TRPC6 Contributes to the Ca^{2+} Leak of Human Erythrocytes

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Key Words

Eryptosis • Calcium • Ion channel • Cell death • Oxidative stress

Abstract

Human erythrocytes express cation channels which contribute to the background leak of Ca^{2+} , Na^{+} and K^{+} . Excessive activation of these channels upon energy depletion, osmotic shock, Cl^{-} depletion, or oxidative stress triggers suicidal death of erythrocytes (eryptosis), characterized by cell-shrinkage and exposure of phosphatidylserine at the cell surface. Eryptotic cells are supposed to be cleared from circulating blood. The present study aimed to identify the cation channels. RT-PCR revealed mRNA encoding the non-selective cation channel TRPC6 in erythroid progenitor cells. Western blotting indicated expression of TRPC6 protein in erythrocytes from man and wildtype mice but not from TRPC6^{-/-} mice. According to flow-cytometry, Ca^{2+} entry into human ghosts prepared by hemolysis in EGTA-buffered solution containing the Ca^{2+} indicator Fluo3/AM was inhibited by the reducing agent dithiothreitol and the erythrocyte cation channel blockers ethylisopropylamiloride and amiloride. Loading of the ghosts with

antibodies against TRPC6 or TRPC3/6/7 but neither with antibodies against TRPM2 or TRPC3 nor antibodies pre-adsorbed with the immunizing peptides inhibited ghost Ca^{2+} entry. Moreover, free Ca^{2+} concentration, cell-shrinkage, and phospholipid scrambling were significantly lower in Cl^{-} -depleted TRPC6^{-/-} erythrocytes than in wildtype mouse erythrocytes. In conclusion, human and mouse erythrocytes express TRPC6 cation channels which participate in cation leak and Ca^{2+} -induced suicidal death.

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Introduction

Human erythrocytes build up and maintain high intracellular K^{+} ($[\text{K}^{+}]_i$) as well as low intracellular Na^{+} ($[\text{Na}^{+}]_i$) concentrations [1]. Despite high intracellular K^{+} concentrations, the resting membrane potential is only around -10 mV [2] suggesting very low basal K^{+} channel activity. Although devoid of intracellular Ca^{2+} stores human erythrocytes maintain a cytosolic free Ca^{2+} concentration ($_{\text{free}}[\text{Ca}^{2+}]_i$) of about 10^{-7} M [3, 4]. This is accomplished by a powerful Ca^{2+} pump and a very low resting Ca^{2+} permeability in the erythrocyte membrane

[5]. Upon opening of Ca^{2+} permeable cation channels, the steep inwardly directed electrochemical gradient drives Ca^{2+} into the erythrocyte. This results in a significant increase in $[\text{Ca}^{2+}]_{\text{free}}$ [6] because of the very low affinity Ca^{2+} buffer in the erythrocyte cytosol [7]. Excess activity of those channels triggers suicidal death of the erythrocytes (eryptosis) which involves the following processes: Elevated $[\text{Ca}^{2+}]_{\text{free}}$ stimulates the protease calpain which digests the cytoskeleton [8] leading to membrane blebbing and triggers cell membrane scrambling [9] with phosphatidylserine (PS) exposure at the cell surface [8]. At the same time, Ca^{2+} activates Ca^{2+} -sensitive GARDOS K^{+} channels (KCNN4, SK4 [10]) leading to hyperpolarisation of the membrane thus driving Cl^{-} out of the cell. The following cellular loss of K^{+} , Cl^{-} , and osmotically obliged water causes cell shrinkage [11–13]. Sustained cell shrinkage in turn activates a sphingomyelinase leading to ceramide formation [14]. Similar to Ca^{2+} , ceramide triggers phospholipid scrambling [14]. The exposure of PS at the outer surface of the cell membrane is followed by binding to PS receptors at macrophages [15] and subsequent phagocytosis of the affected erythrocyte [16–18]. Several stress stimuli such as oxidative stress [19], glucose-depletion, hyperosmotic shock, and decrease of the intra- or extracellular Cl^{-} concentration activate the Ca^{2+} -permeable cation channels [6]. This activation is dependent on the formation of prostaglandin E_2 (PGE_2) by the human erythrocyte, and stimulation of human erythrocytes with PGE_2 induces cation channel activity [8].

