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RESEARCH ARTICLE

Stimulation of erythrocyte phospholipid scrambling by enniatin A

Kashif Jilani, Syed M. Qadri*, Elisabeth Lang, Christine Zelenak, Anand Rotte, Diwakar Bobbala and Florian Lang*

Department of Physiology, University of Tuebingen, Tuebingen, Germany

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Scope: Enniatin A, a peptide antibiotic and common food contaminant, triggers mitochondrial dysfunction and apoptosis. Even though lacking mitochondria, erythrocytes may similarly undergo suicidal cell death or eryptosis. Eryptosis is characterized by cell shrinkage and cell membrane phospholipid scrambling. Triggers of phospholipid scrambling include energy depletion and increase in cytosolic Ca^{2+} activity ($[Ca^{2+}]_i$). The present study explored whether enniatin A triggers phospholipid scrambling.

Methods and results: Phospholipid scrambling was estimated from annexin-V-binding, cell volume from forward scatter (FSC), $[Ca^{2+}]_i$ from Fluo3-fluorescence, cytosolic ATP-concentration ($[ATP]_i$) using a luciferase assay and hemolysis from hemoglobin release. Exposure of erythrocytes for 48 h to enniatin A ($\geq 2.5\,\mu M$) significantly increased $[Ca^{2+}]_i$, decreased $[ATP]_i$, decreased FSC, triggered annexin-V-binding and elicited hemolysis. Annexin-V-binding affected 25%, and hemolysis 2% of treated erythrocytes. Decreased $[ATP]_i$ by glucose depletion for 48 h was similarly followed by increased $[Ca^{2+}]_i$, decreased FSC and annexin-V-binding. Enniatin A augmented the effect on $[Ca^{2+}]_i$ and annexin-V-binding, but not on FSC. Annexin-V-binding was blunted by Ca^{2+} removal, by the cation channel inhibitor amiloride (1 mM), by the protein kinase C inhibitor staurosporine (500 nM) but not by the pancaspase inhibitor zVAD (10 μM).

Conclusion: The food contaminant enniatin A triggers ATP depletion and increases cytosolic Ca^{2+} activity, effects resulting in suicidal erythrocyte death.

Keywords:
Calcium / Cell volume / Enniatin A / Eryptosis / Phosphatidylserine

1 Introduction

Enniatins, cyclic hexadepsipeptide antibiotics [1–4], are common contaminants of grain-based food and feed [1, 5–7]. They are cytotoxic [2, 5, 8], cause cell cycle arrest [1] and trigger suicidal death or apoptosis of mammalian cells involving cell shrinkage, chromatin condensation, DNA fragmentation, and apoptotic body formation [1, 2, 5, 6, 9]. Enniatins do not trigger oxidative stress or DNA damage [5, 6], but have instead been considered to exert their cytotoxic effect at least partially by breakdown of the mitochondrial transmembrane potential, uncoupled oxidative phosphorylation, mitochondrial swelling and decreased ability of mitochondria to sequester Ca^{2+} [1, 9]. Enniatins function as highly selective K^+ ionophores in both, mitochondrial and plasma membranes [9]. The apoptotic effect of enniatins is reversed by the pancaspase inhibitor zVAD [2], pointing to the involvement of caspases in the execution of apoptosis. The signaling triggered by enniatin further involves inhibition of extracellular regulated protein kinase (ERK) and downregulation of NF-κB [7]. In view of their proapoptotic efficacy, enniatins are considered potential anticancer agents [1, 7].

Erythrocytes may, similar to nucleated cells, undergo suicidal death or eryptosis, involving cell shrinkage and cell

Correspondence: Professor Florian Lang, Physiologisches Institut der Universität Tübingen, Gmelinstr. 5, D-72076 Tübingen, Germany
E-mail: florian.lang@uni-tuebingen.de
Fax: +49-7071-29-5618

Abbreviation: FSC, forward scatter

*These authors contributed equally to this work.

membrane scrambling [10]. Eryptosis may be triggered by activation of Ca^{2+}-permeable cation channels leading to increase in cytosolic Ca^{2+} concentration [11–19]. Ca^{2+} entry, increase in cytosolic Ca^{2+} activity and eryptosis may follow energy depletion [20]. Increased cytosolic Ca^{2+} concentration may be followed by activation of Ca^{2+}-sensitive K^+ channels [21, 22] with the subsequent exit of KCl along with osmotically obliged water and thus to cell shrinkage [23]. Ca^{2+} further triggers cell membrane scrambling resulting in the exposure of phosphatidylserine at the cell surface [19, 24–26]. Ca^{2+} sensitivity of cell membrane scrambling is increased by ceramide [27, 28]. Ceramide is generated by a sphingomyelinase, which is in turn stimulated by platelet-activating factor [29]. Erythrocyte cell membrane scrambling could further result from activation of caspases [26, 30], which are in turn stimulated by oxidative stress but are not required for the scrambling effect of Ca^{2+} [24, 28, 31].

