

Die Rolle der PI-3-Kinasen in der Regulation der Sekretion von  
Magensäure

Role of PI-3 kinases in the regulation of gastric acid secretion

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To my  
*Father 'Late' Sri R Rajeshwer Rao,*  
*Mother Srimati R Radha*  
*and rest of the family*  
  
*For all their support and encouragement*

## Table of contents

1	ACKNOWLEDGEMENTS .....	1
2	SUMMARY.....	3
3	ZUSAMMENFASSUNG.....	5
4	INTRODUCTION .....	8
	Significance.....	8
	Gastric gland (structure, location and cells involved in secretion) .....	10
	Parietal cells .....	10
	Enterochromaffin-like cells (ECL cells) .....	11
	G cells.....	12
	D cells.....	12
	Chief cells.....	12
	Mucous producing cells .....	12
	Control of secretion .....	13
	Neuronal .....	13
	Hormonal.....	13
	Secretory machinery.....	14
	H <sup>+</sup> /K <sup>+</sup> -ATPase .....	14
	K <sup>+</sup> channels.....	15
	Cl <sup>-</sup> channels .....	16
	Signal transduction.....	17
	Pathways (gastrinergic, histaminergic, cholinergic) .....	17
	PI3-kinases .....	19
	Physiological role.....	19
	Rationale for the role in gastric acid secretion.....	20
5	AIMS OF THE STUDIES.....	21

6	MATERIALS .....	22
	Equipment .....	22
	Chemicals .....	22
	Kits and Antibodies .....	24
	Animals .....	24
	Diet .....	25
7	METHODS .....	26
	Intracellular pH measurement .....	26
	Gastric H <sup>+</sup> secretion.....	27
	Serum gastrin.....	29
	Serum corticosterone.....	29
	Western blotting .....	29
	In situ hybridization of SGK1 mRNA.....	30
	Immunofluorescence .....	31
	Quantitative real-time polymerase chain reaction measurements.....	32
	Flow cytometric analysis (FACS) of KCNQ1 expression in the isolated parietal cells .....	33
	Statistics .....	33
8	RESULTS .....	34
	i. Role of SGK1 in regulation of acid secretion .....	34
	a. Role of SGK1 in regulation of basal and stimulated acid secretion.....	34
	b. Role of SGK1 in glucocorticoid induced stimulation of acid secretion.....	36
	c. Role of SGK1 in peroxisome proliferator activated receptor gamma (PPAR <sub>γ</sub> ) agonist mediated stimulation of acid secretion.....	43
	d. SGK1 dependant stimulation of gastric acid secretion in mutant APC mice ( <i>apc</i> <sup>Min/+</sup> ) .....	50
	ii. Role of PDK1 in regulation of acid secretion .....	58

9	DISCUSSION.....	66
	i. Role of SGK1 in regulation of acid secretion .....	66
	a. Role of SGK1 in regulation of basal and stimulated acid secretion.....	66
	b. Role of SGK1 in glucocorticoid stimulation of acid secretion .....	67
	c. Role of SGK1 in peroxisome proliferator activated receptor gamma (PPAR <sub>γ</sub> ) agonist mediated stimulation of acid secretion.....	68
	d. SGK1 dependant stimulation of gastric acid secretion in mutant apc mice ( <i>apc<sup>Min/+</sup></i> ). 69	
	ii. Role of PDK1 in regulation of acid secretion .....	71
10	ABBREVIATIONS .....	73
11	REFERENCES .....	75
12	PUBLICATIONS .....	83
13	AKADEMISCHE LEHRER .....	84
14	LEBENS LAUF.....	85

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## 2 SUMMARY

PI-3 kinases (eg. SGK, PDK isoforms) are involved in the trafficking of membrane proteins, which include potassium channels (KCNQ1, Kir 4.1) and are known to be critical for gastric acid secretion. Stimulation of PI-3 kinase signalling could thus stimulate the acid secretion. Inhibition of PI-3 kinase signalling on the other hand leads to increase of cAMP levels which in turn could activate the cAMP dependent kinase PKA and thereby lead to stimulation of acid secretion. Therefore considering the significance of PI-3 kinase signalling in other physiological processes, there is an utmost need to clearly define its role in the regulation of gastric acid secretion.

SGK1 is an important member of this kinase family and is known to be a potent stimulator of membrane KCNQ1 activity. Therefore the first aim of the study was to determine the role of SGK1 in regulation of gastric acid secretion. The rate of Na<sup>+</sup> independent pH recovery after acid load by ammonium pulse (an indicator of H<sup>+</sup>/K<sup>+</sup>-ATPase activity) in the glands from SGK1 wild type and knock out mice, however, showed that rate of acid secretion was similar between the two genotypes under both basal and stimulated conditions. It indicates that lack of SGK1 does not make any difference in the regulation of acid secretion. Next step was to study if the overexpression of SGK1 had any effect on the gastric acid secretion. This was done by studying the gastric acid secretion in the mice treated with clinically used drugs that are known to upregulate SGK1 expression (dexamethasone and pioglitazone) and in the mice with defective cellular handling of  $\beta$ -catenin (*apc*<sup>Min/+</sup> mice), a condition that is known to upregulate genomic SGK1 expression.

In-situ hybridisation or western blotting was employed to determine the SGK1 expression in the stomachs from the mice. The experiments revealed an increased SGK1 abundance in the stomachs from mice treated with dexamethasone or with pioglitazone and from *apc*<sup>Min/+</sup> mice. The rate of H<sup>+</sup> secretion was increased significantly in isolated glands from dexamethasone treated mice as compared to untreated mice. However the increase was significantly blunted in *sgk1*<sup>-/-</sup> mice, clearly demonstrating that dexamethasone induced gastric acid secretion is at least partially dependant on SGK1. Pioglitazone treatment also resulted in significant increase in rate of H<sup>+</sup> secretion in *sgk1*<sup>+/+</sup> but not in *sgk1*<sup>-/-</sup> mice suggesting that pioglitazone treatment could result in SGK1 dependant increase in gastric acid secretion. The rate of H<sup>+</sup> secretion was also enhanced in the *apc*<sup>Min/+</sup> mice. Further experiments showed that the difference between glands from treated (either with dexamethasone or with pioglitazone) and untreated mice of both genotypes was dissipated in

presence of increased local  $K^+$  concentrations pointing to the role of potassium channels. Similar dissipation of difference was seen in glands from *apc<sup>Min/+</sup>* and *apc<sup>+/+</sup>* mice in presence of increased local  $K^+$  concentrations. Experiments conducted by treating the glands with carbachol and/or forskolin also resulted in abrogation of the difference in the rate of  $H^+$  secretion, between treated and untreated SGK1 mice of both genotypes and between both genotypes of APC mice, suggesting that there is no genomic upregulation of the secretory machinery ( $K^+$  channels). This hypothesis was further confirmed by the real time PCR analysis of KCNQ1 expression in the stomach. Immunofluorescence finally confirmed the increased membrane abundance of  $H^+/K^+$ -ATPase and/or KCNQ1 in the glands from dexamethasone or pioglitazone treated SGK1 wild type mice and in the glands from *apc<sup>Min/+</sup>* mice. FACS analysis of the parietal cells was done additionally in APC mice to quantitatively demonstrate the significant increase in membrane abundance of KCNQ1 channel in *apc<sup>Min/+</sup>* mice. The role of SGK1 in the enhanced gastric acid secretion in *apc<sup>Min/+</sup>* mice was established by the experiments in *apc<sup>Min/+</sup>/sgk1<sup>-/-</sup>* mice which showed that the increase in rate of  $H^+$  secretion was abolished by the additional knock out of SGK1. In conclusion, the present studies indicate that overexpression of SGK1 (either due to drug treatment or due to genetic abnormalities) can lead to increase in gastric acid secretion.

The next step of this study was to elucidate the role of PI-3 kinase signalling in the regulation of functioning of parietal cell. Since SGK1 was already shown not to be involved in the regulation of basal acid secretion, a kinase above SGK1 in the signalling cascade, PDK1 was chosen as the candidate. Increased rate of  $H^+$  secretion was seen in glands from *pdk1<sup>hm</sup>* mice suggesting an inhibitory role of PDK1. A clear role overlap of cAMP dependent PKA pathway was seen in the experiments with forskolin, which did not show any additional increase in *pdk1<sup>hm</sup>* mice and with H89, which showed inhibitory effect only in *pdk1<sup>hm</sup>* mice. Involvement of other pathways was ruled out through experiments with carbachol and phorbol ester. In conclusion, the present study suggests the inhibitory role of PDK1 on the cAMP dependent PKA pathway.

The present work thus demonstrates the dual role of the PI-3 kinases in the regulation of gastric acid secretion. On one hand upregulation of these kinases stimulates the secretion by increasing the membrane trafficking of secretory machinery (potassium channels and the proton pump) and on the other hand they inhibit the classical stimulatory pathways (PKA pathway) of acid secretion. The present work has identified that SGK1 is involved in the former effect and PDK1 in the latter effect.

### 3 ZUSAMMENFASSUNG

PI-3-Kinasen sind an Membrantransportvorgängen beteiligt, die Kalium Kanäle (KCNQ1, Kir 4.1) miteinbeziehen und entscheidend für die Magensäuresekretion sind. Eine Stimulation des PI-3-Kinasen Signalwegs könnte einerseits die Säuresekretion verstärken, andererseits könnte eine Hemmung des PI-3-Kinasen Signalwegs zu einer Erhöhung des cAMP Gehalts führen, welcher wiederum über Aktivierung der cAMP abhängigen Kinase PKA auch zur Stimulation der Säuresekretion führt. In Anbetracht der Bedeutung des PI-3-Kinasen Signalwegs in anderen physiologischen Prozessen, besteht ein großer Bedarf seine Rolle in der Regulation der Magensäure genau zu bestimmen.

Die SGK1 ist ein wichtiges Mitglied dieser Kinasenfamilie und ist als ein starker Stimulator der KCNQ1-Aktivität in der Membran bekannt. Daher war das erste Ziel dieser Studie die Rolle der SGK1 in der Regulation der Magensäuresekretion zu bestimmen. Die Bestimmung der  $\text{Na}^+$  unabhängigen Erholung des pH nach Säurebelastung durch Ammoniumpulse als Indikator der  $\text{H}^+/\text{K}^+$ -ATPase Aktivität zeigte, dass die Säuresekretion in Parietaldrüsen von SGK1-Wildtyp und Knock-out Mäusen sowohl unter basalen als auch stimulierten Bedingungen ähnlich war und führte zum Schluss, dass das Fehlen von SGK1 keinen Unterschied in der Regulation der Säuresekretion macht. Der nächste Schritt war, den Effekt der Überexpression von SGK1 auf die Magensäuresekretion zu untersuchen. Dies wurde durch eine Bestimmung der Magensäuresekretion von, mit klinisch gebräuchlichen Medikamenten behandelten, Mäusen, die bekanntermaßen die SGK1 Expression hochzuregulieren steigern (Dexamethason und Pioglitazon). In diesem Zusammenhang wurden auch Mäuse mit dem Gen für die familiäre Adenomatosis polyposis coli (*apc*<sup>Min/+</sup> Mäusen) untersucht, die eine Herunterregulierung von  $\beta$ -catenin aufweisen, was ebenfalls die SGK1 genomisch hochreguliert.

Die SGK1 Expression wurde mittels In-situ Hybridisierung oder Western Blots bestimmt. Dabei zeigte sich eine erhöhte SGK1-Expression in den Mägen von Mäusen, die mit Dexamethason oder mit Pioglitazon behandelt wurden, und von *apc*<sup>Min/+</sup> Mäusen. Im Vergleich zu unbehandelten Mäusen war die Menge der  $\text{H}^+$  Sekretion in isolierten Drüsen von Dexamethason behandelten Mäusen signifikant erhöht, jedoch war die Erhöhung in *sgk1*<sup>-/-</sup> Mäusen signifikant abgeschwächt. Dies bewies, dass eine Dexamethason induzierte Erhöhung der  $\text{H}^+$  Sekretion zumindest teilweise von SGK1 abhängig ist. Eine Pioglitazonbehandlung bewirkte auch eine signifikante Erhöhung der  $\text{H}^+$ -Sekretion in *sgk*<sup>+/+</sup>, aber nicht in *sgk1*<sup>-/-</sup> Mäusen, was nahe legt, dass eine Pioglitazonbehandlung SGK1-abhängige

Magensäuresekretion zur Folge hat. Die Menge an  $H^+$ -Sekretion war auch in *apc*<sup>Min/+</sup> Mäusen erhöht.

Weitere Experimente zeigten, dass die Stimulierbarkeit (entweder mit Dexamethason oder Pioglitazon) in beiden Genotypen in Anwesenheit von lokal erhöhten  $K^+$ -Konzentrationen abgeschwächt war, was auf die Rolle der Kaliumkanäle hinwies. Ähnliche Abschwächungen der Stimulierbarkeit wurden in Drüsen von *apc*<sup>Min/+</sup> und *apc*<sup>+/+</sup> Mäusen in Anwesenheit von lokal erhöhten  $K^+$ -Konzentrationen beobachtet. Die Stimulierbarkeit durch Behandlung mit Carbachol und/oder Forskolin ergaben ebenfalls eine Aufhebung des Unterschieds in der Menge der  $H^+$ -Sekretion zwischen behandelten und unbehandelten SGK1 Mäusen beider Genotypen und zwischen beiden Genotypen von APC Mäusen, was darauf hindeutet, dass es dieser Effekt nicht durch genomische Hochregulation des Sekretionsmechanismus zustande kommt, sondern  $K^+$ -Kanäle einbezieht. Diese Annahme wurde durch Realtime-PCR Analyse der KCNQ1-Expression im Magen weiter bestätigt. Die Immunfluoreszenz bestätigte schließlich die erhöhte Menge an  $H^+/K^+$  ATPase und/oder KCNQ1 in der Membran der Drüsen von, mit Dexamethason oder Pioglitazon behandelten, SGK1 Wildtypmäusen und in den Drüsen von *apc*<sup>Min/+</sup> Mäusen. Eine FACS Analyse von Parietalzellen wurde zusätzlich bei APC Mäusen durchgeführt, um quantitativ die signifikante Erhöhung der Menge an KCNQ1-Kanäle in *apc*<sup>Min/+</sup> Mäusen in der Membran zu demonstrieren. Die Rolle der SGK1 in der erhöhten Magensäuresekretion in *apc*<sup>Min/+</sup> Mäusen wurde durch Experimente in *apc*<sup>Min/+</sup>*sgk*<sup>-/-</sup> Mäusen fundiert, die zeigten, dass die Erhöhung der  $H^+$  Sekretionsrate durch das zusätzliche Hemmung der SGK1 aufgehoben wurde. Schließlich deuten die derzeitigen Studien an, dass die Überexpression von SGK1 (entweder aufgrund der Medikamentenbehandlung oder durch genetische Abnormalität) zu einer erhöhten Magensäuresekretion führen.

Der nächste Schritt dieser Studie war die Rolle des PI-3 Kinase Signalwegs in der Regulation der cAMP Konzentrationen in Parietalzellen aufzuklären. Da schon gezeigt wurde, dass SGK1 nicht an der Regulation der basalen Säuresekretion beteiligt ist, wurde PDK1, eine Kinase oberhalb SGK1 in der Signalkaskade, untersucht. Eine erhöhte  $H^+$  Sekretionsrate wurde in Drüsen von *pdk1*<sup>hm</sup>-Mäusen gefunden, was daraufhin deutet, dass PDK1 eine hemmende Funktion hat. Eine klare Aufgabenüberschneidung des cAMP-abhängigen PKA-Signalwegs wurde in Experimenten mit Forskolin nachgewiesen, die keinen zusätzlichen Anstieg in *pdk1*<sup>hm</sup> Mäusen und mit H89 zeigte. H89 wies einen hemmenden Effekt nur in *pdk1*<sup>hm</sup> Mäusen auf. Die Beteiligung von anderen Signalwegen wurde durch Experimente

mit Carbachol und Phorbol ester ausgeschlossen. Schlussfolgernd deutet die vorliegende Studie auf eine hemmende Rolle der PDK1 auf den cAMP abhängigen PKA Signalweg hin.

Zusammenfassend zeigt die vorliegende Studie die Doppelrolle der PI-3-Kinasen in der Regulation der Magensäuresekretion. Einerseits bewirkt eine Stimulation dieser Kinasen die Sekretion durch eine Erhöhung des Membrantransportvorgangs (Kaliumkanäle und Protonenpumpe) und andererseits hemmen sie die klassischen stimulierenden Signalwege der Säuresekretion (PKA Signalweg). Die vorliegende Arbeit macht deutlich, dass SGK1 einen frühen und PDK1 einen späten Einfluss auf die Magensäuresekretion hat.

## 4 INTRODUCTION

### Significance

Stomach plays a very important role in the digestion of food. The food that is swallowed is mixed, pulverised, disinfected and partly digested in the stomach. While the first two functions are achieved by the muscular layers, the latter two are achieved by the gastric juice produced by the gastric glands of stomach. Gastric juice consists of a proteolytic enzyme precursor 'pepsinogen', concentrated hydrochloric acid (HCl), mucous and water. Inactive pepsinogen is converted to active pepsin in presence of HCl. HCl also serves the function of disinfectant, since most of the harmful pathogens present in the food cannot survive in presence of concentrated HCl. Thus HCl forms the important constituent of the gastric juice. While the secretion of gastric HCl (referred to commonly as 'gastric acid') is physiologically important, concentrated HCl together with active proteolytic pepsin could also digest and destroy the neighbouring mucosal layer leading to the formation of ulcers. Mucous, which is secreted along with HCl and pepsinogen, forms a thick lining and barrier on the inner mucosa and protects it from the harmful effects of gastric juice. The secretion of the gastric acid, on other hand, is also tightly regulated and is secreted in required quantities and only in presence of food in the lumen (1).

However, several physiological conditions like psychological stress or pharmacological interventions like use of steroidal/non steroidal anti-inflammatory drugs could lead to loss of control over the secretion of the gastric acid leading to precipitation of gastric or duodenal ulcers (2; 3).

Clinically, overproduction of acid is treated by two methods: 1. by surgical removal of the vagus nerve innervation of stomach (vagotomy) or total removal of stomach (gastrectomy) 2. by the use of pharmacological inhibitors of acid secretion (histamine 2 receptor antagonists or proton pump inhibitors) (4). However, the former method of surgical intervention has lost its popularity ever since the introduction of histamine 2 receptor antagonists and proton pump inhibitors (most importantly proton pump inhibitors) in the treatment of peptic ulcer (4-6).

Although pharmacological inhibition of the acid secretion by proton pump inhibitors has been quite effective clinically, the chronic and complete inhibition of acid secretion might lead to ineffective disinfection of food in stomach and defective digestion of proteins (4; 7). Apart from that the side effects of proton pump inhibitors have started to gain importance since the recent past (8). Chronic and complete inhibition of gastric acid secretion could lead

to increase in secretion of gastrin, a condition termed as 'hypergastrinemia'. Hypergastrinemia has been reported to cause carcinoid tumors and aggravate already existing cancers in experimental animals (4; 9-14). There is however, no convincing evidence that hypergastrinemia could cause cancer in human beings, but the possibility still exists and cannot be ruled out completely. On the other hand studies indicate that there is a prolonged rebound hypersecretion with increases in both basal and maximal acid output in individuals who stop using proton pump inhibitors, an affect attributed to increased serum gastrin levels (4). Chronic suppression of acid secretion is also reported to lead to malabsorption of vitamin B12 and iron (4). Other potential adverse effects include enteric infections (eg, *Clostridium difficile*), community-acquired pneumonia, and hip fracture (4; 8). The reported adverse effects of Histamine 2 receptor antagonists are gynecomastia, mental confusion and interstitial nephritis (4).

A clear understanding of the mechanisms that lead to overproduction of acid would thus be useful in identifying the defects in the regulatory pathways and thereby help in finding a safer and effective solution to the problem of gastro-intestinal ulcers. Therefore there is a need to study the mechanisms of gastric acid secretion, the regulatory pathways involved in secretion and the conditions that lead to hypersecretion of gastric acid.

## Gastric gland (structure, location and cells involved in secretion)

The internal lining (epithelium) of the stomach is in the form of tubular glands, called as gastric glands. Although the entire internal lining of the stomach consists of tubular glands, the glands located at the beginning and at the terminal portion of the stomach do not produce gastric acid. They mainly serve to produce the mucous and also to dilute the acid. The glands that are located in the middle of the stomach are the ‘true’ gastric glands, which produce the gastric juice (consisting of gastric acid and other enzymes). A typical gastric gland is divided into isthmus, neck and base (Figure 1). Isthmus consists of precursor cells, neck consists of mucous neck cells and parietal cells and base consists of enteroendocrine cells and chief cells.

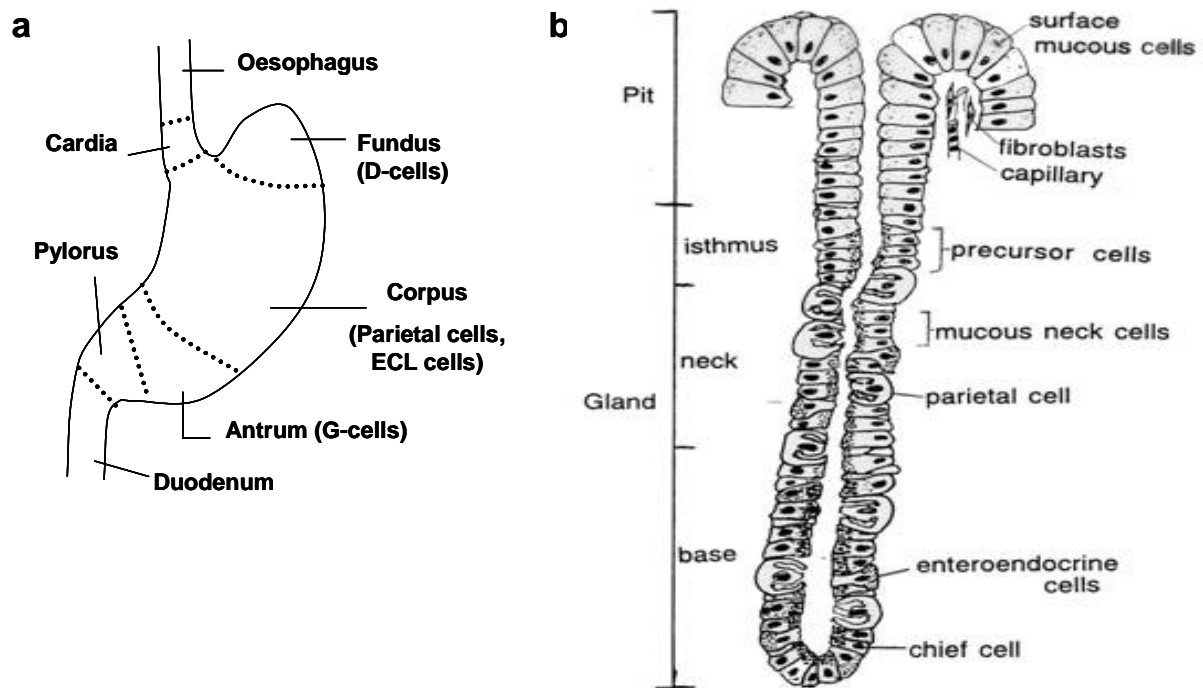


Figure 1: Typical a. stomach and b. gastric gland indicating the location of different cells involved in the gastric acid secretion.

### Parietal cells

The parietal cell is a highly specialized epithelial cell with several morphological characteristics that are required for its function. The cells are large and oval in shape and are stained deeply with eosin. They are also characterized by the presence of large number of mitochondria. A series of small canals (canaliculi) invaginate from the surface of the apical plasma membrane and project throughout the entire cell interior. The cytoplasm consists of membranous structures in the form of vesicles, tubules and cisternal sacs rich in  $H^+/K^+$ -



ATPase (15). Upon activation, parietal cells undergo a profound morphological transition resulting in the fusion of the above cytoplasmic  $H^+/K^+$ -ATPase rich tubulovesicles with the apical plasma membrane (16). The fusion occurs in such a way that the  $H^+/K^+$ -ATPase is exposed into the lumen of the stomach, giving scope for the exchange of the cytoplasmic  $H^+$  with lumenic  $K^+$  Figure 2. Additionally various outwardly projecting potassium and chloride channels are also fused with the apical plasma membrane when the cell is stimulated (17-20). While the purpose of chloride channels is to secrete  $Cl^-$  ions into lumen, potassium channels maintain the concentration of  $K^+$  in the vicinity of  $H^+/K^+$ -ATPase.

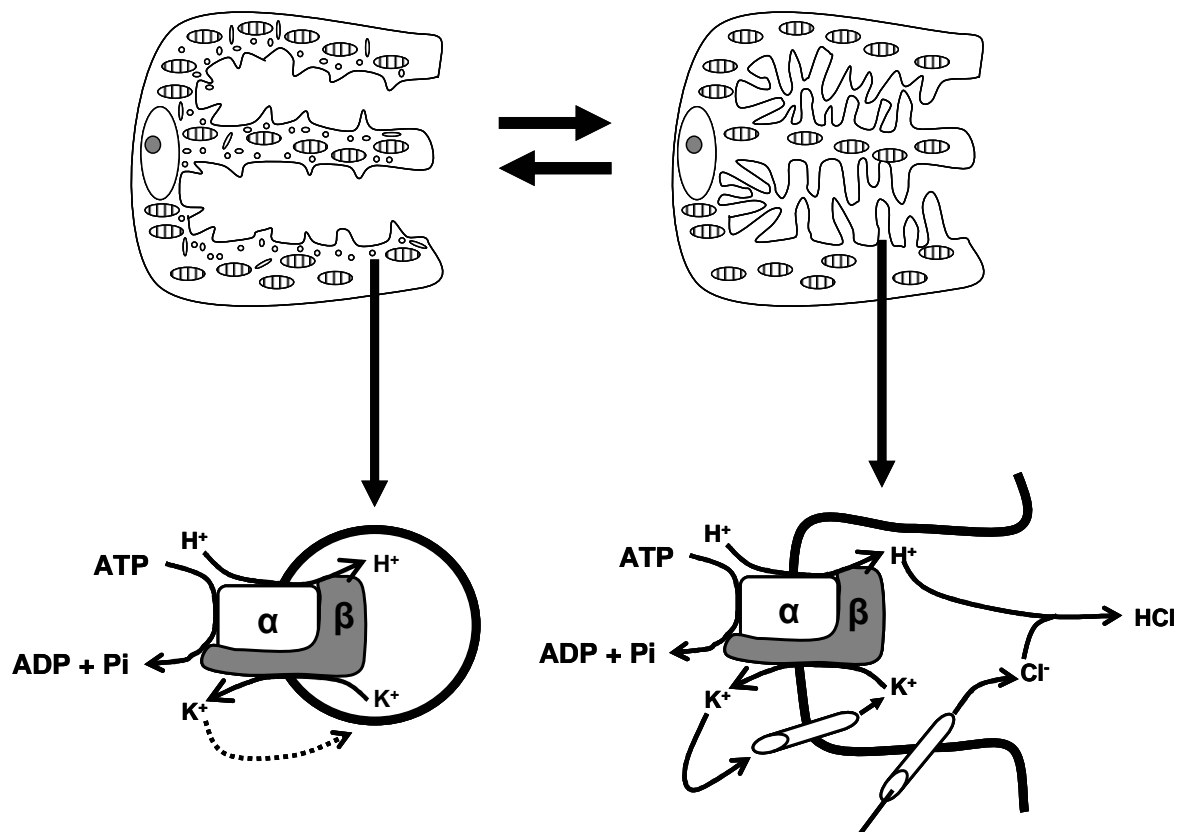


Figure 2: Illustrative diagram showing the parietal cell canaliculus in the resting (left) and active (right) states (16).

