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Original Paper

Stimulation of Eryptosis by Combretastatin A4 Phosphate Disodium (CA4P)

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Key Words
Phosphatidylserine • Cell volume • Eryptosis • Cell membrane scrambling • Calcium

Abstract
Background/Aims: Combretastatin A4 phosphate disodium (CA4P) is utilized for the treatment of malignancy. The substance has previously been shown to trigger suicidal cell death or apoptosis. Similar to apoptosis of nucleated cells, erythrocytes may enter suicidal death or eryptosis, characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Stimulators of eryptosis include increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$), ceramide, oxidative stress and ATP depletion. The present study explored, whether CA4P induces eryptosis and, if so, to gain insight into mechanisms involved. ***Methods:*** Flow cytometry has been employed to estimate phosphatidylserine exposure at the cell surface from annexin-V-binding, cell volume from forward scatter, $[Ca^{2+}]_i$ from Fluo3-fluorescence, reactive oxygen species (ROS) abundance from DCF fluorescence, glutathione (GSH) abundance from CMF fluorescence and ceramide abundance from fluorescent antibodies. In addition cytosolic ATP levels were quantified utilizing a luciferin–luciferase-based assay and hemolysis was estimated from hemoglobin concentration in the supernatant. ***Results:*** A 48 hours exposure of human erythrocytes to CA4P (≥ 50 μM) significantly increased the percentage of annexin-V-binding cells and significantly decreased forward scatter. CA4P did not appreciably increase hemolysis. Hundred μM CA4P significantly increased Fluo3-fluorescence. The effect of CA4P (100 μM) on annexin-V-binding was significantly blunted, but not abolished, by removal of extracellular Ca^{2+}. CA4P (≥ 50 μM) significantly decreased GSH abundance and ATP levels but did not significantly increase ROS or ceramide. ***Conclusions:*** CA4P triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part due to entry of extracellular Ca^{2+} and energy depletion.

Introduction

Combretastatin A4 phosphate disodium (CA4P) is an antiangiogenic drug [1-35] effective against several malignancies [1, 2, 10, 12, 15, 16, 18, 30, 36-61]. The substance has

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been shown to depolymerize microtubules [22], induce autophagy [4, 62], counteract tumor cell migration [63], and trigger apoptosis [64].

Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis [65], the suicidal death of erythrocytes characterized by cell shrinkage [66] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [65]. Eryptosis may be triggered by increase of cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$) [65]. Eryptosis may further involve ceramide formation [67], oxidative stress [65], energy depletion [65], caspases [65, 68, 69], and a variety of kinases including casein kinase 1α, Janus-activated kinase isoform JAK3, protein kinase C, and p38 kinase [65]. Eryptosis is inhibited by AMP activated kinase, cGMP-dependent protein kinase, PAK2 kinase and sorafenib/sunitinib sensitive kinases [65]. Eryptosis may be triggered by a wide variety of small molecules [65, 70-100].

The present study explored, whether CA4P is capable to stimulate eryptosis. To this end, human erythrocytes from healthy volunteers were treated with CA4P and phosphatidylserine surface abundance, cell volume, and $[Ca^{2+}]_i$ determined by flow cytometry.

Materials and Methods

Erythrocytes, solutions and chemicals

Fresh Li-Heparin-anticoagulated blood samples were kindly provided by the blood bank of the University of Tübingen. The study is approved by the ethics committee of the University of Tübingen (184/2003 V). The blood was centrifuged at 120 g for 20 min at 21 °C and the platelets and leukocytes-containing supernatant was disposed. Erythrocytes were incubated *in vitro* at a hematocrit of 0.4% in Ringer solution containing (in mM) 125 NaCl, 5 KCl, 1 $MgSO_4$, 32 N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES; pH 7.4), 5 glucose, 1 $CaCl_2$, at 37°C for 48 hours. Where indicated, $CaCl_2$ was replaced by EDTA and/or erythrocytes were exposed for 48 hours to Combretastatin A4 phosphate disodium (CA4P) (Sigma Aldrich, Hamburg, Germany).

Annexin-V-binding and forward scatter

After incubation under the respective experimental condition, a 100 µl cell suspension was washed in Ringer solution containing 5 mM $CaCl_2$ and then stained with Annexin-V-FITC (1:200 dilution; ImmunoTools, Friesoythe, Germany) in this solution at 37°C for 20 min under protection from light. The annexin-V-abundance at the erythrocyte surface was subsequently determined on a FACS Calibur (BD, Heidelberg, Germany). Annexin-V-binding was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm. A marker (M1) was placed to set an arbitrary threshold between annexin-V-binding cells and control cells. The same threshold was used for untreated and CA4P treated erythrocytes. A dot plot of forward scatter (FSC) vs. side scatter (SSC) was set to linear scale for both parameters. The threshold of forward scatter was set at the default value of "52".

