ION CHANNELS, RECEPTORS AND TRANSPORTERS

Haemolysis induced by α-toxin from *Staphylococcus aureus* requires P2X receptor activation

Marianne Skals · Jens Leipziger · Helle A. Praetorius

Abstract Recently, it was documented that α-haemolysin (HlyA) from *Escherichia coli* uses erythrocyte P2 receptors cause lysis. This finding was surprising as it appeared firmly established that HlyA-dependent pore formation per se is sufficient for full cell lysis. We discovered that HlyA induced a sequential process of shrinkage and swelling and that the final haemolysis is completely prevented by blockers of P2X receptors and pannexin channels. This finding has potential clinical relevance as it may offer specific pharmacological interference to ameliorate hae-molysis inflicted by pore-forming bacterial toxins. In this context, it is essential to know whether this is specific to HlyA-induced cell damage or if other bacterial pore-forming toxins involve purinergic signals to orchestrate haemolysis. Here, we investigate if the haemolysis produced by α-toxin from *Staphylococcus aureus* involves P2 receptor activation. We observed that α-toxin-induced haemolysis is completely blocked by the unselective P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid. Moreover, several selective blockers of $P2X_1$ and $P2X_7$ ionotropic receptors abolished haemolysis in murine and equine erythrocytes. Inhibitors of pannexin channels partially reduced the α-toxin induced lysis. Thus, we conclude that α-toxin, similar to HlyA from *E. coli* produces cell damage by specific activation of a purinergic signalling cascade. These data indicate that pore-forming toxins in general require purinergic signalling to elicit their toxicity.

Keywords Adenosine 5-triphosphate (ATP) · P2-purinergic receptor · Erythrocytes · Cell volume · Membrane · Mouse · Osmotic stress

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Introduction

Staphylococcus aureus is a Gram-positive bacterium prone to form abscesses and known to be responsible for serious infections as pneumonia, endocarditis, brain abscesses and sepsis. The latter occurs with a higher mortality rate than meningococcal septicemia [23]. α-Toxin is known to contribute significantly to the cell damage inflicted by *S. aureus* [3], which might add to the clinical picture of sepsis. The heart is one of the most critical organs affected by sepsis, illustrated by the correlation between depression of myocardial function, sepsis and mortality [29]. The pore-forming α-toxin from *S. aureus* is known to induce myocardial dysfunction in intact animals [35], a situation partially ascribed to apoptosis of the cardiomyocytes [9]. Many hospital infections are caused by multiresistant *S. aureus* strains (for recent reviews, see [24]), increasing the risk of a delayed onset of an effective antibiotic treatment. In this context, it is likely that a reduction of α-toxin-induced cell damage would be beneficial for the overall clinical outcome.

Recently, we discovered that P2 receptor activation is critically involved in the haemolysis induced by the pore-forming toxin α-haemolysin from *Escherichia coli* [38]. We were able to show that it was not merely the haemolysin itself, which was cytotoxic. The insertion of the pore required subsequent activation of P2X receptors and pannexin channels to produce full lysis. The present study

investigates the effect of purinergic receptor activation in α-toxin-induced haemolysis. We are able to show that α-toxin, in a very similar fashion, uses activation of P2 receptors to cause cell damage initialised by this pore-forming toxin as adenosine 5-triphosphate (ATP) potentiates haemolysis, and P2 receptor antagonists completely abolish the α-toxin effect in murine erythrocytes.

Materials and methods

Preparations of erythrocytes

Blood samples were collected in ethylenediaminetetraacetic acid-containing tubes, washed three times ($1,000 \times g$, 5 min, 4°C) in 0.9% NaCl (w/v) and the buffy coat was removed. Defibrinated horse blood was purchased from Statens Serum Institut (Copenhagen, Denmark). Murine blood was obtained from Balb/c mice of either sex after the mice were killed by cervical dislocation.

Transgenic mice

The P2X$_7$ *knockout* (P2X$_{7-/-}$) mice on Balb/c background were compared to wild-type (P2X$_{7+/+}$) littermates obtained from heterozygote breeding. P2X$_{7-/-}$ mice are viable with no apparent abnormalities. They were bred in house according to the Danish animal welfare regulations. The P2X$_{7-/-}$ mice were originally developed by GlaxoSmithKline [36] and bred into the Balb/c background.

