Stimulation of Suicidal Erythrocyte Death by Phosphatase Inhibitor Calyculin A

Mustafa Almasry a Mohamed Jemaà a Morena Mischitelli a,b Caterina Faggio b Florian Lang a,c

aDepartment of Cardiology, Vascular Medicine and Physiology, Eberhard-Karls-University of Tuebingen, Tuebingen, Germany; bDepartment of Chemical, Biological, Pharmaceutical and Environmental Sciences-University of Messina Viale Ferdinando Stagno d’Alcontres, S. Agata-Messina, Italy; cDepartment of Molecular Medicine II, Medical Faculty, Heinrich Heine University, Düsseldorf, Germany

Key Words
Phosphatidylserine • Cell volume • Eryptosis • Calcium

Abstract
Background/Aims: The serine/threonine protein phosphatase 1 and 2a inhibitor Calyculin A may trigger suicidal death or apoptosis of tumor cells. Similar to apoptosis of nucleated cells, erythrocytes may enter eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. Triggers of eryptosis include increase of cytosolic Ca 2+ activity ([Ca 2+ ] i ). Eryptosis is fostered by activation of staurosporine sensitive protein kinase C, SB203580 sensitive p38 kinase, and D4476 sensitive casein kinase. Eryptosis may further involve zVAD sensitive caspases. The present study explored, whether Calyculin A induces eryptosis and, if so, whether its effect requires Ca 2+ entry, kinases and/or caspases
Methods: Phosphatidylserine exposure at the cell surface was estimated from annexin-V-binding, cell volume from forward scatter, and [Ca 2+ ] i from Fluo-3 fluorescence, as determined by flow cytometry.
Results: A 48 hours exposure of human erythrocytes to Calyculin A (≥ 2.5 nM) significantly increased the percentage of annexin-V-binding cells, significantly decreased forward scatter and significantly increased Fluo-3 fluorescence. The effect of Calyculin A on annexin-V-binding was significantly blunted by removal of extracellular Ca 2+, by staurosorine (1 µM), SB203580 (2 µM), D4476 (10 µM), and zVAD (10 µM).
Conclusions: Calyculin A triggers cell shrinkage and phospholipid scrambling of the erythrocyte cell membrane, an effect at least in part requiring Ca 2+ entry, kinase activity and caspase activation.

Introduction
Calyculin A, a toxin isolated from the marine sponge Discodermia calyx [1], is a serine/threonine protein phosphatase 1 and 2a inhibitor thus affecting diverse signaling pathways [1-8]. Calyculin A may promote tumor growth [9]. On the other hand, cellular effects of calyculin A include stimulation of apoptosis [6].
In analogy to apoptosis of nucleated cells, erythrocytes may enter eryptosis [10], the suicidal death of erythrocytes characterized by cell shrinkage [11] and cell membrane scrambling with phosphatidylserine translocation to the cell surface [10]. Signaling involved in the stimulation of eryptosis include increase of cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]\(_i\)) [10], ceramide [12], oxidative stress [10], energy depletion [10], activated caspases [10, 13, 14], stimulation of casein kinase 1α, Janus-activated kinase JAK3, protein kinase C, and p38 kinase [10], as well as inhibition of AMP activated kinase AMPK, cGMP-dependent protein kinase, and sorafenib/sunitinib sensitive kinases [10]. Eryptosis is triggered by multiple xenobiotics [10, 15-56].

In view of the strong influence of kinases on eryptosis, the possibility was considered that the phosphatase 1 and 2a inhibitor Calyculin could – in analogy to its effect on apoptosis - trigger eryptosis. To this end, human erythrocytes from healthy volunteers were exposed to Calyculin A and phosphatidylerosine surface abundance, cell volume, and [Ca\(^{2+}\)], 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, erythrocytes were exposed for 48 hours to Calyculin A (MedChem Express, Princeton, USA). In order to estimate the impact of Ca\(^{2+}\) entry on Calyculin A induced eryptosis, erythrocytes were exposed to Calyculin A in the absence of extracellular Ca\(^{2+}\) and presence of Ca\(^{2+}\) chelator EGTA (1 mM, Merck Millipore, Darmstadt, Germany). To test for an involvement of kinases, erythrocytes were exposed for 48 hours to a combination of Calyculin A and p38 kinase inhibitor SB 203580 (Tocris bioscience, Bristol, UK), protein kinase C inhibitor staurosporine (Sigma Aldrich, Hamburg, Germany) or casein kinase inhibitor D4476 (Sigma Aldrich, Hamburg, Germany). In order to test for a role of caspases, erythrocytes were exposed for 48 hours to a combination of Calyculin A and pan-caspase inhibitor zVAD (Tocris bioscience, Bristol, UK).

