨ is directly linked to ATP production (21). As shown in Fig. 2(a) a rise in the glucose concentration that causes an immediate increase in ATP production is accompanied by a hyperpolarization of ΔΨ indicated by a decrease of the fluorescence signal. The fluorescence signal was lowered from 1501 ± 71 a.u. in the

presence of 0.5 mM glucose to 1283 ± 59 a.u. upon an increase of the glucose concentration to 15 mM. A concentration of 10 µM T0901317 strongly and reversibly depolarized ΔΨ to 1511 ± 75 a.u. After wash-out of the compound ΔΨ repolarized again to 1282 ± 60 a.u. [Fig. 2(b)]. Accordingly, the ATP concentration of islets decreased from 3.3 ± 0.1 pmol/islet in the presence of 15 mM glucose to 2.0 ± 0.2 pmol/islet after addition of 30 µM T0901317 [Fig. 2(c)]. In contrast to the measurements with dispersed islet cells or clusters of cells, these measurements with whole islets, which are encircled by a capsule of connective tissue, were performed with 30 µM T0901317. This capsule may reduce the diffusion of T0901317 (see also MEA measurements and insulin secretion).

*Influence of T0901317 on $K_{ATP}$ current and electrical activity*

As T0901317 influences ΔΨ and thus ATP concentration, it should also affect the $K_{ATP}$ current and the membrane potential of β-cells. Figure 3(a) shows an experiment recorded in the cell-attached configuration. An increase of the glucose concentration from 0.5 to 15 mM was accompanied by a complete block of $K_{ATP}$ channels and action potentials were elicited as indicated by small capacitive artifacts (arrow). The $K_{ATP}$ current was reduced from 1.56 ± 0.25 pA to 0 ± 0.02 pA. Treatment of the cells with T0901317 led to an enormous rise in the $K_{ATP}$ current to 12.81 ± 3.35 pA. Addition of the sulfonylurea tolbutamide almost completely inhibited the current (0.41 ± 0.28 pA) identifying it as current through $K_{ATP}$ channels [summarized in Fig. 3(b)]. In excised patches (inside/out configuration), ATP dramatically reduced $K_{ATP}$ channel activity to almost zero [Fig. 3(c) and summary in Fig. 3(d)]. T0901317 was unable to reopen the channels under this condition showing that the effect of T0901317 is dependent on intact cell metabolism. According to the observations on $K_{ATP}$ current in the cell-attached configuration, T0901317 markedly hyperpolarized Vm from the plateau potential (potential from which action potentials start) in 10 mM glucose of −26 ± 2 mV to −57 ± 5 mV [Fig. 3(e) and 3(f)]. After wash-out of the drug the plateau, potential amounted to −24 ± 2 mV. Similar results were obtained when electrical activity was measured with whole islets on an MEA (Fig. 4). The FOPP was diminished from 64% ± 9% in the presence of 10 mM glucose to 24% ± 6% after addition of 10 µM T0901317 [Fig. 4(a) and (b)]. In 0.5 mM glucose that was used to prove the metabolic integrity of the islets, no electrical activity appeared. A higher concentration of 30 µM T0901317 was necessary to almost completely suppress

electrical activity [Fig. 4(c) and (d)] because MEA measurements were conducted with whole islets.

*Effect of T0901317 on insulin secretion and BGC*

Insulin secretion markedly raised upon an increase of the glucose concentration from substimulatory to stimulatory concentration [0.14 ± 0.03 ng insulin/(islet*h) in 3 mM glucose versus 5.87 ± 0.80 ng insulin/(islet*h) in 15 mM glucose]. Application of 10 and 30 µM T0901317 reduced insulin secretion to 3.73 ± 0.71 ng insulin/(islet*h) and 1.43 ± 0.29 ng insulin/(islet*h), respectively [Fig. 5(a)]. As in the MEA experiments, in secretion studies whole islets were used. Accordingly, 10 µM T0901317 were not as effective as in the experiments performed with dispersed β-cells. To evaluate whether the in vitro observations of acute effects of T0901317 are valid for the situation in vivo, BGC was determined. T0901317 increased BGC after IP injection [Fig. 5(b)]. A tendency of a rise in BGC was already observed within the first 10 minutes after injection of the drug. A significant effect was detected after 20 minutes.

*Effect of T0901317 on ROS*

To further elucidate the mechanisms underlying the T0901317-induced impairment of insulin secretion, ROS formation was determined. DCF fluorescence mainly detects $H_2O_2$ production (and other ROS), and 2-OH-$E^+$ fluorescence detects $O^{2-}$ formation. An increase of the glucose concentration markedly increased DCF fluorescence from 48 ± 3 to 969 ± 67 a.u. [Fig. 6(a)]. T0901317 decreased DCF fluorescence to similar values as obtained at 0.5 mM glucose (46 ± 5 a.u.) showing that T0901317 counteracted the glucose-induced increase in ROS. 2-OH-$E^+$ fluorescence was strongly diminished by a rise in the glucose concentration from 169 ± 4 to 34 ± 1 a.u. [Fig. 6(b)]. T0901317 slightly augmented 2-OH-E+ fluorescence under this condition to 41 ± 2 a.u.

**Discussion**

T0901317 has been used in a variety of in vivo and in vitro studies to evaluate its effects on glucose and lipid homeostasis in peripheral tissues and pancreatic β-cells. The results are at least in part contradictory, which may be due to different concentrations of T0901317, the use of different cell systems (primary human and

murine β-cells, different insulin-secreting tumor cell lines), and animal models (wild-type mice, diabetes-prone mouse models, high fat diet-induced diabetes, and streptozotocin-induced diabetes). Consistently, the effects have been attributed to LXR activation. Likewise, T0901317 was administered chronically in all studies because there was a consensus that LXR activation by T0901317 resulted in transcriptional effects, which need hours to days to become evident. However, proof is still lacking that all effects observed in response to T0901317 administration are due to changes in gene expression. We have now demonstrated that T0901317 induces rapid changes in various parameters of stimulus-secretion coupling (SSC) in mouse and human β-cells. These changes in SSC are not consistent with genomic effects mediated by transcription factors because they occur within minutes. Moreover, the good and rapid reversibility of the effects speaks against transcriptional pathways. In addition, the in vivo data, which show a substantial rise in the BGC already 20 minutes after IP injection of T0901317, support the idea of a nongenomic effect of T0901317 on insulin secretion in β-cells.

Both, LXR and FXR are encoded by the *NR1H* gene subfamily of nuclear receptors and thus exhibit high homology resulting in numerous commonalities on the protein level. A recent paper describes these coordinated actions of both receptors in metabolism (19). In principle, activation of a nuclear receptor can induce rapid nongenomic changes in SSC of β-cells. Recently, we have shown this for the FXR (2). Activation of FXR by bile acids or the synthetic ligand GW4064 leads to enhanced insulin secretion via a rapid effect on $K_{ATP}$ channels and subsequent increase of $[Ca^{2+}]_c$. FXR does not directly affect $K_{ATP}$ channels but by a still unknown cellular factor. In the case of FXR, we could prove that the rapid effects on SSC were mediated by FXR activation because they were blunted in islets of FXR-KO mice. FXR activation results in opposite effects in lean and obese mice. In lean mice, glucose tolerance was impaired by FXR deletion, which fits to our observations with isolated islets (22–25). Surprisingly, glucose tolerance was improved in obese mice (22, 26). Based on the observation of other groups and our own, we hypothesized that this difference between lean and obese mice is caused by an obesity-mediated translocation of the β-cell FXR from the cytosol to the nucleus (22, 27). Noteworthy, a translocation between the cellular compartments is also described for the LXR. Glucose regulates the subcellular distribution of the LXRα in insulin-secreting INS-1

cells (28). At low glucose, LXRα is localized in the cytosol and it is translocated into the nucleus at stimulatory glucose concentration. However, the significance of this observation for β-cell function remains unclear.

Overall, acute administration of the LXR ligand T0901317 to β-cells leads to rapid changes in SSC resulting in inhibition of insulin secretion. These effects are nongenomic, but nevertheless the LXR is presumably involved because two other LXR ligands cause similar acute effects on $[Ca^{2+}]_c$ as T0901317. The rapid reversibility of the T0901317 action and the fact that two other LXR agonists evoke similar effects on $[Ca^{2+}]_c$ speak against an unspecific toxic effect of the drug on β-cells.

Importantly, 10 µM T0901317 that we used for acute application was taken in many studies dealing with consequences of chronic LXR activation on glucose and lipid metabolism (6, 9, 10). It has been postulated that T0901317 also activates the FXR (29) and acts as a reverse agonist at a novel retinoic acid receptor-related orphan receptor-α/γ (RORα and RORγ) (30). According to our observations, FXR activation by T0901317 seems unlikely. Moreover, it would increase and not decrease insulin secretion.

Our data strongly suggest that a primary mode of action of T0901317 is interference with the mitochondrial metabolism: (1) T0901317 strongly and reversibly depolarized ΔΨ. (2) Accordingly, T0901317 decreased the cytosolic ATP concentration. (3) Wash-out of T0901317 led to a drop in $[Ca^{2+}]_c$, which is due to ATP-dependent sarco/endoplasmic reticulum $Ca^{2+}$-ATPase activation. (4) In patch-clamp experiments, opening of $K_{ATP}$ channels by T0901317 is only found in the cell-attached but not in the excised-patch configuration. These data exclude a direct effect of the drug on $K_{ATP}$ channels but rather point to an indirect metabolism-mediated effect. In conclusion, the T0901317-induced depolarization of ΔΨ leads to opening of $K_{ATP}$ channels due to ATP depletion, which in turn reduces $Ca^{2+}$ influx and insulin secretion (21). One should also consider in this context that ATP depletion itself can induce changes in gene expression (e.g., via AMPK activation) (13, 14). Changes in gene expression after T0901317 treatment are described in a variety of papers (6, 9, 10, 31–34) and have always been taken as an argument for genomic effects of LXR activation. Besides its effect on ATP production, T0901317 interferes

with ROS formation, which may also be attributed to the T0901317-induced disturbance of mitochondrial metabolism because $Ca^{2+}$ homeostasis, ATP production, ROS formation, and mitochondria are tightly connected (35). Thus, ROS imbalance may affect mitochondria and finally ΔΨ. T0901317 completely suppressed the glucose-evoked increase in DCF fluorescence. ROS production (36) especially $H_2O_2$ (37) seems to be an important signal for glucose-induced insulin secretion. Lack of this effect may contribute to the inhibitory action of T0901317 on insulin secretion.

It is extremely important to understand the action of T0901317 because fundamental although contradictory conclusions have been drawn from studies with chronic application of this drug: (1) On the one hand, it has been reported that T0901317 leads to improvement of insulin sensitivity (38–40), protection against high fat diet-induced (41) as well as streptozotocin-induced (42) diabetes and increased insulin secretion (11). These studies recommend the development of LXR agonists for clinical use. (2) On the other hand, it has been demonstrated that T0901317 results in β-cell dysfunction via lipid accumulation (6, 9, 43). For valid interpretation of the results, future studies should take into account that T0901317 can exert acute effects of nongenomic nature. Moreover, LXR-KO mice are available (44) and should be used in the future, if possible, to discriminate between LXR-dependent and -independent effects of T0901317.

**Disclosure Summary:** The authors have nothing to disclose

**Author contributions:** J.M., J.S., and C.B. researched data; P.K.-D. evaluated data and edited the manuscript; U.L., C.W., and W.B. contributed to discussion and study design and edited the manuscript; G.D. designed the study, wrote and edited the manuscript, and contributed to discussion.

**Acknowledgments:** We thank Isolde Breuning for her excellent and skillful technical assistance. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (DFG) (to G.D.).

**References**

1. Sugden MC, Holness MJ. Role of nuclear receptors in the modulation of insulin secretion in lipid-induced insulin resistance.Biochem Soc Trans. 2008;36(Pt 5):891–900.

2. Düfer M, Hörth K, Wagner R, Schittenhelm B, Prowald S, WagnerTF, Oberwinkler J, Lukowski R, Gonzalez FJ, Krippeit-Drews P,Drews G. Bile acids acutely stimulate insulin secretion of mouseb-cells via farnesoid X receptor activation and K(ATP) channelinhibition.Diabetes. 2012;61(6):1479–1489.

3. Chertow BS, Blaner WS, Rajan N, Primerano DA, Meda P, CirulliV, Krozowski Z, Smith R, Cordle MB. Retinoic acid receptor,cytosolic retinol-binding and retinoic acid-binding protein mRNAtranscripts and proteins in rat insulin-secreting cells.Diabetes.1993;42(8):1109–1114.

4. Mitro N, Mak PA, Vargas L, Godio C, Hampton E, Molteni V,Kreusch A, Saez E. The nuclear receptor LXR is a glucose sensor.*Nature*. 2007;445(7124):219–223.

5. Efanov AM, Sewing S, Bokvist K, Gromada J. Liver X receptor activation stimulates insulin secretion via modulation of glucose and lipid metabolism in pancreatic beta-cells.*Diabetes*. 2004;53(Suppl 3):S75–S78.

6. Meng ZX, Yin Y, Lv JH, Sha M, Lin Y, Gao L, Zhu YX, Sun YJ,Han X. Aberrant activation of liver X receptors impairs pancreaticbeta cell function through upregulation of sterol regulatory element-binding protein 1c in mouse islets and rodent cell lines.*Diabetologia*. 2012;55(6):1733–1744.

7. Meng ZX, Nie J, Ling JJ, Sun JX, Zhu YX, Gao L, Lv JH, Zhu DY,Sun YJ, Han X. Activation of liver X receptors inhibits pancreatic islet beta cell proliferation through cell cycle arrest.*Diabetologia*.2009;52(1):125–135.

8. Gerin I, Dolinsky VW, Shackman JG, Kennedy RT, Chiang SH, Burant CF, Steffensen KR, Gustafsson JA, MacDougald OA. LXR beta is required for adipocyte growth, glucose homeostasis, and beta cell function.*J Biol Chem*. 2005;280(24):23024–23031.

9. Choe SS, Choi AH, Lee JW, Kim KH, Chung JJ, Park J, Lee KM,Park KG, Lee IK, Kim JB. Chronic activation of liver X receptor induces beta-cell apoptosis through hyperactivation of lipogenesis: liver X receptor-mediated lipotoxicity in pancreatic beta-cells.*Diabetes*. 2007;56(6):1534–1543.

10. Liang H, Zhong Y, Zhou S, Li QQ. Palmitic acid-induced apoptosisin pancreatic b-cells is increased by liver X receptor agonist and attenuated by eicosapentaenoate.*In Vivo*. 2011;25(5):711–718.

11. Ogihara T, Chuang JC, Vestermark GL, Garmey JC, Ketchum RJ,Huang X, Brayman KL, Thorner MO, Repa JJ, Mirmira RG,Evans-Molina C. Liver X receptor agonists augment human islet function through activation of anaplerotic pathways and glycerolipid/free fatty acid cycling.*J Biol Chem.* 2010;285(8):5392–5404.

12. Ketterer C, M¨ussig K, Machicao F, Stefan N, Fritsche A, H¨aringHU, Staiger H. Genetic variation within the NR1H2 gene encoding liver X receptor b associates with insulin secretion in subjects at increased risk for type 2 diabetes.*J Mol Med* (Berl). 2011;89(1):75–81.

13. Hardie DG, Scott JW, Pan DA, Hudson ER. Management of cellular energy by the AMP-activated protein kinase system.*FEBS Lett*. 2003;546(1):113–120.

14. Crawford PA, Schaffer JE. Metabolic stress in the myocardium: adaptations of gene expression. *J Mol Cell Cardiol*. 2013;55:130–138.

15. Gier B, Krippeit-Drews P, Sheiko T, Aguilar-Bryan L, Bryan J,Düfer M, Drews G. Suppression of $K_{ATP}$ channel activity protects murine pancreatic beta cells against oxidative stress.*J Clin Invest*.2009;119(11):3246–3256.

16. National Research Council.Guide for the Care and Use of Laboratory Animals. Washington, DC: National Academies Press;1985. NIH publication no. 85-23.

17. Edalat A, Schulte-Mecklenbeck P, Bauer C, Undank S, Krippeit-Drews P, Drews G, Düfer M. Mitochondrial succinate dehydrogenaseis involved in stimulus-secretion coupling and endogenous ROS formation in murine beta cells.*Diabetologia*. 2015;58(7):1532–1541.

18. Pfeiffer T, Kraushaar U, Düfer M, Schönecker S, Haspel D, Günther E ,Drews G, Krippeit-Drews P. Rapid functional evaluation of beta-cells by extracellular recording of membrane potential oscillations with microelectrode arrays. *Pflugers Arch*. 2011;462(6):835–840.

19. Ding L, Pang S, Sun Y, Tian Y, Yu L, Dang N. Coordinated actions of FXR and LXR in metabolism: from pathogenesis to pharmacological targets for type 2 diabetes.*Int J Endocrinol*.2014;2014:751859.

20. Grapengiesser E, Gylfe E, Hellman B. Dual effect of glucose on cytoplasmic $Ca^{2+}$ in single pancreatic beta-cells.*Biochem BiophysRes Commun*. 1988;150(1):419–425.

21. Drews G, Bauer C, Edalat A, Düfer M, Krippeit-Drews P. Evidence against a $Ca(^{2+})$-induced potentiation of dehydrogenase activity in pancreatic beta-cells.*Pflugers Arch*. 2015;467(11):2389–2397.

22. Schittenhelm B, Wagner R, Kähny V, Peter A, Krippeit-Drews P,Düfer M, Drews G. Role of FXR in b-cells of lean and obese mice.*Endocrinology*. 2015;156(4):1263–1271.

23. Zhang Y, Lee FY, Barrera G, Lee H, Vales C, Gonzalez FJ, WillsonTM, Edwards PA. Activation of the nuclear receptor FXR improves hyperglycemia and hyperlipidemia in diabetic mice.*Proc Natl Acad Sci USA*. 2006;103(4):1006–1011.

24. Cariou B, van Harmelen K, Duran-Sandoval D, van Dijk TH,Grefhorst A, Abdelkarim M, Caron S, Torpier G, Fruchart JC,Gonzalez FJ, Kuipers F, Staels B. The farnesoid X receptor modulates adiposity and peripheral insulin sensitivity in mice.*J BiolChem*. 2006;281(16):11039–11049.

25. Ma K, Saha PK, Chan L, Moore DD. Farnesoid X receptor is essentialfor normal glucose homeostasis.*J Clin Invest*. 2006;116(4):1102–1109.

26. Prawitt J, Abdelkarim M, Stroeve JH, Popescu I, Duez H, Vela-gapudi VR, Dumont J, Bouchaert E, van Dijk TH, Lucas A,Dorchies E, Daoudi M, Lestavel S, Gonzalez FJ, Oresic M, CariouB, Kuipers F, Caron S, Staels B. Farnesoid X receptor deficiency improves glucose homeostasis in mouse models of obesity.*Diabetes*. 2011;60(7):1861–1871.

27. Popescu IR, Helleboid-Chapman A, Lucas A, Vandewalle B,Dumont J, Bouchaert E, Derudas B, Kerr-Conte J, Caron S, PattouF, Staels B. The nuclear receptor FXR is expressed in pancreatic beta-cells and protects human islets from lipotoxicity.*FEBS Lett*.2010;584(13):2845–2851.

28. Helleboid-Chapman A, Helleboid S, Jakel H, Timmerman C,Sergheraert C, Pattou F, Fruchart-Najib J, Fruchart JC. Glucoseregulates LXR alpha subcellular localization and function in rat pancreatic beta-cells.*Cell Res*. 2006;16(7):661–670.

29. Houck KA, Borchert KM, Hepler CD, Thomas JS, Bramlett KS, Michael LF, Burris TP. T0901317 is a dual LXR/FXR agonist. *MolGenet Metab*. 2004;83(1-2):184–187.

30. Kumar N, Solt LA, Conkright JJ, Wang Y, Istrate MA, Busby SA,Garcia-Ordonez RD, Burris TP, Griffin PR. The benzenesulfoamide T0901317 [N-(2,2,2-trifluoroethyl)-N-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide] is a novel retinoic acid receptor-related orphan receptor-alpha/gamma inverse agonist. *Mol Pharmacol.* 2010;77(2):228–236.

31. Høgmoen Åstrand OA, Gikling I, Sylte I, Rustan AC, Thoresen GH,Rongved P, Kase ET. Development of new LXR modulators that regulate LXR target genes and reduce lipogenesis in human cell models*. Eur J Med Chem*. 2014;74:258–263.

32. Hansmannel F, Mordier S, Iynedjian PB. Insulin induction of glucokinase and fatty acid synthase in hepatocytes: analysis of the roles of sterol-regulatory-element-binding protein-1c and liver X receptor.*Biochem J*. 2006;399(2):275–283.

33. Talukdar S, Bhatnagar S, Dridi S, Hillgartner FB. Chenodeoxycholic acid suppresses the activation of acetyl-coenzyme Acarboxylase-alpha gene transcription by the liver X receptor agonist T0-901317. *J Lipid Res*. 2007;48(12):2647–2663.

34. Zhu C, Di D, Zhang X, Luo G, Wang Z, Wei J, Shi Y, Berggren-Söderlund M, Nilsson-Ehle P, Xu N. TO901317 regulating apo-lipoprotein M expression mediates via the farnesoid X receptor pathway in Caco-2 cells. *Lipids Health Dis.* 2011;10:199.

35. Brookes PS, Yoon Y, Robotham JL, Anders MW, Sheu SS. Calcium, ATP, and ROS: a mitochondrial love-hate triangle. *Am J PhysiolCell Physiol.* 2004;287(4):C817–C833.

36. Leloup C, Tourrel-Cuzin C, Magnan C, Karaca M, Castel J,Carneiro L, Colombani AL, Ktorza A, Casteilla L, Penicaud L. Mitochondrial reactive oxygen species are obligatory signals for glucose-induced insulin secretion. *Diabetes.* 2009;58(3):673–681.

37. Pi J, Bai Y, Zhang Q, Wong V, Floering LM, Daniel K, Reece JM,Deeney JT, Andersen ME, Corkey BE, Collins S. Reactive oxygen species as a signal in glucose-stimulated insulin secretion.*Diabetes*.2007;56(7):1783–1791.

38. Dong Y, Gao G, Fan H, Li S, Li X, Liu W. Activation of the liver Xreceptor by agonist TO901317 improves hepatic insulin resistance via suppressing reactive oxygen species and JNK pathway.*PloS One*. 2015;10(4):e0124778.

39. Cao G, Liang Y, Broderick CL, Oldham BA, Beyer TP, Schmidt RJ,Zhang Y, Stayrook KR, Suen C, Otto KA, Miller AR, Dai J,Foxworthy P, Gao H, Ryan TP, Jiang XC, Burris TP, Eacho PI,Etgen GJ. Antidiabetic action of a liver x receptor agonist mediated by inhibition of hepatic gluconeogenesis.*J Biol Chem*. 2003;278(2):1131–1136.

40. Baranowski M, Zabielski P, Błachnio-Zabielska AU, Harasim E,Chabowski A, G´orski J. Insulin-sensitizing effect of LXR agonistT0901317 in high-fat fed rats is associated with restored muscle GLUT4 expression and insulin-stimulated AS160 phosphorylation.*Cell Physiol Biochem*. 2014;33(4):1047–1057.

41. Gao M, Liu D. The liver X receptor agonist T0901317 protects mice from high fat diet-induced obesity and insulin resistance.*AAPS J*. 2013;15(1):258–266.

42. Gao M, Zhang C, Ma Y, Liu D. Cold exposure improves the antidiabetic effect of T0901317 in streptozotocin-induced diabetic mice.*AAPS J*. 2015;17(3):700–710.

43. Chisholm JW, Hong J, Mills SA, Lawn RM. The LXR ligand T0901317 induces severe lipogenesis in the db/db diabetic mouse.*J Lipid Res*. 2003;44(11):2039–2048.

