Original Paper

Effect of Casein Kinase 1 α Activator Pyrvinium Pamoate on Erythrocyte Ion Channels

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Key Words

Ca²⁺-permeable cation channels • Ca²⁺-activated K⁺ channels • Casein kinase 1 α • Eryptosis • Erythrocytes • Phosphatidylserine • Annexin • Cell volume

Abstract

Pharmacological modification of protein kinase CK1 (casein kinase 1) has previously been shown to influence suicidal erythrocyte death or eryptosis, which is triggered by activation of Cl⁻-sensitive Ca²⁺-permeable cation channels. Ca²⁺ entering through those channels stimulates cell membrane scrambling and opens Ca²⁺-activated K⁺-channels resulting in KCl exit and thus cell shrinkage. The specific CK1-inhibitor D4476 (1 μ M) blunted, whereas the specific CK1 α -activator pyrvinium pamoate (10 μ M) enhanced cell membrane scrambling. The substances were at least partially effective through modification of cytosolic Ca²⁺-activity. The present study explored, whether pyrvinium pamoate indeed influences Cl⁻-sensitive cation-channels in erythrocytes. As a result, removal of Cl⁻ increased Fluo3-fluorescence (reflecting cytosolic Ca²⁺-activity), triggered cell membrane scrambling (apparent from annexin-V-binding), and decreased forward scatter (pointing to cell shrinkage). Pyrvinium pamoate significantly augmented the effect of Cl⁻-removal on Fluo3 fluorescence and annexin-V-binding, but blunted the effect on forward scatter. According to whole cell patch clamp recording, Cl⁻-removal activated a cation current, which was significantly enhanced by pyrvinium pamoate. Pyrvinium pamoate inhibited Ca²⁺-activated K⁺-channels. Ca²⁺-ionophore ionomycin (1 μ M) decreased forward scatter, an effect significantly blunted by pyrvinium pamoate. In conclusion, pyrvinium pamoate activates Cl⁻-sensitive Ca²⁺-permeable cation channels with subsequent Ca²⁺-entry and inhibits Ca²⁺-activated K⁺-channels thus blunting the stimulating effect of Ca²⁺ on those channels, K⁺-exit and thus cell shrinkage.

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Introduction

Erythrocytes may be eliminated by eryptosis, a suicidal cell death characterized by cell shrinkage and cell membrane scrambling [1]. Eryptosis may be triggered by a myriad of xenobiotics and endogenous substances [2-14]. Accelerated eryptosis further contributes to anemia in a variety of clinical disorders [1], such as iron deficiency [15], phosphate depletion [16], Hemolytic Uremic Syndrome [17], sepsis [18], malaria [1, 19, 20] or Wilson's disease [21]. Most xenobiotics and clinical disorders trigger eryptosis mainly by activating Ca^{2+} -permeable cation channels, which are stimulated by removal of Cl^- , oxidative stress, osmotic shock or prostaglandin E_2 [1]. The subsequent Ca^{2+} -entry leads to cell membrane scrambling with phosphatidylserine exposure at the cell surface and to activation of Ca^{2+} -sensitive K^+ channels with subsequent K^+ -exit, hyperpolarisation, Cl^- -exit and thus cellular KCl -loss with osmotically obliged exit of water and thus cell shrinkage [1].

According to pharmacological evidence, cellular mechanisms regulating erythrocyte Ca^{2+} -entry and thus eryptosis include the protein kinase $\text{CK1}\alpha$ (casein kinase 1α) [22], a serine/threonine protein kinase expressed in erythrocytes [23, 24] and shown to regulate cytoskeleton proteins [25, 26].

In nucleated cells CK1 regulates a variety of cellular functions including membrane trafficking, cell cycle progression, chromosome segregation, apoptosis and cellular differentiation [27, 28]. CK1 phosphorylates β -catenin and so fosters its degradation [29]. By phosphorylating RhoB and stress fibers [30], CK1 influences actin stress fiber organization [30]. Furthermore, CK1 participates in four-and-a-half LIM (FHL) signaling [31].

$\text{CK1}\alpha$ is activated by pyrvinium pamoate (6-(Dimethylamino)-2-[2-(2,5-dimethyl-1-phenyl-1H-pyrrol-3-yl)ethenyl]-1-methyl-4,4'-methylene-bis[3-hydroxy-2-naphthalenecarboxylate] (2:1)-quinolinium) [32], an antimicrobial drug [32-35]. Several CK1 isoforms including $\text{CK1}\alpha$, are inhibited by D4476 (4-(4-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl)benzamide) [36-39], a substance known to either inhibit [40] or foster [37] apoptosis. Most recent experiments revealed that pyrvinium pamoate increases and D4476 inhibits Ca^{2+} -entry into erythrocytes and subsequent eryptosis [22]. Those experiments suggested an effect of pyrvinium pamoate and D4476 on erythrocyte channels. The present study thus explored, whether pyrvinium pamoate modifies the Cl^- -sensitive Ca^{2+} -permeable cation channels in erythrocytes.