Intracellular Ca^{2+}

After incubation, erythrocytes were washed in Ringer solution and loaded with Fluo-3/AM (Biotium, Hayward, USA) in Ringer solution containing 1 mM $CaCl_2$ and 5 µM Fluo-3/AM. The cells were incubated at 37°C for 30 min. Ca^{2+}-dependent fluorescence intensity was measured with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur.

Hemolysis

After incubation at 37°C, the samples were centrifuged (10 min at 2000 rpm, RT), and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatants was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

Reduced glutathione

The content of reduced glutathione was determined using 5-chloromethylfluorescein diacetate (CMFDA) staining. After treatment, cells were spun down, incubated in Ringer solution containing 1 µM of CMFDA (Santa Cruz Biotechnology, USA) for 45 min, washed once, and resuspended in 200 µl of

Ringer solution. The fluorescence intensity was measured with flow cytometry (FACS-calibur from Becton Dickinson; Heidelberg, Germany) at an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Reactive oxidant species (ROS)

Oxidative stress was determined utilizing 2',7'-dichlorodihydrofluorescein (DCF) diacetate. After incubation, a 100 µl suspension of erythrocytes was washed in Ringer solution and then stained with DCF (Sigma, Schnelldorf, Germany) in Ringer containing DCF diacetate at a final concentration of 10 µM. Erythrocytes were incubated at 37°C for 30 min in the dark and then washed in PBS. The DCF-loaded erythrocytes were resuspended in 200 µl Ringer solution, and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS Calibur (BD).

Ceramide abundance

For the determination of ceramide, a monoclonal antibody-based assay was used. After incubation, cells were stained for 1 hour at 37°C with 1 µg/ml anti ceramide antibody (clone MID 15B4, Alexis, Grünberg, Germany) in PBS containing 0.1% bovine serum albumin (BSA) at a dilution of 1:10. The samples were washed twice with PBS-BSA. Subsequently, the cells were stained for 30 minutes with polyclonal fluorescein isothiocyanate (FITC) conjugated goat anti-mouse IgG and IgM specific antibody (Pharmingen, Hamburg, Germany) diluted 1:50 in PBS-BSA. Unbound secondary antibody was removed by repeated washing with PBS-BSA. The samples were then analyzed by flow cytometric analysis with an excitation wavelength of 488 nm and an emission wavelength of 530 nm.

Intracellular ATP concentration

For the determination of intracellular ATP, 80 µl of erythrocyte pellets were incubated for 24 h at 37°C in Ringer solution (final hematocrit 4.7%). All subsequent manipulations were performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of $HClO_4$ (6%). After centrifugation, an aliquot of the supernatant (400 µl) was adjusted to pH 7.7 by addition of saturated $KHCO_3$ solution. After dilution of the supernatant, the ATP concentrations of the aliquots were determined utilizing the luciferin–luciferase assay kit (Roche Diagnostics) on a luminometer (BertholdBiolumat LB9500, Bad Wildbad, Germany) according to the manufacturer's protocol.

Statistics

Data are expressed as arithmetic means ± SEM. As indicated in the figure legends, statistical analysis was made using ANOVA with Tukey's test as post-test and *t* test as appropriate. n denotes the number of different erythrocyte specimens studied. Since different erythrocyte specimens used in distinct experiments are differently susceptible to triggers of eryptosis, the same erythrocyte specimens have been used for control and experimental conditions.

Results

The present study explored whether Combretastatin A4 phosphate disodium (CA4P) stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Forward scatter was determined by flow cytometry as a measure of erythrocyte volume. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with CA4P (10 - 100 µM). As illustrated in Fig. 1, CA4P decreased erythrocyte forward scatter, an effect reaching statistical significance at 50 and 100 µM CA4P (15%, 25% decrease respectively). Moreover, exposure of erythrocytes to CA4P was followed by a significant increase of the percentage of shrunken erythrocytes (Fig. 1C), an effect reaching statistical significance at 25 µM CA4P (233% increase). CA4P treatment simultaneously increased the percentage of swollen erythrocytes (Fig. 1D), an effect reaching statistical significance at 50 µM CA4P (75% increase).