44. Steffensen KR, Gustafsson JA. Putative metabolic effects of the liverX receptor (LXR).*Diabetes*. 2004;53(Suppl 1):S36–S4

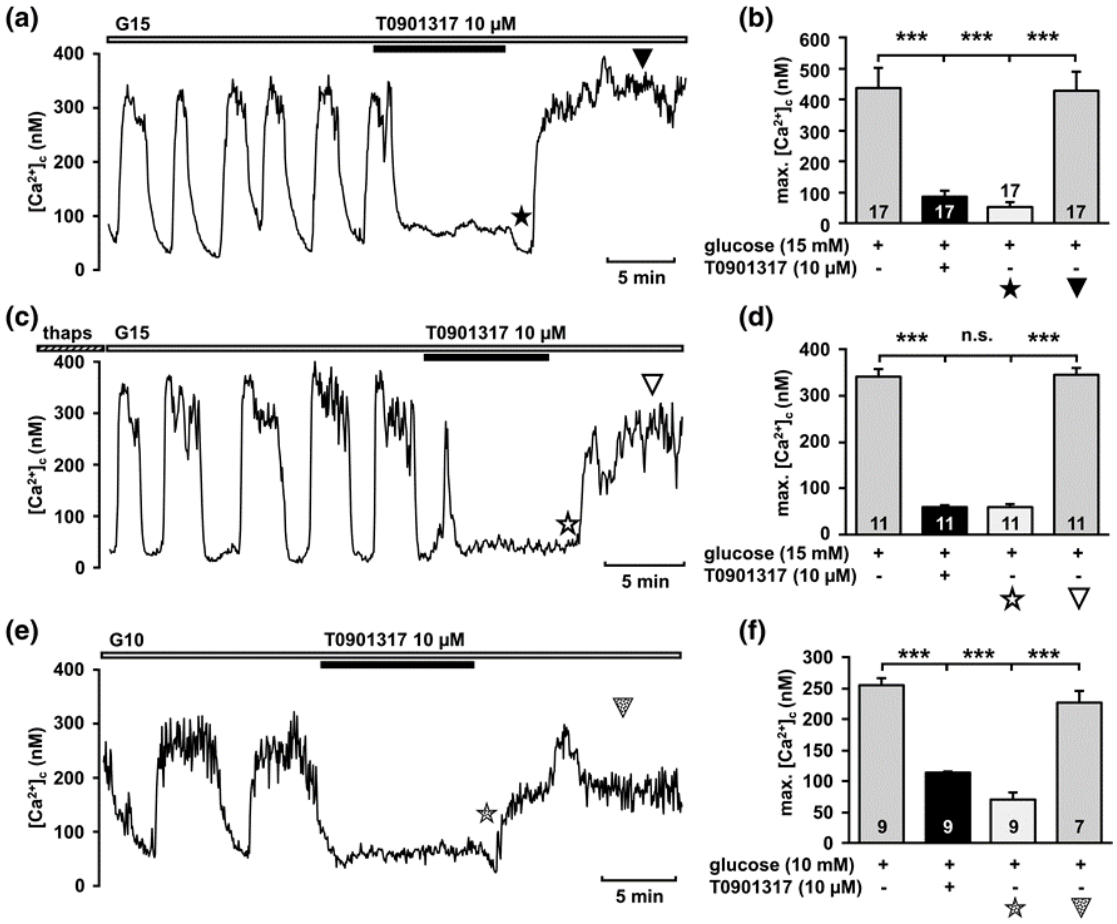



Fig. 1: Effects of 10 µM T0901317 on $[Ca^{2+}]_c$ in the presence of 15 and 10 mM glucose, respectively, in mouse and human β-cells. (a) Representative measurement showing inhibition of glucose-induced oscillations of $[Ca^{2+}]_c$ by T0901317 in a mouse β-cell. Note the drop in $[Ca^{2+}]_c$ after wash-out of T0901317 (asterisk), followed by elevated $[Ca^{2+}]_c$ (arrowhead). (b) Summary of all experiments. The number in the columns gives the number of experiments with different cell clusters from three different mice. This applies to all figures except Fig. 1(f), Fig. 2(c), and Fig. 5. (c, d) Effect of T0901317 after 30 minutes preincubation with thapsigargin [(thaps) 1 µM]. Preincubation with thapsigargin abolished the transient drop occurring after wash-out of T0901317 (open asterisk). (e, f) Effect of T0901317 on glucose-induced oscillations of $[Ca^{2+}]_c$ in human β-cells. ***P ≤ 0.001.

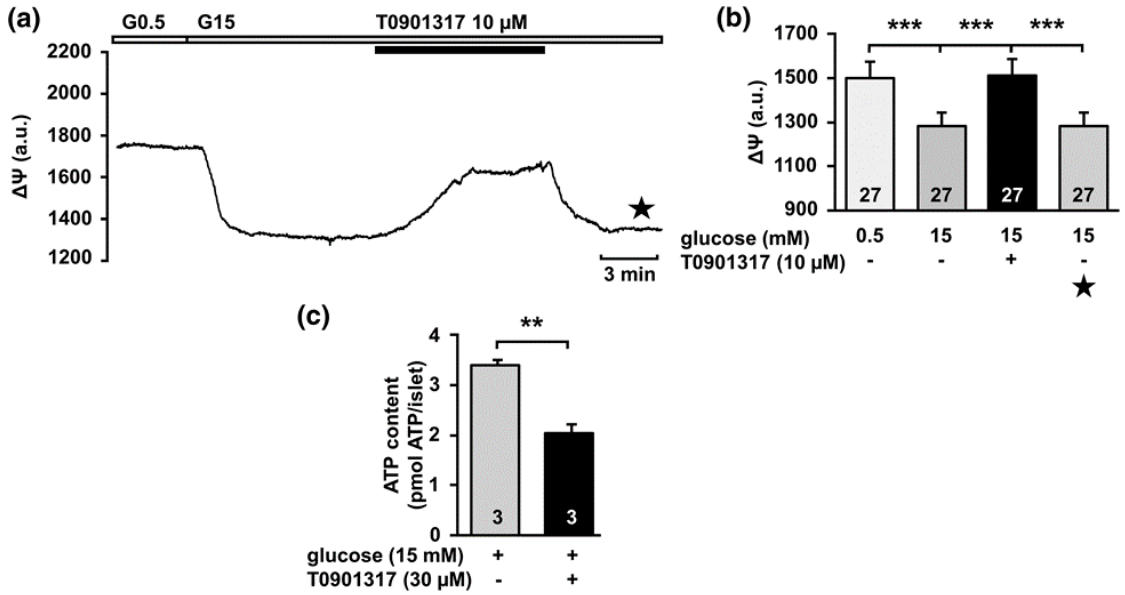



Fig. 2: Influence of T0901317 on ΔΨ in mouse β-cells and ATP concentration of islets in the presence of 15 mM glucose. (a) Representative measurement of ΔΨ. Increasing the glucose concentration from 0.5 to 15 mM led to hyperpolarization of ΔΨ due to enhanced ATP production. Administration of T0901317 (10 µM) resulted in a rapid and reversible depolarization of ΔΨ. (b) Summary of all experiments of this series. (c) Measurement of ATP concentration of intact mouse islets. ATP content of islets was significantly reduced by T0901317 in comparison with control islets kept in 15 mM glucose. The number in the columns gives the number of mice. **P ≤ 0.01; ***P ≤ 0.001.

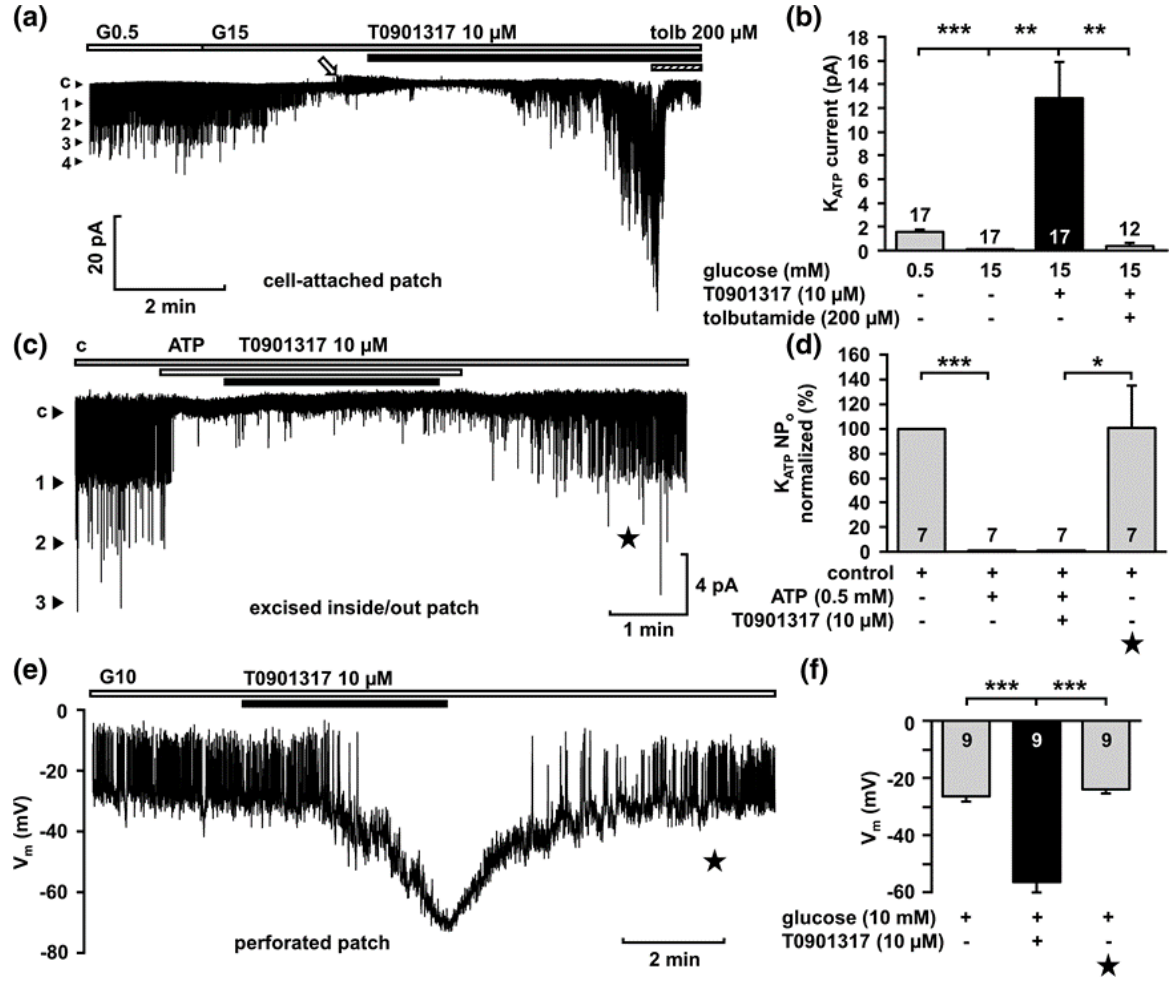



Fig. 3: Ten micromolars T0901317 affects $K_{ATP}$ current and membrane potential of mouse β-cells. (a) Representative experiment of $K_{ATP}$ current measured in the cell-attached configuration of the patch-clamp technique. Administration of T0901317 led to opening of $K_{ATP}$ channels in the presence of a glucose concentration of 15 mM that entirely inhibited $K_{ATP}$ channel current. The arrow marks the occurrence of artifacts due to action potentials, which are provoked by the strong depolarization caused by 15 mM glucose. The current evoked by T0901317 was inhibited by the specific $K_{ATP}$ channel blocker tolbutamide [(tolb) 200 µM]. (b) Summary of all experiments of this series. (c, d) Ten micromolars T0901317 did not affect $K_{ATP}$ channel activity in excised patches. (c) Representative measurement of $K_{ATP}$ current in the inside-out configuration of the patch-clamp technique. ATP (0.5 mM) led to $K_{ATP}$ channel closure, which was not affected by additional administration of T0901317 (10 µM). (d) The open probability (NPo) of the $K_{ATP}$ channel was determined. Control values were normalized. Summary of all experiments of this series. (e) Representative recording of Vm measured with the perforated patch technique. Administration of T0901317 caused immediate hyperpolarization of Vm. (f) Summary of all experiments. *P ≤ 0.05; **P ≤ 0.01; ***P ≤ 0.001.

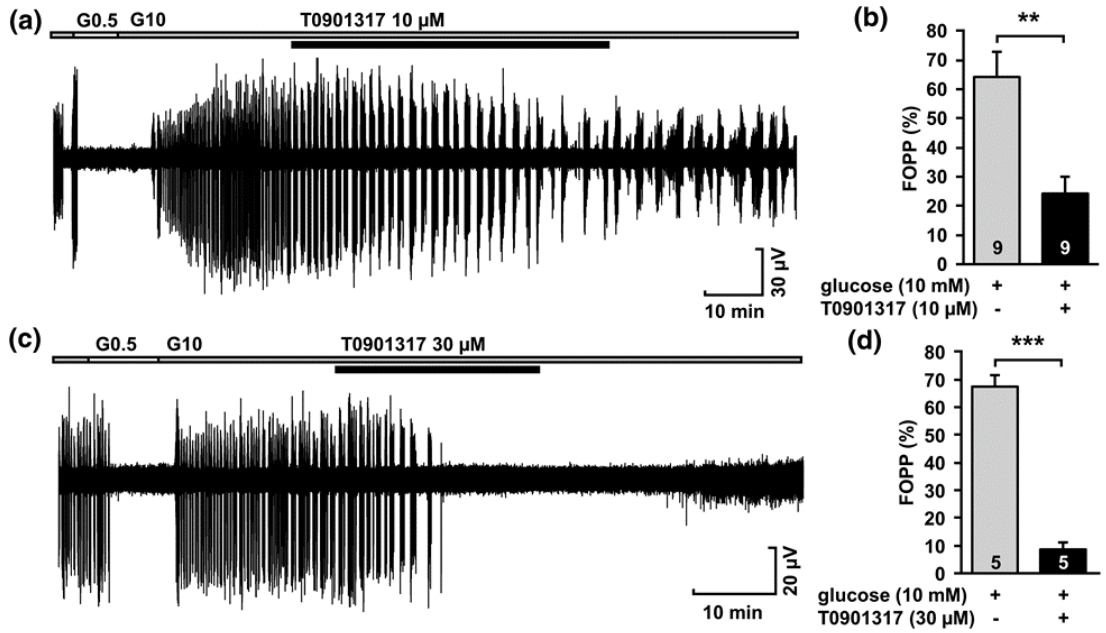



Fig. 4: T0901317 reduces electrical activity of intact mouse islets. (a) Representative registration of electrical activity of an islet placed on an MEA. Administration of 10 µM T0901317 decreased electrical activity and changed the typical pattern of bursts and interbursts. (b) Summary of the FOPP of all experiments of this series. (c, d) Increasing the T0901317 concentration to 30 µM led to almost complete inhibition of the electrical activity. **P ≤ 0.01; ***P ≤ 0.001.

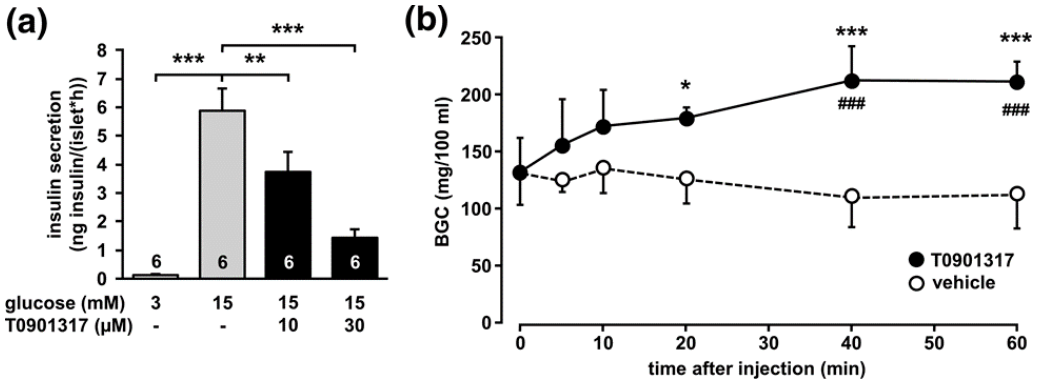



Fig. 5: Acute administration of T0901317 impairs insulin secretion of intact mouse islets and enhances the BGC. (a) Glucose-induced insulin secretion was reduced by T0901317 (10 and 30 µM) compared with control islets in the presence of 15 mM glucose. Metabolic integrity is shown by increased insulin secretion at stimulating glucose concentration (15 mM) compared with a substimulatory concentration (3 mM). The number in the columns gives the number of mice. **P ≤ 0.01; ***P ≤ 0.001. (b) BGC before (0 minutes) and after injection of T0901317 (50 mg/kg body weight) and vehicle, respectively. Four mice were included in each group. *P ≤ 0.05; ***P ≤ 0.001; T0901317 application versus value before drug application (0 minutes). ###P ≤ 0.001; T0901317 application versus solvent application.

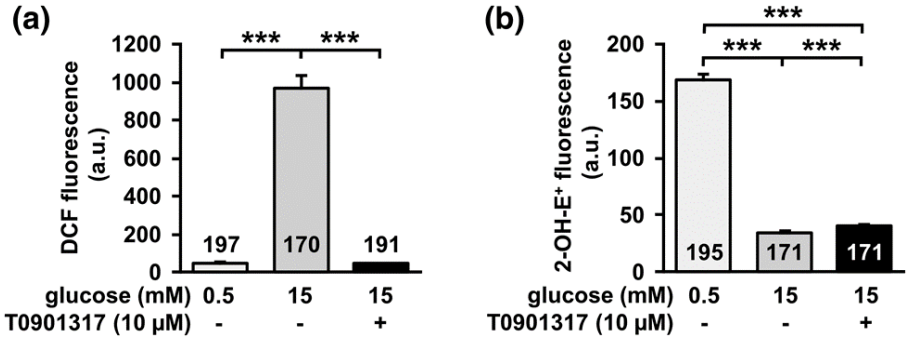
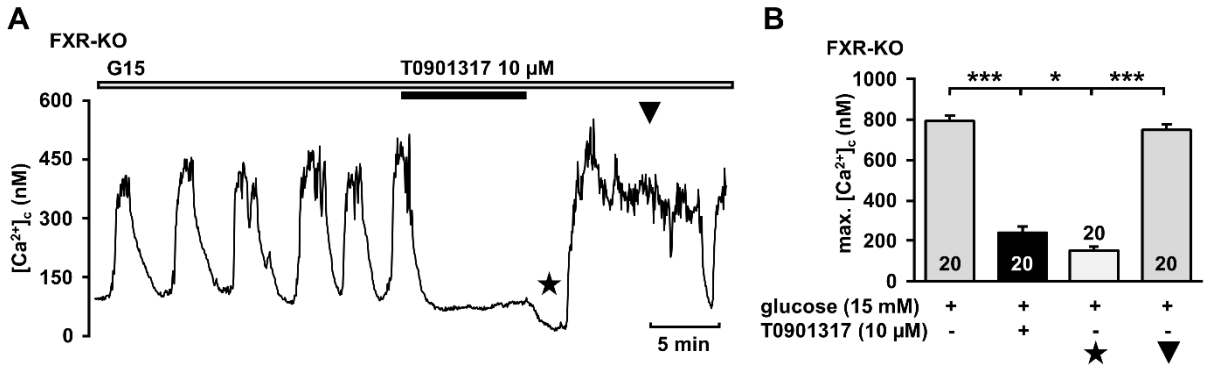
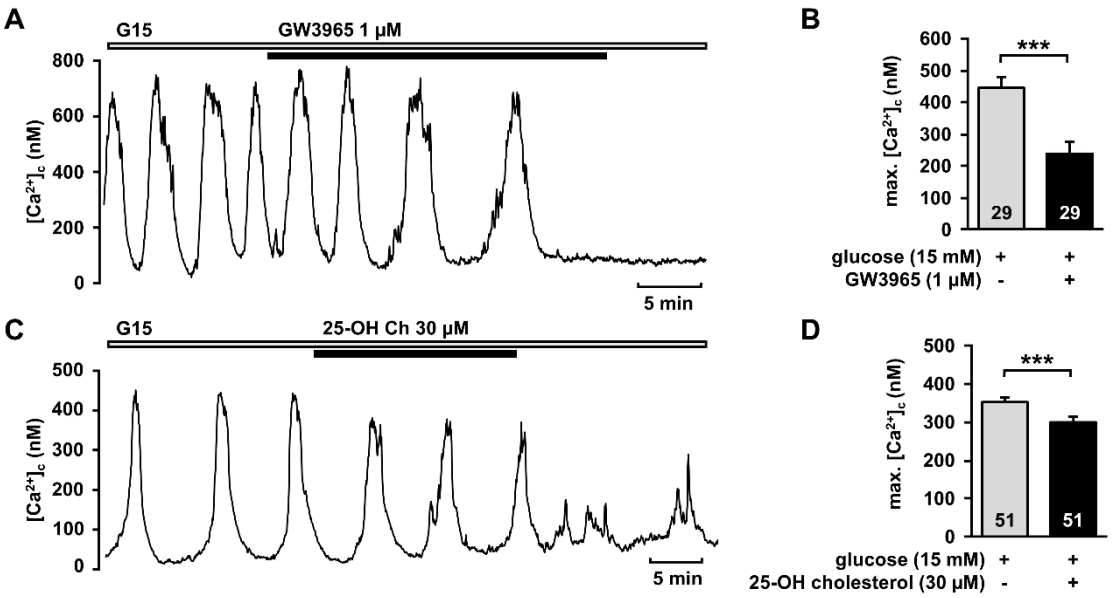



Fig. 6: Influence of T0901317 on ROS in the presence of 15 mM glucose. (a) Measurement of DCDHF-detectable oxidants. Increasing the glucose concentration from 0.5 to 15 mM led to enhanced fluorescence (mainly $H_2O_2$ production). Administration of T0901317 (10 µM) to 15 mM glucose resulted in a massive reduction of DCDHF-detectable ROS to the level of 0.5 mM glucose. (b) Determination of 2-OH-$E^+$-detectable oxidants (mainly $O_2^-$). Increasing the glucose concentration from 0.5 to 15 mM reduced the fluorescence. T0901317 (10 µM) led to a small increase of 2-OH-$E^+$-detectable ROS at high glucose concentration. ***P ≤ 0.001.




Supp. 1: Effects of 10 µM T0901317 on $[Ca^{2+}]_c$ in the presence of 15 mM glucose in beta-cells of FXR-KO mice. A) Representative measurement showing inhibition of glucose-induced oscillations of $[Ca^{2+}]_c$ by T0901317. Note the drop in $[Ca^{2+}]_c$ after wash-out of T0901317 (asterisk). B) Summary of all experiments. The number in the coluns gives the number of experiments with different cell clusters from 3 different mice. *P ≤ 0.05, ***P ≤ 0.001.




Supp. 2: Effects of GW3965 and 25-OH Cholesterol on $[Ca^{2+}]_c$ of WT beta-cells in the presence of 15 mM glucose. A) Representative measurement showing inhibition of glucose-induced oscillations of $[Ca^{2+}]_c$ by 1 µM GW3965. B) Summary of all experiments. C) Respresentative measurements showing inhibition of glucose-induced oscillations of $[Ca^{2+}]_c$ by 30 µM 25-OH cholesterol. D) Summary of all experiments. The number in the columns gives the number of experiments with different cell clusters from 3 different mice. ***P ≤ 0.001.

## Atrial Natriuretic Peptide Affects Stimulus-Secretion Coupling of Pancreatic β-Cells

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*Published:* Diabetes 2017 Nov; 66(11): 2840-2848.