### Enterochromaffin-like cells (ECL cells)

Enterochromaffin-like cells or ECL cells are a type of enteroendocrine cells that are found particularly in the vicinity of parietal cells (1). They synthesize histamine and secrete it when stimulated by hormones (Gastrin, Acetylcholine and Pituitary adenylyl cyclase-activated peptide). The secreted histamine then stimulates the neighbouring parietal cells Figure 3.

**G cells**

G cells are the 'Gastrin' secreting cells found mainly in the antrum region of the stomach. The vagus nerve innervates these cells. During parasympathetic stimulation, 'gastrin-releasing peptide' is released by the post-ganglionic fibers of the vagus nerve onto the G-cell that stimulates gastrin release Figure 3 (1).

**D cells**

D cells (delta cells) are again a type of enteroendocrine cells that secrete a hormone 'somatostatin' which inhibits the G cells from producing Gastrin. They are distributed in the fundus, antrum and pylorus region of the stomach (1).

**Chief cells**

Chief cells are also called as 'Zymogenic cells' as they release a precursor enzyme 'Pepsinogen'. Inactive pepsinogen is converted to active pepsin in presence of acidic pH (1).

**Mucous producing cells**

Mucous producing cells, as their name suggest produce the viscous mucous that forms the barrier between the lumen of the stomach and the corrosive gastric acid (1).

## Control of secretion

The secretion of gastric acid is tightly regulated and is achieved by a coordinated interplay of neuronal and hormonal pathways (Figure 3).

### Neuronal

Central control of the gastric acid secretion is achieved by the vagus nerve that innervates the stomach. The sight or smell of food could result in the release of 'gastrin-releasing peptide' from the vagal nerve endings in the stomach that stimulates the G-cells to secrete 'gastrin' which further stimulates the acid secretion. However, gastrin releasing peptide also stimulates the D-cells to secrete 'somatostatin' which indirectly inhibits the acid secretion (1). The secretion of gastric acid is thus, always tightly regulated and overproduction is prevented.

### Hormonal

Endocrinal control of gastric acid secretion involves the hormones – gastrin, somatostatin and histamine. Gastrin is secreted from the antral G cells in response to stimulus from food intake. Gastrin-releasing peptide, released onto the G cells from the post ganglionic fibers of the vagus nerve also stimulates the secretion of gastrin. Gastrin in turn acts on the 'cholecystochinin type 2' (CCK<sub>2</sub>) receptors of the ECL cells and stimulates the release of histamine. Both histamine and gastrin activate the parietal cell to secrete gastric acid through respective 'histamine type 2' (H<sub>2</sub>) and CCK<sub>2</sub> receptors respectively. The acid/acidic pH in the lumen then stimulates the D-cells to release somatostatin, which acts on the G-cell through 'somatostatin receptor type 2 (SSRT<sub>2</sub>) and inhibits the gastrin release. Apart from that it also inhibits the release of histamine from ECL cells. Thus the 'negative feedback control' of acid secretion is achieved (1).

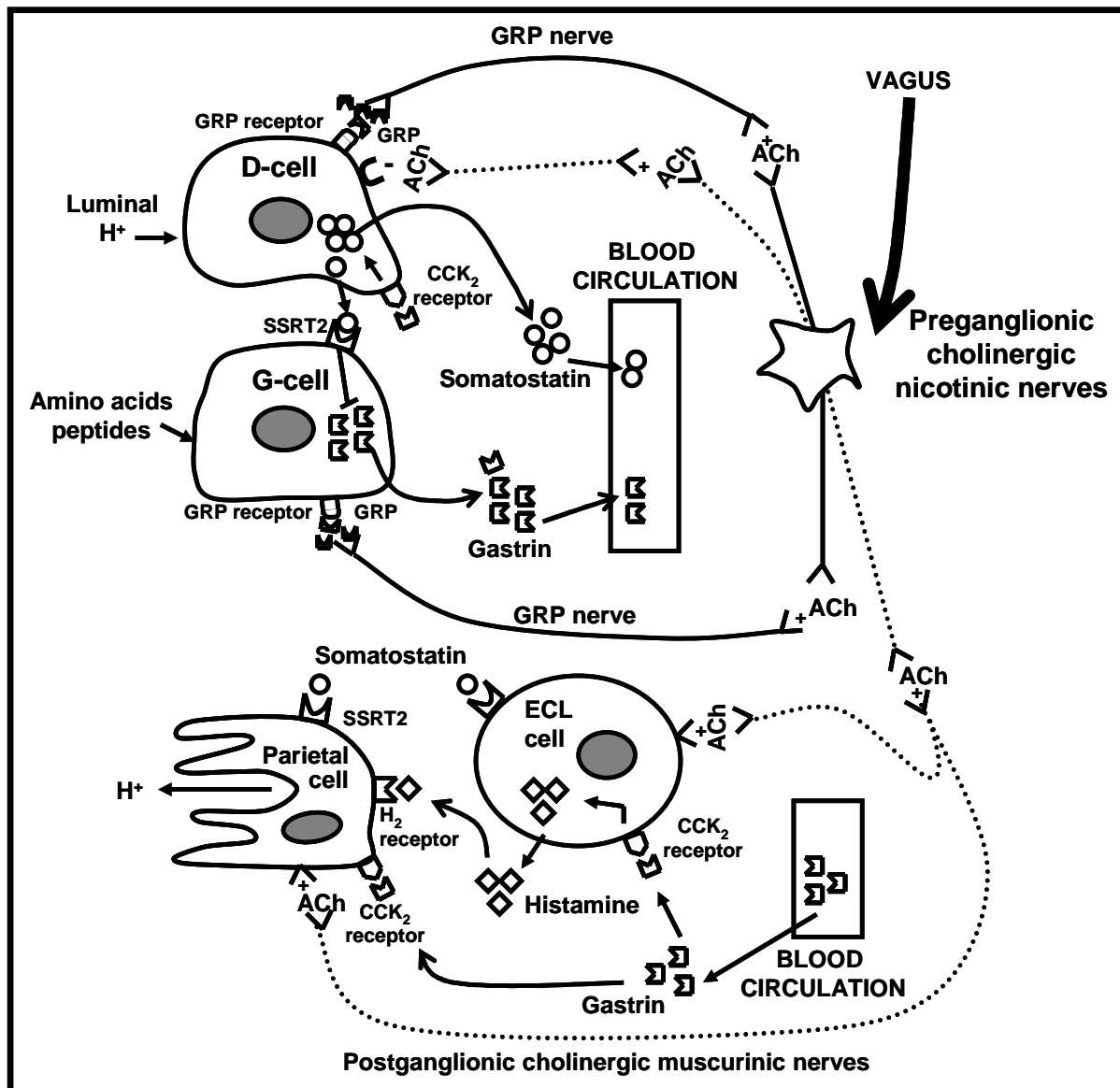


Figure 3: A schematic representation of the control of gastric acid secretion (source (13)).

GRP – gastrin releasing peptide, SSRT2 – somatostatin receptor type 2, ACh – acetyl choline, CCK2 – cholecystinin type 2 receptor, H<sub>2</sub> – histamine type 2 receptor, ECL cell – enterochromaffin like cell.

## Secretory machinery

### H<sup>+</sup>/K<sup>+</sup>-ATPase

The proton pump of stomach, hydrogen potassium ATPase (H<sup>+</sup>/K<sup>+</sup>-ATPase) is the enzyme primarily responsible for the acidification of the contents of stomach. It is a member of the P-type ATPase superfamily, a large family of proteins that transports usually cations across the biological membranes (15; 21). It exchanges one hydrogen ion (H<sup>+</sup>) from the cytoplasm of

parietal cell with one potassium ion ( $K^+$ ) from the gastric lumen. Thus for every 'one ion' of hydrogen secreted, 'one ion' of potassium is retrieved (22).

The  $H^+/K^+$ -ATPase is a heterodimeric protein, the product of two genes. The  $\alpha$  - subunit of  $H^+/K^+$ -ATPase containing approximately 1000 amino acids is encoded by ATP4A. It contains the catalytic sites of the enzyme and it forms the pore through the cell membrane that allows the transport of ions. The  $\beta$ -subunit of  $H^+/K^+$ -ATPase is an approximately 300 amino acid protein with a 36 amino acid N-terminal cytoplasmic domain, a single transmembrane domain and a highly glycosylated extracellular domain. It is encoded by ATP4B. The  $\beta$ -subunit stabilizes the  $\alpha$ -subunit of the enzyme and is also required for the function of the enzyme (15; 21; 23).

### **$K^+$ channels**

Parietal cell  $H^+/K^+$ -ATPase exchanges one cytoplasmic  $H^+$  ion with one lumenic  $K^+$  ion. The concentration of  $K^+$  is quite low in the parietal cell canaliculus where the exchange takes place. Therefore the availability of  $K^+$  ions in the parietal cell canaliculus for the exchange is the limiting factor for the acid secretion. This is overcome by simultaneous pumping out of the  $K^+$  ions into the canaliculus.  $K^+$  is reportedly recycled and enters the parietal cell canaliculus via specific apical  $K^+$  channels (22; 24).

Among the different types of  $K^+$  channels identified so far, KCNQ1 and inwardly rectifying  $K^+$  channels (Kir) have been suggested to serve this critical function (25-28). Basolateral  $K^+$  channels on the other hand that have been functionally characterized in isolated parietal cells of several species are thought to be essential to maintain the negative membrane potential essential for apical  $Cl^-$  secretion (29-31). The mice lacking KCNQ1 demonstrated loss of acid secretion and severe gastric gland hyperplasia indicating its significance in gastric acid secretion (25; 27; 28). The members of Kir channel family (Kir 2.1, 4.1, 5.1, 7.1) are shown to be expressed in gastric mucosa and are believed to be involved in the acid secretion (32; 33). It was shown recently in isolated cultured rabbit parietal cells that stimulation leads to co-localization of KCNQ1 and Kir 4.1 with  $H^+/K^+$ -ATPase (18; 19). However, it is uncertain which of these channels, plays the major role in  $K^+$  efflux and a complete understanding of the  $K^+$  channel(s) involved is not achieved yet.

## Cl<sup>-</sup> channels

The secretion of gastric acid (HCl) from the parietal cell also requires a simultaneous secretion of Cl<sup>-</sup> ions into the lumen along with H<sup>+</sup> ions. This is done by chloride channels of the apical membrane, which pump the cytoplasmic Cl<sup>-</sup> into the gastric lumen. The accumulation of Cl<sup>-</sup> ions inside the parietal cells is achieved by Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger on the basolateral membrane which exchanges the cytoplasmic HCO<sub>3</sub><sup>-</sup> ions with the Cl<sup>-</sup> ions of the blood circulation (see Figure 4). Thus secretion of gastric acid in the stomach is paralleled by alkalization of the blood (34).

Although it is accepted that Cl<sup>-</sup> channels play a vital role in the acid secretion, the exact nature and type of channels that are involved is not known. ClC2-channel was suggested to be the likely candidate (35; 36), but the experiments performed in the respective knockout mice and the studies of the localization of the channel, showed that it might not be involved (37). Recently a chloride transporter Slc26a9 was reported to be crucial for the gastric acid secretion as seen by loss of tubulovesicles in the parietal cells leading to 'achlorhydria' in the corresponding knockout mice (20).

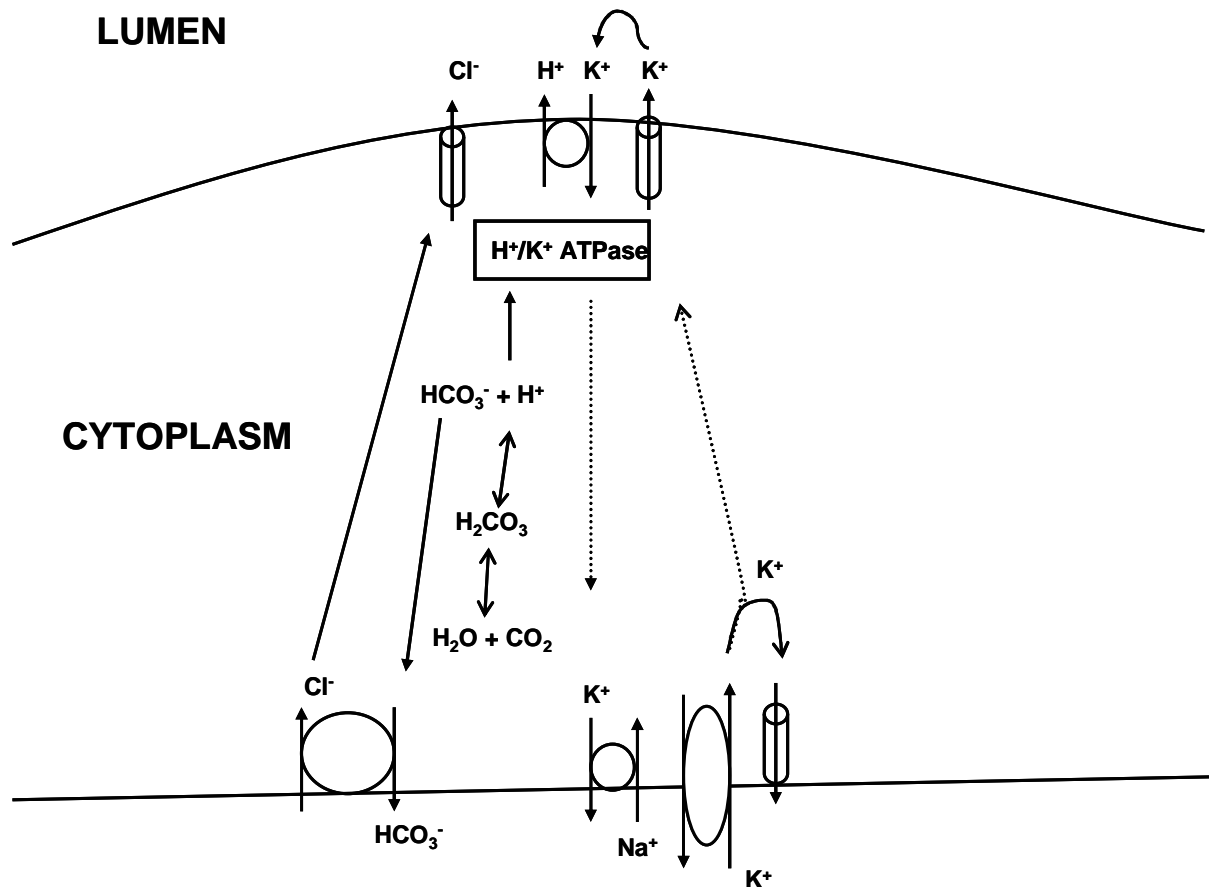


Figure 4: A simplified model for the gastric acid secretion by the parietal cell.

## Signal transduction

### Pathways (gastrinergic, histaminergic, cholinergic)

There are three types of activating receptors on the basolateral membrane of the parietal cell, i.e., histamine  $H_2$ , acetylcholine  $M_3$ , and gastrin  $CCK_2$ .  $H_2$  receptor couples to  $G_s$  to activate adenylate cyclase (ACase) producing adenosine 3', 5'-cyclic monophosphate (cAMP) and cAMP-dependent protein kinase (PKA) is activated. Whereas both  $M_3$  and  $CCK_2$  receptors couple to  $G_q$ , to activate phospholipase C (PLC) producing inositol 1,4,5-trisphosphate (IP3) and diacylglycerol. IP3 acts on the intracellular calcium stores and stimulates the release of  $Ca_2^+$  while diacylglycerol activates protein kinase C (PKC) (16). The stimulation of the parietal cell leading to secretion of gastric acid requires the fusion of the proton pump containing tubulovesicles, the  $K^+$  and the  $Cl^-$  channels with the apical plasma membrane. Therefore all the stimulatory pathways whether histaminergic, cholinergic or gastrinergic, lead to activation of cytoskeletal proteins and docking/fusing proteins which facilitate the

trafficking of the vesicles towards the membrane. Ezrin, a putative membrane-cytoskeletal linker phosphoprotein is the most common downstream effector among them (15; 16). A simplified version of signal transduction inside the parietal cell is shown in Figure 5.

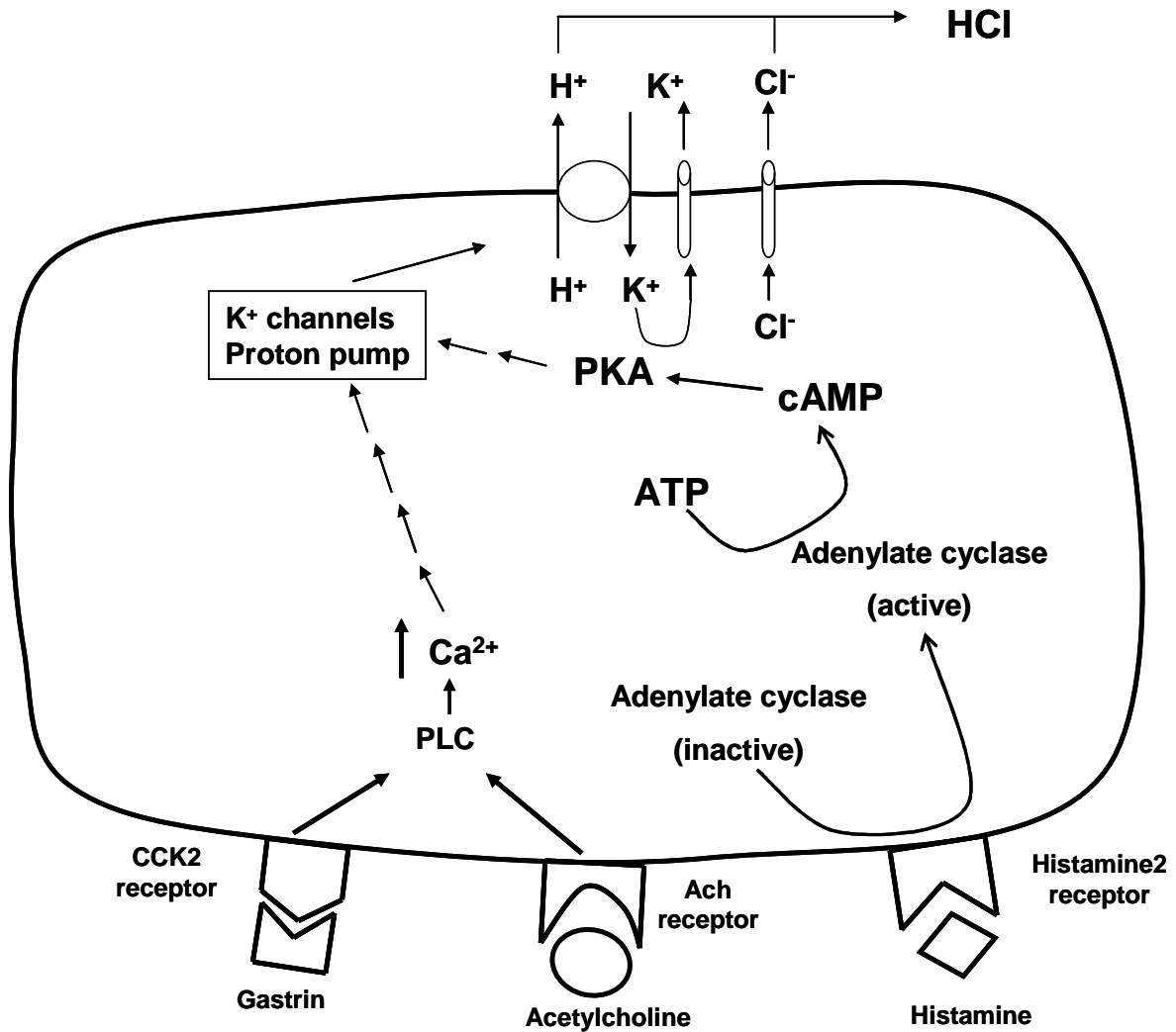


Figure 5: A simplified version of signal transduction inside the parietal cell.



## PI3-kinases

### Physiological role

The PI3-kinases are a group of enzymatic proteins that were shown to be involved in the insulin and growth factor signalling (38). The binding of insulin to its receptor results in the recruitment of a lipid kinase termed PI3-kinase to the plasma membrane of the cells which then phosphorylates 'phosphatidylinositol (4, 5) bisphosphate' (PIP-2) to phosphatidylinositol (3, 4, 5) triphosphate (PIP-3). PIP-3 is a key messenger in the insulin signalling pathway. PIP-3 then facilitates the phosphorylation of protein kinase B (Akt/PKB) by phosphoinositide-dependent protein kinase-1 (PDK1) (39). Apart from Akt, PIP-3 also facilitates the phosphorylation of p70 ribosomal S6 kinase (S6K), and the serum and glucocorticoid induced protein kinase (SGK1, 2 and 3) by PDK1 (40; 41). Phosphorylation of these kinases makes them active and the active Akt/PKB and SGK isoforms reportedly dephosphorylate and inactivate the glycogen synthase kinase 3 (GSK3) (42). S6 kinase (S6K) uncouples further signalling cascades which are involved in biosynthesis of ribosomes and protein synthesis (38). Akt/PKB and SGK isoforms are involved in glucose transport by regulating the membrane expression of glucose transporters (GLUT, SGLT) (40; 41; 43-45). These kinases are also actively involved in the mediation of other cellular activities such as proliferation, differentiation, chemotaxis, survival and trafficking (41; 45).

Additionally insulin signalling inhibits lipid metabolism in fat cells by decreasing cellular concentrations of cyclic AMP in adipocytes. Akt/PKB is believed to be involved in this process via phosphorylation and activation of a cAMP phosphodiesterase isoform, PDE3B (46; 47).

## Rationale for the role in gastric acid secretion

The key second messengers in the PI3-kinase pathway eg. Akt, SGK isoforms are involved in the trafficking of membrane proteins (40; 41; 45; 48-50). Among the proteins whose transport is regulated are the potassium channels (KCNQ1, Kir 4.1) (51-55). Increase in the membrane localization of potassium channels could at least in theory result in the increased availability of  $K^+$  ions on the luminal side of the parietal cells. The luminal  $K^+$  ions are then easily exchanged with cytoplasmic  $H^+$  ions by  $H^+/K^+$ -ATPase resulting in increased acid production. SGK1 is a potent stimulator of the  $K^+$  channel KCNQ1 (51), which is necessary for recycling of  $K^+$  across the apical membrane of gastric parietal cells and thus for gastric acid secretion (25; 28). Stimulation of PI3-kinase signalling could thus stimulate the acid secretion.

Dexamethasone a known stimulator of SGK1 is also known to cause gastric ulcers. Although this effect is believed to be due to inhibition of cyclooxygenase and subsequent reduction of the local PGE2 levels in the stomach, it is also worthwhile to verify if this effect is mediated through SGK1. SGK1 is also upregulated by treatment with ‘peroxisome proliferator activated receptor  $\gamma$ ’ agonist pioglitazone (56). Therefore it is also important to verify if this upregulation also leads to increased gastric acid secretion.

On the other hand, the cellular cAMP levels are regulated by PI3-kinase signalling (46; 47; 57). Inhibition of PI3-kinase signalling reportedly leads to increase of cAMP levels which in turn could activate the cAMP dependent kinase PKA and thereby lead to stimulation of acid secretion. Thus it is clear that PI3-kinase signalling is an important regulator of gastric acid secretion and considering its significance in other physiological processes, there is an utmost need to clearly define its role in the regulation of gastric acid secretion.

## 5 AIMS OF THE STUDIES

The present studies were aimed at identifying the kinases involved in modulation of basal gastric acid secretion with a special emphasis on kinases involved in PI3-kinase pathway. The studies were conducted using gene targeted mice lacking the kinase under study. Known activators and inhibitors of the regulatory pathways of gastric acid secretion were also used as part of the study.

The experiments were carried out with following objectives:

- To identify the PI3-kinases involved in modulation of basal gastric acid secretion.
- To demonstrate the role of serum and glucocorticoid inducible kinase SGK1 in dexamethasone induced stimulation of gastric acid secretion.
- To elucidate the effect of pioglitazone treatment on the SGK1 abundance in stomach and to explore the subsequent effects on gastric acid secretion.
- To study the gastric acid secretion in mice with defective cellular handling of  $\beta$ -catenin (*apc*<sup>Min/+</sup> mice), and to explore the role of sgk1 in the regulation of acid secretion in these mice.
- To define the role of PDK1 in the modulation of gastric acid secretion.

## 6 MATERIALS

### Equipment

Eppendorf Centrifuge 5415R	Hinz GmbH, Hamburg, Germany.
Eppendorf Pipets 1000µl, 200µl, 20µl, 10µl	Eppendorf, Hamburg, Germany.
Fluorescence microscope	Axiovert, Zeiss, Jena, Germany.
Fluorescence Microscopy low light CCD camera	Proxitronic, Bensheim, Germany.
LSM 510 confocal microscope	Zeiss, Jena, Germany.
Lambda 10-2 Sutter Instrument	Novato, USA.
Lamp ebx 75 isolated	Leika, Jena, Germany.
Light cycler	Roche Diagnostics, Mannheim, Germany.
FACS-Calibur (NIR/UV)	Becton Dickinson, Heidelberg, Germany
MagNa Lyser	Roche Diagnostics, Mannheim, Germany
Mastercycler gradient	Eppendorf, Hamburg, Germany.
Metaflour Image Analyzer software	MDS Analytical Technologies, Toronto, Canada.
Microflow Biological Safety Cabinet	Nalge Nunc, Wiesbaden-Bierbach, Germany.
Microscope Stemi 2000	Zeiss, Jena, Germany.
Milli-Q	MILLIPORE, S.A. Molsheim France.
Na-Heparine disposable capillaries	
Petridishes	Greiner Bio-one, Frickenhausen, Germany.
pH Meter 765	Knick, Zweibrücken, Germany.
Preparation material	F.S.T., Heidelberg, Germany.
Vortex	Labnet Abimed, Langenfeld, Germany.

### Chemicals

Amiloride	Sigma, Taufkirchen, Germany
BaCl <sub>2</sub> x2H <sub>2</sub> O	Carl Roth, Karlsruhe, Germany.

BCECF	Molecular Probes, Leiden, Netherlands
(2',7',-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein)	
Buffer –formamide	Sigma, Taufkirchen, Germany.
BSA bovine serum albumine	Sigma, Taufkirchen, Germany.
CaCl <sub>2</sub> x2H <sub>2</sub> O	Carl Roth, Karlsruhe, Germany.
Carbachol	Sigma, Taufkirchen, Germany
Cell Tak adhesive	B D Biosciences, Leiden, Netherlands
Corticosterone acetate	Sigma, Taufkirchen, Germany.
Diethyl ether	Carl Roth, Karlsruhe, Germany.
EDTA	Sigma, Taufkirchen, Germany.
(Ethylenediamine tetraacetic acid)	
Ethanol absolute (99%)	Sigma, Taufkirchen, Germany.
Forskolin	Sigma, Taufkirchen, Germany.
Glucose	Carl Roth, Karlsruhe, Germany.
H89	Sigma, Taufkirchen, Germany.
HCl	Sigma, Taufkirchen, Germany.
HEPES	Sigma, Taufkirchen, Germany.
K <sub>2</sub> HPO <sub>4</sub> x2H <sub>2</sub> O	Sigma, Taufkirchen, Germany.
KCl	Carl Roth, Karlsruhe, Germany.
KH <sub>2</sub> PO <sub>4</sub>	Sigma, Taufkirchen, Germany.
Mannitol	Sigma, Taufkirchen, Germany.
MgSO <sub>4</sub>	Sigma, Taufkirchen, Germany.
NaCl	Sigma, Taufkirchen, Germany.
N-Methyl-d-glucamine (NMDG)	Sigma, Taufkirchen, Germany.
Nigericin	Sigma, Taufkirchen, Germany.
Omeprazole	Sigma, Taufkirchen, Germany.
PFA- Paraformaldehyde	Merck, Darmstadt, Germany.
Phorbol ester	Sigma, Taufkirchen, Germany
Saline 0.9%	Fresenius Kabi, Bad Homburg, Germany.
Sterilium	Carl Roth, Karlsruhe, Germany.
TEA buffer	Sigma, Taufkirchen, Germany.
(triethanolamine acetic anhydride)	
Tris buffer	Sigma, Taufkirchen, Germany.

