Protein Kinase CK1 α Regulates Erythrocyte Survival

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Key Words

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

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 CK1 α , and FACS analysis to determine whether the CK1 inhibitor D4476 and CK1 α 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 μ M) 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 CK1 α specific activator pyrvinium pamoate (10 μ M) 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.

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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].

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Several CK1 isoforms α , β , γ_1 , γ_2 , γ_3 , 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].

CK1 α 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]. CK1 α expression is decreased in erythrocytes from AMPK α 1-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]. CK1 α 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 CK1 α are inhibited by D4476 (4-(4-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-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, Schnelldorf, Germany) was added. The CK1 inhibitors 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 (R)-DRF053 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, Schnelldorf, Germany) were used at the indicated concentrations.

D4476, (R)-DRF053 and pyrvinium pamoate were solubilized in DMSO (Calbiochem). Thus, control experiments of erythrocytes in Ringer and glucose depleted Ringer $\pm$ 5 μ l 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.

	CONTROL RINGER		GLUCOSE-DEPLETED RINGER	
	0 μ l DMSO	5 μ l DMSO	0 μ l DMSO	5 μ l DMSO
PS-exposing cells [%]	2.6 $\pm$ 0.8	2.3 $\pm$ 0.8	44.5 $\pm$ 5.8	38.9 $\pm$ 5.9
FSC [arb. units]	441 $\pm$ 8	453 $\pm$ 6	195 $\pm$ 5	202 $\pm$ 6
Fluo3-fluorescence [arb. units]	13.5 $\pm$ 0.7	16.6 $\pm$ 1.0	29.7 $\pm$ 1.2	26.9 $\pm$ 0.7

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

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.

Western blotting

To examine the expression of protein kinase CK1 α in human erythrocytes 150 μ l 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 μ l lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.5% SDS, 1mM NaF, 1mM Na_3VO_4 , 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 CK1 α . In all cases, 50 μ g 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-CK1 α antibody (1:1000; 34 kDa, Product number #2655 Cell Signaling, Freiburg, Germany), which, according to manufacturer information, may crossreact with CK1 ϵ , CK1 δ or CK1 γ . 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) for 1 h at room temperature. After washing antibody binding was detected with the ECL detection reagent (Amersham, 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 μ l of erythrocytes, incubated in respective experimental conditions, were stained with FITC-conjugated annexin-V-FITC (1:100 dilution; ImmunoTools, Friesoythe, Germany) in 200 μ l Ringer solution containing 5 mM $CaCl_2$. Then the erythrocytes were washed twice and finally resuspended in 50 μ l of Ringer solution containing 5 mM $CaCl_2$. 10 μ l were mixed on a glass slide with 10 μ l 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 $\pm$ 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 CK1 α . As a result, both, human and murine erythrocytes expressed the CK1 α protein (Fig. 1). In murine erythrocytes two bands were observed. The second band may reflect CK1 α modified e.g. by phosphorylation. The CK1 α abundance was significantly lower in erythrocytes from AMPK α 1-deficient mice than in corresponding wild type mice (Fig. 1). Subsequent experiments were performed to explore whether CK1 α 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 μ M 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

V binding following glucose removal, an effect reaching statistical significance at 10 μ M 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 μ M 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 μ M D4476 (Fig. 5).

Fig. 3. Effect of D4476 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 incubation for 48 hours in Ringer in the absence (red line) or presence (green line) of 10 μ M CK1 inhibitor D4476. 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 μ M CK1 inhibitor D4476. C. Arithmetic means $\pm$ SEM ($n = 4 - 11$) of the percentage of annexin V binding of erythrocytes following a 48 hours incubation in the absence (0 μ M D4476) or presence of 1 and 10 μ M D4476 in 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 ($p < 0.001$) difference from glucose-containing Ringer without 0.3 mM tert-butyl-hydroperoxide, ##indicate significant ($p < 0.01$) difference from the absence of D4476 (ANOVA).

A further series of experiments explored whether CK1 inhibition influences the eryptosis following oxidative

Fig. 4. Effect of (R)-DRF053 dihydrochloride on erythrocyte phosphatidylserine exposure. Arithmetic means $\pm$ SEM ($n = 11$) of the percentage of annexin V binding of erythrocytes following a 48 hours incubation in the absence (0 μ M (R)-DRF053) or presence of 0.1 and 1 μ M (R)-DRF053 in control Ringer with glucose (white bars) or in glucose-depleted Ringer (black bars). ***indicates significant ($p < 0.001$) difference from glucose-containing Ringer, ###indicates significant ($p < 0.001$) difference from the absence of (R)-DRF053 (ANOVA).

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 μ M D4476.

Additional experiments were performed elucidating the effect of the CK1 α 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 μ M (in the absence of glucose) and 5 μ M (in the presence of TBOOH).