The present study explored whether enniatin A stimulates phospholipid scrambling and modifies phospholipid scrambling following energy depletion.

2 Materials and methods

2.1 Erythrocytes, solutions and chemicals

Leukocyte-depleted erythrocytes (aged 1–2 wk) were kindly provided by the blood bank of the University of Tübingen. Viability of erythrocytes may depend on the donor and the storage time thus causing some interindividual variability. To avoid any bias potentially introduced by the use of different erythrocyte batches, comparison was always made within a given erythrocyte batch. The study was approved by the ethics committee of the University of Tübingen (184/2003V).

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), 5 glucose, 1 $CaCl_2$; pH 7.4 at 37°C for 48 h. Where indicated, extracellular glucose was removed or enniatin A (Enzo, Lörrach, Germany) added at the indicated concentrations. In Ca^{2+}-free Ringer solution, 1 mM $CaCl_2$ was substituted by 1 mM glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA). Where indicated, the caspase inhibitor zVAD (Enzo, Lörrach, Germany) was used at a concentration of 10 μM, the protein kinase C inhibitor staurosporine (Sigma, Freiburg, Germany) was used at a concentration of 500 nM and oxidative stress was induced using 0.3 mM *tert*-butyl hydroperoxide [*t*-BOOH] (Sigma, Freiburg, Germany).

All incubations (48 h) were carried out in Ringer solution containing 1 mM $CaCl_2$ or, where indicated, in Ca^{2+}-free Ringer solution. Following pretreatment with the respective Ca^{2+}-containing or Ca^{2+}-free solutions for the indicated time periods, the erythrocytes were washed with Ringer solution containing 5 mM $CaCl_2$ and resuspended for 20 min with the same solution containing Annexin V-Fluos. The time period of 20 min is considered too short for the triggering of eryptosis.

2.2 FACS analysis of annexin-V-binding and forward scatter (FSC)

After incubation under the respective experimental conditions, 50 μL cell suspension was washed in Ringer solution containing 5 mM $CaCl_2$ and then stained for 20 min with Annexin-V-Fluos (1:500 dilution; Roche, Mannheim, Germany) under protection from light [32]. In the following, the FSC of the cells was determined and annexin V fluorescence intensity was measured in FL-1 with an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a FACS calibur (BD, Heidelberg, Germany).

2.3 Measurement of intracellular Ca^{2+}

After incubation 50 μL suspension erythrocytes were washed in Ringer solution and then loaded with Fluo-3/AM (Calbiochem, Bad Soden, Germany) in Ringer solution containing 5 mM $CaCl_2$ and 2 μM Fluo-3/AM. The cells were incubated at 37°C for 20 min and washed twice in Ringer solution containing 5 mM $CaCl_2$. The Fluo-3/AM-loaded erythrocytes were resuspended in 200 μL Ringer. Then, Ca^{2+}-dependent fluorescence intensity was measured in fluorescence channel FL-1 in the FACS analysis.

2.4 Measurement of hemolysis

For the determination of hemolysis, the samples were centrifuged (3 min at 400 × *g*, room temperature) after incubation, and the supernatants were harvested. As a measure of hemolysis, the hemoglobin (Hb) concentration of the supernatant was determined photometrically at 405 nm. The absorption of the supernatant of erythrocytes lysed in distilled water was defined as 100% hemolysis.

2.5 Determination of intracellular ATP concentration

For the determination of intracellular ATP, 90 μL of erythrocyte pellets was incubated for 48 h at 37°C in Ringer solution with or without enniatin A (final hematocrit 5%). Additionally, erythrocytes were incubated in glucose-depleted Ringer solution as a positive control. All manipulations were then performed at 4°C to avoid ATP degradation. Cells were lysed in distilled water, and proteins were precipitated by addition of $HClO_4$ (5%). 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 using the luciferin–luciferase assay kit (Roche Diagnostics) on a luminometer (Berthold Biolumat LB9500, Bad Wildbad, Germany) according to manufacturer's protocol. ATP concentrations are expressed in mmol/L cytosol of erythrocytes.

2.6 Confocal microscopy and immunofluorescence

For the visualization of eryptotic erythrocytes, 4 μL of erythrocytes, incubated in respective experimental conditions, was stained with FITC-conjugated Annexin-V-Fluos (1:250 dilution; Roche, Mannheim, Germany) in 200 μL Ringer solution containing 5 mM $CaCl_2$. Then the erythrocytes were washed twice and finally resuspended in 50 μL of Ringer solution containing 5 mM $CaCl_2$. Totally, 20 μL was smeared onto a glass slide, 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.