By applying patch-clamp single-channel recording, ~20 pS non-selective cation channels were identified in the erythrocyte membrane that are activated by strong depolarization of the membrane [20] and stimulated by nicotinic acetylcholine [21] and PGE_2 receptors [22]. These channels are directly activated by clotrimazole and its analogues [23], are permeable to divalent cations such as Ca^{2+} , Ba^{2+} , and Mg^{2+} [22, 24], and exhibit a hysteric-like voltage-dependent gating (i.e., half-maximal voltages of activation and deactivation curves differ by ~25 mV) [24, 25]. The voltage-gated nonselective cation channels are also apparent in whole-cell and nystatin-perforated patch-clamp recordings [26]. Whole-cell recordings in our laboratory have demonstrated additional cation channels that differ from the voltage-gated 20 pS channel by their voltage-independent activity [8]. During continuous whole-cell recordings the voltage-independent cation channels activate reversibly within few minutes upon oxidative stress (1 mM *tert*-butylhydroperoxide; *t*-BHP) [19], hyperosmotic shrinkage [6, 27–29], and replacement of

intracellular and extracellular Cl^{-} by gluconate [6, 8, 28–30]. Cation channel activation also occurs when extracellular Cl^{-} is replaced by further anions or when the ionic strength of the bath solution is lowered by isosmotic substitution of NaCl with sorbitol (i.e. in low ionic strength medium) suggesting half-maximal channel activity at some 30 mM extracellular Cl^{-} concentration [19]. Similar to the voltage-dependent channels these Cl^{-} -dependent and voltage-independent channels are permeable for Ca^{2+} [19] and activated by PGE_2 formation [8]. The voltage-independent cation channels are inhibited by ethylisopropylamiloride (EIPA) or amiloride [19].

The present study aimed to define the molecular identity of the erythrocyte cation channels involved in basal cation leak and eryptotic cell death. The biophysical similarity of the erythrocyte cation channels (cation selectivity, Ca^{2+} permeability, inactivity at resting conditions, current voltage relationship [19]) with members of the transient receptor potential channels (TRP) [31] and the fact that TRPC6 [31] has been detected in erythroid progenitor cells [32] prompted us to define the functional significance of TRPC6 channels for erythrocyte survival.

Material and Methods

Preparation of erythrocyte ghosts

Pelleted human erythrocytes (70 μl) were lysed in a hypotonic buffer containing (in mM) 10 N-2-hydroxyethyl-piperazine-N-2-ethanesulfonic acid (Hepes)/KOH pH 7.4, 30 KCl, 1 MgCl_2 , 3 Mg-ATP , 0.5 EGTA, and 0.002 Fluo3/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 Biotechnology, Inc.; 1 : 200 dilution), the blocking peptide of TRPC 3/6/7 (N-18) antibody (Santa Cruz Biotechnology, 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), TRPM2 antibody (ab11167; Abcam, Cambridge, UK, 1:200 dilution) or dithiothreitol (DTT; 1 mM). The non-lysed cells were separated by centrifugation at 2000 rpm, and the erythrocyte ghosts in the supernatant were spun down at 17000 rpm (4°C, 20 min). The supernatant was discarded, and the erythrocyte ghosts were re-buffered in isosmotic 2 mM CaCl_2 -containing NaCl Ringer (in mM: 125 NaCl, 5 KCl, 1 MgSO_4 , 32 HEPES/NaOH pH 7.4, 5 glucose) at $t = 0$ min. Then, the Ca^{2+} -dependent Fluo3/AM fluorescence was measured in fluorescence channel 1 (FL-1; 488 nm excitation, 530 nm emission) on a FACS-Calibur in the presence or absence of amiloride (1 mM) or ethylisopropylamiloride (EIPA; 50 μM). To test whether the Fluo3/AM fluorescence indeed reflects the intra-ghost Ca^{2+} concentration and not changes in ghost volume or shape the

following control experiments were performed: the ghosts were additionally re-buffered in EGTA (0.5 mM)-containing Ca^{2+} -free NaCl Ringer or in Ca^{2+} ionophore ionomycin (1 μM)- and Ca^{2+} (2 mM)-containing NaCl Ringer. Buffering of the extracellular Ca^{2+} concentration diminished the Fluo3/AM fluorescence change from 2.5 ± 0.7 rel. units/min in Ca^{2+} -containing Ringer to 0.9 ± 0.3 rel. units/min while treatment with Ca^{2+} ionophore increased the Fluo3/AM fluorescence change to 11.4 ± 0.9 rel. units/min ($n = 4-5$; data not shown). This suggests that changes in cytosolic Ca^{2+} concentrations rather than erythrocyte shape/volume changes were monitored.