Materials and Methods

Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes from concentrates provided by the blood bank of the University of Tübingen were used. The volunteers providing erythrocytes gave informed consent. The study has been approved by the Ethical commission of the University of Tübingen. For the *in vitro* experiments on suicidal death of erythrocytes incubations were carried out as indicated in the figure legends 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 at a hematocrit of 0.4%. Where indicated, Cl^- was replaced by gluconate (Sigma, Schnellendorf, Germany). The CK1 inhibitor D4476 (4-(4-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl)benzamide) (Calbiochem, Bad Soden, Germany) and the $\text{CK1}\alpha$ activator pyrvinium pamoate (6-(Dimethylamino)-2-[2-(2,5-dimethyl-1-phenyl-1H-pyrrol-3-yl)ethenyl]-1-methyl-4,4'-methylene-bis[3-hydroxy-2-naphthalenecarboxylate] (2:1)-quinolinium) (Sigma, Schnellendorf, Germany) or the Gardos channel inhibitor TRAM 34 [41, 42] (Sigma, Germany) were used at the indicated concentrations.

Phosphatidylserine exposure and forward scatter

For FACS analysis erythrocytes were washed after incubation once in Ringer solution containing 5 mM CaCl_2 . The cells were then stained with Annexin-V-FITC (ImmunoTools, Friesoythe, Germany) at a 1:200 dilution. After 15 min, samples were measured by flow cytometric analysis (FACS-Calibur from Becton Dickinson, Heidelberg, Germany). Cells were analysed by forward scatter, and annexin-V-fluorescence intensity

was measured in fluorescence channel FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Measurement of intracellular Ca^{2+}

After incubation, 50 μ l suspension of erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM $CaCl_2$ and 2 μ M Fluo-3/AM. The cells were incubated at 37°C for 30 min and then resuspended in 150 μ l Ringer. The Ca^{2+} -dependent fluorescence intensity was then measured in fluorescence channel FL-1 in FACS analysis. The control cell population (Fluo3-stained cells incubated in normal Ringer solution without any treatment) was set to a geomean of ~20 arbitrary units corresponding to the fluorescence range of $10^1 - 10^2$ in the histogram. Alteration of Fluo3-dependent fluorescence intensity was paralleled by the respective shift of the cell population in the histogram. Changes in the geomean (arbitrary units) measured by Fluo3-dependent fluorescence were taken as measure of intracellular Ca^{2+} activity.

Electrophysiology

Patch clamp recordings were performed at room temperature. The patch electrodes were made of borosilicate glass capillaries (150 TF-10, Clark Medical Instruments) using a horizontal DMZ puller (Zeitz). Pipettes with high resistance from 17 to 23 M Ω were connected via an Ag-AgCl wire to the headstage of an EPC 9 patch-clamp amplifier (HEKA). Data acquisition and data analysis were controlled by a computer equipped with an ITC 16 interface (Instrutech) and by using Pulse software (HEKA). For current measurements, erythrocytes were held at a holding potential (V_h) of -10 mV for the non-selective cation channels and 0 mV for the Gardos channels recordings; 200 ms pulses from -100 to +100 mV were applied in increments of +20 mV. The original current traces are depicted without filtering (acquisition frequency of 3 kHz). The currents were analyzed by averaging the current values measured between 90 and 190 ms of each square pulse (current-voltage relationship). The applied voltages refer to the cytoplasmic face of the membrane with respect to the extracellular space. The offset potentials between both electrodes were zeroed before sealing. The liquid junction potentials between bath and pipette solutions, and between the bath solutions and the salt bridge (filled with NaCl bath solution) were calculated according to Barry and Lynch [43]. Data were corrected for liquid junction potentials.

For whole cell recording, the pipette solutions consisted of (in mM): 125 Na-gluconate, 10 NaCl, 1 $MgCl_2$, 1 MgATP, 1 EGTA, 10 HEPES/NaOH (pH 7.4) or 120 K-gluconate, 5 KCl, 1.2 $MgCl_2$, 2 MgATP, 2 EGTA, 2.05 $CaCl_2$, 10 HEPES/NaOH (pH 7.4). The NaCl Ringer bath solution contained (in mM): 145 NaCl, 5 KCl, 2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 HEPES/NaOH (pH 7.4). In the experiments, where Cl^- was substituted with gluconate, the solution contained (in mM): 150 Na-gluconate, 5 glucose, 10 HEPES/NaOH (pH 7.4). The Na-gluconate bath solution used for determination of Gardos channel activity contained in addition 5 mM K-gluconate. The NMDG-Cl bath solution contained (in mM): 180 NMDG, 5 glucose, 10 HEPES (titrated with HCl to pH 7.4). Chemicals were obtained from Sigma (Germany) and were of the highest grade available. Pyrvinium pamoate (10 μ M) was added either acutely (whole-cell Gardos channels activity measurements) or the cells were pre-incubated (3-6 hours, +37 °C) with 10 μ M pyrvinium pamoate (for whole-cell measurements of the non-selective cation channels).

Statistics

Data are expressed as arithmetic means $\pm$ SEM, n is the number of batches used. As different erythrocyte batches used in distinct experiments are differently susceptible to stimulators of eryptosis, comparisons were always made with erythrocytes from the same batches. Paired ANOVA with Tukey's test as post-test or paired *t*-test, as appropriate, were used for statistical analysis. In some cases, a normalization of values to control values was made and denoted in the respective graphs. $p < 0.05$ was considered as statistically significant.