Measurements of haemolytic activity

The haemolytic activity was measured spectrophotometrically (Ultraspec III, LKB Biochrom, Cambridge, UK) at 540 nm. The erythrocyte volume fraction of the final test solution was 1.25% in Ca^{2+}-containing4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-buffered saline. In the haemolysis assay, purified α-toxin was added in increasing concentrations for up to 60 min at 37°C under constant swirl (180 rpm). We confirmed that 100 Uml^{-1} of α-toxin caused 100% haemolysis after 60-min incubation and 50% haemolysis in the given erythrocyte suspension was achieved with ~10 U α-toxin ml^{-1} (60 min). The 1.5-ml test tubes were then centrifuged ($1,000 \times g$, 5 min, 4°C) and the OD$_{540}$ of the supernatant was determined. Normalised data refer to 50% lysis after 60 min of incubation unless otherwise stated in figure legends.

Live cell imaging

The erythrocytes were attached to glass coverslips by BD Cell-Tak™ (BD Bioscience, Franklin Lakes, NJ, USA). The preparation was placed in an incubation chamber on an inverted microscope (Nikon TE2000) at 37°C. Time laps recordings of the erythrocyte appearance in DIC (60X, NA1.2) were collected over 30 min after addition of α-toxin (sampling rate, 0.1 Hz). The erythrocyte shape change was compared to time controls without α-toxin.

For $[Ca^{2+}]_i$ measurements, erythrocytes were incubated with fluo 4-AM (5 μM, 60 min) and attached to glass coverslips by BD Cell-Tak™ and placed in an incubation chamber on a IMIC stage (TILL Photonics, Munich, Germany). The fluorophore was excited at 488 nm by a monochromator (PolychromeV, TILL Photonics), and the emission was collected >510 nm. The preparation was imaged with a 60X, 1.45 NA Plan Apo (Olympus) objective and a charge coupled device camera (Sensicam qe, PCO, Kelheim, Germany). The entire setup was delivered from Bio-Science ApS, Gilleleje, Denmark. The experiments were conducted at 37°C.

Flow cytometry

Flow cytometry analysis was performed on a Quanta SC MPL (Beckman Coulter) using Cell Lab Quanta MPL analysis software for acquisition and analysis. Murine erythrocytes were incubated with fluo 4-AM for 60 min, washed and resuspended in HEPES-buffered saline (~10^6 cells ml^{-1}). Toxin was added at a concentration leading to 50% lysis after 60 min (~10 Uml^{-1}). The fluorescent signal was collected every minute for 10 min. At each time point, 2,000 cells were counted at a flow rate adjusted to ~200 events per second. The data in Fig. 7c represents a single experiment plotting the number of cells at a given fluorescence intensity (exited at 488 nm). The data were further analysed in Igor Pro (WaveMetrics, Lake Oswego, OR, USA) for graphical presentation and each vertical line represent the number of cells for each fluorescent intensity value.

Solutions and materials

The HEPES-buffered saline constituted of (in mM): $[Na^+]$ 138.0, $[Cl^-]$ 132.9, $[K^+]$ 5.3, $[Ca^{2+}]$ 1.8, $[Mg^{2+}]$ 0.8, $[SO_{42-}]$ 0.8, [HEPES] 14, [glucose] 5.6, pH 7.4 at 37°C. For experiments with the enzyme hexokinase (data presented in Fig. 2a), the HEPES-buffered saline was supplemented with 10 mM glucose and 2.5 mM $MgSO_{42-}$ and the concentration of NaCl was decreased to maintain the same osmolality for all experiments.

α-Toxin, carbenoxolone, ATP-2′,3′-dialdehyde (oxidised ATP), mefloquine, hexokinase, pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS), TRAM-34, clotrimazole, and MRS2159 were obtained from Sigma-Aldrich. Brilliant blue G (BBG) was purchased from INC Biomedicals Inc. (Aurora, OH, USA). A438079 was from Tocris Bioscience

(Bristol, UK). Fluo-4-AM was from Invitrogen (Taastrup, Denmark). Standard reagents were dissolved in distilled water and pH adjusted to 7.4 at 37°C. BBG, mefloquine, TRAM-34 and clotrimazole were dissolved in DMSO to a final concentration of maximal 0.5% DMSO. Control experiments for possible DMSO effects alone were included. HlyA was purified from *E. coli* strain ARD6 (Statens Serum Institute, Copenhagen, Denmark) according to [18].

