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TRPC channels in erythrocytes: Role for basal Ca^{2+} leak and suicidal cell death

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1 Introduction

1.1 Apoptosis

1.1.1 Apoptosis in nucleated cells

Programmed cell death (PCD) is a genetically regulated process of self-destruction. Its most frequent phenotype is called apoptosis. Apoptosis can be characterized by a series of stereotyped changes affecting nucleus, cytoplasm and plasma membrane. It leads to the dismantling of the dying cell and to its rapid ingestion by macrophages or other neighboring cells (Bratosin et al., 2001). Hallmarks of apoptosis include nuclear condensation, DNA fragmentation, mitochondrial depolarization, cell shrinkage, and breakdown of phosphatidylserine asymmetry of the plasma membrane (Green and Reed, 1998; Gulbins et al., 2000). In mammalian cells, PCD depends on two major executionary pathways that usually operate together and amplify each other. One involves the proteolytic activation of a family of aspartate-directed cysteine proteinases, the effector caspases. The other pathway involves mitochondrial inner membrane permeabilization. This permeabilization leads to the release of mitochondrial pro-apoptotic proteins into the cytosol. These proteins might either induce caspase activation, such as cytochrome *c* and Smac/Diablo, or might trigger caspase-independent effector pathways such as apoptosis-inducing factor AIF (Bratosin et al., 2001). Most, if not all, pro-apoptotic stimuli appear to require a mitochondrion-dependent step (Bratosin et al., 2001). Therefore, mitochondria have been proposed to play a central role in PCD (Bratosin et al., 2001; Green et al., 1998). Recent knock-out experiments of genes encoding cytochrome c or AIF have indicated that each of these intra-mitochondrial proteins is required for the induction of PCD in response to some but not all pro-apoptotic stimuli. However, the direct caspase 8 activation by the engagement of cell surface death receptors of the CD95/tumor necrosis factor receptor family has been described (Bratosin et al., 2001).

1.1.2 “Apoptosis“ in erythrocytes

1.1.2.1 Features of erythrocyte “apoptosis”

Human mature erythrocytes are terminally differentiated cells of the erythroid lineage. They do not have mitochondria, as well as a nucleus and other organelles. Their normal life span amounts to 120 days (Bratosin et al., 2001).

It has been observed that erythrocyte senescence is associated with cell shrinkage, plasma membrane microvesiculation, a progressive shape change from a discocyte to a spherocyte, cytoskeleton alterations associated with protein (spectrin) degradation, and loss of plasma membrane phospholipid asymmetry leading to the externalization of phosphatidylserine in the erythrocyte membrane (Bratosin et al., 2001; Lang et al., 2005a). The exposure of phosphatidylserine and further eat-me-signals at the cell surface trigger, and the decrease of cell volume facilitates, the engulfment of the dying cells by phagocytes (Boas et al., 1998; Eda and Sherman, 2002).

In vitro storage of erythrocytes leads to the gradual accumulation of these modifications, and *ex vivo*, a very small subpopulation of human erythrocytes with a senescent phenotype can be isolated from the peripheral blood (Boas et al., 1998; Bratosin et al., 2001). These modifications associated with erythrocyte senescence share striking similarities with some cytoplasmic features of apoptosis in nucleated cells. Nevertheless, erythrocytes survive two conditions that induce PCD in all human nucleated cells studied so far, i.e. treatment with the protein kinase inhibitory drug staurosporine, and culture in the absence of serum or other potential survival-promoting factors. Therefore, mature erythrocytes have been considered as the sole mammalian cell lacking the machinery required to undergo PCD (Bratosin et al., 2001).

A wide variety of stimuli has been described to induce apoptosis in nucleated cells. These stimuli include nitric oxide (Ibe et al., 2001), UV radiation (Kulms et al., 1999; Rosette and Karin, 1996), exposure to pathogens (Fillon et al., 2002), osmotic shock (Bortner and Cidlowski, 1998; Bortner and Cidlowski, 1999; Lang

et al., 1998a; Lang et al., 2000), and the activation of defined receptors such as CD95 (Gulbins et al., 2000; Lang et al., 1998b; Lang et al., 1999), TNFα (Lang et al., 2002), and somatostatin (Teijeiro et al., 2002). Erythrocyte “apoptosis” can be similarly induced by some of those stimuli (Lang et al., 2003a) but appears not to require caspase activation.

Taken together these findings indicate that erythrocytes constitutively express a death machinery and suggest that erythrocyte survival may be modulated *in vitro* and *in vivo* by therapeutic intervention (Bratosin et al., 2001; Lang et al., 2003a)

1.1.2.2 The role of cation channels in erythrocyte “apoptosis”

Erythrocyte cell membranes usually show little channel activity. Moreover, the erythrocytes are predominantly permeable to Cl^- (Bernhardt and Ellory, 2003). Osmotic cell shrinkage, however, opens non-selective cation channels in the erythrocyte cell membrane (Huber et al., 2001). The same channels are activated by oxidative stress (Duranton et al., 2002) and are inhibited by intracellular or extracellular Cl^- (Duranton et al., 2002; Huber et al., 2001). Thus, it is necessary to remove Cl^- ions from the medium to observe the cation channels in patch clamp experiments (Fig. 1).

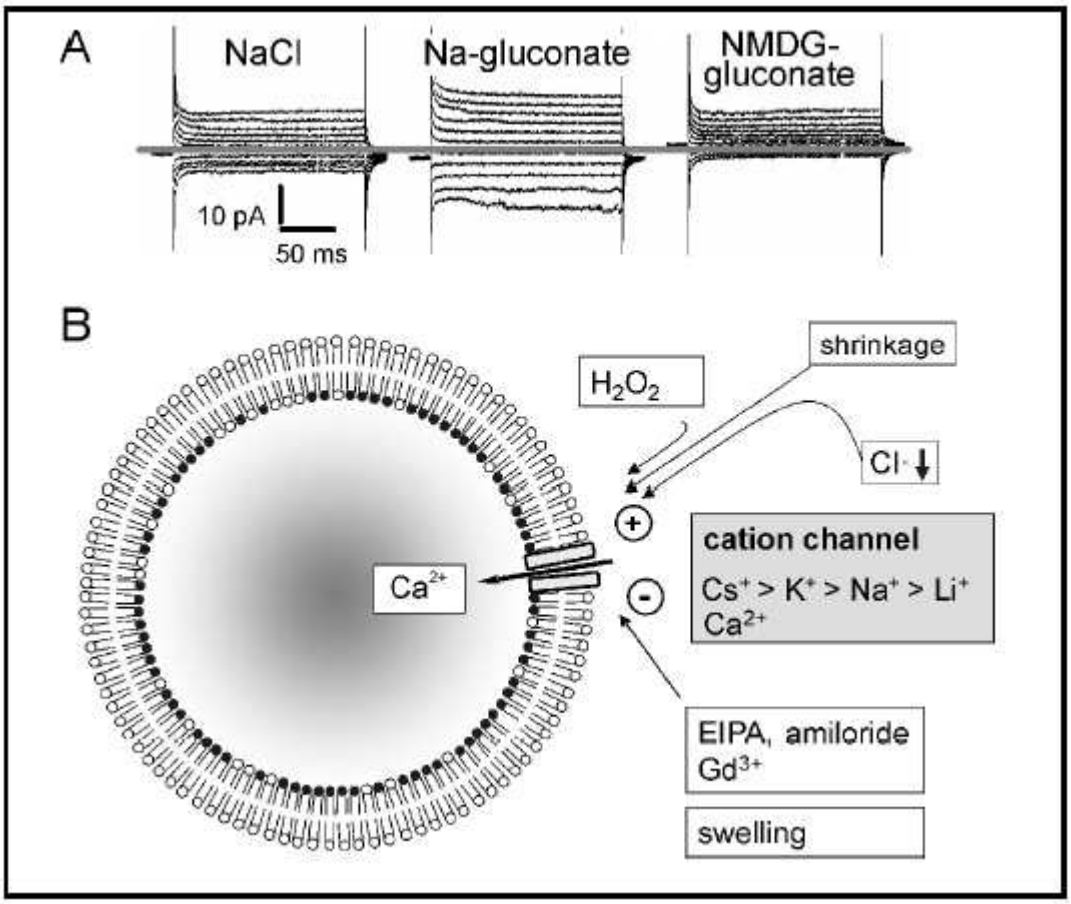

Fig. 1. Erythrocyte cation channels activated by osmotic shock and oxidative stress.
A. Activation of cation channels by Cl^- removal either in the presence (middle traces) or absence (right traces) of a permeable cation.
B. Schematic representation of erythrocyte cation channel regulation. EIPA, ethylisopropylamiloride, and H_2O_2, hydrogen peroxide.

This property is reminiscent of the Na^+ and K^+ permeability activated by incubating human erythrocytes in low ionic strength (LIS) medium (Bernhardt et al., 1991; Jones and Knauf, 1985; LaCelle and Rothsteto, 1966). Similar to what has been shown for the LIS permeability (Culliford et al., 1995; Jones et al., 1985), activation of the volume- and oxidant-sensitive cation channel by removal of extracellular Cl^- is inhibited by the anion channel/transport inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) (Duranton et al., 2002). The cation channels allow the permeation of Ca^{2+} (Lang et al., 2003b). The phosphatidylserine exposure following osmotic shock and oxidative stress is blunted following chelation of extracellular Ca^{2+} (Lang et al., 2003b).

Moreover, the phosphatidylserine exposure is blunted by amiloride (Fig. 2) (Lang et al., 2003b) and ethylisopropylamiloride (EIPA) (Lang et al., 2003c) at concentrations needed to inhibit the cation channel (Lang et al., 2003b; Lang et al., 2003c). Thus, it appears safe to conclude that activation of the cell volume- and oxidant-sensitive cation channel and subsequent Ca^{2+} entry contribute to the stimulation of erythrocyte scramblase following osmotic shock or oxidative stress (Fig. 2). Interestingly, the Na^+/H^+ exchange inhibitor ethylisopropylamiloride (EIPA) is effective at a concentration of 1 µM, whereas amiloride, which inhibits both Na^+/H^+ exchange and cation channels, requires 1 mM to become effective (Lang et al., 2003c).

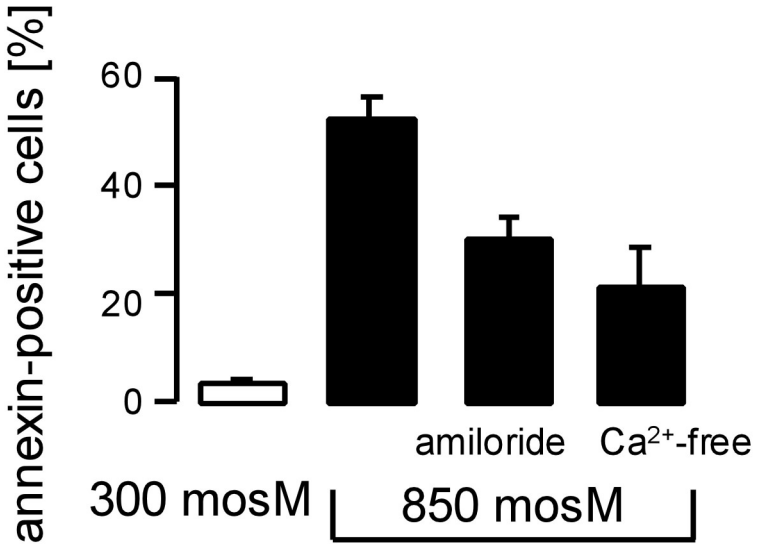

Fig. 2. Cell-shrinkage-induced break down of the erythrocyte membrane phospholipid asymmetry is dependent on extracellular Ca^{2+} and inhibited by amiloride. Mean percentage of annexin binding erythrocytes as measured by flow cytometry. Erythrocytes were cultured for 24 hours at 37℃ either in isotonic (*open bar*) or in hypertonic Ringer solution (*closed bars*; osmolarity increased to 850 mOsm by adding sucrose). In some experiments, incubation in hypertonic Ringer solution was performed in the presence of the cation channel inhibitor amiloride (1 mM) or in the absence of extracellular Ca^{2+}.

Furthermore, energy depletion leads to enhanced phosphatidylserine exposure (Lang et al., 2003b). Presumably, energy depletion impairs the replenishment of GSH and thus weakens the antioxidative defence of the erythrocytes (Bilmen et al., 2001).

The capacity for oxidative defence decreases with erythrocyte age (Imanishi et al., 1985; Piccinini et al., 1995), a phenomenon paralleled by increase of passive cation permeability (Joiner and Lauf, 1978) and cytosolic free [Ca^{2+}] (Aiken et al., 1992; Allan and Raval, 1987; Cameron et al., 1993; Kramer and Swislocki, 1985; Romero et al., 1997; Seidler and Swislocki, 1991). It is thus tempting to speculate that the cation channels sense cell age. Within the ageing erythrocytes, the loss of antioxidative defence can be expected to increase cation channel activity leading to Ca^{2+} entry, increased Ca^{2+} pump activity, ATP depletion, further impairment of antioxidative defence, further activation of cation channels, further Ca^{2+} entry, and eventually activation of the scramblase (Lang et al., 2003a).

1.1.2.3 Prostaglandins stimulate erythrocyte cation channels and "apoptosis"

Intriguing evidence points to a role of prostaglandins in the regulation of erythrocyte "apoptosis". It has been demonstrated that hyperosmotic shock and Cl^--removal trigger the release of prostaglandin E_2 (PGE_2) (Lang et al., 2005b). PGE_2 in turn activates the cation channels (Kaestner and Bernhardt, 2002; Lang et al., 2005b), increases the cytosolic Ca^{2+} concentration (Lang et al., 2005b; Kaestner et al., 2004), and stimulates phosphatidylserine exposure at the erythrocyte surface (Lang et al., 2005b). Subsequently, the activation of the cation channels by Cl^--removal is abolished by blocking the PGE_2 formation either by inhibiting the cyclooxygenase or the phospholipase-A2 (Lang et al., 2005b). PGE_2 further activates the Ca^{2+} dependent cysteine endopeptidase calpain, an effect, however, apparently not required for stimulation of phosphatidylserine exposure (Lang et al., 2005b).

1.1.2.4 Ca^{2+} sensitive K^+ channels mediate shrinkage in erythrocyte "apoptosis"

Ca^{2+} entering erythrocytes does not only activate the scramblase but in addition stimulates the Ca^{2+} sensitive "Gardos" K^+ channels in erythrocytes (Bookchin et al., 1987; Brugnara et al., 1993; Franco et al., 1996). The activation of the channels leads to hyperpolarization of the cell membrane driving Cl^- in parallel to K^+ out of the cell. The cellular loss of KCl favours cell shrinkage. In addtion, the cellular loss of K^+ presumably participates in the triggering of "apoptosis" (Lang et al., 2003e). Increase of extracellular K^+ or pharmacological inhibition of the Gardos channels by clotrimazole or charybdotoxin do not only blunt the cell shrinkage but also decrease the phosphatidylserine exposure following exposure to ionomycin (Lang et al., 2003e). Presumably, cellular loss of K^+ somehow stimulates erythrocyte "apoptosis" as has been shown for apoptosis of nucleated cells (Bortner et al., 1997; Bortner et al., 1999). As PGE_2 increases cytosolic Ca^{2+} activity (Lang et al., 2005b) (see above), it similarly activates the Ca^{2+} sensitive "Gardos" K^+ channels with subsequent cell shrinkage (Allen and Rasmussen, 1971; Li et al., 1996).

