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Cation channels trigger apoptotic death of erythrocytes

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Abstract

Erythrocytes are devoid of mitochondria and nuclei and were considered unable to undergo apoptosis. As shown recently, however, the Ca^{2+}-ionophore ionomycin triggers breakdown of phosphatidylserine asymmetry (leading to annexin binding), membrane blebbing and shrinkage of erythrocytes, features typical for apoptosis in nucleated cells. In the present study, the effects of osmotic shrinkage and oxidative stress, well-known triggers of apoptosis in nucleated cells, were studied. Exposure to 850 mOsm for 24 h, to tert-butyl-hydroperoxide (1 mM) for 15 min, or to glucose-free medium for 48 h, all elicit erythrocyte shrinkage and annexin binding, both sequelae being blunted by removal of extracellular Ca^{2+} and mimicked by ionomycin (1 μM). Osmotic shrinkage and oxidative stress activate Ca^{2+}-permeable cation channels and increase cytosolic Ca^{2+} concentration. The channels are inhibited by amiloride (1 mM), which further blunts annexin binding following osmotic shock, oxidative stress and glucose depletion. In conclusion, osmotic and oxidative stress open Ca^{2+}-permeable cation channels in erythrocytes, thus increasing cytosolic Ca^{2+} activity and triggering erythrocyte apoptosis.

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Keywords: cell volume; Annexin; osmotic cell shrinkage; oxidation; tert-butyl-hydroperoxide; glucose depletion; calcium

Abbreviations: tBOOH, Tert-butyl-hydroperoxide

Introduction

Similar to other cell types, erythrocytes have to be eliminated after their physiological life span.[1] Beyond this, mechanisms are required for the removal of defective erythrocytes. In other cell types, the primary mechanism of clearance is apoptosis.[2,3] Until very recently, erythrocytes have been considered unable to undergo apoptosis, as they lack mitochondria and nuclei, key organelles in the apoptotic machinery of other cells.[1] However, most recent observations revealed that treatment of erythrocytes with the Ca^{2+}-ionophore ionomycin leads to cell shrinkage, cell membrane blebbing and annexin binding, all typical features of apoptosis in other cell types.[1,4,5]

The present study has been performed to test whether erythrocyte annexin binding could be induced by osmotic shock or oxidative stress, well-known triggers of apoptotic death of other cell types.[6–12] It is indeed shown that both challenges lead to annexin binding. Further experiments have been done to elucidate the cellular mechanisms involved. It is shown that the effect of osmotic shock and oxidative stress is dependent on the presence of Ca^{2+} and mimicked by stimulation of Ca^{2+} entry with ionomycin, that osmotic shock and oxidative stress, both, open Ca^{2+}-permeable cation channels and increase cytosolic Ca^{2+} concentration, and that amiloride, an inhibitor of the cation channels, blunts the stimulation of annexin binding following osmotic shock or oxidative stress.

Results

Osmotic shock and oxidative stress activate a calcium-permeable cation channel

Whole-cell recordings of untreated erythrocytes show a low conductance in the range of 0.1–2 nS, reflecting the low resting channel activity within the erythrocyte cell membrane (Figure 1). As shown in Figure 1a and c, both, osmotic cell shrinkage by addition of sucrose and oxidative stress by addition of 1 mM of the oxidant tert-butyl-hydroperoxide (tBOOH) decrease the erythrocyte cell membrane resistance. Figure 1b illustrates the I/V relation of the activated current after cell shrinkage. In additional experiments, the channel characteristics were defined. Figure 1d and e reveal the Ca^{2+} conducting property of the channel. The channel is inhibited by high concentrations (1 mM) of amiloride (Figure 1F). From these results, we conclude that osmotic and/or oxidative shock activate a calcium-permeable cation channel in the erythrocyte cell membrane.

Osmotic shock, oxidative stress and glucose depletion increase cytosolic calcium content

In untreated erythrocytes the total cellular Ca^{2+} content $[Ca_T]_i$ was approximately 2 μmol/10^{13} cells at an extracellular Ca^{2+} concentration of 150 μM (Figure 2). Addition of the Ca^{2+} ionophore ionomycin (1 μM) led to a rapid and sustained increase of cellular $[Ca_T]_i$ (Figure 2a). Increase of the extracellular osmolarity to 850 mOsm led within 30 min to a doubling of $[Ca_T]_i$ (Figure 2b). A similar increase of $[Ca_T]_i$

