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# Cation channels, cell volume and the death of an erythrocyte

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**Abstract** Similar to a variety of nucleated cells, human erythrocytes activate a non-selective cation channel upon osmotic cell shrinkage. Further stimuli of channel activation include oxidative stress, energy depletion and extracellular removal of $Cl^-$. The channel is permeable to $Ca^{2+}$ and opening of the channel increases cytosolic [$Ca^{2+}$]. Intriguing evidence points to a role of this channel in the elimination of erythrocytes by apoptosis. $Ca^{2+}$ entering through the cation channel stimulates a scramblase, leading to breakdown of cell membrane phosphatidylserine asymmetry, and stimulates $Ca^{2+}$-sensitive $K^+$ channels, thus leading to KCl loss and (further) cell shrinkage. The breakdown of phosphatidylserine asymmetry is evidenced by annexin binding, a typical feature of apoptotic cells. The effects of osmotic shock, oxidative stress and energy depletion on annexin binding are mimicked by the $Ca^{2+}$ ionophore ionomycin (1 μM) and blunted in the nominal absence of extracellular $Ca^{2+}$. Nevertheless, the residual annexin binding points to additional mechanisms involved in the triggering of the scramblase. The exposure of phosphatidylserine at the extracellular face of the cell membrane stimulates phagocytes to engulf the apoptotic erythrocytes. Thus, sustained activation of the cation channels eventually leads to clearance of affected erythrocytes from peripheral blood. Susceptibility to annexin binding is enhanced in several genetic disorders affecting erythrocyte function, such as thalassaemia, sickle-cell disease and glucose-6-phosphate dehydrogenase deficiency. The enhanced vulnerability presumably contributes to the shortened life span of the affected erythrocytes. Beyond their role in the limitation of erythrocyte survival, cation channels may contribute to the triggering of apoptosis in nucleated cells exposed to osmotic shock and/or oxidative stress.

**Keywords** Annexin · Scramblase · Osmotic cell shrinkage · Oxidation · Glucose depletion

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## Introduction

Apoptosis is a physiological mechanism eliminating abundant and potentially harmful cells [27, 30]. Hallmarks of apoptosis include nuclear condensation, DNA fragmentation, mitochondrial depolarization, cell shrinkage and breakdown of phosphatidylserine asymmetry of the plasma membrane [27, 30]. The exposure of phosphatidylserine at the cell surface triggers, and the decrease of cell volume facilitates, the engulfment of the dying cells by phagocytes [6, 22]. Thus, apoptosis allows the elimination of the cells without the release of intracellular proteins, which would otherwise cause inflammation [30]. The stimulation of apoptosis modifies the activity of several transport processes at the cell membrane including $K^+$ channels [29, 53, 56, 68, 69], anion channels [56, 70], $Ca^{2+}$ channels [55], taurine release channels [45, 48, 60] and $Na^+/H^+$ exchange [47].

A wide variety of stimuli induce apoptosis, including nitric oxide [33], UV radiation [42, 65], exposure to pathogens [23], osmotic shock [7, 8, 44, 48, 56, 59, 63, 65] and the activation of defined receptors such as CD95 [30, 45, 46], TNF$\alpha$ [50] and somatostatin [71].

Despite their lack of mitochondria and nuclei, intracellular organelles involved in the apoptosis of nucleated cells, erythrocytes exposed to the $Ca^{2+}$ ionophore ionomycin undergo shrinkage, membrane blebbing and break down of cell membrane phosphatidylserine asymmetry, all typical features of apoptosis in nucleated cells [3, 9, 15]. It is thus fair to say that erythrocytes undergo apoptosis upon increase of intracellular [$Ca^{2+}$]. The present brief review presents evidence that entry of $Ca^{2+}$ through a non-selective cation channel is a major mechanism triggering erythrocyte apoptosis.

## Properties of erythrocyte cation channels

Osmotic shock [32] and oxidative stress [21] open non-selective cation channels in the erythrocyte cell membrane. The same channels can be activated by removal of intracellular and extracellular $Cl^-$ (Fig. 1A, B) [21, 32]. This property is reminiscent of the $Na^+$ and $K^+$ permeability activated by incubating human erythrocytes in low ionic strength (LIS) medium [4, 36, 43]. Incubation in LIS medium also induces permeabilities to organic osmolytes, such as taurine and glutamine, which share many properties of the LIS-induced cation permeability [14]. This feature may be of interest as taurine release is a typical feature of apoptosis in nucleated cells [45, 48, 60]. Similar to what has been shown for the LIS permeability [14, 36], activation of the volume- and oxidant-sensitive cation channel by removal of extracellular $Cl^-$ is inhibited by the anion channel/transport inhibitor 4,4′-diisothiocyanostilbene-2,2′-disulphonic acid (DIDS) [21]. The cation channels allow the permeation of $Ca^{2+}$ [21]. Accordingly, exposure to osmotic shock or oxidative stress triggers erythrocyte $Ca^{2+}$ uptake [51].

