PGE₂-induced Apoptotic Cell Death in K562 Human Leukaemia Cells

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Key Words

Prostaglandin • Gene silencing • TRP channels • TRPC7 • Caspase • Annexin binding • Mitochondrial potential • Phosphatidylserine

Abstract

Prostaglandin-E₂ (PGE₂) is known to trigger suicidal death of nucleated cells (apoptosis) and enucleated erythrocytes (eryptosis). In erythrocytes PGE₂ induced suicidal cell death involves activation of nonselective cation channels leading to Ca²⁺ entry followed by cell shrinkage and triggering of Ca²⁺ sensitive cell membrane scrambling with phosphatidylserine (PS) exposure at the cell surface. The present study was performed to explore whether PGE₂ 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, Ca²⁺ activity was estimated from Fluo3 fluorescence, mitochondrial potential from DePispher 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 human leukaemia cells with PGE₂ (50 μM) increased cytosolic Ca²⁺

activity, decreased forward scatter, depolarized the mitochondrial potential, increased annexin binding, led to caspase activation and resulted in DNA fragmentation. Gene silencing of the Ca²⁺-permeable transient receptor potential cation channel TRPC7 significantly blunted PGE₂-induced triggering of PS exposure and DNA fragmentation. In conclusion, K562 cells express Ca²⁺-permeable TRPC7 channels, which are activated by PGE₂ and participate in the triggering of apoptosis.

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Introduction

Osmotic or isotonic cell shrinkage are well known triggers of apoptotic cell death [1]. Most recent experiments revealed that shrinkage of peripheral blood erythrocytes stimulates the formation of prostaglandin E₂ (PGE₂) which in turn triggers phosphatidylserine (PS) exposure at the outer leaflet of the plasma membrane, one of the hallmarks of apoptosis in nucleated cells [2].

In erythrocytes, prostaglandin E₂ (PGE₂) increases cytosolic Ca²⁺ concentration [3, 4]. The Ca²⁺ entry then leads to triggering of Ca²⁺ sensitive cell membrane

scrambling with subsequent breakdown of the membrane phospholipid asymmetry and exposure of PS at the cell surface [2, 5, 6]. The increase of intraerythrocytic Ca^{2+} concentration further activates Ca^{2+} sensitive K^{+} channels resulting in release of K^{+} paralleled by loss of Cl^{-} and osmotically obliged water, cell shrinkage and increased erythrocyte filterability [2, 4, 7-10].

Ca^{2+} may enter erythrocytes through Ca^{2+} permeable cation channels [11-13], which are activated following removal of external Cl^{-} , osmotic shock by increase of extracellular osmolarity, oxidative stress by addition of t-butylhydroperoxide (t-BHP) and energy depletion by removal of extracellular glucose [5, 6, 14]. Accordingly, erythrocyte death induced by oxidative stress, energy depletion or removal of external Cl^{-} is abrogated and hyperosmotic shock-induced erythrocyte death is blunted when the cells were suspended in medium with low free Ca^{2+} concentrations [5, 6]. Suicidal erythrocyte death is similar to apoptosis of nucleated cells and has thus been termed eryptosis [15]. Mechanisms triggering eryptosis may contribute to but do not necessarily account for erythrocyte senescence [15-18] or neocytolysis [19]. Reportedly, eryptosis may contribute to anemia e.g. in iron deficiency [20].

The present study has been performed to explore whether PGE_2 triggers similar mechanisms eventually leading to apoptosis of nucleated cells. To this end, experiments have been performed in K562 human leukaemia cells, which indeed increased cytosolic Ca^{2+} activity and entered apoptosis following exposure to PGE_2 . The present study further aimed to identify the molecular identity of the cation channels involved.