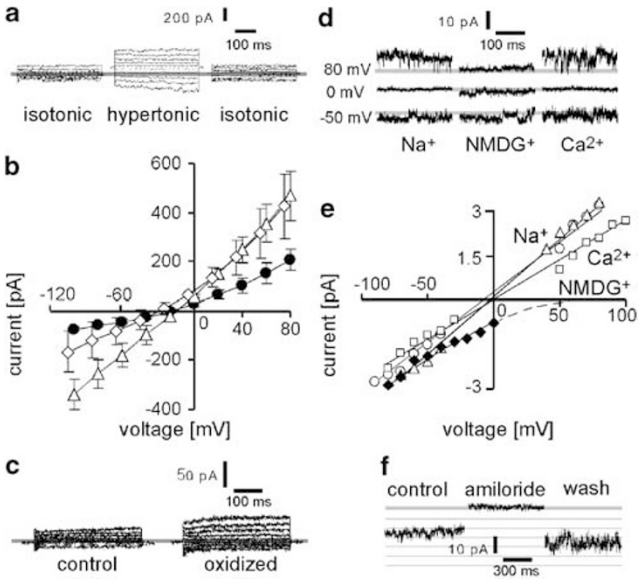

Figure 1 Cell-shrinkage- and oxidative stress-induced nonselective cation channels in human erythrocytes. (**a**) Original whole-cell current traces (K-gluconate/KCl pipette solution) recorded with isotonic bath solution, after addition of 400 mM sucrose to the bath solution (hypertonic), and after wash-out of sucrose by isotonic bath solution. The zero current is indicated by the grey line. (**b**) Current-voltage relationships recorded as in (**a**) with isotonic (closed circles) and hypertonic NaCl bath solution (+400 mM sucrose; open triangles) and after replacement of NaCl by NMDG-Cl (+400 mM sucrose, open diamonds). Data are means $\pm$ S.E.M.; n=3. (**c**) Original whole-cell current traces (NaCl pipette solution) recorded with isotonic NaCl bath solution, before (left) and after applying oxidative stress (1 mM *t*-BOOH for 10 min). (**d**) Single channel current transitions recorded in the excised-patch, inside-out mode at various voltages with KCl pipette and Na-gluconate (Na^+), NMDG-gluconate ($NMDG^+$), and Ca-$(gluconate)_2$ bath solution (Ca^{2+}), respectively. The closed state of the channels is indicated by grey lines. (**e**) Current–voltage relationships as recorded in (**d**) with Na^+ (open circles and triangles), Ca^{2+} (open squares), and $NMDG^+$ (closed diamonds) as principal cation in the bath solution. (**f**) Original current tracings of an outside-out patch recorded at -100 mV voltage (KCl pipette and Na-gluconate bath solution before (control), during (amiloride) and after (wash-out) applying amiloride in the bath solution. Thick grey line indicates zero current

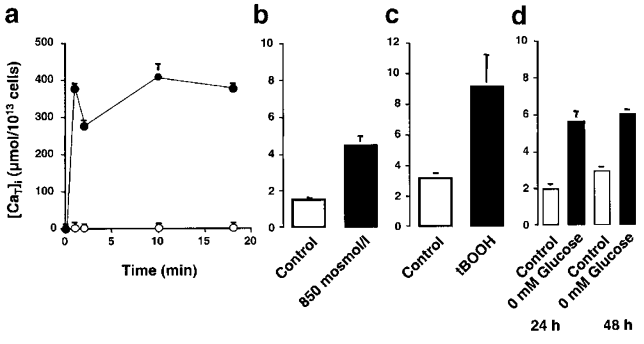

Figure 2 Increase of intracellular Ca^{2+} content $[Ca_T]_i$ following exposure to ionomycin, osmotic shock, oxidative stress or glucose depletion. Arithmetic means $\pm$ S.E.M. (n=3) of intracellular Ca^{2+} content $[Ca_T]_i$ following exposure of erythrocytes to 1 μM ionomycin (**a**, closed symbols), to osmotic shock (**b**, 850 mOsm buffer for 30 min), oxidative stress (**c**, 1 mM tBOOH for 10 min) or to glucose depletion for 24 h or 48 h (**d**). Controls (open symbols in **a**, open columns in **b–d**) indicate respective values in the absence of ionomycin, osmotic shock, oxidative stress and presence of 5 mM glucose. Values of $[Ca_T]_i$ are given as μmol/10^{13} cells

was observed following a 10 min exposure to 1 mM tBOOH (Figure 2c) and a 24 or 48 h exposure to glucose-free buffer (Figure 2d).

Increase of cytosolic calcium by ionomycin induces erythrocyte apoptosis

In order to test the hypothesis that stress-induced opening of calcium-permeable channels in the erythrocyte membrane leads to activation of the apoptotic programme in this cell type, we used the calcium-ionophore ionomycin. As shown in the scatter plots in Figure 3a, the addition of 1 μM ionomycin led within 3 h to marked calcium-dependent cell shrinkage. A decrease of forward scatter from a value of 428 $\pm$ 13 (n=3) in control cells to 121 $\pm$ 2 (n=4) in ionomycin-treated cells was observed. Moreover, exposure of erythrocytes to 1 μM