Erythroid progenitor cell enrichment

Human burst forming units of erythropoiesis (BFU-E) were purified from peripheral blood obtained from healthy volunteers as described recently [33, 34]. 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 of BFU-E by negative selection with MACS^{TM} antibody-coated paramagnetic microbeads (Miltenyi Biotec, Inc., Auburn, CA) bearing CD3 (part of T-Cell-receptor complex), CD11b (integrin αM , part of macrophage-1-antigen), CD15 (3-fucosyl-N-acetyl-lactosamine), and CD45-RA (leucocyte common antigen). 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 erythropoietin, 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 (Ampliphip CYP450, Affymetrix; Wycombe, UK).

Cytosolic free Ca^{2+} -concentration

For measurement of intracellular Ca^{2+} activity, erythrocytes from TRPC6^{-/-} mice and wildtype littermates were washed and incubated for 24 h at 37°C in CaCl_2 (1 mM)-containing NaCl Ringer or in Ca-D -gluconate₂ (1 mM)-containing Na-gluconate solution (in mM: 125 Na-D-gluconate, 5 K-D-gluconate, 1 MgSO_4 , 32 HEPES/NaOH pH 7.4, 5 glucose). The cells were then loaded with Fluo3/AM (Calbiochem; Bad Soden, Germany) in CaCl_2 (1 mM)-containing NaCl or Na-gluconate Ringer solution containing 2 μM Fluo3/AM. The cells were incubated at 37°C for 15 min under shaking, washed twice and resuspended in CaCl_2 (2 mM)-containing NaCl Ringer solution. Then, Ca^{2+} -dependent Fluo3/AM fluorescence intensity was measured in fluorescence channel FL-1.

Phosphatidylserine (PS) exposure

PS in the outer membrane leaflet was determined in FACS analysis. Erythrocytes from TRPC6^{-/-} mice and wildtype littermates were washed and incubated for 24 h at 37°C in CaCl_2 (1 mM)-containing NaCl or Na-gluconate Ringer solution. Cells were washed in annexin-V-binding buffer containing (in mM) 125 NaCl, 10 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°C, 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).

RT-PCR

Total RNA (1-2 μg) from erythroid progenitor cells was isolated and pooled using the 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_2O , and stored at -80°C. 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: TGG ATT TCT GAG CTG TTC CA (binding site at TRPC6 mRNA: 2774-2793 nt); antisense primer: CCC TTC GTT CAC TTC ATC ACT (binding site: 3053-3073 nt)], sterilized distilled water up to 50 μl . The PCR conditions were: 1 min at 94°C, 1 min at 57°C, 1 min at 72°C, for 35 cycles and then final 5 min at 72°C. After amplification, PCR products (expected size: 299 bp) 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°C) 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, 10 μl 2-mercaptoethanol. Lysates (100 μg total protein) were separated by 10% SDS-PAGE and blotted on nitrocellulose membranes. After blocking the blots were probed overnight at 4°C 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 the enhanced chemiluminescence (ECL) kit (Amersham, Freiburg, Germany). For Western blots in mouse erythrocytes a rabbit polyclonal anti TRPC6 antiserum (861) was generated against a C-terminal fragment (19 amino acid residues) of mouse TRPC6 (accession number NM_013838) and affinity-purified. The specificity of the antibodies was confirmed using microsomal membrane protein fractions (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. The Western blots using antibody 861 (Fig. 2) were kindly provided by V. Flockerzi, M. Meissner and M. Freichel (Homburg, Germany).

Fig. 1. 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).

Results

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. 1A-C). These cells expressed mRNAs of erythrocyte markers such as α -globin, β -globin or CD71 (Fig. 1D). In addition, RT-PCR revealed transcript levels of the transient receptor potential C6 (TRPC6) channel (Fig. 1E). 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 kDa band in white membranes prepared from human erythrocytes (Fig. 1F, left lane). No band was seen, when the anti-TRPC6 antibody was pre-adsorbed and co-incubated with the immunizing peptide (Fig. 1F, right lane).