1.1.2.5 Physiological significance of erythrocyte "apoptosis"

During their daily life, erythrocytes are exposed to several stress situations. On average they pass once a minute the lung where they are exposed to oxidative stress. More than once an hour they travel through kidney medulla where they face osmotic shock. Erythrocytes have to squeeze through capillaries which are smaller than themselves. Thus, the integrity of erythrocytes is constantly challenged. Rupture of erythrocyte cell membranes releases hemoglobin into the blood which may be filtered at the glomerula of the kidney, precipitates in the acid lumen of the tubules, obliterates the tubules and thus leads to renal failure. To avoid those complications, erythrocytes, as other cell, require a mechanism allowing them to be disposed without release of intracellular

components (Lang et al., 2005a). The mechanisms described here could well participate in the limitation of erythrocyte survival. Several disorders may decrease the life span of mature erythrocytes by facilitating erythrocyte "apoptosis". As a matter of fact, the sensitivity of sickle cells and of glucose-6-phosphate dehydrogenase deficient cells to osmotic shock and of sickle cells, thalassemic cells, and glucose-6- phosphate dehydrogenase deficient cells to oxidative stress and to glucose depletion is significantly higher than that of control cells (Lang et al., 2005a). This enhanced susceptibility most likely contributes to the decrease of erythrocyte life span in those genetic disorders. Erythrocyte "apoptosis" may further be relevant for the intraerythrocyte survival of the malaria pathogen *Plasmodium falciparum*. The parasite invades erythrocytes to escape the immune system. However, transport across the intact erythrocyte cell membrane is not sufficient to meet the excessive demands of the pathogen. Thus, *Plasmodium falciparum* induces novel permeability pathways (NPP) allowing the uptake of nutrients and the disposal of waste products (Kirk, 2001). Recent experiments revealed that NPP is made up of endogeneous host cell channels which are activated by the pathogen through oxidation of the cell membrane (Duranton et al., 2003; Huber et al., 2002). Activation of the cation channel is required for the cellular accumulation of Na^+ and Ca^{2+} which are both needed by the pathogen. By the same token, however, the activation of the cation channel triggers erythrocyte "apoptosis" (Brand et al., 2003). Presently, it is not entirely clear whether phosphatidylserine exposure of infected host cells is favourable for the host or the pathogen. In other model systems, host cell apoptosis has proven to be a crucial defence mechanism of the host (Grassme et al., 2000). In any case, erythrocyte "apoptosis" favours the recognition of the erythrocytes by macrophages and thus limits the life span of the infected cell. Most importantly, erythrocyte "apoptosis" may serve to prevent hemolysis. Energy depletion, defective Na^+/K^+ATPase or enhanced leakiness of the cell membrane all lead to gain of Na^+ and Cl^- and osmotically obliged water with subsequent cell swelling (Lang et al., 1998a). Initially, the entry of Na^+ may be compensated by cellular loss of K^+, the decrease of the K^+ equilibrium potential will, however, eventually lead to

depolarisation which will favour the entry of Cl^-. The increase of cell volume will lead to rupture of the cell membrane with cellular release of hemoglobin (see above). It is intriguing to speculate that an increase of erythrocyte Ca^{2+} activity is indicative of the inability of the cell to maintain its electrolyte gradients. The activation of the Gardos K^+ channel serves to delay swelling and disruption of defective erythrocytes. Decreasing cytosolic K^+ concentrations accelerate the phosphatidylserine exposure. Paradoxically, many of the cell injuries such as oxidative stress and energy depletion lead initially to cell shrinkage. Thus, erythrocyte “apoptosis” may be an important mechanism to clear erythrocytes prior to detrimental hemolysis (Lang et al., 2005a).

1.2 TRP cation channels

1.2.1 General features of TRP channels

Changes in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) play a central role in many fundamental cellular processes including muscle contraction, transmitter release, cell proliferation, gene transcription, and cell death (Berridge et al., 2000). The family of transient receptor potential (TRP) channels contributes to changes in $[Ca^{2+}]_i$ by providing Ca^{2+} entry pathways and very likely also by providing intracellular pathways for Ca^{2+} release from cellular organelles (Pedersen et al., 2005).

Transient receptor potential (TRP) channels were first described in Drosophila, where photoreceptors carrying trp gene mutations exhibited a transient voltage response to continuous light. Unlike most ion channels, TRP channels are identified by their homology rather than by ligand function or selectivity because their functions are disparate and often unknown (Clapham, 2003).

All TRPs contain six putative transmembrane domains, which are thought to assemble as homo- or hetero-tetramers to form cation selective channels. All TRPs are cation channels, although the permeability for different mono- and divalent cations varies greatly between isoforms (Pedersen et al., 2005). Based on amino acid homologies, the mammalian TRP channel superfamily can be

divided into seven families: TRPC, TRPM, TRPV, TRPA, TRPP, TRPML, and TRPN (Clapham, 2003; Corey, 2003; Montell et al., 2002a; Montell et al., 2002b). The TRPC ('Canonical') and TRPM ('Melastatin') subfamilies consist of seven and eight different channels, respectively (i.e. TRPC1–TRPC7 and TRPM1–TRPM8). The TRPV ('Vanilloid') subfamily presently comprises six members (TRPV1–TRPV6). The TRPML ('Mucolipin') family comprises three members, and the TRPP ('Polycystin') family three channel-like and five non-channel members, respectively. These families are not sufficiently characterized, but gain increasing interest because of their involvement in several human diseases (Pedersen et al., 2005). The most recently proposed subfamily, TRPA ('Ankyrin'), has only one mammalian member, TRPA1, and finally, the TRPN (no mechanoreceptor potential C, or NOMPC) has so far only been detected in *Caenorhabditis elegans*, *Drosophila*, and zebra fish. Fig. 3 shows the phylogentic tree of the TRP superfamily.

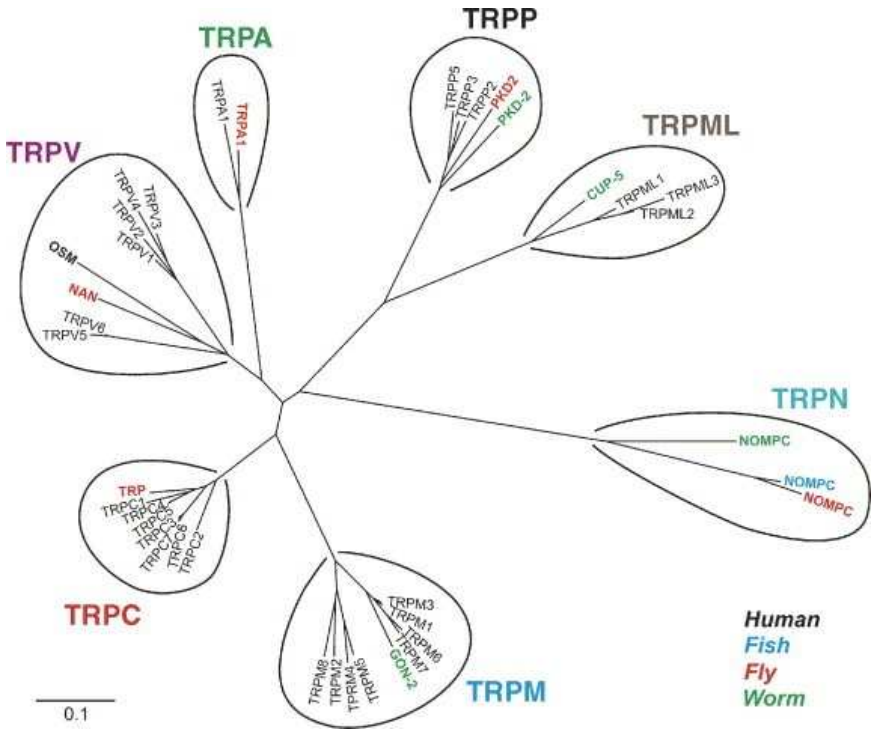

Fig. 3. Phylogenetic tree of the TRP superfamily.

The Ca^{2+} influx channels of the TRP family compromise all the TRPCs, all TRPVs, TRPM1, 2, 3, 6, 7, and 8, TRPA1, TRPP2, 3, and 5 and TRPML1, 2, and 3. The permeability ratios P_{Ca}/P_{Na} for these channels vary considerably, ranging from 0.3 for TRPM2 to >100 for TRPV5 and TRPV6 (Pedersen et al., 2005). Moreover, a number of recent studies indicate that some members of the TRP superfamily could function as intracellular Ca^{2+} release channels, in some cases (e.g. TRPV1 and TRPM8), in addition to their roles as plasmalemmal Ca^{2+} channels (Turner et al., 2003; Zhang and Barritt, 2004). Consistent with roles as Ca^{2+} release channels, some of the less-studied TRP channels, including TRPML1 and TRPP2 (Koulen et al., 2002; Raychowdhury et al., 2004) appear to be mainly localized in intracellular membranes.

TRP channels are activated by a wide range of stimuli including intra- and extracellular messengers, chemical, mechanical, and osmotic stress, and some probably by the filling state of intracellular Ca^{2+} stores (Clapham, 2003). A large number of TRP channel binding partners have recently been described, many of which have been assigned important roles in the regulation and function of TRP channels. TRPs also contain consensus sites for direct phosphorylation by serine/threonine and tyrosine kinases, although the role of phosphorylation in channel function remains to be fully elucidated (Pedersen et al., 2005). Finally, in addition to regulatory modes activating TRP channels resident in the plasma membrane, several TRPs appear to be constitutively open, and may be regulated by vesicular insertion (Bezzerides et al., 2004; Kanzaki et al., 1999; Pedersen et al., 2005).

1.2.2 The TRPC subfamily

1.2.2.1 Members of the TRPC subfamily

Based on structural and functional similarities, the TRPC family can be further subdivided into four different subfamilies: TRPC1, TRPC2, TRPC3, 6, and 7 and TRPC4 and 5 (Fig. 3). TRPC2 is a pseudogene in humans, in old world

monkeys, and apes (Liman and Innan, 2003), but TRPC2 apparently forms fully regulated channels in other mammalian species. TRPC3, 6, and 7 form a closely related subfamily, sharing a high degree of amino acid identity (70–80%) and functional, regulatory and pharmacological similarities. A similarly close structural and apparently functional relationship also exists between TRPC4 and TRPC5 (Vazquez et al., 2004).

1.2.2.2 TRPC 3/6/7 channels

1.2.2.2.1 Molecular structure and tissue distribution of the TRPC3/6/7 channels

Members of the TRPC3/6/7 subfamily display common structural features of the TRPC family: they contain four N-terminal ankyrin repeats, six transmembrane-spanning domains and a putative pore region located between transmembrane domains 5 and 6 (see Fig. 4) (Dietrich et al., 2005a).

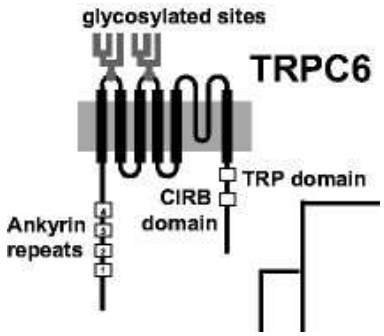

Fig. 4. Structural elements of the TRPC6 cation channel

Evidence for the importance of the highly conserved pore region for TRPC function is derived from site-directed mutagenesis studies resulting not only in the complete loss of channel activity upon heterologous expression, but also in a dominant-negative effect of a mutated channel monomer on functional homo- or heteromeric channel tetramers (Hofmann et al., 2002). However in contrast to e.g. TRPV channels (van Abel et al., 2005), the exact location of a selectivity

filter and the pore helix has not yet been determined in TRPC channels (Dietrich et al., 2005a).

The full-length cDNA of mouse TRPC6 was isolated from brain (Boulay et al., 1997), while human TRPC6 was cloned from placenta (Hofmann et al., 1999). TRPC6 is most prominently expressed in lung tissues as deduced from Northern Blot analysis (Boulay et al., 1997). Three splice variants with shorter amino termini were additionally cloned from rat lung (Zhang et al., 2006). Although TRPC6 expression in brain is lower than that of TRPC3, a TRPC6 splice variant, TRPC6A, is highly expressed in the dentate gyrus of the hippocampus (Bonaventure et al., 2002). By in situ hybridization and histochemistry, TRPC6 expression was found to be exclusively localized in the dentate granule cell layer of the adult mouse brain (Otsuka et al., 1998).

1.2.2.2.2 Pharmacology and electrophysiological properties

TRPC3, 6, and 7 appear to form nonselective cation channels that show both inward and outward rectification at negative and positive voltages, respectively. The current–voltage relationships for these TRPCs is presented in Fig. 5 (Vazquez et al., 2004).

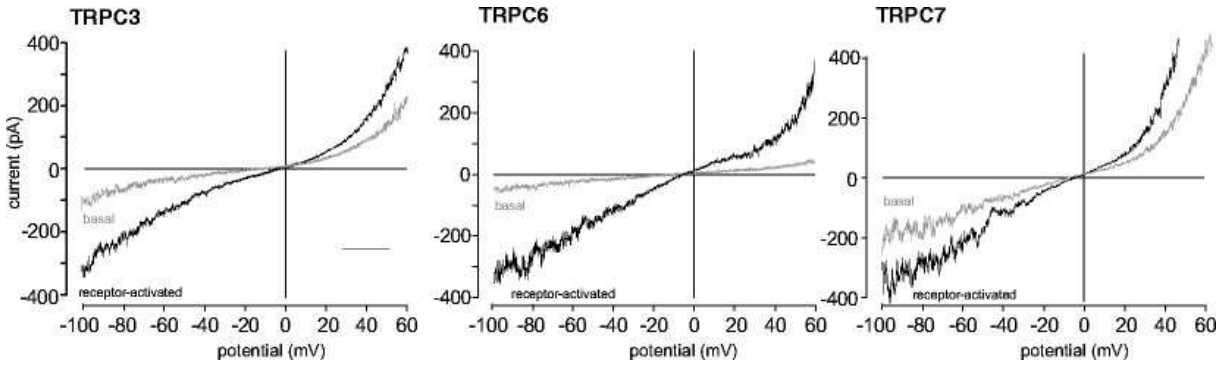

Fig. 5. Current–voltage relationship of TRPC3, TRPC6, and TRPC7 as revealed by electrophysiological recordings of HEK293 cells heterologously expressing these non-selective cation channels.