## Kits and Antibodies

Gastrin RIA kit	DiaSorin, Dietzenbach, Germany
Corticosterone ELISA kit	DRG Instruments, Marburg, Germany
Immobilon Western kit	Millipore, Billerica, MA, USA
DIG-labeling kit	Roche, Mannheim, Germany
PCR purification kit	Qiagen, Hilden, Germany.
Primer mix Search	LC, Heidelberg, Germany.
Rabbit anti-B0AT1	Alpha Diagnostics, San Antonio, TX, USA.
Rabbit anti-EAAC1/ EAAT3	Alpha Diagnostics, San Antonio, TX, USA.
Rabbit anti-SIT	Alpha Diagnostics, San Antonio, TX, USA.
RNAeasy Mini Kit	Qiagen, Hilden, Germany.
Superscript II reverse transcriptase	Invitrogen, Karlsruhe, Germany.
Trizol reagent	Invitrogen, Karlsruhe, Germany.
Polyclonal goat anti-KCNQ1	C20, Santa Cruz Biotechnologies, USA
Monoclonal mouse anti- H <sup>+</sup> /K <sup>+</sup> ATPase (β-subunit)	Affinity bioreagents, USA
Rabbit anti-mouse β-catenin	Cell signalling, USA
Polyclonal monospecific anti SGK1	Dr. Pineda, Berlin, Germany
Mouse monoclonal anti-GAPDH	Sigma, Taurkirchen, Germany
Secondary antibodies conjugated with alkaline phosphatase or horse radish peroxidase	Promega, USA
Donkey anti-goat Alexa-488	Invitrogen, Karlsruhe, Germany
Donkey anti-mouse Alexa-555	Invitrogen, Karlsruhe, Germany

## Animals

All animal experiments were conducted according to the guidelines of the American Physiological Society and the German law for the care and welfare of animals and were approved by local authorities. Age and sex matched mice of more than 3 months age were used for the experiments.

PDK1 hypomorphic mice and mice deficient in SGK1 (*sgk1*<sup>-/-</sup>) were obtained as described previously (58; 59). The mice had a mixed (C57/Bl-6-Sv129J) background. Mice lines were maintained by breeding the respective heterozygote males and females. Genotypes were determined from the samples obtained from ear or tail biopsies using standard PCR protocols.

Mice with mutated APC (*apc*<sup>Min/+</sup>) and their wild type littermates (*apc*<sup>+/+</sup>) with a mixed (C57/Bl-6-Sv129J) background were generated by breeding of male *apc*<sup>Min/+</sup> mice initially obtained from the Jackson Laboratory. Where indicated, additional mice have been generated by crossbreeding *apc*<sup>Min/+</sup> with gene targeted mice lacking functional SGK1 (*sgk1*<sup>-/-</sup>) to generate mice carrying the mutant APC and simultaneously lacking SGK1 (*apc*<sup>Min/+</sup>/*sgk1*<sup>-/-</sup>).

## Diet

Mice were fed with a standard mouse diet (C1310/1314) and tap water.

Standard diet C1310/1314 [0.24% Na<sup>+</sup>, 0.71% K<sup>+</sup>, 0.95% Ca<sub>2</sub><sup>+</sup> (wt/wt)] Altromin, Heidenau, Germany.

Control diet C1000 [0.24% Na<sup>+</sup>, 0.71% K<sup>+</sup>, 0.95% Ca<sub>2</sub><sup>+</sup> (wt/wt)] Altromin, Heidenau, Germany

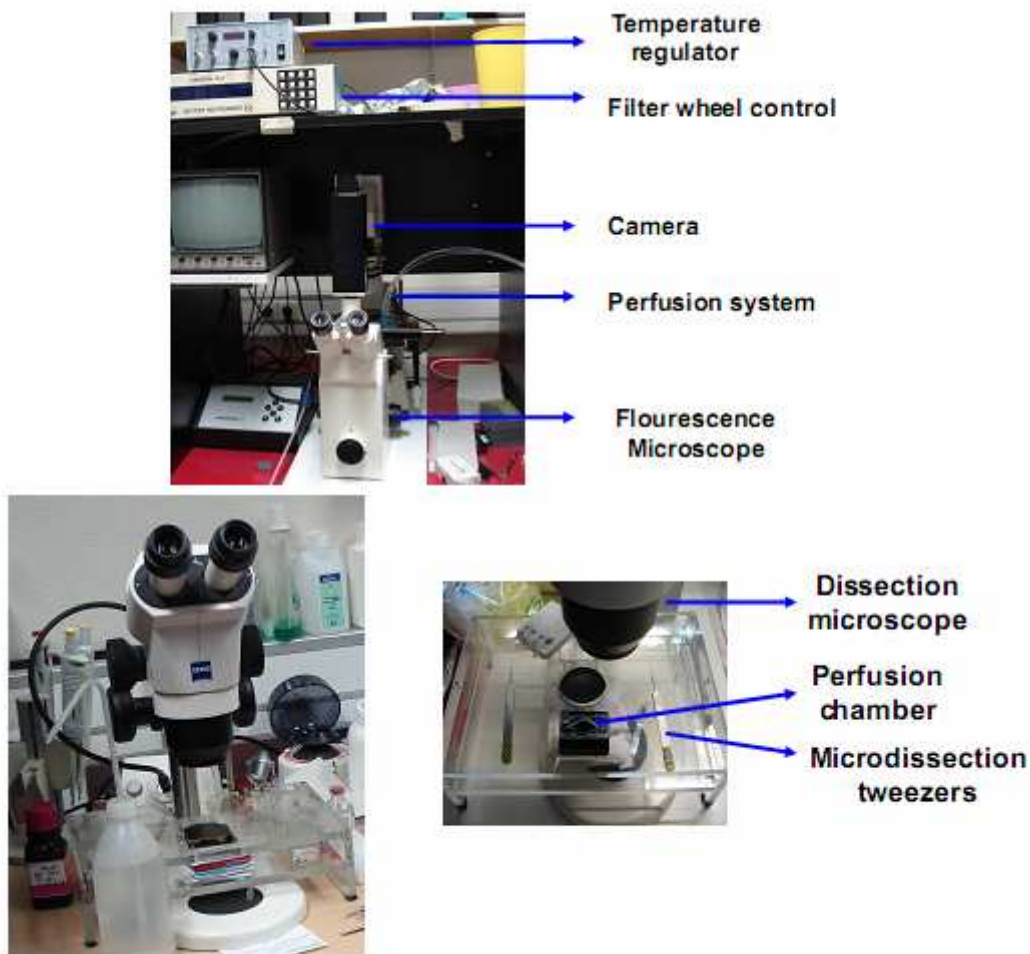
Where indicated, control diet (C1000) was replaced with custom made pellets containing 0.2 % w/w pioglitazone.

## 7 METHODS

### Intracellular pH measurement

For digital imaging of cytosolic pH, isolated individual glands were incubated in a HEPES-buffered Ringer solution containing 10  $\mu$ M BCECF-AM for 15 min at 37°C. After loading, the chamber was flushed for 5 min with Ringer solution to remove any deesterified dye sticking to the outside of the glands. The perfusion chamber was mounted on the stage of an inverted microscope, which was used in the epifluorescence mode with a 40 X oil immersion objective (Figure 6). BCECF was successively excited at 490/10 and 440/10 nm, and the resultant fluorescent signal was monitored at 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software. Between 8 and 20 parietal cells were outlined and monitored during the course of the measurements. Intensity ratio data (490/440) were converted into pH values using the high-K<sup>+</sup>/nigericin calibration technique (60; 61). In the end of each experiment, the glands were perfused for 5 minutes with standard high-K<sup>+</sup>/nigericin (10 $\mu$ g/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the  $r_{\max}$ ,  $r_{\min}$ , pKa values previously generated from calibration experiments performed in isolated gastric glands to generate a standard non-linear curve (pH range 5 to 8.5).





**Figure 6: Fluorescence microscope along with its supporting components required for the isolation of gastric glands and measurement of intracellular pH.**

## Gastric H<sup>+</sup> secretion

For isolation of gastric glands, animals were fasted for 16 hours prior to experiments on wire grids with free access to tap water. After sacrificing the animals the stomach was removed and cut longitudinally. After washing with standard Hepes solution the fundic and pyloric regions were discarded and the gastric corpus was sliced into 0.3 cm<sup>2</sup> sections. The tissues were transferred onto the cooled stage of a dissecting microscope and individual glands were carefully detached from the gastric wall by snapping of the tissue using sharpened microdissection tweezers. Care was taken not to touch the apical part of the glands. The glands were attached to a glass coverslip precoated with Cell-Tak adhesive.

The solutions, flow lines and perfusion chamber were maintained at 37°C by a thermostatically controlled heating system. The volume of the perfusion chamber was 600 µl

and the flow rate was 4 ml/min for all solutions. The glands were first incubated with BCECF for 15 minutes and then the intracellular pH recording was started. They were then loaded with acid by transient exposure to a solution containing 20 mM  $\text{NH}_4\text{Cl}$  leading to marked initial alkalinization of cytosolic pH ( $\text{pH}_i$ ) due to entry of  $\text{NH}_3$  and binding of  $\text{H}^+$  to form  $\text{NH}_4^+$  (62). The acidification of cytosolic pH upon removal of ammonia allowed calculating the mean intrinsic buffering power of the cells (62). Assuming that  $\text{NH}_4^+$  and  $\text{NH}_3$  are in equilibrium in cytosolic and extracellular fluid and that ammonia leaves the cells as  $\text{NH}_3$ :

$$\beta = \Delta[\text{NH}_4^+]_i / \Delta\text{pH}_i,$$

where  $\Delta\text{pH}_i$  is the decrease of cytosolic pH ( $\text{pH}_i$ ) following ammonia removal and  $\Delta[\text{NH}_4^+]_i$  is the decrease of cytosolic  $\text{NH}_4^+$  concentration, which is identical to the concentration of  $[\text{NH}_4^+]_i$  immediately before the removal of ammonia. The pK for  $\text{NH}_4^+/\text{NH}_3$  is 8.9 (63) and at an extracellular pH ( $\text{pH}_0$ ) of 7.4 the  $\text{NH}_4^+$  concentration in extracellular fluid ( $[\text{NH}_4^+]_0$ ) is 19.37  $[20/(1+10^{\text{pH}_0-\text{pK}})]$ . The intracellular  $\text{NH}_4^+$  concentration ( $[\text{NH}_4^+]_i$ ) was calculated from:

$$[\text{NH}_4^+]_i = 19.37 \cdot 10^{\text{pH}_0-\text{pH}_i}.$$

The calculation of buffer capacity requires that  $\text{NH}_4^+$  exits completely. After the initial decline,  $\text{pH}_i$  indeed shows little further change in the absence of  $\text{Na}^+$  and presence of omeprazole, indicating that there is no relevant further exit of  $\text{NH}_4^+$ .

The pH recovery in the absence of sodium is then dependent on the exchange of  $\text{K}^+$  ions in the physiological solution with the cytoplasmic  $\text{H}^+$  ions. The exchange and subsequent pH recovery is thus a direct indicator of the proton pump  $\text{H}^+/\text{K}^+$  ATPase activity. To calculate the  $\Delta\text{pH}/\text{min}$  during re-alkalinization, a manual linear fit was placed over a narrow pH range (pH 6.7 to 6.9) which could be applied to all measured cells.

The solutions were composed of (in mM): standard Hepes: 115 NaCl, 5 KCl, 1  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 2  $\text{NaH}_2\text{PO}_4$ , 10 glucose, 32.2 Hepes; sodium free Hepes: 132.8 NMDG, 3 KCl, 1  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 2  $\text{KH}_2\text{PO}_4$ , 32.2 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM  $\text{NH}_4\text{Cl}$ ); high  $\text{K}^+$  for calibration 105 KCl, 1  $\text{CaCl}_2$ , 1.2  $\text{MgSO}_4$ , 32.2 Hepes, 10 mannitol, 5  $\mu\text{M}$  nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37°C. Where indicated the  $\text{K}^+$  concentration was increased at the expense of  $\text{Na}^+/\text{NMDG}$  to 35 mM.

Omeprazole was added into the luminal side of the parietal cells while incubating with BCECF. The protons released from cells activate the drug by converting it into its active sulfenic acid/sulfenamide derivative which is further taken up into the cell and eventually binds covalently to  $H^+/K^+$  ATPase resulting in the irreversible inhibition of the pump.

### **Serum gastrin**

To determine the serum gastrin levels, the animals were fasted overnight for 16 hours with free access to tap water. About 150  $\mu$ l of blood specimens were withdrawn into heparinized hematocrit capillaries by retro-orbital puncture during light anesthesia with diethylether. Care was taken during the collection of blood to prevent the degradation of serum gastrin. The specimens were collected in ice-cold capillaries and immediately stored at  $-80^{\circ}\text{C}$ . The measurement was also done within 2 days after collection of the specimen. Total serum gastrin concentration was determined by radio immuno assay (RIA) kit according to the manufacturer's instructions.

### **Serum corticosterone**

Corticosterone is the main glucocorticoid in mice; its plasma concentrations show a circadian rhythm with a peak 2-3 h prior to the dark cycle (64). Blood was taken from *sgk1*<sup>+/+</sup> and *sgk1*<sup>-/-</sup> mice at the beginning of the light cycle in the morning at 7 am and prior to the beginning of the dark cycle at 5 pm. Additionally, blood was taken from mice following a 4 day treatment with dexamethasone (10  $\mu$ g/g BW s.c.).

About 150  $\mu$ l of blood specimens were withdrawn into heparinized hematocrit capillaries by retro-orbital puncture during light anesthesia with diethylether. Plasma corticosterone concentrations were determined using an ELISA kit according to the manufacturer's instructions.

### **Western blotting**

Mice were sacrificed by cervical dislocation under ether anaesthesia and the abdomen was cut open. The stomach was then longitudinally cut, and the lumen was cleaned with PBS. 2 ml lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1mM NaF, 1mM  $\text{Na}_3\text{VO}_4$ ,  $\beta$ -mercaptoethanol) containing protease inhibitor cocktail were added to

the tissue and homogenized for 2 min on ice. Samples were centrifuged at 20,000 g for 30 min and supernatants were collected. After measurement of the total protein concentration, 100 µg of protein were solubilized in Laemmli sample buffer at 65 °C for 5 min and resolved by 12% SDS-PAGE. For immunoblotting, proteins were electro-transferred onto a nitrocellulose membrane and blocked in 5% nonfat milk in PBS-0.10% Tween 20 for one hour at room temperature. The membrane was incubated with either affinity purified rabbit anti-mouse β-catenin antibody (1:2000; 92 kDa), or with a polyclonal monospecific antibody against the SGK1 protein (1:100; 55 kDa), raised by a commercial antibody service as previously described (65), or with a mouse monoclonal anti-GAPDH antibody (1: 1000; 42 kDa) overnight at 4 °C. After washing and subsequent blocking, blots were incubated with secondary antibodies conjugated with alkaline phosphatase or horse radish peroxidase (goat anti-rabbit 1: 2000 and donkey anti-mouse 1: 10000), for 1 h at room temperature. Antibody binding was detected with the Immobilon Western kit with the Diana III chemiluminescence detection system. Bands were quantified with the Aida Image Analyzer software.

### **In situ hybridization of SGK1 mRNA**

*sgk1*<sup>+/+</sup> mice were treated with dexamethasone (10 µg/g BW) or vehicle (0.9% NaCl) for four consecutive days and were sacrificed by cervical dislocation under slight ether anaesthesia. The stomach was removed, immediately frozen in -25°C cold isopentane, and sliced on a freezing microtome at 12 µm thickness. Sections were subsequently mounted on silane-coated slides [2% 3-aminopropyltriethoxy-silane in acetone], dried at 60°C for 30 s, and fixed with 4% phosphatebuffered paraformaldehyde for 20 min. After three washes with phosphate-buffered saline (PBS; 0.1 M, pH 7.4), slides were incubated with Tris-ethylenediamine tetraacetic acid (EDTA) buffer (100 mM Tris and 50 mM EDTA; pH 8.0) containing 2 µg/ml proteinase K for 10 min at room temperature and rinsed again three times with PBS. To reduce nonspecific background, slides were acetylated with triethanolamine buffer (0.1 M triethanolamine; pH 8.0) containing 0.25% (v/v) acetic anhydride twice for 5 min. After prehybridization with hybridization buffer [50% formamide, 10% dextran sulfate, 5 mM EDTA, 20 mM Tris (pH 8.0), 10 mM dithiothreitol, 1X Denhardt's solution, 0.05% tRNA and 300 mM NaCl] for 1 h at 62°C, sections were incubated with fresh hybridization buffer containing the denatured DIG-labeled sense or antisense probe (200 ng/ml) overnight at 63°C. DIG-labeled, SGK1- specific, antisense and sense RNA probes were generated by in vitro transcription with a DIG-labeling kit. The SGK1 plasmid containing murine SGK1 sequence

(position 20-1373) was kindly provided by Prof (Dr). K. Klingel. For in vitro transcription, the plasmid was linearized with NotI (antisense probe) or with Sall (sense probe). To enhance the penetration into tissue sections, probes were alkali-hydrolyzed to an average length of 400 bases. After hybridization, slides were briefly rinsed in 2 X standard saline citrate (SSC) at room temperature and three times in 0.1 X SSC for 15 min at 63°C. Detection of the DIG-labeled RNA probe was performed according to the protocol of the DIG nucleic acid detection kit. Tissues were blocked for 30 min with blocking buffer (1% blocking reagent), in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl; pH 7.5), and then incubated with alkaline phosphatase-conjugated antibody solution (anti-DIG antibody 1:2500) in blocking buffer containing 0.1% Triton X-100 for 1 h. After washing for four times with maleic acid buffer for 15 min, slides were equilibrated for 5 min in Tris buffer (0.1 M Tris, 0.1 M NaCl, 50 mM MgCl<sub>2</sub>, pH 9.5). The color development was carried out with freshly prepared substrate solution [nitroblue tetrazolium salt (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate); in Tris buffer]. After washing for three times with PBS, slides were rinsed in distilled water, dried and coverslipped with Kaiser's gelatine solution.

## Immunofluorescence

For immunofluorescence analysis of KCNQ1 protein abundance in the cell membrane of gastric glands, the animals were sacrificed by cervical dislocation under ether anesthesia and the corpus regions of the stomach was isolated and fixed in 4% paraformaldehyde/0.1 M sodium phosphate buffer (pH 7.4) for two hours and then stored in PBS. After immersion in 30% sucrose for 24 h, 15 µm cryostat sections were taken. After a brief rinse, the sections were incubated for 1 h in normal donkey serum with 0.3% Triton X-100. All the following washes and incubations were performed in PBS containing 0.3% Triton X-100 and 1% dimethyl sulfoxide. Without rinsing, primary polyclonal goat anti-KCNQ1 antibody (1:50) and mouse monoclonal anti-H<sup>+</sup>/K<sup>+</sup>-ATPase β-sub unit (1:2000) were applied to the sections for 12 h at 4°C. After three washes at 10 min, the sections were incubated with donkey anti-goat Alexa-488 and donkey anti-mouse Alexa-555, (1:400) secondary antibodies for 1.5 h at room temperature. The slides were analyzed on a Zeiss LSM 510 confocal microscope equipped with a 40 X oil immersion objective (NA 1.3) using an argon laser at 488 and a He/Ne laser at 543 nm excitation sequentially with appropriate filter sets.

## Quantitative real-time polymerase chain reaction measurements

To determine the transcript levels of K<sup>+</sup> channel KCNQ1 and SGK1, gastric tissue was quickly removed and frozen in liquid nitrogen. Automated disruption and homogenization of frozen tissue was achieved using the MagNa Lyser Instrument™. For each sample, oneway special tubes were filled with ceramic beads, 20–30 mg of frozen tissue and 600 µl of RLT-buffer. Cleared cell lysate was transferred for further RNA purification. Subsequently, 1 µg of total RNA was reverse transcribed to complementary DNA (cDNA) utilizing the reverse transcription system with oligo (dT) primers according to the manufacturer's protocol. To determine mRNA levels, quantitative real-time polymerase chain reaction (PCR) with the Light Cycler System™ was established. PCR reactions for KCNQ1 and SGK1 were performed in a final volume of 20 µl containing 2 µl cDNA, 2.4 µl MgCl<sub>2</sub> (3 µM), 1 µl primermix (0.5 µM of both primers), 2 µl cDNA Master SybrGreen I mix, and 12.6 µl diethyl pyrocarbonate (DEPC)-treated water. The transcript levels of the housekeeping gene mGAPDH were determined in each sample using a commercial primer kit. PCR reactions for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were performed in a final volume of 20 µl containing 2 µl cDNA, 2 µl primer mix, 2 µl cDNA Master Sybr Green I mix, and 14 µl DEPC-treated water. Amplification of the target DNA was performed during 35 cycles of 95°C for 10 s, 68°C for 10 s and 72°C for 16 s, each with a temperature transition rate of 20°C/s and a secondary target temperature of 58°C with a step size of 0.5°C. Melting curve analysis was performed at 95°C 0 s, 58°C 10 s and 95°C 0 s to determine melting temperatures of primer dimers and the specific PCR products. Melting curve analysis confirmed the amplified product, which were then separated on 1.5% agarose gels to confirm the expected size (218 bp for KCNQ1 and 406 bp for SGK1). Finally, results were calculated as a ratio of the target vs house keeping gene transcripts. The following primers were used:

KCNQ1 forward 5' CCA TCA TTG ACC TCA TCGTG 3'

KCNQ1 reverse 5' CCA GAA AGC CAA TGT ACAGG 3'

SGK1 forward: 5'-TGT CTT GGG GCT GTC CTG TAT G-3'

SGK1 reverse: 5'-GCT TCT GCT GCT TCC TTC ACA C-3'

Sequence analysis confirmed the amplification of PCR products of the expected gene for KCNQ1 (accession number NM\_008434) and SGK1 (NM\_011361).

## **Flow cytometric analysis (FACS) of KCNQ1 expression in the isolated parietal cells**

To isolate the parietal cells from gastric mucosa, a previously described protocol was modified slightly and used (66). Briefly, the mice were overnight fasted prior to experiments on wire grids with free access to tap water. The mice were then sacrificed and the stomach was cut open longitudinally. The fundic and the pyloric regions were quickly discarded and the gastric corpus was taken onto ice-cold standard Hepes buffer. The entire isolation procedure was carried out on ice (4° C). The tissue was then cleaned with standard Hepes buffer thoroughly and mucous was removed as much as possible. After taking the tissue into fresh standard Hepes buffer, the muscular layer was then separated from the mucosal layer by scraping with a glass slide. The scrapings were then cut into very small pieces with a stainless steel razor and the isolated cells were separated from the tissue debris by gradient centrifugation. The cell pellet that was obtained was resuspended again in standard Hepes buffer and the suspension was passed through stainless steel needle (35 gauge). The cells thus obtained were washed once with standard Hepes buffer and then incubated at 37° C for 30 minutes first with primary polyclonal anti-goat KCNQ1 antibody, 1:100 dilution and then with anti-goat FITC secondary antibody, 1:1000 dilution in an atmosphere with 5 % CO<sub>2</sub>. The cells were washed twice and resuspended in 'annexin binding buffer' for flow cytometric analysis. FACS analysis was performed as described. Cells were analysed by forward scatter, and FITC-fluorescence intensity was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

## **Statistics**

Intracellular pH, pH recovery and buffer capacity of all parietal cells from one gland were averaged and the respective mean value was used for further statistical analysis. Data are provided as arithmetic means  $\pm$  SEM, n represents the number of mice used. All data were tested for significance using Student's t-test with Welch's correction or ANOVA (Dunnets test), where applicable and only results with  $p < 0.05$  were considered statistically significant.

## 8 RESULTS

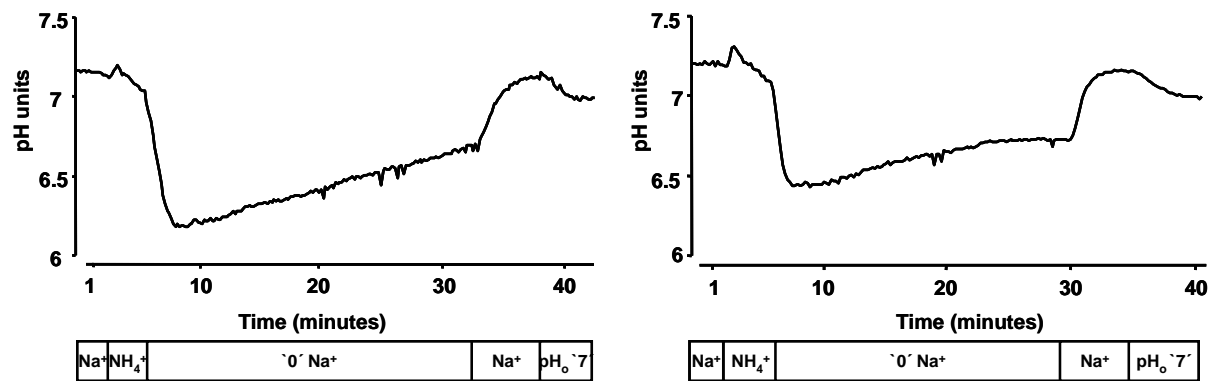
### i. Role of SGK1 in regulation of acid secretion

#### a. Role of SGK1 in regulation of basal and stimulated acid secretion

The stomach was morphologically similar in *sgk1*<sup>-/-</sup> and *sgk1*<sup>+/+</sup> mice. Accordingly, the stomach weight was similar in *sgk1*<sup>+/+</sup> mice ( $0.23 \pm 0.03$  g, n = 3) and *sgk1*<sup>-/-</sup> mice ( $0.28 \pm 0.02$  g, n = 3). pH recovery following an ammonium pulse in the absence of Na<sup>+</sup> ( $\Delta$ pH/min) which is an indicator of membrane H<sup>+</sup>/K<sup>+</sup> ATPase activity, was measured in gastric glands isolated from gene targeted mice lacking SGK1 (*sgk1*<sup>-/-</sup>) and their wild type littermates (*sgk1*<sup>+/+</sup>).

The cytosolic pH in freshly isolated gastric glands was similar in *sgk1*<sup>+/+</sup> mice ( $7.13 \pm 0.01$ , n = 6) and *sgk1*<sup>-/-</sup> mice ( $7.13 \pm 0.02$ , n = 6). The apparent buffer capacity was also similar in *sgk1*<sup>+/+</sup> mice ( $44.9 \pm 4.7$  mM/pH, n = 6) and *sgk1*<sup>-/-</sup> mice ( $43.7 \pm 7.3$  mM/pH, n = 6). The pH recovery following an ammonium pulse in the absence of Na<sup>+</sup> ( $\Delta$ pH/min) was again similar in *sgk1*<sup>+/+</sup> ( $0.027 \pm 0.005$ , n = 6 mice, 52 cells) and *sgk1*<sup>-/-</sup> mice ( $0.030 \pm 0.005$ , n = 6 mice, 68 cells) thus indicating that SGK1 is not critically important in the regulation of basal acid secretion (Figure 7). Addition of 100  $\mu$ M omeprazole virtually abolished the Na<sup>+</sup>-independent pH recovery to  $0.006 \pm 0.001$   $\Delta$ pH/min (n = 3) in *sgk1*<sup>+/+</sup> mice and to  $0.004 \pm 0.010$   $\Delta$ pH/min (n = 4) in *sgk1*<sup>-/-</sup> mice.