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. 2).

To study functional expression of TRPC6 in human erythrocytes, ghosts were prepared from human

Fig. 2. TRPC6 protein expression in mouse erythrocytes: 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 antibody 861. The antibody recognizes at least two TRPC6 variants in COS cells transfected with the mTRPC6 cDNA (lane 1 in A); like in TRPC6 expressing HEK cells [37] these variants apparently represent recombinant TRPC6 proteins differing in their glycosylation patterns. No TRPC6 protein is detected in non-transfected COS 7 cells (lane 2 in A), whereas in microsomal membrane protein fractions from wildtype mouse lung (B, lane 1) and wildtype erythrocytes (B, lane 2) the antibody recognizes the TRPC6 protein, which is absent in TRPC6^{-/-} erythrocytes (B, lane 3).

erythrocytes by hypoosmotic hemolysis. The ghosts maintained a redox-sensitive and EIPA- or amiloride-

Fig. 3. 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 the reducing agent dithiothreitol to prevent oxidizing conditions (DTT; 1 mM) and incubated for 90 min in Ca^{2+} -containing medium. B-C. Time-dependence of the mean Fluo3-fluorescence ($\pm$ 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 ($\pm$ 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). ***: values significantly ($p = 0.001$; two-tailed one sample t-test) different from 1.0.

inhibited Ca^{2+} influx as measured by Fluo3 fluorescence in flow cytometry similar to intact erythrocytes (Fig. 3). As shown in Fig 3A, B, Ca^{2+} uptake of ghosts was blunted in the presence of the reducing agent DTT pointing to the maintenance of an oxidation-activated Ca^{2+} uptake as present in intact erythrocytes. Thus, cation channel function was preserved in the ghost membrane. In K562 human leukemia cells which display certain characteristics of erythrocytes activation of TRPM2 led to increase of intracellular Ca^{2+} concentration resulting in apoptosis [35]. Moreover, upregulation of TRPC3 channels has been observed in smooth muscle cells of TRPC6^{-/-} mice [36]. Therefore, additional experiments were performed with antibodies against these channels. 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. 4). In further experiments, we tested whether the effect of antiTRPC3/6/7-antibody on the Ca^{2+} uptake of ghosts was additive or not to that of amiloride and EIPA. Amiloride (1 mM) and EIPA (50 μM) together decreased the Ca^{2+} increase to $38 \pm 8\%$ ($n = 11$) when normalized to that of the control ($100 \pm 10\%$; $n = 11$; not shown). This inhibition was not different from the inhibitory efficacy of EIPA or amiloride alone (see Fig. 3D, 3rd and 4th bar).

Treating the amiloride + EIPA samples in addition with antiTRPC3/6/7-antibody did not result in a further inhibition of the ghost Ca^{2+} uptake ($34 \pm 6\%$ of the control; $n = 11$ -12) while in these series of experiments antiTRPC3/6/7-antibody alone decreased the Ca^{2+} uptake by the ghost significantly to $79 \pm 12\%$ of the control ($n = 12$; $p = 0.05$, ANOVA; not shown). Taken together, these data strongly suggest that TRPC6 contributes to the amiloride- and EIPA-sensitive cation leak through the human erythrocyte membrane. The non-additivity of the effects does not allow to discriminate between a non-quantitative inhibition of a TRPC channel type by the antibody or the presence of a further amiloride- and EIPA-sensitive channel that is not TRPC3/6/7.

In accordance with the proposed low resting activity of TRPC6 channels [37], erythrocytes from TRPC6^{-/-} and wildtype mice exhibited a similar basal cytosolic free Ca^{2+} concentration as assessed after 24 h of incubation in NaCl Ringer solution by Fluo3/AM fluorescence in flow cytometry (Fig. 5A-C). Decreasing extracellular Cl^- concentration (24 h Cl^- -free) to activate the erythrocyte cation channels increased the cytosolic free Ca^{2+} concentrations in erythrocytes from wildtype mice while having only little effect on cells from TRPC6^{-/-} mice (Fig. 5A-C). In addition, Cl^- depletion (24 h) decreased the forward scatter in flow cytometry of erythrocytes from both genotypes, an effect signifi-

Fig. 4. Effect of inclusion of TRP-specific antibodies by human ghosts on the Ca^{2+} uptake. **A.** Scheme showing the inclusion of TRP-specific antibodies, immunizing blocking peptides, and Fluo3/AM-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 ($\pm$ 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 ($\pm$ 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 ($p = 0.01$; two-tailed one sample t-test) different from 1.0.

cantly blunted in TRPC6^{-/-} erythrocytes as compared to TRPC6^{+/+} erythrocytes (Fig. 5D-F). In contrast, TRPC6^{-/-} and TRPC6^{+/+} erythrocytes had identical forward scatters when incubated for 24 h in NaCl Ringer solution.