Results

The Ca^{2+} -permeable cation channels are activated by Cl^- removal. To activate the channels, Cl^- was thus replaced by gluconate. The experiments were performed in the absence and presence of the CK1 inhibitor D4476. In a first series of experiments Fluo3 fluorescence has been utilized to estimate cytosolic Ca^{2+} activity. In the presence of Cl^- , 10 μ M D4476 slightly but

Fig. 1. Effect of D4476 on erythrocyte Fluo3-fluorescence, forward scatter and phosphatidylserine exposure following Cl⁻ removal. A. Histograms of Fluo3-fluorescence reflecting cytosolic Ca²⁺-activity in representative experiments of erythrocytes exposed for 48 hours to Ringer with (black line) or without Cl⁻ in the absence (red line) or presence (green line) of CK1 inhibitor D4476 (10 μM). B. Arithmetic means ± SEM (n = 7) of the of Fluo3-fluorescence in erythrocytes following a 48 hours incubation in the absence or presence of D4476 (1 or 10 μM). The erythrocytes were exposed 48 hours to Cl⁻-containing control Ringer (white bars) or Cl⁻-depleted Ringer (black bars) *** indicate significant (p < 0.001) difference from Cl⁻ containing Ringer, #, ### significant (p < 0.05, p < 0.001) difference from the absence of D4476 (ANOVA). C. Histograms of forward scatter in representative experiments of erythrocytes exposed for 48 hours to Ringer with (black line) or without Cl⁻ in the absence (red line) or presence (green line) of CK1 inhibitor D4476 (10 μM). D. Arithmetic means ± SEM (n = 8) of the forward scatter of erythrocytes following a 48 hours incubation in the absence or presence of D4476 (1 or 10 μM). The erythrocytes were exposed 48 hours to Cl⁻-containing control Ringer (white bars) or Cl⁻-depleted Ringer (black bars) *** indicate significant (p < 0.001) difference from Cl⁻-containing Ringer, ### indicate significant (p < 0.001) difference from absence of D4476 (ANOVA). E. Histograms of annexin-V-binding reflecting phosphatidylserine exposure in representative experiments of erythrocytes exposed for 48 hours to Ringer with (black line) or without Cl⁻ in the absence (red line) or presence (green line) of CK1 inhibitor D4476 (10 μM). F. Arithmetic means ± SEM (n = 8) of the of annexin-V-binding of erythrocytes following a 48 hours incubation in the absence or presence of 1 or 10 μM D4476. The erythrocytes were exposed 48 hours to Cl⁻ containing control Ringer (white bars) or Cl⁻ depleted Ringer (black bars) *** indicate significant (p < 0.001) difference from Cl⁻ containing Ringer, ### indicate significant (p < 0.001) difference from absence of D4476 (ANOVA).

significantly, decreased Fluo3 fluorescence (Fig. 1A,B). Cl⁻ removal was followed by a marked increase of Fluo3 fluorescence (Fig. 1A,B). D4476 blunted the increase of Fluo3-fluorescence following Cl⁻-removal, an effect reaching statistical significance at 1 μM D4476.

Fig. 2. Effect of pyrvinium pamoate on erythrocyte Fluo3 fluorescence, forward scatter and phosphatidylserine exposure following Cl⁻ removal. A. Histograms of Fluo3 fluorescence reflecting cytosolic Ca²⁺ activity in representative experiments of erythrocytes exposed for 24 hours to Ringer with (black line) or without Cl⁻ in the absence of CK1α activator pyrvinium and during additional 24 hours in the absence (red line) or presence (green line) of CK1α activator pyrvinium pamoate (10 μM). B. Arithmetic means ± SEM (n = 6 - 21) of the of Fluo3 fluorescence in erythrocytes following a 24 hours incubation in the absence of pyrvinium pamoate and then further 24 hours in the absence or presence of pyrvinium pamoate (1, 5, 10 μM). The erythrocytes were exposed 48 hours to Cl⁻ containing control Ringer (white bars) or Cl⁻ depleted Ringer (black bars) *** indicate significant (p < 0.001) difference from Cl⁻ containing Ringer, #,### indicate significant (p < 0.001) difference from absence of pyrvinium pamoate (ANOVA). C. Histograms of forward scatter in representative experiments of erythrocytes exposed for 24 hours to Ringer with (black line) or without Cl⁻ in the absence of CK1α activator pyrvinium and during additional 24 hours in the absence (red line) or presence (green line) of CK1α-activator pyrvinium pamoate (10 μM). D. Arithmetic means ± SEM (n = 16) of the forward scatter of erythrocytes following a 24 hours incubation in the absence of pyrvinium pamoate and then further 24 hours in the absence or presence of pyrvinium pamoate (1, 5, 10 μM). The erythrocytes were exposed 48 hours to Cl⁻ containing control Ringer (white bars) or Cl⁻ depleted Ringer (black bars) *** indicate significant (p < 0.001) difference from Cl⁻-containing Ringer, #,### indicate significant (p < 0.05, p < 0.01) difference from the absence of pyrvinium pamoate (ANOVA). E. Histograms of annexin-V-binding reflecting phosphatidylserine exposure in representative experiments of erythrocytes exposed for 24 hours to Ringer with (black line) or without Cl⁻ in the absence of CK1α activator pyrvinium and during additional 24 hours in the absence (red line) or presence (green line) of CK1α activator pyrvinium pamoate (10 μM). F. Arithmetic means ± SEM (n = 16) of the of annexin-V-binding of erythrocytes following a 24 hours incubation in the absence of pyrvinium pamoate and then further 24 hours in the absence or presence of pyrvinium pamoate (1, 5, 10 μM). The erythrocytes were exposed 48 hours to Cl⁻ containing control Ringer (white bars) or Cl⁻ depleted Ringer (black bars) *, *** indicate significant (p < 0.05, p < 0.001) difference from Cl⁻-containing Ringer, ### indicate significant (p < 0.001) difference from the absence of pyrvinium pamoate (ANOVA).