### Abstract

Atrial natriuretic peptide (ANP) influences glucose homeostasis and possibly acts as a link between the cardiovascular system and metabolism, especially in metabolic disorders like diabetes. The current study evaluated effects of ANP on β-cell function by the use of a β-cell–specific knockout of the ANP receptor with guanylate cyclase activity (βGC-A-KO). ANP augmented insulin secretion at the threshold glucose concentration of 6 mmol/L and decreased KATP single-channel activity in β-cells of control mice but not of βGC-A-KO mice. In wild-type β-cells but not β-cells lacking functional $K_{ATP}$ channels (SUR1-KO), ANP increased electrical activity, suggesting no involvement of other ion channels. At 6 mmol/L glucose, ANP readily elicited $Ca^{2+}$ influx in control β-cells. This effect was blunted in β-cells of βGC-A-KO mice, and the maximal cytosolic $Ca^{2+}$ concentration was lower. Experiments with inhibitors of protein kinase G (PKG), protein kinase A (PKA), phosphodiesterase 3B (PDE3B), and a membrane-permeable cyclic guanosine monophosphate (cGMP) analog on $K_{ATP}$ channel activity and insulin secretion point to participation of the cGMP/PKG and cAMP/PKA/Epac (exchange protein directly activated by cAMP) directly activated by cAMP Epac pathways in the effects of ANP on β-cell function; the latter seems to prevail. Moreover, ANP potentiated the effect of glucagon-like peptide 1 (GLP-1) on glucose-induced insulin secretion, which could be caused by a cGMP-mediated inhibition of PDE3B, which in turn reduces cAMP degradation.

**Introduction**

ANP plays an important role in the regulation of blood volume and blood pressure (1). ANP also is involved in the regulation of food intake and lipid and glucose homeostasis. Cellular effects of ANP are mediated by a plasma membrane–associated receptor with guanylate cyclase activity (GC-A receptor). Thus, activation of the receptor results in an increased cyclic guanosine monophosphate (cGMP) concentration. GC-A receptors are expressed in murine β- and α-cells and in the insulin-secreting tumor cell line INS-1E (2). However, functional studies about effects of ANP on β-cells are controversial. In cultured mouse islets, ANP increases glucose-stimulated insulin secretion (GSIS), and the effect is suggested to be mediated by closure of KATP channels and an increase of the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) (3). In the current study, β-cells of a global GC-A receptor knockout mouse (GC-A-KO) have been used to investigate the link between this receptor and islet function. The interpretation of the data is somewhat limited because a global GC-A-KO can alter systemic parameters, including blood pressure and lipid and glucose homeostasis (e.g., through effects on insulin resistance), which can retroact on the functional status of β-cells before islet isolation. A weak insulinotropic effect also has been observed in perfused rat pancreas (4), and another study reported marked hypoglycemia after intravenous ANP infusion in rats (5). On the contrary, acute incubation of isolated rat islets with ANP did not influence insulin secretion (6,7), and long-term culture with ANP even inhibited insulin production and GSIS (7). In healthy male volunteers, infusion of ANP slightly elevated plasma insulin and moderately increased blood glucose concentrations (8–10). Taken together, the mode of action on pancreatic islets remains to be elucidated.

In vitro data with isolated islets and/or β-cells are sparse, and the experiments with ANP infusion are difficult to interpret because one cannot discriminate between effects on β-cells and effects on peripheral organs or blood flow. Long-term effects were studied in mouse models lacking the GC-A receptor; however, the data are inconsistent. Global homozygous GC-A receptor deletion has led to enhanced fasted blood glucose concentration, whereas glucose tolerance and insulin sensitivity remained unchanged in GC-A-KO mice after 12 weeks of high-fat or standard diet compared with control mice (3). In contrast, mice with heterozygous receptor deletion that were not hypertensive developed impaired glucose tolerance after a high-fat diet

(11). Humans with genetic variants predisposing to low plasma concentrations of ANP and brain natriuretic peptide (BNP) (mainly secreted by the heart) exhibit a high risk for the development of hypertension and heart hypertrophy (12,13). Epidemiological studies also found an association between low ANP (and BNP) plasma concentrations and obesity, insulin resistance, and the metabolic syndrome (14–16). According to the concept of Gruden et al. (17), the lack of the beneficial effects of the natriuretic peptides on adipose tissue, skeletal muscle, and β-cells promotes the development of type 2 diabetes. The current study takes advantage of a β-cell–specific GC-A-KO (βGC-A-KO) mouse to clarify the effects of ANP on stimulus-secretion coupling of β-cells.

**Research Design and Methods**

*Mice.* C57BL/6N mice (wild type [WT]) were bred in the animal facility of the Department of Pharmacology at the University of Tübingen in Germany. GC-A-KO mice and their WT littermates (l-WT) were provided by Dr. M. Kuhn (Physiological Department, University of Würzburg, Würzburg, Germany). As previously described (18), βGC-A-KO mice were generated by crossing rat insulin II promoter (RIP)-Cre mice (RIP-Cre founders of the Herrera strain) (19) with floxed GC-A mice of a C57BL/6/Sv129 background (20). Deletion of GC-A protein in islet cells was verified by immunohistochemistry (18). The Guide for the Care and Use of Laboratory Animals and German laws were followed.

*Cell and islet preparation.* The details for cell and islet preparation have been previously described (21). Briefly, collagenase was injected into the ductus pancreaticus, and exocrine tissue was digested for ~5 min. Islets were handpicked, and clusters/single cells were made by trypsin digestion of islets.

*Solutions and chemicals.* Standard whole-cell and cell-attached recordings were done with a bath solution that contained (in mmol/L) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, or 10 $CaCl_2$ (for measurements of membrane potential), glucose as indicated, and 10 HEPES, pH adjusted to 7.4 with NaOH. The same bath solution was used for the determination of $[Ca^{2+}]_c$ and the mitochondrial membrane potential ($\Delta\Psi$). The pipette solution for standard whole-cell measurements of $K_{ATP}$ currents contained (in mmol/L) 130 KCl, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 20 HEPES, and $Na_2ATP$ as indicated,

pH adjusted to 7.15 with KOH. For cell-attached recordings, the pipette solution contained (in mmol/L) 130 KCl, 1.2 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, and 20 HEPES, pH adjusted to 7.4 with KOH. Cell membrane potential recordings were performed with amphotericin B (250 µg/mL) in the pipette solution, which contained (in mmol/L): 10 KCl, 10 NaCl, 70$K_2SO4$, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, and 20 HEPES, pH adjusted to 7.15 with KOH. Fura-2 acetoxymethylester was obtained from Molecular Probes (Eugene, OR). RPMI1640 medium was from PromoCell (Heidelberg, Germany), penicillin/streptomycin from Gibco BRL (Karlsruhe, Germany), atrial natriuretic factor (1-28) (mouse, rabbit, rat) trifluoroacetate salt from Bachem (Weil am Rhein, Germany), Rp-8-Br-PET-cGMPS from Biolog (Bremen, Germany), and PKI 14-22 amide, myristoylated (myr-PKI) from Tocris Bioscience (Wiesbaden, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany) or Merck (Darmstadt, Germany) in the purest form available.

*Patch-Clamp recordings.* $K_{ATP}$ currents and membrane potentials were recorded with an EPC-9 patch-clamp amplifier by using PATCHMASTER software (HEKA Elektronik, Lambrecht, Germany). Channel activity of the single channels was measured at the actual membrane potential in the cell-attached mode. Point-by-point analysis of the current traces reveals an open probability ($P_o$) owing to all active channels (N) in the patch and is thus presented as NPo. Whole-cell $K_{ATP}$ current was evoked by 300-ms voltage steps from −70 to −60 mV and −80 mV. Under these conditions, the current is completely blockable by $K_{ATP}$ channel inhibitors (22).

*Measurements of the mitochondrial ΔΨ.* Mitochondrial ΔΨ was measured as Rh123 fluorescence at 480-nm excitation wavelength as previously described (23).

*Measurement of $[Ca^{2+}]_c$.* Details have been previously described (21). In brief, cells were loaded with 5 µmol/L Fura-2 acetoxymethylester for 30 min at 37°C. Fluorescence was excited at a 340- and 380-nm wavelength, and fluorescence emission was filtered (LP515) and measured by a digital camera. $[Ca^{2+}]_c$ was calibrated in vitro by using Fura-2 pentapotassium salt (24).

*Measurement of Insulin Secretion.* Details for steady-state incubations have been previously described (21). For perifusions, 50 islets were placed in a bath chamber

and perifused with 3 mmol/L glucose for 60 min before the beginning of the experiment. Samples for the determination of insulin were taken every 2 min.

*Statistics.* Each series of experiments was performed with islets or islet cells of at least three independent preparations. Mean ± SEM is given for the indicated number of experiments. Statistical significance of differences was assessed by Student t test for paired values. Multiple comparisons were made by ANOVA followed by Student-Newman-Keuls test. $P \leq 0.05$ was considered significant.

**Results**

*ANP increases insulin secretion and $[Ca^{2+}]_c$ at a threshold glucose concentration*

Insulin secretion of isolated mouse islets was measured in vitro to evaluate whether GC-A receptor stimulation by ANP results in changed insulin secretion. First, ANP was added in the second phase of insulin release after increasing the glucose concentration from 3 to 10 mmol/L (Fig. 1A). Under these conditions ANP increased insulin secretion induced by 10 mmol/L glucose from 7.6 ± 1.2 to 8.7 ± 1.3 pg insulin/(islet · min) ($P \leq 0.001$) and insulin area under the curve (AUC) (Fig. 1B). For these experiments, islets from C57BL/6N mice (WT) were used. ANP effects at a threshold glucose concentration of 6 mmol/L were tested in steady-state incubation. In islets of l-WT, ANP significantly increased insulin secretion from 0.24 ± 0.06 to 0.33 ± 0.07 ng insulin/(islet · h) ($P \leq 0.05$), whereas it was ineffective in islets from βGC-A-KO mice [0.24 ± 0.04 vs. 0.23 ± 0.06 ng insulin/(islet · h)] (Fig. 1C). The stimulating effect of ANP also was absent in islets from SUR1-KO mice that lacked functional $K_{ATP}$ channels (Supplementary Fig. 1A).

$[Ca^{2+}]_c$ was measured in the presence of 6 mmol/L glucose in islet cell clusters of l-WT and βGC-A-KO mice. In 6 mmol/L glucose, $[Ca^{2+}]_c$ was at basal values in most cells (i.e., no oscillations occurred). These cells with basal Ca2+ concentration were selected for investigation of the effect of ANP (Fig. 1D). Glucose at a concentration of 15 mmol/L was added at the end of each experiment to test whether cells were metabolically intact and thus able to show a response to ANP. Maximal $[Ca^{2+}]_c$ ($max[Ca^{2+}]_c$) before (basal) and after application of ANP was calculated for each of the 76 and 74 experiments performed with l-WT and βGC-A-KO cells, respectively. The mean of the $max[Ca^{2+}]_c$ is an indirect measure for the percentage of ANP-

responsive cells because it mirrors the number of responsive cells (Fig. 1E). Figure 1D shows the typical response to ANP for cell clusters of each genotype. The summary of the data is shown in Fig. 1E. In l-WT β-cells, ANP increased max[Ca2+]c from 68 ± 4 to 270 ± 33 nmol/L (P ≤ 0.001). Switching to the bath solution with ANP also augmented max$[Ca^{2+}]_c$ in βGC-A-KO β-cells from 68 ± 4 to 135 ± 23 nmol/L (P ≤ 0.01). This increase in the βGC-A-KO β-cells is significantly lower than that in the l-WT β-cells. Of note, the mean value for max$[Ca^{2+}]_c$ for βGC-A-KO cells in Fig. 1E is not 0, which may be a result of spontaneous $Ca^{2+}$ transients occurring sporadically in single cells or small clusters after a change in bath solution from a stimulatory glucose concentration to a threshold concentration for induction of $Ca^{2+}$ oscillations. To emphasize the significance of the data, we also calculated the percentage of cells for each of the three mouse preparations per genotype, which was 42 ± 12% for l-WT cells and 11 ± 5% for β-GC-A-KO cells. Because of the high variability between days and the small and limited number of mice, this data evaluation was not statistically significant (P = 0.08).

*ANP decreases $K_{ATP}$ channel activity in a GC-A receptor–dependent manner*

$K_{ATP}$ channel activity was measured with β-cells from WT mice in the cell-attached mode in the presence of 0.5 mmol/L glucose to test whether ANP affects these channels. ANP at a concentration of 10 nmol/L reduced the NPo from 100% under control conditions to 62 ± 10% (P ≤ 0.01) (Fig. 2A and B). Changes in $K_{ATP}$ channel activity can be caused by altered mitochondrial metabolism (25). Therefore, the effects of ANP on the mitochondrial ΔΨ were measured, which can be taken to estimate mitochondrial ATP production (26). Neither in the presence of 15 mmol/L glucose nor in the presence of 4 mmol/L glucose did 10 nmol/L ANP alter ΔΨ (Supplementary Fig. 2). These data argue against an influence of ANP on ATP formation. To examine whether the GC-A receptor is involved in the inhibitory effect of ANP, β-cells from βGC-A-KO mice and l-WT mice were used. In l-WT β-cells, NPo was reduced from 100% to 29 ± 11% (P ≤ 0.01) (Fig. 2C and D) upon addition of 10 nmol/L ANP. In contrast, ANP was without effect in βGC-A-KO β-cells (100% vs. 119 ± 15%) (Fig. 2E and F). In accordance with $K_{ATP}$ single-channel current measurements, ANP increased the electrical activity of β-cells of WT mice. The fraction of plateau phase (FOPP), which is the percentage of time with spike activity, increased from 47 ± 5% to 65 ± 5% (P ≤ 0.05) (Fig. 3A and B). However, ANP did not

change electrical activity in β-cells obtained from SUR1-KO mice (Fig. 3C and D). In these experiments, β-cells did not oscillate because the membrane potential is more depolarized in this genotype. Therefore, data were analyzed by determining the number of action potentials 2 min before change of the bath solution.

The global GC-A-KO leads to reduced expression of the $K_{ATP}$ channel subunits SUR1 and Kir6.2 in β-cells (3). To elucidate a possible difference in $K_{ATP}$ current density between the two genotypes, the maximal $K_{ATP}$ current that developed after formation of the standard whole-cell configuration was measured without ATP in the patch pipette in l-WT (Fig. 4A) and βGC-A-KO β-cells (Fig. 4B). The data revealed no difference in the $K_{ATP}$ current density (21 ± 2 vs. 23 ± 1 pA/pF in l-WT and βGC-A-KO, respectively) (Fig. 4C).

*Involvement of the cGMP/PKG and cAMP/PKA pathways in theeEffect of ANP in β-cells*

Because GCs synthesize the second messenger cGMP (27), we tested whether cGMP can mimic the effect of ANP on $K_{ATP}$ channels. The membrane-permeable analog 8-Br-cGMP reduced NPo of β-cells from l-WT mice in the cell-attached configuration from 100% to 72 ± 8% ($P \leq 0.05$) (Fig. 5A and B). Preincubation of the cells with the PKG inhibitor Rp-8 completely blunted the effect of ANP on $K_{ATP}$ channel activity of β-cells from WT mice measured in the cell-attached configuration (100% vs. 110 ± 23%) (Fig. 5C and D), indicating the dependence of the effect of ANP on $K_{ATP}$ channels on this protein kinase. However, ANP still increased insulin secretion in the presence of Rp-8 and 6 mmol/L glucose [0.14 ± 0.03 to 0.19 ± 0.03 ng insulin/(islet · h); $P \leq 0.05$] (Fig. 5E). Besides stimulating the PKG, cGMP can activate or inhibit various types of phosphodiesterases (PDEs) (28). With respect to insulin secretion, PDE3B is the most important PDE in β-cells (29), which is inhibited by cGMP (28). Because inhibition of PDE3B should inhibit the degradation of cAMP, cGMP and cAMP signaling pathways may converge on this PDE. Thus, ANP may affect the cAMP concentration and, consequently, insulin secretion by a PKA-dependent pathway. The specific PDE3B blocker cilostamide markedly increased insulin secretion in the presence of 10 mmol/L glucose, showing that this pathway is present in β-cells (Supplementary Fig. 3A). After inhibition of PDE3B by cilostamide, ANP no longer was able to increase insulin secretion (Supplementary Fig. 3B),

pointing to a significant role of cAMP in the ANP effect on insulin secretion. Treatment of the cells with the PKA inhibitor myr-PKI did not suppress the inhibitory effect of ANP on $K_{ATP}$ channel activity of β-cells from WT mice (85 ± 15% vs. 28 ± 12% with myr-PKI vs. with myr-PKI and ANP, respectively; P ≤ 0.01) (Fig. 5F and G). Insulin secretion was not significantly enhanced by ANP in the presence of myr-PKI, but a tendency was discernible (Fig. 5H). Figure 6 demonstrates the well-known effect that glucagon-like peptide 1 (GLP-1) potentiates GSIS. Of note, Fig. 6 also shows that the action of GLP-1 on secretion is augmented by 90-min preincubation of the cells with ANP [1.7 ± 0.2 vs. 2.3 ± 0.4 ng insulin/(islet · h) with GLP-1 vs. with GLP-1 and ANP, respectively; P ≤ 0.05]. Ten-minute preincubation with ANP was ineffective [3.2 ± 0.4 vs. 3.2 ± 0.6 ng insulin/(islet · h); n = 6]; after 20 min preincubation, a tendency to increase the action of GLP-1 appeared [1.7 ± 0.4 vs. 2.0 ± 0.5 ng insulin/(islet · h); n = 12]. The potentiating action of ANP on the GLP-1 effect was absent in islets from SUR1-KO mice, pointing to a significant role of $K_{ATP}$ channels in this intensification (Supplementary Fig. 1B). The potentiating effect is most likely a result of inhibition of the PDE3B by cGMP (see above) and increased cAMP concentration. It can be mimicked by cilostamide. Insulin secretion amounted to 1.6 ± 0.2 ng insulin/(islet · h) with GLP-1 alone versus 3.8 ± 0.2 ng insulin/(islet · h) with GLP-1 and cilostamide (P ≤ 0.001) (Supplementary Fig. 3A).

## Discussion

*Effects of ANP at the threshold concentration of glucose*

An ANP-induced increase in insulin secretion was easier to detect at 6 mmol/L glucose, the threshold concentration for the induction of insulin secretion, than at 10 mmol/L glucose. Identical steady-state incubation experiments with 10 mmol/L glucose did not reveal a significant increase in insulin secretion (data not shown). However, with 10 mmol/L glucose, an effect of ANP could be detected in perifusion experiments. This kind of experiment allows discrimination between the effects of a drug on first and second phase of secretion. The results revealed an increase of GSIS by ANP in the second phase. Ropero et al. (3) showed an augmentation of insulin secretion by ANP in steady-state experiments in the presence of the threshold concentration of 7 mmol/L glucose but did not mention whether other glucose concentrations were tried. ANP seems to be less effective on insulin secretion than

on other parameters of the stimulus-secretion coupling, which may be due to insulin secretion being measured with whole islets and, for example, membrane potential or $[Ca^{2+}]_c$ with dispersed cells. The capsule of connective tissue that surrounds the islets can restrain access of drugs to islet cells. Therefore, one should keep in mind that in vivo ANP reaches the cells through the capillaries, not across the capsule.

The particular effectiveness of ANP at the threshold glucose concentration also is assessed with $[Ca^{2+}]_c$ measurements. ANP considerably increased max$[Ca^{2+}]_c$ above the basal values obtained at 6 mmol/L. The special physiological role of ANP in β-cells possibly augments GSIS in a coordinated action, with incretins at blood glucose concentrations occurring at the beginning of a meal. In humans with metabolic disorders, low plasma concentrations of ANP (14–16) may contribute to the impairment of insulin secretion in addition to a reduced incretin effect.

*Involvement of the GC-A receptor/cGMP/PKG pathway in effects of ANP on β-cell function*

The patch-clamp data clearly demonstrate that ANP-mediated inhibition of $K_{ATP}$ single-channel activity is caused by activation of GC-A receptors on β-cells because the effect is abolished in βGC-A-KO cells. $K_{ATP}$ channel current density was the same in both genotypes, which contrasts findings in β-cells with a global GC-A-KO, showing diminished $K_{ATP}$ channel activity and reduced expression of both $K_{ATP}$ channel subunits compared with WT cells (3). The findings may explain the higher rate of insulin secretion in islets of the global GC-A receptor KO compared with WT islets, an effect not observed in the β-cell–specific KO model (Fig. 1C). As expected, the inhibitory effect of ANP on $K_{ATP}$ channel activity was accompanied by a depolarization of the plasma membrane and an increase in $[Ca2+]_c$ and insulin secretion attributable to GC-A activation. ANP did not affect membrane potentials of cells from SUR1-KO mice, suggesting that ANP does not influence the activity of other ion channels than $K_{ATP}$ channels. Stimulation of GC-A receptors should result in an increase in the cGMP concentration. Dankworth (18) showed that the ANP-induced increase in cGMP is much higher in islets of l-WT mice than in those of βGC-A-KO mice. The inhibitory effect of ANP on $K_{ATP}$ channels was mimicked by a membrane-permeable cGMP analog and completely blocked by the PKG inhibitor Rp-8, strongly suggesting the involvement of the GC-A/cGMP/PKG signaling

pathway in the action of ANP on β-cell $K_{ATP}$ channels (Fig. 7). This pathway also is proposed for the rapid action of estrogen (30) and the effect caused by small amounts of nitric oxide (31) on β-cell function. Which PKG is involved in this pathway in β-cells is unknown, and Rp-8 is not isoform specific. Because two studies exclude the expression of PKGI in β-cells (32,33), PKGII is the most likely candidate. Pancreatic $K_{ATP}$ channels comprise SUR1 (sulfonylurea receptor) and Kir6.2 (inwardly rectifying $K^+$ channel) subunits. PKG is proposed to have dual effects in regulating SUR1/Kir6.2 channels: indirect activation of the channels by phosphorylation of cellular compounds not directly linked to $K_{ATP}$ channels and inhibition by phosphorylation of channel proteins or tightly coupled proteins (34). In pancreatic β-cells, we, like Ropero et al. (35), found that the latter pathway seems to prevail. After preincubation with Rp-8, ANP still significantly augmented insulin secretion (Fig. 5E), which apparently contrasts with the patch-clamp data. Considering that cGMP inhibits the PDE3B (28), an increase in insulin secretion could be explained by increased cAMP concentration and activation of PKA (see below). The data even suggest that the cGMP/cAMP signaling pathway is more important for the final ANP effects on β-cell function than the cGMP/$K_{ATP}$ pathway.

*Effects of ANP on the cAMP signaling pathway*

ANP can increase the cAMP concentration through GC-A receptor activation, cGMP formation, and inhibition of the PDE3B by reducing cAMP degradation. Inhibition of PKA did not influence the inhibitory effect of ANP on $K_{ATP}$ channel activity, making a direct link between PKA and $K_{ATP}$ channels unlikely (Fig. 5F and G). From these patch-clamp experiments, one would expect that ANP still activates insulin secretion when PKA is blocked. In our experiments, there was a tendency but no significant effect (Fig. 5H). However, PKA directly interferes with exocytosis (e.g., by increasing the $Ca^{2+}$ sensitivity of the exocytotic machinery) (36). This effect may be alleviated by PKA inhibition. The experiments with cilostamide support the hypothesis that the increase of the cAMP concentration is an essential step in the action of ANP. In the presence of cilostamide, ANP did not enhance insulin secretion, most likely because PDE3B is fully inhibited and cAMP maximally increased under these conditions. The current data suggest that ANP augments cAMP through cGMP-mediated inhibition of the PDE3B. cAMP activates the PKA, which influences exocytosis, and exchange protein activated by cAMP (Epac), which interferes with $K_{ATP}$ channels by rendering

them more sensitive to ATP (37) (Fig. 7). The cAMP/Epac/$K_{ATP}$ pathway seems to be indispensable for the action of ANP on β-cell function and to prevail in the cAMP/PKA/exocytosis pathway because ANP did not increase insulin secretion in islets from SUR1-KO mice (Supplementary Fig. 1A). The cGMP/cAMP pathway is mediated by the ANP-induced activation of the GC-A receptor because ANP-induced augmentation of insulin secretion is completely blunted in islets from βGC-A-KO mice (Fig. 1C).