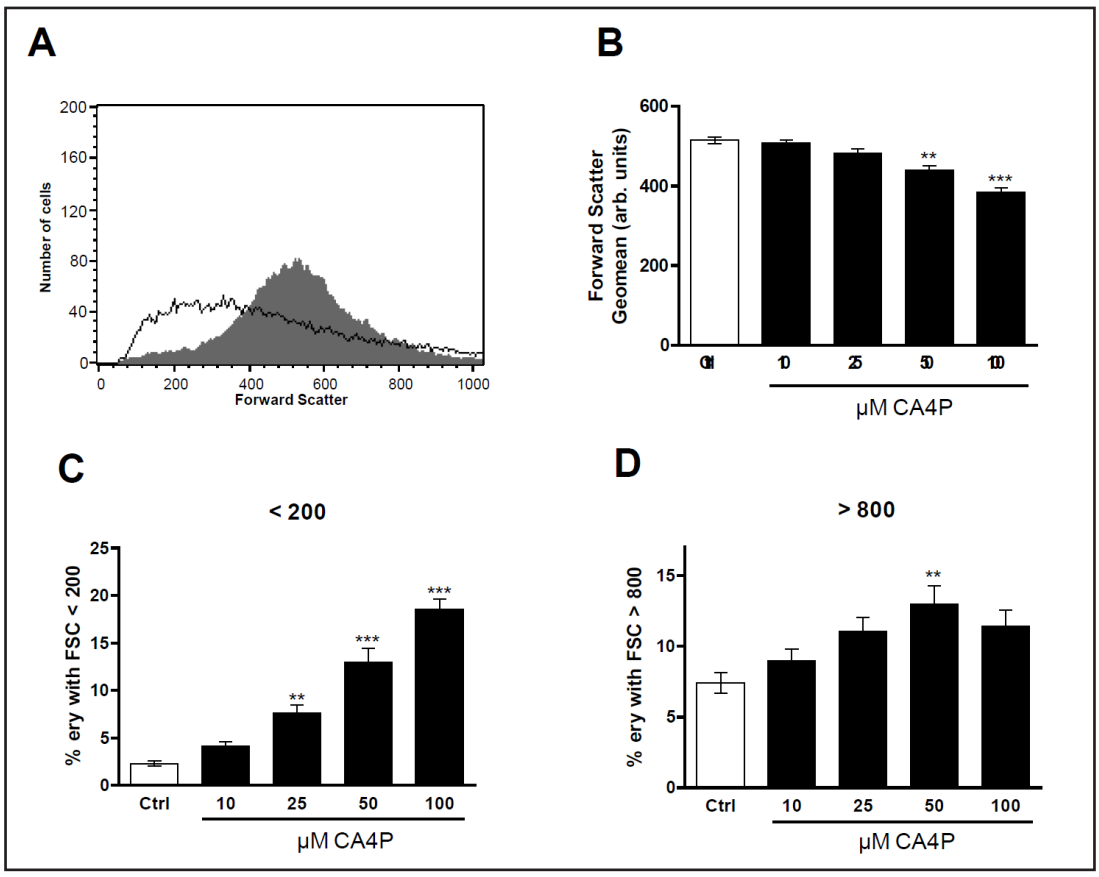

Fig. 1. Effect of CA4P on erythrocyte forward scatter. A. Original histogram of forward scatter of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 100 μM CA4P. B. Arithmetic means ± SEM (n = 13) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) CA4P (10 – 100 μM). C. Arithmetic means ± SEM (n = 13) of the percentage erythrocytes with forward scatter (FSC) <200 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) CA4P (10 – 100 μM). D. Arithmetic means ± SEM (n = 13) of the percentage erythrocytes with forward scatter (FSC) >800 following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) CA4P (10 – 100 μM). **(p<0.01),***(p<0.001) indicates significant difference from the absence of CA4P (ANOVA).

Phosphatidylserine exposing erythrocytes were identified utilizing annexin-V-binding, as determined by flow cytometry. Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with CA4P (10 - 100 μM). As shown in Fig. 2, a 48 hours exposure to CA4P increased the percentage of phosphatidylserine exposing erythrocytes, an effect reaching statistical significance at 50 μM CA4P (212% increase).

In order to test whether CA4P induces hemolysis, the hemoglobin concentration was determined in the supernatant. As a result, the percentage of hemolytic erythrocytes was not significantly different between exposure to Ringer solution (1.9 ± 0.2 %, n = 13), and following exposure to 10 μM (1.8 ± 0.2 %, n = 13), 25 μM (1.9 ± 0.3 %, n = 13), 50 μM (2.5 ± 0.3 %, n = 13), and 100 μM (2.7 ± 0.3 %, n = 13) CA4P.