Fig. 3. Effect of pyrvinium pamoate on ionomycin induced decrease of forward scatter. A. Histogram showing the changes in forward scatter induced by ionomycin (1 μ M) within 11 minutes in absence of pyrvinium pamoate. B. Histogram showing the changes in forward scatter induced by ionomycin (1 μ M) within 11 minutes in the presence of pyrvinium pamoate (10 μ M). C. Time-dependent decrease of the forward scatter after treatment with ionomycin with (red line) and without (black line) pyrvinium pamoate. *** indicate significant ($p < 0.001$) difference from absence of pyrvinium pamoate (t-test).

An increase of cytosolic Ca^{2+} is expected to activate Ca^{2+} -sensitive K^{+} -channels with subsequent K^{+} -exit, hyperpolarization, Cl^{-} -exit and cell shrinkage. Forward scatter was utilized to estimate cell volume. Cl^{-} -removal was followed by a significant decrease of forward scatter (Fig. 1C,D). The addition of the specific CK1 inhibitor D4476 did not significantly alter forward scatter in the presence of Cl^{-} but at 10 μM D4476 significantly blunted the decrease of forward scatter following Cl^{-} -removal (Fig. 1C,D).

Cl^{-} -removal further triggered cell membrane scrambling with exposure of phosphatidylserine at the erythrocyte surface, which could be quantified by annexin-V-binding. Cl^{-} -removal was followed by a significant increase in annexin-V-binding (Fig. 1E,F). Addition of D4476 did not significantly modify annexin-V-binding in the presence of Cl^{-} , but blunted the increase of annexin-V-binding following Cl^{-} -removal, an effect reaching statistical significance at 10 μM D4476 (Fig. 1E,F).

A further series of experiments explored the effect of the CK1 α -activator pyrvinium pamoate. As illustrated in Fig. 2A,B, pyrvinium pamoate (10 μM) slightly but significantly increased Fluo3-fluorescence in the presence of Cl^{-} . Moreover, pyrvinium pamoate significantly augmented the increase of Fluo3-fluorescence following Cl^{-} removal (Fig. 2A,B), an effect reaching statistical significance at 10 μM . Pyrvinium pamoate did not significantly alter forward scatter in the presence of Cl^{-} but at 5 and 10 μM significantly blunted the decrease of forward scatter following Cl^{-} -removal (Fig. 2C,D). Addition of pyrvinium pamoate tended to increase annexin-V-binding in the presence of Cl^{-} , an effect, however, not reaching statistical significance. Pyrvinium pamoate augmented the increase of annexin-V-binding following Cl^{-} -removal, an effect reaching statistical significance at 10 μM pyrvinium pamoate (Fig. 2E,F).

The effect of pyrvinium pamoate on Fluo3-fluorescence suggested a stimulating effect of pyrvinium pamoate on Cl^{-} -sensitive Ca^{2+} -permeable cation channels, the blunting effect of pyrvinium pamoate on the decline of forward scatter following Cl^{-} -removal pointed to an inhibitory effect on Ca^{2+} -activated K^{+} - or Cl^{-} -channels. To further explore the effect of pyrvinium pamoate on cell volume, forward scatter was determined prior to and during

Fig. 4. Effect of pyrvinium pamoate on Cl⁻ sensitive cation channels. A. Whole-cell current tracings (Na-gluconate pipette and NaCl Ringer bath solution) recorded in bath Ringer (left), 180 mM NMDG-chloride (middle) and 150 mM Na-gluconate (right) in the absence (upper panel) and presence (lower panel) of 10 μ M pyrvinium pamoate. B. Arithmetic means ($\pm$ SEM, n = 11) of the whole cell current as a function of voltage (I-V relationship) in erythrocytes containing 150 mM Na-gluconate in the pipette and either Ringer (open triangles), 180 mM NMDG-chloride (closed triangles) or 150 mM Na-gluconate (closed squares) in the absence of pyrvinium pamoate. C. Arithmetic means ($\pm$ SEM, n = 11) of the whole cell current as a function of voltage (I-V relationship) in erythrocytes containing 150 mM Na-gluconate in the pipette and either Ringer (open triangles), 180 mM NMDG-chloride (closed triangles) or 150 mM Na-gluconate (closed squares) in the presence of pyrvinium pamoate (10 μ M). D. Arithmetic means ($\pm$ SEM, n = 11) of the whole cell conductance (as calculated from the data in (B, C) for the inward currents by linear regression) in erythrocytes containing 150 mM Na-gluconate in the pipette and either Ringer (Ringer), Na-gluconate (NaGlu) or 180 mM NMDG-chloride (NMDG-Cl) in the absence (white bars) or presence (black bars) of pyrvinium pamoate (10 μ M). E. Arithmetic means ($\pm$ SEM, n = 11) of Cl⁻-dependent sodium conductance, calculated as a difference of the conductances in Na-gluconate and NaCl bath media in the bath in the absence (white bar) or presence (black bar) of pyrvinium pamoate (10 μ M). *indicates significant (p < 0.05) difference from absence of pyrvinium pamoate (t-test).