The incretin hormone GLP-1 increases insulin secretion by increasing the cAMP concentration. This effect is potentiated by preincubation with ANP. A significant potentiating effect was seen after 90 min of preincubation with ANP at room temperature. Twenty minutes of preincubation led at least to a tendency to potentiate the action of the incretin hormone. We assume that penetration through the capsule is slow (see above), especially at room temperature. Although we cannot entirely rule out a genomic effect for this potentiation, a cytosolic interaction of the hormones seems much more likely. The ANP/cGMP/PDE3B/cAMP pathway may also be involved in the additive effect of ANP and GLP-1 in β-cells. This assumption is confirmed by the observation that the PDE3B inhibitor cilostamide potentiates the GLP-1 effect on GSIS. Our hypothesis is in accordance with earlier findings. Overexpression of PDE3B in insulin-secreting rat insulinoma cells leads to a decrease of cAMP concentration and GSIS. Furthermore, the ability of GLP-1 to potentiate insulin secretion is impaired (38). Thus, we assume that ANP inhibits PDE3B, which leads to reduced degradation of cAMP and finally increases the effectiveness of GLP-1 (Fig. 7). Because this potentiation requires $K_{ATP}$ channels, it is most likely mediated by the cAMP/Epac/$K_{ATP}$ pathway.

In conclusion, the data point to a dual action of ANP in pancreatic β-cells (Fig. 7): 1) ANP activates the GC-A/cGMP/PKG pathway wherein phosphorylation by PKG blocks $K_{ATP}$ channels and 2) ANP inhibits PDE3B and thus increases cAMP concentration, which positively influences insulin secretion through the PKA and Epac pathway. The second pathway seems to be essential for ANP-mediated enhancement of insulin secretion.

**Duality of Interest:** No potential conflicts of interest relevant to this article were reported.

**Funding:** This work was supported by Deutsche Forschungsgemeinschaft grant DR 225/9-1 (to G.D.).

**Author Contributions:** S.U., J.K., and J.S. researched data. M.D. contributed to the discussion and study design and edited the manuscript. P.K.-D. evaluated data and edited the manuscript. G.D. designed the study, wrote and edited the manuscript, and contributed to the discussion. G.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Acknowledgments:** The authors thank Isolde Breuning, Institute of Pharmacy, University of Tübingen, for excellent technical support. The authors thank Dr. Michaela Kuhn, Physiological Department, University of Würzburg for kindly providing the knockout mice and corresponding littermates.

**Prior Presentation:** Parts of this study were presented in abstract form at the 51st European Association for the Study of Diabetes Annual Meeting, Stockholm, Sweden, 14–18 September 2015.

**References**

1. Kuhn M. Molecular physiology of membrane guanylyl cyclase receptors. Physiol Rev 2016;96:751–804pmid:27030537

2. You H, Laychock SG. Atrial natriuretic peptide promotes pancreatic islet beta-cell growth and Akt/Foxo1a/cyclin D2 signaling. Endocrinology 2009;150:5455–5465pmid:19837876

3. Ropero AB, Soriano S, Tudurí E, et al. The atrial natriuretic peptide and guanylyl cyclase-A system modulates pancreatic beta-cell function. Endocrinology 2010;151:3665–3674pmid:20555029

4. Fehmann HC, Noll B, Göke R, Göke B, Trautmann ME, Arnold R. Atrial natriuretic factor has a weak insulinotropic action in the isolated perfused rat pancreas. Res Exp Med (Berl) 1990;190:253–258pmid:2145621

5. Carlsson PO, Andersson A, Jansson L. Cardiac natriuretic peptides and pancreatic islet blood flow in anesthetized rats. Horm Metab Res 2001;33:181–185pmid:11355754

6. Verspohl EJ, Ammon HP. Atrial natriuretic peptide (ANP) acts via specific binding sites on cGMP system of rat pancreatic islets without affecting insulin release. Naunyn Schmiedebergs Arch Pharmacol 1989;339:348–353pmid:2542810

7. You H, Laychock SG. Long-term treatment with atrial natriuretic peptide inhibits ATP production and insulin secretion in rat pancreatic islets. Am J Physiol Endocrinol Metab 2011;300:E435–E444pmid:20959527

8. Birkenfeld AL, Budziarek P, Boschmann M, et al. Atrial natriuretic peptide induces postprandial lipid oxidation in humans. Diabetes 2008;57:3199–3204pmid:18835931

9. Uehlinger DE, Weidmann P, Gnädinger MP, et al. Increase in circulating insulin induced by atrial natriuretic peptide in normal humans. J Cardiovasc Pharmacol 1986;8:1122–1129pmid:2434736

10. Birkenfeld AL, Boschmann M, Moro C, et al. Beta-adrenergic and atrial natriuretic peptide interactions on human cardiovascular and metabolic regulation. J Clin Endocrinol Metab 2006;91:5069–5075pmid:16984990

11. Miyashita K, Itoh H, Tsujimoto H, et al. Natriuretic peptides/cGMP/cGMP-dependent protein kinase cascades promote muscle mitochondrial biogenesis and prevent obesity. Diabetes 2009;58:2880–2892pmid:19690065

12. Kato N, Sugiyama T, Morita H, et al. Genetic analysis of the atrial natriuretic peptide gene in essential hypertension. Clin Sci (Lond) 2000;98:251–258pmid:10677382

13. Rubattu S, Bigatti G, Evangelista A, et al. Association of atrial natriuretic peptide and type a natriuretic peptide receptor gene polymorphisms with left ventricular mass in human essential hypertension. J Am Coll Cardiol 2006;48:499–505pmid:16875975

14. Das SR, Drazner MH, Dries DL, et al. Impact of body mass and body composition on circulating levels of natriuretic peptides: results from the Dallas Heart Study. Circulation 2005;112:2163–2168pmid:16203929

15. Khan AM, Cheng S, Magnusson M, et al. Cardiac natriuretic peptides, obesity, and insulin resistance: evidence from two community-based studies. J Clin Endocrinol Metab 2011;96:3242–3249pmid:21849523

16. Wang TJ, Larson MG, Levy D, et al. Impact of obesity on plasma natriuretic peptide levels. Circulation 2004;109:594–600pmid:14769680

17. Gruden G, Landi A, Bruno G. Natriuretic peptides, heart, and adipose tissue: new findings and future developments for diabetes research. Diabetes Care 2014;37:2899–2908pmid:25342830

18. Dankworth B. Charakterisierung der dynamischen Interaktion des Guanyl Cyclase-A (GC-A)-Rezeptors mit den Transient Receptor Potential Canonical Type 3 und Type 6 (TRPC3/C6)-Kanälen und Generierung von β-Zell-spezifischen GC-A-knockout-Mäusen sowie die Analyse der Bedeutung von ANP für die Insulin-Homöostase unter pathophysiologischen Bedingungen. Würzburg, Germany, University of Würzburg, 2013

19. Herrera PL. Adult insulin- and glucagon-producing cells differentiate from two independent cell lineages. Development 2000;127:2317–2322pmid:10804174

20. Holtwick R, Gotthardt M, Skryabin B, et al. Smooth muscle-selective deletion of guanylyl cyclase-A prevents the acute but not chronic effects of ANP on blood pressure. Proc Natl Acad Sci U S A 2002;99:7142–7147pmid:11997476

21. Gier B, Krippeit-Drews P, Sheiko T, et al. Suppression of KATP channel activity protects murine pancreatic beta cells against oxidative stress. J Clin Invest 2009;119:3246–3256pmid:19805912

22. Garrino MG, Plant TD, Henquin JC. Effects of putative activators of K+ channels in mouse pancreatic beta-cells. Br J Pharmacol 1989;98:957–965pmid:2531623

23. Edalat A, Schulte-Mecklenbeck P, Bauer C, et al. Mitochondrial succinate dehydrogenase is involved in stimulus-secretion coupling and endogenous ROS formation in murine beta cells. Diabetologia 2015;58:1532–1541pmid:25874444

24. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca2+ indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–3450pmid:3838314

25. Drews G, Krippeit-Drews P, Düfer M. Electrophysiology of islet cells. In Islets of Langerhans. Vol. 1, 2nd ed. Islam MS, Ed. New York, Springer, 2015, p. 249–303

26. Krippeit-Drews P, Düfer M, Drews G. Parallel oscillations of intracellular calcium activity and mitochondrial membrane potential in mouse pancreatic B-cells. Biochem Biophys Res Commun 2000;267:179–183pmid:10623595

27. Kuhn M. Function and dysfunction of mammalian membrane guanylyl cyclase receptors: lessons from genetic mouse models and implications for human diseases. Handb Exp Pharmacol 2009;(191):47–69pmid:19089325

28. Potter LR, Abbey-Hosch S, Dickey DM. Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. Endocr Rev 2006;27:47–72pmid:16291870

29. Pyne NJ, Furman BL. Cyclic nucleotide phosphodiesterases in pancreatic islets. Diabetologia 2003;46:1179–1189pmid:12904862

30. Soriano S, Ropero AB, Alonso-Magdalena P, et al. Rapid regulation of K(ATP) channel activity by 17beta-estradiol in pancreatic beta-cells involves the estrogen receptor beta and the atrial natriuretic peptide receptor. Mol Endocrinol 2009;23:1973–1982pmid:19855088

31. Sunouchi T, Suzuki K, Nakayama K, Ishikawa T. Dual effect of nitric oxide on ATP-sensitive K+ channels in rat pancreatic beta cells. Pflugers Arch 2008;456:573–579pmid:18239934

32. Lutz SZ, Hennige AM, Feil S, et al. Genetic ablation of cGMP-dependent protein kinase type I causes liver inflammation and fasting hyperglycemia. Diabetes 2011;60:1566–1576pmid:21464444

33. Leiss V, Friebe A, Welling A, Hofmann F, Lukowski R. Cyclic GMP kinase I modulates glucagon release from pancreatic α-cells. Diabetes 2011;60:148–156pmid:20978093

34. Chai Y, Lin YF. Dual regulation of the ATP-sensitive potassium channel by activation of cGMP-dependent protein kinase. Pflugers Arch 2008;456:897–915pmid:18231807

35. Ropero AB, Fuentes E, Rovira JM, Ripoll C, Soria B, Nadal A. Non-genomic actions of 17beta-oestradiol in mouse pancreatic beta-cells are mediated by a cGMP-dependent protein kinase. J Physiol 1999;521:397–407pmid:10581311

36. Skelin M, Rupnik M. cAMP increases the sensitivity of exocytosis to $Ca^{2+}$ primarily through protein kinase A in mouse pancreatic beta cells. Cell Calcium 2011;49:89–99pmid:21242000

37. Kang G, Leech CA, Chepurny OG, Coetzee WA, Holz GG. Role of the cAMP sensor Epac as a determinant of KATP channel ATP sensitivity in human pancreatic beta-cells and rat INS-1 cells. J Physiol 2008;586:1307–1319pmid:18202100

38. Härndahl L, Jing XJ, Ivarsson R, et al. Important role of phosphodiesterase 3B for the stimulatory action of cAMP on pancreatic beta-cell exocytosis and release of insulin. J Biol Chem 2002;277:37446–37455pmid:12169692

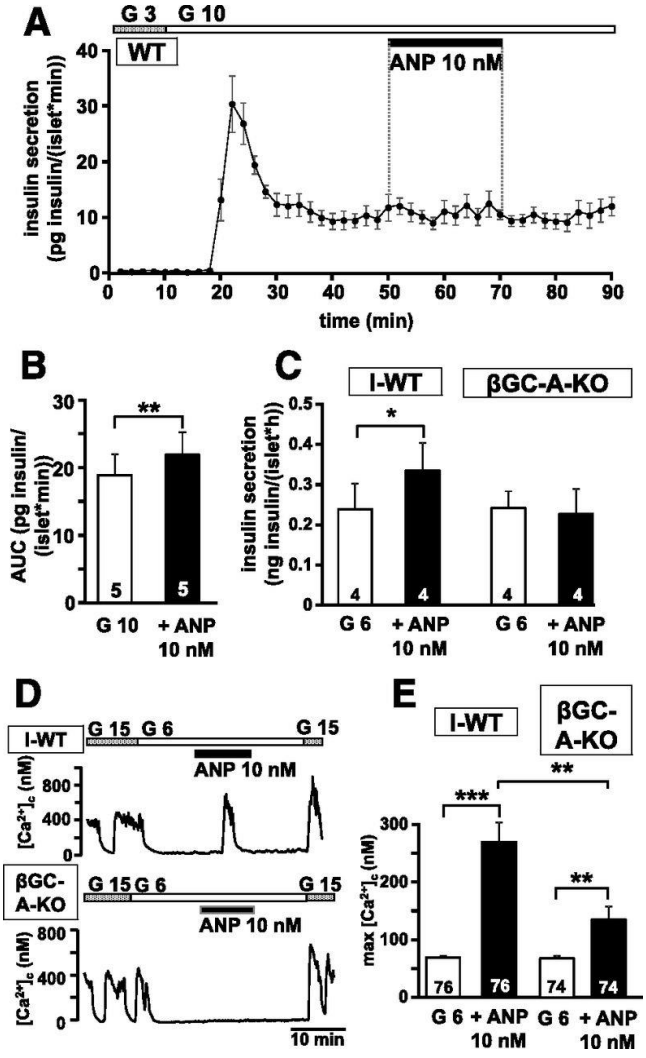



Fig. 1: Effects of ANP on insulin secretion and $[Ca^{2+}]_c$. *A*: Perifusion experiments with WT C57BL/6 islets showing the typical biphasic response of the insulin secretion after augmentation of the glucose concentration from 3 (G 3) to 10 mmol/L (G 10). ANP was added during the second phase as indicated. *B*: Evaluation of the AUCs, which were calculated between minutes 40 and 48 (control) and 60 and 68 (ANP application). *C*: Steady-state insulin secretion measurements at the threshold glucose concentration for the stimulation of insulin secretion with islets from *I*-WT and βGC-A-KO mice. *D*: Measurements of $[Ca^{2+}]_c$ at 6 mmol/L glucose (G 6). The top panel shows a typical recording for an *I*-WT β-cell, and the bottom panel shows a recording for a βGC-A-KO β-cell. Each experiment was started at 15 mmol/L glucose (G 15), where β-cells exhibit oscillations of $[Ca^{2+}]_c$. Lowering the glucose concentration to 6 mmol/L decreased $[Ca^{2+}]_c$ in most cells to basal values. Cells in which oscillations continued were discarded. *E*: Summary of the ANP-induced increase of $[Ca^{2+}]_c$ in *I*-WT and βGC-A-KO β-cells. The numbers within the columns in *B* and *C* are the number of different mice. The numbers within the columns in *E* are the number of different experiments with three different mice. **P* ≤ 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

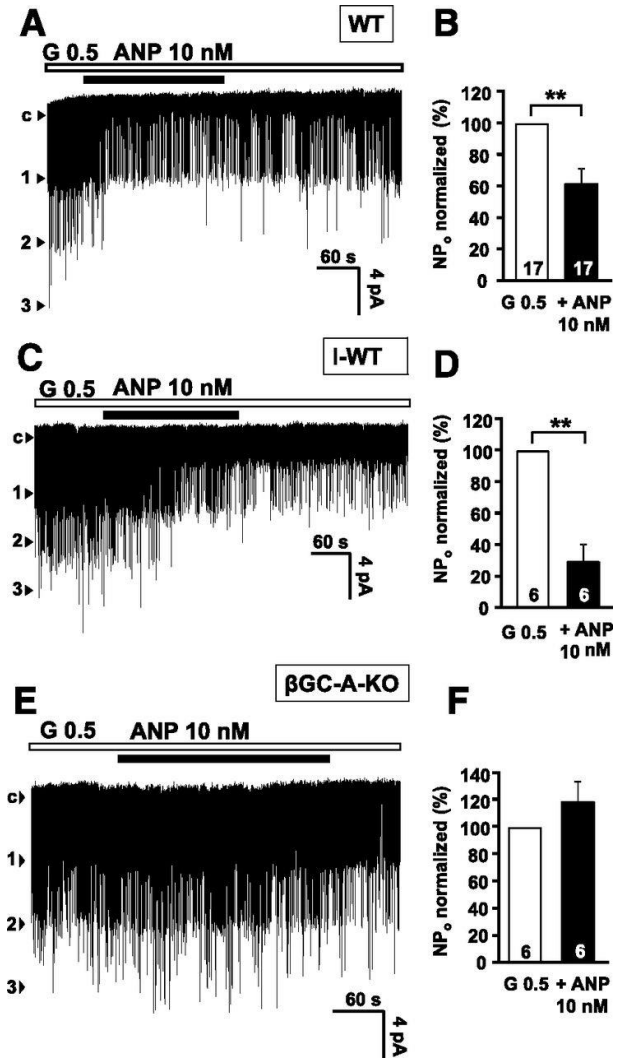



Fig. 2: Effects of ANP on $NP_o$ of single $K_{ATP}$ channels. *A*: Typical $K_{ATP}$ single-channel recording of a WT β-cell in the cell-attached mode, demonstrating the effect of ANP application in the presence of 0.5 mmol/L glucose (G 0.5). *B*: Mean $NP_o$ of all experiments conducted under this condition. $NP_o$ before addition of ANP was normalized to 100%. *C* and *D*: Same experimental protocol with *I*-WT β-cells. *E* and *F*: Same experimental protocol with βGC-A-KO β-cells. The numbers within columns are the number of different cells used for the experiments. In each series, cells of at least three different mice were used. $^{**}P \leq 0.01$. c, closed state of the channel.

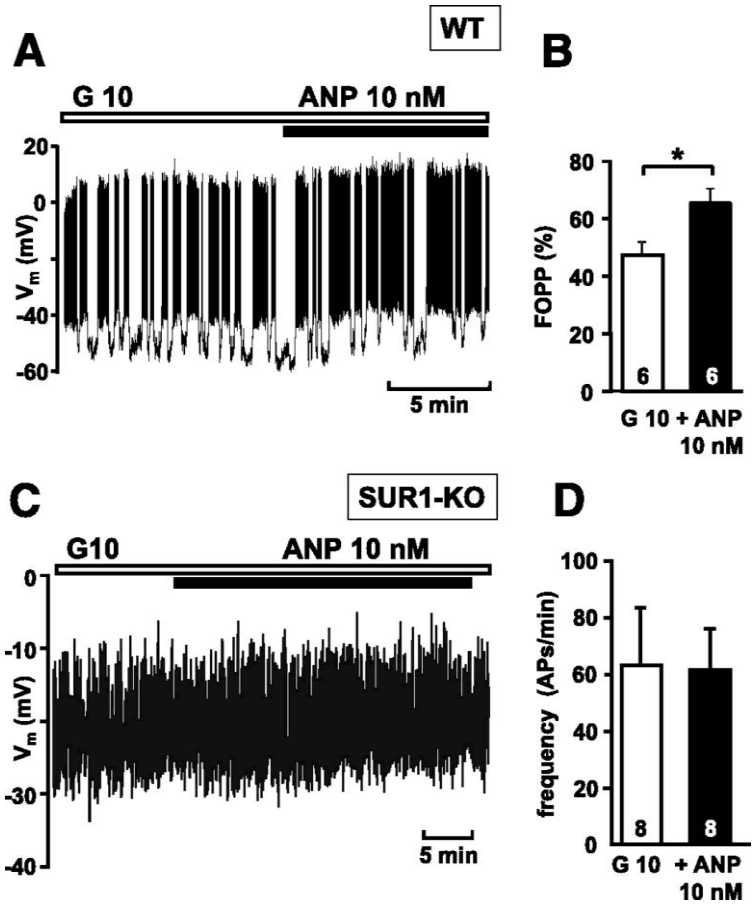



Fig. 3: Influence of ANP on the membrane potential ($V_m$) in cells from WT and SUR1-KO mice. *A*: Typical recording of $V_m$ in the perforated-patch mode, showing the effect of ANP in the presence of 10 mmol/L glucose (G 10) in a WT β-cell. *B*: Calculation of the mean FOPP (percentage of time with spike activity) for all experiments conducted under this condition. *C*: Typical recording of $V_m$ in the perforated-patch mode, showing the effect of ANP in the presence of G 10 in an SUR1-KO β-cell. *D*: Calculation of the number of action potentials (APs) during the 2 min before change of bath solution for all experiments conducted under this condition. The numbers within columns are the number of different cells used for the experiments. In each series, cells of at least three different mice were used. *$P \leq 0.05$.

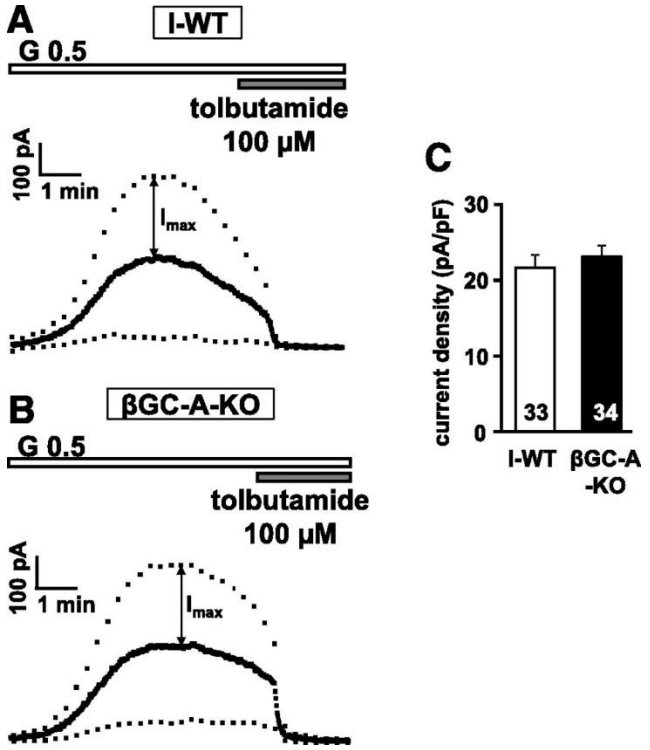



Fig. 4: Maximum $K_{ATP}$ current density evoked by the washout of ATP in the standard whole-cell configuration. After gigaseal formation, the patch under the patch-pipette is ruptured, establishing the standard whole-cell configuration. The cell is dialyzed by the ATP-free pipette solution, completely removing ATP from the cytosol. Thus, the $K_{ATP}$ current measured at −70 mV (solid line) or during voltage steps to −60 mV (upper dotted line) increased, whereas the current at −80 mV did not greatly change because this potential is close to the $K^+$ equilibrium potential. After a maximum, the current in ATP-free solution usually decreases as a result of an unspecific phenomenon called rundown. *A*: Typical recording from an *I*-WT β-cell. At the end of each experiment, tolbutamide was added to ensure that the entire current is sensitive to ATP. The current elicited by a voltage step from −70 to −60 mV was taken for evaluation. *B*: Typical recording from a βGC-A-KO β-cell. *C*: Summary of the results obtained by all experiments conducted with this protocol. Current density = current / cell capacitance. The numbers within columns are the number of different cells used for the experiments. In each series, cells of at least three different mice were used. G 0.5, 0.5 mmol/L glucose.