Data analysis and statistics

Data are presented as mean±SEM. For the majority of the experiments, the *n* value indicates number of animals but for Figs. 2b, 3e and supplementary Fig. 1b, two experiments were conducted per animal. The data were tested for normality by Kolmogorov–Smirnov test. Significant differences were determined by paired or unpaired Student's *t* test or one way analysis of variance (Tukey's post test) for multiple comparisons as appropriate. In both cases, a *p* value less than 0.05 was considered statistically significant.

Results

α-Toxin from *S. aureus* is a classic haemolysin, which induces lysis much more potently in murine compared to human erythrocytes. This feature has been ascribed to the lack of receptors for the toxin on human erythrocytes [10]. Therefore, the present study primarily addressed the response in murine erythrocytes and selected experiments verified in equine erythrocytes. We have previously shown that another pore-forming bacterial toxin (HlyA from *E. coli*) requires P2 receptor activation to produce haemolysis. Here, we investigated whether this is a specific finding for HlyA or if a similar mechanism also implies for α-toxin-induced haemolysis.

Figure 1 illustrates the well-documented α-toxin-induced release of haemoglobin to the supernatant of murine erythrocytes. Haemoglobin release is reduced considerably by the nonselective P2 receptor antagonist PPADS. Figure 1a shows that PPADS concentration dependently reduces the α-toxin-induced haemolysis of murine erythrocytes at various toxin concentrations. In the presence of 200 μM PPADS, the α-toxin-induced haemolysis was completely inhibited at all toxin concentrations. In Fig. 1b, the PPADS responsiveness was tested at an α-toxin concentration that produced 50% haemolysis after 60 min (~10 $U ml^{-1}$) and the data are normalised. PPADS also significantly reduced the haemolysis of equine erythrocytes (Fig. 1b). These data indicate an involvement of P2 receptor activation during α-toxin-induced haemolysis.

Another way of addressing whether purinergic signalling is required for the full haemolytic effect of α-toxin is to rapidly degrade ATP released from the cells. We tested the effect of the ATPases apyrase and hexokinase on α-toxin-induced haemolysis. Figure 2 shows a clear concentration-dependent effect of hexokinase on the α-toxin-induced haemolysis. This effect could not be confirmed by apyrase, which similarly to what has previously been shown for bovine serum albumin [11] severely potentiates the α-toxin induced haemolysis (suppl. Fig. 2). Both hexokinase and apyrase is known to inhibit HlyA-induced haemolysis [38], so we cannot offer any straightforward explanation for the discrepancy between the two enzymes in the present study. We assume the effect of apyrase to be unspecific as it does not fit with any of the other findings in the study.

We also tested the effect of merely adding ATP together with the toxin (Fig. 2b). ATP has a clear biphasic effect. In the early phases of haemolysis (2.5 min), ATP potentiates effect of HlyA at all the used concentrations. After 15 and 30 min, this effect reverses to an inhibition at the higher ATP concentrations (3 and 5 mM). From these experiments,
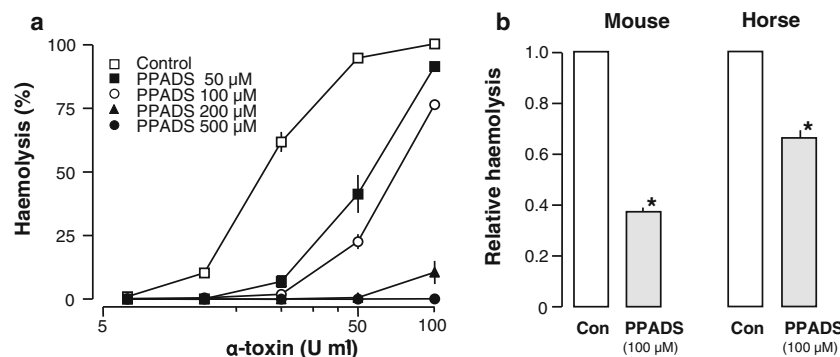