20 minutes following treatment with the Ca²⁺-ionophore ionomycin (1 μ M). As illustrated in Fig. 3, administration of ionomycin was followed by a decline of forward scatter, which was significantly blunted in the presence of 10 μ M pyrvinium pamoate from 5 minutes onwards.

Whole cell patch clamp was employed to closely define the channels involved in Ca²⁺-entry and in cell shrinkage. The Cl⁻-sensitive unspecific Ca²⁺-permeable cation channels were recorded with Na-gluconate in the pipette and NaCl-Ringer, NMDG-Cl or Na-gluconate in the bath solution. As illustrated in Fig. 4, whole cell currents in the presence of Na-gluconate in the pipette increased by replacement of Ringer with Na-gluconate in the bath, an effect

Fig. 5. Effect of pyrvinium pamoate on Gardos channel activity. A. Current tracings (K-gluconate pipette and Na-K-gluconate bath solutions) in the absence of channel inhibitors (left), in the presence of 10 μ M pyrvinium pamoate (middle) and in the presence of 1 μ M Gardos channel inhibitor TRAM 34 (right). B. Current-voltage (I-V) relationships (arithmetic means $\pm$ SEM; $n = 8$) of human erythrocytes recorded with 120 mM K-gluconate pipette solution and Na-K-gluconate bath solution in the absence of inhibitors (closed squares), upon acute application of 10 μ M pyrvinium pamoate (open triangles) or in the presence of 1 μ M TRAM 34 (closed triangles). C. Normalized ($I_{+80 \text{ mV}}$) current-voltage (I-V) relationships (arithmetic means $\pm$ SEM; $n = 8$) of human erythrocytes recorded with 120 mM K-gluconate pipette solution and Na-K-gluconate bath solution in the absence of inhibitors (open squares), upon acute application of 10 μ M pyrvinium pamoate (closed triangles) or in the presence of 1 μ M TRAM 34 (open diamonds).

D. Arithmetic means ($\pm$ SEM, $n = 8$) of the whole cell conductance in erythrocytes recorded with K-gluconate in the pipette and Na-K-gluconate in the bath solutions (as calculated for the linear part of I-V dependences between +20 mV and +60 mV) in the absence of inhibitors (white bar), in the presence of 10 μ M pyrvinium pamoate (black bar) or in the presence of 1 μ M TRAM 34 (stippled bar). *indicates significant ($p < 0.05$) difference from absence of pyrvinium pamoate (paired t-test).

augmented in the cells exposed to pyrvinium pamoate (10 μ M, after 3 - 6 hours incubation at 37°C).

The inhibition of Ca^{2+} -induced cell shrinkage by pyrvinium pamoate could, at least in theory, result from inhibition of the Ca^{2+} -activated (Gardos) K^{+} -channels, which are sensitive to the channel inhibitor TRAM 34 (1 μ M). Whole-cell patch clamp experiments with K-gluconate pipette solution confirmed the presence of TRAM 34 sensitive Ca^{2+} -activated K^{+} -channels with characteristics similar to Gardos channels (Fig. 5). The Gardos channels activity was stimulated by addition of CaCl_2 (180 μ M intracellular free Ca^{2+}) to the K-gluconate pipette bath solution. Acute application of pyrvinium pamoate (10 μ M) resulted in a significant decline in the Gardos channel activity during the first 10 min of incubation (Fig. 5).

Discussion

The present observations provide the novel evidence that the casein kinase 1 α (CK1 α) activator pyrvinium pamoate [32] stimulates the Cl^{-} -sensitive Ca^{2+} -cation channel and by the same token inhibits the Ca^{2+} -activated K^{+} -channel in erythrocytes. Accordingly, pyrvinium pamoate augments the increase in Fluo3-fluorescence and blunts the decrease of forward

scatter following Cl⁻ removal. Inhibition of CK1 α with the specific CK1 inhibitor D4476 [36, 39] blunts the increase of Fluo3-fluorescence following Cl⁻-removal, an observation supporting the view that the effect of pyrvinium pamoate on the Cl⁻-sensitive Ca²⁺-permeable cation channel is at least partially due to activation of CK1 α . The activation of the channel leads to the expected stimulation of cell membrane scrambling, an effect again blunted by D4476 and augmented by pyrvinium pamoate. Similar to pyrvinium pamoate, D4476 blunts the effect of Cl⁻-removal on forward scatter. Thus, the inhibitory effect of pyrvinium pamoate on the Ca²⁺-activated K⁺-channels is presumably not related to its stimulating effect on CK1 α .

Apparently, in the presence of Cl⁻ the activity of the Cl⁻-sensitive Ca²⁺-permeable cation channel is low and the Ca²⁺ entry through this channel is negligible. Under those conditions, inhibition of CK1 with D4476 decreased Fluo3-fluorescence with no effect on forward scatter and annexin-V-binding. Pyrvinium pamoate, on the other hand, increased Fluo3-fluorescence significantly in the presence and absence of Cl⁻. It did not increase cell membrane scrambling in the presence of Cl⁻, but significantly enhanced cell membrane scrambling following Cl⁻-removal. Thus, the effects of D4476 and pyrvinium pamoate on eryptosis become only apparent, when the Cl⁻-sensitive Ca²⁺-permeable cation channel is activated by Cl⁻-removal. Along those lines, CK1 activity may not be relevant for erythrocyte survival under conditions, when the channel is not activated by oxidative stress or energy depletion, as under those conditions, CK1 inhibition is without appreciable effect on cell membrane scrambling [22]. Instead, activated CK1 α augments cell membrane scrambling following glucose depletion and oxidative stress.