2.7 Statistical analysis

Data are expressed as arithmetic mean ± SEM. Statistical analysis was made using paired ANOVA with Tukey's test as post-test, as appropriate. *n* denotes the number of different erythrocyte specimens studied. The batches of erythrocytes differed moderately in their susceptibility to eryptosis. Thus, the control values were not identical in all series of experiments. To avoid any bias potentially introduced by the use of different erythrocyte batches, comparison was always made within a given erythrocyte batch.

3 Results

In a first series of experiments, Fluo3 fluorescence was employed to estimate the cytosolic Ca^{2+} concentration. As illustrated in Fig. 1A and B, exposure of erythrocytes for 48 h to Ringer solution containing enniatin A was followed by an increase in the cytosolic Ca^{2+} concentration. When compared to erythrocytes exposed for 48 h to Ringer solution without enniatin A, the cytosolic Ca^{2+} concentration was

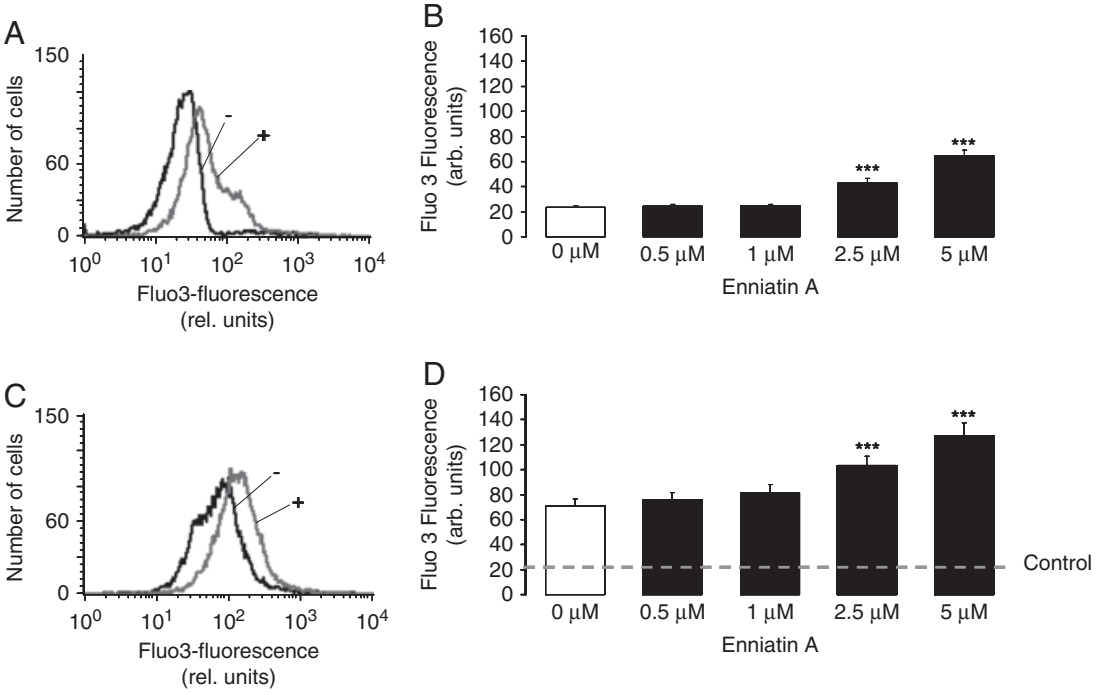

Figure 1. Effect of enniatin A on erythrocyte cytosolic Ca^{2+} concentration. (A) Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, gray line) the presence of 5 μM enniatin A. (B) Arithmetic mean ± SEM ($n = 12$) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 fluorescence in erythrocytes exposed for 48 h to Ringer solution without (white bar) or with (black bars) enniatin A. *** indicates significant difference ($p < 0.001$) from the respective value in the absence of enniatin A (ANOVA). (C) Original histogram of Fluo3 fluorescence in erythrocytes following exposure for 48 h to Ringer solution without glucose, without (−, black line) and with (+, gray line) the presence of 5 μM enniatin A. (D) Arithmetic mean ± SEM ($n = 12$) of the geo means (geometric mean of the histogram in arbitrary units) of Fluo3 fluorescence in erythrocytes exposed for 48 h to Ringer solution without glucose in the absence (white bar) or in the presence (black bars) of enniatin A. The broken line indicates control Fluo3 fluorescence in the presence of glucose and absence of enniatin A. *** indicates significant difference ($p < 0.001$) from the respective value in the absence of enniatin A (ANOVA).

significantly higher at $\geq 2.5\,\mu M$ enniatin A concentration. Additional experiments explored the effect of enniatin A during glucose depletion. As illustrated in Fig. 1C and D, glucose withdrawal similarly increased the cytosolic Ca^{2+} activity, an effect augmented in the presence of enniatin A. The difference in the cytosolic Ca^{2+} activity of glucose-depleted erythrocytes between the presence and absence of enniatin A reached statistical significance at $\geq 2.5\,\mu M$ enniatin A concentration.