Materials and Methods

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 $\mu\text{g}/\text{ml}$, 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 (50 μM) or cells treated with 40 $\mu\text{g}/\text{ml}$ 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°C 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 [2]. 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 and then loaded with Fluo-3/AM (Calbiochem; Bad Soden, Germany) in NaCl Ringer solution containing 1 μM Fluo-3/AM. The cells were incubated at room temperature for 15 minutes under shaking and washed twice in NaCl Ringer solution. 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_2O , and stored at -80 °C. The primers used are listed in Table 1. 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), sterilized distilled water up to 50 μl . The PCR conditions used in this

Table 1. PCR primers

template	accession number	sense 5' - 3'	position	antisense 5' - 3'	position	fragment [bp]
TRPC3	NM_003305	GTTGTGGAATGTGC TTGACT	1825- 1844	TGAAAGGTGGAGGTA ATGTT	2527- 2546	721
TRPC7	NM_020389	ACCGAGGTGCCAAA TACAAC	1811- 1830	GCGAACTTCCATTCC ACATC	2050- 2069	259
TRPM2	NM_003307	TCGGACCCAACCAC ACGCTGTA	4125- 4146	CGTCATTCTGGTCTCG GAAGTG	4442- 4463	338
TRPM7	NM_017672	TGAAGCAAAGCAGA GTGACCT	2230- 2350	TATTCAGCCTT CCCAT CCAC	2476- 2495	266
ACCN2	NM_020039	ACCGAGGTGCCAAA TACAAC	879-896	GCGAACTTCCATTCC ACATC	1130- 1151	272

Table 2. PCR experimental conditions

template	denaturing	annealing	extension	number of cycles
TRPC3	94 °C for 30 sec.	57 °C for 30 sec.	72 °C for 30 sec.	35
TRPC7	94 °C for 60 sec.	56 °C for 60 sec.	72 °C for 120 sec.	35
TRPM2	94 °C for 30 sec.	57 °C for 30 sec.	72 °C for 30 sec.	35
TRPM7	94 °C for 45 sec.	56 °C for 60 sec.	72 °C for 60 sec.	30
ACCN2	94 °C for 45 sec.	56 °C for 60 sec.	72 °C for 60 sec.	30

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

study are summarized in Table 2. 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. 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°C, 5 % CO₂ 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°C. 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 μ g 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 at 4 °C, the blots were probed overnight at 4 °C 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 [21].

Statistics

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

Results

To test whether PGE₂ increases the cytosolic free Ca²⁺ concentration of K562 cells, Fluo-3 fluorescence measurements have been performed in cells incubated for 48 hours either in the absence or presence of PGE₂ (50 μ M). As illustrated in Fig. 1, the incubation in PGE₂ increased significantly the Fluo-3 fluorescence, pointing to an increase of cytosolic free Ca²⁺ concentration (Fig. 1A, B).

K562 cells express Ca²⁺-activated hSK4 K⁺ channels [22] suggesting that elevated cytosolic Ca²⁺ 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. PGE₂ (50 μ M for 48 h) indeed decreased cell volume as

Fig. 1. PGE₂ increases cytosolic free Ca²⁺ 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 and PGE₂ (50 μ M for 48 h)-stimulated K562 cells. B. Mean ($\pm$ SE, $n=9-12$) Fluo-3 fluorescence (as calculated from the geometrical means of the histograms) recorded in control cells (open bar), and in PGE₂ (50 μ M for 48 h)-stimulated cells (closed bar). C. Forward scatter distribution in FACS histograms of control and PGE₂ (50 μ M for 48 h)-stimulated cells. D. Mean forward scatter ($\pm$ SE; $n=5-6$; forward scatter in geometrical mean) of control K562 cells (open bar) and cells stimulated for 48 h with increasing concentrations of PGE₂ (closed bars; **: $p=0.01$; ANOVA).

Fig. 2. PGE₂ decreases the mitochondrial membrane potential. A. FACS histogram of DePsipher fluorescence as a measure of mitochondrial activity recorded from control and PGE₂ (50 μ M for 48 h)-stimulated K562 cells. B. Mean ($\pm$ SE, $n=5$) DePsipher fluorescence (as calculated from the geometrical means of the histograms) recorded in control cells (open bar), and in PGE₂ (50 μ M for 48 h)-stimulated cells (closed bar).