Zhu et al. (Zhu et al., 1998) first described TRPC3 as an agonist-activated channel when expressed in HEK293 cells and demonstrated that it was relatively insensitive to low concentrations of Gd^{3+}, but was blocked by concentrations in the 100 μM range. Hofmann et al. (Hofmann et al., 1999) later demonstrated that TRPC3 could also be activated directly by DAG analogues.

TRPC3 channels have also been shown to have two potential conductance states when expressed in HEK293 cells, one of 66 pS and one of 17 pS (Kiselyov et al., 1998). In this study it was shown that these channels were activated by calcium store depletion in intact cells, consistent with an earlier report by Preuβ et al. (Preuss et al., 1997), or by addition of recombinant IP_3 receptors in excised patches. In a subsequent study, however, activation of TRPC3 was shown to be dependent solely upon DAG with no requirement for either IP_3 or the IP_3 receptor (Trebak et al., 2003a).

Since their discovery, these channels have been shown to be sensitive to a variety of agents with relative sensitivities varying dramatically depending upon the expression system. In HEK293 cells, TRPC3 was inhibited by high concentrations of: SKF96365, verapamil, La^{3+}, Gd^{3+}, and Ni^{2+} (Zhu et al., 1998) and was partially inhibited by moderate (30 μM) concentrations of 2-aminoethoxydiphenyl borane (2APB) (Trebak et al., 2002); in DT40 cells by low concentrations of Gd^{3+} (Trebak et al., 2002); and in CHO cells they are reported to be sensitive to low concentrations of La^{3+}, Gd^{3+}, and SKF96365 (Halaszovich et al., 2000). However, in the latter case, it appeared that the lanthanides acted by first entering the cell and blocking from the inside. This requirement for penetration, which does not normally occur with lanthanides, may explain the relative insensitivity of TRPC3 to lanthanides in other cellular systems (Vazquez et al., 2004).

TRPC6 has also been shown to be a DAG-activated channel (Hofmann et al., 1999) and is very similar to TRPC3 and 7 with regard to its current–voltage relationship. The single channel conductance was 35 pS, with no second conductance state observed (Hofmann et al., 1999). TRPC6 does, however, possess a unique characteristic to this TRPC subfamily. When expressed in

HEK293 cells, TRPC6 currents are enhanced by the cation channel blocker flufenamate while TRPC3 and 7 currents are not (Inoue et al., 2001).

TRPC7, the final member of this subfamily, demonstrates properties very similar to TRPC3 and 6 with regard to its voltage–current relationship, and activation by DAG (Okada et al., 1999). TRPC7 has demonstrable sensitivity to SKF96365 and, in a similar fashion to TRPC3 and 6, is relatively insensitive to lanthanides (Vazquez et al., 2004).

The main differences between the three channel types may lie in their ion selectivity, in that TRPC6 is reported to be somewhat Ca^{2+}-selective, while TRPC3 and TRPC7 do not appear to be. Reported values for (P_{Na}:P_{Ca}) are 1 : 1.5, 1 : 5 and 1 : 2 for TRPC3, 6, and 7, respectively (Clapham et al., 2001).

1.2.2.2.3 Regulation of TRPC6

Single-cell Ca^{2+} imaging experiments measuring carbachol- and thapsigargin-induced Ca^{2+} entry allowed the characterization of TRPC6 as a receptor-regulated but not store-regulated cation channel (Boulay et al., 1997); a typical I–V relationship illustrating double rectification is shown in Fig 5. Addition of IP_3 activated recombinant TRPC6 channels neither in the whole-cell mode nor in excised inside-out patches, although the G protein activator AlF_4^- stimulated and the PLC inhibitor U73122 blocked agonist-induced activation of TRPC6, thus pointing to a G protein- and PLC-dependent activation mechanism. Along these lines of reasoning, 1-oleoyl-1-acetyl-*sn*-glycerol (OAG), a membrane-permeable analogue of diacylglycerol (DAG), as well as the DAG lipase inhibitor RHC80267 markedly increased TRPC6 activity (Hofmann et al., 1999). TRPC6 was the first ion channel identified that is activated by DAG in a membrane-delimited fashion, independently of protein kinases C. The exact location of a putative binding site for diacylglycerols in the TRPC6 protein is still elusive because an OAG-insensitive splice variant of TRPC6, (TRPC6B; (Zhang et al., 2006)) characterized by means of fluorometry turned out to be activated by DAG when analyzed by electrophysiological methods (Jung et al., 2003). In contrast to the

scenario with TRPC6 which is a tightly receptor-regulated store-independent cation channel, TRPC3 and TRPC7 display considerable basal activity (see Fig. 5, (Hofmann et al., 1999; Okada et al., 1999)). To identify potential molecular correlates accounting for the functional difference, the glycosylation pattern of TRPC6 was compared with that of TRPC3 (Dietrich et al., 2003). TRPC6 carries two extracellular N-linked glycosylation sites, while TRPC3 is a monoglycosylated protein (Vannier et al., 1998). Elimination of the extra glycosylation site missing in TRPC3 was sufficient to transform the tightly receptor-regulated TRPC6 into a constitutively active, TRPC3-like ion channel. Conversely, engineering of an additional glycosylation site in TRPC3 to imitate the situation in TRPC6, markedly reduced TRPC3 basal activity. Thus, the glycosylation pattern is a critical determinant for the tight regulation of TRPC6 by PLC-coupling receptors (Dietrich et al., 2003).

The regulation of TRPC6 by calmodulin was carefully analyzed leading to an overall picture different from that of TRPC3. Calmodulin inhibitors like calmidazolium and trifluoperazine had an inhibitory effect on receptor-operated calcium influx into TRPC6-expressing HEK293 cells, indicating a stimulatory impact of Ca^{2+}/calmodulin on TRPC6 channel activity (Boulay, 2002). The latter concept was further extended by the observation that TRPC6 activation and its acceleration by the extracellular calcium concentration ($[Ca^{2+}]_o$) most probably involves phosphorylation by calmodulin-dependent kinase II, an effect that was not noted for the closely related TRPC7 protein (Shi et al., 2004). To conclude, TRPC6 and 7 are subject to a complex regulation by Ca^{2+} on both sides of the plasma membrane involving calmodulin-dependent and calmodulin-independent mechanisms (Shi et al., 2004).

Like TRPC3, the TRPC6 ion channel is regulated by protein phosphorylation. PKC appears to contribute to channel inactivation (Trebak et al., 2003b). Fyn, a member of the Src family of protein tyrosine kinases, increases TRPC6 channel activity. Stimulation of EGF receptor entails tyrosine phosphorylation of TRPC6, and Fyn and TRPC6 physically interact in mammalian brain as well as after heterologous expression in COS-7 cells (Hisatsune et al., 2004).

1.2.2.2.4 Physiological role of TRPC6

The physiological role of TRPC6 is still largely unknown. However, there is growing evidence that TRPC6 is an intrinsic constituent of receptor-operated cation entry involved in numerous physiological processes. In human platelets thrombin-activated cation influx is independent of store depletion, consistent with the observation that TRPC6 is highly expressed in these cells. In this model system, TRPC6 does not serve as a substrate of tyrosine kinases, but was phosphorylated in a cAMP-dependent manner (Hassock et al., 2002). It is well documented that phosphoinositide 3-kinase (PI3K) activation resulting in the production of phosphatidylinositol 3,4,5-trisphosphate (PIP_3) triggers platelet aggregation by inducing a Ca^{2+} influx (Lu et al., 1998). In accord with this concept, TRPC6 was recently identified as the putative molecular correlate of a PIP_3-sensitive calcium entry system in platelets, Jurkat T cells, and RBL-2H3 mast cells (Tseng et al., 2004).

There are numerous studies providing evidence for an important role of TRPC6 in vascular and pulmonary smooth muscle cells. By comparative biophysical characterization and gene suppression using antisense oligonucleotides, TRPC6 was suggested to be the molecular correlate of the α_1-adrenoceptor-activated non-selective cation channel in vascular smooth muscle cells (Inoue et al., 2001) and the vasopressin-activated cation channel in an aortic smooth muscle cell line (Jung et al., 2002). In addition, TRPC6 has been proposed to play a critical role in the intravascular pressure-induced depolarization and constriction of small arteries and arterioles (Welsh et al., 2002) known as the Bayliss effect. Myogenic constriction of resistance arteries results from Ca^{2+} influx through voltage-gated Ca^{2+} channels subsequent to membrane depolarization. Apart from TRPC6, the Ca^{2+}-activated cation channel TRPM4 has recently been implicated in myogenic vasoconstriction (Earley et al., 2004). The precise location of either TRPC6 or TRPM4 in the signaling pathway elicited by elevated intravascular pressure still remains poorly understood.

Recently, expression studies revealed that PDGF-mediated proliferation of pulmonary artery smooth muscle cells (PASMC) is associated with c-*jun*/STAT3-induced upregulation of TRPC6 expression (Yu et al., 2003). In this context it is intriguing to note that excessive PASMC proliferation, a major cause of the elevated pulmonary vascular resistance in patients with idiopathic pulmonary arterial hypertension (IPAH), also correlates with overexpression of TRPC6 and TRPC3 proteins in these tissues. In line with these data, downregulation of TRPC6 by TRPC6 specific small interfering RNAs resulted in attenuated IPA-PASMC proliferation (Yu et al., 2004). Moreover, TRPC6 expression is upregulated in pulmonary arteries of rats kept under chronic hypoxic conditions to induce pulmonary hypertension. As expected, OAG-induced cation entry was significantly increased in hypoxia-treated PASMC as compared to control cells (Lin et al., 2004).

Recently, initial results on the phenotype of mice deficient in TRPC6 were published (Dietrich et al., 2005a). It could have been assumed that loss of TRPC6 function would lead to diminished vascular smooth muscle tone and hypotension. Unexpectedly, a higher agonist-induced contractility in aortic rings prepared from these mice was observed, and elevated systemic blood pressure that was further increased by inhibition of nitric oxide (NO) synthase (Dietrich et al., 2005b). These effects could be explained by in vivo replacement of TRPC6 by TRPC3-type channels which are closely related, but constitutively active resulting in enhanced basal and agonist-induced cation entry into smooth muscle cells leading to increased smooth muscle contractility (Dietrich et al., 2005a). As the expression pattern of TRPC3 and TRPC6 overlaps in most and that of TRPC6 and TRPC7 in some smooth muscle cell tissues (Beech et al., 2004), a heterotetrameric TRPC3/6 or TRPC6/7 channel complex might be the real molecular correlate of the nonselective cation influx into smooth muscle cells (Gudermann et al., 2004). These findings imply that TRPC3, 6, and 7 are functionally non-redundant and that TRPC6 plays a unique role in the control of airway and vascular smooth muscle contractility (Dietrich et al., 2005a).

1.3 Objective of this study

This study aims to define the molecular identity of the erythrocyte cation channel involved in basal cation leak and apoptotic cell death. As K562 human leukaemia cells and erythrocytes share common features in many respects (Andersson et al., 1979; Gahmberg and Andersson, 1981), K562 cellls were chosen as a cell model for erythrocytes. First, experiments in K562 cells have been performed to explore whether PGE_2 triggers similar mechanisms leading to apoptosis in these cells. Subsequently, a TRP channel expression profile of K562 cells was made by RT-PCR in order to identify possible candidates for the cation channel involved in PGE_2 triggered apoptosis. The role of the TRP channels identified in K562 cells for apoptosis was elucidated by siRNA-based silencing of these channels. As a result, specific silencing of TRPC7 significantly blunted PGE_2 triggered annexin-binding as a measure of phospatidylserine exposure at the outer leaflet of the cell membrane and DNA-fragmentation, two hallmarks of apoptosis in nucleated cells. The involvement of TRPC7 in the PGE_2 triggered apoptosis of K562 cells prompted me to define the functional significance of TRPC channels for erythrocyte apoptosis and basal cation leak. To this end, the expression of TRPC channels in human erythrocytes was investigated by immunoblots. As TRPC6 was found in human erythrocytes, the functional significance of TRPC6 was explored in human erythrocytes ghosts. To confirm the involvement of TRPC6 channels in erythrocyte apoptosis and basal cation leak further experiments were performed in erythocytes from mice lacking functional TRPC6 ($TRPC6^{-/-}$) and their wild type littermates ($TRPC6^{+/+}$).

2 Materials and Methods

2.1 Investigation of PGE_2 triggered apoptosis and the cation channel involved in human leukaemia K562 cells

Cell culture and solutions. K562 human leukaemia cells were cultivated in RPMI 1640 medium + L-glutamine (Gibco, Karlsruhe, Germany) supplemented with 4-10% FCS and 1% penicillin/streptomycin. Where indicated, ethylisopropylamiloride (EIPA; 50 µM), PGE_2 (0.1 - 50 µM) or carboplatin (40 µg/ml, all from Sigma, Taufkirchen, Germany) were added to the cell culture medium.

Caspase activity. The CaspACE *In Situ* Marker (FITC-VAD-FMK; Promega, Mannheim, Germany) was used to measure the caspase activity by FACS analysis. According to the manufacturer´s protocol, the fluorescence intensity was measured in fluorescence channel FL-1. Briefly, cells were stimulated with PGE_2 (50µM) or cells treated with 40 µg/ml carboplatin served as positive control. All samples were incubated for 48 h at 37°C and 5% CO_2. Then, CaspACE FITC-VAD-FMK was added (1 µl) to 500 µl of the cell suspension. The cells were incubated for 20 min under protection from light and washed with phosphate buffered saline (PBS). Then, 150 µl PBS were added, and FACS analysis was performed (FACS-Calibur, Becton Dickinson, Heidelberg, Germany) using the fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Phosphatidylserine (PS) exposure. PS in the outer membrane leaflet was determined in immunofluorescence microscopy by estimating the binding of annexin-V Fluos. K562 cells were stimulated for 48 h at 37℃ with PGE_2 (50 µM) diluted in RPMI 1640 medium + L-glutamine (Gibco, Karlsruhe, Germany) supplemented with 4% FCS and 1% penicillin/streptomycin. Cells were washed in annexin-V-binding buffer containing (in mM) 125 NaCl, 10 N-2-

hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), and 5 $CaCl_2$. Cells were suspended in a solution composed of Annexin-V-Fluos (Roche Diagnostics, Mannheim, Germany) and annexin-V buffer (dilution of 1:50). After 10 minutes of incubation, samples were finally diluted 1:5 in annexin-V-binding buffer and analyzed by fluorescence microscopy. In addition, the annexin-V-Fluos binding of these cells was assessed by FACS analysis as described previously (Lang et al., 2005b) The annexin-V-fluorescence intensity was measured in FL-1 on a FACS-Calibur (Becton and Dickinson, Heidelberg, Germany).