At least in theory, CK1 may play a similar role in nucleated cells. A role of CK1 in the regulation of cell survival has been reported before, but considered to be due to interference with transcriptional regulation [37, 39] and modification of β -catenin [29]. Those mechanisms are not likely to participate in the effect of CK1 on cell membrane scrambling, as erythrocytes are devoid of nuclei and the transcriptional or translational machinery.

The effect of CK1 on the cell survival could involve other protein kinases linked to and activated by CK1. CK1 was shown to phosphorylate PKC [44, 45] and PKC in turn phosphorylates CK1 [46]. PKC is known to regulate several TRP channels [47-51]. Moreover, PKC up-regulates endothelin-1 receptors coupled to TRPC1/5/6 channels [51], which participate in the erythrocyte cation channels [52]. In addition, endogenous PKC was shown to modulate the Gardos channels activity in human erythrocytes [53].

In conclusion, the present observations disclose that CK1 α stimulator pyrvinium pamoate is a positive regulator of the Cl⁻-sensitive Ca²⁺-permeable cation channel, and thus of Ca²⁺ entry and cell membrane scrambling in erythrocytes.

Acknowledgements

The authors acknowledge the valuable input of Boris Macek, the friendly support by Mehrdad Ghashghaeinia and the meticulous preparation of the manuscript by Tanja Loch and Sari Rbe. This study was supported by the Deutsche Forschungsgemeinschaft, GRK 1302, SFB 773, La 315/13-3.

References

- 1 Foller M, Bobbala D, Koka S, Huber SM, Gulbins E, Lang F: Suicide for survival-death of infected erythrocytes as a host mechanism to survive malaria. *Cell Physiol Biochem* 2009;24:133-140.
- 2 Bhavsar SK, Gu S, Bobbala D, Lang F: Janus kinase 3 is expressed in erythrocytes, phosphorylated upon energy depletion and involved in the regulation of suicidal erythrocyte death. *Cell Physiol Biochem* 2011;27:547-556.