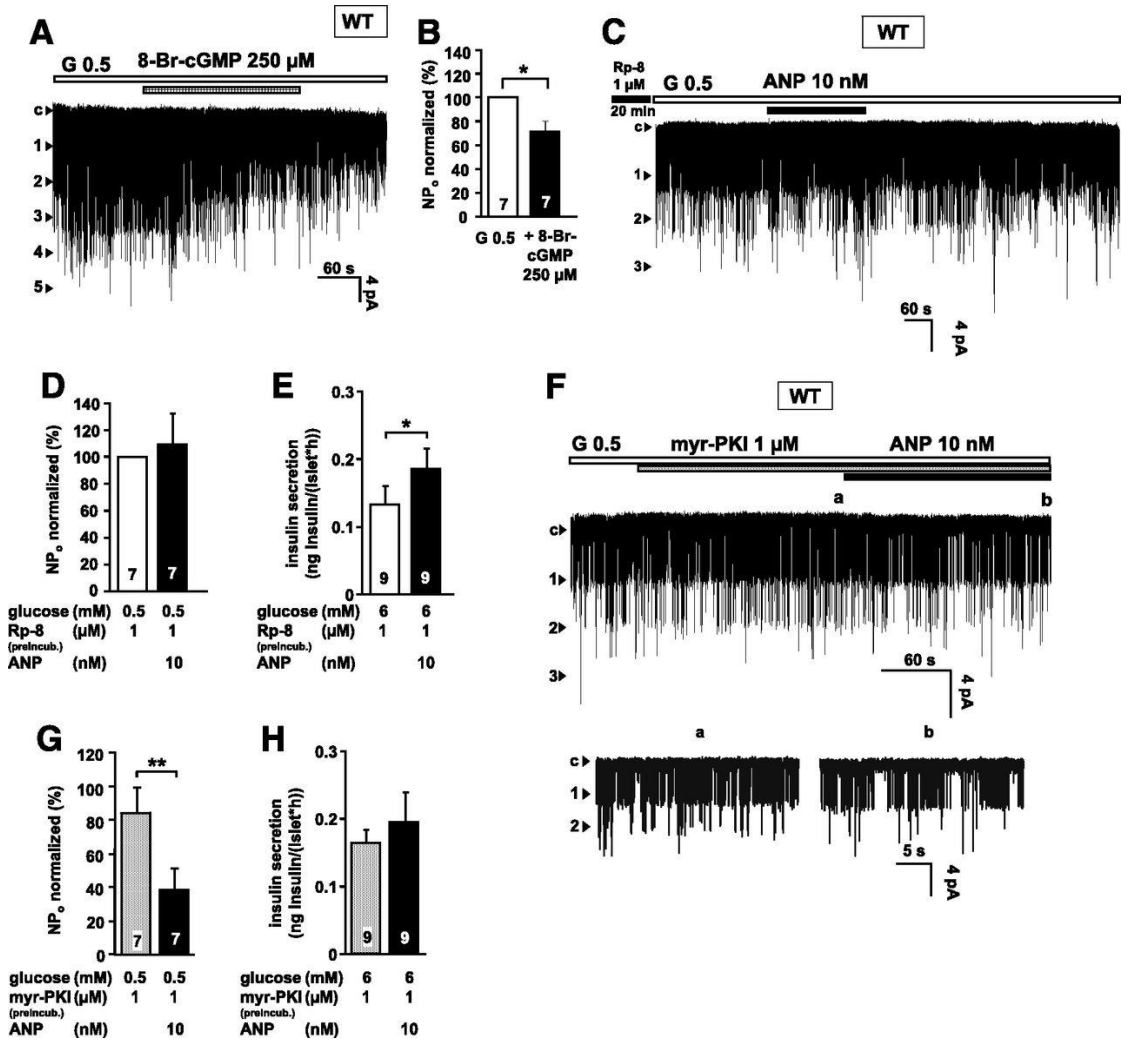



Fig. 5: Involvement of cGMP/PKG and cAMP/PKA signaling pathways in the effect of ANP on the $K_{ATP}$ current and insulin secretion. *A*: Typical recording showing the effect of 8-Br-cGMP on the $NP_o$ of single $K_{ATP}$ channels recorded in the cell-attached mode with a WT β-cell. *B*: Summary of the results. *C*: Typical recording showing the lack of effect of ANP after preincubation with Rp-8 on the $NP_o$ of single $K_{ATP}$ channels recorded in the cell-attached mode of a WT β-cell. *D*: Summary of the results. *E*: ANP effect on insulin secretion in the presence of 6 mmol/L glucose after preincubation of WT islets with Rp-8. *F*: Typical recording showing the effect of ANP in the presence of myr-PKI on the $NP_o$ of single $K_{ATP}$ channels recorded in the cell-attached mode of a WT β-cell. *G*: Summary of the results. *H*: ANP effect on insulin secretion in the presence of 6 mmol/L glucose and after treatment of WT islets with myr-PKI. The numbers within the columns are the number of different experiments with at least three different mice. *$P \leq 0.05$, **$P \leq 0.01$. c, closed state of the channel; G 0.5, 0.5 mmol/L glucose.

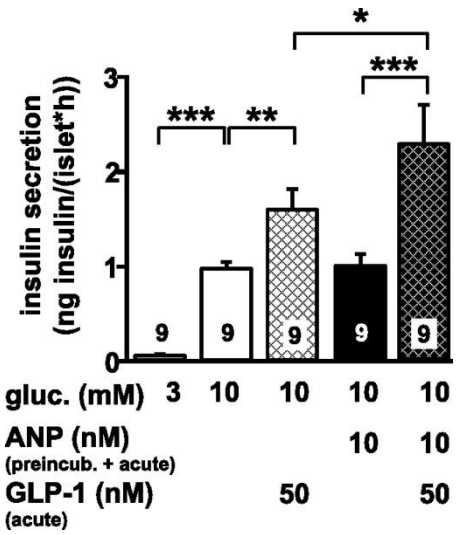



Fig. 6: Enhancement of the cGMP effect by cAMP. Potentiation of GLP-1–induced increase in insulin secretion by 90 min preincubation at room temperature with ANP. The experiments were performed with islets from WT mice. The numbers within columns are the number of different experiments. In each series, islets of at least three different mice were used. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$.

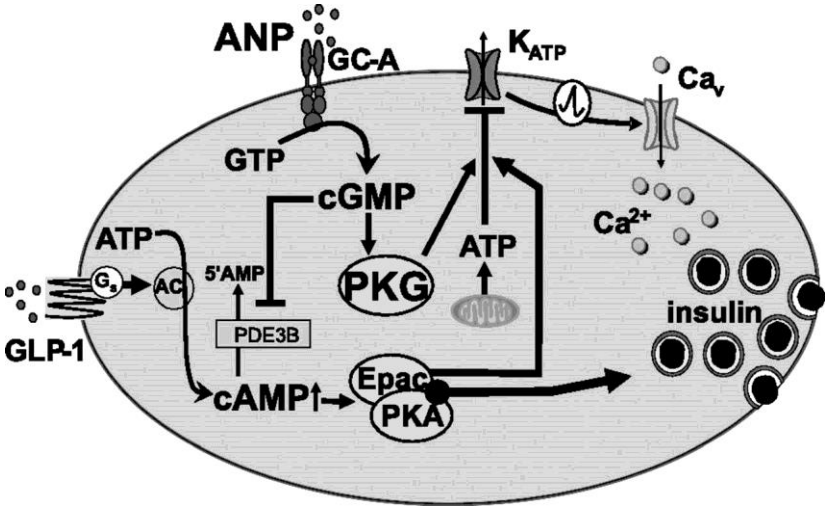
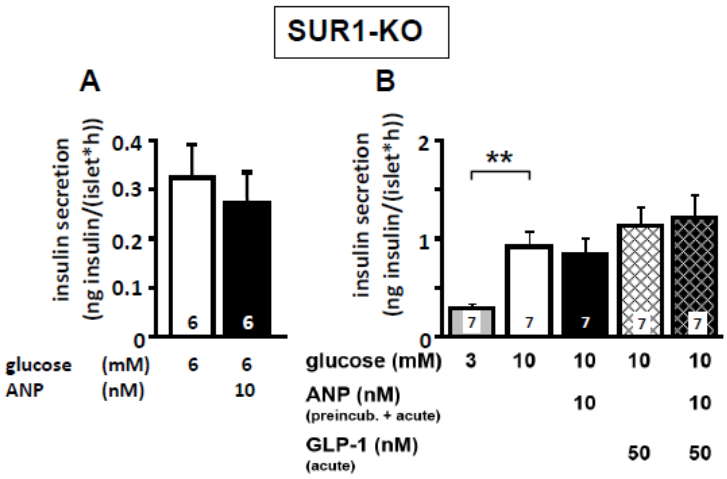
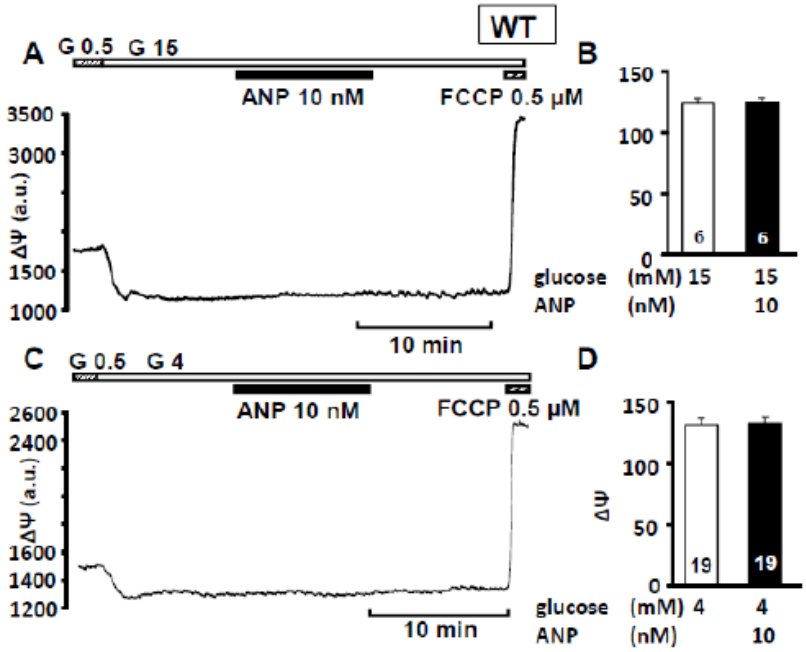


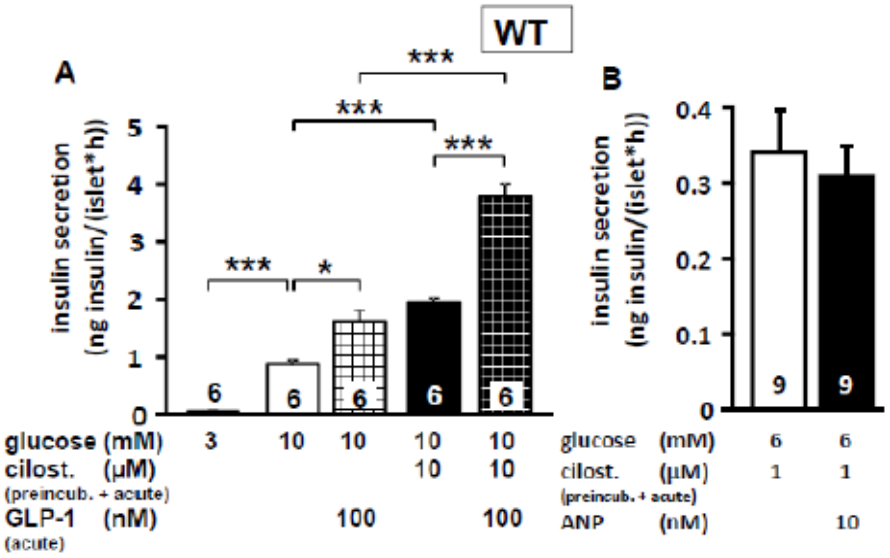

Fig. 7: Model showing how ANP affects stimulus-secretion coupling in β-cells. For details, see discussion. AC, adenylyl cyclase; $Ca_v$, voltage-dependent Ca channel; $G_s$, stimulative regulative G-protein.




**Supplementary Figure S1:** Lack of effects of ANP in SUR1-KO mice. A) Lack of stimulating effect of ANP on insulin secretion in the presence of 6 mM glucose. B) Lack of potentiation of the GLP-1 effect on insulin secretion in the presence of 10 mM glucose. Islets were preincubated with ANP for 90 min at room temperature. **P≤0.01




**Supplementary Figure S2:** Lack of effect of 10 nM ANP on the mitochondrial membrane potential ΔΨ in the presence of 15 or 4 mM glucose. A,C) Representative measurements showing the hyperpolarization induced by an increase of the glucose concentration from 0.5 to 15 and 4 mM glucose, respectively, indicating ATP production. Addition of ANP was without effect. At the end of each experiment, FCCP was added to determine the maximal depolarization of ΔΨ. B,D) Summary of all experiments conducted under the condition in A and C, respectively. The number in the columns gives the number of experiments with different cell clusters from 3 different WT mice.




**Supplementary Figure S3:** Effect of cilostamide on insulin secretion. A) Preincubation of the islets with the PDE3 inhibitor cilostamide potentiated the effect of GLP-1 on glucose-stimulated insulin secretion. B) In the presence of cilostamide ANP does not increase insulin secretion in 6 mM glucose. The experiments were performed with islets from WT mice. The number in the columns gives the number of different mice. *P≤0.05, ***P≤0.001

**ATP mediates a negative autocrine signal on stimulus-secretion coupling in mouse pancreatic β-cells**

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*Published:* Endocrine volume 63, pages270–283(2019

**Abstract**

Purpose: The role of ATP, which is secreted by pancreatic β-cells, is still a matter of debate. It has been postulated that extracellular ATP acts as a positive auto- or paracrine signal in β-cells amplifying insulin secretion. However, there is rising evidence that extracellular ATP may also mediate a negative signal.

Methods: We evaluated whether extracellular ATP interferes with the $Ca^{2+}$-mediated negative feedback mechanism that regulates oscillatory activity of β-cells.

Results: To experimentally uncover the $Ca^{2+}$-induced feedback we applied a high extracellular $Ca^{2+}$ concentration. Under this condition ATP (100 µM) inhibited glucose-evoked oscillations of electrical activity and hyperpolarized the membrane potential. Furthermore, ATP acutely increased the interburst phase of $Ca^{2+}$ oscillations and reduced the current through L-type $Ca^{2+}$ channels. Accordingly, ATP (500 µM) decreased glucose-induced insulin secretion. The ATP effect was not mimicked by AMP, ADP, or adenosine. The use of specific agonists and antagonists and mice deficient of large conductance $Ca^{2+}$-dependent $K^+$ channels revealed that P2X, but not P2Y receptors, and $Ca^{2+}$-dependent $K^+$ channels are involved in the underlying signaling cascade induced by ATP. The effectiveness of ATP to interfere with parameters of stimulus-secretion coupling is markedly reduced at low extracellular $Ca^{2+}$ concentration.

Conclusion: It is suggested that extracellular ATP which is co-secreted with insulin in a pulsatile manner during glucose-stimulated exocytosis provides a negative feedback signal driving β-cell oscillations in co-operation with $Ca^{2+}$ and other signals.

**Introduction**

ATP is the energy source of the cell, but serves as a signaling molecule, too. ATP enriched in insulin-containing granules via a vesicular nucleotide transporter [1] is thus secreted to the cell surface. The ATP concentration within the granules is around 3.5 mM [2]. ATP is co-released with insulin [3] or secreted without insulin in a process called kiss-and-run exocytosis [4] where only small molecules are discharged. Extracellular ATP is degraded by specific ecto-nucleotidases (for review see [5]) which contribute to the regulation of the extracellular ATP content. ATP exerts numerous important physiological effects in many mammalian cell types including pancreatic islets [6, 7] via activation of purinergic P2 receptors divided in the P2X and P2Y families with seven and eight subtypes, respectively. P2X receptors are ligand-operated cation channels while P2Y receptors belong to the large group of G-protein-coupled receptors. All these subtypes have been identified in the endocrine pancreas (for review see [7]). The effects of extracellular ATP on β-cell function are numerous, but often controversial depending on the species, cell systems, and experimental conditions. Moreover, activation of different receptor subtypes may induce various and even opposed effects which complicates the understanding of the action of extracellular ATP on β-cell function. Studies with murine β-cells mainly report an inhibitory effect of extracellular ATP on stimulus-secretion coupling (SSC), but the underlying mechanism is far from being clear. Two studies reported that extracellular ATP reduced insulin secretion of isolated mouse islets despite a reduction of $K^+$ conductance and KATP current, respectively [8, 9]. Petit et al. [8] assumed that the inhibitory effect of ATP can be attributed to the degradation of ATP to adenosine while Poulsen et al. [9] suggested that it is caused by an interaction with the exocytotic machinery via $P2Y_1$-induced activation of calcineurin. Other groups observed stimulation and inhibition of insulin secretion in dependence of different receptor subtypes [10,11,12]. Studies with P2Y receptor knockout (KO) mice did not solve the discrepancies since $P2Y_1$ receptor-KO mice show increased glucose-induced insulin secretion while $P2Y_{14}$ receptor-KO mice exhibit decreased secretion [13, 14]. In rats the results of extracellular ATP on β-cell function are less

controversial and in contrast to mice a stimulatory effect of ATP is supported by most studies (e.g. [15,16,17] although participating receptors and involved mechanisms are not entirely clear. IP3-induced $Ca^{2+}$ mobilization with subsequent activation of $Ca^{2+}$ influx via CRAC channels are mechanisms discussed in this context. ATP-evoked $Ca^{2+}$ release from the endoplasmic reticulum (ER) is observed in mouse, rat, and human β-cells; however, in mouse β-cells this seems not to result in CRAC channel activation [18]. Hellman and colleagues [19,20,21] suggested that extracellular ATP is involved in the control of the rhythmic activity of β-cells and in the propagation of the oscillations from cell to cell. In the proposed model ATP has a time-dependent dual effect on β-cell function: prompt activation followed by inhibition.

In contrast to other studies, we focused on the influence of the nucleotide on bursting activity of β-cells. In particular, we investigated effects of ATP on β-cell function at conditions with enhanced $Ca^{2+}$-mediated negative feedback on SSC, especially reflecting the burst phases with action potentials.

**Materials and methods**

*Animals and islet preparation.* Islets of Langerhans were isolated from adult C57Bl/6N mice or C57Bl/6N mice with a global knockout of BK channels (BK-KO). The mice were bred in the animal facility of the Department of Pharmacology at the University of Tübingen. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and German laws were followed. Isolation and culture were performed as described previously [22], except, islets of Langerhans were dispersed to single cells or cell clusters by trypsin treatment.

*Solutions and chemicals.* Recordings of $[Ca^{2+}]_c$ were performed with a bath solution which contained (in mM): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 10 HEPES; $CaCl_2$, and glucose as indicated, pH 7.4 adjusted with NaOH. The same bath solution was used for the determination of the mitochondrial membrane potential (Δψ) and for measurements of membrane potential (Vm) in the perforated-patch configuration. For this purpose, the pipette solution was composed of (in mM): 10 KCl, 10 NaCl, 70 $K_2SO4$, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 20 HEPES, 0.27 amphotericin B, pH adjusted to 7.15 with KOH. For perforated-patch measurements of $Ca^{2+}$ currents a bath solution of the following composition was used (mM): 115 NaCl, 1.2 $MgCl_2$, 10 $CaCl_2$, 10 TEA,

10 glucose, 10 HEPES, pH 7.4 adjusted with NaOH. The respective pipette solution contained (in mM): 10 KCl, 10 NaCl, 70 $Cs_2SO_4$, 7 MgCl2, 10 HEPES, 0.27 amphotericin B, pH adjusted to 7.15 with NaOH. Krebs–Ringer–Hepes solution for insulin secretion was composed of (in mM): 122 NaCl, 4.7 KCl, 1.1 $MgCl_2$, 10 $CaCl_2$, glucose as indicated, 10 HEPES, 0.5 % bovine serum albumin and pH 7.4 adjusted with NaOH.

Adenosine 5′-triposphate (ATP) was obtained from Carl Roth (Karlsruhe, Germany) or Sigma-Aldrich (Taufkirchen, Germany), the $P2X_{1,3}$-agonist α,β-methyleneadenosine 5′-triphosphate (αβ-MeATP) from Tocris Bioscience (Bristol, United Kingdom). Fura-2/AM was either purchased from Biotrend (Köln, Germany) or Sigma-Aldrich (Schnelldorf, Germany). Rhodamine, RPMI 1640 medium, and penicillin/streptomycin was from Invitrogen (Karlsruhe, Germany). All other chemicals were purchased from Sigma-Aldrich or Carl Roth in the purest form available.

*Measurements of the mitochondrial membrane potential.* ΔΨ was measured as rhodamine 123 fluorescence at 480 nm excitation wave length as described in [23]. To evaluate the effects, the values were averaged for 60 s at the end of each interval before solution change.

*Measurement of $[Ca^{2+}]_c$.* $[Ca^{2+}]_c$ was measured by the fura-2 method as described by Grynkiewicz et al. [24]. Details are described in [22]. In brief, cells were loaded with 5 µM fura-2-AM for 35 min at 37 °C. Fluorescence was excited at 340 and 380 nm, emission was filtered (LP515), and measured by a digital camera. $[Ca^{2+}]_c$ was calculated according to an in vitro calibration with fura2-5K-salt. The area under the curve (AUC) was taken to reveal the effect of ATP or αβ-MeATP on oscillations of the cytosolic $Ca^{2+}$ concentration. The AUC was evaluated in the steady state before the switch to ATP or αβ-MeATP and between min 0 to 5 and 5 to 10 in the presence of the nucleotide. In the experiments with NF-279 the AUC was calculated for 10 min after addition of the drug. In fura-2 measurements with whole islets the fluorescence ratio F340/F380 is given instead of $[Ca^{2+}]_c$.

*Patch-clamp measurements.* Membrane currents and potentials were recorded with an EPC-9 patch-clamp amplifier using “Patchmaster” software (HEKA, Lambrecht, Germany). For Vm measurements, the plateau potential under control conditions was

compared to the maximal hyperpolarization induced by ATP. Where applicable, action potential frequency was determined during a period of 2.5 min before ATP application and separately during the first and second 2.5 min period after addition of ATP. At the high $Ca^{2+}$ concentration of 10 mM the membrane potential oscillated, i.e., burst phases with action potentials changed with silent interburst phases. Under this condition the fraction of plateau phase (FOPP ~ percentage of time with spike activity) was calculated for 2 min before and during min 3 and 5 after drug application. Currents through L-type $Ca^{2+}$ channels were measured using the perforated-patch configuration in the voltage-clamp mode by 50 ms pulses from –70 to 0 mV. The last three currents prior to solution change were used for analysis of the maximum peak current (Ipeak), the AUC to determine charge movement, and τ to characterize current inactivation.

*Insulin secretion.* After preparation islets were kept overnight in RPMI 1640 culture medium with 11.1 mM glucose. Details for steady-state incubations are described in [22]. Briefly, insulin secretion under steady-state conditions was measured for 1 h at 37 °C under conditions as indicated. For perifusion experiments 50 islets of Langerhans were perifused continuously with bath solution as described in [23] and test substances as indicated and a sample was taken every 2 min. Levels of insulin were determined by radioimmunoassay (Merck Millipore, Darmstadt, Germany).

*Statistics.* Each series of experiments was performed with islets of Langerhans or cell clusters from at least three independent preparations unless otherwise indicated. Means ± SEM are given for the indicated number of experiments. Statistical significance of differences was assessed by a Student's t-test for paired values. Multiple comparisons were made by ANOVA followed by Student–Newman–Keuls test. P-values ≤0.05 were considered significant.