As Ca^{2+} activates K^+ channels with the subsequent exit of KCl and cell shrinkage, FSC was determined to reveal a possible effect of enniatin A on cell volume. As shown in Fig. 2A and B, exposure of erythrocytes for 48 h to Ringer solution containing enniatin A was indeed followed by a decrease in FSC, an effect reaching statistical significance at 1 μM enniatin A. As shown in Fig. 2C and D, glucose withdrawal was again followed by a decrease in FSC, reflecting cell shrinkage. The administration of 1 μM enniatin A was followed by a further significant decrease in FSC. However, at a concentration of 5 μM enniatin A, the FSC of the glucose-depleted erythrocytes was significantly higher than the FSC of glucose-depleted erythrocytes in the absence of enniatin A.

Ca^{2+} further stimulates cell membrane scrambling with phosphatidylserine exposure at the cell surface. To estimate an effect of enniatin A on cell membrane scrambling, phosphatidylserine-exposing erythrocytes were identified using annexin-V-binding. In a first approach, annexin-V-binding cells were visualized using confocal microscopy (Fig. 3). The number of annexin-V-positive erythrocytes was higher following treatment (for 48 h) with enniatin A than following incubation (for 48 h) in Ringer solution without enniatin A.

To quantify the effect of enniatin A on cell membrane scrambling, annexin-V-binding erythrocytes were counted in the FACS analysis. As displayed in Fig. 4A and B, the percentage of annexin-V-binding erythrocytes was significantly higher following the exposure of erythrocytes for 48 h to Ringer solution containing $\geq 2.5\,\mu M$ enniatin A than following the exposure of erythrocytes for 48 h to Ringer solution without enniatin A. Glucose withdrawal further enhanced the percentage of annexin-V-binding erythrocytes. As shown in Fig. 4C and D, the percentage of annexin-V-binding erythrocytes was higher in glucose-depleted cells exposed to enniatin A than in glucose-depleted cells without enniatin A exposure. The difference in the percentage annexin-V-binding erythrocytes between the presence and absence of enniatin A was statistically significant at $\geq 1\,\mu M$ enniatin A.

Further experiments were performed to reveal a possible hemolytic action of enniatin A. To this end, Hb release was