**Figure 7: Original tracings illustrating alterations of pH in typical experiments in *sgk1*<sup>+/+</sup> (left panels) and *sgk1*<sup>-/-</sup> (right panels) mice.** Alterations of cytosolic pH ( $\Delta$ pH) in parietal cells following an ammonium pulse. To load the cells with  $H^+$ , 20 mM  $NH_4Cl$  was added and  $Na^+$  removed (replaced by NMDG) in a first step (see bars below each original tracing),  $NH_4Cl$  removed in a second step,  $Na^+$  added in a third step and nigericin (Nig.) applied in a fourth step to calibrate each individual experiment.

To further study the role of SGK1 in stimulated acid secretion, the isolated gastric glands were stimulated with 100  $\mu$ M carbachol (cholinergic pathway) or with 5  $\mu$ M forskolin (histaminergic pathway) and the rate of pH recovery was studied. As illustrated in Figure 8, both carbachol and forskolin led to a strong stimulation of gastric acid secretion ( $Na^+$ -independent pH recovery) in *sgk1*<sup>+/+</sup> and *sgk1*<sup>-/-</sup> mice. The  $Na^+$  independent pH recovery after stimulation with carbachol or forskolin was, however, not significantly different between the genotypes. The absence of difference between genotypes when stimulated with carbachol and forskolin indicates that SGK1 is virtually not required in both the cholinergic and histaminergic stimulation of gastric acid secretion either.

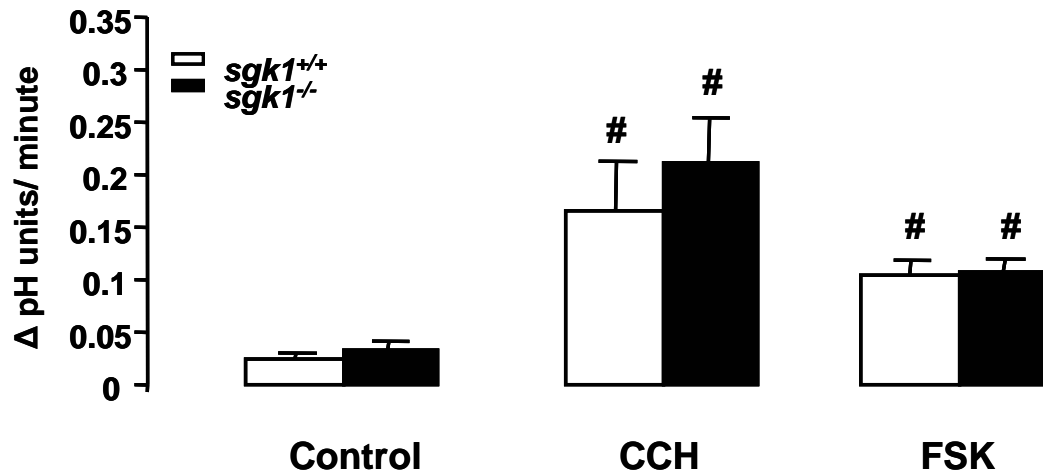


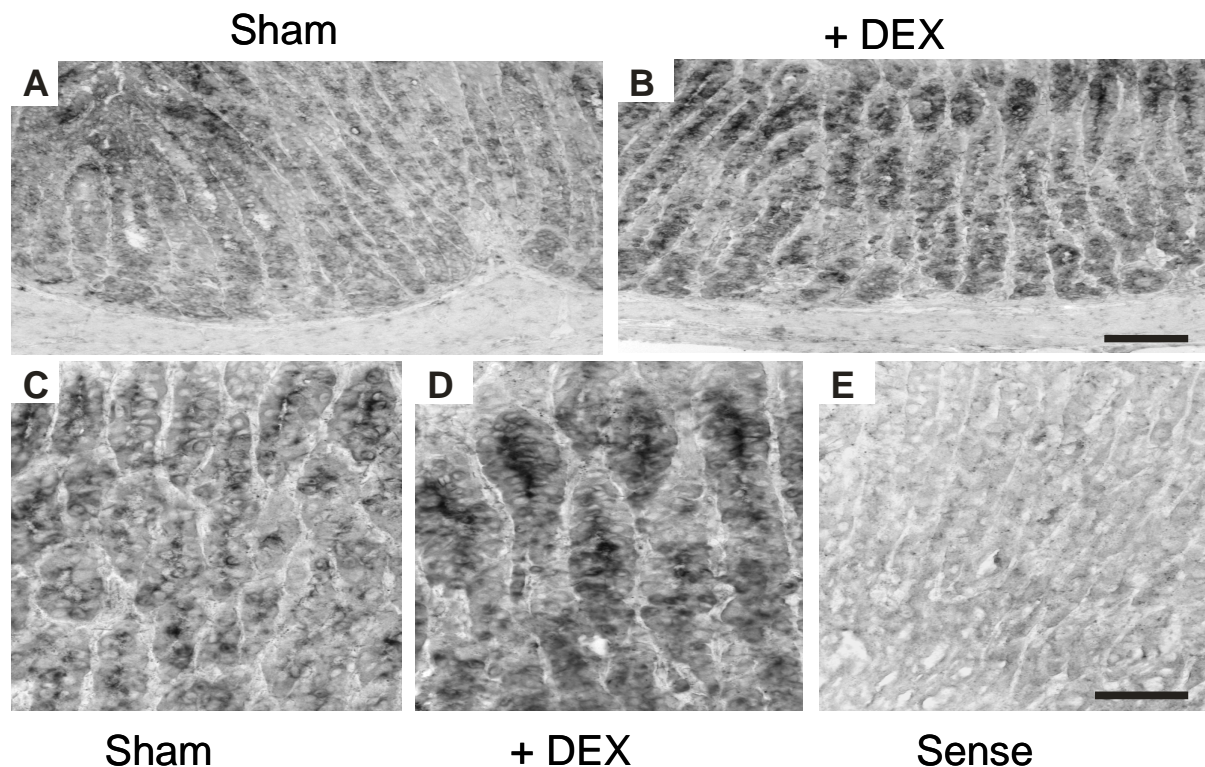
Figure 8: Rate of Na<sup>+</sup>-independent pH recovery in glands from SGK1 mice under basal and stimulated conditions.

# - indicates significant difference to respective basal/control conditions.

### b. Role of SGK1 in glucocorticoid induced stimulation of acid secretion

To study the role of SGK1 in the glucocorticoid induced stimulation of acid secretion, *sgk1*<sup>+/+</sup> and *sgk1*<sup>-/-</sup> mice were treated sub-cutaneously with dexamethasone (10 μg/g body weight) for four days and then gastric acid secretion was analysed in them.

In situ hybridization was employed to elucidate the affect of dexamethasone treatment on the abundance of SGK1 mRNA in gastric tissue. As illustrated in Figure 9 dexamethasone enhanced the expression of SGK1 in gastric glands.



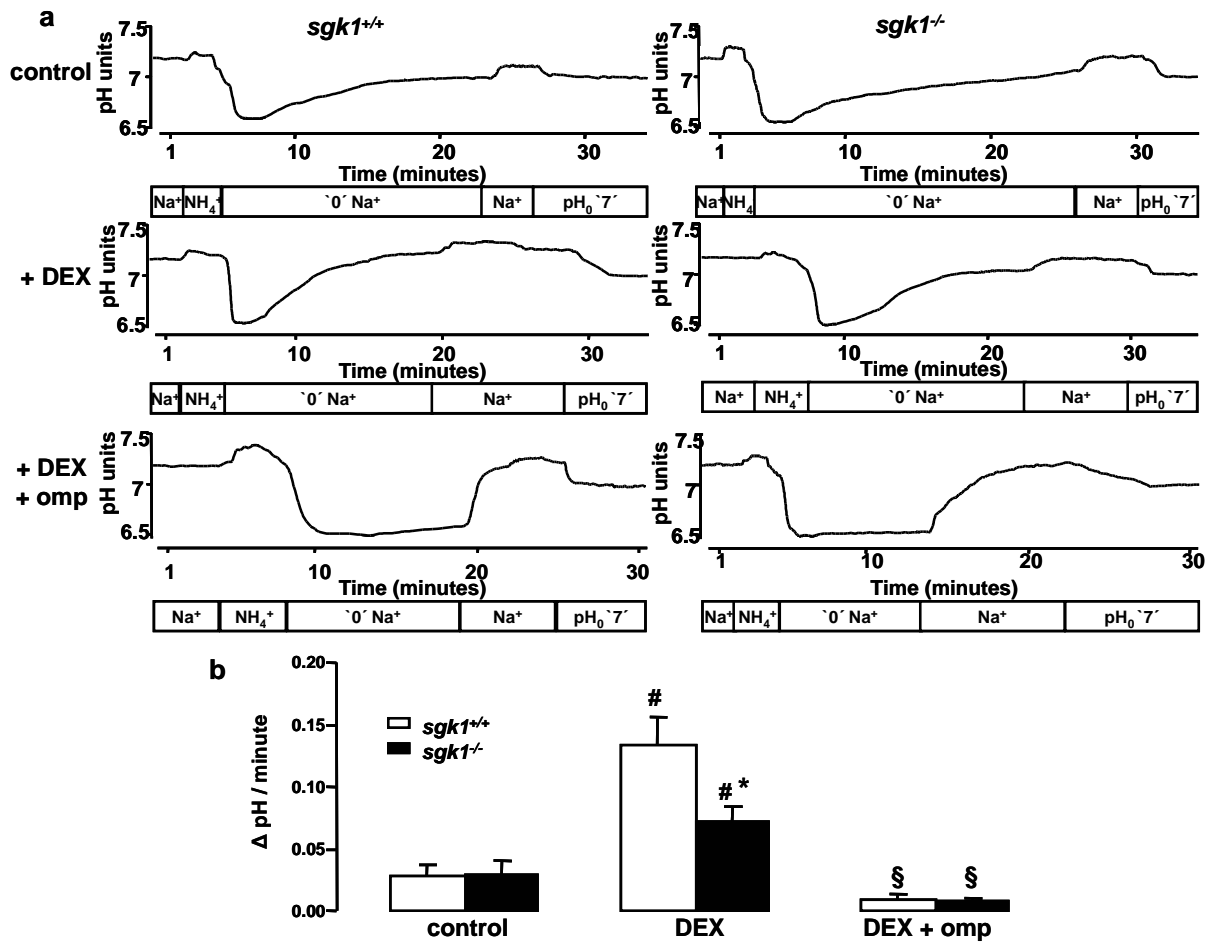
**Figure 9: SGK1 mRNA abundance in gastric tissue of wild-type mice**

The SGK1 mRNA abundance in gastric tissue of wild type mice following a 4 day treatment with dexamethasone (10  $\mu\text{g/g}$  BW) (B, D, DEX) or left untreated (A, C, control). No signal was obtained by using a sense probe (E, sense control).

Plasma concentrations of corticosterone, the main glucocorticoid in mice were then measured. At the beginning of the light cycle in the morning at 7 am, plasma corticosterone concentrations were  $401 \pm 52$  nM in *sgk1*<sup>+/+</sup> and  $489 \pm 80$  nM in *sgk1*<sup>-/-</sup> mice (n = 10-12 each). There was no significant difference between both genotypes. Towards the end of the light cycle prior to the beginning of the dark cycle at 5 pm, plasma corticosterone concentrations increased significantly to  $640 \pm 90$  nM in *sgk1*<sup>+/+</sup> and to  $985 \pm 112$  nM in *sgk1*<sup>-/-</sup> mice, respectively (n = 10-12 each). This value was significantly higher in *sgk1*<sup>-/-</sup> mice. A 4 day treatment with dexamethasone suppressed corticosterone concentrations in both genotypes to the same low level ( $87 \pm 31$  nM in *sgk1*<sup>+/+</sup> and  $91 \pm 29$  nM in *sgk1*<sup>-/-</sup> mice, n = 4-5 each).

Dexamethasone treatment did not affect the cytosolic pH of parietal cells from *sgk1*<sup>+/+</sup> ( $7.15 \pm 0.01$  (n = 8)) and *sgk1*<sup>-/-</sup> ( $7.17 \pm 0.01$  (n = 10)) mice, i.e. values similar to untreated mice and again not significantly different between both genotypes. Apart from that apparent buffer capacity was also not affected by treatment with dexamethasone and was similar in *sgk1*<sup>+/+</sup> mice ( $46.4 \pm 3.1$  mM/pH, n = 8) and in *sgk1*<sup>-/-</sup> mice ( $46.4 \pm 2.4$  mM/pH, n = 10). pH

recovery after ammonium pulse in the absence of  $\text{Na}^+$  was however, increased significantly in both the genotypes but the affect was blunted in *sgkl*<sup>-/-</sup> mice. Treatment with dexamethasone led to a ~ 4 fold increase of  $\text{Na}^+$  independent realkalinization in *sgkl*<sup>+/+</sup> mice ( $0.133 \pm 0.022$   $\Delta\text{pH}/\text{min}$ , n = 8) and ~ 2 fold increase in *sgkl*<sup>-/-</sup> mice ( $0.071 \pm 0.012$   $\Delta\text{pH}/\text{min}$ , n = 10), values significantly different between the two genotypes (Figure 10). Treatment of the isolated glands with omeprazole led to decrease of the  $\text{Na}^+$  independent pH recovery to  $0.009 \pm 0.003$   $\Delta\text{pH}/\text{min}$  (n = 5) in *sgkl*<sup>+/+</sup> mice and to  $0.007 \pm 0.002$   $\Delta\text{pH}/\text{min}$  (n = 5) in *sgkl*<sup>-/-</sup> mice thus indicating that the stimulation by dexamethasone is dependent on the activity of  $\text{H}^+/\text{K}^+$ -ATPase.



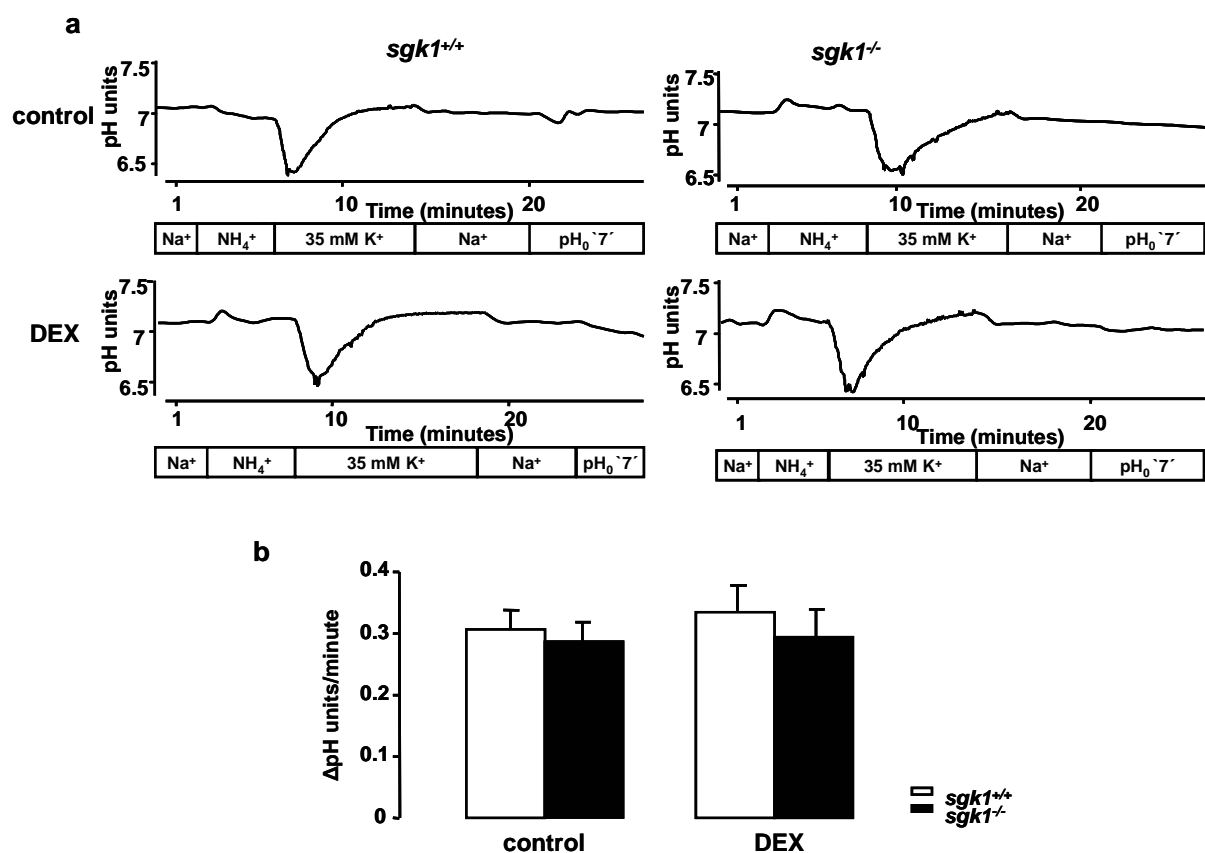
**Figure 10: pH recovery following an ammonium pulse in parietal cells from dexamethasone treated mice in presence and absence of omeprazole**

a Original tracings illustrating alterations of pH in typical experiments in *sgk1<sup>+/+</sup>* (left panels) and *sgk1<sup>-/-</sup>* (right panels) in untreated animals (upper panels), in animals treated with 10  $\mu$ g/g BW dexamethasone for four consecutive days (middle panels) and in dexamethasone treated mice in the presence of 100  $\mu$ M omeprazole (lower panels).

b Arithmetic means  $\pm$  SEM (n = 5 – 10 mice each group) of Na<sup>+</sup> independent  $\Delta$ pH in parietal cells from *sgk1<sup>+/+</sup>* mice (open columns) and *sgk1<sup>-/-</sup>* mice (closed columns) treated with dexamethasone (DEX) or left untreated (control).  $\Delta$ pH was determined in dexamethasone treated animals both, in the absence (DEX) or presence of omeprazole (DEX + omeprazole).

\* indicates significant difference between *sgk1<sup>-/-</sup>* and *sgk1<sup>+/+</sup>* mice, # indicates significant difference between DEX treatment and control, § significant difference between omeprazole and DEX treatment to DEX treatment alone.

The experiments were then conducted under conditions that are known to enhance the  $\text{Na}^+$  independent pH recovery to study the effect of dexamethasone in stimulated acid secretion. In presence of enhanced extracellular  $\text{K}^+$  concentration (35 mM), the pH recovery was significantly increased in both the genotypes and the difference between the genotypes disappeared (Figure 11). This effect again underscores the importance of outwardly projecting  $\text{K}^+$  channels in the stimulation of gastric acid secretion. When the availability of  $\text{K}^+$  in the canaliculus is no longer a rate limiting step due to enhanced local  $\text{K}^+$  concentrations, the gastric acid secretion is no longer dependent on the activity of the  $\text{K}^+$  channels and thus the difference disappears.



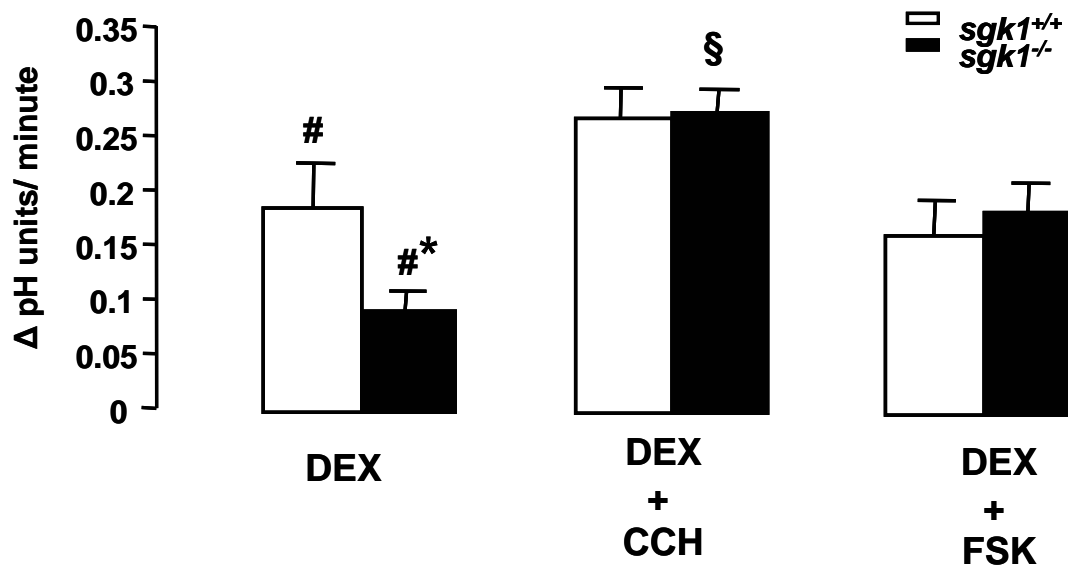
**Figure 11: Influence of enhanced extracellular  $\text{K}^+$  concentration on pH recovery in parietal cells**

Alterations of cytosolic pH ( $\Delta\text{pH}$ ) in parietal cells following an ammonium pulse at enhanced extracellular  $\text{K}^+$  concentration.

a. Original tracings illustrating alterations of pH at enhanced extracellular  $\text{K}^+$  concentration (35 mM) in  $\text{sgk1}^{+/+}$  (left panels) and  $\text{sgk1}^{-/-}$  (right panels) mice left untreated (upper panels) or treated with dexamethasone (lower panels).

b. Arithmetic means  $\pm$  SEM ( $n = 7 - 8$  mice each group) of  $\text{Na}^+$  independent  $\Delta\text{pH}$  at enhanced extracellular  $\text{K}^+$  concentration in parietal cells from  $\text{sgk1}^{+/+}$  mice (open columns) and  $\text{sgk1}^{-/-}$  mice (closed columns) either left untreated (control) or treated with dexamethasone (DEX).

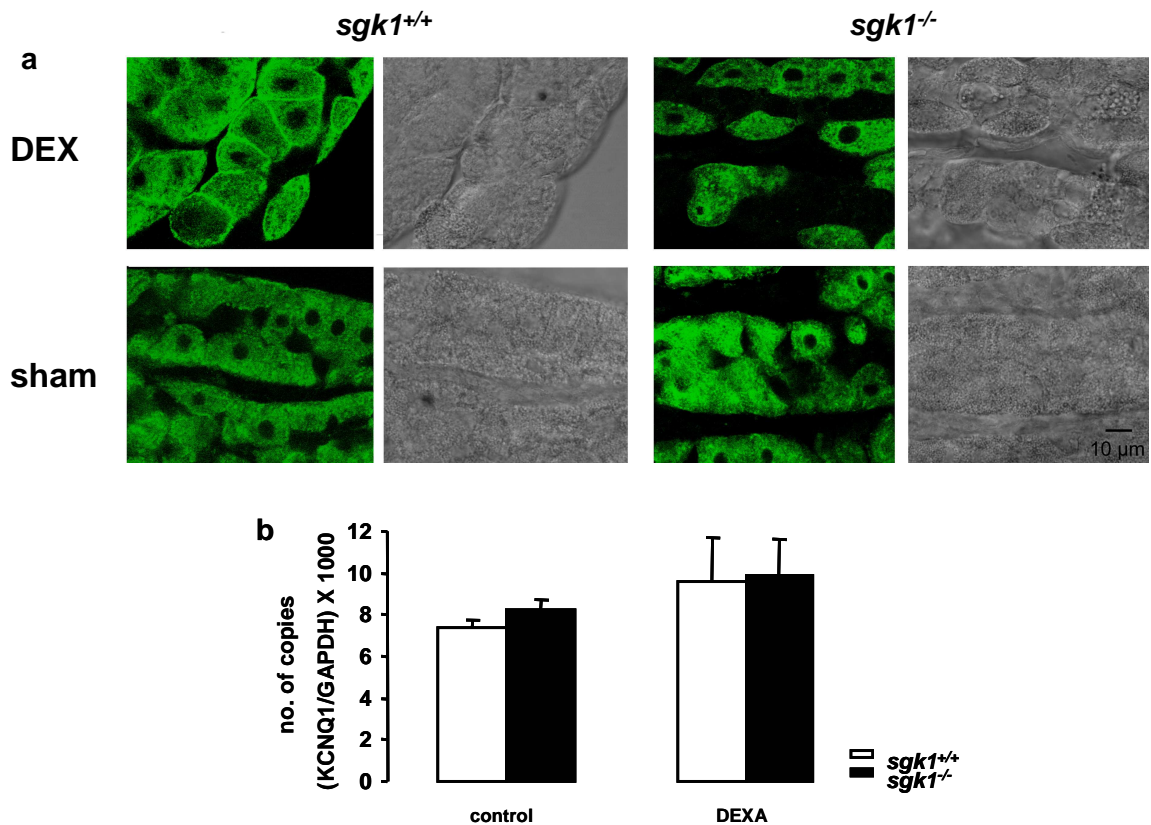
Treatment of the glands from dexamethasone treated mice additionally with carbachol (100  $\mu$ M) and forskolin (5  $\mu$ M) tended to further increase gastric acid secretion without reaching statistical significance (n = 4-6 each). Again, there was no significant difference between in *sgk1*<sup>+/+</sup> and *sgk1*<sup>-/-</sup> mice (Figure 12).



**Figure 12: Rate of Na<sup>+</sup>-independent pH recovery in glands from dexamethasone treated SGK1 mice under basal and stimulated conditions.**

\* - indicates significant difference to *sgk1*<sup>+/+</sup> mice. # - indicates significant difference to respective sham treatment (see Figure 8). § - indicates significant difference to respective basal condition.

Further, real-time PCR and immunohistochemistry was applied to analyze the expression of the potassium channel, KCNQ1 in gastric glands from untreated and dexamethasone treated mice. As shown in Figure 13, there was no difference in transcript levels of KCNQ1 between *sgk1*<sup>+/+</sup> and *sgk1*<sup>-/-</sup> mice both under control conditions and after dexamethasone treatment thus excluding genomic upregulation of the channel due to dexamethasone treatment. Immunohistochemistry showed intense staining for KCNQ1 in both genotypes. After dexamethasone treatment, stainings for KCNQ1 showed enrichment of the channel at the plasma membrane. Overall, stainings tended to be stronger in dexamethasone treated *sgk1*<sup>+/+</sup> mice (Figure 13).



**Figure 13: Expression of the potassium channel KCNQ1 in parietal cells**

a Immunohistochemistry of isolated glands showing KCNQ1 expression in *sgk1<sup>+/+</sup>* (left panels) and *sgk1<sup>-/-</sup>* (right panels) mice following a 4 day treatment with dexamethasone (upper panels) or sham treatment (lower panels).

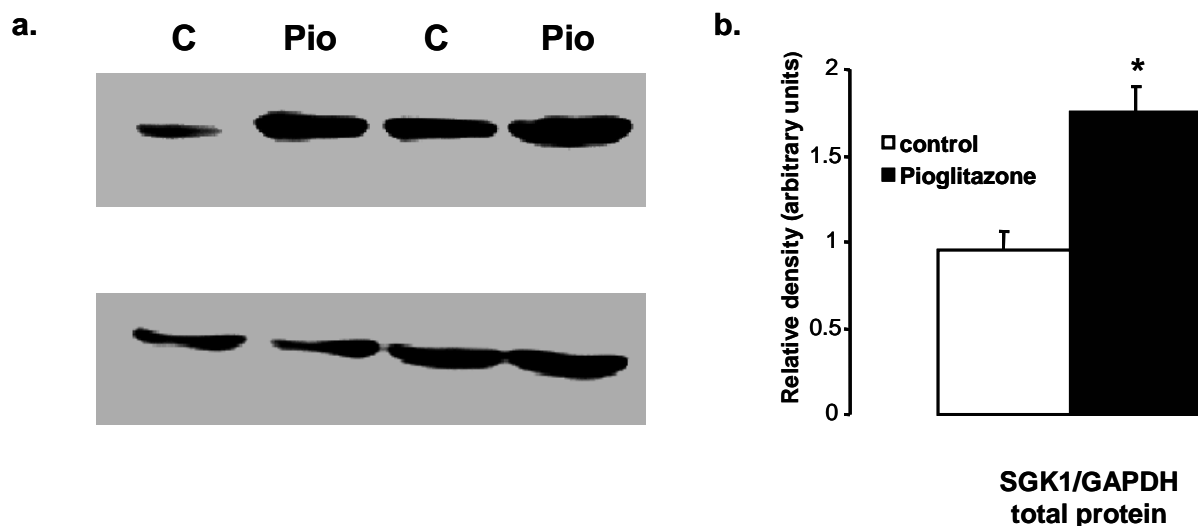
b Arithmetic means  $\pm$  SEM (n = number of experiments) of KCNQ1 over GAPDH transcript levels in gastric corpus as determined by quantitative real time PCR in *sgk1<sup>+/+</sup>* (open columns) and *sgk1<sup>-/-</sup>* mice (closed columns) either sham treated (sham) or treated with dexamethasone (DEX).