Cytosolic free Ca^{2+}-concentration. For measurement of intracellular Ca^{2+} activity, K562 cells were washed in NaCl Ringer solution (in mM: 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 HEPES/NaOH pH 7.4, 5 glucose, 1 $CaCl_2$) and then loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in NaCl Ringer solution containing 2 µM Fluo-3/AM and 2 mM $CaCl_2$. The cells were incubated at room temperature for 15 minutes under shaking and washed twice in NaCl Ringer solution plus 2 mM $CaCl_2$. Then, the Ca^{2+}-dependent Fluo-3 fluorescence was measured in FL-1 on a FACS-Calibur.

Mitochondrial membrane potential. The DePsipher Kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) was used to measure the mitochondrial membrane potential in a flow cytometry assay according to the manufacturer's instructions.

RT-PCR. Total RNA (1-2 µg) from 10 million K562 cells was isolated and pooled using Tri Reagent (Biozol, Eching, Germany). The RNA was reverse transcribed with the Super Script II kit from Invitrogen (Karlsruhe, Germany). cDNA was precipitated, washed, diluted in H_20, and stored at -80 ℃. The primers used are listed in Table 1.

Table 1. PCR primers

template	accession number	sense 5' - 3'	position	antisense 5' - 3'	position	fragment [bp]
TRPC3	NM_003305	GTTGTGGAATG TGCTTGACT	1825-1844	TGAAAGGTGGA GGTAATGTT	2527-2546	721
TRPC7	NM_020389	ACCGAGGTGCC AAATACAAC	1811-1830	GCGAACTTCCA TTCCACATC	2050-2069	259
TRPM2	NM_003307	TCGGACCCAAC CACACGCTGTA	4125-4146	CGTCATTCTGG TCCTGGAAGTG	4442-4463	338
TRPM7	NM_017672	TGAAGCAAAGC AGAGTGACCT	2230-2350	TATTCAGCCTT CCCATCCAC	2476-2495	266
ACCN2	NM_020039	ACCGAGGTGCC AAATACAAC	879-896	GCGAACTTCCA TTCCACATC	1130-1151	272

PCR was performed using the TaKaRa LA Taq polymerase (TAKARA BIO Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions. Briefly, one sample contained in a total volume of 50 µl: 0.5 µl TaKaRa LA Taq polymerase (2.5 units), 5 µl 10X LA PCR Buffer II (Mg^{2+} free), 5 µl 25 mM $MgCl_2$, 8 µl dNTP mixture (2,5 mM each), 1 µl sense primer (50 µM), 1 µl antisense primer (50 µM), and sterilized distilled water up to 50 µl. The PCR conditions used in this study are summarized in Table 2.

Table 2. PCR experimental conditions

template	denaturing	Annealing	extension	Number of cycles
TRPC3	94 ℃ for 30 sec.	57 ℃ for 30 sec.	72 ℃ for 30 sec.	35
TRPC7	94 ℃ for 60 sec.	56 ℃ for 60 sec.	72 ℃ for 120 sec.	35
TRPM2	94 ℃ for 30 sec.	57 ℃ for 30 sec.	72 ℃ for 30 sec.	35
TRPM7	94 ℃ for 45 sec.	56 ℃ for 60 sec.	72 ℃ for 60 sec.	30
ACCN2	94 ℃ for 45 sec.	56 ℃ for 60 sec.	72 ℃ for 60 sec.	30

After amplification, PCR products were analyzed using ethidium bromide-stained agarose gels and the PCR products were verified by sequencing.

siRNA based gene silencing. siRNA expression cassettes were prepared in a PCR-based method with the *Silencer* ™ Express Kit from Ambion (Cambridgeshire, UK) according to the manufacturer's instructions. Precursor SEC were amplified based on Human H1 promoter from two template oligonucleotide primers. The PCR-primers are summarized in Table 3.

Table 3. PCR primers for siRNA constructs

siRNA	accession number	sense primer	antisense primer	position
TRPC7a	NM_020389	AAG CTA CAC AAA CTT AAA TTC AGT CTC AAT GCC GGT GTT TCG TCC TTT CCA CAA G	CGG CGA AGC TTT TTC CAA AAA ACA TTG AGA CTG AAT TTA AGC TAC ACA AAC TTA	762-780
TRPC7c	NM_020389	ATG CTA CAC AAA CAT GAA CTT GAA GAT ATC TCG GTG TTT CGT CCT TTC CAC AAG	CGG CGA AGC TTT TTC CAA AAA AAG ATA TCT TCA AGT TCA TGC TAC ACA AAC ATG	1731-1749
TRPC7negative	NM_020389	AGA CTA CAC AAA TCT TTT CAC ATT GGA GAT TCG GTG TTT CGT CCT TTC CAC AAG	CGG CGA AGC TTT TTC CAA AAA AAA TCT CCA ATG TGA AAA GAC TAC ACA AAT CTT	-
ACNN2	NM_020039	ATA CTA CAC AAA TAT GTA TTG CTC AGA TTT GCC GGT GTT TCG TCC TTT CCA CAA G	CGG CGA AGC TTT TTC CAA AAA ACA AAT CTG AGC AAT ACA TAC TAC ACA AAT ATG	1416-1432

K562 cells were transfected with siRNA expression cassettes in RPMI 1640 medium + L-Glutamine (Gibco) supplemented with 10% FCS and 1% penicillin/streptomycin in a 37℃, 5% CO_2 incubator using the siPORT XP-1 transfection agent from Ambion. Briefly, 1 µg of the respective siRNA Expression Cassette and 7 µl siPORT XP in 200 µl Opti Mem Medium (Gibco)

were added to 50,000 K562 cells in 1 ml RPMI 1640 medium + L-glutamine supplemented with 10 % FCS and 1% penicillin/streptomycin. The cells were incubated for 72 h to downregulate the desired gene product.

Immunoblotting. To study the efficacy of TRPC7 and ACCN2 channel downregulation, K562 cells transfected with siRNA expression cassettes and control cells were incubated for 72 h at 37℃. Cells were harvested and washed once with PBS and then solubilized in 125 mM NaCl, 25 mM HEPES/NaOH (pH 7.4), 10 mM EDTA, 10 mM Na-pyrophosphate, 10 mM NaF, 0.1% SDS, 0.5% deoxycholic acid, 1% Triton X-100, and 10 µl β-mercaptoethanol. The protein concentration of the samples was determined using the Bradford method (Biorad, Munich, Germany) with bovine serum albumin (Sigma) as standard. Equal amounts of lysate protein (50 µg per lane) were separated by 8% or 10% SDS-PAGE, and proteins were transferred to Protan BA83 nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany). After blocking with 5% non-fat milk at room temperature for 1 h, or overnight at4 ℃, the blots were probed overnight at 4℃ with a commercial polyclonal goat anti-human TRPC 3/6/7 (A-15) antibody against the C-Terminus (Santa Cruz, Heidelberg, Germany; 1:500 dilution in PBS, 0.1% Tween 20, 5% non-fat milk) or a polyclonal goat anti-human TRPC 3/6/7 (N-18) antibody against the N-Terminus (Santa Cruz Biotechnology, Inc.; 1:500 dilution in PBS, 0.1% Tween 20, 5% non-fat milk). Further blots were probed for 2 h at room temperature with a commercial rabbit anti-rat ASIC1 (AN-02) antibody directed against the C-Terminus (Alomone Labs., Jerusalem, Israel; 1:200 dilution in 5% non-fat milk). After washing, the blots were incubated with a secondary donkey anti-goat antibody (1:2000 dilution) or anti-rabbit antibody (1:1000), conjugated with horseradish peroxidase (Amersham, Freiburg, Germany) for 1 h at room temperature. After washing, antibody binding was detected with the enhanced chemiluminescence (ECL) kit from Amersham (Freiburg, Germany).

DNA fragmentation. Cytoplasmic histone-associated DNA fragments were quantified by a photometric enzyme immunoassay (Cell Death Detection

ELISA) from Roche Diagnostics (Basel, Switzerland) according to the protocol supplied by the manufacturer and as described and evaluated in a previous study (Wieder et al., 1998).

Statistics. Data were expressed as means ± SE. Statistical analysis was made by ANOVA or unpaired two-tailed t-test, as appropriate.

2.2 *Identification of the erythrocyte cation channel participating in erythrocyte apoptosis and basal cation leak*

Preparation of erythrocyte ghosts. Pelleted human erythrocytes (70 µl) were lysed in a hypotonic buffer containing 10 mM Hepes/K pH 7.4, 30 mM KCl, 1 mM $MgCl_2$, 3 mM Mg-ATP, 0.5 mM EGTA, and 2 µM Fluo-3/AM (Calbiochem; Bad Soden, Germany). As indicated, the buffer contained in addition TRPC 3/6/7 (N-18) antibody against the N-Terminus (Santa Cruz Biotechnologie, Inc.; 1 : 200 dilution), the blocking peptide of TRPC 3/6/7 (N-18) antibody (Santa Cruz Biotechnologie, Inc.; 1:200 dilution), TRPC6-antibody (Alomone Labs, Jerusalem, Israel, 1:200 dilution), the blocking peptide of TRPC6 antibody (1:100 dilution), TRPC3-antibody (Alomone Labs, Jerusalem, Israel, 1:200 dilution), the blocking peptide of TRPC3 antibody (1:100 dilution) or dithiothreitol (DTT; 1 mM). The non-lysed cells were separated by centrifugation with 2000 rpm (RT, 3 min), and the erythrocyte ghosts in the supernatant were spinned down with 17000 rpm (4℃, 20 min). The supernatant was discarded, and the erythrocyte ghosts were resuspended in 2 mM $CaCl_2$-containing NaCl Ringer (in mM: 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 HEPES/NaOH pH 7.4, 5 glucose, 2 $CaCl_2$) at t = 0min. Then, the Ca^{2+}-dependent Fluo-3 fluorescence was measured in FL-1 on a FACS-Calibur in the presence or absence of amiloride (1 mM) or ethylisopropylamiloride (EIPA; 50 µM), as indicated.

Erythroid progenitor cell enrichment. Human burst forming units of erythropoiesis (BFU-E) were purified from peripheral blood obtained from healthy volunteers as described recently (Jacobs-Helber et al., 1998; Wickrema et al., 1999). Whole blood (500 ml) was separated over Ficoll-Hypaque (1.077 g/ml) to obtain mononuclear cells. Platelets were depleted by washing the cells and spinning down twice at 700 g, followed by adherent cell depletion in polystyrene tissue culture flasks. The cell population was enriched for BFU-E by negative selection with MACS™ antibody-coated paramagnetic microbeads (Miltenyi Biotec, Inc., Auburn, CA) bearing CD3, CD11b, CD15, and CD45-RA.

The purified BFU-E cells were cultured for 7 or 8 days (in 15% fetal calf serum, 15% human AB serum, Isocove's modified Dulbecco's medium, 1% penicillin/streptomycin,, 10 ng/ml interleukin-3, 2 units/ml erythropoetin, 50 units/ml insulin-like growth factor-1, 50 ng/ml stem cell factor, 0.8% methylcellulose) to obtain a highly enriched population of erythroid progenitors that were at the colony forming unit of erythropoiesis (CFU-E) stage. Messenger RNA expression of erythroid progenitor cells was assessed by microarray analysis (Amplichip CYP450, Affymetrix; Wycombe, UK).

Phosphatidylserine (PS) exposure. PS in the outer membrane leaflet was determined in FACS analysis. Erythrocytes were incubated for 24 h at 37℃ in NaCl Ringer solution (in mM: 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 HEPES/NaOH pH 7.4, 5 glucose, 1 $CaCl_2$). Cells were washed in annexin-V-binding buffer containing (in mM) 125 NaCl, 10 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4), and 5 $CaCl_2$. Cells were suspended in a solution composed of Annexin-V-Fluos (Roche Diagnostics, Mannheim, Germany) and annexin-V buffer (dilution of 1:50). After 20 minutes of incubation at 37℃, the annexin-V binding to PS-exposing cells was assessed by the annexin-V-fluorescence intensity as measured in FL-1 on a FACS-Calibur (Becton and Dickinson, Heidelberg, Germany).

Cytosolic free Ca^{2+}-concentration. For measurement of intracellular Ca^{2+} activity, mouse erythrocytes were washed in NaCl Ringer solution and then loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in NaCl Ringer solution containing 2 µM Fluo-3/AM. The cells were incubated at 37 ℃ for 15 min under shaking and washed twice in 2 mM $CaCl_2$-containing NaCl Ringer solution. For flow cytometry, Fluo-3/AM-loaded erythrocytes were resuspended in 5 ml Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1.

RT-PCR. Total RNA (1-2 µg) from erythroid progenitor cells was isolated and pooled using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA

was reverse transcribed with the Super Script II kit from Invitrogen (Karlsruhe, Germany). cDNA was precipitated, washed, diluted in H_20, and stored at -80 ℃. PCR was performed using the TaKaRa LA Taq polymerase (TAKARA BIO Inc., Otsu, Shiga, Japan) according to the manufacturer's instructions. Briefly, one sample contained in a total volume of 50 µl: 0.5 µl TaKaRa LA Taq polymerase (2.5 units), 5 µl 10X LA PCR Buffer II (Mg^{2+} free), 5 µl 25 mM $MgCl_2$, 8 µl dNTP mixture (2,5 mM each), 1 µl sense primer (50 µM), 1 µl antisense primer (50 µM) (sense primer: TGGATTTCTGAGCTGTTCCA; antisense primer: CCCTTCGTTCACTTCATCACT), and sterilized distilled water up to 50 µl. The PCR conditions were: 1 min at 94℃, 1 min at 57℃, 1 min at 72℃, for 35 cycles, and then final 5 min at 72℃. After amplification, PCR products were analyzed using ethidium bromide-stained agarose gels and the PCR products were verified by sequencing.

Western blotting. Human erythrocytes were hemolyzed in 20 mM HEPES/NaOH (pH 7.4) containing a cocktail of protease inhibitors (Roche, Mannheim, Germany). Ghosts membranes were pelleted (15,000 g for 20 min at 4℃) and lysed in (mM) 125 NaCl, 25 HEPES/NaOH (pH 7.3), 10 EDTA, 10 Na-pyrophosphate,10 NaF, 0.1% SDS, 0.5% deoxycholic acid, 1% triton-X, and 10 µl 2-mercaptoethanol. Lysates were separated by 10% SDS-PAGE and blotted on nitrocellulose membranes. After blocking, the blots were probed overnight at 4℃ with a polyclonal rabbit anti-TRPC6 peptide antibody (Alomone Labs, Jerusalem, Israel; 1:200 dilution in 1% BSA) raised against the residues 24-38 of mouse TRPC6. After washing, the blots were incubated with secondary anti-rabbit antibody (1:1000 dilution), conjugated with horseradish peroxidase (Amersham, Freiburg, Germany) for 1 h at room temperature and antibody binding was detected with enhanced chemiluminescence (ECL) kit (Amersham, Freiburg, Germany). For Western blots in mouse erythrocytes a rabbit polyclonal anti TRPC6 antiserum (861) was generated and affinity-purified. Specificity of antibodies was confirmed using microsomal membrane protein fractions (up to 150 µg per lane) from WT mice and mice deficient in TRPC6 as

well as from non-transfected or mouse TRPC6-transfected COS cells. Anti-β-actin antibody was used as control for protein loading.