## Results

*Extracellular ATP affects key parameters of SSC*

*Cytosolic $Ca^{2+}$ concentration and cell membrane potential*

The cell membrane potential ($V_m$) takes a prominent position within SSC as it connects glucose metabolism to insulin secretion by determining the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$). Within the SSC, opening of voltage-dependent L-type $Ca^{2+}$ channels and $Ca^{2+}$ influx represent the decisive trigger for secretion of insulin from storage vesicles. For this reason, alterations of the cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_c$) are crucial for insulin secretion. The recording in Fig. 1a shows periodic oscillations of $[Ca^{2+}]_c$ in the presence of 10 mM glucose and 2.5 mM $Ca^{2+}$. Addition of ATP in a concentration of 100 µM interrupted this regular pattern so that the next oscillation appeared later and exhibited a smaller amplitude. To quantify this observation, the AUC of $[Ca^{2+}]_c$ was determined before and after addition of ATP (Fig. 1b). The AUC was reduced from 384 ± 27 nM × 5 min under control conditions to 300 ± 29 nM × 5 min during the first 5 min period in the presence of ATP. The AUC amounted to 353 ± 24 nM × 5 min in the second 5 min period of ATP treatment indicating a transient effect of ATP.

The plasma membrane potential Vm was measured in the perforated-patch configuration under the same conditions. The record (Fig. 1c) and the bar chart (Fig. 1d) show that ATP had no effect on Vm (−37 ± 3 mV under control conditions vs. −39 ± 3 mV in the first 2.5 min and −38 ± 4 mV in the second 2.5 min period in the presence of ATP). Likewise, action potential frequency did not change (Fig. 1c, e). It amounted to 3.3 ± 0.6 Hz before ATP application, to 2.9 ± 0.6 Hz during the first 2.5 min period with ATP (n = 4, n.s.), and to 3.4 ± 0.5 Hz during the second 2.5 min period with ATP (n = 4, n.s.). Under these conditions characteristic slow waves consisting of electrically silent phases (interbursts) and active phases (bursts with action potentials) are hardly detectable [25]. Bursting activity can be achieved by increasing the extracellular $Ca^{2+}$ concentration (compare Fig. 1c and Fig. 2c). It is known that $[Ca^{2+}]c$ affects its own entry via opening of $K_{ATP}$ channels, i.e., it exerts an important feedback control on insulin secretion [26, 27]. Increasing the extracellular $Ca^{2+}$ concentration which enhances the $Ca^{2+}$ gradient and thus $Ca^{2+}$ entry augments

this feedback mechanism under experimental conditions. To evaluate whether extracellular ATP interferes with this $Ca2^{+}$-mediated feedback mechanism, it was amplified in the following experiments by applying 10 mM $Ca^{2+}$ in the presence of 10 mM glucose (G10/Ca10). Treatment with 100 µM ATP strongly reduced the AUC of $[Ca^{2+}]c$ from 414 ± 26 nM × 5 min under control conditions to 188 ± 19 and 319 ± 34 nM × 5 min, respectively, during the first and second 5 min period of ATP addition (Fig. 2a, b). Under these conditions with 10 mM Ca2+ the effect of ATP was much larger in the first 5 min period of ATP addition compared to conditions with 2.5 mM $Ca^{2+}$ and remained significant in the second application period. Furthermore, the interburst phase directly after addition of ATP was markedly longer compared to the mean interburst phase under control condition (247±35 vs. 117±21 s, n = 20, P ≤ 0.001). The representative measurement of electrical activity in Fig. 2c shows characteristic slow waves in the presence of G10/Ca10 as a result of the $Ca^{2+}$ feedback described above. Addition of ATP (100 µM) led to a sustained hyperpolarization of Vm from a plateau potential of −44.2 ± 1.4 to −68.0 ± 3.5 mV (Fig. 2d).

*Mitochondrial membrane potential*

Hyperpolarization of $V_m$ can be caused by opening of $K_{ATP}$ channels due to a reduction in ATP production. The mitochondrial membrane potential ($\Delta\Psi$) is directly linked to glucose metabolism and ATP production [27] because the electrochemical proton gradient across the inner mitochondrial membrane determines the activity of the F1/F0-ATPase. Increasing glucose concentration caused a hyperpolarization of $\Delta\Psi$, which is indicated by a decrease in rhodamine 123 fluorescence signal and reflects ATP production. On average, the fluorescence signal was lowered from 502 ± 47 a.u. in the presence of 0.5 mM glucose to 412 ± 32 a.u. upon an increase of the glucose concentration to 10 mM (P ≤ 0.001, n = 28). ATP (100 µM) had no effect on $\Delta\Psi$ (409±32 a.u.) (n = 28, n.s., data not shown). The experiment was repeated with 500 µM ATP and additionally in 5 mM glucose to create conditions where $\Delta\Psi$ is not that hyperpolarized but ATP did not show any effect (G10/Ca10: 530±35 a.u., G10/Ca10 + 500 µM ATP: 528±34 a.u., n = 27, n.s.; G5/Ca10: 550±45 a.u., G5/Ca10 + 500 µM ATP: 552±45 a.u., n = 16, n.s., data not shown). Thus, extracellular ATP seems not to affect mitochondrial ATP production.

*Current through voltage-dependent $Ca^{2+}$ channels*

Next, a possible influence of ATP on $Ca^{2+}$ currents was studied. In Fig. 3a, a typical $Ca^{2+}$ current is shown which was elicited by a 50 ms voltage step from −70 to 0 mV. Under control conditions (black curve) opening of L-type $Ca^{2+}$ channels led to a marked and rapid $Ca^{2+}$ influx followed by a slow current decay due to $Ca^{2+}$-dependent current inactivation. Addition of ATP (100 µM, dotted curve) reduced the peak $Ca^{2+}$ current (Ipeak) from −87 ± 8 to −72 ± 9 pA (Fig. 3b) and the charge movement, measured as area under the curve (AUC), from 2.6 ± 0.3 to 2.1 ± 0.2 pC (Fig. 3c). The $Ca^{2+}$-dependent inactivation of the channels [28] which was determined by calculating the time constant τ was not influenced by ATP (22 ± 3 ms under control conditions vs. 21 ± 2 ms with ATP) (Fig. 3d).

*Extracellular ATP reduces insulin secretion*

The effect of ATP on insulin secretion was studied in steady state as well as in perifusion experiments. As seen in Fig. 4a, after 1 h steady-state incubation in G15/Ca10, ATP (500 µM and 1 mM) reduced glucose-induced insulin secretion by 26% and 33%, respectively. ATP in a concentration of 100 µM was without effect in these experiments. In contrast to the experiments described before, insulin secretion measurements are performed with whole islets which are encircled by a capsule of connective tissue. Evidently, this capsule impedes diffusion of ATP to the islets cells, an observation that confirms earlier findings [29]. To test for this, the $Ca^{2+}$ experiments illustrated in Fig. 2a, b were repeated with whole islets instead of dispersed cells. Indeed, 100 µM ATP did not influence $[Ca^{2+}]_c$ measured with whole islets (as fluorescence ratio F340/F380) (10 mM glucose: 0.48 ± 0.09; after ATP application: 0.48 ± 0.09, n = 6 different islets, not shown). This suggestion is supported by the finding that pre-treatment of islets for 1 h with 100 and 200 µM ATP, respectively, reduced the response to a subsequent glucose stimulus (Fig. 4b).

The inhibitory effect of ATP on insulin secretion was not mimicked by AMP, ADP, or adenosine (Suppl. Figure 1). In contrast to ATP, concentrations of adenosine higher than 100 µM increased insulin secretion. Perifusion experiments disclosed that ATP diminished the first and second phase of insulin secretion. Rising the glucose concentration from 3 to 15 mM glucose in the absence (solid curve) and presence of

ATP (dotted curve) revealed that the first phase of insulin secretion was markedly reduced by ATP (Fig. 5a). The quantitative analyses of the AUC demonstrated that ATP decreased the first phase of insulin secretion from 61 ± 14 ng insulin/(50 islets × 30 min) to 41 ± 10 ng insulin/(50 islets × 30 min) (Fig. 5b). In Fig. 5c the glucose concentration was first raised from 3 to 15 mM glucose showing the typical biphasic pattern of insulin secretion. The figure shows the typical steep, transient rise during the first phase followed by a lower but still elevated plateau in the second phase. Addition of ATP at the steady state during the second phase reduced insulin secretion. For further quantification the AUC was calculated for the last 10 min in the absence and presence of ATP, respectively. It decreased from 15 ± 2 ng insulin/(50 islets × 10 min) to 12 ± 2 ng insulin/(50 islets × 10 min) (Fig. 5d).

*Extracellular ATP mediates its effects by activation of P2X receptors*

The $P2X_{1,3}$ agonist αβ-MeATP was used to test whether ATP-induced effects on SSC are mediated via P2X receptors. As shown in Fig. 6a αβ-MeATP mimicked the effect of ATP on $[Ca^{2+}]_c$ oscillations. αβ-MeATP led to a decrease in the AUC of $[Ca^{2+}]_c$ (Fig. 6b) from 345 ± 22 nM × 5 min under control conditions to 264 ± 33 and 287 ± 30 nM × 5 min, respectively, during the first and second 5 min period in the presence of αβ-MeATP. As observed with ATP, duration of the interburst phase directly after addition of αβ-MeATP was prolonged compared to the mean interburst phase before application of the $P2X_{1,3}$ agonist (240±54 vs. 123±17 s, n = 16, P≤0.05). Furthermore, αβ-MeATP reduced glucose-induced insulin secretion by 36% (from 3.1 ± 0.4 ng insulin/(islet × h) under control conditions to 2.0 ± 0.1 ng insulin/(islet × h) in the presence of αβ-MeATP) after 1 h steady-state incubation in 15 mM glucose and 10 mM $Ca^{2+}$ (Fig. 6c). Insulin secretion experiments with the $P2X_1$ antagonist NF-279 revealed that ATP is no longer able to reduce insulin secretion when $P2X_1$ channels are blocked (Fig. 6d). Insulin secretion in the presence of NF-279 was 2.2 ± 0.2 ng insulin/(islet × h) before and 2.2 ± 0.1 ng insulin/(islet × h) after addition of ATP. In contrast, the $P2X_3$ antagonist RO-3 was not able to suppress the inhibitory effect of extracellular ATP (Fig. 6e). Insulin secretion in the presence of RO-3 was 3.9 ± 0.7 ng insulin/(islet × h) before and 2.4 ± 0.3 ng insulin/(islet × h) after addition of ATP.

The most prominent receptor of the P2Y family in β-cells is the $P2Y_1$ receptor [12, 13, 30]. This receptor family is Gq protein-coupled and thus affects intracellular $Ca^{2+}$

stores. We observed a short $Ca^{2+}$ transient after ATP administration (Fig. 2a). This was also present when L-type $Ca^{2+}$ channels were blocked (Suppl. Figure 2A, B) and is due to ER store depletion (Suppl. Figure 2C, D). However, pre-treatment of β-cells with the SERCA inhibitor thapsigargin did not affect the inhibitory effect of ATP on insulin secretion (Suppl. Figure 2E). Moreover, the inhibitory effect of ATP on insulin secretion was not influenced by the specific $P2Y_1$ receptor antagonist MRS-2179 (Suppl. Figure 2F). Obviously, the P2Y receptor family does not essentially contribute to the inhibitory effect of ATP.

*Possible involvement of $Ca^{2+}$-dependent potassium channels*

As P2X receptors are unspecific cation channels leading to a depolarizing cation influx, activation of these receptors seems hard to reconcile with the above-mentioned negative influences of ATP and αβ-MeATP on parameters of SSC. In β-cells several types of $Ca^{2+}$-dependent $K^+$ channels are expressed mediating $K^+$ outflux upon activation including BK channels with large conductance and SK4 channels with intermediate conductance. Both channel types are involved in the regulation of β-cell function [31, 32]. To test whether extracellular ATP activates these channels to mediate a negative feedback on SSC, we used β-cells from BK-KO mice and TRAM-34 as a pharmacologic inhibitor of SK4 channels.

Figure 7a shows a measurement of Vm in the perforated-patch configuration with BK-KO β-cells. After SK4 channel inhibition the BK channel-deficient β-cells are rather depolarized and show continuous spike activity. Administration of ATP (100 µM) only led to a transient hyperpolarization of −10.7 ± 2.6 mV in six out of nine experiments (from −39 ± 3 mV in the presence of TRAM-34 in 10 mM glucose and 10 mM Ca2+ to −50 ± 3 mV after application of ATP (Fig. 7b)). A slight depolarization (from −34 ± 3 mV in the presence of TRAM-34 to −29 ± 3 mV) was observed in all nine measurements during the last 3 min of ATP addition (Fig. 7c).

In addition, the effect of ATP on $Ca^{2+}$ oscillations is reduced after inhibition of SK4 channels in BK-KO cells. Figure 7d shows $Ca^{2+}$ oscillations in β-cells from BK-KO mice in the presence of TRAM-34. Under these conditions ATP reduced the AUC of $[Ca^{2+}]_c$ only transiently despite of the high extracellular $Ca^{2+}$ concentration (Fig. 7e) which fits to the transient effect of ATP on electrical activity. The AUC decreased

from 472 ± 29 nM × 5 min under control conditions with TRAM-34 to 339 ± 20 nM × 5 min in the presence of ATP during the first 5 min application period. In the second 5 min period of ATP addition the AUC of $[Ca^{2+}]_c$ amounted to 439 ± 21 nM × 5 min.

In agreement with the influence of BK and SK4 channels on ATP-induced alterations in $[Ca^{2+}]_c$, the effect of ATP on insulin secretion was reduced after inhibition of these channels. Five hundred micromolar ATP led to a significant reduction of glucose-stimulated insulin secretion which amounted to 34% under control conditions but only to 23% after pharmacological blockage of BK channels with 100 nM iberiotoxin and SK4 channels with 10 µM TRAM-34. In this series of experiments, insulin secretion (15 mM glucose) was reduced by ATP from 4.5 ± 0.5 to 2.9 ± 0.1 ng insulin/(islet × h) ($n = 6$, $P \leq 0.05$). In the presence of TRAM-34 and iberiotoxin the effect of ATP was lower (reduction from 4.2 ± 0.3 to 3.1 ± 0.2 ng insulin/(islet × h), $n = 6$, $P \leq 0.05$, data not shown).

*Blockage of $P2X_1$ channels increased electrical activity and $[Ca^{2+}]_c$*

According to our hypothesis inhibition of the $P2X_1$ channels should increase electrical activity, the AUC of $[Ca^{2+}]_c$, and insulin secretion. Figure 8a, b reveals that NF-279 indeed augmented the AUC of $[Ca^{2+}]_c$ from 592 ± 35 nM × 10 min under control conditions to 632 ± 45 nM × 10 min in the presence of 5 µM NF-279. Accordingly, the FOPP increased from 46 ± 9 to 56 ± 11% after addition of NF-279 (Fig. 8c, d). Paradoxically, insulin secretion was reduced by NF-279 from 2.81 ± 0.30 to 2.16 ± 0.18 ng insulin/(islet × h) ($n = 6$, $P \leq 0.05$). Since this is unexpected and does not fit to the results obtained with NF-279 on $[Ca^{2+}]_c$ and $V_m$, it is suggested that this effect is an unspecific $Ca^{2+}$-independent interaction with the exocytotic machinery. To further strengthen this conclusion, we tested suramin on $[Ca^{2+}]_c$, another frequently used blocker of $P2X_1$ channels, although less specific. Suramin enhanced the AUC of $[Ca^{2+}]_c$ during a 5 min application period in 9 out of 13 cells from 323 ± 29 nM × 5 min to 404 ± 42 nM × 5 min. Subsequent addition of ATP for 5 min in the presence of suramin did not significantly alter the AUC of $[Ca^{2+}]_c$ (Fig. 8e, f).

**Discussion**

*The inhibitory signaling pathway of extracellular ATP*

It is well known that signaling through purinergic receptors affects insulin secretion and that ATP released from secretory granules or nerve endings can activate these receptors. Effects on β-cells are mediated by P2X and P2Y receptors but not by P1 receptors (for review see [33]). The physiological significance of activation of purinergic receptors is still debated; stimulation and inhibition of insulin secretion have been reported. The situation is complex because each receptor has many subtypes and subtype expression varies between species and between primary β-cells and insulin-secreting tumor cells.

We have chosen a protocol for our experiments where we stimulated β-cells and islets by glucose and raised $Ca^{2+}$ influx by increasing the electrochemical gradient for $Ca^{2+}$. This protocol enhances the $Ca^{2+}$ -mediated negative feedback on β-cell function [26] which is most prominent during burst phases with $Ca^{2+}$ action potentials. Under these conditions, extracellular ATP inhibited SSC of β-cells as evidenced by hyperpolarization of $V_m$, reduced $Ca^{2+}$ influx and AUC of $[Ca^{2+}]c$ and decreased insulin secretion. This effect was specific for ATP and not mimicked by AMP, ADP, or adenosine suggesting interference of ATP with specific receptors. Our results point to the involvement of $P2X_1$ receptors under these conditions: (1) The $P2X_{1,3}$ agonist αβ-MeATP mimicked the effect of extracellular ATP on insulin secretion. (2) The $P2X_1$ receptor antagonist NF-279 suppressed the effect of ATP on insulin release but not the $P2X_3$ receptor antagonist RO-3. (3) The $P2Y_1$ antagonist MRS-2179 did not influence the effect of ATP on insulin secretion.

The $P2X_1$ receptor is a non-specific cation channel with a relatively high permeability to $Ca^{2+}$ [34]. $P2X_1$ receptor activation by extracellular ATP leads to $Ca^{2+}$ influx and the local enhancement of $Ca^{2+}$ in the sub-membrane space obviously influences the activity of other ion channels: (1) Extracellular ATP reduced the charge movement through L-type $Ca^{2+}$ channels. This may be explained by a reduction of the driving force for $Ca^{2+}$ due to the local increase of the intracellular $Ca^{2+}$ concentration after $P2X_1$ receptor activation. Since V influx through L-type $Ca^{2+}$ channels constitutes the major trigger signal for insulin secretion [35], reduced influx would diminish secretion.

(2) The effectiveness of ATP to inhibit SSC was reduced after genetic and pharmacological deletion of two $Ca^{2+}$ -activated $K^+$ channels present in β-cells, the BK and SK4 channels. An increase in the current through these channels after $P2X_1$ receptor activation would hyperpolarize the membrane and in turn lower insulin secretion contributing to the negative feedback induced by ATP.

Since the early paper of Gylfe and Hellman [36], who demonstrated that extracellular ATP induces $Ca^{2+}$ release from ER $Ca^{2+}$ stores, a variety of studies has shown similar results. These observations are confirmed by our experiments. Notably, store depletion does not affect the effectiveness of ATP to inhibit insulin secretion. This indicates that the sub-membrane increase in $Ca^{2+}$ is decisive for the effect of ATP.

*Physiological significance of extracellular ATP for β-cell oscillations*

Oscillatory activity of β-cells is a prerequisite for normal action of insulin and glycemic control. Disturbances in the fluctuations of the parameters of SSC profoundly impair insulin signaling in peripheral tissues and are early events in diabetes [37]. We have extensively studied the mechanisms underlying these oscillations earlier [27, 31]. In the present paper we suggest a negative feedback for extracellular ATP in synergism with $Ca^{2+}$, i.e., a role for ATP in the termination of burst phases with action potentials. Partial inhibition of this feedback by blockade of $P2X_1$ channels indeed prolonged burst phases and thus increased electrical activity and the AUC of $[Ca^{2+}]_c$.

An extracellular ATP concentration of 100 µM, as used in the present work, seems not to be extraordinarily high considering that the ATP concentration within exocytotic granules of β-cells is around 3.5 mM [2]. In 1998 Hazama and co-workers [38] demonstrated that the ATP concentration at the cell surface is above 25 µM after stimulation of rat β-cells with glucose. They concluded that ATP can reach concentrations high enough to stimulate purinergic receptors during insulin secretion. Therefore, we used 100 µM ATP to realize an effect of ATP additional to that evoked by endogenous ATP from the granules. In experiments with whole islets, even higher concentrations of ATP (500 µM) were needed, which is most likely due to the capsule of connective tissue surrounding the islets which can form a barrier for molecules like ATP (see also Extracellular ATP reduces insulin secretion). Interestingly, Weitz et al. [6] recently demonstrated that tissue-resident macrophages of islets sense the

interstitial ATP concentration. It is concluded that macrophages use ATP as a signal to estimate the activity state of the β-cells via purinergic receptors in order to balance their secretion products which influence β-cell proliferation. To achieve maximum $Ca^{2+}$ signals in macrophages concentrations of ATP up to 1 mM were used.

In most experiments we have used a high extracellular $Ca^{2+}$ concentration of 10 mM to augment the negative feedback mediated by $Ca^{2+}$ which is most important during the burst phases. This feedback is thought to contribute to the initiation of burst termination. Exocytotic vesicles contain 120 mM $Ca^{2+}$ which is mostly bound. Anyhow, the free granular $Ca^{2+}$ concentration reaches 10 mM [2] suggesting that the local $Ca^{2+}$ concentration on the surface of the cells is higher than the bulk $Ca^{2+}$ concentration of the extracellular space. Enhanced local $Ca^{2+}$ concentration steepens the gradient driving $Ca^{2+}$ entry.

Ion flux through P2X receptors is dependent on the extracellular $Ca^{2+}$ concentration, i.e., $Ca^{2+}$ influx through P2X increases concomitantly with rising extracellular $Ca^{2+}$ concentration [39] initiating an inhibitory signaling pathway (see above).

It has been demonstrated that a rise in the mitochondrial $Ca^{2+}$ concentration during a burst phase increases KATP current and hyperpolarizes Vm to initiate the interburst [40]. Based on our current results, it can be concluded that extracellular ATP and fluctuations of the intracellular $Ca^{2+}$ concentration act synergistically to exert a negative feedback that terminates the burst phases and perpetuate oscillatory activity.

*Proposed model*

The negative effects of extracellular ATP on SSC described in the present paper are in accordance with numerous reports in the literature, e.g. [8, 9, 19, 41]. This contrasts with findings about stimulating effects of ATP on β-cells in mouse and other species [11, 15, 16, 42,43,44]. To reconcile these observations it is hypothesized that short ATP pulses stimulate β-cell SSC [42], while longer exposure and higher concentrations induce a negative feedback [19,20,21, 30]. The authors suggest that this dual effect of extracellular ATP supports the coordination of $Ca^{2+}$ oscillations. Furthermore, it is assumed that short pulses of ATP evoked by kiss-and run exocytosis [45] (where only small molecules like ATP but no insulin is released) or by

exocytosis at the beginning of a burst phase activate P2Y receptors. Activation of these receptors increases $[Ca^{2+}]_c$ via $Ca^{2+}$ release from the ER, an effect also seen in our experiments. However, this is not sufficient to potentiate the first phase of insulin release.

Our observation that the effect of extracellular ATP depends on the concentration of extracellular $Ca^{2+}$ underlines the complexity of the action of ATP. At a low extracellular $Ca^{2+}$ concentration ATP hardly affects β-cell SSC. Obviously, an increased extracellular $Ca^{2+}$ concentration is necessary to unveil the ATP-induced negative feedback, i.e. extracellular ATP amplifies the negative feedback on β-cell function induced by high $Ca^{2+}$, thus contributing to the termination of bursts and the maintenance of β-cell oscillations.

According to our model (Fig. 9) the effect of ATP essentially depends on Vm and activity of $Ca^{2+}$-dependent ion channels during burst phases. During burst phases quickly enhanced extracellular ATP and $Ca^{2+}$ concentrations and thus marked ATP-mediated $Ca^{2+}$ influx through $P2X_1$ receptors prevail, exerting a negative feedback limiting the burst phase. This involves opening of SK4 and BK channels. The novel proposed feedback mechanism of ATP may offer new approaches to influence the regulation of β-cell activity.