To test for an impact of TRPC-dependent Ca^{2+} entry on eryptosis, PS exposure of the erythrocytes was determined by annexin V binding in both, TRPC6^{-/-} and TRPC6^{+/+} erythrocytes. After 24 h of incubation in

NaCl Ringer, TRPC6^{-/-} erythrocytes tended ($p = 0.073$; two-tailed t-test) to have a higher percentage of PS exposing cells ($11 \pm 1\%$; $n = 19$) than TRPC6^{+/+} erythrocyte ($8 \pm 1\%$; $n = 19$; not shown; see also Fig. 5G, open bars). Cl^- depletion increased the percentage PS exposing cells in both, TRPC6^{-/-} and TRPC6^{+/+} erythrocytes (Fig. 5G). This effect was again significantly blunted in TRPC6^{-/-} erythrocytes as compared to TRPC6^{+/+} erythrocytes (Fig. 5G, individual

Fig. 5. 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 24 h in Cl⁻-containing (control) or Cl⁻-depleted medium (chloride-free). C. Mean normalized Fluo3 fluorescence ($\pm$ SE, n = 33-37) of wildtype and TRPC6^{-/-} erythrocytes incubated as in (A) in Cl⁻-containing (control, open bars) or Cl⁻-depleted medium (chloride-free, closed bars) (***: p = 0.001; ANOVA; n.s.: not significantly different). D,E. Histograms showing the forward scatter of erythrocytes drawn from wildtype (D) and TRPC6^{-/-} mice (E) incubated for 24 h in Cl⁻-containing (control) or Cl⁻-depleted medium (chloride-free). F. Mean forward scatter ($\pm$ 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 = 0.01 ANOVA). G. Mean percentage of PS-exposing wildtype and TRPC6^{-/-} erythrocytes ($\pm$ SE; n = 6) incubated for 24 h in Cl⁻-containing (open bars) or in Cl⁻-free medium (closed bars) (***: p = 0.001; ANOVA). H, I. Normalized total PS exposure (H) and Cl⁻-removal-sensitive fraction of PS exposure (I) of wildtype and TRPC6^{-/-} erythrocytes (means $\pm$ SE; n = 19) incubated for 24 h in Cl⁻-free medium (***: p = 0.001; two-tailed t-test).

experiment and 5H). Fig. 5I shows the normalized fractions of PS exposing cells in TRPC6^{-/-} and TRPC6^{+/-} erythrocytes that resulted from Cl⁻ depletion. Taken together, these data indicate functional significance of TRPC6 for the erythrocyte Ca^{2+} leak, erythrocyte volume, and PS exposure.

Discussion

The present paper 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 [32], (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.

Several TRPC channels including TRPC6 have previously been shown to allow the permeation of Ca^{2+} (for review see [38]). 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 [39] and the activation of TRPC3 and TRPC4 by oxidative stress [40]. Anti-TRPC6 and anti-TRPC3/6/7 antibodies, however, only partially inhibit the amiloride/EIPA-sensitive fraction of the ghost Ca^{2+} uptake (compare Figs. 3 and 4). This might be either due to an insufficient inhibition of the channels by the antibodies or to the presence of further amiloride/EIPA-sensitive Ca^{2+} -permeable channels that are different from

TRPC3/6/7. Patch-clamp recordings have clearly indicated more than one type of Ca^{2+} -permeable channel in human erythrocytes [41]. Cl^- -removal shrunk TRPC6^{-/-} and TRPC6^{+/+} erythrocytes differently, suggesting that effluxes of cations through TRPC6 contribute to the loss of $\text{K}(\text{Na})\text{Cl}$ and osmotically obliged H_2O in Cl^- -free medium. The anion conductance charges the erythrocyte membrane to high positive voltages when the cells were suspended in Cl^- -free medium. Positive voltages reportedly activate a Ca^{2+} -permeable non-selective cation channel [20, 24] which might be different from TRPC6. Accordingly, in the present study, TRPC6^{-/-} erythrocytes shrank considerably in Cl^- -free medium hinting to a TRPC6-independent voltage-gated cation leak.

