Protein Kinase CK1α Regulates Erythrocyte Survival

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Abstract
Protein kinase CK1 (casein kinase 1) isoforms are involved in the regulation of various physiological functions including apoptosis. The specific CK1 inhibitor D4476 may either inhibit or foster apoptosis. Similar to apoptosis of nucleated cells, eryptosis, the suicidal death of erythrocytes, is paralleled by cell shrinkage and cell membrane scrambling with phosphatidylserine exposure at the cell surface. Triggers of eryptosis include increase of cytosolic Ca²⁺ activity following energy depletion (removal of glucose) or oxidative stress (exposure to the oxidant tert-butyl hydroperoxide [TBOOH]). Western blotting was utilized to verify that erythrocytes express the protein kinase CK1α, and FACS analysis to determine whether the CK1 inhibitor D4476 and CK1α activator pyrvinium pamoate modify forward scatter (reflecting cell volume), annexin V binding (reflecting phosphatidylserine exposure), and Fluo3 fluorescence (reflecting cytosolic Ca²⁺ activity). As a result, both, human and murine erythrocytes express CK1 isoform α. Glucose depletion (48 hours) and exposure to 0.3 mM TBOOH (30 minutes) both decreased forward scatter, increased annexin V binding and increased Fluo3 fluorescence. CK1 inhibitor D4476 (10 µM) significantly blunted the decrease in forward scatter, the increase in annexin V binding and the increase in Fluo 3 fluorescence. (R)-DRF053, another CK1 inhibitor, similarly blunted the increase in annexin V binding upon glucose depletion. The CK1α specific activator pyrvinium pamoate (10 µM) significantly enhanced the increase in annexin V binding and Fluo3 fluorescence upon glucose depletion and TBOOH exposure. In the presence of glucose, pyrvinium pamoate slightly but significantly increased Fluo3 fluorescence. In conclusion, CK1 isoform α participates in the regulation of erythrocyte programmed cell death by modulating cytosolic Ca²⁺ activity.

Key Words
Casein kinase 1α • Eryptosis • Oxidative stress • Phosphatidylserine • Annexin • Cell volume

Introduction
Protein kinase CK1 (casein kinase I) is a monomeric serine/threonine protein kinase involved in the regulation of a wide variety of cellular functions including membrane trafficking, cell cycle progression, chromosome segregation, apoptosis and cellular differentiation [1, 2].
Several CK1 isoforms α, β, γ1, γ2, γ3, δ, and ε and additional splice variants are expressed in mammalian cells [2].

Genetic defects or dysregulation of CK1 have been implicated in neurodegeneration and cancer [1, 2]. CK1 may suppress tumor growth in part by phosphorylation of β-catenin, which fosters β-catenin degradation [3], by phosphorylating RhoB and stress fibers [4] thus interacting with the actin stress fiber organization [4] or by participating in four-and-a-half LIM (FHL) signaling [5].

CK1α is expressed in erythrocytes [6-10] and found in the cytosol as well as in the membrane as “membrane bound casein kinase 1” (MBCK1) [11]. CK1α expression is decreased in erythrocytes from AMPKα1-deficient mice [12]. Both, the cytosolic and the membrane-bound form of CK1 in erythrocytes are known to affect the assembly of cytoskeleton proteins by phosphorylation [11]. The membrane-bound form of CK1 is known to phosphorylate β-spectrin resulting in decreased mechanical stability of the membrane [13]. The cytosolic and the membrane-bound form of CK1 have been identified as “very similar but not identical” [14].

CK1 is regulated by (auto)phosphorylation/dephosphorylation and interaction with other proteins or small allosterically active molecules [2]. CK1α is stimulated 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) [15], an antimicrobial drug used e.g. in the therapy against pinworms (Enterobius vermicularis) [15, 16], Plasmodium falciparum [17] and Cryptosporidium parvum [18]. Several CK1 isoforms including CK1α are inhibited by D4476 (4-(4-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1Himidazol-2-yl)benzamide) [19-22], which has been reported to either inhibit [23] or foster [19] apoptosis.

Erythrocytes, similar to apoptosis of nucleated cells, are able to enter suicidal death, which is then called eryptosis [24]. In analogy to apoptosis, eryptosis is paralleled by cell shrinkage, cell membrane blebbing and cell membrane scrambling with exposure of phosphatidylserine at the erythrocyte surface [24]. Eryptosis may be triggered by a wide variety of xenobiotics and endogenous substances [25-35]. On the other hand, eryptosis may be blunted by pharmacological inhibition of erythrocyte AMPA receptor [36]. Moreover, accelerated eryptosis contributes to or even accounts for anemia in several clinical disorders [24], such as iron deficiency [37], phosphate depletion [38], Hemolytic Uremic Syndrome [39], sepsis [24], malaria [40] or Wilson’s disease [41].