Fluo3-fluorescence was employed to estimate cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with CA4P (10 - 100 μM). As shown in Fig. 3, a 48 hours exposure to 100 μM CA4P significantly increased the Fluo3-fluorescence (54% increase).

A next series of experiments explored, whether the CA4P-induced cell membrane scrambling required entry of extracellular Ca^{2+}. To this end, erythrocytes were incubated

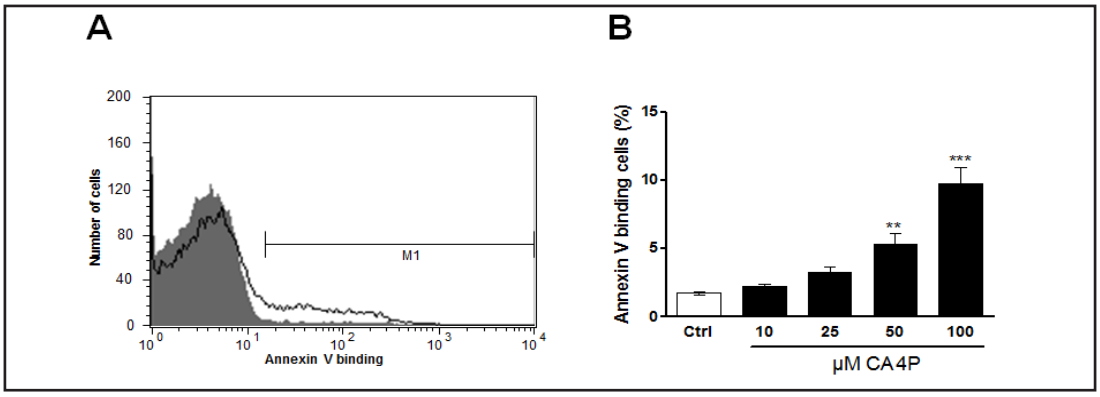

Fig. 2. Effect of CA4P on phosphatidylserine exposure. A. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 100 μM CA4P. B. Arithmetic means ± SEM (n = 13) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) CA4P (10 - 100 μM). **(p<0.01),***(p<0.001) indicates significant difference from the absence of CA4P (ANOVA).

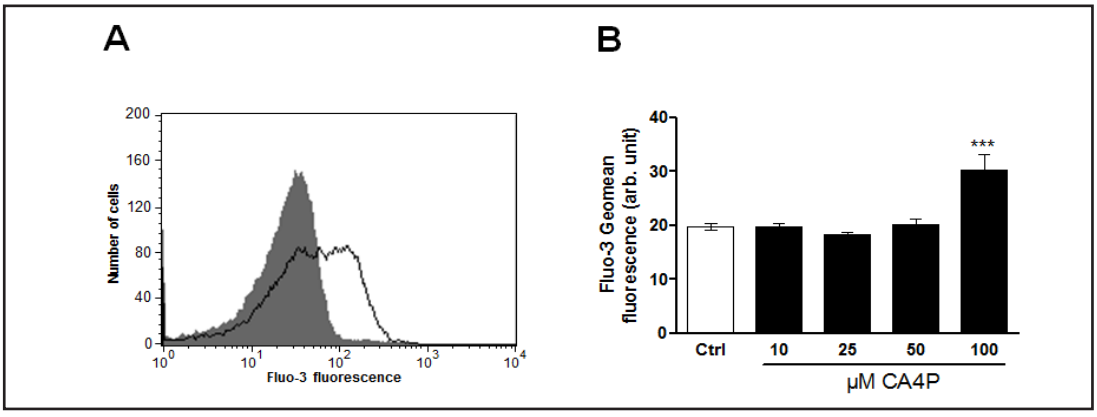

Fig. 3. Effect of CA4P on Fluo3-fluorescence. A. Original histogram of Fluo3-fluorescence reflecting cytosolic Ca^{2+} activity in erythrocytes following exposure for 48 hours to Ringer solution without (grey area) and with (black line) presence of 100 μM CA4P. B. Arithmetic means ± SEM (n = 13) of erythrocyte Fluo3-fluorescence following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) CA4P (10 - 100 μM). ***(p<0.001) indicates significant difference from the absence of CA4P (ANOVA).

for 48 hours in the absence or presence of 50 or 100 μM CA4P in the presence or nominal absence of extracellular Ca^{2+}. As illustrated in Fig. 4, CA4P again profoundly increased the percentage of annexin-V-binding erythrocytes. The effect of 100 μM CA4P was significantly blunted following removal of extracellular Ca^{2+}. However, even in the absence of extracellular Ca^{2+}, CA4P significantly increased the percentage of annexin-V-binding erythrocytes. Thus, CA4P-induced cell membrane scrambling was in large part but not exclusively due to mechanisms dependent on entry of extracellular Ca^{2+}.