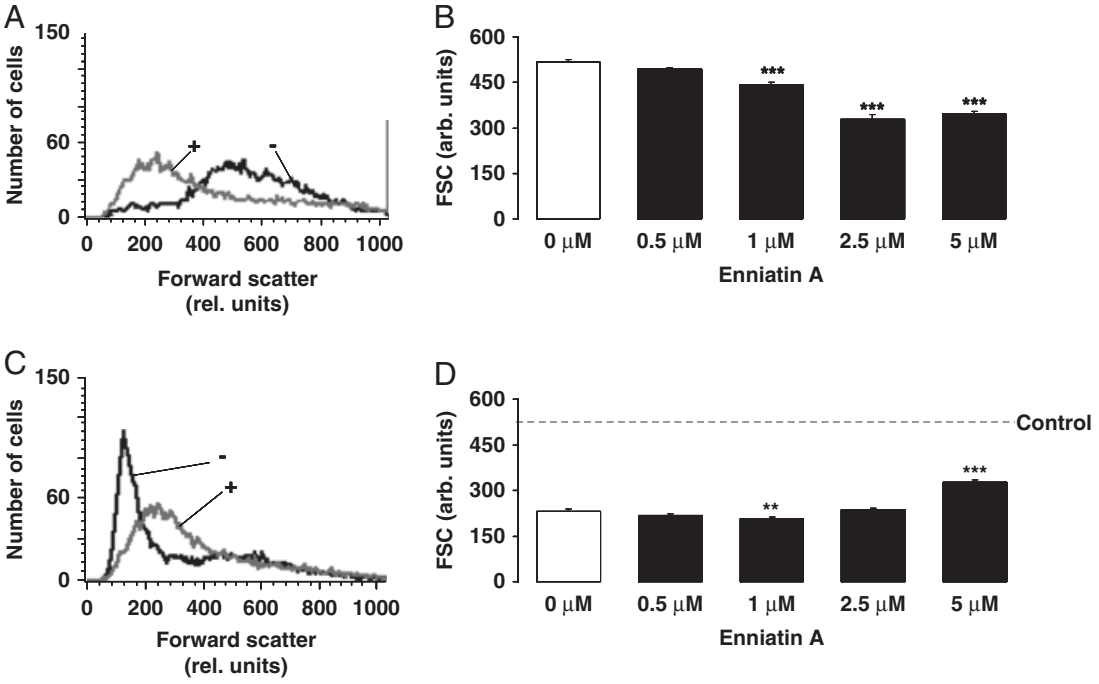

Figure 2. Effect of enniatin A on erythrocyte FSC. (A) Original histogram of FSC of erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, gray line) the presence of 5 μM enniatin A. (B) Arithmetic mean ± SEM ($n = 12$) of the erythrocyte FSC following incubation for 48 h in Ringer solution without (white bar) or with (black bars) 1–5 μM enniatin A. ***$p < 0.001$ indicate significant difference from the absence of enniatin A (ANOVA). (C) Original histogram of FSC of erythrocytes following exposure for 48 h to Ringer solution without glucose, without (−, black line) and with (+, gray line) the presence of 5 μM enniatin A. (D) Arithmetic mean ± SEM ($n = 12$) of the erythrocyte FSC following incubation for 48 h in Ringer solution without glucose, in the absence (white bar) or presence (black bars) of 1–5 μM enniatin A. The broken line indicates control FSC in the presence of glucose and absence of enniatin A. **, *** ($p < 0.01$, $p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA).

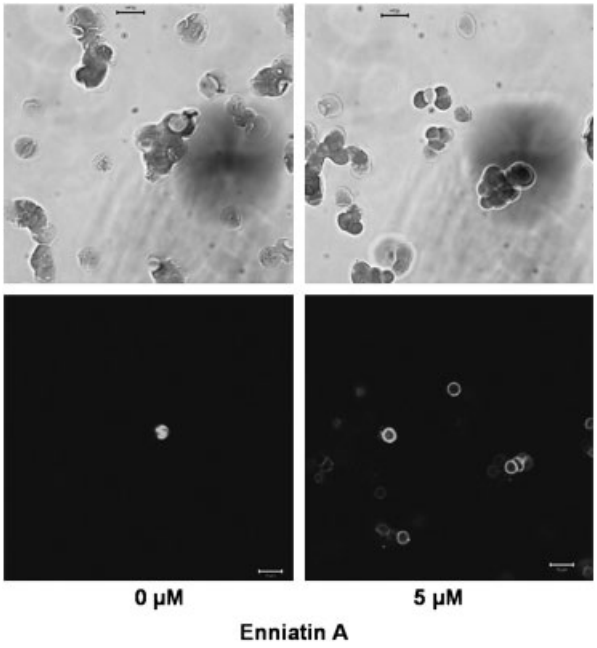

Figure 3. Confocal images of PS-exposing erythrocytes exposed to enniatin A. Confocal microscopy of FITC-dependent fluorescence (lower panels) and light microscopy (upper panels) of human erythrocytes stained with FITC-conjugated Annexin-V-Fluos following 48 h incubation in Ringer solution without (left panels) and with (right panels) 5 μM enniatin A.

determined following exposure of the erythrocytes to Ringer solution for 48 h without or with 1–5 μM enniatin A. As indicated in Fig. 4B, the exposure to enniatin was followed by hemolysis, an effect reaching statistical significance at 2.5 μM enniatin A. Following enniatin A exposure, the percentage of hemolysed erythrocytes was, however, one magnitude smaller than the percentage of phosphatidylserine-exposing erythrocytes. Collectively, the above results reveal that enniatin A triggers phospholipid scrambling and to a clearly lesser extent hemolysis.

The parallel alteration of the cytosolic Ca^{2+} activity and cell membrane scrambling was suggestive for a causal role of Ca^{2+} in the triggering of cell membrane scrambling following enniatin A exposure. To test whether enniatin A-induced cell membrane scrambling was indeed dependent on Ca^{2+}, erythrocytes were treated with enniatin A in the presence and nominal absence of extracellular Ca^{2+}. As shown in Fig. 5A, the effect of enniatin A on annexin-V-binding was significantly blunted but not fully abolished in the nominal absence of Ca^{2+}. Furthermore, the cation channel inhibitor amiloride (1 mM) blunted but did not abolish the effect of enniatin A on annexin-V-binding (Fig. 5B). Further experiments were performed to test whether enniatin A-induced cell membrane scrambling required the activation of caspases. To this end, erythrocytes were treated with enniatin A in the absence and presence of the pancaspase inhibitor zVAD. As shown in Fig. 5D, the effect of enniatin A on annexin-V-binding was not significantly modified by zVAD (10 μM). As a positive control, erythrocytes were treated with zVAD (10 μM) for 48 h and then exposed to oxidative stress using *t*-BOOH (0.3 mM) for 30 min. zVAD treatment significantly attenuated the enhanced annexin-V-binding induced by oxidative stress (Fig. 5E). Further experiments explored the impact of kinases on enniatin A-induced cell membrane scrambling. The kinase inhibitor staurosporine (500 nM) blunted the effect of enniatin A on annexin-V-binding (Fig. 5C).

Phospholipid scrambling could be triggered by energy depletion. Accordingly, additional experiments were performed to determine whether enniatin A exposure influences intracellular ATP concentrations in erythrocytes. As shown in Fig. 6A, 48 h exposure of human erythrocytes to enniatin A ($\geq$2.5 μM) significantly reduced the cellular ATP concentration. Intracellular ATP concentration in erythrocytes incubated in glucose-depleted Ringer solution was similarly decreased significantly.