The present study explored whether casein kinase 1 expressed in erythrocytes influences eryptosis.

**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 isolation of murine erythrocytes, density centrifugation of full blood with two polysucrose solutions of different concentrations (Histopaque 1083 and 1119, Sigma, Freiburg, Germany) was used according to the protocol provided by the company with slight modifications. Briefly, erythrocytes were pelleted at the bottom of the tube by centrifugation, whereas other cells were captured in layers over the histopaque solutions. The erythrocyte pellet was isolated and washed three times with PBS.

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 MgSO4, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 5 glucose, 1 CaCl2; pH 7.4 at a hematocrit of 0.4%. Where indicated, glucose was removed or 0.3 mM tert-butyl-hydroperoxide (TBOOH) (Sigma, Schnellendorf, Germany) was added. The CK1 inhibitors D4476 (4-(4-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1Himidazol-2-yl)benzamide) (Calbiochem, Bad Soden, Germany) and (R)-DVF053 dihydrochloride (Tocris, Bristol, UK) and the CK1 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) were used at the indicated concentrations.

D4476, (R)-DVF053 and pyrvinium pamoate were solubilized in DMSO (Calbiochem). Thus, control experiments of erythrocytes in Ringer and glucose depleted Ringer ± 5 µl DMSO were performed. As a result, DMSO exposure did not significantly modify the number of phosphatidylserine-exposing erythrocytes nor erythrocyte forward scatter, nor intracellular calcium activity (Table 1).

**Phosphatidylserine exposure and forward scatter**

For FACS analysis erythrocytes were washed after incubation once in Ringer solution containing 5 mM CaCl2. 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 µl suspension of erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 5 mM CaCl₂ and 2 µM Fluo-3/AM. The cells were incubated at 37°C for 30 min and then resuspended in 150 µl Ringer. The Ca^{2+}-dependent fluorescence intensity was then measured in fluorescence channel FL-1 in FACS analysis.

Western blotting

To examine the expression of protein kinase CK1α in human erythrocytes 150 µl erythrocyte pellet were lysed in 50 ml of 20 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES)/NaOH (pH 7.4). Ghost membranes were pelleted (15,000 g for 20 min at 4°C) and lysed in 200 µl lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1mM NaF, 1mM Na₃VO₄, 0.4% β-mercaptoethanol) containing protease inhibitor cocktail (Sigma, Taufkirchen, Germany). Isolated murine erythrocytes were lysed in the same lysis buffer. K562 cells were used as positive control for the expression of CK1α. In all cases, 50 µg of protein were solubilized in Laemmli sample buffer at 95°C for 5 min and resolved by 10% SDS-PAGE. For immunoblotting proteins were electro-transferred onto a PVDF membrane and blocked with 5% nonfat milk in TBS-0.10% Tween 20 (TBST) at room temperature for 1 h. Then, the membrane was incubated at 4°C overnight with affinity purified rabbit anti-CK1α antibody (1:1000; 34 kDa, Product number #2655 Cell Signaling, Freiburg, Germany), which, according to manufacturer information, may crossreact with CK1ε, CK1δ, or CK1γ. After washing 3 times with TBST (10 min each) the blots were incubated with horseradish peroxidase conjugated secondary anti-rabbit antibody (1:2000; Cell Signaling, Freiburg, Germany). Antibody-binding was quantified densitometrically with Quantity One Software (Biorad, München, Germany). Rabbit anti-GAPDH antibody (1:1000; 37 kDa, Cell Signaling, Freiburg, Germany) was used for detecting a loading control.

Confocal microscopy

For the visualisation of eryptotic erythrocytes, 20 µl of erythrocytes, incubated in respective experimental conditions, were stained with FITC-conjugated annexin-V-FITC (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 µl Ringer solution containing 5 mM CaCl₂. Then the erythrocytes were washed twice and finally resuspended in 50 µl of Ringer solution containing 5 mM CaCl₂, 10 µl were mixed on a glass slide with 10 µl of Prolong Gold antifade reagent (Invitrogen, Darmstadt, Germany), covered with a coverslip, and images were subsequently taken on a Zeiss LSM 5 EXCITER confocal laser scanning microscope (Carl Zeiss MicroImaging, Oberkochen, Germany) with a water immersion Plan-Neofluar 63/1.3 NA DIC.