3 Results

3.1 Investigation of PGE_2 triggered apoptosis and the cation channel involved in human leukaemia K562 cells

To test whether PGE_2 increases the cytosolic free Ca^{2+} concentration of K562 cells, Fluo-3 fluorescence measurements have been performed in cells incubated for 48 hours either in the absence or presence of PGE_2 (50 µM). As illustrated in Fig. 6, the incubation in PGE_2 increased significantly the Fluo-3 fluorescence, pointing to an increase of cytosolic free Ca^{2+} concentration (Fig. 6A, B).

K562 cells express Ca^{2+}-activated hSK4 K^+ channels (Lang et al., 2003d) suggesting that elevated cytosolic Ca^{2+} activity triggers the loss of KCl and osmotically obliged water and thus leads to cell shrinkage, which should be apparent from a decrease of forward scatter in FACS analysis. PGE_2 (50 µM for 48 h) indeed decreased cell volume as determined by a significant decrease of forward scatter (Fig. 6C, D). The effect was dependent on the PGE_2 concentration (Fig. 6D).

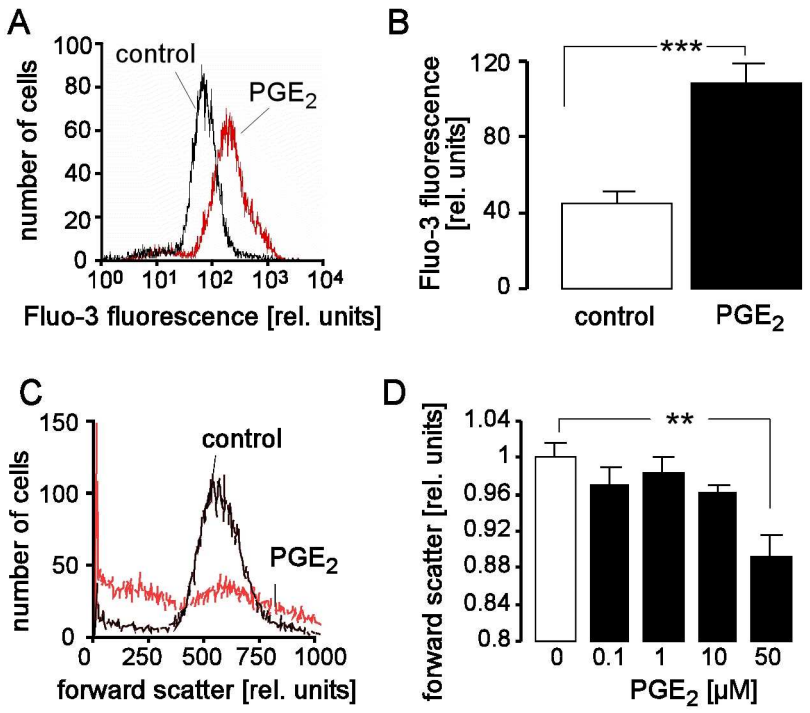

Fig. 6. PGE_2 increases cytosolic free Ca^{2+} concentration ($i[Ca^{2+}]_{free}$) and decreases cell volume.
A. FACS histogram of Fluo-3 fluorescence as a measure of $i[Ca^{2+}]_{free}$ as recorded from control (black line) and PGE_2 (50 µM for 48 h)-stimulated K562 cells.
B. Mean (± SE, n=9-12) Fluo-3 fluorescence (as calculated from the geometrical means of the histograms) recorded in control cells (open bar), and in PGE_2 (50µM for 48h)-stimulated cells (closed bar).
C. Forward scatter distribution in FACS histograms of control (black line) and PGE_2 (50 µM for 48h)-stimulated cells.
D. Mean forward scatter (± SE; n=5-6; geometrical mean of forward scatter) of control K562 cells (open bar) and cells stimulated for 48 h with increasing concentrations of PGE_2 (closed bars; **: $p \leq 0.01$; ANOVA).

As illustrated in Fig. 7, PGE_2 (50 µM for 48h) led to a significant increase of DePsipher fluorescence, thus pointing to depolarization of the mitochondrial membrane potential.

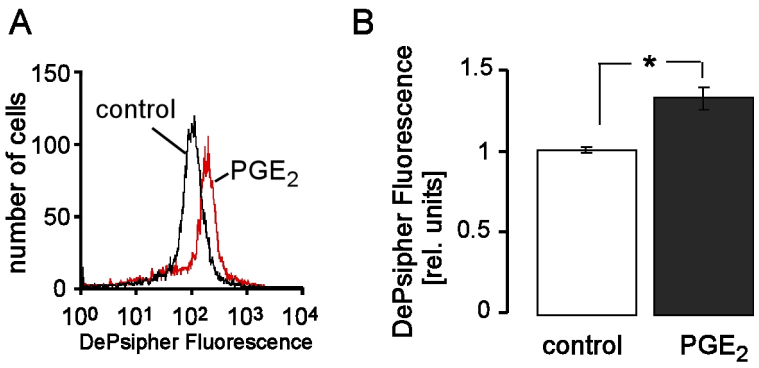

Fig. 7. PGE_2 decreases the mitochondrial membrane potential.
A. FACS histogram of DePsipher fluorescence as a measure of mitochondrial activity recorded from control (black line) and PGE_2 (50 µM for 48 h)-stimulated K562 cells (red line).
B. Mean (± SE, n=5) DePsipher fluorescence (as calculated from the geometrical means of the histograms) recorded in control cells (open bar), and in PGE_2 (50µM for 48h)-stimulated cells (closed bar).

To elucidate, whether those effects result in apoptotic cell death, total caspase activity was determined in K562 cells by the use of a fluorescent caspase substrate. The potent caspase activator carboplatin was applied as a positive control. As shown in Fig. 8, PGE_2 (50 µM) triggered within 48 h an about two-fold increase in caspase activity. For comparison, carboplatin (40 µg/ml) led within 48 h to an about 5-fold caspase activity (Fig. 8C).

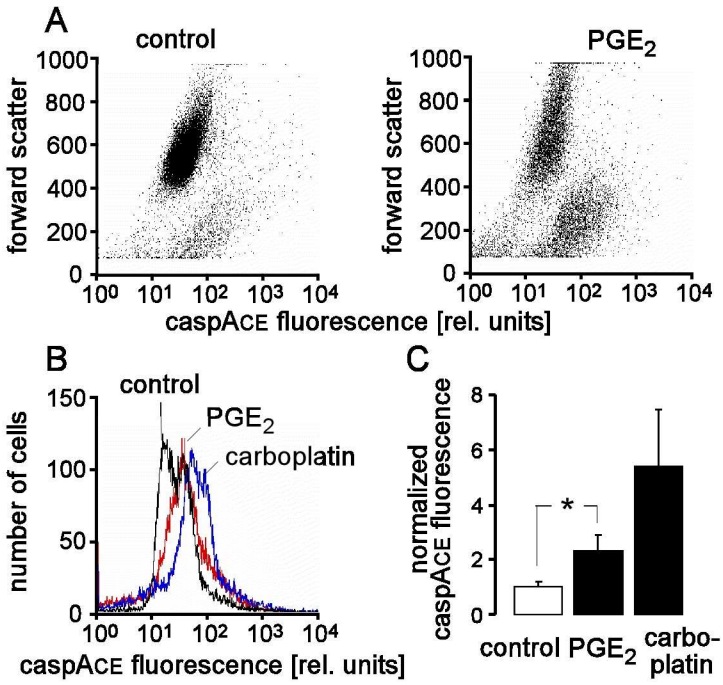

Fig. 8. PGE_2 activates caspases in K562 leukaemic cells.

A. Two-dimensional flow cytometry histograms in FACS analysis of control (left) and PGE_2-stimulated cells (50 µM for 48 h; right). The plots show the relationship between cell forward scatter and caspAce fluorescence as a measure of cell volume and caspase activity, respectively.
B. FACS histogram of the caspAce fluorescence intensity recorded in control cells (black line), in PGE_2- (50 µM for 48 h; red line), or in carboplatin-stimulated cells (40 µg/ml for 48 h; blue line).
C. Mean caspAse activity (± SE; n=6; *: $p \leq 0.05$; ANOVA) of cells incubated as in (B) in the absence or presence of PGE_2 or carboplatin.

Furthermore, PGE_2 (48 h) dose-dependently induced the breakdown of the phospholipid asymmetry in the plasma membrane as analyzed by annexin-V binding to externalized phosphatidylserine (PS) in fluorescence microscopy (Fig. 9A) and flow cytometry (Fig. 9B, C). A significant increase of annexin binding was observed at a PGE_2 concentration of 1 µM (Fig. 9C). In erythrocytes the PGE_2 activated cation channels are inhibited by ethylisopropylamiloride (EIPA) (Lang et al., 2005b). Therefore, additional experiments have been performed to determine the EIPA sensitivity of PGE_2-induced PS exposure of K562 cells. To this end, the cells were incubated for 48 h with PGE_2 (50 µM) in the presence or absence of EIPA (50 µM). As shown in Fig. 9D and E, EIPA blunted the PGE_2 effect on PS exposure by some 40 %.

Taken together, the data suggest the stimulation of K562 apoptosis by PGE_2 in an EIPA-sensitive manner.

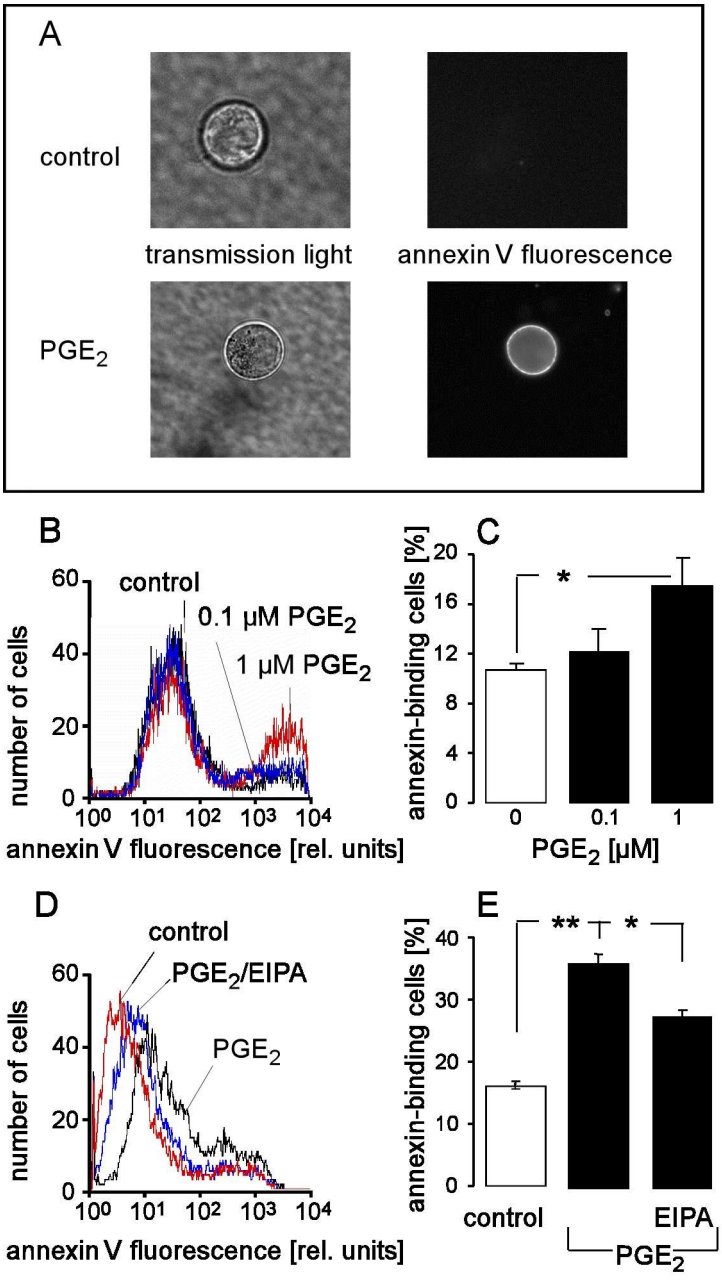

Fig. 9. PGE_2 triggers phosphatidylserine (PS) exposure.
A. Light micrograph (left column) and corresponding immunfluorescence (right column) showing PS exposure of control (upper row) and PGE_2 (50 µM for 48 h; lower row)-stimulated K562 cells. PS exposure is indicated by binding of the fluorescent annexin-V fluos.
B. FACS histogram showing annexin-V fluos binding of K562 cells stimulated for 48 h without (control; black), or with 0.1 µM (blue), and 1 µM of PGE_2 (red).

C. Mean percentage (± SE; n=9) of annexin-binding cells treated as in (B) with increasing concentrations of PGE_2 (*: $p \leq 0.05$; ANOVA).
D, E. Effect of EIPA (50 µM) on the PGE_2 (50 µM)-stimulated annexin-V binding in K562 cells. Annexin-V binding (FACS histogram in D) and mean percentage (± SE; n=6) of annexin-V-binding K562 cells (bar diagram in E) grown for 48 h in the absence (D, control, red line; E, open bar) and presence of PGE_2 alone (D, black line; E, middle) or in the presence of PGE_2 and EIPA (D, blue line; E, right; **: $p \leq 0.01$, *: $p \leq 0.05$; ANOVA).

To screen for possible candidates of Ca^{2+}-permeable cation channels, DNA fragments specific for the classical short (TRPC) and the melastatin long (TRPM) subfamilies of transient receptor potential channels (Clapham, 2003) were amplified from K562 cDNA by RT-PCR. As confirmed by sequencing, K562 cells expressed TRPC3, -C7, and -M7 mRNA (data not shown). In addition, mRNA specific for the amiloride-sensitive sodium-selective ACCN2 channel was detected (data not shown). To define the functional significance of TRPC3 and 7 for the PGE_2-stimulated apoptosis in K562 cells, these channel subtypes were down-regulated by RNA interference. In addition, ACCN2 was silenced (negative control). Down-regulation of ACCN2 or TRPC7 by one or two constructs (siTRPC7a and -c), respectively, was monitored by immunoblotting using an ACCN2-specific (Fig. 10A) or two TRPC3/6/7-specific antibodies (one directed to the N terminus and the second directed to the C terminus, (Fig. 10B). The construct siTRPC7c decreased TRPC3/6/7 protein expression by 33% (Fig. 10C).