4 Discussion

The present observations reveal a novel action of enniatin A, i.e. ATP depletion, triggering of Ca^{2+} entry, cell shrinkage and cell membrane scrambling in human erythrocytes. Moreover, the present observations reveal that enniatin A augments the Ca^{2+} entry and cell membrane scrambling following energy depletion. The concentrations required for those effects are well in the range of those triggering apoptosis of nucleated cells [7] and to be toxic in vivo [33].

As indicated earlier [20], Ca^{2+} entry into erythrocytes is stimulated by phorbol esters and inhibited by staurosporine and chelerythrine, pointing to regulation of Ca^{2+} entry by protein kinase C (PKC). The kinase is activated and thus Ca^{2+} entry stimulated by ATP depletion [20]. The effects of energy depletion were blunted but not abolished in the presence of kinase inhibitors staurosporine or calphostin C and mimicked by PKC stimulator phorbol-12-myristate-13-acetate or the phosphatase inhibitor okadaic acid [20]. The enniatin A-induced Ca^{2+} entry thus presumably results in large part from the severe ATP depletion. Along those lines the cell membrane scrambling was blunted by staurosporine.

An increase in the cytosolic Ca^{2+} concentration is well known to trigger erythrocyte membrane scrambling [24, 26]. According to the present observations, enniatin A further augments the increase in the cytosolic Ca^{2+} concentration following energy depletion. The increase in Ca^{2+} concentration following enniatin A exposure presumably results from stimulation of Ca^{2+} entry, which is accomplished by Ca^{2+} permeable cation channels involving TRPC6 [14]. Accordingly, the Ca^{2+} entry is blunted by the addition of cation channel blocker amiloride. The effect of enniatin A on cell membrane scrambling is, however, only blunted but not fully abolished in the nominal absence of extracellular Ca^{2+} indicating that the effect of enniatin A on cell