In the present study, erythrocytes from TRPC6^{-/-} and wildtype mice exhibited a similar percentage of PS exposing cells when incubated for 24 h in NaCl Ringer solution. This is in accordance with the observed comparable free Ca^{2+} concentration and forward scatter of these cells. Removal of extracellular Cl^- induced PS exposure in erythrocytes from both mouse lines, pointing to an TRPC6-independent trigger of PS exposure. The Cl^- -depletion-induced increase in cytosolic free Ca^{2+} did not reach significance in TRPC6^{-/-} erythrocytes while increased significantly in wildtype erythrocytes. Steady state free Ca^{2+} concentrations result from the equilibrium between Ca^{2+} leak and Ca^{2+} pump activity and pump activity increases with increasing Ca^{2+} leak [42]. Therefore, unchanged free Ca^{2+} concentrations in TRPC6^{-/-} cells do not necessarily indicate unchanged Ca^{2+} leak and pump activities. Nevertheless, the TRPC6-independent trigger of PS exposure is rather independent of high cytosolic free Ca^{2+} concentrations. PS exposure in shrunken TRPC6^{-/-} erythrocytes (and also partially in wildtype erythrocytes) in Cl^- -free medium was probably mediated through sphingomyelinase activation. The resulting ceramide formation reportedly stimulates PS exposure in a largely Ca^{2+} -independent manner. [43]. The most important finding of the present study, however, is that Cl^- -depletion elicited PS exposure in an extremely significant higher percentage of wildtype than TRPC6^{-/-} erythrocytes indicating that TRPC6 participates in the Ca^{2+} -mediated stimulation of eryptotic cell death.

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 [36]. In the present study antibodies against TRPC3 did not delay Ca^{2+}

increase when included into the human ghosts while the same antibodies inhibited whole-cell and single channel currents in rabbit ear-artery-myocytes [44] arguing against a compensatory upregulation of TRPC3. Finally, TRPC form heteromultimeric channels [45] 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 eryptosis. TRPC6 channels reportedly have a very low or even missing basal activity [37]. Similarly, cation channels are almost not detectable in unstressed and unstimulated human erythrocytes by electrophysiological means [8]. Accordingly, the basal Ca^{2+} permeability of human erythrocytes measured by $^{45}\text{Ca}^{2+}$ tracer flux is many orders of magnitude lower than the basal Ca^{2+} permeability of nucleated cells [46]. Since erythrocytes lack a high affinity Ca^{2+} buffer [47], the stress-stimulated activation of TRPC6 channels may result in a significant increase in erythrocytic free Ca^{2+} concentration.

It is intriguing to speculate that a prolonged increase in erythrocyte-free Ca^{2+} concentration is indicative of the inability of the cell to rebuild and maintain its electrolyte gradients. Eryptosis may serve to prevent colloid osmotic hemolysis since Ca^{2+} -stimulated activation of the Gardos K^+ channel delays colloid osmotic swelling of defective erythrocytes [11]. Moreover, Ca^{2+} triggers scrambling of the cell membrane with exposure of phosphatidylserine at the cell surface. Phosphatidylserine in the external membrane leaflet reportedly is an 'eat-me signal' contributing to the recognition and phagocytosis of apoptotic nucleated cells by macrophages which are equipped with phosphatidylserine receptors [15, 48]. Phosphatidylserine exposure parallels the time-dependent clearance of biotin-labeled rabbit erythrocytes *in vivo* [16]. Moreover, erythrocytes are engulfed rapidly by macrophages *in vitro* [49] and cleared *in vivo* from peripheral blood after inserting a synthetic fluorescent phosphatidylserine analogue into the erythrocyte membrane [50].

Thus, eryptosis may be an important mechanism to clear erythrocytes prior to detrimental hemolysis. Therefore, stress-stimulated TRPC6 activation may prevent colloid osmotic hemolysis of, e.g., injured, energy-depleted, or oxidized erythrocytes prior to their recognition by macrophages [51]. On the other hand, pathologically enhanced eryptosis might contribute to anemia [51-60].

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 eryptosis. 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.

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