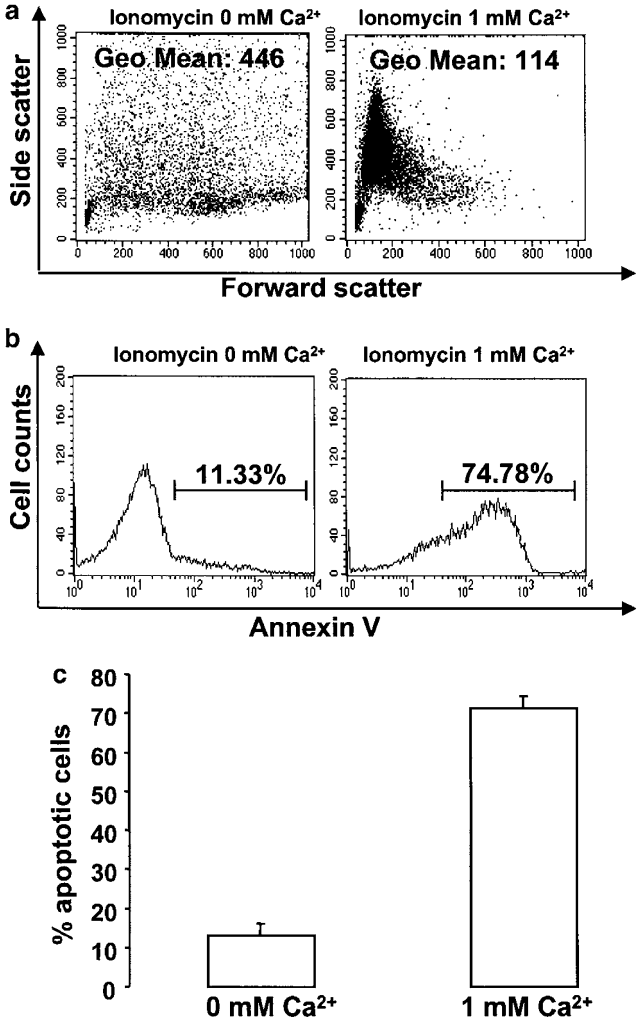

Figure 3 Ionomycin induced erythrocyte apoptosis. (**a**) Effects of 1 μM ionomycin after 3 h of incubation on cell volume without Ca^{2+} (left panel) and with Ca^{2+} (right panel) in the extracellular solution as evidenced from forward scatter analysis. Geometric mean values are given for a single experiment. (**b**) Phosphatidylserine asymmetry after 3 h of ionomycin treatment, as evidenced from annexin binding without Ca^{2+} (left panel) and with Ca^{2+} (right panel) in the extracellular solution (**c**). Arithmetic means ($\pm$S.E.M.) of annexin binding in cells exposed to 1 μM ionomycin for 3 h without and with Ca^{2+} in the extracellular fluid

ionomycin enhanced the annexin binding from 4.1±0.4% (n=3) in control cells to 71.3±3.2% (n=4) in ionomycin-treated cells. A typical experiment is shown in Figure 3b. Ionomycin-induced cell shrinkage and annexin binding are both significantly blunted in the nominal absence of extracellular calcium (Figure 3a,b). The respective values were 337±16 (n=4) for the forward scatter and 12.9±3.1% (n=4) for the annexin binding (Figure 3c). Thus, treatment of erythrocytes with ionomycin elicits two effects typical for apoptosis, i.e, phosphatidylserine exposure and cell shrinkage.

Osmotic shock induces erythrocyte annexin binding

Osmotic shock has been shown to induce apoptosis in different nucleated cell types.[6,8–12] To test for triggering of apoptotic cell shrinkage and annexin binding, cells were exposed to osmotic shock (preincubation in 850 mOsm by addition of sucrose for 24 h). Following this incubation, the cells were incubated in isotonic solution for 20 min containing the fluorescent annexin and measured in the FACS Calibur for forward scatter, side scatter and annexin binding. Following this procedure the cells remained slightly shrunken (Figure 4a), as reflected by a decrease of the forward scatter from 421±25 (n=5) to 336±20 (n=5). Removal of extracellular Ca^{2+} did not blunt cell shrinkage (327±21, n=3). In the presence of amiloride (1 mM) cell volume approached 378±11 (n=3).

Osmotic shock also increased annexin binding (Figure 4b–d). Exposure to 850 mOsm/l led to an increase of annexin-binding cells from 3.9±0.3% (n=4) to 51.4±3.8% (n=9) within 24 h. A typical experiment is depicted in Figure 4b. In the nominal absence of calcium, the effect of osmotic shock