Statistics

Data are expressed as arithmetic means ± SEM. n is the number of batches used. As different erythrocyte batches used in distinct experiments are differently susceptible to eryptotic effects, paired ANOVA with Tukey’s test as post-test was used for statistical analysis. In some cases, a normalization of values to control values was appropriate and is denoted in the respective graphs. p < 0.05 was considered as statistically significant.

Results

Western blotting was utilized to verify whether erythrocytes express the protein kinase CK1α. As a result, both, human and murine erythrocytes expressed the CK1α protein (Fig. 1). In murine erythrocytes two bands were observed. The second band may reflect CK1α modified e.g. by phosphorylation. The CK1α abundance was significantly lower in erythrocytes from AMPKα1-deficient mice than in corresponding wild type mice (Fig. 1). Subsequent experiments were performed to explore whether CK1α is involved in the regulation of suicidal erythrocyte death or eryptosis.

In the first series of experiments, eryptosis was triggered by energy depletion. According to forward scatter, glucose depletion was followed by a significant decrease of cell volume (Fig. 2). The addition of the specific CK1 inhibitor D4476 did not significantly alter forward scatter in the presence of glucose but significantly blunted the decrease of forward scatter following glucose depletion, an effect reaching statistical significance at 10 µM D4476 (Fig. 2).

Glucose removal further triggered cell membrane scrambling with exposure of phosphatidylserine at the erythrocyte surface, which could be quantified by annexin V binding. Glucose removal was followed by a significant increase in annexin V-binding (Fig. 3). Addition of D4476 did not significantly modify annexin V binding in the presence of glucose but blunted the increase of annexin.

| Measure | CONTROL RINGER | GLUCOSE-DEPLETED RINGER |
|---------|----------------|------------------------|
| PS-exposing cells [%] | 2.6 ± 0.8 | 2.3 ± 0.8 | 4.4 ± 5.8 | 38.9 ± 5.9 |
| FSC [arb. units] | 441 ± 8 | 453 ± 6 | 195 ± 5 | 202 ± 6 |
| Fluo3-fluorescence [arb. units] | 13.5 ± 0.7 | 16.6 ± 1.0 | 29.7 ± 1.2 | 26.9 ± 0.7 |

Table 1. Effect of DMSO on phosphatidylserine (PS) exposure, forward scatter (FCS) and Fluo3-fluorescence (arithmetic means ± SEM, n = 5).

CK1α and Eryptosis
Expression of CK1α in human and murine erythrocytes. A. Western blot demonstrating the expression of CK1α protein in human whole blood and purified human erythrocytes. K562 cells were used as positive control. B. Western blot demonstrating the expression of CK1α protein in histopaque isolated erythrocytes from wild type mice and from AMPKα1-deficient mice. C. Arithmetic means ± SEM (n = 3) of the densitometric western blot analysis showing the expression of CK1α in wild type (white bar) and in AMPKα1-deficient erythrocytes (black bar). ***indicates significant (p < 0.001) difference from wild type animals.

Fig. 1. Expression of CK1α in human and murine erythrocytes. A. Western blot demonstrating the expression of CK1α protein in human whole blood and purified human erythrocytes. K562 cells were used as positive control. B. Western blot demonstrating the expression of CK1α protein in histopaque isolated erythrocytes from wild type mice and from AMPKα1-deficient mice. C. Arithmetic means ± SEM (n = 3) of the densitometric western blot analysis showing the expression of CK1α in wild type (white bar) and in AMPKα1-deficient erythrocytes (black bar). ***indicates significant (p < 0.001) difference from wild type animals.

V binding following glucose removal, an effect reaching statistical significance at 10 µM D4476 (Fig. 3). Similarly (R)-DRF053, a non specific CK1 inhibitor, blunted the increase of annexin V-binding upon energy depletion (Fig. 4). As all pharmacological inhibitors and activators were dissolved in DMSO, additional experiments were performed to rule out an effect of the vehicle on annexin V-binding. As a result, neither in control Ringer nor in glucose depleted Ringer did DMSO significantly modify cytosolic Ca²⁺ activity, forward scatter or phosphatidylserine exposure (Table 1).