**Annexin-V-binding and forward scatter**

After incubation under the respective experimental condition, a 150 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 15 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 Calyculin A 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 5 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.

**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. 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 Calyculin A stimulates eryptosis, the suicidal erythrocyte death characterized by cell shrinkage and phospholipid scrambling of the cell membrane with phosphatidylserine translocation to the cell surface.

Erythrocyte volume was estimated from forward scatter which was determined utilizing flow cytometry. Prior to measurements, the erythrocytes were incubated for 48 hours in Ringer solution without or with Calyculin A (2.5, 5 and 10 nM). As illustrated in Fig. 1, Calyculin A significantly decreased erythrocyte forward scatter at each concentration tested (2.5, 5 and 10 nM Calyculin A).

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 Calyculin A (2.5, 5 and 10 nM). As shown in Fig. 2, 48 hours exposure to Calyculin A significantly increased the percentage of phosphatidylserine exposing erythrocytes at each concentration tested (2.5, 5 and 10 nM Calyculin A).

Fluo-3 fluorescence was taken as a measure of cytosolic Ca$^{2+}$ activity ([Ca$^{2+}$]). Prior to measurements, the erythrocytes were again incubated for 48 hours in Ringer solution without or with Calyculin A (2.5, 5 and 10 nM). As shown in Fig. 3, 48 hours exposure to Calyculin A significantly increased the Fluo-3 fluorescence at each concentration tested (2.5, 5 and 10 nM Calyculin A).

**Fig. 1.** Effect of Calyculin A 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 10 nM Calyculin A. B. Arithmetic means ± SEM (n = 12) of the erythrocyte forward scatter (FSC) following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Calyculin A (2.5, 5 and 10 nM). For comparison, the effect of the solvent DMSO is shown (grey bar). ***(p<0.001) indicates significant difference from the absence of Calyculin A (ANOVA).**

**Fig. 2.** Effect of Calyculin A 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 10 nM Calyculin A. B. Arithmetic means ± SEM (n = 12) of erythrocyte annexin-V-binding following incubation for 48 hours to Ringer solution without (white bar) or with (black bars) Calyculin A (2.5, 5 and 10 nM). For comparison, the effect of the solvent DMSO is shown (grey bar). ***(p<0.001) indicates significant difference from the absence of Calyculin A (ANOVA).**
A next series of experiments explored whether the Calyculin A-induced translocation of phosphatidylserine or erythrocyte shrinkage required entry of extracellular Ca²⁺. To this end, erythrocytes were incubated for 48 hours in the absence or presence of 10 nM Calyculin A in the presence or nominal absence of extracellular Ca²⁺. As illustrated in Fig. 4, removal of extracellular Ca²⁺ significantly blunted the effect of Calyculin A on the percentage of annexin-V-binding erythrocytes. However, even in the absence of extracellular Ca²⁺, Calyculin A significantly decreased the percentage of annexin-V-binding erythrocytes. Thus, Calyculin A-induced cell membrane scrambling was in part triggered by entry of extracellular Ca²⁺.

To explore, whether the effects of Calyculin A were sensitive to kinase activity, the influence of Calyculin A on annexin-V-binding was tested in the presence of protein kinase C inhibitor staurosporine (1 µM), p38 kinase inhibitor SB 203580 (2 µM) or casein kinase inhibitor D4476 (10 µM). As illustrated in Fig. 5, the effect of Calyculin A (10 nM) on phosphatidylserine exposure was significantly blunted by each, staurosporine, SB203580
and D4476. In the presence of SB203580 (2 µM) Calyculin A (10 nM) still slightly, but significantly, stimulated phosphatidylserine exposure (Fig. 5).

In order to test for the involvement of caspases, the influence of Calyculin A on annexin-V-binding and forward scatter was tested in the presence of pan caspase inhibitor zVAD (10 µM). As illustrated in Fig. 5, the effect of Calyculin A (10 nM) on phosphatidylserine exposure was significantly blunted by zVAD. In the presence of zVAD (10 µM) Calyculin A (10 nM) still slightly, but significantly, stimulated phosphatidylserine exposure (Fig. 5).