- 3 Felder KM, Hoelzle K, Ritzmann M, Kilchling T, Schiele D, Heinritzi K, Groebel K, Hoelzle LE: Hemotrophic mycoplasmas induce programmed cell death in red blood cells. *Cell Physiol Biochem* 2011;27:557-564.
- 4 Gatidis S, Zelenak C, Fajol A, Lang E, Jilani K, Michael D, Qadri SM, Lang F: p38 MAPK Activation and Function following Osmotic Shock of Erythrocytes. *Cell Physiol Biochem* 2011;28:1279-1286.
- 5 Ghashghaieinia M, Toulany M, Saki M, Bobbala D, Fehrenbacher B, Rupec R, Rodemann HP, Ghoreschi K, Rocken M, Schaller M, Lang F, Wieder T: The NFκB pathway inhibitors Bay 11-7082 and parthenolide induce programmed cell death in anucleated Erythrocytes. *Cell Physiol Biochem* 2011;27:45-54.
- 6 Lang E, Jilani K, Zelenak C, Pasham V, Bobbala D, Qadri SM, Lang F: Stimulation of suicidal erythrocyte death by benzethonium. *Cell Physiol Biochem* 2011;28:347-354.
- 7 Nguyen DB, Wagner-Britz L, Maia S, Steffen P, Wagner C, Kaestner L, Bernhardt I: Regulation of phosphatidylserine exposure in red blood cells. *Cell Physiol Biochem* 2011;28:847-856.
- 8 Qadri SM, Bauer J, Zelenak C, Mahmud H, Kucherenko Y, Lee SH, Ferlinz K, Lang F: Sphingosine but not sphingosine-1-phosphate stimulates suicidal erythrocyte death. *Cell Physiol Biochem* 2011;28:339-346.
- 9 Qadri SM, Kucherenko Y, Zelenak C, Jilani K, Lang E, Lang F: Dicoumarol Activates Ca-permeable Cation Channels Triggering Erythrocyte Cell Membrane Scrambling. *Cell Physiol Biochem* 2011;28:857-864.
- 10 Bhavsar SK, Eberhard M, Bobbala D, Lang F: Monensin induced suicidal erythrocyte death. *Cell Physiol Biochem* 2010;25:745-752.
- 11 Bobbala D, Alesutan I, Foller M, Huber SM, Lang F: Effect of anandamide in Plasmodium Berghei-infected mice. *Cell Physiol Biochem* 2010;26:355-362.
- 12 Eberhard M, Ferlinz K, Alizzi K, Cacciato PM, Faggio C, Foller M, Lang F: FTY720-induced suicidal erythrocyte death. *Cell Physiol Biochem* 2010;26:761-766.
- 13 Lang F, Gulbins E, Lang PA, Zappulla D, Foller M: Ceramide in suicidal death of erythrocytes. *Cell Physiol Biochem* 2010;26:21-28.
- 14 Siraskar B, Ballal A, Bobbala D, Foller M, Lang F: Effect of amphotericin B on parasitemia and survival of plasmodium berghei-infected mice. *Cell Physiol Biochem* 2010;26:347-354.
- 15 Kempe DS, Lang PA, Duranton C, Akel A, Lang KS, Huber SM, Wieder T, Lang F: Enhanced programmed cell death of iron-deficient erythrocytes. *FASEB J* 2006;20:368-370.
- 16 Birka C, Lang PA, Kempe DS, Hoefling L, Tanneur V, Duranton C, Nammi S, Henke G, Myssina S, Krikov M, Huber SM, Wieder T, Lang F: Enhanced susceptibility to erythrocyte "apoptosis" following phosphate depletion. *Pflügers Arch* 2004;448:471-477.
- 17 Lang PA, Beringer O, Nicolay JP, Amon O, Kempe DS, Hermle T, Attanasio P, Akel A, Schafer R, Friedrich B, Rislér T, Baur M, Olbricht CJ, Zimmerhackl LB, Zipfel PF, Wieder T, Lang F: Suicidal death of erythrocytes in recurrent hemolytic uremic syndrome. *J Mol Med* 2006;84:378-388.
- 18 Kempe DS, Akel A, Lang PA, Hermle T, Biswas R, Muresanu J, Friedrich B, Dreischer P, Wolz C, Schumacher U, Peschel A, Gotz F, Doring G, Wieder T, Gulbins E, Lang F: Suicidal erythrocyte death in sepsis. *J Mol Med* 2007;85:269-277.
- 19 Koka S, Bobbala D, Lang C, Boini KM, Huber SM, Lang F: Influence of paclitaxel on parasitemia and survival of Plasmodium berghei infected mice. *Cell Physiol Biochem* 2009;23:191-198.
- 20 Lang PA, Kasinathan RS, Brand VB, Duranton C, Lang C, Koka S, Shumilina E, Kempe DS, Tanneur V, Akel A, Lang KS, Foller M, Kun JF, Kremsner PG, Wesselborg S, Laufer S, Clemen CS, Herr C, Noegel AA, Wieder T, Gulbins E, Lang F, Huber SM: Accelerated clearance of Plasmodium-infected erythrocytes in sickle cell trait and annexin-A7 deficiency. *Cell Physiol Biochem* 2009;24:415-428.
- 21 Lang PA, Schenck M, Nicolay JP, Becker JU, Kempe DS, Lupescu A, Koka S, Eisele K, Klarl BA, Rubben H, Schmid KW, Mann K, Hildenbrand S, Heftner H, Huber SM, Wieder T, Erhardt A, Haussinger D, Gulbins E, Lang F: Liver cell death and anemia in Wilson disease involve acid sphingomyelinase and ceramide. *Nat Med* 2007;13:164-170.
- 22 Zelenak C, Eberhard M, Jilani K, Qadri SM, Macek B, Lang F: Protein kinase CK1a regulates erythrocyte survival. *Cell Physiol Biochem* 2012;in press.
- 23 Hosey MM, Tao M: Protein kinases of rabbit and human erythrocyte membranes. Solubilization and characterization. *Biochim Biophys Acta* 1977;482:348-357.
- 24 Zelenak C, Foller M, Velic A, Krug K, Qadri SM, Viollet B, Lang F, Macek B: Proteome analysis of erythrocytes lacking AMP-activated protein kinase reveals a role of PAK2 kinase in eryptosis. *J Proteome Res* 2011;10:1690-1697.
- 25 Brockman JL, Anderson RA: Casein kinase I is regulated by phosphatidylinositol 4,5-bisphosphate in native membranes. *J Biol Chem* 1991;266:2508-2512.
- 26 Manno S, Takakuwa Y, Nagao K, Mohandas N: Modulation of erythrocyte membrane mechanical function by beta-spectrin phosphorylation and dephosphorylation. *J Biol Chem* 1995;270:5659-5665.
- 27 Knippschild U, Wolff S, Giamas G, Brockschmidt C, Wittau M, Wurl PU, Eismann T, Stoter M: The role of the casein kinase 1 (CK1) family in different signaling pathways linked to cancer development. *Onkologie* 2005;28:508-514.
- 28 Knippschild U, Gocht A, Wolff S, Huber N, Lohler J, Stoter M: The casein kinase 1 family: participation in multiple cellular processes in eukaryotes. *Cell Signal* 2005;17:675-689.