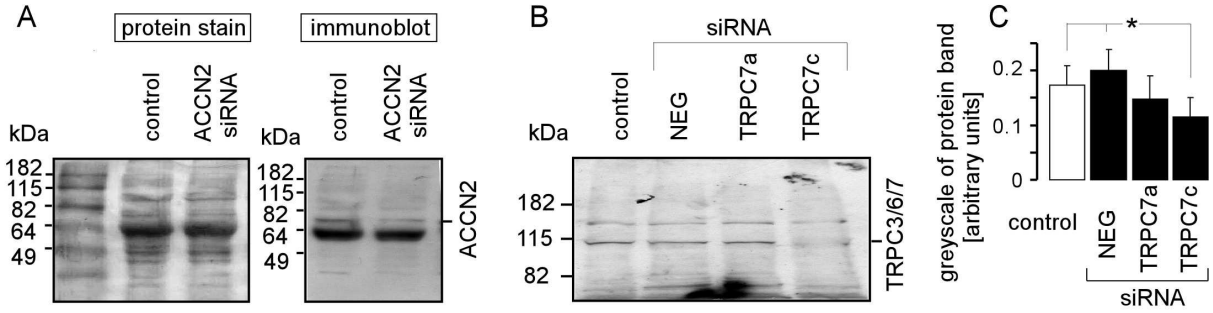

Fig. 10. ACCN2 and TRPC7 RNA interference downregulate ACCN2 and TRPC7 protein, respectively.
A. Ponceau red staining of blotted proteins (left) and immunoblot (right) probed against ACCN2 protein (right) following 72 h incubation in the absence (vehicle control; left lanes) or presence of ACCN2-specific siRNA (construct in Table 3; right lanes).

B, C. Immunoblot of TRPC3/6/7 protein expression (B) and quantitative analysis (C) of TRPC3/6/7 protein expression following 72 h treatment with TRPC7-specific siRNA (constructs a and c in Table 3), with vehicle (control), or with a non-specific construct (NEG). The nonspecific band at about 150 kDa in (A) indicates equal protein loading. Data in (C) are means ± SE (n= 4-6; *: p≤0.05, two-tailed paired t-test).

As illustrated in Fig. 11, siTRPC7 treatment significantly inhibited PGE_2 (50 µM for 48 h)-induced PS exposure of K562 cells by 65 % (Fig. 11B, C). In contrast, the construct directed against ACCN2 did not affect PGE_2-induced PS exposure (Fig. 11A, C). A nonsense construct (siNEG) which was used as further negative control was similarly not capable to significantly interfere with PGE_2-induced PS exposure (Fig. 11C).

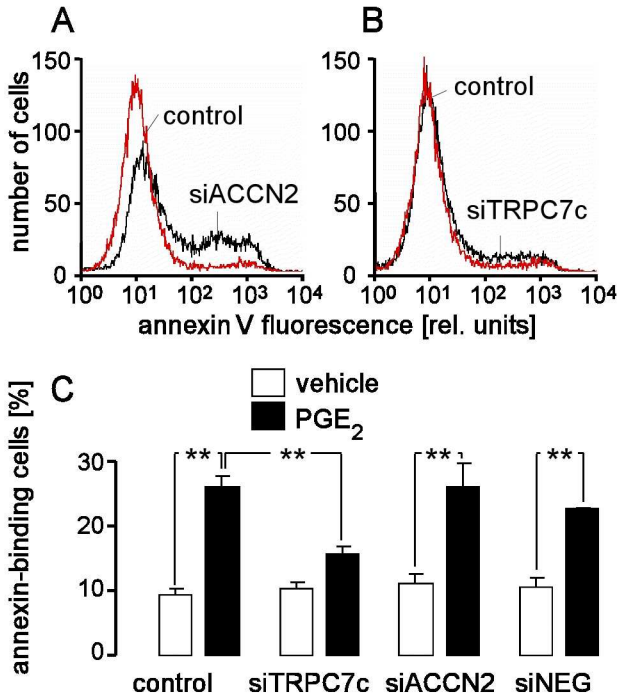

Fig. 11. TRPC7 RNA interference blunts PGE_2-stimulated PS exposure.
A, B. FACS histograms showing annexin-V fluos binding of K562 cells stimulated for 48 h with PGE_2 (50 µM) in the absence (A, B, control; red lines) or presence of ACCN2 (A, black line) and TRPC7c siRNA (B, black line).
C. Mean percentage of annexin-V binding cells cultivated for 48 h in the absence (vehicle; open bars) or presence of PGE_2 (50 µM; closed bars). Cells were coincubated without (control) or with siRNAs (as indicated). Data are means ± SE (n = 4-6; **,*: p ≤ 0.01 and 0.05, respectively; ANOVA).

Downregulation of TRPC7 further blunted PGE_2 (50 µM for 48 h)-induced DNA-fragmentation significantly by 53% as compared to the nonsense construct siNEG (Fig. 12).

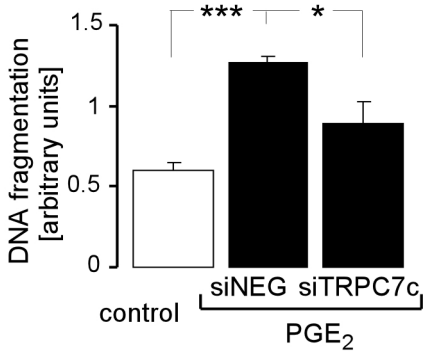

Fig. 12. TRPC7 RNA interference blunts PGE_2-stimulated DNA fragmentation.
DNA fragmentation of non-stimulated (control) and PGE_2 (50 µM for 48h)-stimulated K562 cells was assessed by quantitative determination of cytoplasmic histone-associated DNA fragments using a photometric enzyme immunoassay. PGE_2-stimulated cells were either treated with TRPC7-specific siRNA (siTRPC7) or with a non-specific construct (siNEG). Data are means ± SE (n=3; *, ***: $p \leq$ 0.05 and 0.001, respectively; ANOVA).

In summary, the present study provides several lines of experimental evidence for triggering of K562 cell apoptosis by PGE_2 through activation of TRPC7 cation channels, subsequent increase in intracellular Ca^{2+} activity, cell shrinkage, mitochondrial depolarization, caspase activation, DNA fragmentation, and phosphatidylserine exposure.

After gainig evidence of functional significance of TRPC7 for PGE_2 – induced apoptosis in K562 cells the role of TRP channels for programmed cell death of erythrocytes had to be elucidated.

3.2 Identification of the erythrocyte cation channel participating in erythrocyte apoptosis and basal cation leak

To explore, whether TRP channels are expressed in erythroid progenitor cells, human haematopoietic stem cell progenitors were prepared from peripheral blood (negative selection for CD3, CD11b, CD15, and CD45-RA and positive selection for CD71) and differentiated to erythroid progenitors (Fig. 13A-C). These cells expressed mRNAs of erythrocyte markers such as α-globin, β-globin or, CD71 (Fig. 13D). In addition, RT-PCR revealed transcript levels of the transient receptor potential C6 (TRPC6) channel (Fig. 13E). Since TRPC6 generates a Ca^{2+}-permeable non-selective cation channel, TRPC6 might be a candidate for the erythrocyte cation channel. Moreover, a commercial polyclonal antibody raised against a peptide specific for the N-terminus of the TRPC6 protein bound to a ~100 kD band in white membranes prepared from human erythrocytes (Fig. 13F, left lane). No band was seen when the anti-TRPC6 antibody was pre-adsorbed and co-incubated with the immunizing peptide (Fig. 13F, right lane).

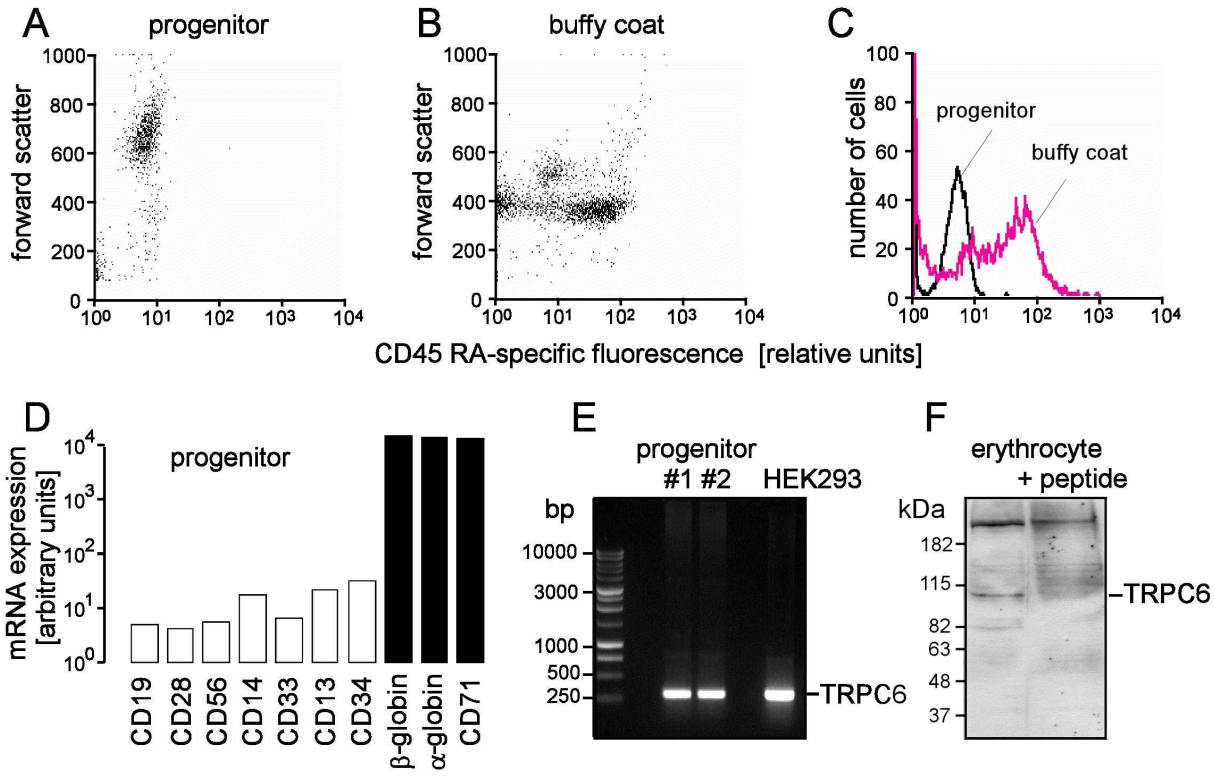

Fig. 13. TRPC6 expression in human erythroid progenitor cells and human erythrocytes.

A-D. Differentiation of human erythroid progenitors. Dot blot (A) and histogram (C) indicating that the erythroid progenitors were negative for CD45-RA, a marker of T lymphocytes, NK cells, B lymphocytes, monocytes, macrophages, and granulocytes. The dot blot of (B) shows the CD45-RA expression of buffy coat used as a positive control. Erythroid progenitors express erythrocyte markers such as α-globin, β-globin, and CD71 (D) as analyzed by microarray.
E-F. mRNA expression (E) in erythroid progenitors and (F) protein expression in human erythrocytes of the transient receptor potential C6 (TRPC6) non-selective cation channel. The gel in (E) shows two individual progenitor cell preparations and HEK cells as a positive control. The left and the right lane of the immunoblot in (F) were probed with anti-TRPC6 antibody (left), or antibody pre-adsorbed and co-incubated with the immunizing peptide (right).

Further experiments have been performed in erythrocytes from mice lacking functional TRPC6 ($TRPC6^{-/-}$) and their wildtype littermates ($TRPC6^{+/+}$). TRPC6 protein was detected in erythrocytes from $TRPC6^{+/+}$ but not from $TRPC6^{-/-}$ mice by Western blotting using the mTRPC6-specific antibody 861 (Fig. 14A-C). The lack of TRPC6 protein in the mouse $TRPC6^{-/-}$ erythrocyte membrane was not compensated by upregulation of TRPC3 (Fig.14D,E) as demonstrated for TRPC6-deficient smooth muscle cells (Dietrich et al., 2005b).

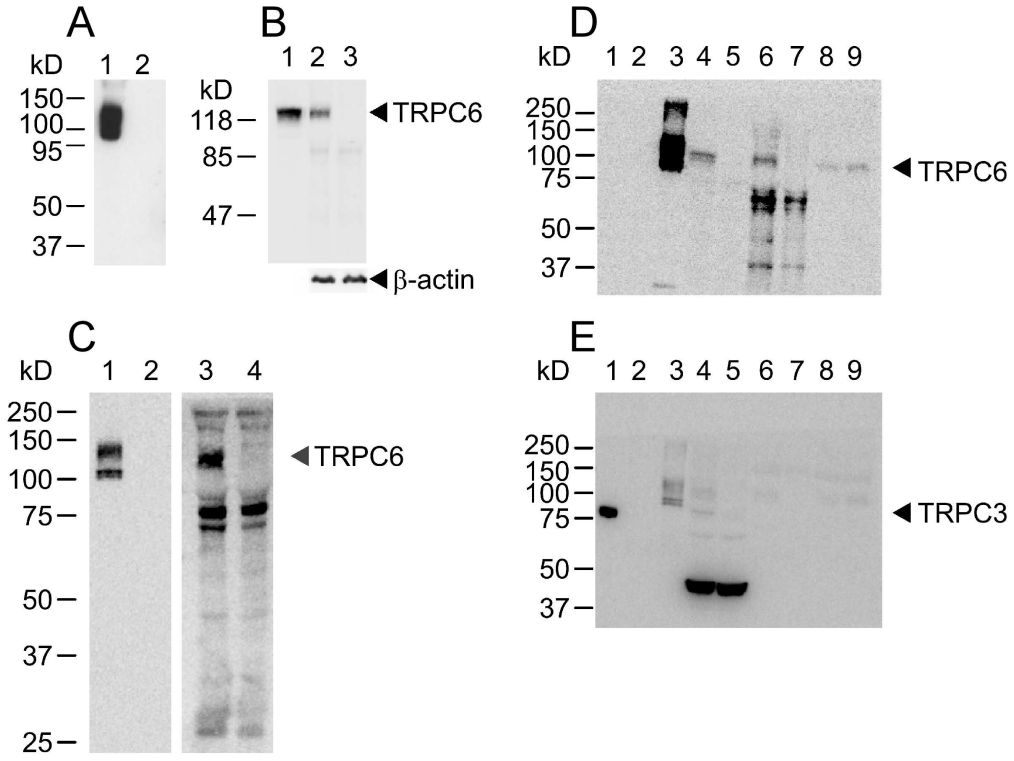

Fig. 14. TRPC6 protein expression in mouse erythrocytes.