It is thereby evident that dexamethasone stimulated acid secretion is at least in part dependent on presence of SGK1.



### c. Role of SGK1 in peroxisome proliferator activated receptor gamma (PPAR<sub>γ</sub>) agonist mediated stimulation of acid secretion

Treatment with PPAR<sub>γ</sub> agonists (pioglitazone) was also reported to upregulate the expression of SGK1 (56). Therefore the gastric acid secretion was studied in the mice treated with pioglitazone. The body weights of the mice were similar in gene targeted mice lacking serum and glucocorticoid inducible kinase (*sgk1*<sup>-/-</sup>, 31.03 ± 0.84 g, n = 6) and their wild type littermates (*sgk1*<sup>+/+</sup>, 30.21 ± 0.93 g, n = 6). A 7 day pioglitazone treatment did not lead to any significant increase in body weight in either *sgk1*<sup>+/+</sup> mice (31.60 ± 1.02 g, n = 6) or *sgk1*<sup>-/-</sup> mice (31.90 ± 0.66 g, n = 6). Macroscopic inspection of the stomachs of *sgk1*<sup>-/-</sup> and *sgk1*<sup>+/+</sup> mice did not reveal apparent morphological differences. Accordingly, the stomach weight was similar in *sgk1*<sup>+/+</sup> mice (0.23 ± 0.03 g, n = 3) and *sgk1*<sup>-/-</sup> mice (0.28 ± 0.02 g, n = 3). Treatment with pioglitazone did not significantly alter the stomach weight in neither *sgk1*<sup>+/+</sup> mice (0.21 ± 0.01 g, n = 3) nor *sgk1*<sup>-/-</sup> mice (0.24 ± 0.01 g, n = 3). According to Western blotting from gastric tissue, pioglitazone treatment was followed by a significant increase of SGK1 protein abundance (Figure 14).



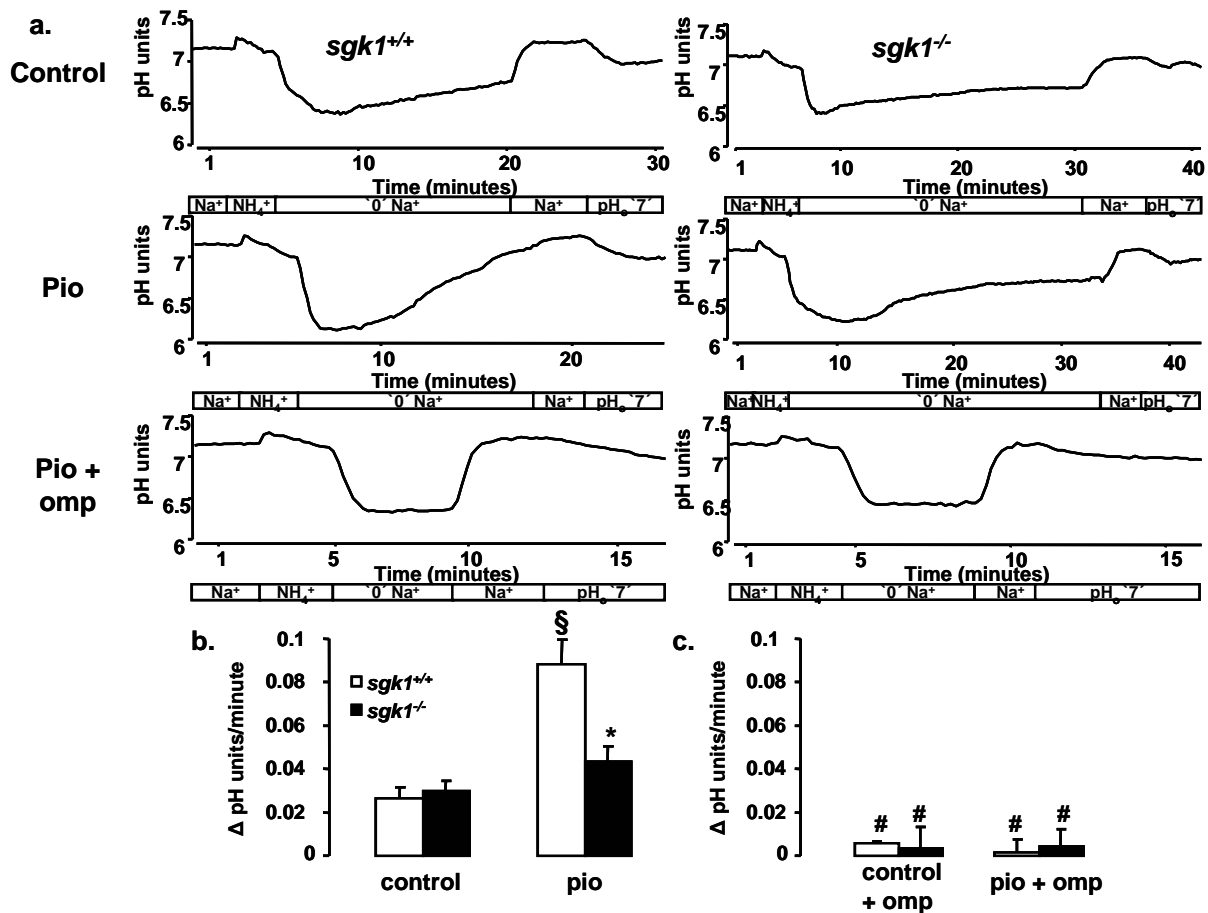
**Figure 14: SGK1 protein abundance in stomach.**

a. Original Western Blot illustrating the SGK1 (upper panel) and GAPDH (lower panel) protein abundance in stomach from *sgk1*<sup>+/+</sup> mice without (C) and with (PIO) prior pioglitazone treatment

b. Arithmetic means ± SEM (n = 4 mice in each group) of relative densities of SGK1 protein expression in stomach from *sgk1*<sup>+/+</sup> mice without (C) and with (PIO) prior pioglitazone treatment. \* indicates significant difference to absence of pioglitazone treatment (p<0.05).

Pioglitazone treatment also did not significantly alter cytosolic pH in *sgkl*<sup>+/+</sup> mice ( $7.16 \pm 0.02$ ,  $n = 11$ ) and *sgkl*<sup>-/-</sup> mice ( $7.13 \pm 0.02$ ,  $n = 11$ ). The apparent buffer capacity was also not significantly different in pioglitazone treated *sgkl*<sup>+/+</sup> mice ( $44.4 \pm 4.9$  mM/pH,  $n = 11$ ) and *sgkl*<sup>-/-</sup> mice ( $53.6 \pm 5.3$  mM/pH,  $n = 11$ ).

As illustrated in Figure 15, pH recovery following an ammonium pulse in the absence of Na<sup>+</sup> ( $\Delta$ pH/min) was similar in untreated *sgkl*<sup>+/+</sup> and *sgkl*<sup>-/-</sup> mice. Pioglitazone treatment was followed by a marked significant increase of  $\Delta$ pH/min in *sgkl*<sup>+/+</sup> mice but not in *sgkl*<sup>-/-</sup> mice (Table 1, Figure 15). Accordingly, following pioglitazone treatment,  $\Delta$ pH/min was significantly higher in *sgkl*<sup>+/+</sup> mice than in *sgkl*<sup>-/-</sup> mice (Figure 15). Addition of omeprazole virtually abolished the Na<sup>+</sup>-independent pH recovery to  $0.006 \pm 0.001$   $\Delta$ pH/min ( $n = 3$ ) in *sgkl*<sup>+/+</sup> mice and to  $0.004 \pm 0.010$   $\Delta$ pH/min ( $n = 4$ ) in *sgkl*<sup>-/-</sup> mice. The same low values were obtained in pioglitazone treated *sgkl*<sup>+/+</sup> mice ( $0.001 \pm 0.006$   $\Delta$ pH/min,  $n = 3$ ) and *sgkl*<sup>-/-</sup> mice ( $0.004 \pm 0.008$   $\Delta$ pH/min,  $n = 3$ ). Thus, Na<sup>+</sup>-independent pH recovery required H<sup>+</sup>/K<sup>+</sup>-ATPase activity in untreated and pioglitazone treated *sgkl*<sup>+/+</sup> and *sgkl*<sup>-/-</sup> mice.



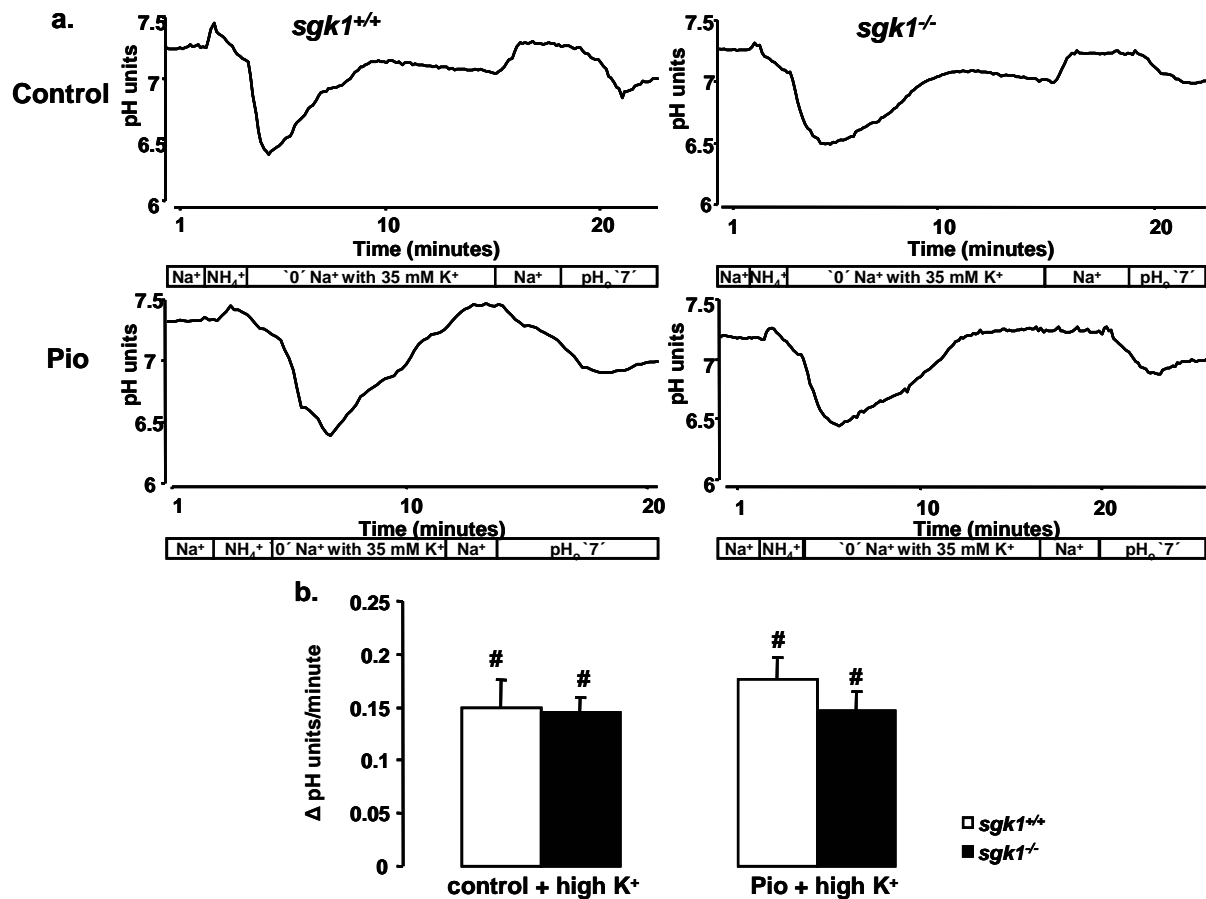
**Figure 15: Effect of pioglitazone on pH recovery following an ammonium pulse in parietal cells from pioglitazone treated mice in the presence and absence of omeprazole**

a. Original representative tracings illustrating alterations of pH in parietal cells from *sgk1*<sup>-/-</sup> (right panels) and *sgk1*<sup>+/+</sup> (left panels) mice without treatment (upper panels), following treatment with pioglitazone for 7 days (middle panels) as well as following prior pioglitazone treatment and local application of omeprazole (omp) (lower panels).

b. Arithmetic means  $\pm$  SEM of Na<sup>+</sup>-independent alterations of cytosolic pH ( $\Delta$ pH/min) in parietal cells from *sgk1*<sup>+/+</sup> (open bars) and *sgk1*<sup>-/-</sup> (closed bars) mice without treatment (left bars) (n = 6 mice in each group) and with prior pioglitazone treatment (right bars) (n = 11 mice in each group). \* indicates significant difference from *sgk1*<sup>+/+</sup> mice, § indicates significant difference from untreated mice (control) (p<0.05).

c. Arithmetic means  $\pm$  SEM (n = 3-4 mice in each group) of Na<sup>+</sup>-independent alterations of cytosolic pH ( $\Delta$ pH/min) in the presence of omeprazole (100 $\mu$ M) in parietal cells from *sgk1*<sup>+/+</sup> (open bars) and *sgk1*<sup>-/-</sup> (closed bars) mice without treatment (left bars) and with prior pioglitazone treatment (right bars). # indicates significant difference from absence of omeprazole.

As illustrated in Figure 16, an increase in extracellular  $K^+$  concentration to 35 mM by replacing  $Na^+$  led to a significant increase in  $Na^+$ -independent pH recovery to similarly high levels in  $sgk1^{+/+}$  and  $sgk1^{-/-}$  mice. In the presence of 35 mM extracellular  $K^+$ , the treatment with pioglitazone did not lead to a further increase of  $\Delta pH/min$ . Thus, enhanced extracellular  $K^+$  concentration dissipated the differences in  $H^+$  secretion between pioglitazone treated  $sgk1^{+/+}$  and  $sgk1^{-/-}$  mice.

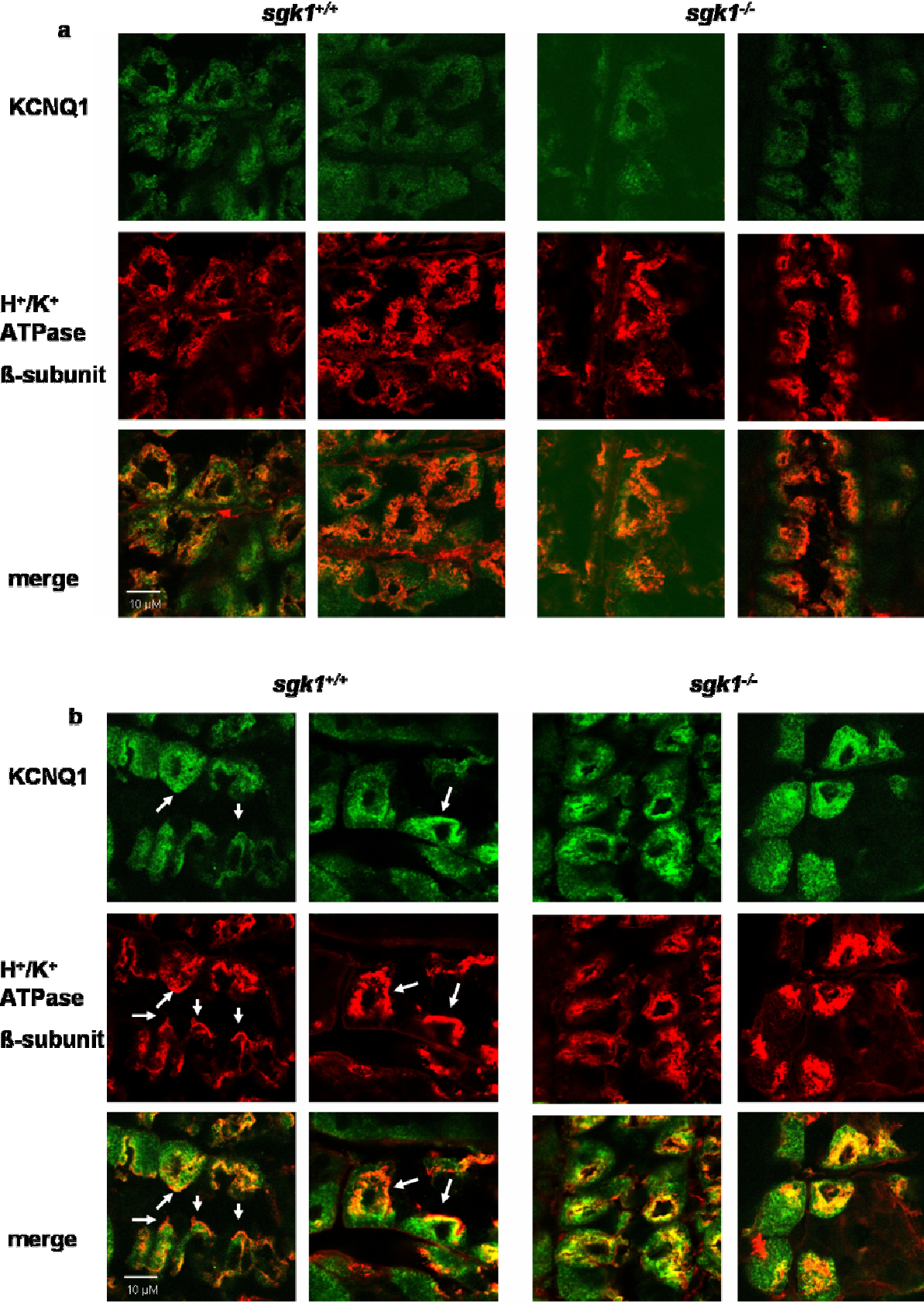


**Figure 16: Influence of enhanced extracellular  $K^+$  concentration on pH recovery in parietal cells**

a. Original representative tracings illustrating alterations of pH at enhanced extracellular  $K^+$  concentration (35 mM) in parietal cells from  $sgk1^{-/-}$  (right panels) and  $sgk1^{+/+}$  (left panels) mice without treatment (upper panels) and with prior pioglitazone treatment (lower panels).

b. Arithmetic means  $\pm$  SEM ( $n = 4-5$  mice in each group) of  $Na^+$ -independent alterations of cytosolic pH ( $\Delta pH/min$ ) at enhanced extracellular  $K^+$  concentration (35 mM) in parietal cells from  $sgk1^{+/+}$  (open bars) and  $sgk1^{-/-}$  (closed bars) mice without treatment (left bars) and with prior pioglitazone treatment (right bars). # indicates significant difference from control physiological solution containing 3 mM  $K^+$  (refer Figure 15).

It was reported earlier that stimulation of the parietal cell results in trafficking of the vesicles containing the proton pump  $H^+/K^+$ -ATPase and KCNQ1 channel towards the apical membrane in a tubulovesicular network (18). Therefore, immunohistochemistry was employed to elucidate, whether pioglitazone influenced KCNQ1 and  $H^+/K^+$  ATPase protein abundance in the cell membranes of *sgkl*<sup>+/+</sup> and *sgkl*<sup>-/-</sup> mice. As illustrated in Figure 17a both the  $H^+/K^+$ -ATPase were diffusely distributed throughout the cytoplasm in the parietal cells for control treated *sgkl*<sup>+/+</sup> and *sgkl*<sup>-/-</sup> mice. As expected, there was no difference between the two genotypes under control conditions. Pioglitazone treatment however, resulted in a clear colocalization of the channel and the pump proteins and in clustering of the immunoreactivities within the tubulovesicular network mostly towards the apical membrane in the parietal cells from *sgkl*<sup>+/+</sup> mice (Figure 17 b). In contrast, in parietal cells from *sgkl*<sup>-/-</sup> mice both proteins remained in the perinuclear space.



**Figure 17: Localization of KCNQ1 and H<sup>+</sup>/K<sup>+</sup>-ATPase in parietal cells from a. control mice, b. pioglitazone treated mice.** Immunofluorescence of KCNQ1 (green) and H<sup>+</sup>/K<sup>+</sup>-ATPase (red) expression in parietal cells from *sgk1*<sup>-/-</sup> mice (right panels) and *sgk1*<sup>+/+</sup> mice (left panels). Two different microscopic fields are shown for each genotype.

**Table1: Gastric acid secretion in sham/pioglitazone treated *sgk1*<sup>-/-</sup> and *sgk1*<sup>+/+</sup> mice under basal and stimulated conditions.**

			pH recovery (Δ pH Units/minute)	intracellular pH (units)	buffer capacity (mM/pH unit)	Number of mice	Number of cells
<b>control (untreated)</b>	<b>sham</b>	<i>sgk1</i> <sup>+/+</sup>	0.027 ± 0.005	7.13 ± 0.01	44.87 ± 4.7	6	52
		<i>sgk1</i> <sup>-/-</sup>	0.030 ± 0.005	7.13 ± 0.02	43.66 ± 7.3	6	68
	<b>pio</b>	<i>sgk1</i> <sup>+/+</sup>	0.088 ± 0.012 <sup>b</sup>	7.16 ± 0.02	44.37 ± 4.9	11	140
		<i>sgk1</i> <sup>-/-</sup>	0.044 ± 0.007 <sup>a</sup>	7.13 ± 0.02	53.56 ± 5.3	11	160
<b>omeprazole</b>	<b>sham</b>	<i>sgk1</i> <sup>+/+</sup>	0.006 ± 0.001 <sup>c</sup>	7.19 ± 0.04	31.43 ± 1.5	3	52
		<i>sgk1</i> <sup>-/-</sup>	0.004 ± 0.010 <sup>c</sup>	7.18 ± 0.03	30.05 ± 2.8	4	44
	<b>pio</b>	<i>sgk1</i> <sup>+/+</sup>	0.001 ± 0.006 <sup>c</sup>	7.21 ± 0.02	32.57 ± 7.3	3	50
		<i>sgk1</i> <sup>-/-</sup>	0.004 ± 0.008 <sup>c</sup>	7.12 ± 0.03	44.25 ± 9.9	3	33
<b>High K<sup>+</sup></b>	<b>sham</b>	<i>sgk1</i> <sup>+/+</sup>	0.149 ± 0.027 <sup>c</sup>	7.19 ± 0.01	32.10 ± 5.5	4	41
		<i>sgk1</i> <sup>-/-</sup>	0.145 ± 0.015 <sup>c</sup>	7.23 ± 0.04	39.10 ± 3.8	4	53
	<b>pio</b>	<i>sgk1</i> <sup>+/+</sup>	0.176 ± 0.021 <sup>c</sup>	7.24 ± 0.04	33.09 ± 7.8	5	45
		<i>sgk1</i> <sup>-/-</sup>	0.147 ± 0.017 <sup>c</sup>	7.19 ± 0.05	46.12 ± 4.6	4	61

a - indicates significant difference to wild type mice.

b - indicates significant difference to respective sham treatment.

c - indicates significant difference to respective basal condition.

#### d. SGK1 dependant stimulation of gastric acid secretion in mutant APC mice (*apc<sup>Min/+</sup>*)

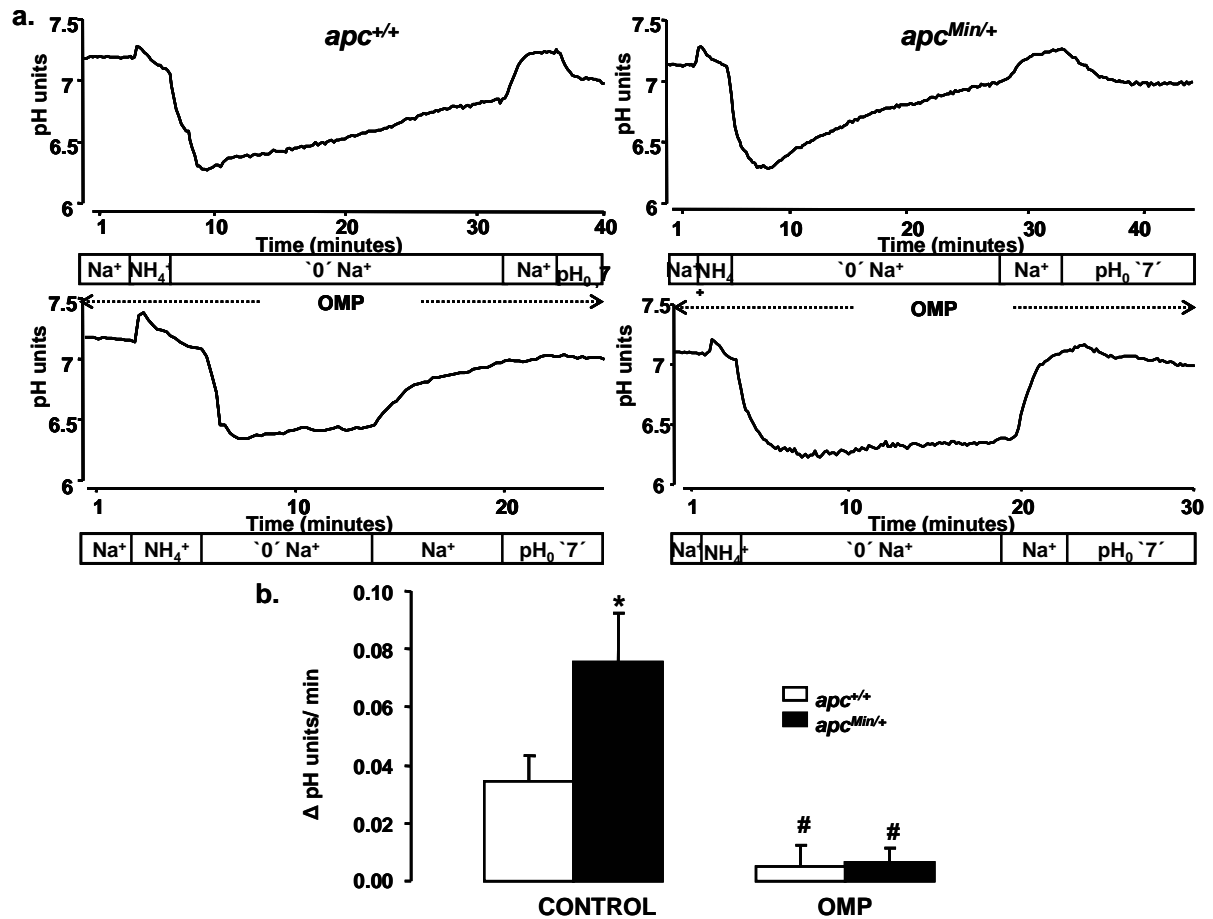
It was reported earlier that nuclear translocation of  $\beta$ -catenin leads to upregulation of SGK1 (67). The present study has thus been performed to elucidate whether gastric acid secretion is altered in *apc<sup>Min/+</sup>* mice and if it is affected by the presence or absence of SGK1. To this end, gastric acid secretion was studied in isolated gastric glands from *apc<sup>Min/+</sup>* mice and their wild type littermates (*apc<sup>+/+</sup>*) and then the role of SGK1 was demonstrated through studies in *apc<sup>Min/+</sup>sgk1<sup>-/-</sup>* mice.

The stomach was morphologically similar in heterozygous mice with defective APC (*apc<sup>Min/+</sup>*) and their wild type littermates (*apc<sup>+/+</sup>*). Accordingly, the stomach weight was similar in both genotypes ( $0.22 \pm 0.01$  g, n = 5 in *apc<sup>Min/+</sup>* mice and  $0.25 \pm 0.02$  g, n = 8 in *apc<sup>+/+</sup>* mice).

