Suicidal Erythrocyte Death Following Cellular K^+ Loss

Juliane Schneider, Jan P. Nicolay, Michael Föller, Thomas Wieder* and Florian Lang

Department of Physiology, University of Tuebingen, *present address: Department of Dermatology, University of Tuebingen

Key Words

Cell volume • Apoptosis • Scramblase • Phosphatidylserine • Red blood cell • Eryptosis

Abstract

Hallmarks of apoptosis include cell shrinkage, which is at least partially due to cellular K^+ loss. The decline of cellular K^+ concentration has been suggested to participate in the triggering of apoptosis. Suicidal erythrocyte death or eryptosis is triggered by increased cytosolic Ca^{2+} activity leading to activation of Ca^{2+} -sensitive K^+ channels with subsequent cellular K^+ loss and cell shrinkage, and to Ca^{2+} -sensitive scrambling of the cell membrane with subsequent phosphatidylserine (PS) exposure at the cell surface. Phosphatidylserine exposing erythrocytes are recognized by macrophages, engulfed, degraded and thus cleared from circulating blood. The present study explored whether cellular loss of K^+ and/or cell shrinkage actively participate in the triggering of cell membrane phospholipid scrambling. Cellular K^+ loss was achieved by treatment of human erythrocytes with the K^+ ionophore valinomycin (1 nM) at different extracellular K^+ concentrations (5-125 mM) and osmolarities (300-550 mOsm). Cell volume was estimated from

forward scatter and PS exposure from annexin V binding in FACS analysis. Treatment with 1 nM valinomycin indeed decreased forward scatter and increased annexin V binding. The effect was significantly blunted in the presence of staurosporine (1 μ M). Increase of extracellular K^+ concentration gradually blunted the decrease of forward scatter but inhibited annexin V binding only at extracellular K^+ concentrations $\geq$ 75 mM. An increase of extracellular osmolarity (+150 mM or 250 mM sucrose) reversed the protective effect of 75 mM KCl during valinomycin treatment. A correlation between forward scatter and annexin binding at different osmolarities and K^+ concentrations suggests that the cellular K^+ content determines the rate of suicidal erythrocyte death primarily through its influence on cell volume.

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Introduction

Hallmarks of apoptosis include cell shrinkage [1], resulting from activation of K^+ and/or Cl^- channels, cellular loss of K^+ and Cl^- and osmotically obliged water [1-12]. Intriguing evidence suggests that the loss of K^+ may

contribute to the triggering of apoptosis by stimulation of nucleases and caspases [13, 14].

Similar to apoptosis of nucleated cells, erythrocytes may undergo suicidal death [15]. In those cells, the Ca^{2+} ionophore ionomycin leads to Ca^{2+} -mediated erythrocyte shrinkage, membrane blebbing and breakdown of cell membrane phosphatidylserine asymmetry, all features typical for programmed cell death or apoptosis of nucleated cells [16-23]. The cell shrinkage results from activation of Ca^{2+} -sensitive K^+ channels [24], the membrane blebbing from Ca^{2+} -dependent activation of calpain [25] and the breakdown of phosphatidylserine asymmetry from Ca^{2+} -sensitive scrambling of the cell membrane [26, 27]. Stimulation of suicidal erythrocyte death may further involve activation of sphingomyelinase [28], formation of PAF [29], and release of PGE_2 [25]. In view of the similarities of suicidal erythrocyte death to apoptosis of nucleated cells the term eryptosis has been coined recently [15]. Eryptosis is enhanced in some inherited erythrocyte disorders [21], phosphate depletion [30], iron deficiency [31], Hemolytic Uremic Syndrome [32], Sepsis [33], malaria infection [34], and Wilson disease [35]. Eryptosis could further be triggered by intoxication with hemolysin [36], paclitaxel [37], chlorpromazine [38], mercury [39] and lead [40]. Eryptosis is similar but not identical to erythrocyte senescence [25, 41-43] and neocytolysis [44]. In contrast to apoptosis of nucleated cells, eryptosis occurs in the absence of caspase activation, DNA fragmentation and mitochondrial depolarisation [22].

Ca^{2+} -induced eryptosis has been demonstrated to be significantly blunted by an increase of the extracellular K^+ concentration and by specific inhibition of the Ca^{2+} -sensitive K^+ channels [24]. Those observations pointed to K^+ -sensitive mechanisms in the stimulation of eryptosis but did not allow to draw conclusions about the question, whether K^+ depletion may be sufficient for the triggering of eryptosis.