A-C. Specificity of the anti-TRPC6 antibody 861 and immunodetection of the TRPC6 protein in mouse erythrocytes. (A) Western blots of cell lysates from mouse TRPC6-transfected (lane 1) and non-transfected COS 7 cells (lane 2) and (B) of microsomal membrane protein fractions from lung (lane 1), erythrocytes (lane 2), and TRPC6$^{-/-}$ erythrocytes (lane 3) using the mTRPC6-specific antibody 861. (C) Similar experiment as in (A) and (B) using independently prepared cell lysates from mouse TRPC6-transfected (lane 1) and non-transfected COS 7 cells (lane 2) and of microsomal membrane protein fractions from wildtype erythrocytes (lane 3) and TRPC6$^{-/-}$ erythrocytes (lane 4, 150 µg). The antibody recognizes at least two TRPC6 variants in COS cells transfected with the mTRPC6 cDNA (lanes 1 in A and C); like in TRPC6 expressing HEK cells (Dietrich et al., 2003) these variants apparently represent TRPC6 proteins differing in their glycosylation patterns. No TRPC6 protein is detected in non-transfected COS 7 cells (lanes 2 in A and C). In microsomal membrane protein fractions from wildtype mouse lung (B, lane 1) and wildtype erythrocytes (B, lane 2 and C, lane 3) the antibody recognizes the TRPC6 protein, which is absent in TRPC6$^{-/-}$ erythrocytes (B, lane 3 and C, lane 4).
D,E. Expression of the TRPC3 protein is not detectable in wildtype erythrocytes or TRPC6$^{-/-}$ erythrocytes. Microsomal membrane proteins from skeletal muscle (lane 1), non-transfected COS cells (lane 2), TRPC6-transfected COS cells (lane 3), wildtype lung (lane 4), TRPC6$^{-/-}$ lung (lane 5), wildtype erythrocytes from three additional and independent preparations (lanes 6, 8 and 9), and from TRPC6$^{-/-}$ erythrocytes (lane 7) were blotted and the TRPC6 protein visualized by the anti-TRPC6 antibody (D). (E) This blot was stripped and reused using the anti-TRPC3 antibody FP306 described in (Philipp et al., 2003). Proteins of the size of TRPC3 are readily detected in skeletal muscle (lane 1) but not in other tissues (lanes 2 to 9). The pattern of the faint bands in (E) resemble the staining patterns in (D) and represent remaining anti-TRPC6 antibodies not stripped off the blot. Amount of microsomal membrane proteins applied per lane were 10 µg (skeletal muscle) and 150 µg (all others); percentage of SDS-PAGE was 7% (A, B,D,E) and 8.5% (C) (The data presented in this figure has been generated in the laboratory of Prof. Veit Flockerzi and Prof. Marc Freichel, University of Saarland, Germany.)

To study functional expression of TRPC6 in human erythrocytes, ghosts were prepared from human erythrocytes by hypoosmotic hemolysis. The ghosts maintained a redox-sensitive and EIPA- or amiloride-inhibited Ca^{2+} influx as measured by Fluo3 fluorescence in flow cytometry (Fig. 15).

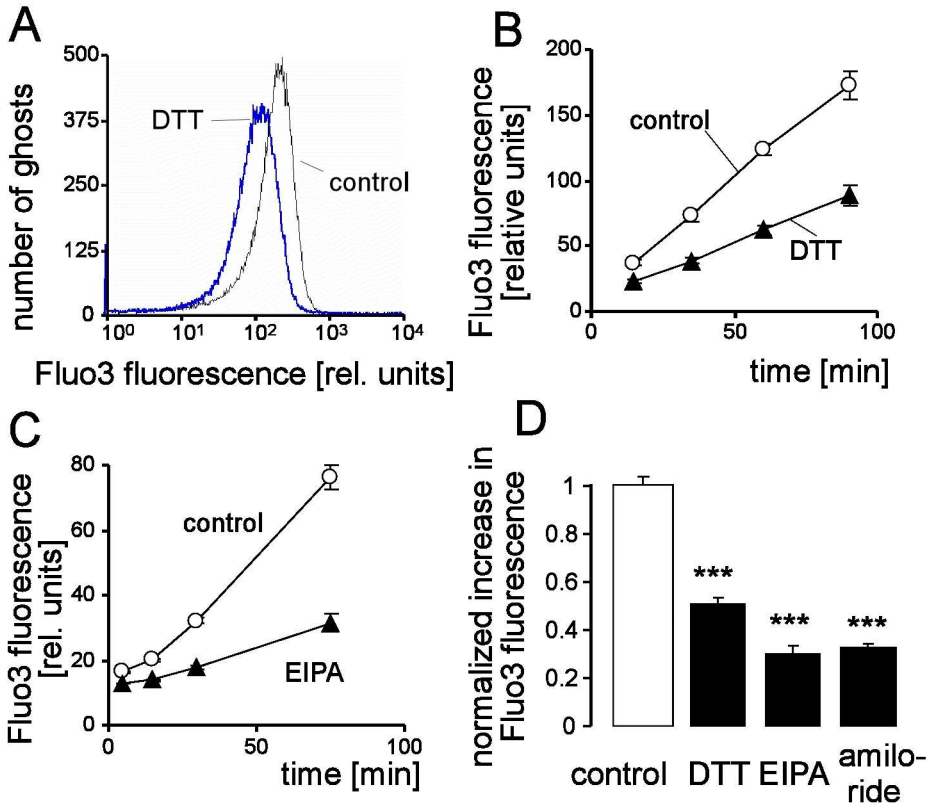

Fig. 15. Cation channel activity in ghosts prepared from human erythrocytes.
A. Histogram showing the Fluo3 fluorescence in human ghosts as a measure of the cytosolic free Ca^{2+} concentration. Ghosts were prepared either in the absence (control) or presence of dithiothreitol (DTT; 1 mM) and incubated for 90 min in Ca^{2+}-containing medium.
B-C. Time-dependence of the mean Fluo3-fluorescence (± SE; n = 3) of ghosts incubated in Ca^{2+}-containing medium under control conditions (B,C, open circles), in the presence of intracellular DTT (1 mM; B, closed triangles) or extracellular EIPA (50 µM; C, closed triangles).
D. Mean normalized increase in Fluo3 fluorescence (± SE; n = 6) of ghosts incubated as in (A-C) in the absence (open bar) or presence (closed bars) of intracellular DTT (1 mM) or extracellular EIPA (50 µM) or amiloride (1 mM) (***: $p \leq 0.001$).

Thus, cation channel function was preserved in the ghost membrane. Incorporation of a non-specific antibody directed against TRPC3/6/7 or a specific antibody against TRPC6 but not incorporation of these antibodies pre-adsorbed with the respective immunizing peptides or of antibodies directed against TRPC3 or TRPM2 blunted the Ca^{2+} uptake by human ghosts (Fig. 16). This strongly suggests that TRPC6 contributes to the cation leak through the human erythrocyte membrane.

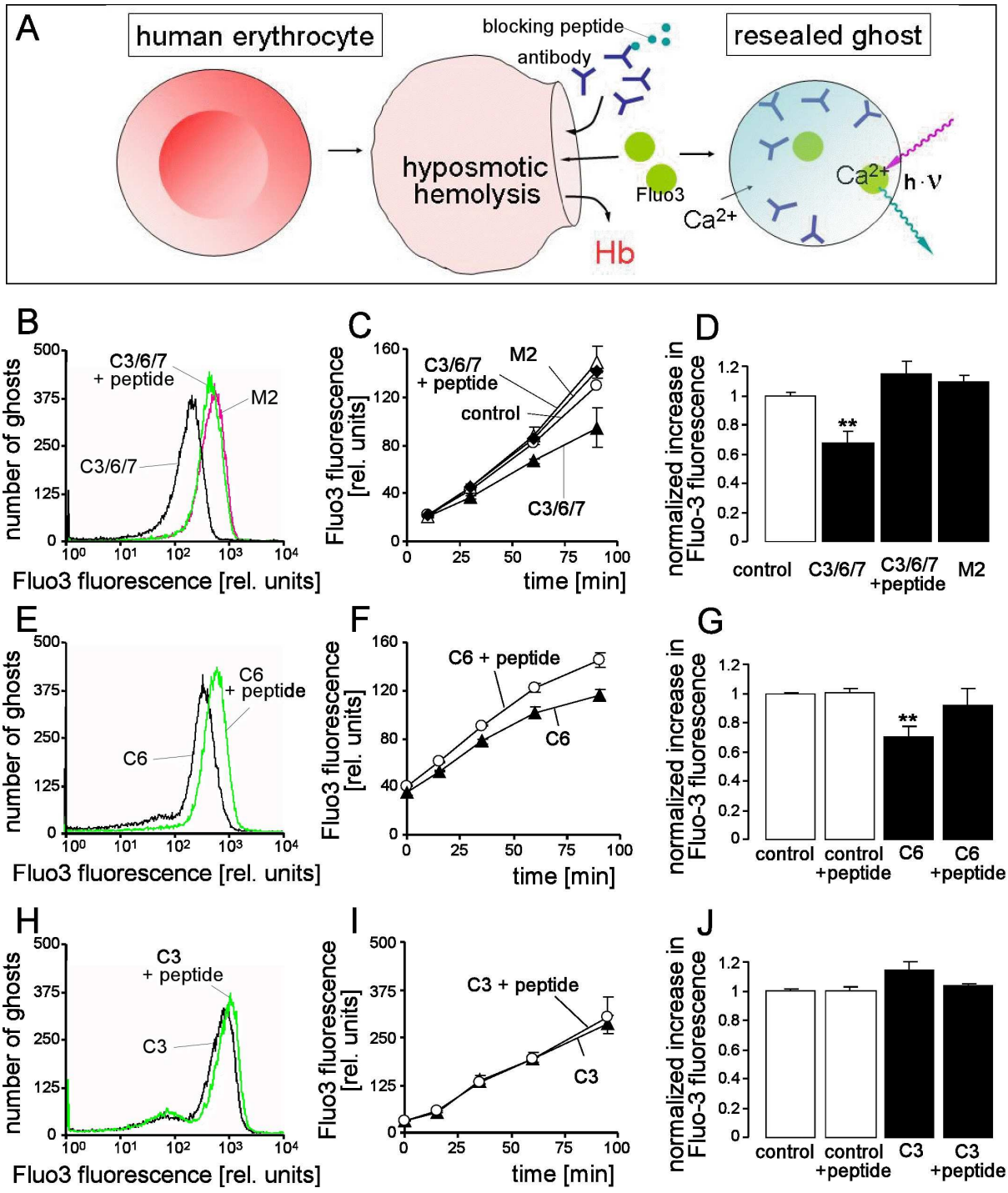

Fig. 16. Effect of inclusion of TRP-specific antibodies on the Ca^{2+} uptake by human ghosts.
A. Scheme showing the inclusion of TRP-specific antibodies, immunizing blocking peptides, and Fluo3-dye during ghost preparation by hypoosmotic hemolysis.
B,E,H. Histograms indicating the Fluo3 fluorescence of ghosts incubated for 90 min in Ca^{2+}-containing medium. Ghosts contained either anti-TRPC3/6/7-antibody, anti-TRPC3/6/7-antibody pre-adsorbed with the immunizing peptide, anti-TRPM2-antibody (B), anti-TRPC6-antibody, anti-TRPC6-antibody pre-adsorbed with the immunizing peptide (E), anti-TRPC3-antibody, or anti-TRPC3-antibody pre-adsorbed with the immunizing peptide (H).
C,F,I. Time-dependence of the mean Fluo3-fluorescence (± SE; n = 3) of ghosts incubated in Ca^{2+}-containing medium. Ghosts contained either no antibody (C, open circles), anti-TRPC3/6/7-antibody

(C, closed triangles), anti-TRPC3/6/7-antibody pre-adsorbed with the immunizing peptide (C, open triangles), anti-TRPM2-antibody (C, closed diamonds), anti-TRPC6-antibody (F, closed triangles), anti-TRPC6-antibody pre-adsorbed with the immunizing peptide (F, open circles), anti-TRPC3-antibody (I, closed triangles), or anti-TRPC3-antibody pre-adsorbed with the immunizing peptide (I, open circles).
D,G,J. Mean normalized increase in Fluo3 fluorescence (± SE; n = 6-22) of ghosts incubated as in (C,F,I) in the absence (open bar) or presence (closed bar) of intracellular anti-TRPC3/6/7-, anti-TRPM2- (D), anti-TRPC6- (G), or anti-TRPC3-antibody (J), or antibodies pre-adsorbed with the immunizing peptides (closed bars). In addition, some controls are shown where the respective immunizing peptides alone were incorporated into the ghosts (G, J; open bars). ** values significantly ($p \leq 0.01$; two-tailed t-test) different from 1.0.

In accordance with the proposed TRPC6-Ca^{2+} permeability, erythrocytes from TRPC6$^{-/-}$ mice exhibited a lower basal cytosolic free Ca^{2+} concentration than wildtype erythrocytes as assessed by Fluo3 fluorescence in flow cytometry (Fig. 17A-C). Decreasing extracellular Cl^- concentration to activate the erythrocyte cation channels increased the cytosolic free Ca^{2+} concentrations in erythrocytes from wildtype mice while having no significant effect in cells from TRPC6$^{-/-}$ mice (Fig. 17A-C). In addition, Cl^- depletion further decreased the forward scatter in flow cytometry, an effect significantly blunted in TRPC6$^{-/-}$ erythrocytes as compared to TRPC6$^{+/+}$ erythrocytes (Fig. 17D-F).

To test for an impact of TRPC dependent Ca^{2+} entry on erythrocyte “apoptosis”, PS exposure of the erythrocytes was determined by annexin V binding in both, TRPC6$^{-/-}$ and TRPC6$^{+/+}$ erythrocytes. Fig. 17G-I shows that the percentage of PS-exposing erythrocytes was higher in TRPC6$^{+/+}$ than in TRPC6$^{-/-}$ mice. Taken together, these data indicate functional significance of TRPC6 for the erythrocyte Ca^{2+} leak, erythrocyte volume, and PS exposure.