Fig. 3. PGE₂ activates caspases in K562 leukaemic cells. A. Two-dimensional flow cytometry histograms in FACS analysis of control (left) and PGE₂-stimulated cells (50 μ M for 48 h; right). The plots show the relationship between cell forward scatter and caspase activity, respectively. B. FACS histogram of the caspase fluorescence intensity recorded in control cells (black line), in PGE₂- (50 μ M for 48 h; red line) or in carboplatin-stimulated cells (40 μ g/ml for 48 h; blue line). C. Mean caspase activity ($\pm$ SE; n=6; *: p = 0.05; ANOVA) of cells incubated as in (B) in the absence or presence of PGE₂ or carboplatin.

determined by a significant decrease of forward scatter (Fig. 1C, D). The effect was dependent on the PGE₂ concentration (Fig. 1D).

As illustrated in Fig. 2, PGE₂ (50 μ M for 48h) led to a significant increase of DePsipher fluorescence, thus pointing to depolarization of the mitochondrial membrane potential.

To determine, 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. 3, PGE₂ (50 μ M) triggered within 48 h an about two-fold increase in caspase activity. For comparison, carboplatin (40 μ g/ml) led within 48 h to an about 5-fold caspase activity (Fig. 3C).

Furthermore, PGE₂ (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

Fig. 4. PGE₂ triggers phosphatidylserine (PS) exposure. A. Light micrograph (left column) and corresponding immunofluorescence (right column) showing PS exposure of control (upper row) and PGE₂ (50 μ M 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 μ M (blue), and 1 μ M of PGE₂ (red). C. Mean percentage ($\pm$ SE; n=9) of annexin-binding cells treated as in (B) with increasing concentrations of PGE₂ (*: p = 0.05; ANOVA). D, E. Effect of EIPA (50 μ M) on the PGE₂ (50 μ M)-stimulated annexin-V binding in K562 cells. Annexin-V binding (FACS histogram in D) and mean percentage ($\pm$ 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 PGE₂ alone (D, black line; E, middle) or in the presence of PGE₂ and EIPA (D, blue line; E, right; **: p = 0.01, *: p = 0.05; ANOVA).

microscopy (Fig. 4A) and flow cytometry (Fig. 4B, C). A significant increase of annexin binding was observed at a PGE_2 concentration of 1 μM (Fig. 4C). In erythrocytes the PGE_2 activated cation channels are inhibited by ethylisopropylamiloride (EIPA) [2]. 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. 4D and E, EIPA blunted the PGE_2 effect on PS exposure by some 40 %. Taken together, the data suggest stimulation of K562 apoptosis by PGE_2 in an EIPA-sensitive manner.

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 [23] 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 for 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. 5A) or two TRPC3/6/7-specific antibodies (one directed to the N terminus and the second directed to the C terminus, (Fig. 5B)). The construct siTRPC7c decreased TRPC3/6/7 protein expression by 33 % (Fig. 5C).

Fig. 6. TRPC7 RNA interference blunts PGE_2 -stimulated PS exposure. **A.** FACS histogram showing annexin-V fluos binding of K562 cells stimulated for 48 h with PGE_2 (50 μM) in the absence (A, B, control) or presence of ACCN2 (A) and TRPC7c siRNA (B). **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 $\pm$ SE (n = 4-6; **, *: p = 0.01 and 0.05, respectively; ANOVA).

Fig. 7. TRPC7 RNA interference blunts PGE₂-stimulated DNA fragmentation. DNA fragmentation of non-stimulated (control) and PGE₂ (50 μ M for 48h)-stimulated K562 cells was assessed by quantitative determination of cytoplasmic histone-associated DNA fragments using a photometric enzyme immunoassay. PGE₂-stimulated cells were either treated with TRPC7-specific siRNA (siTRPC7) or with a non-specific construct (siNEG). Data are means $\pm$ SE (n=3; *, ***: p = 0.05 and 0.001, respectively; ANOVA).

As illustrated in Fig. 6, siTRPC7 treatment significantly inhibited PGE₂ (50 μ M for 48 h)-induced PS exposure of K562 cells by 65 % (Fig. 6B, C). In contrast, the construct directed against ACCN2 did not affect PGE₂-induced PS exposure (Fig. 6A, C). A nonsense construct (siNEG), which was used as further negative control, was similarly not capable to significantly interfere with PGE₂-induced PS exposure (Fig. 6C).