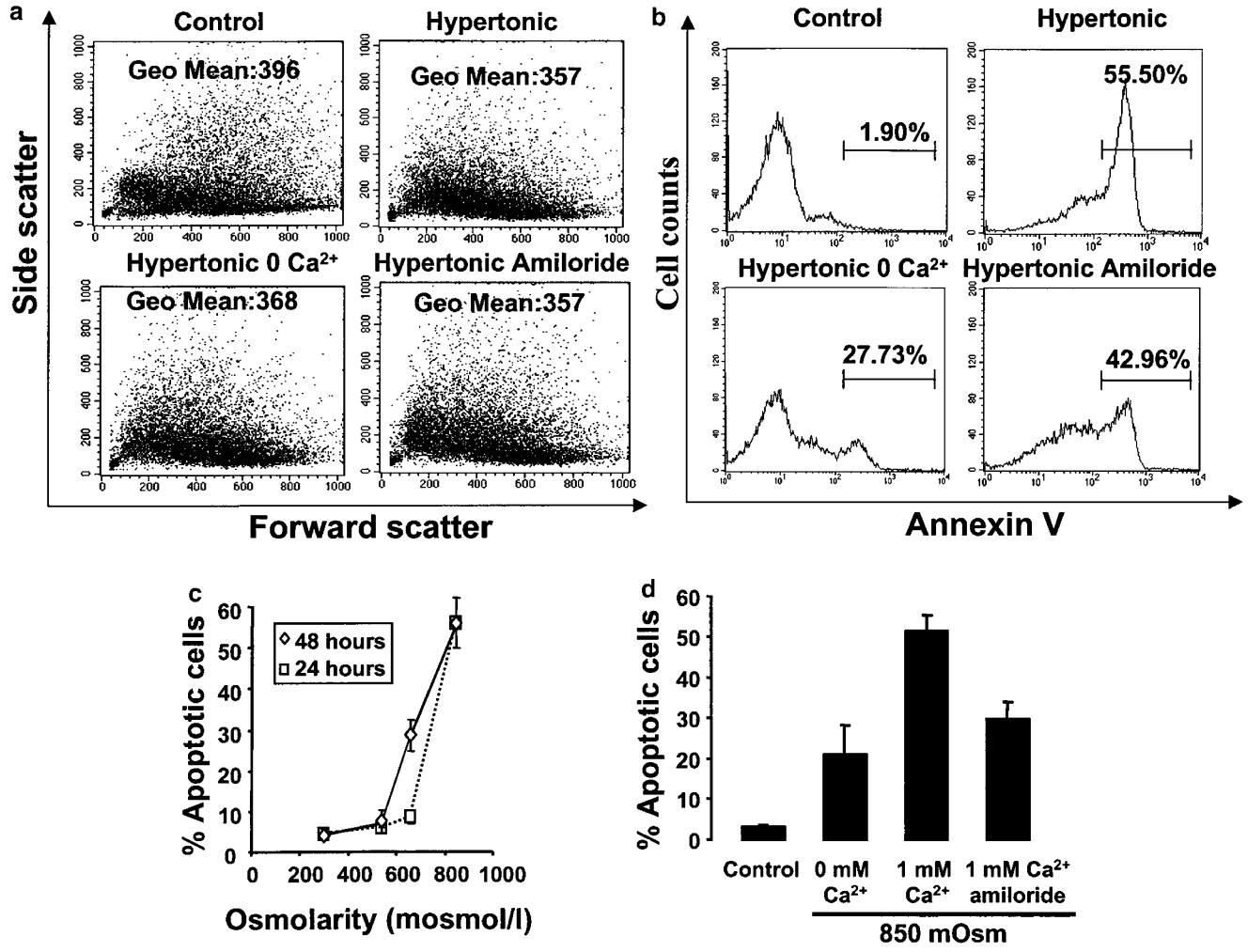

Figure 4 Erythrocyte apoptosis induced by osmotic cell shrinkage. (**a**) Effects of preincubation in hyperosmolar solution on cell volume with Ca^{2+} (upper right panel), without Ca^{2+} (lower left panel) and with amiloride (lower right panel) in the extracellular solution. After 24 h incubation cells were incubated in isotonic solution for 20 min and measured in the FACS calibur for forward scatter and side scatter. Geometric mean values are given for single experiments. (**b**) Annexin binding of erythrocytes in isotonic medium (upper left panel) and in 850 mOsm medium after adding sucrose for 24 h is illustrated (upper right panel). Cells were shrunken without Ca^{2+} (lower left panel) or with 1 mM Ca^{2+} and 1 mM Amiloride (lower right panel). (**c**) Arithmetic means (±S.E.M.) of annexin binding in cells at different osmolarities for 24 h and 48 h. (**d**) Arithmetic means (±S.E.M.) of annexin binding in cells exposed for 24 h to 850 mOsm with or without Ca^{2+} or in the presence of Ca^{2+} with 1 mM amiloride. Control refers to erythrocytes exposed to an isotonic buffer solution for 24 h

on annexin binding was significantly blunted to 21.1±7.2% (n=4, see Figure 4b for a typical experiment and Figure 4d for arithmetic means±S.E.M). Moreover, the cation channel blocker amiloride (1 mM) decreased the number of annexin binding cells significantly to 29.8±4.0% (n=5, see Figure 4b for a typical experiment and Figure 4d for arithmetic means±s.e.m.).

Oxidative stress induces erythrocyte apoptosis

Induction of oxidative stress by addition of 0.66 mM tBOOH or 1 mM tBOOH led to marked shrinkage of erythrocytes, as reflected by a decrease of the forward scatter from 370±13 (n=4) to 314±51 (n=4) and 167±14 (n=4), respectively. Figure 5a depicts typical scatter plots after oxidation of cells. In another set of experiments, we could show that treatment of erythrocytes for 15 min with 0.66 mM tBOOH and further incubation for 24 h induced significant annexin binding (Figure 5b). As shown in Figure 5c, 0.66 mM and 1 mM tBOOH increased the number of annexin binding cells from 3.2±0.5% (n=4) in control cells to 26±7.5% (n=4) and 68.5±5.3% (n=4), respectively. Interestingly, the oxidation-induced cell shrinkage and annexin binding were both blunted in the nominal absence of extracellular calcium (Figure 5a,b). The values for forward scatter in calcium-free incubation media approached 339±22 (n=4) (0.66 mM tBOOH) and 307±15 (n=4) (1 mM tBOOH), as compared with 314±51 (n=4) and 167±14 (n=4) in the presence of calcium, respectively. In this line, the number of annexin-positive cells in the absence of calcium amounted to only 11.0±1.4% (n=4) (0.66 mM tBOOH) and 34.1±2.6% (n=4) (1 mM tBOOH). Accordingly, removal of extracellular Ca^{2+} inhibited tBOOH-induced phosphatidylserine exposure by about 66% and 53%, respectively (Figure 5c). Similarly, the presence of 1 mM