**Author's contribution:** C.B., J.K., and J.S. researched data; P.K.-D. evaluated data and edited the manuscript; M.D. contributed to discussion and study design and edited the manuscript; G.D. designed the study, wrote and edited the manuscript, and contributed to discussion. G.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Acknowledgements:** We are grateful to Isolde Breuning for excellent technical assistance. BK-KO mice were kindly provided by Prof. Dr. Peter Ruth, Institute of Pharmacy, University of Tübingen.

**References**

1.K. Sawada, N. Echigo, N. Juge, T. Miyaji, M. Otsuka, H. Omote, A. Yamamoto, Y. Moriyama, Identification of a vesicular nucleotide transporter. Proc. Natl. Acad. Sci. USA 105(15), 5683–5686 (2008). https://doi.org/10.1073/pnas.0800141105

2. J.C. Hutton, E.J. Penn, M. Peshavaria, Low-molecular-weight constituents of isolated insulin-secretory granules. Bivalent cations, adenine nucleotides and inorganic phosphate. Biochem. J. 210(2), 297–305 (1983)

3. J.W. Leitner, K.E. Sussman, A.E. Vatter, F.H. Schneider, Adenine nucleotides in the secretory granule fraction of rat islets. Endocrinology 96(3), 662–677 (1975). https://doi.org/10.1210/endo-96-3-662

4. S. Obermuller, A. Lindqvist, J. Karanauskaite, J. Galvanovskis, P. Rorsman, S. Barg, Selective nucleotide-release from dense-core granules in insulin-secreting cells. J. Cell. Sci. 118(Pt 18), 4271–4282 (2005). https://doi.org/10.1242/jcs.02549

5. M. Cieslak, K. Roszek, Purinergic signaling in the pancreas and the therapeutic potential of ecto-nucleotidases in diabetes. Acta Biochim. Pol. 61(4), 655–662 (2014)

6. J.R. Weitz, M. Makhmutova, J. Almaca, J. Stertmann, K. Aamodt, M. Brissova, S. Speier, R. Rodriguez-Diaz, A. Caicedo, Mouse pancreatic islet macrophages use locally released ATP to monitor beta cell activity. Diabetologia 61(1), 182–192 (2018). https://doi.org/10.1007/s00125-017-4416-y

7. P. Petit, A.D. Lajoix, R. Gross, P2 purinergic signalling in the pancreatic beta-cell: control of insulin secretion and pharmacology. Eur. J. Pharm. Sci. 37(2), 67–75 (2009). https://doi.org/10.1016/j.ejps.2009.01.007

8. P. Petit, G. Bertrand, W. Schmeer, J.C. Henquin, Effects of extracellular adenine nucleotides on the electrical, ionic and secretory events in mouse pancreatic beta-cells. Br. J. Pharmacol. 98(3), 875–882 (1989)

9. C.R. Poulsen, K. Bokvist, H.L. Olsen, M. Hoy, K. Capito, P. Gilon, J. Gromada, Multiple sites of purinergic control of insulin secretion in mouse pancreatic beta-cells. Diabetes 48(11), 2171–2181 (1999)

10. S. Amisten, S. Meidute-Abaraviciene, C. Tan, B. Olde, I. Lundquist, A. Salehi, D. Erlinge, ADP mediates inhibition of insulin secretion by activation of P2Y13 receptors in mice. Diabetologia 53(9), 1927–1934 (2010). https://doi.org/10.1007/s00125-010-1807-8

11. M. Ohtani, K. Ohura, T. Oka, Involvement of P2X receptors in the regulation of insulin secretion, proliferation and survival in mouse pancreatic beta-cells. Cell. Physiol. Biochem. 28(2), 355–366 (2011). https://doi.org/10.1159/000331752

12. M. Ohtani, J. Suzuki, K.A. Jacobson, T. Oka, Evidence for the possible involvement of the P2Y(6) receptor in Ca (2+) mobilization and insulin secretion in mouse pancreatic islets. Purinergic Signal 4(4), 365–375 (2008). https://doi.org/10.1007/s11302-008-9122-2

13. C. Leon, M. Freund, O. Latchoumanin, A. Farret, P. Petit, J.P. Cazenave, C. Gachet, The P2Y(1) receptor is involved in the maintenance of glucose homeostasis and in insulin secretion in mice. Purinergic Signal 1(2), 145–151 (2005). https://doi.org/10.1007/s11302-005-6209-x

14. J. Meister, D. Le Duc, A. Ricken, R. Burkhardt, J. Thiery, H. Pfannkuche, T. Polte, J. Grosse, T. Schoneberg, A. Schulz, The G protein-coupled receptor P2Y14 influences insulin release and smooth muscle function in mice. J. Biol. Chem. 289(34), 23353–23366 (2014). https://doi.org/10.1074/jbc.M114.580803

15 F. Blachier, W.J. Malaisse, Effect of exogenous ATP upon inositol phosphate production, cationic fluxes and insulin release in pancreatic islet cells. Biochim. Biophys. Acta 970(2), 222–229 (1988)

16. P. Petit, D. Hillaire-Buys, M. Manteghetti, S. Debrus, J. Chapal, M.M. Loubatieres-Mariani, Evidence for two different types of P2 receptors stimulating insulin secretion from pancreatic B cell. Br. J. Pharmacol. 125(6), 1368–1374 (1998). https://doi.org/10.1038/sj.bjp.0702214

17. P. Petit, M. Manteghetti, R. Puech, M.M. Loubatieres-Mariani, ATP and phosphate-modified adenine nucleotide analogues. Effects on insulin secretion and calcium uptake. Biochem. Pharmacol. 36(3), 377–380 (1987)

18. Y.F. Zhao, R. Xu, M. Hernandez, Y. Zhu, C. Chen, Distinct intracellular Ca2+ response to extracellular adenosine triphosphate in pancreatic beta-cells in rats and mice. Endocrine 22(3), 185–192 (2003). https://doi.org/10.1385/ENDO:22:3:185

19. E. Grapengiesser, H. Dansk, B. Hellman, Pulses of external ATP aid to the synchronization of pancreatic beta-cells by generating premature Ca(2+) oscillations. Biochem. Pharmacol. 68(4), 667–674 (2004). https://doi.org/10.1016/j.bcp.2004.04.018

20. B. Hellman, H. Dansk, E. Grapengiesser, Pancreatic beta-cells communicate via intermittent release of ATP. Am. J. Physiol. Endocrinol. Metab. 286(5), E759–765 (2004). https://doi.org/10.1152/ajpendo.00452.2003

21. E. Gylfe, E. Grapengiesser, H. Dansk, B. Hellman, The neurotransmitter ATP triggers $Ca2+$ responses promoting coordination of pancreatic islet oscillations. Pancreas 41(2), 258–263 (2012). https://doi.org/10.1097/MPA.0b013e3182240586

22. B. Gier, P. Krippeit-Drews, T. Sheiko, L. Aguilar-Bryan, J. Bryan, M. Düfer, G. Drews. Suppression of $K_{ATP}$ channel activity protects murine pancreatic beta-cells against oxidative stress. J. Clin. Invest. 119(11), 3246–3256 (2009). https://doi.org/10.1172/JCI38817

23. A. Edalat, P. Schulte-Mecklenbeck, C. Bauer, S. Undank, P. Krippeit-Drews, G. Drews, M. Düfer. Mitochondrial succinate dehydrogenase is involved in stimulus-secretion coupling and endogenous ROS formation in murine beta cells. Diabetologia 58(7), 1532–1541 (2015). https://doi.org/10.1007/s00125-015-3577-9

24. G. Grynkiewicz, M. Poenie, R.Y. Tsien, A new generation of Ca2+ indicators with greatly improved fluorescence properties. J. Biol. Chem. 260(6), 3440–3450 (1985)

25. M. Düfer, D. Haspel, P. Krippeit-Drews, L. Aguilar-Bryan, J. Bryan, G. Drews, Oscillations of membrane potential and cytosolic $Ca^{2+}$ concentration in $SUR1^{-/-}$ beta cells. Diabetologia 47(3), 488–498 (2004). https://doi.org/10.1007/s00125-004-1348-0

26. J.C. Henquin, Glucose-induced electrical activity in beta-cells. Feedback control of ATP-sensitive K+ channels by Ca2+? [corrected]. Diabetes 39(11), 1457–1460 (1990)

27. P. Krippeit-Drews, M. Düfer, G. Drews, Parallel oscillations of intracellular calcium activity and mitochondrial membrane potential in mouse pancreatic B-cells. Biochem. Biophys. Res. Commun. 267(1), 179–183 (2000). doi:10.1006/bbrc.1999.1921 S0006-291X(99)91921-6 [pii]

28. T.D. Plant, Properties and calcium-dependent inactivation of calcium currents in cultured mouse pancreatic B-cells. J. Physiol. 404, 731–747 (1988)

29. Maczewsky, J., Sikimic, J., Bauer, C., Krippeit-Drews, P., Wolke, C., Lendeckel, U., Barthlen, W., Drews, G. The LXR ligand T0901317 acutely inhibits insulin secretion by affecting mitochondrial metabolism. Endocrinology (2017). https://doi.org/10.1210/en.2016-1941

30. A. Salehi, S.S. Qader, E. Grapengiesser, B. Hellman, Inhibition of purinoceptors amplifies glucose-stimulated insulin release with removal of its pulsatility. Diabetes 54(7), 2126–2131 (2005)

31. M. Düfer, B. Gier, D. Wolpers, P. Krippeit-Drews, P. Ruth, G. Drews, Enhanced glucose tolerance by SK4 channel inhibition in pancreatic beta-cells. Diabetes 58, 1835–1843 (2009). doi:db08-1324 [pii] 10.2337/db08-1324

32. M. Düfer, Y. Neye, K. Hörth, P. Krippeit-Drews, A. Hennige, H. Widmer, H. McClafferty, M.J. Shipston, H.U. Häring, P. Ruth, G. Drews, BK channels affect glucose homeostasis and cell viability of murine pancreatic beta cells. Diabetologia 54(2), 423–432 (2011). https://doi.org/10.1007/s00125-010-1936-0

33. G. Burnstock, Purinergic signalling in endocrine organs. Purinergic Signal 10(1), 189–231 (2014). https://doi.org/10.1007/s11302-013-9396-x

34. G. Burnstock, Purine and pyrimidine receptors. Cell. Mol. Life Sci. 64(12), 1471–1483 (2007). https://doi.org/10.1007/s00018-007-6497-0

35. S.N. Yang, P.O. Berggren, The role of voltage-gated calcium channels in pancreatic beta-cell physiology and pathophysiology. Endocr. Rev. 27(6), 621–676 (2006). https://doi.org/10.1210/er.2005-0888

36. E. Gylfe, B. Hellman, External ATP mimics carbachol in initiating calcium mobilization from pancreatic beta-cells conditioned by previous exposure to glucose. Br. J. Pharmacol. 92(2), 281–289 (1987)

37. N. Porksen, M. Hollingdal, C. Juhl, P. Butler, J.D. Veldhuis, O. Schmitz, Pulsatile insulin secretion: detection, regulation, and role in diabetes. Diabetes 51(Suppl 1), S245–254 (2002)

38. A. Hazama, S. Hayashi, Y. Okada, Cell surface measurements of ATP release from single pancreatic beta cells using a novel biosensor technique. Pflug. Arch. 437(1), 31–35 (1998)

39. K. Nakazawa, K. Fujimori, A. Takanaka, K. Inoue, An ATP-activated conductance in pheochromocytoma cells and its suppression by extracellular calcium. J. Physiol. 428, 257–272 (1990)

40. J.F. Rolland, J.C. Henquin, P. Gilon, Feedback control of the ATP-sensitive $K^+$ current by cytosolic $Ca^{2+}$ contributes to oscillations of the membrane potential in pancreatic beta-cells. Diabetes 51(2), 376–384 (2002)

41. Q. Gong, M. Kakei, N. Koriyama, M. Nakazaki, S. Morimitsu, K. Yaekura, C. Tei, P2Y-purinoceptor mediated inhibition of L-type Ca2+ channels in rat pancreatic beta-cells. Cell Struct. Funct. 25(5), 279–289 (2000)

42. M.C. Jacques-Silva, M. Correa-Medina, O. Cabrera, R. Rodriguez-Diaz, N. Makeeva, A. Fachado, J. Diez, D.M. Berman, N.S. Kenyon, C. Ricordi, A. Pileggi, R.D. Molano, P.O. Berggren, A. Caicedo, ATP-gated P2X3 receptors constitute a positive autocrine signal for insulin release in the human pancreatic beta cell. Proc. Natl. Acad. Sci. USA 107(14), 6465–6470 (2010). doi:0908935107 [pii] 10.1073/pnas.0908935107

43. J. Fernandez-Alvarez, D. Hillaire-Buys, M.M. Loubatieres-Mariani, R. Gomis, P. Petit, P2 receptor agonists stimulate insulin release from human pancreatic islets. Pancreas 22(1), 69–71 (2001)

44. Khan, S., Yan-Do, R., Duong, E., Wu, X., Bautista, A., Cheley, S., MacDonald, P.E., Braun, M. Autocrine activation of P2Y receptors couples Ca influx to Ca release in human pancreatic beta cells. Diabetologia (2014). https://doi.org/10.1007/s00125-014-3368-8

45. P.E. MacDonald, M. Braun, J. Galvanovskis, P. Rorsman, Release of small transmitters through kiss-and-run fusion pores in rat pancreatic beta cells. Cell. Metab. 4(4), 283–290 (2006). https://doi.org/10.1016/j.cmet.2006.08.011

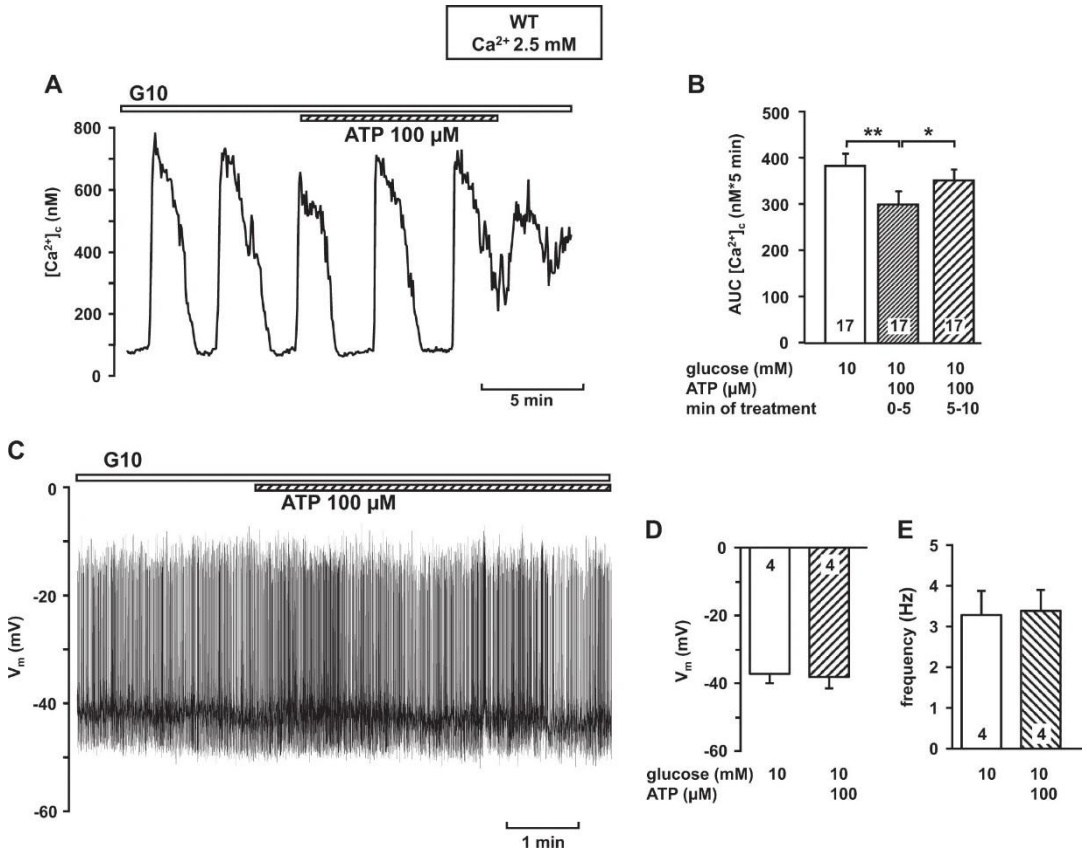



Fig.1: Extracellular ATP slightly affects $V_m$ and $[Ca^{2+}]_c$ in the presence of 10 mM glucose and 2.5 mM $Ca^{2+}$. **a** Representative recording presenting regular oscillations of $[Ca^{2+}]_c$. ATP application results in transiently reduced AUC of $[Ca^{2+}]_c$. **b** Summary of the quantitative analysis of the AUC of $[Ca^{2+}]_c$ before and after addition of ATP. **c** ATP does not affect $V_m$ in the presence of 2.5 mM $Ca^{2+}$. Representative recording showing continuous spike activity in response to 10 mM glucose and 2.5 mM $Ca^{2+}$, measured in the perforated-patch configuration. **d** Summary of the quantitative analysis of the membrane potential measurements. Values under control condition and after addition of ATP were taken at the plateau potential 2.5 min before solution change. **e** Action potential frequency determined during a period of 2.5 min before ATP application and between min 2.5 and 5 in the presence of ATP. The numbers in the columns indicate the number of experiments with different cell clusters from two to three different preparations. *$P \leq 0.05$, **$P \leq 0.01$

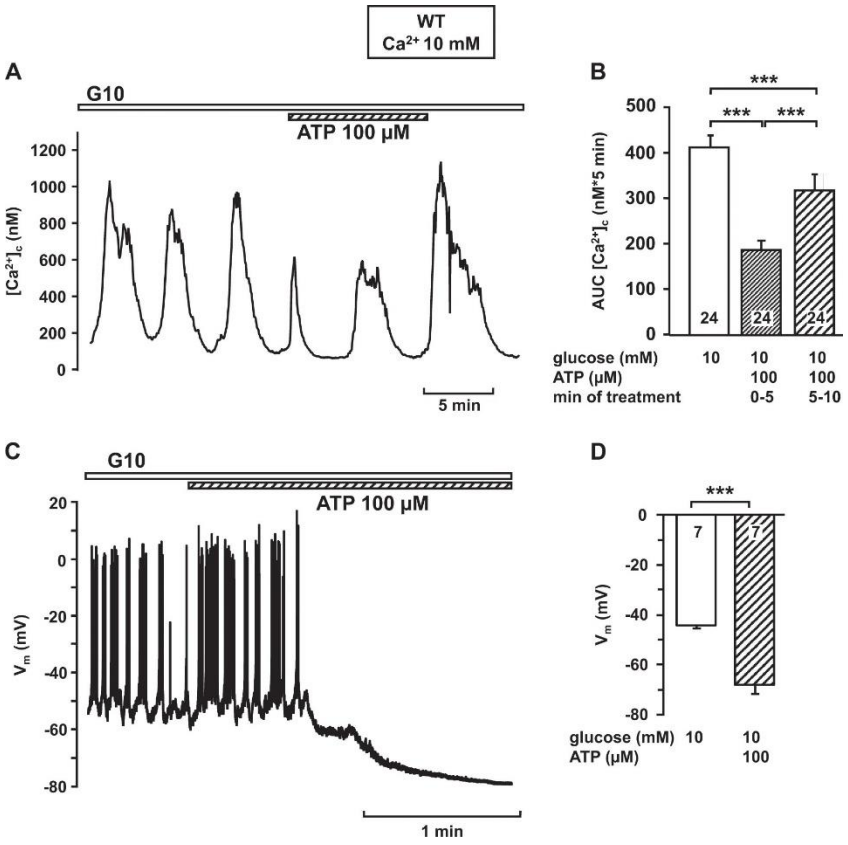



Fig.2: Enhancing the negative feedback of $Ca^{2+}$ on SSC augments the effectiveness of extracellular ATP on $V_m$ and $[Ca^{2+}]_c$. ATP clearly affects oscillations of $[Ca^{2+}]_c$ and $V_m$ in the presence of 10 mM glucose and 10 mM $Ca^{2+}$. **a** Representative recording showing regular oscillations of $[Ca^{2+}]_c$. ATP addition leads to a reduction in the AUC of $[Ca^{2+}]_c$. **b** Summary of the quantitative analysis of the AUC of $[Ca^{2+}]_c$ before and after addition of ATP. **c** ATP hyperpolarizes the cell membrane potential ($V_m$). Representative recording measured in the perforated-patch configuration showing slow waves in response to 10 mM glucose and 10 mM $Ca^{2+}$. **d** Summary of the quantitative analysis of $V_m$ measurements. Values under control condition were taken at the plateau potential, values after application of ATP at the maximal hyperpolarization. The numbers in the columns indicate the number of experiments with different cell clusters from at least three different mice. ***$P \leq 0.001$

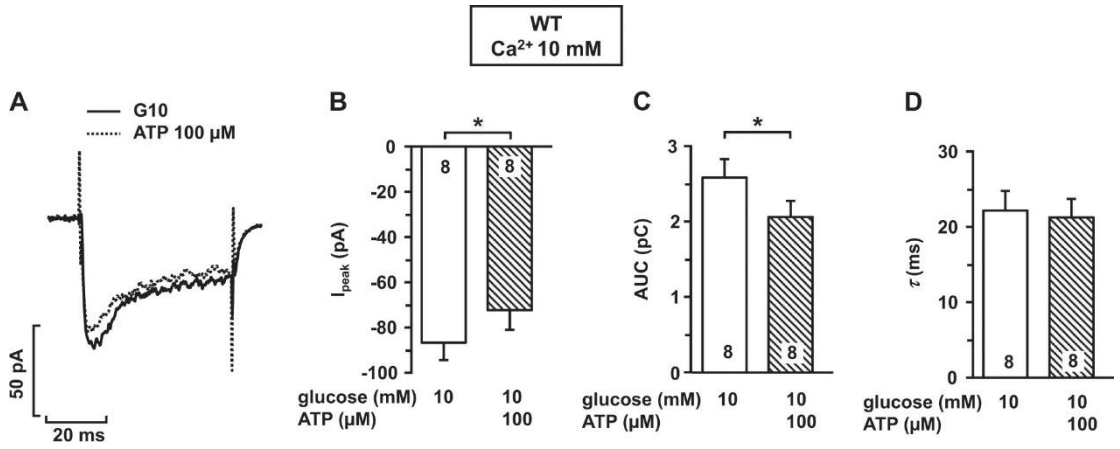



Fig.3: Extracellular ATP influences currents through L-type $Ca^{2+}$ channels. **a** Representative recordings of $I_{Ca}$ under control conditions (black curve) and after addition of ATP (dotted curve). Currents were elicited by 50 ms voltage steps from –70 to 0 mV. **b**–**d** Summary of the quantitative analysis of the peak current ($I_{peak}$) (**b**) and the charge movement (determined by the area under the curve (AUC)) (**c**) as well as the inactivation of the currents determined by the time constant *τ* (**d**). The numbers in the columns indicate the number of experiments with different cell clusters from at least three different mice. *$P \leq 0.05$