Downregulation of TRPC7 further blunted PGE₂ (50 μ M for 48 h)-induced DNA-fragmentation significantly by 53 % as compared to the nonsense construct siNEG (Fig. 7).

In summary, the present study provides several lines of experimental evidence for triggering of K562 cell apoptosis by PGE₂ through activation of TRPC7 cation channels, subsequent increase in intracellular Ca²⁺ activity, cell shrinkage, mitochondrial depolarization, caspase activation, DNA fragmentation and phosphatidylserine exposure.

Discussion

Both, cell proliferation and apoptotic cell death critically depend on the activity of ion channels in the plasma membrane [24]. Specifically, apoptosis has been shown to be paralleled by activation of Cl⁻ channels in several cell types [10, 25-28]. Other channels may play a dual role in the triggering of apoptosis. Apoptosis has been shown to be favored or paralleled by activation of K⁺ channels [29-34]. On the other hand, apoptosis may be paralleled or favoured by inhibition of K⁺ channels [27, 35, 36]. Apoptosis may be paralleled by inhibition of store operated Ca²⁺ channels [37, 38]. On the other hand, sustained increase of cytosolic Ca²⁺ activity has been shown to trigger apoptosis [39-44]. Apparently, the influence of K⁺ channels and Ca²⁺ channels in apoptosis depends on the temporal pattern and amplitude of channel activity as well as the interaction with other channels, transporters and signaling pathways [24].

The present study reveals that PGE₂ stimulates Ca²⁺ entry in nucleated K562 leukaemic cells. Presumably through increase of cytosolic Ca²⁺ activity PGE₂

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 Ca²⁺ sensitive K⁺ channels [22] with subsequent hyperpolarization of the cell membrane and cellular loss of KCl [8, 9, 45].

The treatment of K562 leukaemia cells with PGE₂ 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 [40, 46]. The PS exposure at the cell surface is thought to stimulate uptake of apoptotic bodies by macrophages [47, 48].

Hyperosmotic cell shrinkage has similarly been demonstrated to activate nonselective cation channels in human erythrocytes [49] and to trigger erythrocyte death [5] via the formation of PGE₂ [2]. Hyperosmotic shrinkage triggers apoptotic death in a wide variety of nucleated cells [1, 25, 50-53]. In addition, cellular loss of K⁺ could contribute to the triggering of apoptosis [22, 54-62].

The concentration of PGE₂ (1 μ M) 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 PGE₂ receptors [63]. Since PGE₂ was applied in FCS-containing medium and PGE₂ was presumably bound to serum proteins [64], the free, i.e. effective PGE₂ concentrations were lower than the calculated concentration of 1 μ M. In addition, PGE₂ was applied only once at the beginning of the 48 h incubation period suggesting that time-dependent degradation of PGE₂ further lowered its effective concentration. Similarly high concentrations of PGE₂ were applied in other *in vitro* studies [65, 66]. Those high concentrations could be reached *in vivo*, as PGE₂ released from monocyte-derived dendritic cells has been demonstrated to almost reach micromolar concentrations [67].

PGE₂ has previously been reported to participate in the induction of apoptosis [68] and to protect from

apoptosis [41, 69-74]. 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 [75, 76], 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 [75]. In the present study we were not able to detect TRPM2 mRNA pointing to differences in the sub-clones of K562 cells used in different laboratories.

TRPC3, -C6, and -C7 channels share about 75% identity, exhibit relatively low selectivity for Ca^{2+} over Na^+ , are sensitive to intracellular free Ca^{2+} , and are activated by diacylglycerol [23]. 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 it is highly expressed in cerebral tissue [77]. TRPC7 channels have been shown to participate in the regulation of Ca^{2+} entry [78, 79]. However, to our knowledge 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 [2]. However, in erythrocytes PGE_2 does not activate caspases [2], even though caspases are expressed in erythrocytes [80, 81] and are activated by oxidative stress [80] and during erythrocyte ageing [81]. On the other hand, no activation of erythrocyte caspases has been observed after hyperosmotic shrinkage [14] or after ionomycin treatment [82].

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 phosphatidyl-serine 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.

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