Fig. 1 α-Toxin-induced haemolysis of murine and equine erythrocytes is inhibited by purinergic antagonism. **a** The concentration–response for the P2 receptor blocker PPADS at increasing concentrations of α-toxin from *S. aureus* (*n*=3–5 animals). The erythrocytes were subjected to α-toxin for 60 min at 37°C at a constant swirl of 180. **b** Compares the effect of PPADS (100 μM) on the α-toxin-induced haemolysis in murine and equine erythrocytes. In the experiments, a toxin concentration leading to 50% lysis after 60 min was used (~10 $U ml^{-1}$) and the values are normalised. Haemolysis was measured as OD540 and all values are presented as mean±SEM
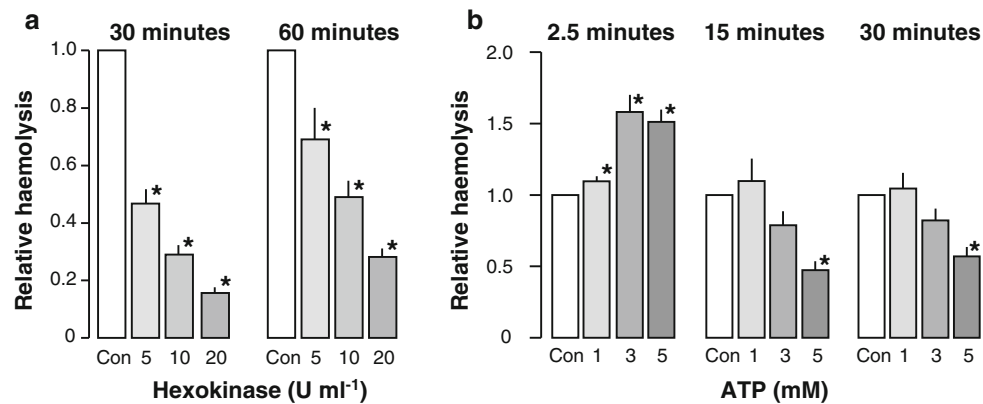
Fig. 2 The effect of ATP and rapid ATP degradation on α-toxin-induced haemolysis. **a** Hexokinase decreases α-toxin-induced lysis of murine erythrocytes. Murine erythrocytes incubated with α-toxin (~10 Uml^{-1}) in increasing concentrations of hexokinase in a HEPES-buffered saline containing 10 mM glucose and 2.5 mM Mg^{2+}. All values are normalised and presented as mean±SEM ($n=8$ animals). The 10 Uml^{-1} α-toxin resulted in 40% lysis after 30 min. **b** Effect of ATP on α-toxin-induced lysis of murine erythrocytes. Murine erythrocytes incubated with α-toxin (15 Uml^{-1}) at increasing concentration of ATP (1, 3 and 5 mM) for 2.5, 15 and 30 min. All values are normalised and presented as mean±SEM, $n=12$ (blood from six animals). The 15 Uml^{-1} α-toxin resulted in ~4%, ~50%, ~70% lysis after 2.5, 15 and 30 min, respectively. *Asterisk* denotes a statistically significant difference from the normalised control

we conclude that ATP degradation inhibits the α-toxin-induced haemolysis and that addition of excess ATP potentiates haemolysis in the early phases, supporting the notion of P2 receptor activation in α-toxin-induced haemolysis.