Both, cell shrinkage and cell membrane scrambling are triggered by increase of cytosolic Ca²⁺ activity. Accordingly, Fluo3 fluorescence was employed to estimate cytosolic Ca²⁺ activity. In the presence of glucose, 10 µM D4476 slightly but significantly decreased Fluo3 fluorescence (Fig. 5). Glucose depletion was followed by a marked increase of Fluo3 fluorescence (Fig. 5). Addition of D4476 blunted the increase of Fluo3 fluorescence following glucose removal, an effect reaching statistical significance at 1 µM D4476 (Fig. 5).
stress. To this end, erythrocytes were exposed for 30 minutes to 0.3 mM tert-butyl-hydroperoxide (TBOOH). As shown in Figures 2, 3, and 5, TBOOH exposure significantly decreased forward scatter (Fig. 2), triggered cell membrane scrambling resulting in the respective increase of the percentage of annexin V binding erythrocytes (Fig. 3) and increased Fluo3 fluorescence (Fig. 5). The addition of D4476 significantly blunted all three effects of TBOOH, i.e. the decrease in forward scatter (Fig. 2), the increase of annexin V binding (Fig. 3) and the increase of Fluo3 fluorescence (Fig. 5). The effects of D4476 in TBOOH treated erythrocytes each reached statistical significance at 10 µM D4476.

Additional experiments were performed elucidating the effect of the CK1α activator pyrvinium pamoate. As illustrated in Fig. 6, pyrvinium pamoate did not significantly alter erythrocyte forward scatter in the presence of glucose and absence of TBOOH. Moreover, pyrvinium pamoate did not significantly alter forward scatter in the absence of glucose and in the presence of TBOOH (Fig. 6).

Addition of pyrvinium pamoate did not significantly modify the percentage of annexin V positive erythrocytes in the presence of glucose and in the absence of TBOOH but augmented the increase of annexin V binding following glucose depletion (Fig. 7) or oxidative stress (Fig. 7). The effects of pyrvinium pamoate on annexin V binding reached statistical significance at 10 µM (in the absence of glucose) and 5 µM (in the presence of TBOOH).

Pyrvinium pamoate enhanced Fluo3 fluorescence in the presence of glucose and in the absence of TBOOH,
an effect reaching statistical significance at 10 µM. Moreover, pyrvinium pamoate augmented the increase of Fluo 3 fluorescence following glucose depletion (Fig. 8) or oxidative stress (Fig. 8). Both, in the absence of glucose and in the presence of TBOOH the effect of pyrvinium pamoate on Fluo 3 fluorescence reached statistical significance at 5 µM.

The effect of D4476 and of pyrvinium pamoate on cell membrane scrambling of glucose depleted erythro-
Fig. 7. Effect of pyrvinium pamoate on erythrocyte phosphatidylserine exposure. A. Histograms of annexin V binding in representative experiments of erythrocytes exposed for 30 minutes to Ringer in the absence (black line) or presence of 0.3 mM tert-butyl-hydroperoxide after 24 hours incubation in Ringer without (red line) or with (green line) 10 µM CK1α activator pyrvinium pamoate. B. Histograms of annexin V binding in representative experiments of erythrocytes exposed for 48 hours to Ringer with (black line), or without glucose in the absence (red line) or presence (green line) of 10 µM CK1α activator pyrvinium pamoate for the second 24 hours. C. Arithmetic means ± SEM (n = 4 - 14) of the percentage of annexin V binding erythrocytes following a 24 hours incubation in the absence of pyrvinium pamoate and then further 24 hours in the absence (0 µM pyrvinium pamoate) or presence of 1, 5 and 10 µM pyrvinium pamoate. The erythrocytes were exposed 48 hours to control Ringer (presence of glucose and absence of tert-butyl-hydroperoxide [TBOOH]) (white bars), in glucose depleted Ringer (black bars) or in glucose containing Ringer with addition of 0.3 mM tert-butyl-hydroperoxide for the final 30 minutes (grey bars). * indicates significant (p < 0.05), ** indicates significant (p < 0.01), *** indicates significant (p < 0.001) difference from Ringer without 0.3 mM tert-butyl-hydroperoxide, ### indicates significant (p < 0.001) difference from absence of pyrvinium pamoate (ANOVA).

cytes was further visualized by confocal microscopy. As illustrated in Fig. 9, glucose depletion increased the number of annexin V positive erythrocytes, an effect blunted in the presence of the CK1 inhibitor D4476 (10 µM) and augmented in the presence of the CK1α activator pyrvinium pamoate (10 µM).