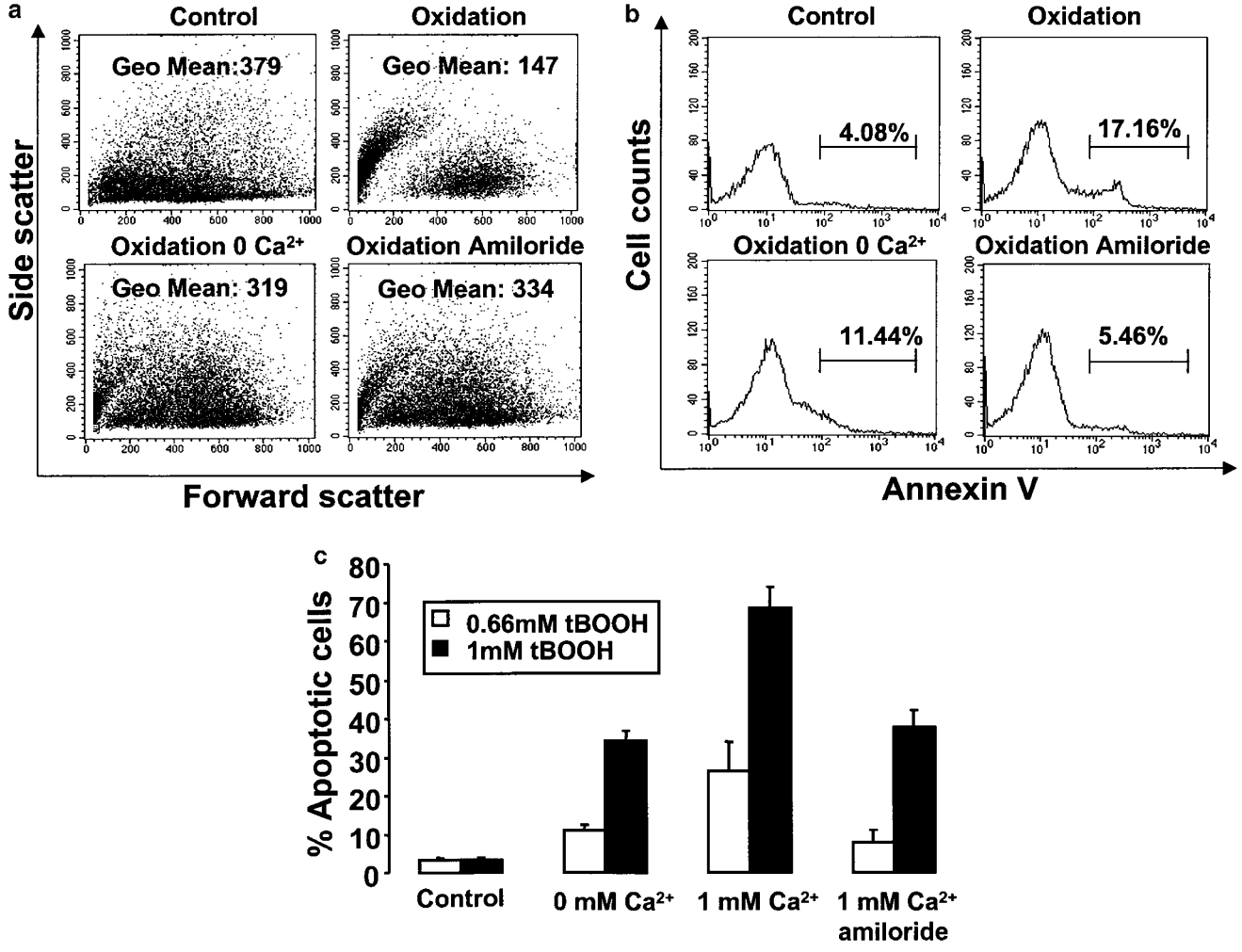

Figure 5 Erythrocyte apoptosis induced by oxidation. (**a**) Effects of oxidation with 1 mM tBOOH for 15 min and further incubation for 24 h on cell volume with Ca^{2+} (upper right panel), without Ca^{2+} (lower left panel) and with amiloride (lower right panel) in the extracellular solution as evidenced from forward scatter analysis. Geometric mean values are given for single experiments. (**b**) Effects on annexin binding without (upper left panel) and with oxidation (upper right panel) with 0.66 mM tBOOH for 15 min and further incubation for 24 h. Erythrocytes were oxidized in Ringer solution with 1 mM Ca^{2+} (upper right panel) or without Ca^{2+} (lower left panel) or in the presence of 1 mM Ca^{2+} and 1 mM amiloride (lower right panel). (**c**) Arithmetic means (±S.E.M.) of annexin binding in cells after exposure to 1 mM tBOOH or 0.66 mM tBOOH for 15 min and further incubation for 24 h, either in the presence or in the absence of 1 mM Ca^{2+} or in the presence of 1 mM Ca^{2+} with 1 mM amiloride are given. Control refers to erythrocytes exposed to oxidant free buffer solution for 24 h

amiloride blunted the effect of tBOOH on cell volume (Figure 5a) and annexin binding (Figure 5b) even in the presence of 1mM Ca^{2+}. The respective values of forward scatter amounted to 356±24.0 (n=4) (0.66 mM tBOOH) and 291±27 (n=4) (1mM tBOOH). In the presence of 1 mM amiloride, annexin-positive cells were reduced to 7.9±3.2% (0.66 mM tBOOH) and 37.7±4.4% (1 mM tBOOH), which reflects an inhibition of tBOOH-induced annexin binding by 80 and 48%, respectively (Figure 5c).

Glucose depletion induces erythrocyte apoptosis

As antioxidative defence requires energy and thus depends on glucose supply to erythrocytes,[13,14] the effect of glucose removal has been tested (see Figure 6a for individual experiments). In the presence of glucose, 3.1±0.6% (n=4) of the erythrocytes bound annexin. Exposure to glucose-free medium increased the number of annexin binding cells to 11.2±2.2% (n=4) after 24 h and to 49.4±5.7% (n=4) after 48 h (Figure 6b). The increase of annexin binding was significantly blunted in the nominal absence of calcium. The respective values were 10.4±2.5% (n=4) after 24 h and 10.0±1.9% (n=4) after 48 h. Similarly, the effect of glucose depletion was inhibited in the presence of 1 mM amiloride. The respective values were 5.7±1.8% (n=4) after 24 h and 11.7±1.9% (n=4) after 48 h (Figure 6b).