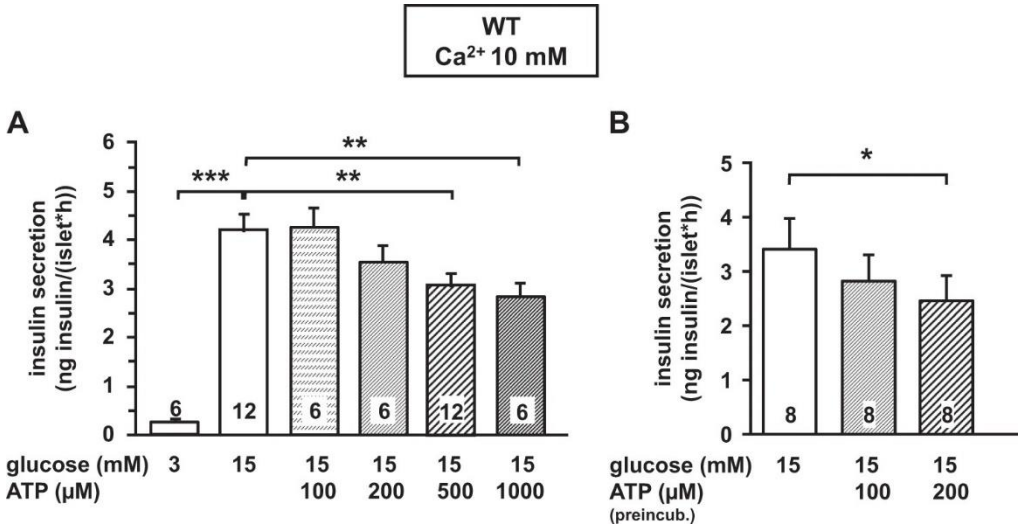



Fig.4: Extracellular ATP reduces steady-state insulin secretion. **a** ATP dose-dependently decreases insulin secretion in the presence of 15 mM glucose after 1 h incubation under steady-state conditions. **b** Pre-treatment with ATP for 1 h reduces the response to a subsequent glucose stimulus. The numbers in the columns indicate the number of mice. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$

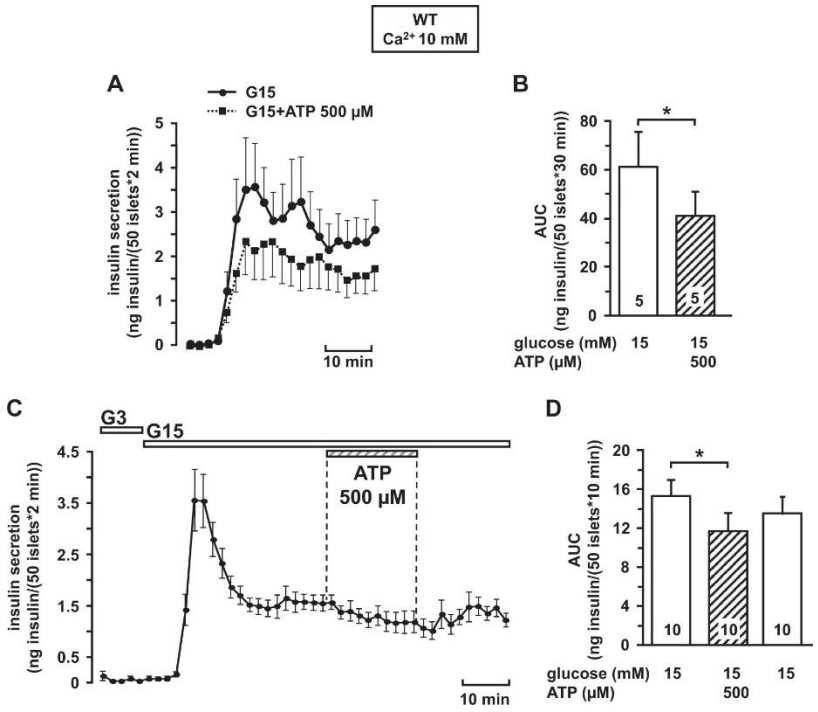



Fig.5: Extracellular ATP diminishes first and second phase insulin secretion. **a** ATP reduces the first phase of insulin secretion. Curves represent the mean values of first phase insulin secretion. Samples were taken every 2 min. Isolated islets were either perifused with 15 mM glucose without (black curve) or with ATP (dotted curve). In both cases, islets were silenced with 3 mM glucose (±ATP 500 µM) before solution change. **b** Summary of the quantitative analysis determined by the AUC of the first 30 min after changing the glucose concentration from 3 to 15 mM (±ATP 500 µM). **c** ATP reduces the second phase of insulin secretion. The curve represents the mean values of the first and second phase of insulin secretion under control conditions. Administration of ATP in the second phase leads to reduction of insulin secretion. Samples were taken every 2 min. **d** Summary of the quantitative analysis determined by the AUC of the last 10 min under control conditions and ATP administration, respectively. The numbers in the columns indicate the number of mice. *$P \leq 0.05$

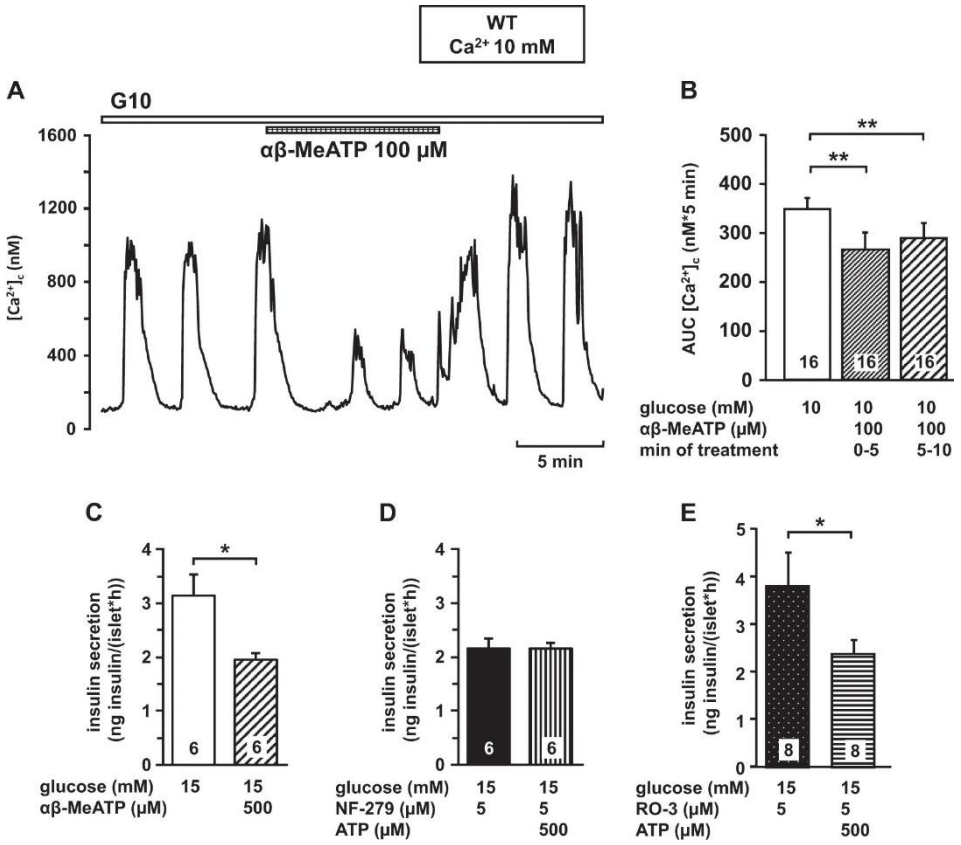



Fig.6: Extracellular ATP mediates its effects by activating P2X receptors. The $P2X_{1,3}$ agonist αβ-MeATP (100 µM) mimics the effect of ATP on oscillations of $[Ca^{2+}]_c$. **a** Representative recording showing regular oscillations of $[Ca^{2+}]_c$. αβ-MeATP (100 µM) reduced the AUC of $[Ca^{2+}]_c$. **b** Summary of the quantitative analysis of the AUC of $[Ca^{2+}]_c$ before and after application of αβ-MeATP. **c** αβ-MeATP (500 µM) reduces insulin secretion in the presence of 15 mM glucose after 1 h incubation under steady-state conditions. **d** After inhibition of $P2X_1$ channels with the specific antagonist NF-279 (5 µM) ATP has no effect on insulin secretion in 15 mM glucose. **e** The $P2X_3$ receptor blocker RO-3 does not alter the effect of ATP on insulin secretion. The numbers in the columns indicate the number of experiments with different cell clusters from three different mice (**a**, **b**) or the number of mice used for islet preparations (**c**–**e**). *$P \leq 0.05$, **$P \leq 0.01$

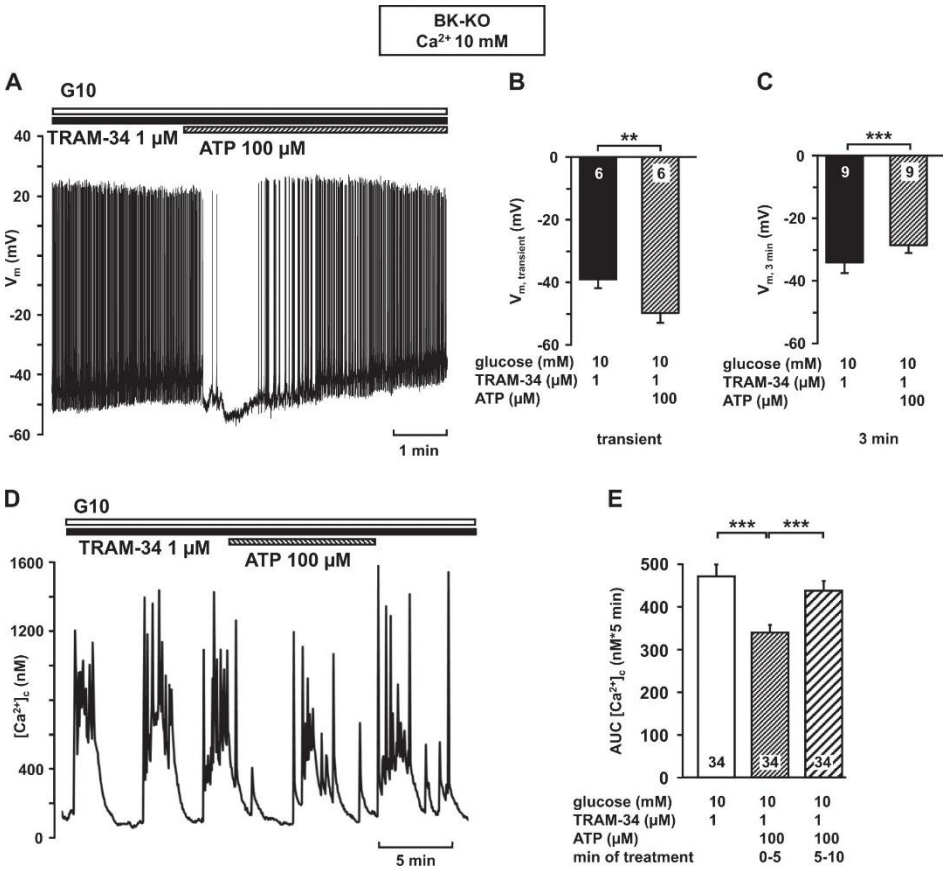



Fig.7: Possible involvement of $Ca^{2+}$-dependent potassium channels. **a** The effect of ATP on $V_m$ is reduced after elimination of BK and SK4 channels. Representative recording showing continuous spike activity in β-cells of BK-KO mice after addition of the SK4 channel blocker TRAM-34 measured in the perforated-patch configuration. ATP hyperpolarizes $V_m$ only transiently and leads to a slight depolarization during the last period of administration. **b**, **c** Summary of the quantitative analysis of $V_m$ measurements. Values under control condition were taken at the plateau potential; values under ATP were determined at the minimum of hyperpolarization (**b**) and over the last 3 min of ATP administration (**c**). **d** The effect of ATP on $Ca^{2+}$ oscillations is reduced after inhibition of SK4 channels in BK-KO β-cells. Representative recording showing regularly oscillations of $[Ca^{2+}]_c$ in β-cells of BK-KO mice treated with the SK4 channel blocker TRAM-34. ATP does not cause a significant delay of the start of the next oscillation after ATP administration and reduces its amplitude less pronounced compared to untreated WT β-cells. **e** Summary of the quantitative analysis of the AUC of $[Ca^{2+}]_c$ before and after addition of ATP. The numbers in the columns indicate the number of experiments with different cell clusters from three different mice. $^{**}P \leq 0.01$, $^{***}P \leq 0.001$

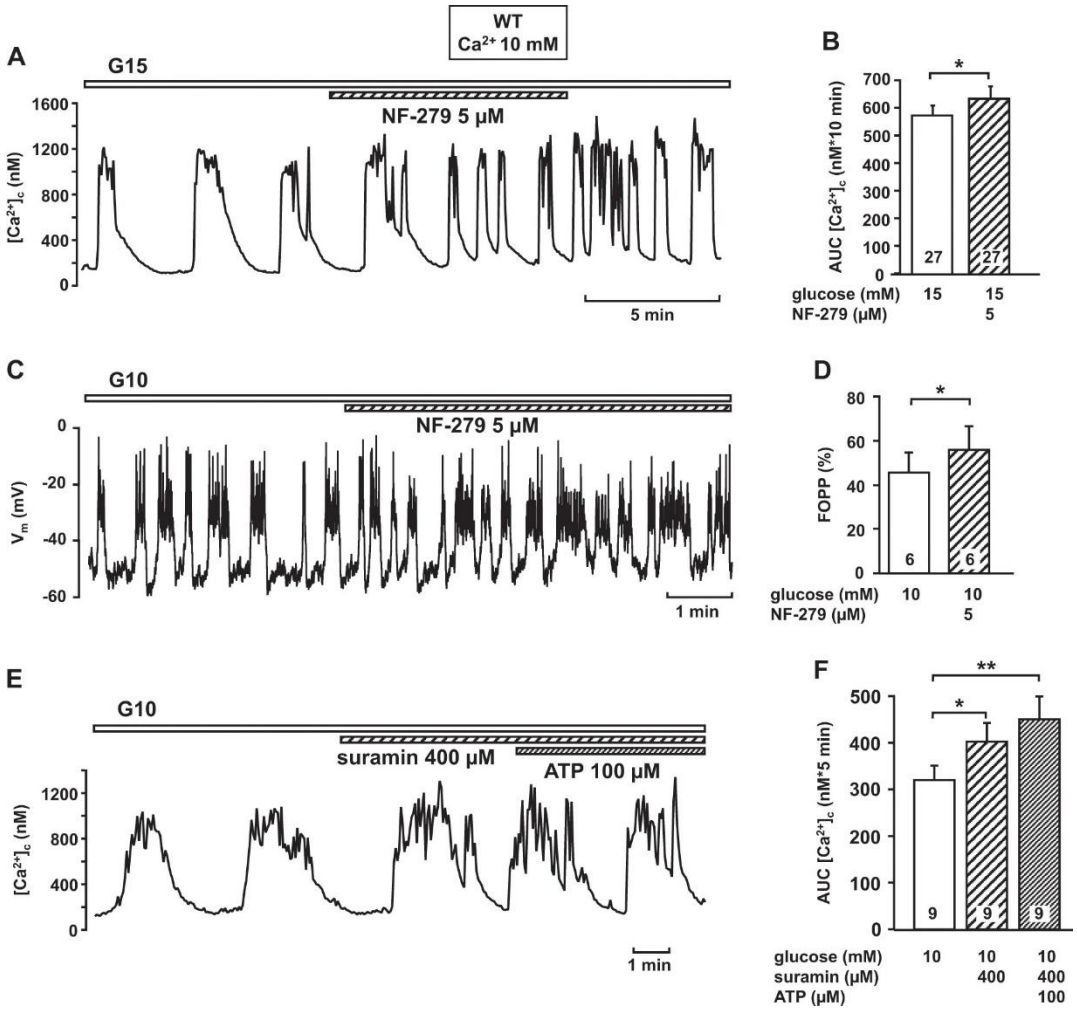



Fig.8: Blockage of $P2X_1$ channels augments the fraction of plateau phase and the AUC of $[Ca^{2+}]_c$. **a** Representative recording showing regular oscillations of $[Ca^{2+}]_c$. NF-279 (5 µM) increases $[Ca^{2+}]_c$. **b** Summary of the quantitative analysis of the AUC of $[Ca^{2+}]_c$ before and after application of NF-279. **c** Representative recording showing oscillations of $V_m$ with burst and interburst phases. Application of NF-279 (5 µM) augments the fraction of plateau phase (FOPP). **d** Summary of the quantitative analysis of the FOPP before and after application of NF-279. **e** Representative recording demonstrating the effect of suramin (400 µM) on the AUC of $[Ca^{2+}]_c$. **f** Quantitative analysis of the data of this series. The numbers in the columns indicate the number of experiments with different cell clusters from three different mice. $*P \leq 0.05$, $**P \leq 0.01$

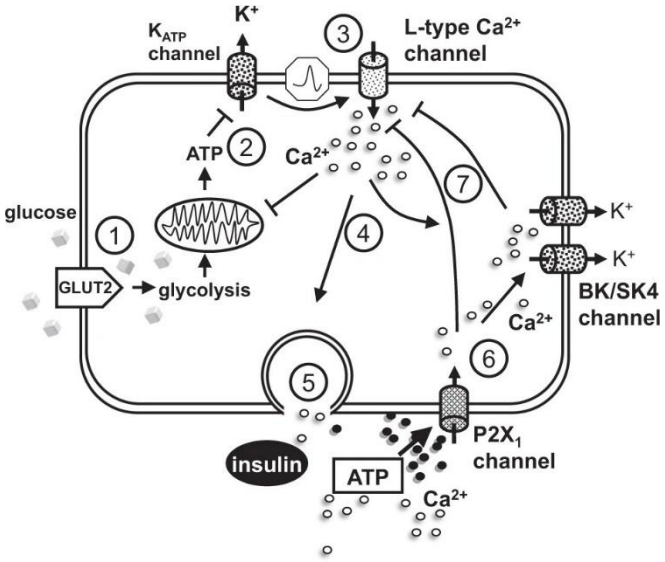
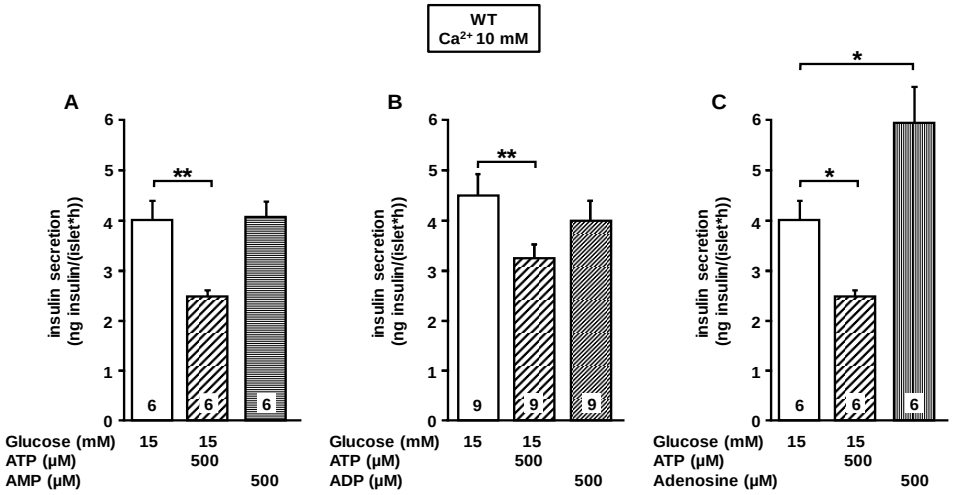
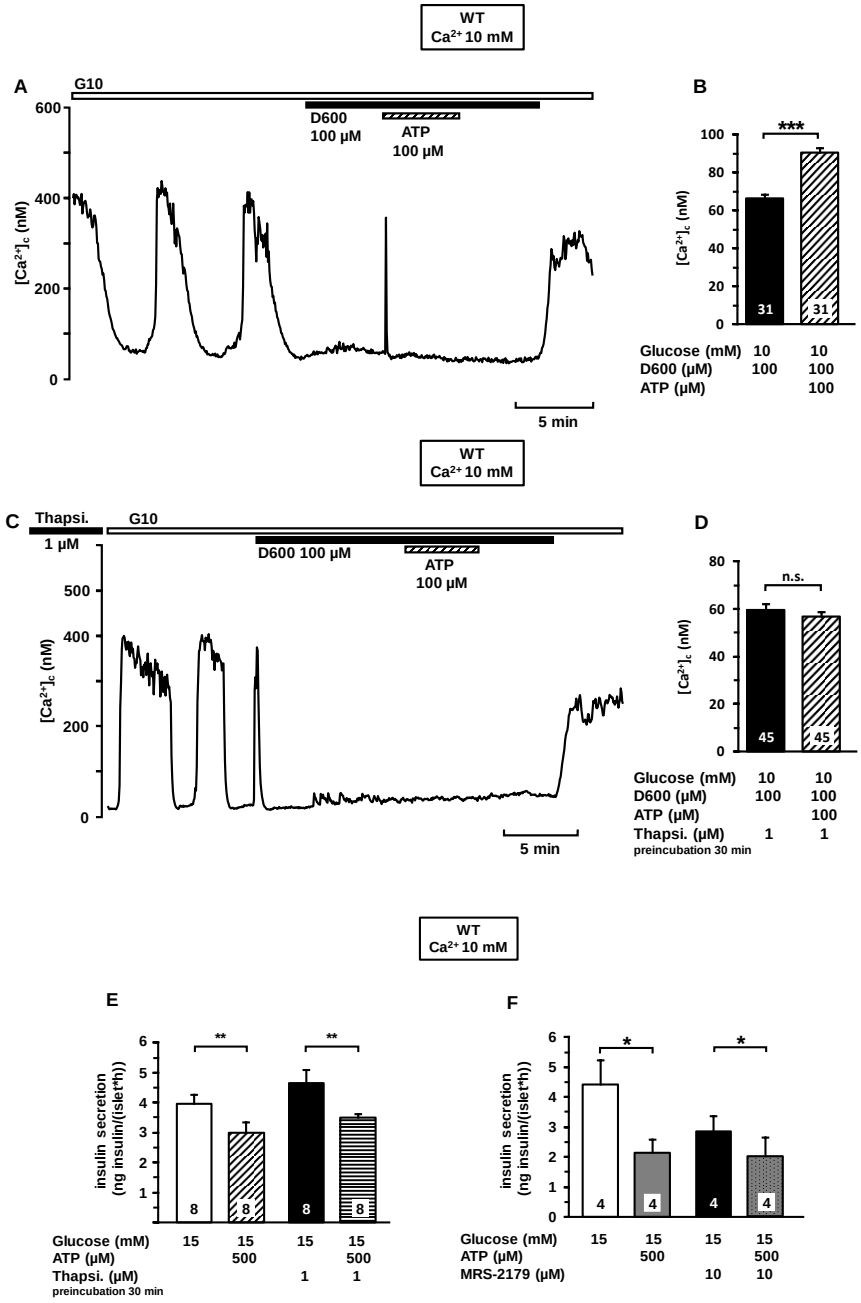



Fig.9: Proposed model. For details see text




Suppl. Fig. 1: AMP, ADP and adenosine do not mimic the effect of ATP. Comparison of the effects of ATP with AMP (A), ADP (B) and adenosine (C) on insulin secretion in the presence of 15 mM glucose. The numbers in the columns indicate the number of mice. *P ≤ 0.05, **P ≤ 0.01




Suppl. Fig. 2: ATP-induced ER store depletion does not influence the ATP effect on insulin secretion. A) ATP-evoked $Ca^{2+}$ peak in the presence of the L-type $Ca^{2+}$ channel blocker D600. B) Summary of the results. For quantification the basal $Ca^{2+}$ concentration in the presence of D600 and the peak $Ca^{2+}$ concentration after addition of ATP was analyzed. C) Suppression of the ATP-induced $Ca^{2+}$ peak by preincubation of the cells with the SERCA blocker thapsigargin. D) Summary of the results. E) Effect of ATP on insulin secretion in the presence of 15 mM glucose without and with preincubation with thapsigargin. F) Effect of ATP on insulin secretion in the presence of 15 mM glucose without and with MRS-2179. The numbers in the columns indicate the number of experiments with different cell clusters from 3 different mice (B,D) or the number of mice (E,F). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001