The present study thus explored whether K^+ depletion by itself is capable to trigger eryptosis and if so, to explore whether the K^+ loss per se or the concomitant cell shrinkage determine the extent of phosphatidylserine exposure.

Materials and Methods

Solutions

Erythrocyte concentrates were obtained by centrifugation/filtration procedures from the blood of healthy volunteers as described in detail [29]. Patients and healthy donors gave

their informed consent, and the study was approved by the Ethic Committee of the University of Tübingen (project number: 184/2003V). 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)/NaOH (pH 7.4), 5 glucose, 1 CaCl_2 . Where indicated, NaCl was partially replaced by KCl to increase extracellular K^+ concentration up to 125 mM, and extracellular osmolality was increased by addition of up to 250 mM sucrose.

FACS analysis

FACS analysis was performed as described [22]. After incubation in the presence or absence of valinomycin (1 nM, 10 nM) at different K^+ concentrations, cells were washed in annexin-binding buffer containing (in mM) 125 NaCl, 10 HEPES/NaOH (pH 7.4), and 5 CaCl_2 . Erythrocytes were suspended in a solution composed of Annexin-V-Fluos (Roche Diagnostics, Mannheim, Germany) and annexin buffer (dilution of 1:50). After 10 min of incubation, samples were finally diluted 1:5 in annexin-binding buffer and measured by flow cytometric analysis on a FACS-Calibur from Becton Dickinson (Heidelberg, Germany). Cells were analysed by forward scatter and annexin-fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Statistics

Data are expressed as arithmetic means $\pm$ SEM and statistical analysis was made by paired or unpaired t-test or ANOVA with Dunnett's or Tukey's tests as the post-hoc tests, where appropriate. $P < 0.05$ was considered as statistically significant.

Results

As illustrated in Fig. 1A, the treatment of erythrocytes with valinomycin was followed by a decrease of the forward scatter thereby indicating a loss of cellular volume. The decrease of forward scatter was dependent on the extracellular K^+ concentration. An increase of extracellular K^+ concentration gradually blunted, and an increase to 125 mM extracellular K^+ concentration almost completely abolished the decrease of the forward scatter following treatment with 1 nM valinomycin (Fig. 1B). An increase of the valinomycin concentration to 10 nM gave similar results (Fig. 1B).

The decrease of forward scatter following valinomycin treatment was paralleled by an increase of annexin V binding, a measure of phosphatidylserine exposure at the cell surface (Fig. 2A). As shown in Fig. 2B, the increase of annexin V binding was again dependent on the extracellular K^+ concentration. However, an increase of extracellular K^+ concentration up to 50 mM had only little effect on annexin V binding, whereas a further increase to 75 mM significantly blunted and an increase to

Fig. 1. Effect of valinomycin on the forward scatter of erythrocytes. A. Original histograms of the forward scatter of human erythrocytes following a 24h treatment in the absence (control, left panel) or presence of 1 nM valinomycin (right panel). B. Arithmetic means $\pm$ SEM ($n = 8$) of the erythrocyte forward scatter following a 24h treatment with different extracellular K^+ concentrations (5-125 mM) in the absence of valinomycin (white bars), and in the presence of 1 nM (grey bars) or 10 nM (black bars) valinomycin. * indicates significant ($p < 0.05$) difference from absence of valinomycin, # indicates significant ($p < 0.05$) difference from 5 mM K^+ in the presence of valinomycin (ANOVA using Tukey's test as post-hoc test).

125 mM extracellular K^+ concentration completely abolished the valinomycin-induced increase of annexin V binding. Again, 1 nM of valinomycin was similarly effective as 10 nM valinomycin (Fig. 2B).