Fig. 8. Effect of pyrvinium pamoate on erythrocyte cytosolic Ca²⁺ concentration. A. Histograms of Fluo3 fluorescence in representative experiments of erythrocytes exposed for 30 minutes to Ringer in the absence (black line) or presence of 0.3 mM tert-butyl-hydroperoxide after 24 hours incubation in Ringer without (red line) or with (green line) 10 µM CK1α activator pyrvinium pamoate. B. Histograms of Fluo3 fluorescence in representative experiments of erythrocytes exposed for 48 hours to Ringer with (black line), or without glucose in the absence (red line) or presence (green line) of 10 µM CK1α activator pyrvinium pamoate for the second 24 hours. C. Arithmetic means ± SEM (n = 4 - 17) of the Fluo3 fluorescence of erythrocytes following a 24 hours incubation in the absence of pyrvinium pamoate and then further 24 hours in the absence (0 µM pyrvinium pamoate) or presence of 1, 5 and 10 µM pyrvinium pamoate. The erythrocytes were exposed 48 hours to control Ringer (presence of glucose and absence of tert-butyl-hydroperoxide [TBOOH]) (white bars), in glucose depleted Ringer (black bars) or in glucose containing Ringer with addition of 0.3 mM tert-butyl-hydroperoxide for the final 30 minutes (grey bars). * indicates significant (p < 0.05), ** indicates significant (p < 0.01), *** indicates significant (p < 0.001) difference from Ringer without 0.3 mM tert-butyl-hydroperoxide, ### indicates significant (p < 0.001) difference from absence of pyrvinium pamoate (ANOVA).
In nucleated cells, CK1 affects cell survival by interference with transcriptional regulation [19, 22], an effect in part due to modification of β-catenin degradation [3]. Those mechanisms are unlikely to participate in the antieryptotic effect, as erythrocytes are devoid of nuclei and lack the transcriptional or translational machinery. Instead, CK1α inhibition blunts and CK1α activation enhances Ca2+ entry following glucose depletion and oxidative stress, which may point to a role of CK1α in the regulation of ion channels. Casein kinase II has previously been shown to participate in the regulation of NMDA receptors [43] and Ca(V)1.1 Ca2+ channels [44]. To the best of our knowledge, regulation of Ca2+ channels by CK1α has never been shown.

Glucose depletion and oxidative stress trigger eryptosis in only a fraction of erythrocytes. Thus, only a fraction of circulating erythrocytes appear to be sensitive to those challenges. The susceptibility may be influenced by erythrocyte age, as senescent erythrocytes may have a decreased capacity to counteract oxidative stress [45-47]. On the other hand, newly formed erythrocytes may be particularly vulnerable, as shown following return from high altitude or a space flight [48] or in patients with paroxysmal nocturnal hemoglobinuria and myelodysplastic syndrome [49]. The increase of cytosolic Ca2+ activity following oxidative stress or glucose depletion is presumably a function of the abundance of functional cation channels on the one hand and the power of Ca2+ extrusion mechanisms on the other. Either one may, at least in theory, be a function of erythrocyte age.

The impact of CK1 on erythrocyte survival may be relevant for the life span of circulating erythrocytes. Phosphatidylserine-exposing cells adhere to [50] and are engulfed by [51] macrophages. Accordingly, they are rapidly cleared from circulating blood [35]. Along those lines, accelerated eryptosis may lead to anemia [24]. Apparently, under control conditions, CK1α activity is not required for the maintenance of cell survival, as under those conditions, CK1α inhibition is without appreciable effect on cell membrane scrambling. In contrast, activated CK1α fosters the cell membrane scrambling following glucose depletion and oxidative stress.

Eryptosis may further influence microcirculation. Phosphatidylserine-exposing erythrocytes may adhere to the vascular wall [52-56] and stimulate the assembly of prothrombinase and tenase, the generation of thrombin and thus blood clotting [52, 57, 58]. Along those lines, enhanced trapping of eryptotic erythrocytes has previously been observed in renal medulla following ischemia [59]. Accordingly, suicidal erythrocytes may participate in...
vascular injury of metabolic syndrome [60]. Moreover, oxidative stress has been implicated in the ageing of stored erythrocytes [61]. In conclusion, the present observations disclose that CK1α is a positive regulator of Ca\(^{2+}\) entry and cell membrane scrambling following glucose depletion and oxidative stress in erythrocytes.

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