The cytosolic pH in freshly isolated gastric glands (Figure 18) was similar in untreated *apc<sup>+/+</sup>* mice ( $7.13 \pm 0.02$ , n = 10) and *apc<sup>Min/+</sup>* mice ( $7.14 \pm 0.01$ , n = 10). The apparent buffer capacity was again similar in *apc<sup>+/+</sup>* mice ( $37.7 \pm 3.8$  mM/pH, n = 10) and *apc<sup>Min/+</sup>* mice ( $39.4 \pm 5.2$  mM/pH, n = 10).

As illustrated in Table 2, Figure 18, pH recovery following an ammonium pulse in the absence of  $\text{Na}^+$  was significantly ( $\sim 2$  -fold) higher in *apc<sup>Min/+</sup>* mice ( $0.076 \pm 0.010$   $\Delta\text{pH}/\text{min}$ , n = 10) than in *apc<sup>+/+</sup>* mice ( $0.034 \pm 0.008$   $\Delta\text{pH}/\text{min}$ , n = 10). Addition of 100  $\mu\text{M}$  omeprazole decreased the  $\text{Na}^+$ -independent pH recovery to  $0.006 \pm 0.006$   $\Delta\text{pH}/\text{min}$  (n = 5) in *apc<sup>Min/+</sup>* mice and to  $0.006 \pm 0.005$   $\Delta\text{pH}/\text{min}$  (n = 4) in *apc<sup>+/+</sup>* mice. Thus, in both genotypes,  $\text{Na}^+$ -independent pH recovery was abolished following pharmacological inhibition of  $\text{H}^+/\text{K}^+$ -ATPase activity (Figure 18).



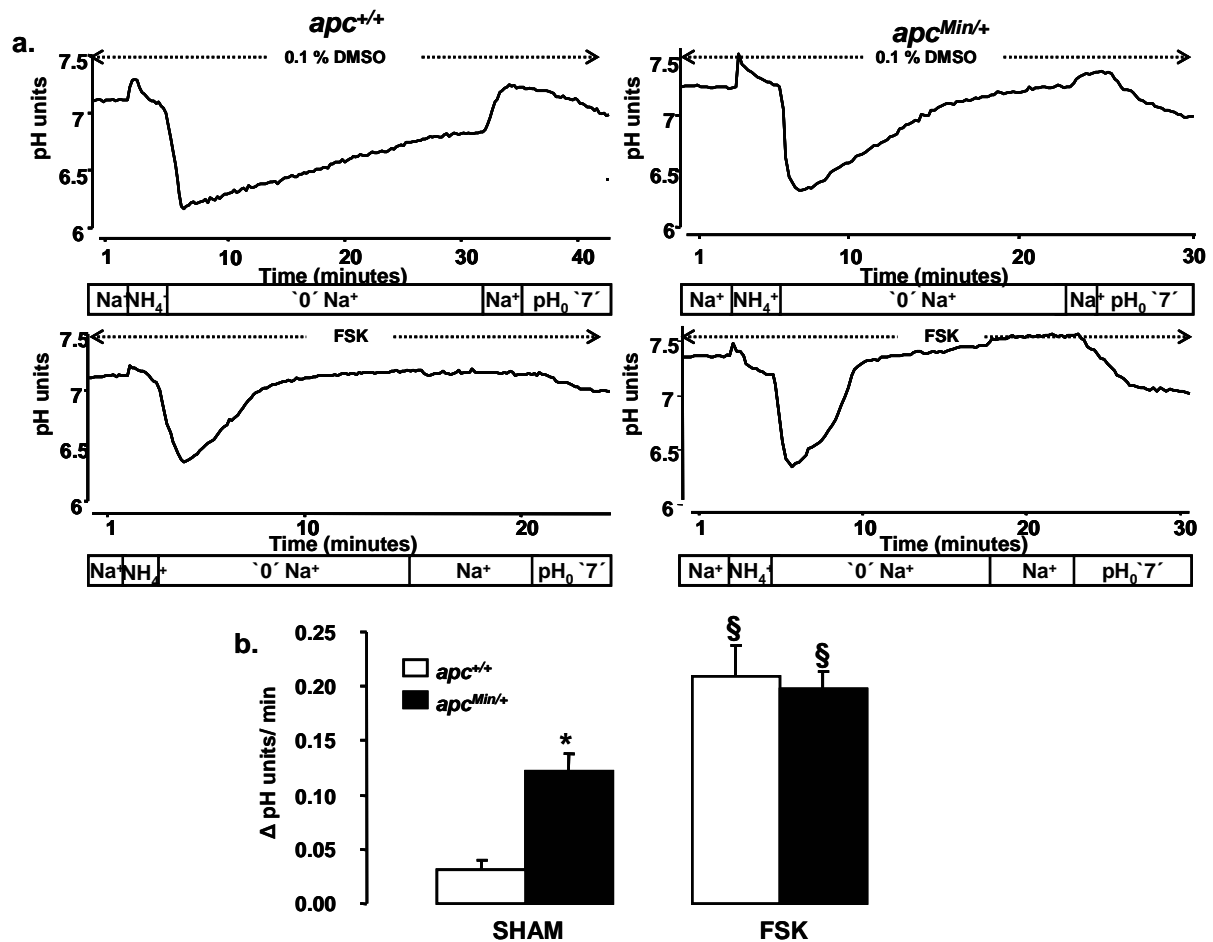


**Figure 18: pH recovery following an ammonium pulse in parietal cells from APC mice in the presence or absence of omeprazole**

a. Original tracings illustrating alterations of pH in typical experiments in *apc<sup>Min/+</sup>* mice (right panels) and *apc<sup>+/+</sup>* mice (left panels) in the absence (upper panels) and presence (lower panels) of 100  $\mu$ M omeprazole (OMP).

b. Arithmetic means  $\pm$  SEM of Na<sup>+</sup>-independent alterations of cytosolic pH ( $\Delta$ pH/min) in parietal cells from *apc<sup>Min/+</sup>* mice (closed bars) and *apc<sup>+/+</sup>* mice (open bars) in the absence (left bars) and presence (right bars) of omeprazole. \* indicates significant difference to *apc<sup>+/+</sup>* mice, # indicates significant difference to absence of omeprazole (control).

As illustrated in Figure 19, forskolin (5  $\mu$ M) led to a strong stimulation of gastric acid secretion in *apc<sup>+/+</sup>* mice ( $0.209 \pm 0.028$   $\Delta$ pH/min, n = 7) and *apc<sup>Min/+</sup>* mice ( $0.198 \pm 0.016$   $\Delta$ pH/min, n = 6). Notably, forskolin treatment increased the Na<sup>+</sup>-independent acidification ( $\Delta$ pH/min) to similar values in both genotypes. Thus, following treatment with forskolin there was no significant difference between *apc<sup>+/+</sup>* and *apc<sup>Min/+</sup>* mice.

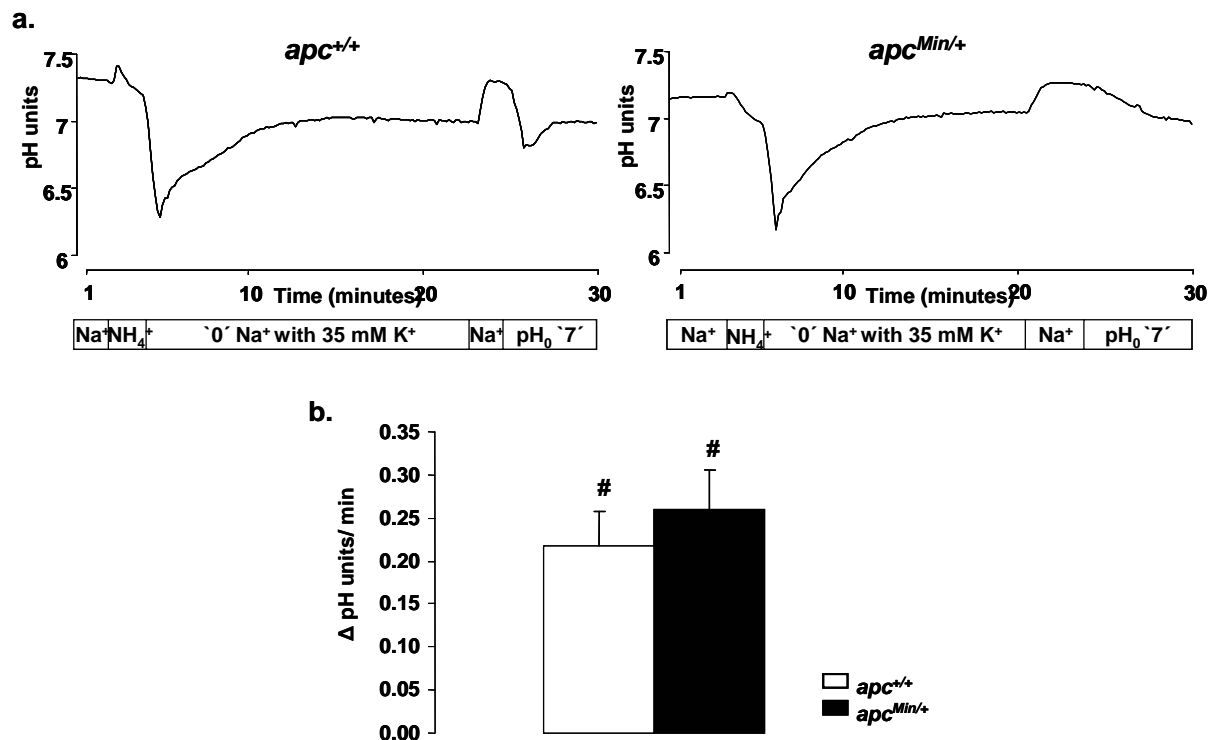


**Figure 19: Effects of forskolin on pH recovery in parietal cells from APC mice.**

a. Original tracings illustrating alterations of pH in typical experiments in *apc*<sup>Min/+</sup> mice (right panels) and *apc*<sup>+/+</sup> mice (left panels) in the absence (upper panels) and presence (lower panels) of forskolin (FSK, 5  $\mu$ M).

b. Arithmetic means  $\pm$  SEM of Na<sup>+</sup>-independent alterations of cytosolic pH ( $\Delta$ pH/min) in parietal cells following an ammonium pulse in the absence (sham) and presence of forskolin (FSK, 5  $\mu$ M) in *apc*<sup>Min/+</sup> (closed bars) and *apc*<sup>+/+</sup> mice (open bars). § indicates significant difference to respective sham treatment, \* indicates significant difference to *apc*<sup>+/+</sup> mice.

An increase in the extracellular K<sup>+</sup> concentration to 35 mM by replacing Na<sup>+</sup>/NMDG led to a significant increase in Na<sup>+</sup>-independent pH recovery to  $0.259 \pm 0.048$   $\Delta$ pH/min (n = 6) in *apc*<sup>Min/+</sup> mice and to  $0.218 \pm 0.039$   $\Delta$ pH/min, (n = 5) in *apc*<sup>+/+</sup> mice (Figure 20). Again, following increase in the extracellular K<sup>+</sup> concentration pH recovery ( $\Delta$ pH/min) increased to similar values in both genotypes. Thus, an increased extracellular K<sup>+</sup> concentration dissipated the differences in H<sup>+</sup> secretion between *apc*<sup>+/+</sup> and *apc*<sup>Min/+</sup> mice.



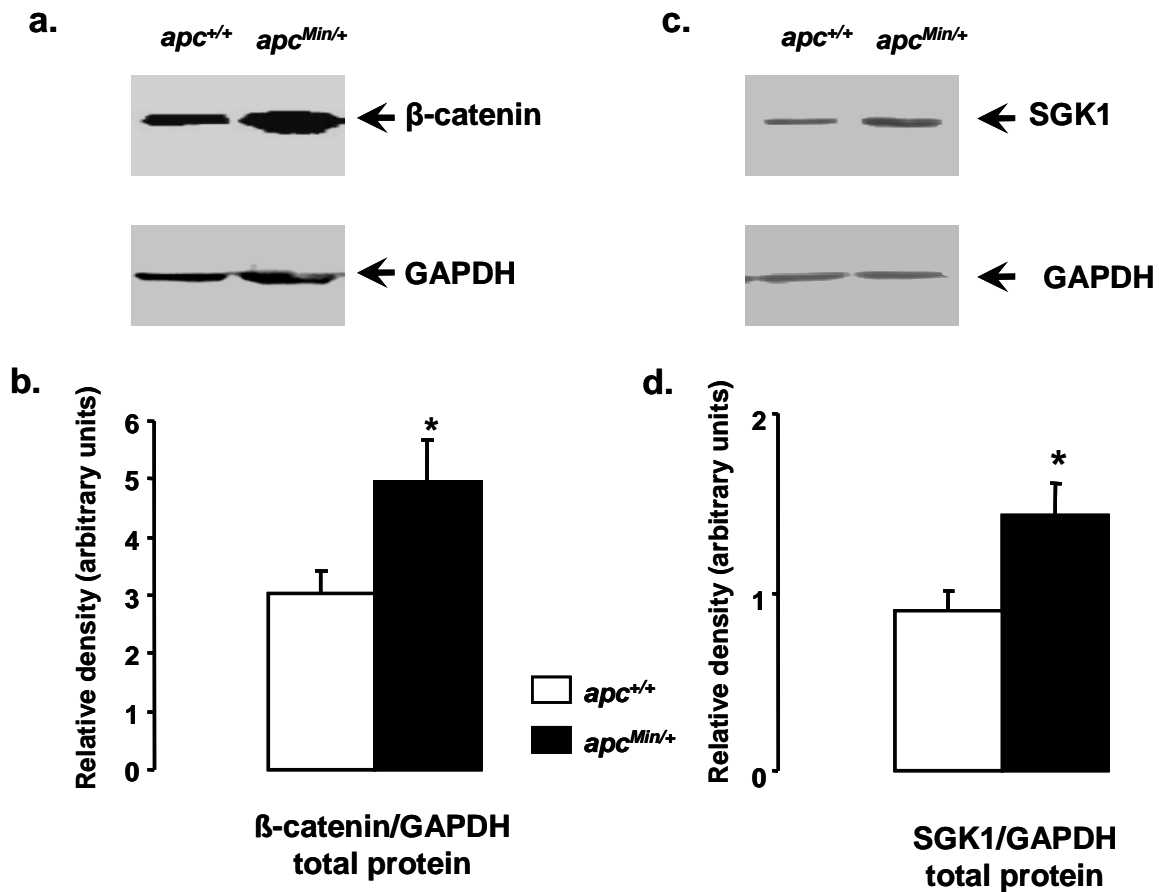
**Figure 20: Influence of enhanced extracellular K<sup>+</sup> concentration on pH recovery in parietal cells**

a. Original tracings illustrating alterations of pH at enhanced extracellular K<sup>+</sup> concentration (35 mM) in *apc*<sup>Min/+</sup> mice (right panels) and *apc*<sup>+/+</sup> mice (left panels).

b. Arithmetic means ± SEM of Na<sup>+</sup>-independent ΔpH in parietal cells from *apc*<sup>Min/+</sup> mice (closed bars) and *apc*<sup>+/+</sup> mice (open bars) at high (35 mM K<sup>+</sup>) extracellular K<sup>+</sup> concentration. # indicates significant difference to normal K<sup>+</sup> concentration (3 mM K<sup>+</sup>, control, see **Figure 18**).

Total serum gastrin concentrations were measured to explore, whether the observed differences between *apc*<sup>+/+</sup> and *apc*<sup>Min/+</sup> parietal cells were due to differences in gastrin levels. Serum gastrin levels were, however, similar in *apc*<sup>Min/+</sup> mice (35.6 ± 2.4 pM/L, n = 8) and *apc*<sup>+/+</sup> mice (36.3 ± 1.8 pM/L, n = 15).

Western blotting was performed to elucidate, whether the functional differences between *apc*<sup>+/+</sup> and *apc*<sup>Min/+</sup> parietal cells could have been related to differences in protein abundance of β-catenin and/or SGK1. As illustrated in Figure 21, the protein abundance for both, β-catenin and SGK1 was indeed significantly larger in *apc*<sup>Min/+</sup> than in *apc*<sup>+/+</sup> mice. Quantitative real time PCR analysis further revealed significantly higher expression of SGK1 in *apc*<sup>Min/+</sup> mice (50.0 ± 6.0 copies/GAPDH X 1000, n = 9) as compared to *apc*<sup>+/+</sup> mice (31.7 ± 4.6 copies/GAPDH X 1000, n = 11).



**Figure 21:  $\beta$ -catenin and SGK1 protein abundance in gastric glands.**

a. Original Western Blot illustrating the  $\beta$ -catenin (upper panel) and GAPDH (lower panel) protein abundance in  $apc^{Min/+}$  mice and  $apc^{+/+}$  mice.

b. Arithmetic means  $\pm$  SEM of relative densities of  $\beta$ -catenin protein expression in stomach from  $apc^{Min/+}$  mice (closed bars) and  $apc^{+/+}$  mice (open bars). \* indicates significant difference between  $apc^{Min/+}$  and  $apc^{+/+}$  mice ( $p < 0.05$ ).

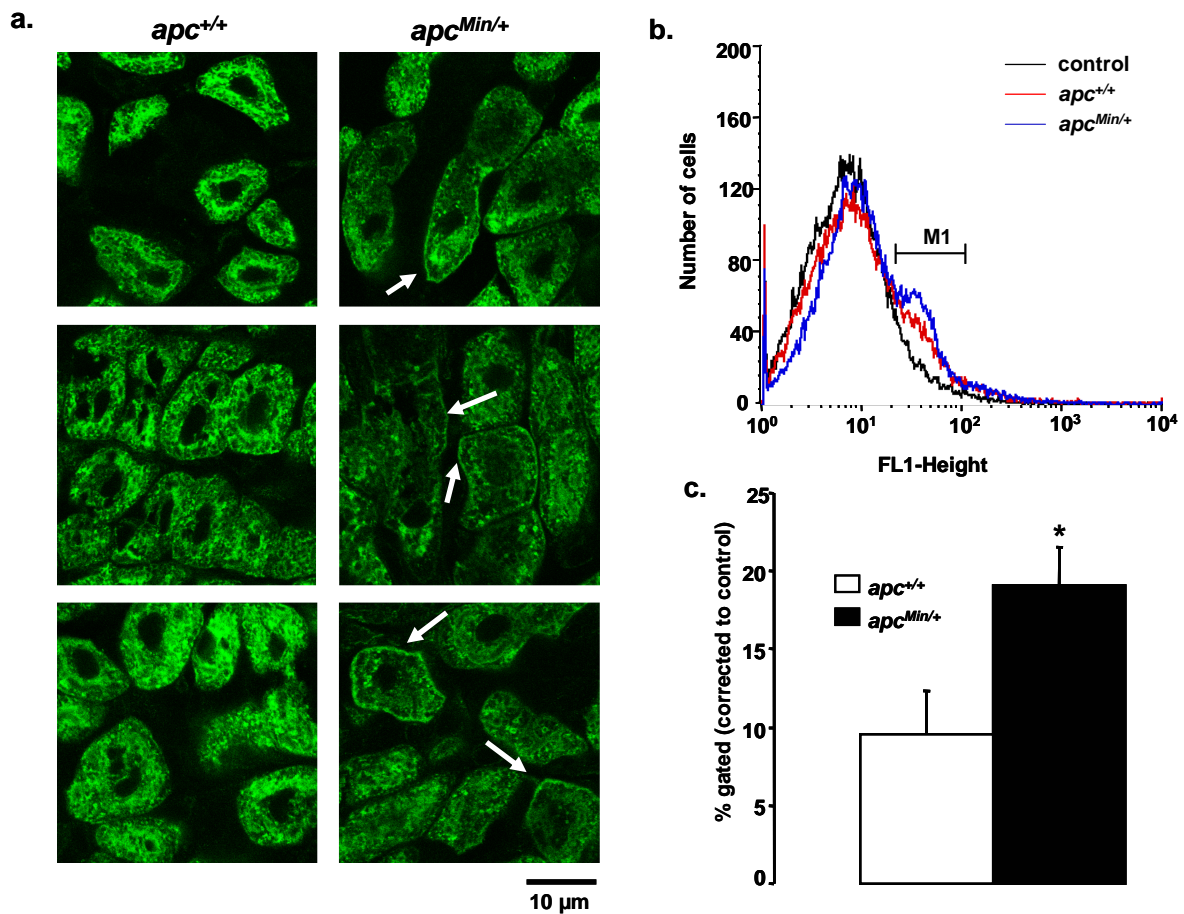
c. Original Western Blot illustrating the SGK1 (upper panel) and GAPDH (lower panel) protein abundance in  $apc^{Min/+}$  mice and  $apc^{+/+}$  mice.

d. Arithmetic means  $\pm$  SEM of relative densities of SGK1 protein expression in stomach from  $apc^{Min/+}$  mice (closed bars) and  $apc^{+/+}$  mice (open bars). \* indicates significant difference between  $apc^{Min/+}$  and  $apc^{+/+}$  mice.

Immunohistochemistry and real time PCR analysis were performed to elucidate, whether the enhanced expression of  $\beta$ -catenin and SGK1 influenced KCNQ1 transcription, expression or protein abundance in the cell membranes of  $apc^{Min/+}$  mice. According to real time PCR, KCNQ1 transcript levels in the gastric glands were not significantly different between  $apc^{+/+}$  ( $2.7 \pm 0.5$  copies/GAPDH X 1000,  $n = 9$ ) and  $apc^{Min/+}$  ( $3.3 \pm 0.4$  copies/GAPDH X 1000,  $n = 9$ ) mice. As shown in Figure 22, the total KCNQ1 protein

abundance was again similar in  $apc^{+/+}$  and  $apc^{Min/+}$  parietal cells. The localization of the protein was however, visibly different between the genotypes. In the parietal cells from  $apc^{+/+}$ , KCNQ1 was diffusely distributed throughout the cytoplasm, whereas in the parietal cells from  $apc^{Min/+}$  the protein was seen to be localized towards the plasma membrane.

To quantitatively analyze the membrane abundance of KCNQ1, the parietal cells were isolated, stained and analyzed in FACS. As shown in the Figure 22, the KCNQ1 abundance in the cell membrane was significantly larger in  $apc^{Min/+}$  than in  $apc^{+/+}$  parietal cells.



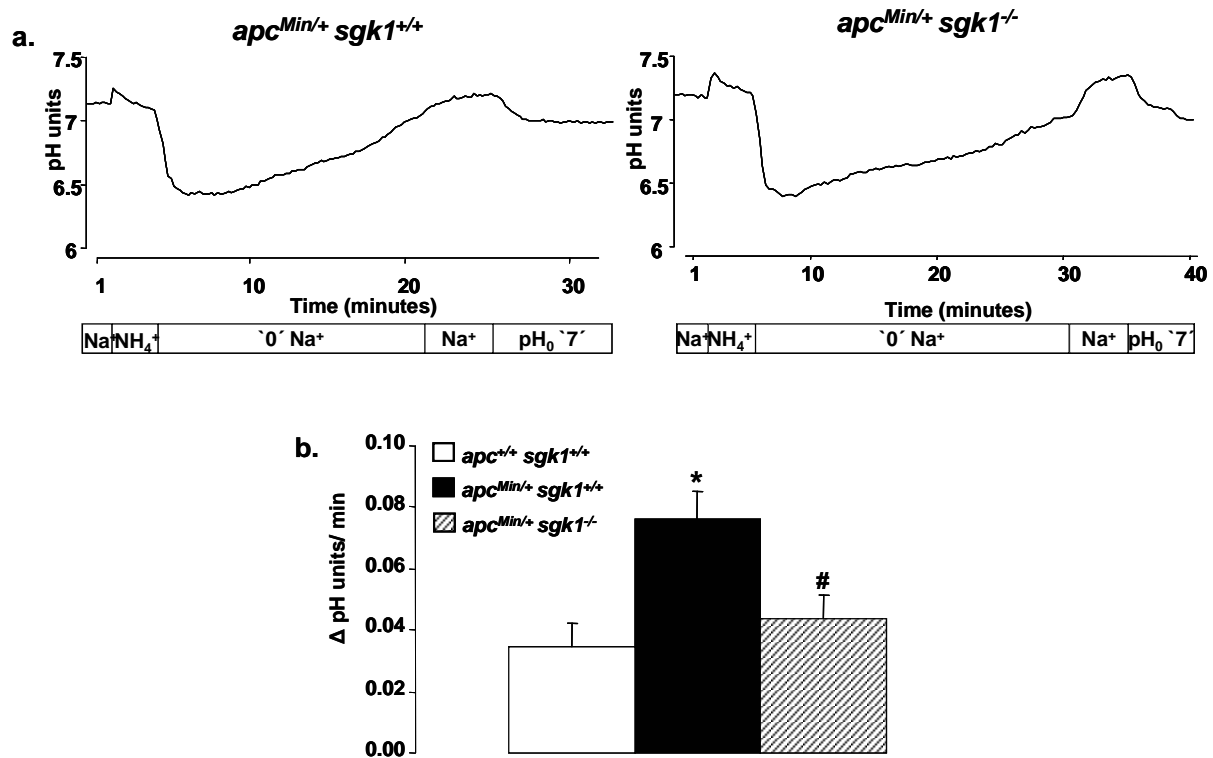
**Figure 22: Cell membrane KCNQ1 protein expression in parietal cells**

a. Immunofluorescence of KCNQ1 expression in parietal cells from  $apc^{Min/+}$  mice (right panels) and  $apc^{+/+}$  mice (left panels). Antibodies are directed against KCNQ1 (green fluorescence). Three different microscopic fields are shown for each genotype.

b. Representative histogram from Flow cytometric (FACS) analysis of the membrane KCNQ1 expression in isolated parietal cells.

c. Arithmetic means  $\pm$  SEM of the percentage of gated cells (corrected to control) from  $apc^{Min/+}$  mice (closed bar) and  $apc^{+/+}$  mice (open bar). \* indicates significant difference between  $apc^{Min/+}$  and  $apc^{+/+}$  mice ( $p < 0.05$ ).

To explore, whether the stimulation of  $H^+$  secretion in  $apc^{Min/+}$  mice indeed required the presence of SGK1, additional experiments were performed in mice carrying the mutant APC and at the same time lacking functional SGK1 ( $apc^{Min/+}/sgk1^{-/-}$ ). Comparison was made to both,  $apc^{+/+}/sgk1^{+/+}$  and  $apc^{Min/+}/sgk1^{+/+}$  littermates. As illustrated in Figure 23, the enhanced  $H^+$  secretion in  $apc^{Min/+}$  mice was absent in mice lacking SGK1 ( $apc^{Min/+}/sgk1^{-/-}$ ). The rate of pH recovery in  $apc^{Min/+}/sgk1^{-/-}$  ( $0.043 \pm 0.008 \Delta pH/min$ ,  $n = 10$ ) was not significantly different from that of  $apc^{+/+}/sgk1^{+/+}$  ( $0.034 \pm 0.008 \Delta pH/min$ ,  $n = 10$ ) mice, but significantly smaller than that of  $apc^{Min/+}/sgk1^{+/+}$  ( $0.076 \pm 0.009 \Delta pH/min$ ,  $n = 10$ ) mice.



**Figure 23: SGK1 requirement for accelerated pH recovery in parietal cells from APC-deficient mice**

a. Original tracings illustrating alterations of pH in typical experiments in  $apc^{Min/+}/sgk1^{+/+}$  mice (left panel) and  $apc^{+/+}/sgk1^{-/-}$  mice (right panel).

b. Arithmetic means  $\pm$  SEM of  $Na^+$ -independent alterations of cytosolic pH ( $\Delta pH/min$ ) in parietal cells from  $apc^{+/+}/sgk1^{+/+}$  mice (open bar)  $apc^{Min/+}/sgk1^{+/+}$  mice (closed bar) and  $apc^{Min/+}/sgk1^{-/-}$  mice (hatched bar). \* indicates significant difference to  $apc^{+/+}/sgk1^{+/+}$  mice, # indicates significant difference to  $apc^{Min/+}/sgk1^{+/+}$  mice.