Osmotic cell shrinkage has previously been shown to trigger ceramide formation [28]. Thus, additional experiments have been performed to explore the effect of

Fig. 2. Effect of valinomycin on annexin V binding of erythrocytes. A. Original histograms of annexin V binding of human erythrocytes following a 24h treatment in the absence (control, left panel) or presence of 1 nM valinomycin (right panel). B. Arithmetic means $\pm$ SEM ($n = 8$) of the erythrocyte annexin V binding following a 24h treatment with different extracellular K^+ concentrations (5 - 125 mM) in the absence of valinomycin (white bars), and in the presence of 1 nM (grey bars) or 10 nM (black bars) valinomycin. * indicates significant ($p < 0.05$) difference from absence of valinomycin, # indicates significant ($p < 0.05$) difference from 5 mM K^+ in the presence of valinomycin (ANOVA using Tukey's test as post-hoc test).

valinomycin treatment on ceramide formation. The number of ceramide exposing cells was $4.0 \pm 0.3\%$ ($n = 8$) in untreated and $3.6 \pm 0.4\%$ ($n = 8$) following a one hour treatment with 1 nM valinomycin, values not significantly different. Bacterial sphingomyelinase was followed by the expected significant ($p < 0.001$) increase of ceramide formation to $14.1 \pm 1.3\%$ ($n = 8$). Thus, valinomycin did

Fig. 3. Effect of valinomycin on forward scatter and annexin V binding of erythrocytes in the absence and presence of staurosporine. A. Arithmetic means $\pm$ SEM ($n = 6-8$) of the erythrocyte forward scatter following a 24h treatment at 5 mM extracellular K^+ concentration in the absence of valinomycin (white bars), and in the presence of 10 nM valinomycin (black bars) in the absence (left bars) and presence (right bars) of 1 μ M staurosporine. * indicates significant ($p < 0.05$) difference from absence of valinomycin, # indicates significant ($p < 0.05$) difference from absence of staurosporine (ANOVA using Tukey's test as post-hoc test). B. Arithmetic means $\pm$ SEM ($n = 6-8$) of the erythrocyte annexin V binding following a 24 h treatment at 5 mM extracellular K^+ concentration in the absence of valinomycin (white bars), and in the presence of 10 nM valinomycin (black bars) in the absence (left bars) and presence (right bars) of 1 μ M staurosporine. * indicates significant ($p < 0.05$) difference from absence of valinomycin, # indicates significant ($p < 0.05$) difference from absence of staurosporine (ANOVA using Tukey's test as post-hoc test).

Fig. 4. Interaction of extracellular K^+ concentration and osmolarity on forward scatter of valinomycin treated erythrocytes. A. Original histograms of forward scatter of human erythrocytes exposed to 1 nM valinomycin at 5 mM (upper panels) and 75 mM K^+ (lower panels) at isotonic conditions (left panels) and at hypertonic conditions (addition of 250 mM sucrose, right panels). B. Arithmetic means $\pm$ SEM ($n = 8$) of the erythrocyte forward scatter following a 24h treatment with 1 nM valinomycin at 5 mM (white bars) and 75 mM K^+ (black bars) at isotonic conditions (290 mOsm, left) or hypertonic conditions (addition of 150 mM (450 mOsm, middle) or 250 mM (550 mOsm, right) sucrose). * indicates significant ($p < 0.05$) difference from absence of valinomycin, # indicates significant ($p < 0.05$) difference from 5 mM K^+ in the presence of valinomycin, ** indicates significant ($p < 0.05$) difference from isotonic conditions (290 mOsm) (ANOVA using Tukey's test as post-hoc test).

not appreciably stimulate ceramide formation.

The effect of osmotic cell shrinkage on annexin binding was partially mediated by PGE_2 formation and blunted by cyclooxygenase inhibitors [25]. Thus, additional experiments have been performed to explore, whether the effects of valinomycin were dependent on cyclooxygenase. Prior to valinomycin treatment, the average forward scatter approached 510 ± 5 ($n = 8$) in the absence and 496 ± 7 ($n = 8$) in the presence of cyclooxygenase inhibitor acetylsalicylic acid (50 μ M). Treatment of erythrocytes with valinomycin (1 nM) for

Fig. 5. Interaction of extracellular K⁺ concentration and osmolarity on annexin V binding of valinomycin treated erythrocytes. A. Original histograms of annexin V binding human erythrocytes exposed to 1 nM valinomycin at 5 mM (upper panels) and 75 mM K⁺ (lower panels) at isotonic conditions (left panels) and at 550 mOsm (addition of 250 mM sucrose, right panels). B. Arithmetic means $\pm$ SEM (n = 8) of the erythrocyte annexin V binding following a 24 h treatment with 1 nM valinomycin at 5 mM (white bars) and 75 mM K⁺ (black bars) at isotonic conditions (290 mOsm, left) or hypertonic conditions (addition of 150 mM (450 mOsm, middle) or 250 mM (550 mOsm, right) sucrose). * indicates significant (p<0.05) difference from absence of valinomycin, # indicates significant (p<0.05) difference from 5 mM K⁺ in the presence of valinomycin, ** indicates significant (p<0.05) difference from isotonic conditions (290 mOsm) (ANOVA using Tukey's test as post-hoc test).