## Impact of cation channels on cell volume

In general, $Na^+$ entry through cation channels leads to cell membrane depolarization, which should favour cell swelling [44]. Depolarization decreases the electrical driving force for extrusion of negatively charged $Cl^-$ and thus leads to accumulation of $Cl^-$ in parallel to $Na^+$. In most cells cytosolic $Cl^-$ concentration ($[Cl^-]_i$) is below 20 mM and thus less than 20% of the extracellular $[Cl^-]$ ($[Cl^-]_o$). Thus, the equilibrium potential for $Cl^-$ ($E_{Cl}(\text{in mV}) = 60 \log \frac{[Cl^-]_i}{[Cl^-]_o}$) is more negative than −40 mV, i.e. any depolarization below −40 mV would drive $Cl^-$ into the cells. $[Cl^-]_i$ in erythrocytes, however, is of the order of 80 mM [76] and the membrane resting potential is at $E_{Cl}$, which is close to −10 mV [13, 18]. The selectivity of the cation channel is some twofold higher for $K^+$ than for $Na^+$ [21]. In view of the intracellular $Na^+$ and $K^+$ concentrations of approximately 10–20 mM [16, 26, 37, 40] and 140 mM [37], respectively, and the extracellular concentrations of 145 mM and 5 mM, respectively, the equilibrium potential for the channel approaches some −18 mV, i.e. a value more negative than the actual cell membrane potential. Accordingly, the activation of the channel should hyperpolarize the cell membrane and shrink rather than swell the erythrocyte. Moreover, $Ca^{2+}$ entering the cells though the cation channel will activate $Ca^{2+}$-sensitive $K^+$ channels (KCNN4) in the erythrocyte cell membrane [31, 53], leading to hyperpolarization of the cell membrane and subsequent loss of KCl from the erythrocyte [17, 20, 25, 28, 54, 61, 67], again rather favouring hyperpolarization and cell shrinkage. Thus, the erythrocyte cation channels probably do not mediate regulatory cell volume increase, even though they are up-regulated by cell shrinkage.