A further series of experiments was performed to elucidate the effect of CA4P on the content of reduced Glutathione (GSH). To this end, erythrocytes were incubated for 48 hours in the absence or presence of 50 and 100 μM CA4P. As illustrated in Fig. 5, CA4P (50, 100 μM CA4P) significantly decreased the content of GSH (by 47% and 64%, respectively).

To explore the effect of CA4P on oxidative stress, reactive oxygen species (ROS) was estimated utilizing 2′,7′-dichlorodihydrofluorescein (DCF) diacetate. As a result, the DCF fluorescence was significantly (p<0.001) lower following exposure to 50 μM (16.3 ± 0.9 a.u., n = 13) or 100 μM (15.6 ± 0.8 a.u., n = 13) CA4P concentration than following exposure to Ringer solution (23.1 ± 1.1 a.u., n = 13). Accordingly, a 48 hours exposure to CA4P decreased but did not increase ROS formation.

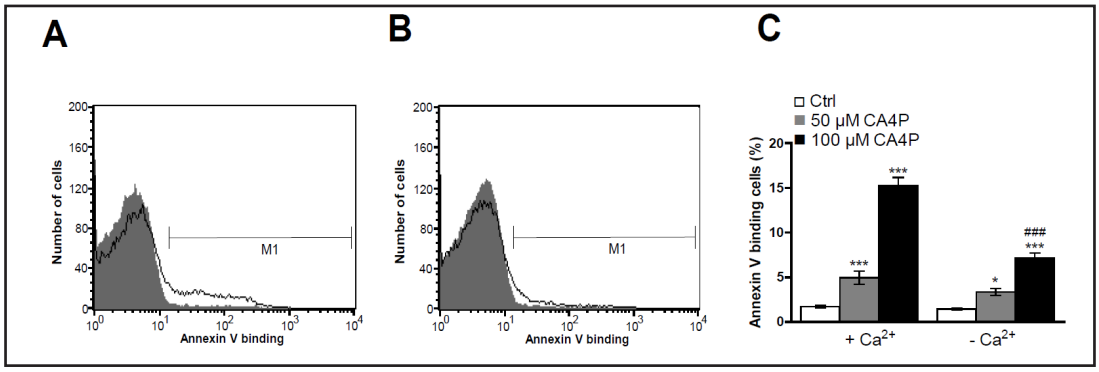

Fig. 4. Ca^{2+} sensitivity of CA4P -induced phosphatidylserine exposure. A,B. Original histogram of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) CA4P (100 µM) in the presence (A) and absence (B) of extracellular Ca^{2+}. C. Arithmetic means ± SEM (n = 10) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (grey, black bars) 50 and 100 µM CA4P, respectively, in the presence (left bars, + Ca^{2+}) and absence (right bars, - Ca^{2+}) of Ca^{2+}. *(p<0.05),***(p<0.001) indicates significant difference from the absence of CA4P, ###(p<0.001) indicates significant difference from the presence of Ca^{2+} (ANOVA).

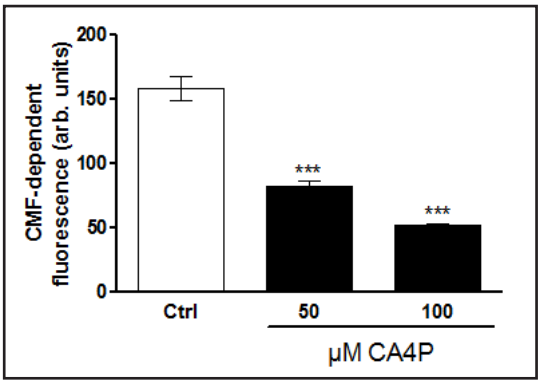

Fig. 5. Effect of CA4P on erythrocyte glutathione (GSH) abundance. Arithmetic means ± SEM (n = 10) of the CMF fluorescence (arbitrary units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) 50 and100 µM CA4P. ***(p<0.001) indicates significant difference from the absence of CA4P (ANOVA).