Ionomycin, osmotic shock, oxidative stress and glucose depletion all decrease erythrocyte number

The number of erythrocytes was significantly decreased by an exposure to 1 μM ionomycin for 16 h, by a 24 h exposure to 850 mOsm, by a 15 min exposure to 1 mM tBOOH and further incubation for 24 h in oxidant-free buffer and by a 48 h exposure to glucose-free buffer. In the absence of extracellular Ca^{2+}, the decline of cell number was significantly blunted (Figure 7) thereby confirming the results of the annexin-binding assay.

Discussion

The present study demonstrates that oxidative and osmotic stresses, well-known triggers of apoptotic death of nucleated cells,[3,10,12] are similarly powerful stimuli of erythrocyte apoptosis. Even though erythrocytes lack nuclei and mitochondria, they are capable of undergoing some of the morphological features of apoptosis, such as external exposure of phosphatidylserine, membrane blebbing and cell shrinkage.[1] All these events are triggered by increase of cytosolic calcium activity,[4,5] while erythrocytes are resistant to serum deprivation and staurosporine, known triggers of apoptosis in nucleated cells.[1]

The present paper further provides evidence for the involvement of amiloride sensitive, cell volume regulated cation channels in the induction of apoptotic cell death by both osmotic cell shrinkage and oxidative stress. The channels have previously been characterized and shown to be inhibited by amiloride.[15,16] Both, osmotic and oxidative stresses open the channel. The effect of both osmotic and oxidative stress is

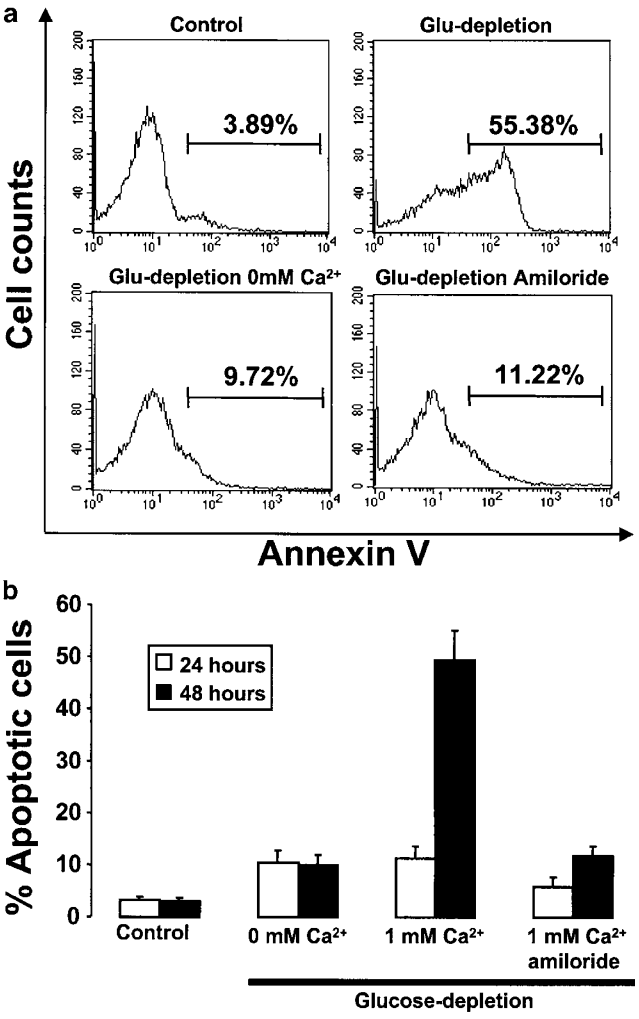

Figure 6 Erythrocyte apoptosis induced by glucose depletion. (**a**) Annexin binding of erythrocytes after 48 h of incubation in the presence (upper left panel) or in the absence of glucose (upper right panel). Cells were additionally incubated in the absence of glucose either without Ca^{2+} (lower left panel) or with 1 mM Ca^{2+} and 1 mM amiloride (lower right panel). (**b**) Arithmetic means (±S.E.M.) of annexin binding in cells incubated for 24 h and 48 h in the absence of glucose, either with or without Ca^{2+} depletion or in the presence of 1 mM Ca^{2+} with 1 mM amiloride. Control refers to cells incubated in the presence of glucose and Ca^{2+} for 24 h and 48 h

mimicked by the addition of the Ca^{2+} ionophore iomomycin in the presence, but not the absence of extracellular Ca^{2+}. Moreover, amiloride and decrease of extracellular Ca^{2+} blunt the effects of osmotic and oxidative stress on annexin binding. Thus, it appears safe to conclude that osmotic and oxidative stresses trigger erythrocyte apoptosis at least in part by stimulating the cation channel and thus increasing cytosolic Ca^{2+} activity.