- 29 Sinnberg T, Menzel M, Kaesler S, Biedermann T, Sauer B, Nahnsen S, Schwarz M, Garbe C, Schitteck B: Suppression of casein kinase 1alpha in melanoma cells induces a switch in beta-catenin signaling to promote metastasis. *Cancer Res* 2010;70:6999-7009.
- 30 Tillement V, Lajoie-Mazenc I, Casanova A, Froment C, Penary M, Tovar D, Marquez R, Monsarrat B, Favre G, Pradines A: Phosphorylation of RhoB by CK1 impedes actin stress fiber organization and epidermal growth factor receptor stabilization. *Exp Cell Res* 2008;314:2811-2821.
- 31 Ding L, Wang Z, Yan J, Yang X, Liu A, Qiu W, Zhu J, Han J, Zhang H, Lin J, Cheng L, Qin X, Niu C, Yuan B, Wang X, Zhu C, Zhou Y, Li J, Song H, Huang C, Ye Q: Human four-and-a-half LIM family members suppress tumor cell growth through a TGF-beta-like signaling pathway. *J Clin Invest* 2009;119:349-361.
- 32 Thorne CA, Hanson AJ, Schneider J, Tahinci E, Orton D, Cselenyi CS, Jernigan KK, Meyers KC, Hang BI, Waterson AG, Kim K, Melancon B, Ghidu VP, Sulikowski GA, LaFleur B, Salic A, Lee LA, Miller DM, III, Lee E: Small-molecule inhibition of Wnt signaling through activation of casein kinase 1alpha. *Nat Chem Biol* 2010;6:829-836.
- 33 Downey AS, Chong CR, Graczyk TK, Sullivan DJ: Efficacy of pyrvinium pamoate against *Cryptosporidium parvum* infection in vitro and in a neonatal mouse model. *Antimicrob Agents Chemother* 2008;52:3106-3112.
- 34 Hempelmann E: Hemozoin biocrystallization in *Plasmodium falciparum* and the antimalarial activity of crystallization inhibitors. *Parasitol Res* 2007;100:671-676.
- 35 TURNER JA, JOHNSON PE, Jr: Pyrvinium pamoate in the treatment of pinworm infection (enterobiasis) in the home. *J Pediatr* 1962;60:243-251.
- 36 Bain J, Plater L, Elliott M, Shpiro N, Hastie CJ, McLauchlan H, Klevernic I, Arthur JS, Alessi DR, Cohen P: The selectivity of protein kinase inhibitors: a further update. *Biochem J* 2007;408:297-315.
- 37 Huart AS, MacLaine NJ, Meek DW, Hupp TR: CK1alpha plays a central role in mediating MDM2 control of p53 and E2F-1 protein stability. *J Biol Chem* 2009;284:32384-32394.
- 38 MacLaine NJ, Hupp TR: The regulation of p53 by phosphorylation: a model for how distinct signals integrate into the p53 pathway. *Aging (Albany NY)* 2009;1:490-502.
- 39 Rena G, Bain J, Elliott M, Cohen P: D4476, a cell-permeant inhibitor of CK1, suppresses the site-specific phosphorylation and nuclear exclusion of FOXO1a. *EMBO Rep* 2004;5:60-65.
- 40 Li C, MacDonald JI, Hryciw T, Meakin SO: Nerve growth factor activation of the TrkA receptor induces cell death, by macropinocytosis, in medulloblastoma Daoy cells. *J Neurochem* 2010;112:882-899.
- 41 Grgic I, Kaistha BP, Paschen S, Kaistha A, Busch C, Si H, Kohler K, Elsasser HP, Hoyer J, Kohler R: Disruption of the Gardos channel (KCa3.1) in mice causes subtle erythrocyte macrocytosis and progressive splenomegaly. *Pflugers Arch* 2009;458:291-302.
- 42 Tharp DL, Bowles DK: The intermediate-conductance Ca²⁺-activated K⁺ channel (KCa3.1) in vascular disease. *Cardiovasc Hematol Agents Med Chem* 2009;7:1-11.
- 43 Barry PH, Lynch JW: Liquid junction potentials and small cell effects in patch-clamp analysis. *J Membr Biol* 1991;121:101-117.
- 44 Okano M, Yokoyama T, Miyanaga T, Ohtsuki K: Activation of C-kinase eta through its cholesterol-3-sulfate-dependent phosphorylation by casein kinase I in vitro. *Biol Pharm Bull* 2004;27:109-112.
- 45 Vila J, Walker JM, Itarte E, Weber MJ, Sando JJ: Phosphorylation of protein kinase C by casein kinase-1. *FEBS Lett* 1989;255:205-208.
- 46 Giamas G, Hirner H, Shoshiashvili L, Grothey A, Gessert S, Kuhl M, Henne-Bruns D, Vorgias CE, Knippschild U: Phosphorylation of CK1delta: identification of Ser370 as the major phosphorylation site targeted by PKA in vitro and in vivo. *Biochem J* 2007;406:389-398.
- 47 Adcock JJ: TRPV1 receptors in sensitisation of cough and pain reflexes. *Pulm Pharmacol Ther* 2009;22:65-70.
- 48 Earley S, Straub SV, Brayden JE: Protein kinase C regulates vascular myogenic tone through activation of TRPM4. *Am J Physiol Heart Circ Physiol* 2007;292:H2613-H2622.
- 49 Harteneck C, Gollasch M: Pharmacological modulation of diacylglycerol-sensitive TRPC3/6/7 channels. *Curr Pharm Biotechnol* 2011;12:35-41.
- 50 Peng H, Lewandrowski U, Muller B, Sickmann A, Walz G, Wegierski T: Identification of a Protein Kinase C-dependent phosphorylation site involved in sensitization of TRPV4 channel. *Biochem Biophys Res Commun* 2010;391:1721-1725.
- 51 Saleh SN, Albert AP, Large WA: Activation of native TRPC1/C5/C6 channels by endothelin-1 is mediated by both PIP3 and PIP2 in rabbit coronary artery myocytes. *J Physiol* 2009;587:5361-5375.
- 52 Foller M, Mahmud H, Gu S, Kucherenko Y, Gehring EM, Shumilina E, Floride E, Sprengel R, Lang F: Modulation of suicidal erythrocyte cation channels by an AMPA antagonist. *J Cell Mol Med* 2009;13:3680-3686.
- 53 Del Carlo B, Pellegrini M, Pellegrino M: Modulation of Ca²⁺-activated K⁺ channels of human erythrocytes by endogenous protein kinase C. *Biochim Biophys Acta* 2003;1612:107-116.