Fig. 6. Correlation between forward scatter and annexin V binding in valinomycin- treated erythrocytes at different K⁺ concentrations and osmolarities. Arithmetic means $\pm$ SEM (n = 8) of the percentage annexin V binding erythrocytes following a 24 h treatment in the absence or presence of 1 nM valinomycin, at 5 mM (white symbols), 75 mM K⁺ (grey symbols) or 100 mM K⁺ (black symbols), at isotonic conditions (circles), at 450 mOsm (squares) and at 550 mOsm (triangles). The equation of an exponential fit and the r² value of the correlation coefficient are included.

one hour significantly (p<0.001) decreased the forward scatter to 339 ± 5 (n = 8) in the absence and to 335 ± 6 (n = 8) in the presence of cyclooxygenase inhibitor acetylsalicylic acid (50 μ M). No significant differences were observed between presence and absence of acetylsalicylic acid. Prior to valinomycin treatment, the percentage of annexin binding erythrocytes averaged 2.3 ± 0.3 % (n = 8) in the absence and 2.6 ± 0.4 % (n = 8) in the presence of cyclooxygenase inhibitor acetylsalicylic acid (50 μ M). Treatment of erythrocytes with valinomycin (1 nM) for one hour significantly (p<0.005) increased the percentage of annexin V binding erythrocytes to 13.0 ± 1.7 % (n = 8) in the absence and to 12.6 ± 1.8 % (n = 8) in the presence of cyclooxygenase inhibitor acetylsali-

cyclic acid (50 μ M). Again, no significant differences were observed between presence and absence of acetylsalicylic acid. Thus, the effect of valinomycin was not sensitive to cyclooxygenase inhibitor acetylsalicylic acid at the applied concentrations.

Eryptosis has previously been shown to be activated by phorbol esters and inhibited by staurosporine [45]. Thus, additional experiments have been performed in the absence and presence of staurosporine. As illustrated in Fig. 3, the effect of valinomycin on both, annexin binding and forward scatter was significantly blunted in the presence of staurosporine.

The effect of valinomycin on phosphatidylserine exposure could have been due to cellular K^+ loss and/or a decrease of the cell volume. To be able to discriminate between these two typical events during eryptosis, valinomycin-treated erythrocytes were exposed to different extracellular K^+ concentrations (5 mM and 75 mM) and osmolarities (300, 450 and 550 mOsm). As illustrated in Fig. 4, the significant effect on the forward scatter of increased extracellular K^+ concentration was partially reversed by an increase of extracellular osmolarity.

Moreover, the effect of valinomycin on phosphatidylserine exposure was modified by increasing the extracellular K^+ concentrations and osmolarities. As illustrated in Fig. 5, the effect of 75 mM extracellular K^+ concentration on annexin V binding was partially reversed by an increase of extracellular osmolarity.

The effects of variations in extracellular K^+ concentration and osmolarity allowed establishing a correlation between erythrocyte forward scatter and annexin V binding before and after treatment with valinomycin. As illustrated in Fig. 6, annexin V binding was negatively correlated with forward scatter irrespective of the extracellular K^+ concentration or osmolarity.

Discussion

The present observations demonstrate that valinomycin decreases cell volume and triggers eryptosis, the suicidal death of erythrocytes. At low extracellular K^+ concentrations, the K^+ ionophore hyperpolarizes the cell membrane thus driving Cl^- out of the cell. The cellular loss of KCl and osmotically obliged water then leads to cell shrinkage. As previously demonstrated, the cellular loss of K^+ contributes to the stimulation of Ca^{2+} -mediated eryptosis [24]. Treatment with valinomycin led to variable stimulation of annexin binding and decrease of forward scatter indicating that the sensitivity of individual

erythrocytes to the eryptotic effects of valinomycin is not homogeneous. As indicated earlier [25], the sensitivity of individual erythrocytes to triggers of suicidal cell death may depend on erythrocyte age. Moreover, previous injury of the individual erythrocytes may affect their viability.