Similar to osmotic stress, oxidative stress leads to marked erythrocyte shrinkage, an effect probably resulting from activation of the Ca^{2+}-sensitive K^+ channel in the erythrocyte cell membrane, which leads to hyperpolarization of the cell membrane and subsequent erythrocyte loss of KCl.[17–19]

The mechanisms described here could well participate in the limitation of erythrocyte survival. The phosphatidylserine

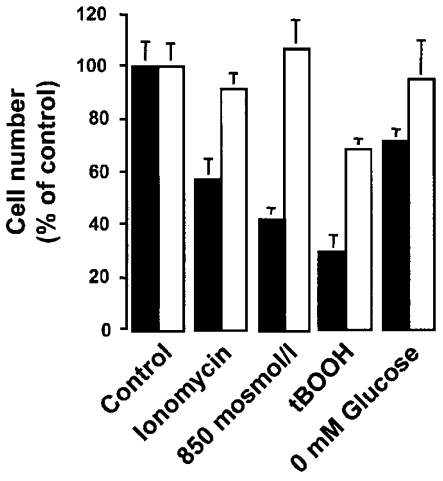

Figure 7 Decrease of erythrocyte number by exposure to ionomycin, osmotic shock, oxidative stress and glucose depletion. Arithmetic means ± S.E.M. (n=3) of erythrocyte number in % of control following exposure to ionomycin (1 μM) for 16 h, to hyperosmolarity of 850 mOsm for 24 h, to tBOOH (1 mM) for 15 min and subsequent incubation for 24 h or to glucose-free buffer for 48 h, in the presence (closed bars) or absence (open bars) of 1 mM Ca^{2+}. Erythrocyte numbers under control conditions in the presence and absence of Ca^{2+} were $(1.7 \pm 0.3) \times 10^8$ cells/ml and $(1.6 \pm 0.16) \times 10^8$ cells/ml, respectively

exposure at the cell surface is thought to stimulate the uptake by macrophages.[20,21] Thus, to the extent that calcium triggers the breakdown of phosphatidylserine asymmetry, an increase of cytosolic Ca^{2+} activity is expected to trigger the clearance of the affected erythrocytes.[1] This may be important for erythrocyte ageing, which is paralleled by increase of cytosolic Ca^{2+} activity.[21,22] Moreover, according to the present results, oxidative stress or defects of antioxidative defence[23] clearly enhance Ca^{2+} entry via the cation channels. This leads to higher intracellular Ca^{2+} concentrations and thus accelerates erythrocyte apoptosis and clearance. During passage of the renal medulla, erythrocytes are exposed to excessive osmolarities sufficient to activate the cation channel. Normally, the exposure is too short, though, to trigger apoptosis. Nevertheless, it is noteworthy that during acute renal failure erythrocytes may be trapped in renal medulla.[24] The subsequent erythrocyte apoptosis may then contribute to the derangement of microcirculation. Beyond this any erythrocyte disorder facilitating erythrocyte shrinkage, such as sickle cell disease,[8,25] thalassemia[26] or iron deficiency,[27] could, to the extent as it leads to activation of the cell volume regulatory cation channels, trigger premature apoptosis and thus accelerate erythrocyte death.

The volume regulatory cation channels are not only expressed in erythrocytes but in several nucleated cells.[28–34] As an increase of cytosolic Ca^{2+} could similarly induce apoptotic cell death in nucleated cells,[2] activation of the volume regulated cation channels could similarly participate in the triggering of apoptosis in nucleated cells exposed to an osmotic shock.[3,9–12]

In summary, we conclude from our results that erythrocyte apoptosis can be induced by different stimuli, such as osmotic shock or oxidative stress, an effect at least partially due to activation of calcium-permeable cation channels. The present data thus disclose a physiological mechanism that may indeed be relevant for the half-life and the turnover of this highly specialised cell type.

Materials and Methods

Solutions

Erythrocytes were drawn from healthy volunteers. Erythrocytes were either used without purification or after separation by centrifugation for 25 min; 2000 g over Ficoll (Biochrom KG, Berlin, Germany). Experiments with nonpurified or experiments with Ficoll-separated erythrocytes yielded the same results (data not shown). Experiments were performed at 37°C in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES), 5 glucose, 1 $CaCl_2$; pH 7.4. For the nominally calcium-free solution $CaCl_2$ was replaced by 1mM ethylene glycol-bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA). Osmolarity was increased to 850 mM by adding sucrose. Ionomycin was used at a concentration of 1 μM, amiloride at a concentration of 1 mM. The final concentration of the solvent dimethyl sulfoxide DMSO was in both cases 0.1%. Ionomycin, amiloride and leupeptin were purchased from Sigma (Taufkirchen, Germany). $^{45}Ca^{2+}$ was from ICN Biomedicals GmbH (Eschwege, Germany) and delivered as $CaCl_2$ in aqueous solution (specific activity: 0.185–1.11 TBq/g Ca).

Patch clamp

Patch-clamp experiments were performed according to Hamill *et al.*[35] RBCs were recorded at 35°C. A continuous superfusion was applied through a flow system inserted into the dish. The bath was grounded via a 2% agarose bridge filled with pipette solution (see below). Borosilicate glass pipettes (9 MΩ tip resistance; GC 150 TF-10, Clark Medical Instruments, Pangbourne, UK) manufactured by a microprocessor-driven DMZ puller (Zeitz, Augsburg, Germany) were used in combination with an MS314 electrical micromanipulator (MW, Märzhäuser, Wetzlar, Germany). The currents were recorded in voltage-clamp mode in fast-whole-cell, inside-out and outside-out configuration, respectively, by an EPC-9 amplifier (Heka, Lambrecht, Germany) using Pulse software (Heka) and an ITC-16 Interface (Instrutech, Port Washington, NY, USA). The whole-cell currents were evoked by a pulse protocol, clamping the voltage in 11 successive 400-ms square pulses from the −10 mV holding potential to potentials between −100 mV and +100 mV.