Fig. 5. Inhibitor sensitivity of Calyculin A-induced phosphatidylserine exposure. A-E. Original histograms of annexin-V-binding of erythrocytes following exposure for 48 hours to Ringer solution without (grey areas) and with (black lines) Calyculin A (10 nM) in the absence (A) and presence of 1 µM staurosporine (B), 2 µM SB203580 (C), 10 µM D4476 (D), and 10 µM zVAD (E). Arithmetic means ± SEM (n = 10) of annexin-V-binding of erythrocytes after a 48 hours treatment with Ringer solution without (white bars) or with (black bars) Calyculin A (10 nM) in the absence (left bars, control) and presence (right bars) of staurosporine (+staurosporine), SB203580 (+SB203580), D4476 (+D4476), or zVAD (+Z-VAD-FMK). *(p<0.05) and ***(p<0.001) indicates significant difference from the absence of Calyculin A, ###(p<0.001) indicates significant difference from the absence of inhibitors (ANOVA).
Discussion

The present study discloses a novel effect of Calyculin A, i.e. the stimulation of suicidal erythrocyte death or eryptosis. Treatment of human erythrocytes drawn from healthy individuals with Calyculin A results in the two hallmarks of eryptosis, i.e. cell shrinkage and cell membrane scrambling with phosphatidylserine translocation to the erythrocyte surface. The full effect of Calyculin A on cell membrane scrambling required Ca\(^{2+}\) entry from the extracellular space, as removal of extracellular Ca\(^{2+}\) significantly blunted the Calyculin A induced eryptosis. As apparent from Fluo-3 fluorescence Ca\(^{2+}\) entry increased cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)), a known stimulator of cell membrane scrambling [10]. The effect of Calyculin A on cell volume was similarly at least in part due to increase of [Ca\(^{2+}\)]\(i\), which activates Ca\(^{2+}\) sensitive K\(^+\) channels, thus leading to cell shrinkage due to K\(^+\) exit, cell membrane hyperpolarization, Cl\(^-\) exit and thus cellular loss of KCl with water [10].

The effect of Calyculin A was significantly blunted by p38 kinase inhibitor SB203580, and virtually abrogated by protein kinase C inhibitor staurosporine, as well as casein kinase inhibitor D4476. Thus, the stimulating effect of Calyculin A on cell membrane scrambling could well be related to its inhibitory effect on the serine/threonine protein phosphatase 1 and 2a. The p38 kinase, protein kinase C and casein kinase have previously been shown to participate in the orchestration of eryptosis [10]. The stimulating effect of Calyculin A on cell membrane scrambling was further significantly blunted by caspase inhibitor zVAD. Caspases participate in the triggering of eryptosis by some, but not by all stimulators [10].

Stimulation of eryptosis is followed by rapid clearance of defective erythrocytes from circulating blood [10]. Failure of defective erythrocytes to enter eryptosis may lead to hemolysis with release of hemoglobin, which passes the renal glomerular filter, precipitates in the acidic lumen of renal tubules, occludes nephrons and thus may lead to renal failure [57]. The clearance of phosphatidylserine exposing erythrocytes from circulating blood may result in anemia as soon as the loss of erythrocytes surpasses the formation of new erythrocytes by erythropoiesis [10]. Enhanced eryptosis contributes to the anemia in a variety of clinical conditions, such as iron deficiency [10], dehydration [58], hyperphosphatemia [59], chronic kidney disease (CKD) [60-63], hemolytic-uremic syndrome [64], diabetes [65], hepatic failure [66], malignancy [10], sepsis [67], sickle-cell disease [10], beta-thalassemia [10], Hb-C and G6PD-deficiency [10], as well as Wilsons disease [68]. Phosphatidylserine exposing erythrocytes further adhere to the vascular wall [69], stimulate blood clotting and trigger thrombosis [70-72], and thus impair microcirculation [12, 70, 73-76].

In conclusion, Calyculin A triggers eryptosis with cell shrinkage and cell membrane scrambling, an effect involving Ca\(^{2+}\) entry, kinases and caspases.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Tanja Loch. The study was supported by the Deutsche Forschungsgemeinschaft, and Open Access Publishing Fund of Tuebingen University.

Disclosure Statement

None.

References