**Table 2: Effect of stimulators and inhibitors of gastric acid secretion on the rate of pH recovery, intracellular pH and buffer capacity in isolated gastric glands from *apc* mice.**

		Rate of pH recovery ( $\Delta$ pH units/minute)	Initial pH (Units)	Buffer capacity (mM/pH unit)	Number of mice	Number of cells
<b>Untreated (control)</b>	<i>apc</i> <sup>+/+</sup>	0.034 $\pm$ 0.008	7.13 $\pm$ 0.02	37.66 $\pm$ 3.8	10	115
	<i>apc</i> <sup>Min/+</sup>	0.076 $\pm$ 0.010 <sup>a</sup>	7.15 $\pm$ 0.01	39.38 $\pm$ 5.2	10	122
	<i>apc</i> <sup>Min/+</sup> / <i>sgk1</i> <sup>-/-</sup>	0.043 $\pm$ 0.008 <sup>b</sup>	7.19 $\pm$ 0.05	35.9 $\pm$ 5.2	10	140
<b>0.1 % DMSO (sham)</b>	<i>apc</i> <sup>+/+</sup>	0.031 $\pm$ 0.009	7.08 $\pm$ 0.04	28.67 $\pm$ 6.3	3	32
	<i>apc</i> <sup>Min/+</sup>	0.124 $\pm$ 0.024 <sup>a</sup>	7.14 $\pm$ 0.04	27.35 $\pm$ 6.4	4	36
<b>Omeprazole</b>	<i>apc</i> <sup>+/+</sup>	0.006 $\pm$ 0.006 <sup>c</sup>	7.12 $\pm$ 0.01	42.58 $\pm$ 3.9	5	87
	<i>apc</i> <sup>Min/+</sup>	0.006 $\pm$ 0.005 <sup>c</sup>	7.14 $\pm$ 0.01	44.66 $\pm$ 4.9	4	60
<b>35mM K<sup>+</sup></b>	<i>apc</i> <sup>+/+</sup>	0.218 $\pm$ 0.039 <sup>c</sup>	7.20 $\pm$ 0.06	30.17 $\pm$ 6.1	5	73
	<i>apc</i> <sup>Min/+</sup>	0.259 $\pm$ 0.048 <sup>c</sup>	7.19 $\pm$ 0.02	37.52 $\pm$ 2.0	6	82
<b>Forskolin</b>	<i>apc</i> <sup>+/+</sup>	0.209 $\pm$ 0.028 <sup>d</sup>	7.19 $\pm$ 0.05	37.61 $\pm$ 5.9	7	84
	<i>apc</i> <sup>Min/+</sup>	0.198 $\pm$ 0.016 <sup>d</sup>	7.31 $\pm$ 0.06 <sup>d</sup>	33.38 $\pm$ 4.2	6	70

a - significant difference with respect to *apc*<sup>+/+</sup> mice.

b - significant difference with respect to *apc*<sup>Min/+</sup> mice

c - significant difference to respective control treatment.

d - significant difference to respective sham treatment.

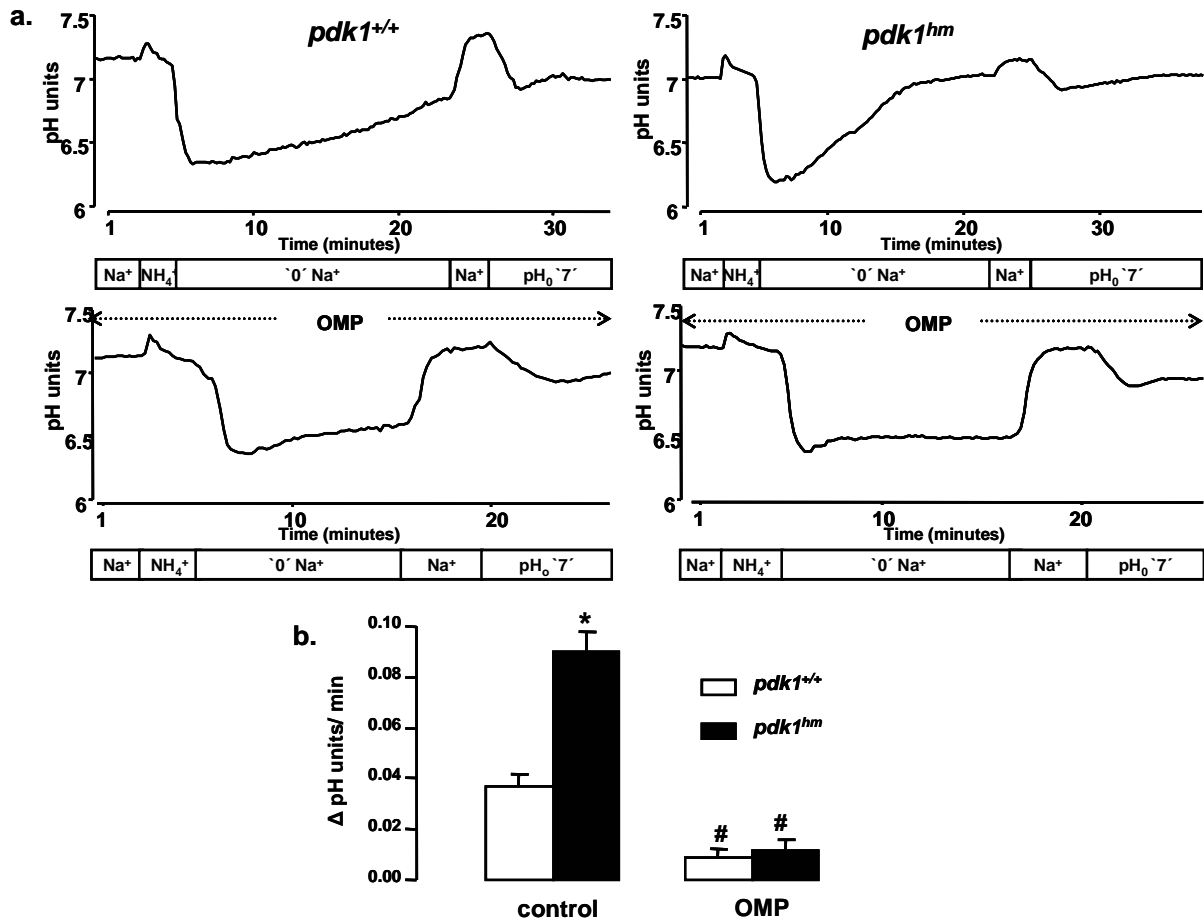
## ii. Role of PDK1 in regulation of acid secretion

To study the role of PDK1 in regulation of acid secretion, pH recovery following an ammonium pulse in the absence of  $\text{Na}^+$  ( $\Delta\text{pH}/\text{min}$ ) was measured in gastric glands isolated from gene targeted mice expressing only 20 % of PDK1 gene (*pdki<sup>hm</sup>*) and their wild type littermates (*pdki<sup>wt</sup>*). As reported previously, the PDK1 hypomorphic mice (*pdki<sup>hm</sup>*) were smaller ( $22.5 \pm 0.9$  g,  $n = 12$ ) than their wild type littermates (*pdki<sup>wt</sup>*, b.w.  $32.7 \pm 1.3$  g,  $n = 12$ ) (68). Food and fluid intake were similar in both genotypes (68).

In isolated gastric glands, cytosolic pH and buffer capacity, determined by utilizing BCECF-fluorescence, was similar in *pdki<sup>hm</sup>* and in *pdki<sup>wt</sup>* mice (Table 3, Figure 24). Accordingly,  $\text{Na}^+$ -independent pH recovery following an ammonium pulse ( $\Delta\text{pH}/\text{min}$ ) was significantly ( $\sim 2.5$ -fold) faster in *pdki<sup>hm</sup>* than in *pdki<sup>wt</sup>* mice. As shown in Figure 24,  $\text{H}^+/\text{K}^+$ -ATPase inhibitor omeprazole (100  $\mu\text{M}$ ) virtually abolished the pH recovery in both genotypes.

Fasting serum gastrin levels were measured to verify if the increased acid secretion is due to increased serum gastrin. Serum gastrin levels were similar in *pdki<sup>hm</sup>* mice ( $39.5 \pm 3.8$  pM,  $n = 3$  mice) and in *pdki<sup>wt</sup>* mice ( $39.9 \pm 3.4$  pM,  $n = 3$  mice).





**Figure 24: pH recovery following an ammonium pulse in parietal cells from PDK1 mice in presence and absence of omeprazole**

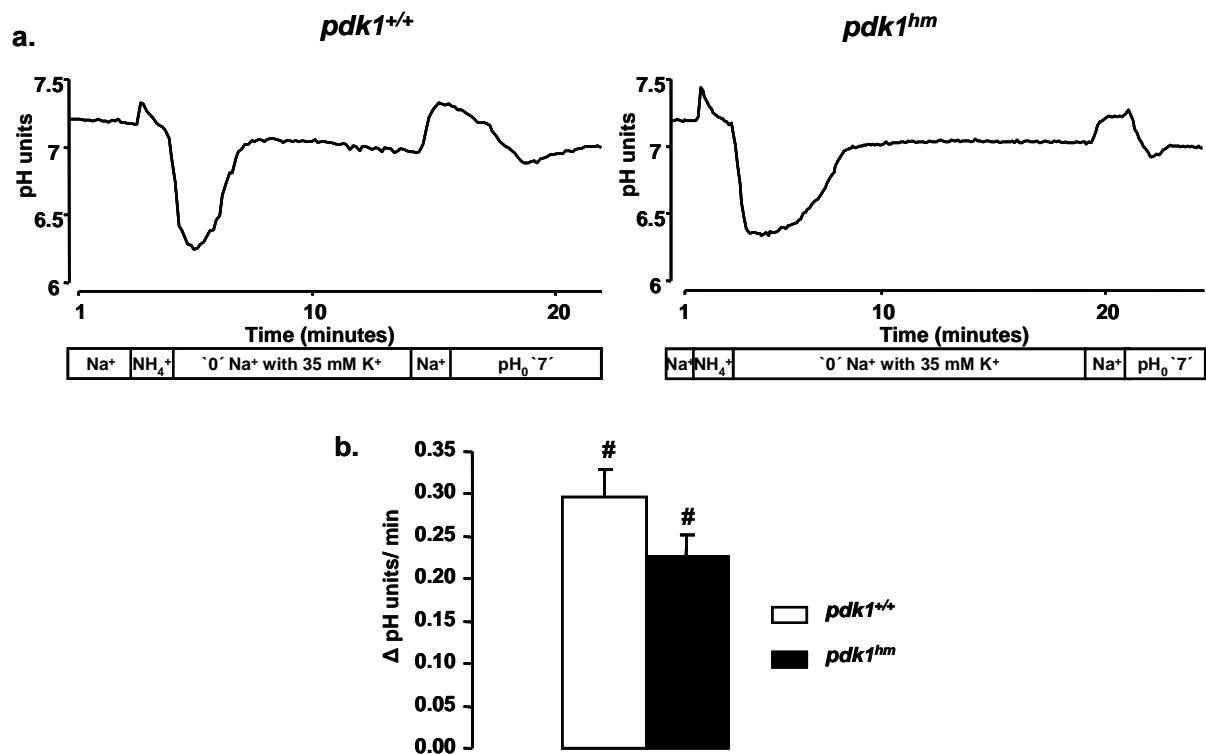
a. Original tracings illustrating alterations of pH in typical experiments in *pdk1<sup>wt</sup>* mice (left panels) and *pdk1<sup>hm</sup>* mice (right panels) in the absence (upper panels) and presence (lower panels) of 100  $\mu$ M omeprazole.

b. Arithmetic means  $\pm$  SEM of Na<sup>+</sup> independent alterations of cytosolic pH ( $\Delta$ pH/min) in parietal cells from *pdk1<sup>hm</sup>* mice (closed bars) and *pdk1<sup>wt</sup>* mice (open bars) in the absence (left bars) and presence (right bars) of 100  $\mu$ M omeprazole. \* indicates significant difference between *pdk1<sup>hm</sup>* mice and *pdk1<sup>wt</sup>* mice, # indicates significant difference between absence (control) and presence of omeprazole.

To identify the specific pathway responsible for the increased acid secretion, the isolated gastric glands from the mice were treated with known stimulators and inhibitors of acid secretion and the rate of H<sup>+</sup> ion secretion was studied. As shown in Table 3, both the intracellular pH and buffer capacity were not affected by different experimental conditions.

To explore the role of potassium (channels) in the increased rate of H<sup>+</sup> ion secretion in *pdk1<sup>hm</sup>* mice, pH recovery was studied under enhanced local K<sup>+</sup> concentrations. An increase of local K<sup>+</sup> concentrations to 35 mM (replacing Na<sup>+</sup>/NMDG) increased  $\Delta$ pH/min to similar

values in *pdk1<sup>hm</sup>* and *pdk1<sup>wt</sup>* mice (Figure 25). The glands from *pdk1<sup>wt</sup>* mice showed a faster recovery in presence of increased extracellular  $K^+$  as compared to glands from *pdk1<sup>hm</sup>* mice.

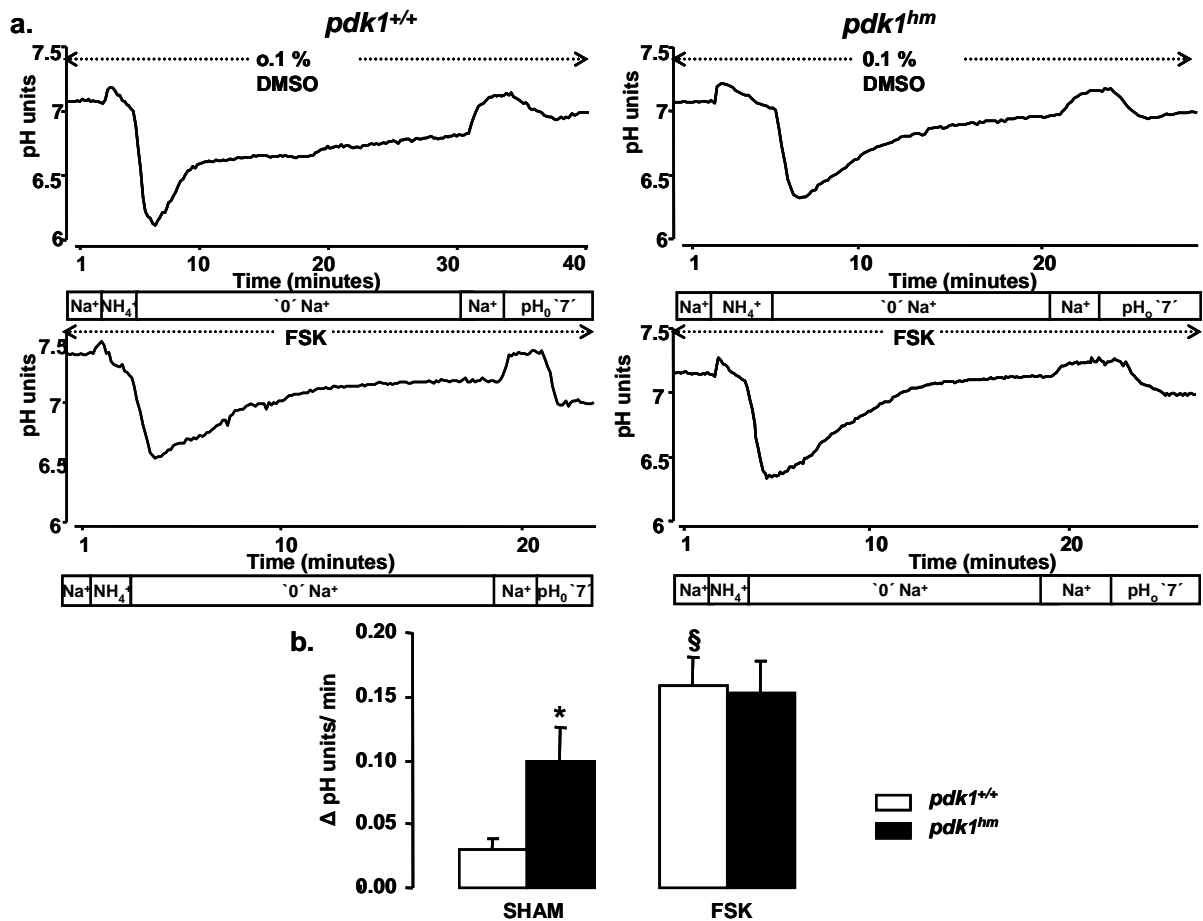


**Figure 25: Influence of enhanced extracellular  $K^+$  concentration on pH recovery in parietal cells**

a. Original tracings illustrating alterations of pH at enhanced extracellular  $K^+$  concentration (35 mM) in *pdk1<sup>wt</sup>* mice (left panels) and *pdk1<sup>hm</sup>* mice (right panels).

b. Arithmetic means  $\pm$  SEM of  $Na^+$  independent  $\Delta pH$  at enhanced (35 mM) extracellular  $K^+$  concentration in parietal cells from *pdk1<sup>hm</sup>* mice (closed bars) and *pdk1<sup>wt</sup>* mice (open bars). # indicates significant difference between high or low  $K^+$  (control, see Figure 24) concentration.

To further study if the 'histaminergic pathway (PKA)' is involved in the increased gastric acid secretion, the gastric glands were stimulated with adenylate cyclase activator, forskolin (5  $\mu M$ ). Treatment of gastric glands with forskolin significantly increased  $\Delta pH/min$  again to values similar in *pdk1<sup>hm</sup>* mice and in *pdk1<sup>wt</sup>* mice (Figure 26). Again, stimulation with forskolin was effective only in the *pdk1<sup>wt</sup>* mice as seen by the significant increase in pH recovery as compared to sham (0.1% DMSO treatment). On the other hand, the pH recovery in glands from *pdk1<sup>hm</sup>* mice was not significantly different between sham and forskolin treatment. The difference between the genotypes however disappeared by the treatment with forskolin.

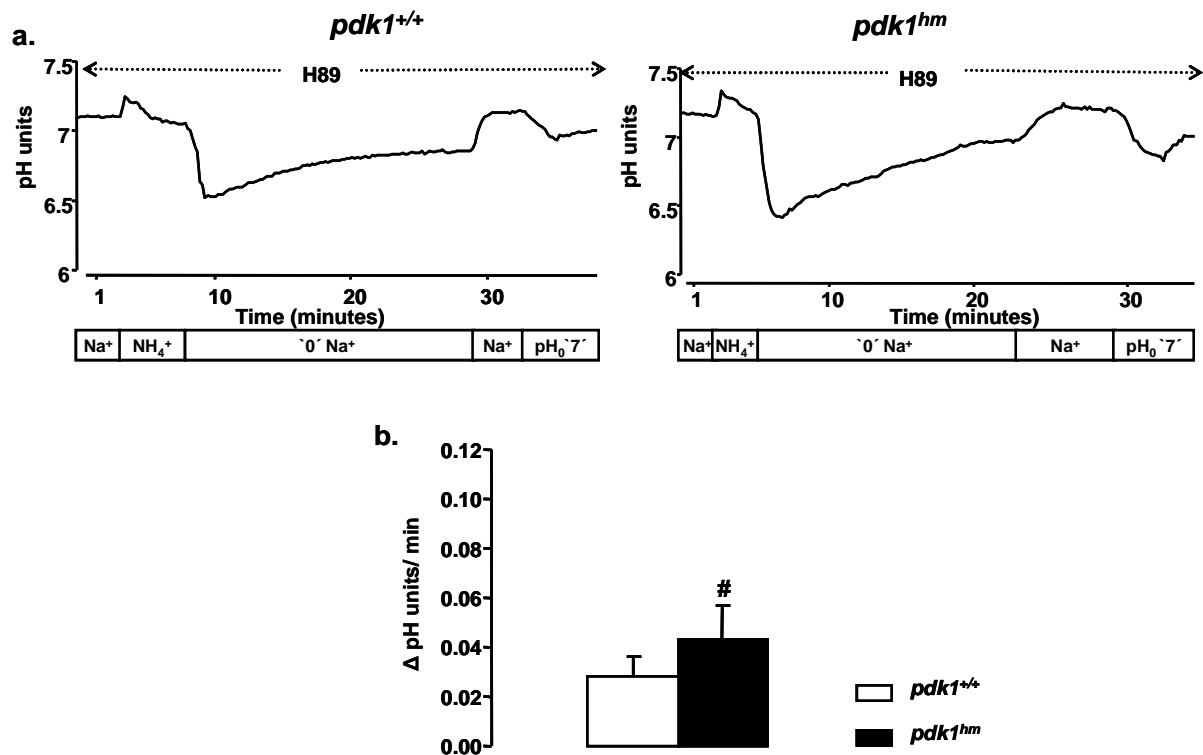


**Figure 26: Effects of forskolin on pH recovery in parietal cells**

a. Original tracings illustrating alterations of pH in typical experiments in *pdk1<sup>wt</sup>* mice (left panels) and *pdk1<sup>hm</sup>* mice (right panels) in the absence (upper panels) and presence (lower panels) of forskolin (FSK, 5 μM).

b. Arithmetic means  $\pm$  SEM of  $\text{Na}^+$  independent alterations of cytosolic pH ( $\Delta \text{pH}/\text{min}$ ) in parietal cells following an ammonium pulse in the absence (left bars) and presence (right bars) of forskolin (FSK, 5 μM) in gastric glands from *pdk1<sup>hm</sup>* mice (closed bars) and *pdk1<sup>wt</sup>* mice (open bars). \* indicates significant difference between *pdk1<sup>hm</sup>* mice and *pdk1<sup>wt</sup>* mice, § indicates significant difference between absence (sham) and presence of forskolin.

The glands were then treated with Protein kinase A inhibitor H89 (50 nM), in order to elucidate the role of cAMP dependent PKA in the increased gastric acid secretion in *pdk1<sup>hm</sup>* mice. H89 treatment resulted in the significant decrease in the  $\Delta \text{pH}/\text{min}$  in *pdk1<sup>hm</sup>* mice but was without significant effect in *pdk1<sup>wt</sup>* mice. Thus the difference between the two genotypes was again abrogated by the H89 treatment of the gastric glands (Figure 27).

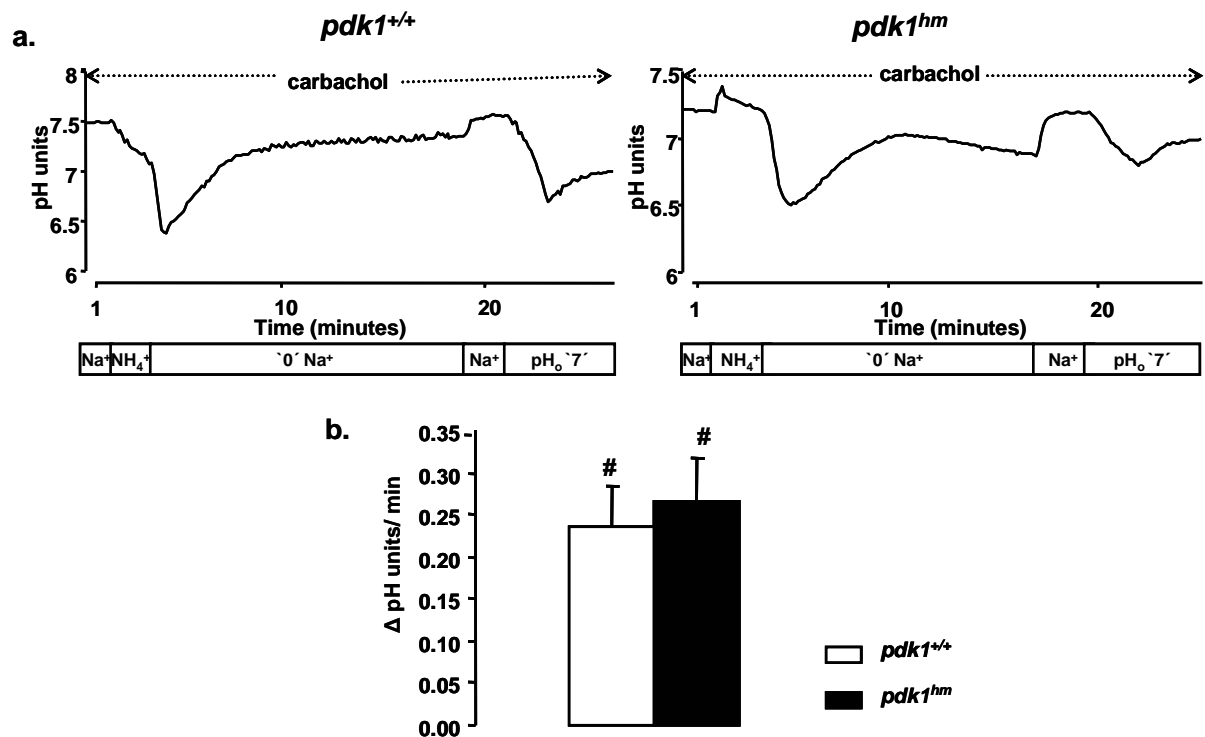


**Figure 27: Effects of H89 on pH recovery in parietal cells**

**a.** Original tracings illustrating alterations of pH in typical experiments in *pdk1<sup>wt</sup>* mice (left panels) and *pdk1<sup>hm</sup>* mice (right panels) in the presence of PKA inhibitor H89 (H89, 50 nM).

**b.** Arithmetic means  $\pm$  SEM of Na<sup>+</sup> independent alterations of cytosolic pH ( $\Delta$ pH/min) in parietal cells following an ammonium pulse in the presence of H89 (H89, 50 nM) in gastric glands from *pdk1<sup>hm</sup>* mice (closed bars) and *pdk1<sup>wt</sup>* mice (open bars). # indicates significant difference between absence (control, see Figure 24) and presence of H89.

The gastric glands were further stimulated with carbachol (100  $\mu$ M) to study if the cholinergic pathway is also involved in the increased acid secretion in *pdk1<sup>hm</sup>* mice. Carbachol significantly enhanced  $\Delta$ pH/min in both *pdk1<sup>hm</sup>* mice and in *pdk1<sup>wt</sup>* mice again resulting in abrogation of the difference between *pdk1<sup>hm</sup>* and in *pdk1<sup>wt</sup>* mice (Figure 28). Carbachol enhanced the acid secretion in mice from both genotypes indicating that cholinergic pathway was still susceptible for stimulation and thus was not involved in the increased acid secretion in *pdk1<sup>hm</sup>* mice.