Whole-cell currents were recorded first in standard isotonic bath solution [containing in mM: 115 NaCl, 10 HEPES, 5 KCl, 5 $CaCl_2$, 10 $MgCl_2$, titrated with NaOH to pH 7.4] in combination with a pipette solution containing (in mM): 60 K-D-gluconate, 80 KCl, EGTA, 1 $MgCl_2$, 1 Mg-ATP, and 10 HEPES, titrated to pH 7.2 with KOH. The whole-cell currents were further recorded during cell shrinkage after addition of 400 mM sucrose to the bath and after replacement of Na^+ in the bath by the impermeable cation $NMDG^+$.

In a further series of whole-cell experiments, a pipette solution containing (in mM) 120 NaCl, 5 HEPES/NaOH, 1 EGTA, 1 Mg-ATP; pH 7.2 was combined with the standard NaCl bath solution. Currents were measured at room temperature before and during oxidative stress applied by adding 1 mM tBOOH to the bath solution.

Excised patch, inside out and outside-out recordings were obtained with a pipette solution containing (in mM) 133 KCl, 3 EGTA, 1.78 $MgCl_2$, 1.13 $CaCl_2$, 1 K_2ATP, and 10 HEPES, titrated to pH 7.2 with KOH combined with standard isotonic NaCl bath solution. Currents through the excised

patches were characterized by replacing NaCl in the bath by equiosmolar amounts of Na-gluconate, NMDG-gluconate, and Ca-$(gluconate)_2$ or by applying amiloride (1 mM) to the bath solution.

The offset potentials between both electrodes were zeroed before sealing. The potentials were corrected for liquid junction potentials as estimated according to Barry and Lynch.[36] The original whole-cell current traces are depicted after 500 Hz low-pass filtering and currents of the individual voltage square pulses are superimposed. The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The inward currents, defined as flow of positive charge from the extracellular to the cytoplasmic membrane face, are negative currents and depicted as downward deflections of the original current traces.

Measurement of calcium uptake

Calcium uptake was measured as described in detail elsewhere.[37,38] Erythrocytes were washed four times by centrifugation ($2000 \times g$ for 5 min) and resuspended in five volumes of solution A containing in mM: 80 KCl, 70 NaCl, 10 HEPES, 0.2 $MgCl_2$, 0.1 EGTA; pH 7.5 to remove extracellular Ca^{2+}. The cell pellet was then washed twice in solution B to remove EGTA from the medium. Solution B had the same composition as solution A, but without EGTA. The cells were suspended at 10% haematocrit and preincubated for 20 min at 37°C in the final incubation solution B supplemented with 10 mM inosine and 1 mM sodium orthovanadate. Then $^{45}Ca^{2+}$ was added from a 100 mM $CaCl_2$ stock solution with a specific activity of about 10^7 cpm μmol to reach an end concentration of 150 μM. After different times, 100 μl aliquots were delivered into 1.2 ml of ice-cold solution B with 0.2 mM $CoCl_2$ and 1 mM amiloride. The cells were collected by centrifugation in an Eppendorf centrifuge (14 000 rpm for 0.5 min, 4°C) and the cell pellet was washed twice using 1 ml of the same medium. The supernatant was discarded and the cells were lysed and the proteins precipitated by addition of 0.6 ml 6% trichloroacetic acid (TCA). After a further spin (14 000 rpm for 2 min, 4°C), 0.5 ml of clear supernatant was used for measuring $^{45}Ca^{2+}$ radioactivity by scintillation counting. $^{45}Ca^{2+}$-specific activity was determined by addition of 0.6 ml 6% TCA to 100 μl suspension samples and centrifugation as described above. Then, 100 μl of clear supernatant were taken for scintillation counting. The total calcium content of the cells $[Ca_T]_i$ was calculated by dividing the activity of the samples by the specific activity of $^{45}Ca^{2+}$ and by the number of cells.

Ionomycin (1 μM) and 1 mM tBOOH were added to the cell suspensions together with $^{45}Ca^{2+}$. Exposure of erythrocytes to 850 mOsm was achieved by addition of sucrose to solution B during 20 min of preincubation and 10 min of $^{45}Ca^{2+}$ uptake. Note that the delivery medium for washing the cells after radioactive labelling was also adjusted to 850 mOsml/l by addition of sucrose. Glucose depletion was achieved by preincubating the cells in Ringer solution (5% haematocrit) for 24 and 48 h at 37°C in the absence of glucose. Control cells were preincubated in the presence of 5 mM glucose.

FACS analysis

FACS analysis was performed essentially as described.[39] After incubation, cells were washed in annexin-binding buffer containing (in mM) 125 NaCl, 10 HEPES, pH 7.4, and 5 $CaCl_2$. Erythrocytes were stained with Annexin-Flous (Böhringer Mannheim, Germany) at a 1 : 100 dilution. After 15 min, samples were diluted 1 : 5 and measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson). Cells were analysed by forward and sideward scatter and annexin-fluorescence intensity was measured in FL-1.

Determination of cell numbers

Erythrocytes were suspended at 2% haematocrit and incubated under different control and stress conditions (1 μM ionomycin, osmotic and oxidative stress, glucose depletion). After incubation, the cell number was determined using a hemocytometer as described previously.[40]

Statistics

Data are expressed as arithmetic means $\pm$ S.E.M. and statistical analysis was made by paired or unpaired *t*-test, where appropriate.

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