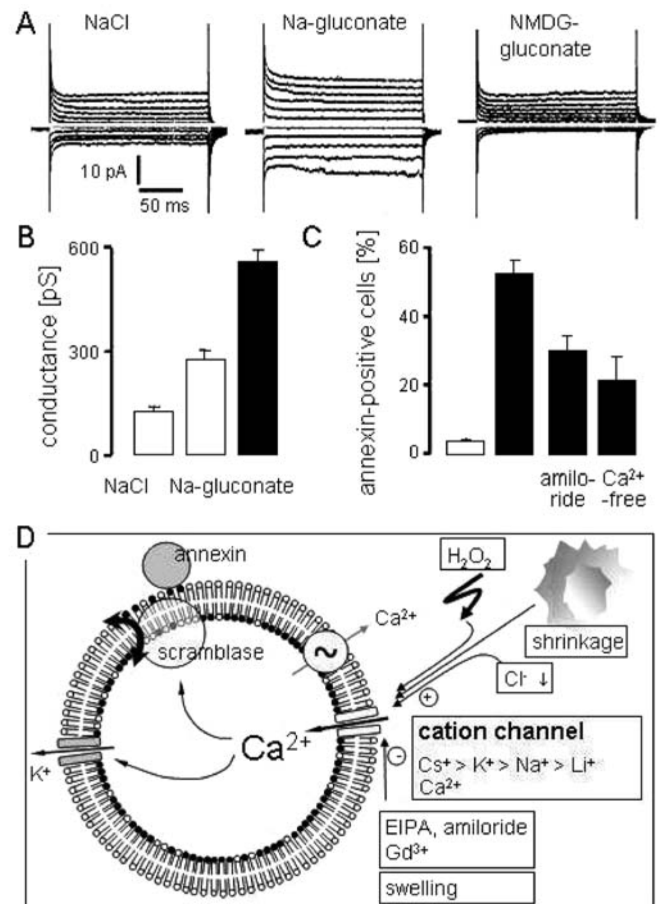


**Fig. 1A–D** Activation of cell volume-sensitive, non-selective cation channels in the erythrocyte membrane induces annexin binding. **A** Cation-selective ion channels are activated by removal of extracellular $Cl^-$. Patch-clamp current traces recorded with Na-D-gluconate pipette solution and isotonic NaCl bath solution (*left*), isotonic Na-D-gluconate bath solution (*middle*), and after replacement of bath $Na^+$ by the impermeant cation *N*-methyl-D-glucamine (*NMDG*$^+$, *right*). Currents were recorded in the fast, whole-cell, voltage-clamp mode; membrane potential was held at −10 mV and currents were elicited by 400-ms square pulses to test potentials between −100 and +100 mV; currents of the individual voltage sweeps are superimposed; zero current is indicated by the *grey line*. **B** Mean (±SE; *n*=3–16) slope conductance of human erythrocytes recorded under isotonic conditions as in **A** prior to (NaCl; *open bar*) and upon activation of the cation channels by extracellular $Cl^-$ removal (Na-gluconate; *open bar*). Hypertonic cell shrinkage (by adding 250 mM sucrose to the bath solution) further activates the cation channels (Na-gluconate; *solid bar*). **C** Cell-shrinkage-induced break down of the erythrocyte membrane phospholipid asymmetry is dependent on extracellular $Ca^{2+}$ and inhibited by amiloride. Mean percentage of annexin binding erythrocytes (±SE; *n*=3–16) as measured by flow cytometry. Erythrocytes were cultured for 24 h at 37°C either in isotonic (*open bar*) or in hypertonic Ringer solution (*closed bars*; osmolarity increased to 850 mOsm by adding sucrose). In some experiments, incubation in hypertonic Ringer solution was performed in the presence of the cation channel inhibitor amiloride (1 mM) or in the absence of extracellular $Ca^{2+}$. **D** Summary of the experimental manoeuvres inducing activation or inactivation/inhibition of the $Ca^{2+}$-permeable non-selective cation channel in human erythrocytes (*EIPA* ethylisopropylamiloride). Increased channel activity leads to elevated cytosolic free $[Ca^{2+}]$ and subsequently to scramblase activation

## Impact of cation channels on erythrocyte apoptosis

Compelling evidence points to a role of the volume-sensitive cation channels in the induction of erythrocyte apoptosis. Besides triggering cell shrinkage (see above), an increase of $[Ca^{2+}]_i$ stimulates a scramblase, thus leading to the breakdown of phosphatidylserine asymmetry [3, 9, 15, 51]. Exposure of phosphatidylserine is detected by determination of annexin binding, together with cell shrinkage, a typical feature of apoptosis in nucleated cells [30].

Erythrocyte annexin binding is triggered by osmotic shock (Fig. 1C) and oxidative stress [51], both manoeuvres that activate the cation channel [21, 32]. Furthermore, energy depletion leads to enhanced annexin binding [51]. Presumably energy depletion impairs the replenishment of GSH and thus weakens the antioxidative defence of the erythrocytes [5, 58]. The annexin binding following osmotic shock and oxidative stress is blunted following chelation of extracellular $Ca^{2+}$ [51]. Moreover, the annexin binding is blunted by amiloride (Fig. 1C) [51] and ethylisopropylamiloride (EIPA) [52] at concentrations needed to inhibit the cation channel [51, 52]. Thus, it appears safe to conclude that activation of the cell volume- and oxidant-sensitive cation channel and subsequent $Ca^{2+}$ entry contribute to the stimulation of erythrocyte scramblase following osmotic shock or oxidative stress (Fig. 1D). Interestingly, the $Na^+/H^+$ exchange inhibitor ethylisopropylamiloride (EIPA) is effective at a concentration of 1 μM, whereas amiloride, which inhibits both $Na^+/H^+$ exchange and cation channels, requires 1 mM to become effective [52].

Further experiments have revealed the enhanced sensitivity of erythrocytes from patients with thalassaemia, sickle-cell anaemia and glucose-6-phosphate dehydrogenase deficiency [49]. Similarly, increased scramblase activity and phosphatidylserine exposure has been demonstrated for erythrocytes in mouse models of sickle cell disease and thalassaemia [38].

## Impact of cation channels on erythrocyte ageing

Aged erythrocytes expose more phosphatidylserine, which contributes to the elimination of the senescent cells [6]. The capacity for oxidative defence decreases with erythrocyte age [34, 62] a phenomenon paralleled by increase of passive cation permeability [35] and cytosolic free $[Ca^{2+}]$ [1, 2, 11, 41, 64, 66]. It is thus tempting to speculate that the cation channels sense cell age. Within the ageing erythrocytes, the loss of antioxidative defence can be expected to increase cation channel activity leading to $Ca^{2+}$ entry, increased $Ca^{2+}$ pump activity, ATP depletion, further impairment of antioxidative defence, further activation of cation channels and further $Ca^{2+}$ entry and eventually activation of the scramblase.

## Volume-sensitive cation channels in nucleated cells

Cell volume-sensitive cation channels are not only expressed in erythrocytes but are found in a wide variety of nucleated cells, such as airway epithelial cells [12], mast cells [10], macrophages [24], vascular smooth muscle, colon carcinoma and neuroblastoma cells [39], cortical collecting duct [73] and hepatocytes [74, 75]. Moreover, cation channels activated by $Cl^-$ removal have been identified in salivary and lung epithelial cells [19, 57, 72]. $Cl^-$ influences the channels via a pertussis toxin-sensitive G protein [19]. It is intriguing to speculate that non-selective cation channels are similarly involved in apoptosis of nucleated cells.

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