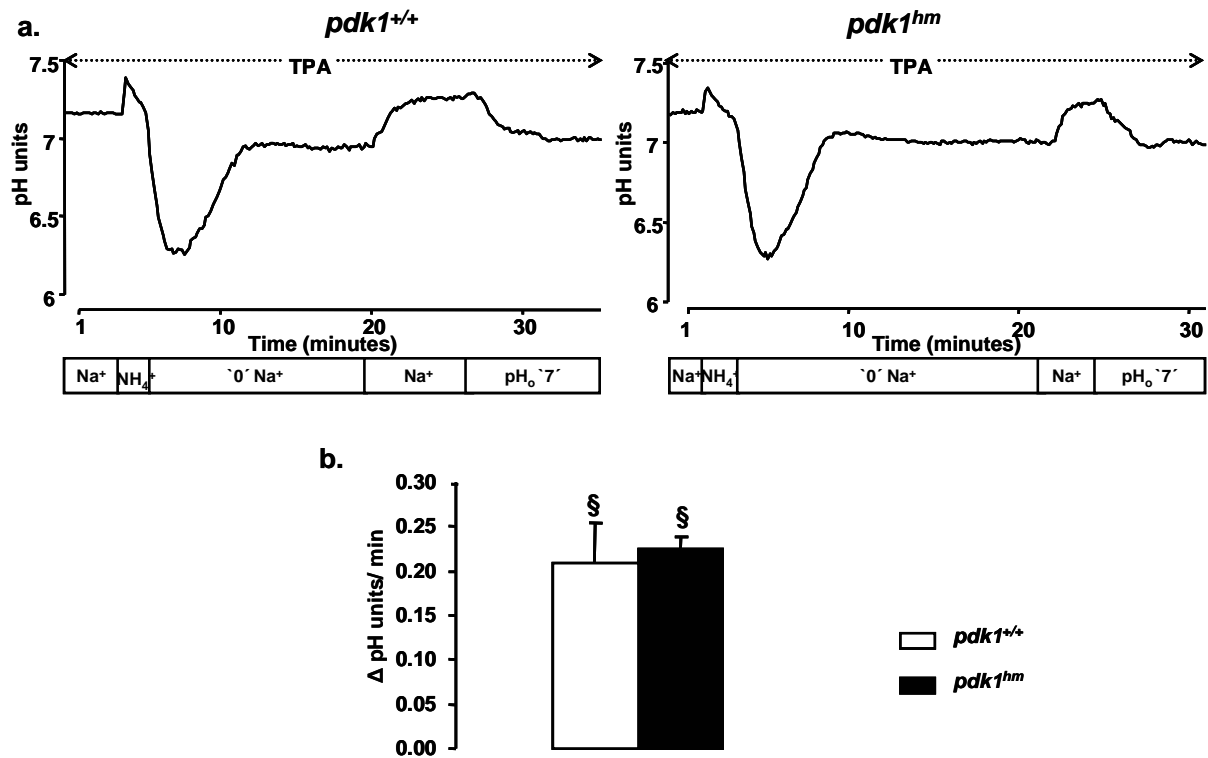


**Figure 28: Effects of carbachol on pH recovery in parietal cells**

**a.** Original tracings illustrating alterations of pH in typical experiments in *pdk1<sup>wt</sup>* mice (left panels) and *pdk1<sup>hm</sup>* mice (right panels) in the presence of carbachol (100  $\mu$ M).

**b.** Arithmetic means  $\pm$  SEM of Na<sup>+</sup> independent alterations of cytosolic pH ( $\Delta$ pH/min) in parietal cells following an ammonium pulse in the presence of carbachol (100  $\mu$ M) in gastric glands from *pdk1<sup>hm</sup>* mice (closed bars) and *pdk1<sup>wt</sup>* mice (open bars). # indicates significant difference between absence (control, see Figure 24) and presence of carbachol.

To rule out the possibility of defective activation of protein kinase C in *pdk1<sup>hm</sup>* as a reason for increased gastric acid secretion, the glands were finally stimulated with activator of protein kinase C, phorbol 12 myristate 13 acetate (Phorbol ester, TPA) (100 nM) (69; 70). Stimulation of protein kinase C with Phorbol ester (TPA) enhanced  $\Delta$ pH/min in *pdk1<sup>hm</sup>* mice and *pdk1<sup>wt</sup>* mice and  $\Delta$ pH/min was again similar in *pdk1<sup>hm</sup>* and in *pdk1<sup>wt</sup>* mice (Figure 29).



**Figure 29: Effects of phorbol ester on pH recovery in parietal cells**

**a.** Original tracings illustrating alterations of pH in typical experiments in *pdk1<sup>wt</sup>* mice (left panels) and *pdk1<sup>hm</sup>* mice (right panels) in the presence of phorbol ester, phorbol 12 myristate 13 acetate (TPA, 100 nM).

**b.** Arithmetic means  $\pm$  SEM of Na<sup>+</sup> independent alterations of cytosolic pH ( $\Delta$ pH/min) in parietal cells following an ammonium pulse in the presence of phorbol ester (TPA, 100 nM) in *pdk1<sup>hm</sup>* mice (closed bars) and *pdk1<sup>wt</sup>* mice (open bars). § indicates significant difference between absence (sham, see Figure 26) and presence of phorbol ester.

**Table 3: Effect of stimulators and inhibitors of gastric acid secretion on the rate of pH recovery, intracellular pH and buffer capacity in isolated gastric glands from PDK1 mice**

		Rate of pH recovery ( $\Delta$ pH units/ minute)	Intracellular pH (units)	Buffer capacity (mM/pH unit)	Number of mice	Number of cells
<b>Untreated (control)</b>	<i>pdk1<sup>wt</sup></i>	0.037 $\pm$ 0.005	7.16 $\pm$ 0.03	42.11 $\pm$ 5.83	15	156
	<i>pdk1<sup>hm</sup></i>	0.090 $\pm$ 0.008 <sup>a</sup>	7.19 $\pm$ 0.02	37.93 $\pm$ 5.51	16	181
<b>Sham (0.1 % DMSO)</b>	<i>pdk1<sup>wt</sup></i>	0.030 $\pm$ 0.009	7.15 $\pm$ 0.03	39.69 $\pm$ 4.75	5	41
	<i>pdk1<sup>hm</sup></i>	0.099 $\pm$ 0.027 <sup>a</sup>	7.18 $\pm$ 0.06	36.75 $\pm$ 6.51	4	72
<b>Omeprazole</b>	<i>pdk1<sup>wt</sup></i>	0.009 $\pm$ 0.004 <sup>b</sup>	7.19 $\pm$ 0.04	31.83 $\pm$ 2.22	3	30
	<i>pdk1<sup>hm</sup></i>	0.011 $\pm$ 0.005 <sup>b</sup>	7.15 $\pm$ 0.02	37.25 $\pm$ 18.60	3	50
<b>35 mM K<sup>+</sup></b>	<i>pdk1<sup>wt</sup></i>	0.296 $\pm$ 0.032 <sup>b</sup>	7.21 $\pm$ 0.05	30.73 $\pm$ 3.60	6	98
	<i>pdk1<sup>hm</sup></i>	0.227 $\pm$ 0.025 <sup>b</sup>	7.25 $\pm$ 0.02	35.33 $\pm$ 4.42	6	63
<b>Forskolin</b>	<i>pdk1<sup>wt</sup></i>	0.159 $\pm$ 0.021 <sup>c</sup>	7.28 $\pm$ 0.03 <sup>c</sup>	31.95 $\pm$ 3.74	6	65
	<i>pdk1<sup>hm</sup></i>	0.153 $\pm$ 0.026	7.25 $\pm$ 0.03	35.24 $\pm$ 4.02	6	71
<b>H89</b>	<i>pdk1<sup>wt</sup></i>	0.028 $\pm$ 0.008	7.16 $\pm$ 0.06	44.65 $\pm$ 8.85	5	61
	<i>pdk1<sup>hm</sup></i>	0.044 $\pm$ 0.013 <sup>b</sup>	7.10 $\pm$ 0.01 <sup>b</sup>	49.79 $\pm$ 6.81	5	40
<b>Carbachol</b>	<i>pdk1<sup>wt</sup></i>	0.237 $\pm$ 0.046 <sup>b</sup>	7.30 $\pm$ 0.08 <sup>b</sup>	36.15 $\pm$ 8.26	5	57
	<i>pdk1<sup>hm</sup></i>	0.266 $\pm$ 0.050 <sup>b</sup>	7.27 $\pm$ 0.06	54.12 $\pm$ 12.83	5	49
<b>Phorbol ester</b>	<i>pdk1<sup>wt</sup></i>	0.209 $\pm$ 0.045 <sup>c</sup>	7.24 $\pm$ 0.06	41.60 $\pm$ 7.96	5	61
	<i>pdk1<sup>hm</sup></i>	0.224 $\pm$ 0.014 <sup>c</sup>	7.31 $\pm$ 0.03 <sup>c</sup>	31.70 $\pm$ 5.50	5	51

a - significant difference with respect to *pdk1<sup>+/+</sup>* mice.

b - significant difference to respective control treatment.

c - significant difference to respective sham treatment.

## 9 DISCUSSION

### i. Role of SGK1 in regulation of acid secretion

#### a. Role of SGK1 in regulation of basal and stimulated acid secretion

Serum and glucocorticoid inducible kinase 1 (SGK1) is potent regulator of KCNE1/KCNQ1 trafficking and activity (51; 54; 71). KCNQ1 channels are known to be upregulated by SGK1 in the presence and absence of coexpressed KCNE1 (51). It can thus be assumed that the effect of SGK1 does not depend on KCNE1. SGK1 is partially effective through phosphorylation of Nedd4-2, which decreases the affinity of this ubiquitin ligase to its target proteins (72-75). SGK1 does apparently not affect the transcription of KCNQ1 but may be effective through increase of protein abundance in the cell membrane.

KCNQ1 is reported to play a critical role in the gastric acid secretion as seen by the complete absence of secretion in the KCNQ1 knock out mice (25; 28). Thus it is expected that absence of SGK1 would lead to decreased membrane abundance of KCNQ1 and thus impaired gastric acid secretion. However, pH recovery in the absence of Na<sup>+</sup>, an indicator of H<sup>+</sup> secretion was similar in SGK1 knock out and wild type mice, clearly demonstrating that gastric acid secretion is absolutely normal in *sgk1*<sup>-/-</sup> mice. Moreover, even under conditions that are known to activate the classical pathways, the Na<sup>+</sup>-independent pH recovery is not different between the genotypes.

The apparently normal H<sup>+</sup> secretion in the *sgk1*<sup>-/-</sup> mice raises the question which mechanism could replace SGK1 in regulating the membrane abundance of KCNQ1. Candidate kinases possibly replacing SGK1 include the isoforms SGK2 and SGK3 which have been cloned from homology screening (42) and share the ability of SGK1 to enhance the protein abundance and/or activity of several channels and transporters (45). Experiments in *Xenopus* oocytes indeed disclosed the ability of SGK3 to up-regulate KCNE1/KCNQ1 (51).

The present observations thus indicate that SGK1 might not be trivial for the regulation of gastric acid secretion and its absence is possibly easily compensated. But nevertheless the importance of SGK1 in the regulation of membrane abundance of KCNQ1 cannot be undermined. Therefore further experiments were aimed at exploring the effect of upregulation of SGK1 on the gastric acid secretion.



## **b. Role of SGK1 in glucocorticoid stimulation of acid secretion**

Treatment with corticosteroids is known to increase gastric acid secretion and thereby lead to ulcerations (2; 3). The present observations provide evidence for an involvement of SGK1 in the corticosteroid induced gastric acid secretion.

At least in theory, SGK1 could enhance  $H^+$  secretion by stimulating KCNQ1 channels, which are required for recycling of  $K^+$  into the lumen (25; 28). The  $K^+$  recycling provides the  $H^+/K^+$ -ATPase with luminal  $K^+$  and the KCNQ1 channels are thus critically important for gastric  $H^+$  secretion (25; 28).

The critical role of  $K^+$  channels is suggested by the potent stimulation of pH recovery following increase in extracellular  $K^+$  concentration to 35 mM. The increase of extracellular  $K^+$  concentration may enhance luminal  $K^+$  concentration by depolarization of the basolateral cell membrane and through circular current of the apical cell membrane with subsequent activation of apical KCNQ1. In any case those experiments suggest that under the experimental conditions extracellular  $K^+$  concentration is indeed limiting for  $H^+/K^+$ -ATPase activity. SGK1 dependent upregulation of KCNQ1 channels would accelerate  $K^+$  recycling into the lumen and thus provide the  $H^+/K^+$ -ATPase with  $K^+$ .

Notably, prior to treatment with dexamethasone, the acid secretion is similar in SGK1 knockout animals (*sgk1*<sup>-/-</sup>) and their wild type littermates (*sgk1*<sup>+/+</sup>). Moreover, the effect of carbachol and forskolin is similar in *sgk1*<sup>-/-</sup> and *sgk1*<sup>+/+</sup> mice. Thus, stimulation of protein kinase A apparently overrides the lack of SGK1. Under our experimental conditions the role of SGK1 becomes apparent only after stimulation with the glucocorticoid dexamethasone.

Basal corticosterone levels at the beginning of the light cycle were similar in *sgk1*<sup>+/+</sup> and *sgk1*<sup>-/-</sup> mice. However, *sgk1*<sup>-/-</sup> showed significantly higher peak corticosterone levels which were suppressible to the same extent as in *sgk1*<sup>+/+</sup> mice by dexamethasone treatment. The increased peak corticosterone levels of *sgk1*<sup>-/-</sup> mice does, however, not translate into an enhanced basal gastric acid secretion, again pointing to a blunted glucocorticoid sensitivity of gastric acid secretion in those mice.

In conclusion, in the absence of glucocorticoids little differences are observed between *sgk1*<sup>-/-</sup> and *sgk1*<sup>+/+</sup> mice in gastric  $H^+$  secretion. Thus, basal  $H^+$  secretion does not depend on SGK1. However, the glucocorticoid induced stimulation of  $H^+$  secretion is at least partially dependent on SGK1 and upregulation of SGK1 could possibly lead to increased gastric acid secretion.

### **c. Role of SGK1 in peroxisome proliferator activated receptor gamma (PPAR $\gamma$ ) agonist mediated stimulation of acid secretion**

The present observations disclose a stimulating effect of pioglitazone on gastric acid secretion. Pioglitazone treatment increased gastric expression of the serum and glucocorticoid inducible kinase SGK1, which obviously accounts for this side effect of the PPAR $\gamma$  agonist.

As shown previously (56), *sgk1*<sup>+/+</sup> and *sgk1*<sup>-/-</sup> mice do not differ in PPAR $\gamma$  expression or pioglitazone plasma levels. The latter were in the lower micromolar range (10-15  $\mu$ M), which is comparable to levels measured in volunteers after oral ingestion of 30 mg pioglitazone (3  $\mu$ M, (76)). At those concentrations, pioglitazone does increase extracellular fluid volume, as previously shown for PPAR $\gamma$  agonists (77-79).

The stimulation of gastric acid secretion by pioglitazone requires the H<sup>+</sup>/K<sup>+</sup>-ATPase, as it is abrogated in the presence of H<sup>+</sup>/K<sup>+</sup>-ATPase blocker omeprazole. Interestingly, pioglitazone treatment leads to increased colocalization of the proton pump H<sup>+</sup>/K<sup>+</sup>-ATPase and the potassium channel KCNQ1 in both genotypes, but the membrane translocation of the vesicles containing the channel and the pump is seen only in SGK1 wild type mice. Thus, SGK1 does not play a role in the colocalization but is mainly important for the trafficking of the vesicles towards the membrane. H<sup>+</sup> secretion is markedly enhanced following an increase in extracellular K<sup>+</sup> concentration and the difference between *sgk1*<sup>+/+</sup> and *sgk1*<sup>-/-</sup> mice and between untreated and pioglitazone treated mice from either genotype is dissipated. This finding again supports the view that stimulation of gastric acid secretion is at least in part by enhancement of K<sup>+</sup> recycling.

Previous experiments have shown that glucocorticoids stimulate gastric acid secretion by a mechanism similarly sensitive to extracellular K<sup>+</sup> concentration and at least in part through the serum and glucocorticoid inducible kinase SGK1. Along those lines, the stimulation of gastric acid secretion by dexamethasone is inhibited by blockage of phosphatidylinositol PI-3 kinase, a kinase upstream of SGK1 (80).

Stimulation of gastric acid secretion would be expected to favour the formation of gastric ulcers. However, PPAR $\gamma$  agonists have been reported to foster ulcer healing (81) and protect against gastric ischemia-reperfusion damage (82). Possibly, the stimulating effect on gastric acid secretion is overridden by the simultaneous stimulating effect on trefoil factor expression (83), which are known to exert protective effects (84).

In conclusion, PPAR $\gamma$  agonists upregulate the gastric expression of SGK1, which in turn stimulates gastric acid secretion. The effect adds to the known side effects of those clinically valuable drugs.

#### **d. SGK1 dependant stimulation of gastric acid secretion in mutant *apc* mice (*apc*<sup>Min/+</sup>)**

The present observations provide evidence for involvement of 'wnt' signalling in the stomach. Na<sup>+</sup>-independent omeprazole-sensitive realkalinization of gastric gland cells following an ammonium pulse was significantly more rapid in heterozygous mice with defective APC (*apc*<sup>Min/+</sup>) than in their wild type littermates (*apc*<sup>+/+</sup>). Accordingly, in the absence of stimulation, H<sup>+</sup>/K<sup>+</sup>-ATPase activity was about two fold as fast in *apc*<sup>Min/+</sup> mice as in *apc*<sup>+/+</sup> mice. Notably, the marked difference is observed between heterozygous and wild type mice. Thus, the moderate decrease of APC expression in heterozygous mice is sufficient to substantially modify gastric acid secretion.

The results presumably do not reflect a direct stimulation of H<sup>+</sup>/K<sup>+</sup>-ATPase activity. Instead, APC deficiency could enhance H<sup>+</sup> secretion more indirectly by upregulation of SGK1 which in turn is shown to enhance K<sup>+</sup> recycling through KCNQ1 channels (51; 54; 71). The K<sup>+</sup> recycling from the cell into the lumen provides the H<sup>+</sup>/K<sup>+</sup>-ATPase with luminal K<sup>+</sup> and is thus critically important for gastric H<sup>+</sup> secretion (25; 28). Accordingly, an increase in the extracellular K<sup>+</sup> concentration leads to marked increase in H<sup>+</sup>/K<sup>+</sup>-ATPase activity. Under those conditions, the extracellular K<sup>+</sup> concentration is no more limiting for H<sup>+</sup>/K<sup>+</sup>-ATPase activity. The disappearance of the difference between *apc*<sup>Min/+</sup> mice and *apc*<sup>+/+</sup> mice at enhanced extracellular K<sup>+</sup> concentration supports an effect of APC on K<sup>+</sup> recycling. Previous experiments in SGK1 mice have shown that glucocorticoids and pioglitazone stimulate gastric acid secretion by a mechanism similarly sensitive to extracellular K<sup>+</sup> concentration.

Western blotting and quantitative real time PCR experiments indeed disclosed higher SGK1 expression levels in gastric glands from *apc*<sup>Min/+</sup> mice than in those from *apc*<sup>+/+</sup> mice. Most recently, SGK1 expression has been shown to be stimulated by  $\beta$ -catenin through binding of TCF/ $\beta$ -catenin complexes to the SGK1 promoter (67). As a matter of fact  $\beta$ -catenin expression is similarly higher in *apc*<sup>Min/+</sup> than in *apc*<sup>+/+</sup> gastric glands and immunohistochemistry reveals enhanced KCNQ1 protein abundance in the plasma membrane of parietal cells.

The difference between *apc*<sup>Min/+</sup> mice and *apc*<sup>+/+</sup> mice was further dissipated by prior treatment with forskolin, which leads to stimulation of protein kinase A (PKA) and, similar to SGK1, to upregulation of KCNQ1 activity (85-87). Apparently, similar to SGK1, protein kinase A increases KCNQ1 trafficking to the cell membrane.

Furthermore, the central role of SGK1 is clearly demonstrated by the experiments conducted in the *apc*<sup>Min/+</sup> mice lacking additionally SGK1. The additional knock out of SGK1 in the *apc*<sup>Min/+</sup> mice led to abrogation of enhanced gastric acid secretion in these mice and the H<sup>+</sup>/K<sup>+</sup>-ATPase activity was comparable to that of *apc*<sup>+/+</sup> mice thus indicating that upregulation of SGK1 due to abnormal 'wnt' signalling is responsible for increased gastric acid secretion in the respective mice.

In conclusion, basal gastric acid secretion is markedly increased in mice carrying the defective APC gene and the present studies suggest the upregulation of SGK1 as the possible reason for the observed effect.

## ii. Role of PDK1 in regulation of acid secretion

The present observations provide evidence for an involvement of phosphoinositide dependent kinase 1 (PDK1) in the regulation of gastric acid secretion and underscore the conclusions of the previous study suggesting that gastric acid secretion is suppressed by phosphoinositide 3 kinase (PI3K) dependent signalling (57). Moreover, these experiments define the PI3K dependent pathway involved. PI3K is apparently effective through PDK1, which activates the serum and glucocorticoid inducible kinase (SGK) or protein kinase B (PKB/Akt) isoforms (41; 52; 88-93).

The present study did not attempt to define the PDK1 dependent signalling, which may involve any of the SGK or PKB/Akt isoforms. In the previous study, pharmacological PI3K inhibition disrupted the phosphorylation of PKB/Akt, a finding suggesting that PKB/Akt could have been the downstream target of PI3K signalling (57). According to the previous observations, the basal gastric acid secretion is not significantly altered by genetic knockout of SGK1. Thus, defective activation of SGK1 hardly accounts for the observed stimulation of basal gastric acid secretion in the PDK1 deficient mouse or following pharmacological PI3K inhibition.

The present observations further demonstrate that increase of extracellular  $K^+$  concentration enhances gastric acid secretion to a greater extent in wild type mice (*pdk1<sup>wt</sup>*) than in PDK1 hypomorphic mice (*pdk1<sup>hm</sup>*). Accordingly, the increase of extracellular  $K^+$  concentration abrogated the differences between the genotypes. Although the difference did not reach statistical significance, the experiment did demonstrate that PDK1 dependent signalling influences gastric acid secretion by affecting the availability of luminal  $K^+$  for the  $H^+/K^+$ -ATPase. In presence of surplus  $K^+$  ions in the canaliculus, availability of  $K^+$  ions for exchange is no more a rate limiting step and thus  $H^+$  ion secretion is no longer dependant on the activity of  $K^+$  channels. Therefore glands from *pdk1<sup>wt</sup>* mice showed faster recovery values as compared to *pdk1<sup>hm</sup>* mice.

The recycling of  $K^+$  from the cell into the lumen is accomplished by KCNQ1 channels, which indeed play a decisive role in gastric acid secretion (25; 28). The channels are activated not only by SGK1 (51; 54; 71), but also by protein kinase A (94-97).

Previous studies indicated the negative regulation of cellular cAMP levels by PI3K signalling and accordingly inhibition of PI3-kinase signalling lead to increased of cellular cAMP levels and eventually to enhanced activation of protein kinase A (46; 47). More importantly this mechanism was shown to be effective in the parietal cells as seen by the

increased gastric acid secretion in the wortmannin and LY294002 (PI3 kinase inhibitors) treated cells (57). Accordingly, the protein kinase A inhibitor H89 substantially decreased the gastric acid secretion in *pdk1<sup>hm</sup>* mice. Inhibitory effect of H89 on the acid secretion only in *pdk1<sup>hm</sup>* mice clearly confirms the role of the cAMP dependent PKA in the increased gastric acid secretion.

Conversely, activation of protein kinase A by forskolin stimulated gastric acid secretion only in *pdk1<sup>wt</sup>* mice suggest that PKA is already active in *pdk1<sup>hm</sup>* mice and could not be further activated. Both, H89 and forskolin virtually abolished the differences of gastric acid secretion between the genotypes.

Carbachol enhanced the acid secretion in mice from both genotypes indicating that cholinergic pathway was still susceptible for stimulation and thus was not involved in the increased acid secretion in *pdk1<sup>hm</sup>* mice. Similarly stimulation of gastric acid secretion by phorbol esters in both the genotypes indicates that PKC is not involved in the enhanced gastric acid secretion in *pdk1<sup>hm</sup>* mice.

In conclusion, basal gastric acid secretion is significantly stronger in *pdk1<sup>hm</sup>* than in *pdk1<sup>wt</sup>* mice. Thus, PDK1 exerts an inhibitory action on basal gastric acid secretion.

## 10 ABBREVIATIONS

ADP	Adenosine Di Phosphate
AMP	Adenosine Mono Phosphate
APC	Adenomatous polyposis coli
ATP	Adenosine Tri Phosphate
Ach	Acetyl choline
µg	Microgram
µl	Microliter
µM	Micromolar
CCK <sub>2</sub>	Cholecystochinin type 2 receptor
Bw	Body weight
BSA	Bovine serum albumin
cAMP	Cyclic Adenosine Mono phosphate
CCH	Carbachol
cDNA	complementary Deoxyribonucleic Acid
cRNA	complementary Ribonucleic Acid
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribo-Nucleotidetriphosphate
DEX	Dexamethasone
ECL cells	Enterochromaffin-like cells
EDTA	Ethylenediamine tetraacetic acid
ELISA	Enzyme-Linked Immunoabsorbent Assay
ENaC	Epithelial sodium channel
FSK	Forskolin
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GLUT1 / 4	Glucose Transporter isoforms 1 /4
GRP	Gastrin releasing peptide
H <sub>2</sub>	Histamine type 2 receptor
HEPES	N-(2-Hydroxyethyl) piperazine-N-(2-ethanesulfonic acid)
IGF1	Insulin- like growth factor 1
Min	Multiple intestinal neoplasms
Nedd4-2	Neuronal cell expressed developmentally down regulated 4-2

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NHE3	Na/H-exchanger isoform 3
NMDG	N-methyl-D-glucamine
°C	Degree(s) Celsius (centigrade)
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase isoform 1
PFA	Paraformaldehyde
PI3K	Phosphatidyl- inositol -3-kinase
PKA	Protein Kinase A
PKB (Akt)	Protein Kinase B; oncogene from Akt mouse
PKC	Protein Kinase C
kDa	Kilo Dalton
Ringer	Buffer Solution
ROMK	Renal outer medullary K <sup>+</sup> channel
SEM	Standard Error of Mean
SGKs	Serum and Glucocorticoid inducible protein Kinase isoforms
SGLT	Sodium-glucose cotransporter
SSRT <sub>2</sub>	Somatostatin receptor type 2
TPA	Phorbol 12 myristate 13 acetate (phorbol ester)
TRIS	Tris (hydroxymethyl) aminomethane



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## 12 PUBLICATIONS

**Rotte A**, Mack AF, Bhandaru M, Kempe DS, Beier N, Scholz W, Dicks E, Pötzsch S, Kuhl D, Lang F: Pioglitazone induced gastric acid secretion. *Cell Physiol Biochem* 2009;24(3-4):193-200.

Rexhepaj R\*, **Rotte A\***, Kempe DS, Sopjani M, Föller M, Gehring EM, Bhandaru M, Grunner I, Mack AF, Rubio-Aliaga I, Näßl AM, Daniel H, Kuhl D, Lang F: Stimulation of electrogenic intestinal dipeptide transport by the glucocorticoid dexamthasone. *Pflugers Arch*. 2009 Nov;459(1):191-202. (\* - shared first authorship).

Bhandaru M, Kempe DS, **Rotte A**, Rexhepaj R, Kuhl D, Lang F: Hyperaldosteronism, hypervolemia, and increased blood pressure in mice expressing defective APC. *Am J Physiol Regul Integr Comp Physiol*. 2009 Sep;297(3):R571-5.

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Sandu C, Artunc F, Grahammer F, **Rotte A**, Boini KM, Friedrich B, Sandulache D, Metzger M, Just L, Mack A, Skutella T, Rexhepaj R, Risler T, Wulff P, Kuhl D, Lang F: Role of the serum and glucocorticoid inducible kinase SGK1 in glucocorticoid stimulation of gastric acid secretion. *Pflugers Arch*. 2007 Dec;455(3):493-503.

## 13 AKADEMISCHE LEHRER

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## 14 LEBENS LAUF

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### Education

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- 1994-1996 Board of Intermediate Education, Vijay Junior College,  
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- 1997-2001 **Bachelor of Pharmacy**, Vaagdevi College of Pharmacy, Kakatiya University,  
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- 2002-2004 **Master of Pharmacy** (Pharmacology), University College of Pharmaceutical  
sciences, Kakatiya University, Warangal, Andhra Pradesh, India.
- Thesis: Anti-cancer activity and quantitative DNA binding studies of some novel  
palladium organic complexes.
- Supervisor: Prof Dr. Devarakonda R Krishna
- 2006-2009 Ph.D., Institute of Physiology. University of Tuebingen, Tuebingen.
- Dissertation: **Role of PI-3 kinases in the regulation of gastric acid secretion.**
- Supervisor: Prof Dr F. Lang