Pyrvinium pamoate enhanced Fluo3 fluorescence in the presence of glucose and in the absence of TBOOH,

Fig. 5. Effect of D4476 on erythrocyte cytosolic Ca^{2+} 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 incubation for 48 hours in Ringer in the absence (red line) or presence (green line) of 10 μ M CK1 inhibitor D4476. 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 μ M CK1 inhibitor D4476. C. Arithmetic means $\pm$ SEM ($n = 4 - 7$) of the Fluo3 fluorescence of erythrocytes following a 48 hours incubation in the absence (0 μ M D4476) or presence of 1 and 10 μ M D4476 in 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). *, *** indicate significant ($p < 0.05$, $p < 0.001$) difference from glucose-containing Ringer without 0.3 mM tert-butyl-hydroperoxide, #, ### indicate significant ($p < 0.05$, $p < 0.001$) difference from the absence of D4476 (ANOVA).

an effect reaching statistical significance at 10 μ M. 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

Fig. 6. Effect of pyrvinium pamoate on erythrocyte forward scatter. A. Histograms of forward scatter 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 μ M CK1 α activator pyrvinium pamoate. B. Histograms of forward scatter 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 μ M CK1 α activator pyrvinium pamoate for the second 24 hours. C. Arithmetic means $\pm$ SEM ($n = 4 - 14$) 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 (0 μ M pyrvinium pamoate) or presence of 1, 5 and 10 μ M 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). *** indicate significant ($p < 0.001$) difference from Ringer without 0.3 mM tert-butyl-hydroperoxide, (ANOVA).

and in the presence of TBOOH the effect of pyrvinium pamoate on Fluo 3 fluorescence reached statistical significance at 5 μ M.

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 $\pm$ 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). *, ** indicate significant ($p < 0.05$, $p < 0.001$) difference from Ringer without 0.3 mM tert-butyl-hydroperoxide, ### indicate significant ($p < 0.001$) difference from the 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

Fig. 8. Effect of pyrvinium pamoate on erythrocyte cytosolic Ca^{2+} 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 $\pm$ 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). *, **, *** indicate significant ($p < 0.05$, $p < 0.01$, $p < 0.001$) difference from Ringer without 0.3 mM tert-butyl-hydroperoxide. #, ### indicate significant ($p < 0.05$, $p < 0.001$) difference from absence of pyrvinium pamoate (ANOVA).

the presence of the CK1 inhibitor D4476 (10 μM) and augmented in the presence of the CK1α activator pyrvinium pamoate (10 μM).

Fig. 9. Confocal images of PS-exposing erythrocytes following treatment with D4476 or pyrvinium pamoate. Confocal images of erythrocytes binding to FITC-conjugated annexin-V (lower panels) and the corresponding transmission light photographs (upper panels).

Discussion

The present observations reveal the expression of the casein kinase 1 alpha (CK1 α) isoform in human and murine erythrocytes and the downregulation of CK1 α in AMPK α 1-deficient murine erythrocytes. As erythrocytes lack nuclei and are thus unable to express proteins, the effect of AMPK on the CK1 protein abundance may result from altered expression of CK1 in erythrocyte progenitor cells. Alternatively, AMPK could have an effect on the stability of the CK1 protein. Further experiments will be required to elucidate the underlying mechanisms.

The present study further uncovers a novel function of CK1 α , namely the participation in the regulation of erythrocyte survival. The specific CK1 inhibitor D4476 [21, 22] blunts and the CK1 α activator pyrvinium pamoate [15] augments the effect of glucose removal and oxidative stress on cytosolic Ca²⁺ and cell membrane scrambling. Since the CK1 α activator pyrvinium pamoate is specific for CK1 α and does not affect the activity of other CK1 isoforms, and since D4476 has the opposite effects to pyrvinium pamoate, the present experiments are most likely reflecting effects of CK1 α . The concentrations of pyrvinium pamoate employed in the present study are in the range of those required for antihelminthic efficacy [18, 42]. During *in vivo* treatment, 61 μ mol/kg bodyweight/day were administered in humans and 22 μ mol/kg bodyweight/day in mice [18]. The D4476 concentrations employed are again similar to those shown effective in previous studies [19]. D4476 has, to our best knowledge, not yet been used *in vivo*.

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, CK1 α inhibition blunts and CK1 α activation enhances Ca²⁺ entry following glucose depletion and oxidative stress, which may point to a role of CK1 α 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 CK1 α 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, CK1 α activity is not required for the maintenance of cell survival, as under those conditions, CK1 α inhibition is without appreciable effect on cell membrane scrambling. In contrast, activated CK1 α 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 CK1 α is a positive regulator of Ca²⁺ entry and cell membrane scrambling following glucose depletion and oxidative stress in erythrocytes.

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