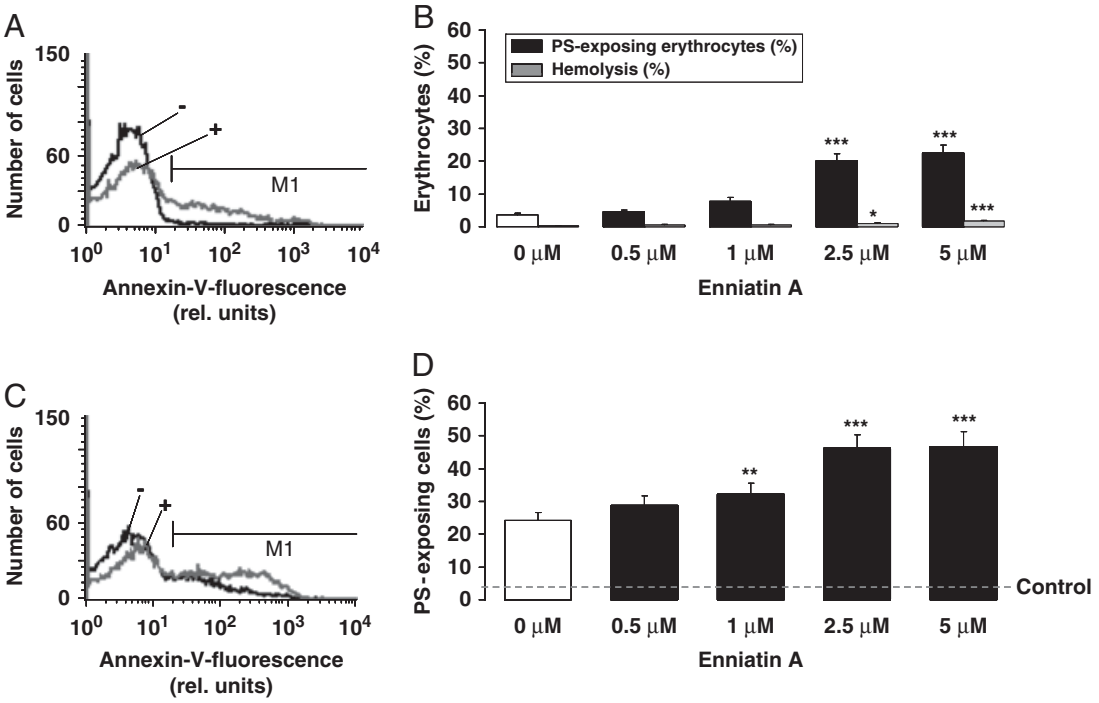

Figure 4. Effect of enniatin A on phosphatidylserine exposure and erythrocyte membrane integrity. (A) Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without (−, black line) and with (+, gray line) the presence of 5 μM enniatin. (B) Arithmetic mean ± SEM ($n = 12$) of erythrocyte annexin-V-binding following incubation for 48 h in Ringer solution without (white bar) or with (black bars) the presence of 1–5 μM enniatin A. For comparison, arithmetic mean ± SEM ($n = 4$) of the percentage of hemolysis is shown as gray bars. *, *** ($p < 0.05$, $p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA). (C) Original histogram of annexin-V-binding of erythrocytes following exposure for 48 h to Ringer solution without glucose in the absence (−, black line) and presence (+, gray line) of 5 μM enniatin A. (D) Arithmetic mean ± SEM ($n = 12$) of erythrocyte annexin-V-binding following incubation for 48 h in the absence of glucose (white bar) or presence (black bars) of 1–5 μM enniatin A. The broken line indicates control annexin-V-binding in the presence of glucose and absence of enniatin A. **, *** ($p < 0.01$, $p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA).

membrane scrambling is in part but not completely accounted for by its effect on Ca^{2+} entry.

The effect of enniatin A is not significantly modified by the pancaspase inhibitor zVAD. This result contrasts the observations in nucleated cells, where the apoptotic effect of enniatins appears to be at least partially dependent on caspase activation [2]. Erythrocytes do express caspases [26, 30], which could be activated by oxidative stress but do not participate in the stimulation of cell membrane scrambling by Ca^{2+} [24, 28, 31]. As illustrated in Fig. 5E, zVAD does significantly blunt the annexin-V-binding following exposure of erythrocytes to oxidative stress, indicating that the substance was able to inhibit erythrocyte caspases.

The increase in erythrocyte Ca^{2+} concentration is expected to activate Ca^{2+}-sensitive K^+ channels [21, 22] leading to the subsequent cell shrinkage due to exit of K^+, hyperpolarization of the cell membrane, exit of Cl^- and exit of osmotically obliged water [23]. Enniatin A exposure indeed decreased the erythrocyte FSC and thus led to cell shrinkage. Somewhat surprisingly, higher concentrations of enniatin A blunted the effect of glucose depletion on cell volume. Possibly, the severe ATP depletion following enniatin A treatment in the absence of glucose impaired the Na^+/K^+ ATPase activity leading to a decline of cellular K^+ concentration and thus to dissipation of the K^+ gradient across the cell membrane. Following loss of K^+ gradient across the cell membrane, the activation of Ca^{2+}-sensitive K^+ channels fails to trigger K^+ exit, hyperpolarization and Cl^- exit. Thus, in K^+-depleted erythrocytes Ca^{2+} entry fails to trigger cell shrinkage. The swelling following exposure of ATP-depleted erythrocytes to high (5 μM) concentrations of enniatin A could be explained by Na^+ entry through the Ca^{2+} permeable cation channels.

Similar to glucose depletion, other triggers of phospholipid scrambling could compound the effect of enniatin A. Excessive phospholipid scrambling is observed in a variety of clinical disorders [10] including iron deficiency [34], phosphate depletion [35], hemolytic uremic syndrome [36], sepsis [37], sickle cell disease [38], malaria [38–42], Wilson's disease [43] and possibly metabolic syndrome [44]. Moreover, phospholipid scrambling is triggered by a variety of further xenobiotics and endogeneous substances [45–51]. Accelerated erythrocyte phospholipid