In view of the present observations, any stimulation of the endogenous K^+ channels would be prone to trigger eryptosis and thus to jeopardize the survival of circulating erythrocytes. Along those lines, activation of Ca^{2+} -sensitive K^+ channels participates in the cell shrinkage and deformation of desoxygenized sickle cells [46-50] which are highly sensitive to stress-induced eryptosis [21]. Moreover, accelerated eryptosis has previously been demonstrated in several further anemic conditions, including beta-thalassemia or glucose-6-phosphate dehydrogenase (G6PD)-deficiency [21], phosphate depletion [30], iron deficiency anemia [31] and intoxication with lead [40] or mercury [39]. In all those conditions, cellular loss of K^+ due to activation of K^+ channels may participate in the triggering of suicidal erythrocyte death.

Surprisingly, though, the effects of valinomycin did not trigger appreciable ceramide formation and the valinomycin-induced eryptosis was seemingly insensitive to cyclooxygenase inhibitor acetylsalicylic acid. Previously, osmotic shock was shown to stimulate the formation of ceramide [28] and PGE_2 [25], which both participated in the triggering of eryptosis. The present experiments do not provide a clue to this seeming discrepancy. Possibly, valinomycin is effective downstream of ceramide and PGE_2 .

According to the present observations, the effects of valinomycin were significantly blunted by staurosporine. This observation may point to the involvement of protein kinase C, which has previously been shown to stimulate erythrocyte Ca^{2+} entry [16] and phosphatidylserine exposure [45, 51]. $PKC\alpha$, $PKC\iota$, $PKC\mu$ and $PKC\zeta$ have been reported to be expressed in erythrocytes [52]. Upon activation, they phosphorylate cytoskeletal proteins, such as band 4.1, 4.9 and adducin [53] as well as the human Na^+/H^+ antiporter NHE 1 [54]. Additional experiments will be needed to allow insight into the signaling of valinomycin-induced eryptosis.

Eryptosis may not only lead to accelerated clearance of erythrocytes and thus anemia, but may in addition foster adherence of erythrocytes to the vascular wall [55] leading to impairment of microcirculation [56-58]. Enhanced adhesiveness of erythrocytes could further support hemostasis [59-63] and enhance the risk to develop thrombosis [64]. Trapping of eryptotic erythrocytes has indeed been observed in the renal medulla following re-

nal ischemia [22]. According to the present observations, activation of erythrocyte K⁺ channels could participate in vascular effects of circulating erythrocytes.

The present observations may be relevant for apoptosis of nucleated cells as well. At least in theory, cellular K⁺ loss could contribute to the altered apoptosis in a wide variety of conditions [2, 8, 10-13, 65-71], including stimulation of TNF receptors [72], CD95 activation [73, 74], glucocorticoid [75-78], GABA [79], or dopamine [80] treatment, growth factor depletion [81], thyroid status [82], sulfonylureas [83], etoposide treatment [84], transformation [85], choline deficiency [86], glutamine depletion [87], oxidative stress [6, 88-90], hypoxia [91], radiation [92], sphingosine treatment [93], amyloid treatment [94], staurosporine [95], urea [96], Cl⁻ channel blockers [97] and K⁺ channel blockers [98, 99].

In erythrocytes, however, the cellular K⁺ loss is apparently by itself not a major determinant of suicidal cell death. Instead, the release of cellular K⁺ leads to cell shrinkage, which at a certain threshold triggers eryptosis. An increase of extracellular K⁺ concentration is in large part protective through blunting of the cellular shrinkage. Those observations do not challenge the view that the cellular K⁺ concentration is by itself a determinant of

suicidal cell death of nucleated cells because in those systems K⁺ mainly regulates apoptotic enzymes, e. g. caspases or nucleases [13, 14], which apparently do not play a significant role in the cell death machinery of erythrocytes. Nevertheless, our present data highlight the importance of K⁺-dependent cell volume changes in the regulation of suicidal erythrocyte death.

In conclusion, valinomycin triggers eryptosis, a form of suicidal death of erythrocytes. The effect is dependent on erythrocytic K⁺ loss and in part due to erythrocyte shrinkage.

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