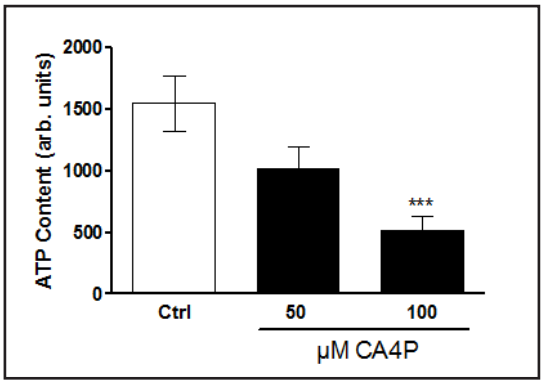

Fig. 6. Effect of CA4P on erythrocyte ATP concentration. Arithmetic means ± SEM (n = 5) of the cytosolic ATP concentrations (arb. units) in erythrocytes exposed for 48 hours to Ringer solution without (white bar) or with (black bars) 50 and 100 µM CA4P. *** (p<0.001) indicate significant difference from the absence of CA4P (ANOVA).

Eryptosis could further be stimulated by ceramide. Ceramide abundance at the erythrocyte surface was thus quantified with flow cytometry utilizing specific antibodies. As a result, the ceramide abundance was similar following exposure to 50 µM (9.9 ± 0.1 a.u., n = 10) or 100 µM (10.6 ± 0.2 a.u., n = 10) CA4P concentration and following exposure to Ringer solution (10.4 ± 0.1 a.u., n = 10). Thus, a 48 hours exposure to CA4P (50 and 100 µM) did not significantly modify ceramide abundance.

In order to explore whether CA4P triggers energy depletion, ATP levels were determined utilizing a luciferin–luciferase assay. As illustrated in Fig. 6, a 48 hours exposure to 100 µM CA4P significantly decreased the cytosolic ATP levels (by 66%).

Discussion

The present observations reveal a novel effect of CA4P, i.e. the triggering of suicidal erythrocyte death or eryptosis. Exposure of human erythrocytes to CA4P results in cell shrinkage

and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The CA4P concentrations required for the stimulation of eryptosis are in the range of concentrations encountered in the plasma of patients [41]. The higher concentrations employed here (50 and 100 μM) would be approached, however, only following intake of toxic drug doses.

The effect of CA4P on cell membrane scrambling was in large part dependent on Ca^{2+} entry from the extracellular space, as removal of extracellular Ca^{2+} significantly blunted CA4P induced phosphatidylserine translocation. Nevertheless, even in the absence of extracellular Ca^{2+}, CA4P was able to significantly trigger cell membrane scrambling. The effect of CA4P on cell shrinkage was again presumably in part due to Ca^{2+} entry. An increase of $[Ca^{2+}]_i$ is expected to activate Ca^{2+} sensitive K^+ channels with subsequent K^+ exit, cell membrane hyperpolarization, Cl^- exit and thus cellular loss of KCl with water [65].

Eryptosis is a physiological mechanism accomplishing clearance of defective erythrocytes from circulating blood prior to hemolysis [65]. Hemolysis of defective erythrocytes leads to release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and may thus trigger renal failure [101]. Eryptosis is further pivotal for the elimination of erythrocytes infected with the malaria pathogen *Plasmodium* [65].

As phosphatidylserine exposing erythrocytes are rapidly cleared from circulating blood, stimulation of eryptosis may lead to anemia as soon as the loss of erythrocytes outcasts the formation of new erythrocytes by erythropoiesis [65]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [102], stimulate blood clotting and trigger thrombosis [103-105], thus impairing microcirculation [67, 103, 106-109].

The sensitivity of erythrocytes to CA4P treatment is presumably enhanced in several eryptosis-inducing clinical conditions, such as dehydration [110], hyperphosphatemia [111], chronic kidney disease (CKD) [112-115], hemolytic-uremic syndrome [116], diabetes [117], hepatic failure [118], malignancy [65], sepsis [119], sickle-cell disease [65], beta-thalassemia [65], Hb-C and G6PD-deficiency [65], as well as Wilsons disease [120]. The possibility of enhanced CA4P toxicity in those clinical conditions should be taken into consideration.

In conclusion, CA4P triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect apparently in large part due to stimulation of Ca^{2+} entry.

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Disclosure Statement

The authors of this manuscript state that they have no conflicts of interest to declare.

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