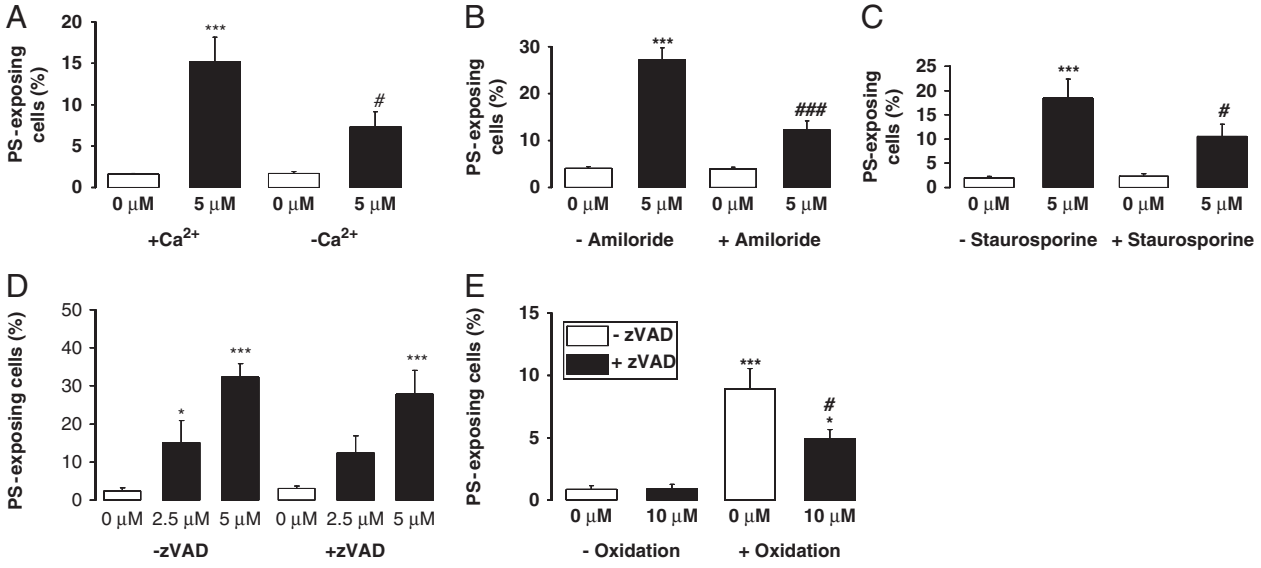

Figure 5. Effect of Ca^{2+} withdrawal, cation channel, caspase and protein kinase C inhibition on enniatin A-induced phosphatidylserine exposure. (A) Arithmetic mean ± SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 5 μM enniatin A in the presence (left bars, + Ca^{2+}) and absence (right bars, − Ca^{2+}) of calcium. *** ($p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA), ♯ indicates significant difference ($p < 0.05$) from the respective values in the presence of Ca^{2+}. (B) Arithmetic mean ± SEM ($n = 5$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 5 μM enniatin A in the absence of amiloride (1 mM) (left bars) and presence of amiloride (1 mM) (right bars). *** ($p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA), ### indicates significant difference ($p < 0.001$) from the respective values in the absence of amiloride. (C) Arithmetic mean ± SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 5 μM enniatin A in the absence (left bars, − staurosporine) and presence (right bars, + staurosporine) of staurosporine (500 nM). *** ($p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA). ♯ indicates significant difference ($p < 0.05$) from the respective value in the absence of staurosporine. (D) Arithmetic mean ± SEM ($n = 4$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 2.5 μM and 5 μM enniatin A in the absence (left bars, − zVAD) and presence (right bars, + zVAD) of zVAD (10 μM). *, *** ($p < 0.05$, $p < 0.001$) indicate significant difference from the absence of enniatin A (ANOVA). (E) Arithmetic mean ± SEM ($n = 9$) of the percentage of annexin-V-binding erythrocytes after a 48 h treatment with Ringer solution without (white bars) or with (black bars) 10 μM zVAD in the absence (left bars, − oxidation) and presence (right bars, + oxidation) of *t*-BOOH (0.3 mM). *, *** ($p < 0.05$, $p < 0.001$) indicate significant difference from the absence of *t*-BOOH (ANOVA). ; indicates significant difference ($p < 0.05$) from the absence of zVAD.

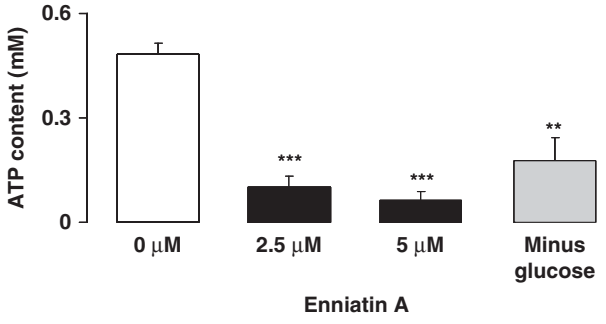

Figure 6. Effect of enniatin A on erythrocyte cytosolic ATP content. Arithmetic mean ± SEM ($n = 4$) of the ATP concentration after a 48 h incubation in Ringer solution without (white bar) or with (black bars) enniatin A at the indicated concentrations, or in glucose-depleted Ringer solution (gray bar, minus glucose). **, *** ($p < 0.01$, $p < 0.001$) indicates significant difference from control (absence of enniatin A and presence of glucose) (ANOVA).

scrambling may result in the development of anemia [10]. Clinically overt anemia will however develop only, if the accelerated clearance of eryptotic erythrocytes cannot be compensated by increased production of new erythrocytes. The loss of circulating erythrocytes is followed by release of erythropoietin and subsequent hormonal stimulation of erythrocyte formation, which is apparent from the increase in the reticulocyte number [15, 52]. In addition to triggering anemia, erythrocyte phospholipid scrambling may impede microcirculation due to adherence of phosphatidylserine-exposing erythrocytes to the vascular wall [53–57]. Moreover, eryptotic erythrocytes may stimulate blood clotting [53, 58, 59].

In conclusion, the present observations disclose that enniatin A stimulates erythrocyte phospholipid scrambling and augments phospholipid scrambling during energy depletion. Those effects presumably contribute to the toxicity of this mycotoxin food contaminant.

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The authors have declared no conflict of interest.

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