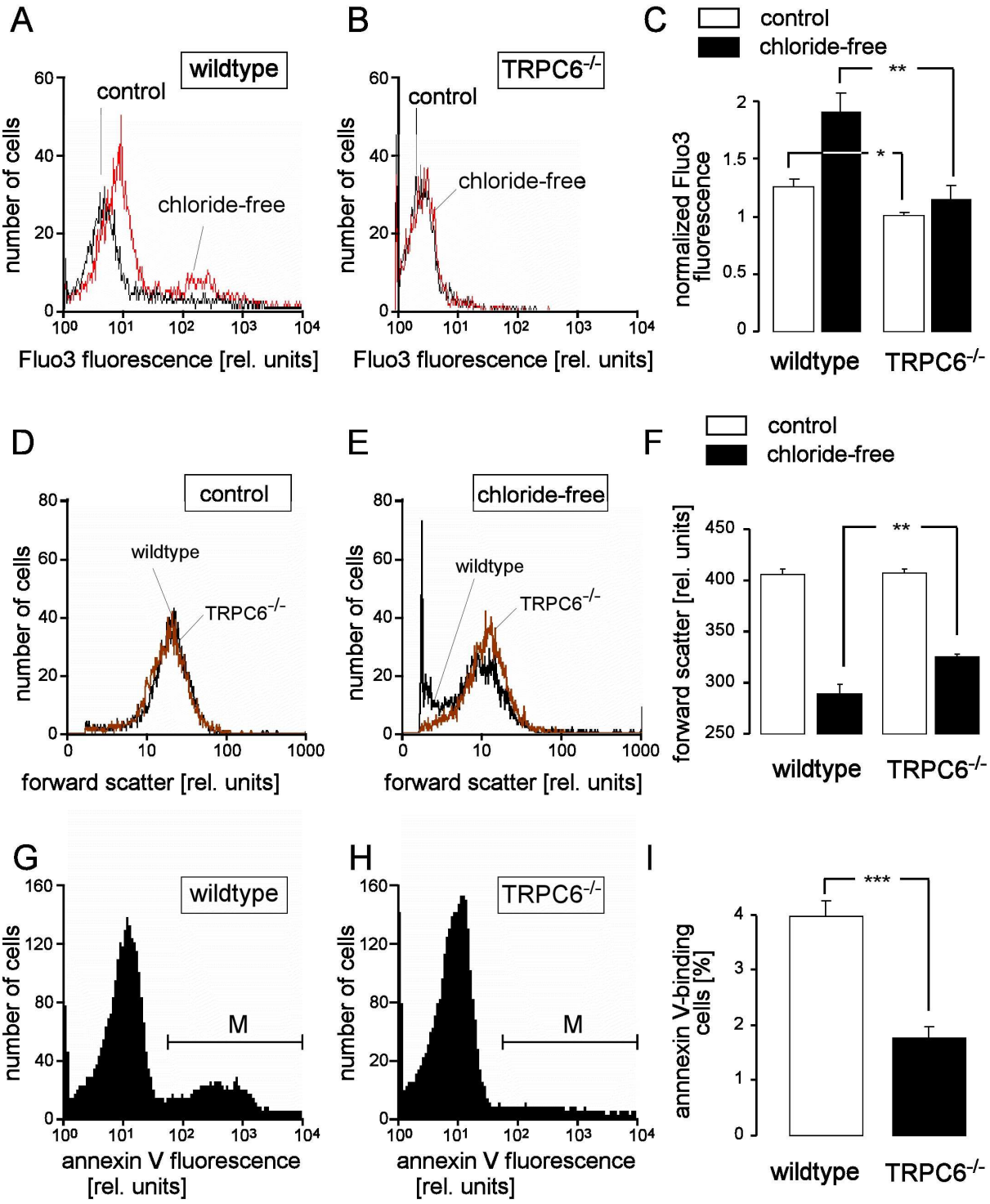

Fig. 17. Cytosolic free Ca^{2+} concentration, forward scatter, and PS exposure of erythrocytes from TRPC6$^{-/-}$ and wildtype mice.

A,B. Histograms showing the Ca^{2+}-specific Fluo3 fluorescence of erythrocytes drawn from wildtype (A) and TRPC6$^{-/-}$ mice (B) incubated for 24h in Cl^--containing (control) or Cl^--depleted medium (chloride-free).

C. Mean normalized Fluo3 fluorescence (± SE, n = 24-28) of wildtype and TRPC6$^{-/-}$ erythrocytes incubated as in (A) in Cl^--containing (control, open bars) or Cl^--depleted medium (chloride-free, closed bars) (*: $p \leq 0.05$;**: $p \leq 0.01$ ANOVA).

D,E. Histograms showing the forward scatter of erythrocytes drawn from wildtype (D) and TRPC6$^{-/-}$ mice (E) incubated for 24h in Cl^--containing (control) or Cl^--depleted medium (chloride-free).
F. Mean forward scatter (± SE, n = 8) of wildtype and TRPC6$^{-/-}$ erythrocytes incubated as in (D,E) in Cl^--containing (control, open bars) or Cl^--depleted medium (chloride-free, closed bars) (**: $p \leq 0.01$ ANOVA).
G,H. Histograms showing the annexin V binding of PS-exposing erythrocytes drawn from wildtype (G) and TRPC6$^{-/-}$ mice (H) post incubated for 3h in Cl^--containing medium.
I. Mean percentage of PS-exposing wildtype (open bar) and TRPC6$^{-/-}$ (closed bar) erythrocytes (± SE; n = 8) incubated as in (G,H) (***: $p \leq 0.001$; two-tailed t-test).

4 Discussion

The present study reveals that PGE_2 stimulates Ca^{2+} entry in nucleated K562 leukaemic cells. Presumably through increase of cytosolic Ca^{2+} activity, PGE_2 decreases cell volume of K562 cells, as evidenced from the decline of forward scatter in flow cytometry. The effect most likely results from activation of the Ca^{2+} sensitive K^+ channels (Lang et al., 2003d) with subsequent hyperpolarization of the cell membrane and cellular loss of KCl (Bookchin et al., 1987; Brugnara et al., 1993; Franco et al., 1996).

The treatment of K562 leukaemia cells with PGE_2 further leads to several key events of apoptosis, such as decline of the mitochondrial membrane potential, activation of caspases, DNA fragmentation, and breakdown of phosphatidylserine asymmetry of the cell membrane (Green et al., 1998; Thornberry and Lazebnik, 1998). The PS exposure at the cell surface is thought to stimulate uptake of apoptotic bodies by macrophages (Boas et al., 1998; Romero and Romero, 1999).

The concentration of PGE_2 (1 µM) required in the present study to trigger PS exposure of K562 cells was rather high in comparison to the nanomolar K_Ds reported for the PGE_2 receptors (Wu-Wang et al., 1990). Since PGE_2 was applied in FCS-containing medium and PGE_2 was presumably bound to serum proteins (Raz, 1972), the free, i.e. effective PGE_2 concentrations were lower than the calculated concentration of 1 µM. In addition, PGE_2 was applied only once at the beginning of the 48 h incubation period suggesting that time-dependent degradation of PGE_2 further lowered its effective concentration. Similarly high concentrations of PGE_2 were applied in other *in vitro* studies (Holla et al., 2005; Tang et al., 2005). Those high concentrations could be reached *in vivo*, as PGE_2 released from monocyte-derived dendritic cells has been demonstrated to almost reach micromolar concentrations (Legler et al., 2006).

PGE_2 has previously been reported to participate in the induction of apoptosis (Sheu et al., 2005) and to protect from apoptosis (Backlund et al., 2005; Hendrickx et al., 2003; Chu et al., 2004; Jia et al., 2004; Liu et al., 2005;

Nishihara et al., 2003; Tessner et al., 2004). In none of those reports the involvement of TRP channels has been shown. In K562 cells activation of Ca^{2+}-permeable TRPM2 channels reportedly initiates apoptosis induced by oxidative stress (Zhang et al., 2005; Zhang et al., 2003), suggesting that TRPM2 and C3/6/7 channels may play a similar role in the induction of apoptosis. In contrast to those findings, a truncated splice isoform of TRPM2 (TRPM2-S) has been reported to co-localize with TRPM2 in erythroblasts and to inhibit the oxidation-stimulated Ca^{2+} entry through TRPM2 and subsequent cell death (Zhang et al., 2003). In the present study TRPM2 mRNA could not be detected pointing to differences in the sub-clones of K562 cells used in different laboratories.

The K562 cells studied here expressed TRPC3 and –C7 mRNA as demonstrated by RT-PCR. Silencing of the TRPC7 but not of TRPC3 decreased TRPC3/6/7-specific proteins suggesting that TRPC7 is the principal TRPC channel expressed in K562 cells. TRPC7 has originally been isolated from foetal brain and is highly expressed in cerebral tissue (Nagamine et al., 1998). TRPC7 channels have been shown to participate in the regulation of Ca^{2+} entry (Lievremont et al., 2004; Shi et al., 2004). However, the involvement of TRPC7 in apoptosis has never been shown so far. In the present study silencing of TRPC7 blunted the PGE_2- induced apoptosis of K562 cells, pointing to involvement of the TRPC7 channel in Ca^{2+} entry.

The effect of PGE_2 on K562 cells is reminiscent to its effects on circulating erythrocytes in which the eicosanoid similarly increases cytosolic Ca^{2+} activity and triggers PS exposure.

In conclusion, PGE_2-stimulated activation of Ca^{2+} permeable TRPC channels results in Ca^{2+} entry, cell shrinkage, caspase activation, DNA fragmentation, decline of the mitochondrial membrane potential, and phosphatidylserine exposure of K562 leukaemic cells. The stimulation of PGE_2-induced cell death may serve to remove defective, infected, and thus potentially harmful cells. Moreover, TRPC7 channels may participate in the regulation of cell survival under the control of further stimulators or inhibitors of apoptosis.

The study in human or mouse erythrocytes and erythrocyte ghosts resp. provides several lines of evidence for the functional expression of TRPC6 in erythrocytes. (i) TRPC6 mRNA was expressed in human erythroid progenitor cells confirming previous reports (Chu et al., 2004), (ii) the immunoblot probed against TRPC6 suggested TRPC6 protein expression in human erythrocytes, (iii) anti-TRPC6 and anti-TRPC3/6/7 antibodies inhibited the Ca^{2+} entry into human ghosts, and (iv) the Ca^{2+} entry was blunted in erythrocytes from gene targeted mice lacking functional TRPC6 ($TRPC6^{-/-}$). Taken together, those observations strongly suggest the presence of TRPC6 in erythrocytes of humans and wildtype mice.

In the present study Ca^{2+} uptake by human ghosts was inhibited by amiloride or its derivate EIPA and was dependent on the redox state. Previous observations disclosed the inhibition of TRPC6 by amiloride (Inoue et al., 2001) and the activation of TRPC3 and TRPC4 by oxidative stress (Yao and Garland, 2005). Anti-TRPC6 and anti-TRPC3/6/7 antibodies, however, only partially inhibited the ghost Ca^{2+} uptake (compare Fig. 16). Thus, TRPC6 may not be the only channel allowing Ca^{2+} entry into erythrocytes. Similarly, additional channels may substitute partially the function of TRPC6 in TRPC6-deficient mice. Possibly, lack of TRPC6 might lead to upregulation of other cation channels, and thus, the contribution of TRPC6 may be underestimated from the comparison of wildtype and TRPC6 knockout mice. As a matter of fact, upregulation of TRPC3 channels has been observed in smooth muscle cells of $TRPC6^{-/-}$ mice (Dietrich et al., 2005c). However, we did not observe upregulation of TRPC3 in $TRPC6^{-/-}$ erythrocytes. TRPC forms heterotetrameric channels (Hofmann et al., 2002) and the cation channel functionally expressed in erythrocytes could well be composed of different TRPC channels. Moreover, the participation of channels from other channel families cannot be excluded.

The present observations point to a functional role of TRPC6, i.e. its involvement in the machinery leading to erythrocyte “apoptosis”.

In conclusion, evidence is provided for the expression of TRPC6 in circulating erythrocytes and its involvement in basal Ca^{2+} leak and stress-stimulated Ca^{2+} entry. Ca^{2+} entering through TRPC6 contributes to the triggering of erythrocyte

“apoptosis“. Thus, TRPC6 participates in the regulation of erythrocyte survival. The present observations demonstrate, however, that lack of TRPC6 does not completely abrogate Ca^{2+} entry suggesting that further cation channels are operating in erythrocytes. The molecular identity of those channels remains to be established.

5 Summary

Prostaglandin-E_2 (PGE_2) is known to trigger suicidal death of nucleated cells and enucleated erythrocytes (apoptosis). In erythrocytes Cl^- depletion, energy depletion, osmotic shock, or oxidative stress also induce suicidal cell death via the formation of PGE_2. The triggering of erythrocyte apoptosis involves the activation of nonselective cation channels leading to Ca^{2+} entry followed by cell shrinkage and triggering of Ca^{2+} sensitive cell membrane scrambling with phosphatidylserine (PS) exposure at the cell surface. As a cell model for erythrocytes, K562 human leukaemia cells were chosen due to their erythroid features. Experiments were performed in K562 cells to explore whether PGE_2 induces apoptosis of nucleated cells similarly through cation channel activation and to possibly disclose the molecular identity of the cation channels involved. To this end, Ca^{2+} activity was estimated from fluorescence of the Ca^{2+}-sensitive fluorescence dye Fluo3, mitochondrial potential from DePsipher fluorescence, phosphatidylserine exposure from annexin binding, caspase activation from caspAce fluorescence, cell volume from FACS forward scatter, and DNA fragmentation utilizing a photometric enzyme immunoassay. Stimulation of K562 cells with PGE_2 (50 µM) increased cytosolic Ca^{2+} activity, decreased forward scatter, depolarized the mitochondrial potential, increased annexin V binding, led to caspase activation, and resulted in DNA fragmentation. Gene silencing of the Ca^{2+}-permeable transient receptor potential cation channel TRPC7 significantly blunted PGE_2-induced triggering of PS exposure and DNA fragmentation. In conclusion, K562 cells express Ca^{2+}-permeable TRPC7 channels which are activated by PGE_2 and participate in the triggering of apoptosis.

The results in nucleated K562 cells suggest the investigation of the involvement of TRPC channels in the triggering of erythrocyte “apoptosis”. RT-PCR revealed that erythroid progenitor cells differentiated from human peripheral blood expressed messenger RNA encoding for the non-selective cation channel TRPC6. Western blotting indicated expression of TRPC6 protein in erythrocyte

membranes from man and wild type mice but not from TRPC6 deficient mice (TRPC6$^{-/-}$). According to flow-cytometry, Ca^{2+} entry into human erythrocyte ghosts prepared by hemolysis in EGTA-buffered solution containing Fluo3 was inhibited by the reducing agent dithiothreitol (1 mM) and the erythrocyte cation channel blockers ethylisopropylamiloride (50 µM) and amiloride (1 mM). Loading of the ghosts with antibodies against TRPC6 or TRPC3/6/7 but neither with antibodies against TRPM2 or TRPC3 nor with antibodies pre-adsorbed with the respective immunizing peptides inhibited human ghost Ca^{2+} entry. Steady state Ca^{2+} activity as well as Cl^- sensitive Ca^{2+} entry, cell shrinkage, and phospholipid scrambling were significantly blunted in erythrocytes from TRPC6$^{-/-}$ mice as compared to erythrocytes from their wildtype littermates. In conclusion, human and mouse erythrocytes express TRPC6 non-selective cation channels which participate in both, constitutive cation leak and Ca^{2+} induced suicidal death.

6 References

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7 Publications

First authorships/shared first authorship:

Foller,M., R.S.Kasinathan, C.Duranton, T.Wieder, S.M.Huber, and F.Lang. 2006. PGE2-induced apoptotic cell death in K562 human leukaemia cells. Cell Physiol Biochem 17:201-210.

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Foller M., E.Shumilina, R.S.Lam, W.Mohamed, R.S.Kasinathan, S.M.Huber, T.Chakraborty, F.Lang. 2007. Induction of suicidal erythrocyte death by listeriolysin from Listeria monocytogenes (in press).

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Abstracts:

Feil, R., S. Feil, P. Franken, Y. Emmenegger, M. Tafti, K. Weindl, S.M. Hölter, W. Wurst, S. Langmesser, U. Albrecht, M. Föller, F. Lang, S. Weber, F. Hofmann. 2007. New mouse models for the analysis of cGMP signalling. 3rd International Conference on cGMP Dresden, Germany. 2007

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