The P2 receptor candidates responsible for the HlyA-induced haemolysis were previously identified from the inhibition pattern of various P2 receptor antagonists. We found the $P2X_7$ to be mainly responsible for HlyA-induced haemolysis in human erythrocytes with some contribution from $P2X_1$. In murine erythrocytes, the predominant effect is caused by the $P2X_1$ with support from the $P2X_7$ receptor [38]. The effect of PPADS on the α-toxin-induced haemolysis suggests that P2 receptor activation is similarly important for α-toxin-induced haemolysis. Therefore, it is likely to use the same P2 receptor subtypes found to be important for HlyA-induced haemolysis. We tested this by antagonists with relative selectivity towards the $P2X_1$ and $P2X_7$ receptors. Figure 3 shows the effect of $P2X_7$ receptor antagonists on α-toxin-induced haemolysis. These inhibitors (BBG, oxidised ATP (OxATP) and A438079) have previously been shown to concentration-dependently reduce HlyA-induced haemolysis in murine erythrocytes. Figure 3a shows the effect of BBG at various concentrations of α-toxin. BBG concentration-dependently inhibited the α-toxin-induced haemolysis in all concentrations of the toxin. Figure 3b shows that α-toxin-induced haemolysis is also reduced by OxATP and A438079. These results indicate a possible role for the $P2X_7$ receptor in α-toxin-induced haemolysis in murine erythrocytes. Therefore, we tested the effect of the toxin in $P2X_7$ receptor-deficient mice. Figure 3c shows that $P2X_7$ receptor-deficient mice have a reduced response (~25%) to α-toxin at a concentration near EC_{50} (~10 Uml^{-1}) as compared to controls. Considering the full concentration response curve there is,

however, no difference at higher concentrations of the toxin (Fig. 3d). We also tested the effect of adding ATP together with α-toxin in $P2X_7$-deficient erythrocytes and did not find any significant potentiation of ATP on haemolysis within 2.5 min (Fig. 3e) compared to controls (Fig. 2b). At the later time points (15 and 30 min), ATP reduced the α-toxin-induced lysis in $P2X_{7+/+}$ erythrocytes, which is similar to what we found in wild type (Fig. 2b). These data further suggest the $P2X_7$ receptor to play a role in the lytic process.

As $P2X_7$ receptor antagonists are not selective in higher concentrations, the results certainly leave room for other P2 receptors. Since the $P2X_1$ receptor has previously been suggested to be important for HlyA-induced haemolysis in murine erythrocytes [38], we tested the blocker MRS2159 with relative selectivity towards the $P2X_1$ receptor. This antagonist showed a marked concentration-dependent blockage of α-toxin-induced haemolysis (Fig. 4a). This finding supports our previous data that the $P2X_1$ receptor seems to be the receptor with preferential importance for the purinergic amplification of haemolysis induced by HlyA in mice. Previously, we found that $P2X_7$ receptor-deficient mice showed enhanced expression of the $P2X_1$ receptor in the erythrocyte membrane compared to wild-type controls [38]. Therefore, we tested the relative effect of MRS2159 on α-toxin-induced haemolysis in $P2X_7$-deficient mice (Fig. 4b). Again, we showed that $P2X_7$ receptor-deficient mice showed a lower degree of haemolysis at a toxin concentration of ~10 Uml^{-1} as compared to controls. Normalising for this difference revealed a very similar response to MRS2159 between the two genotypes. This means that there do not seem to be functionally more $P2X_1$ receptor activity in the $P2X_7$ receptor-deficient mice compared to controls.

Fig. 3 α-Toxin-induced haemolysis is inhibited by P2X$_7$ receptor antagonists. **a** The concentration dependent effect of brilliant blue G (BBG) at various concentrations of α-toxin ($n=5$ animals). **b** Effect of a single high concentration of ATP-2′,3′-dialdehyde (OxATP, 500 μM) and A438079 (500 μM) at ~10 Uml^{-1} of α-toxin. **c** Depicts the haemolysis in control and P2X$_7$-deficient mice induced by ~10 Uml^{-1} α-toxin, which results in ~50% lysis in WT ($n=7$ animals). **d** The haemolysis induced by various concentrations of α-toxin in P2X$_7$-deficient and control mice ($n=7$ animals). All values are mean±SEM. **e** The effect of ATP (1 and 3 mM) on P2X$_7$-deficient erythrocytes, which can be compared to controls in Fig. 2b. All values are normalised and presented as mean±SEM, $n=6$ (blood from three animals) for 2.5 and $n=4$ (blood from two animals) for 15 and 30 min. The 15 Uml^{-1} α-toxin resulted in ~4%, ~40%, ~50% lysis after 2.5, 15 and 30 min, respectively. *Asterisk* denotes a statistically significant difference from the normalised control
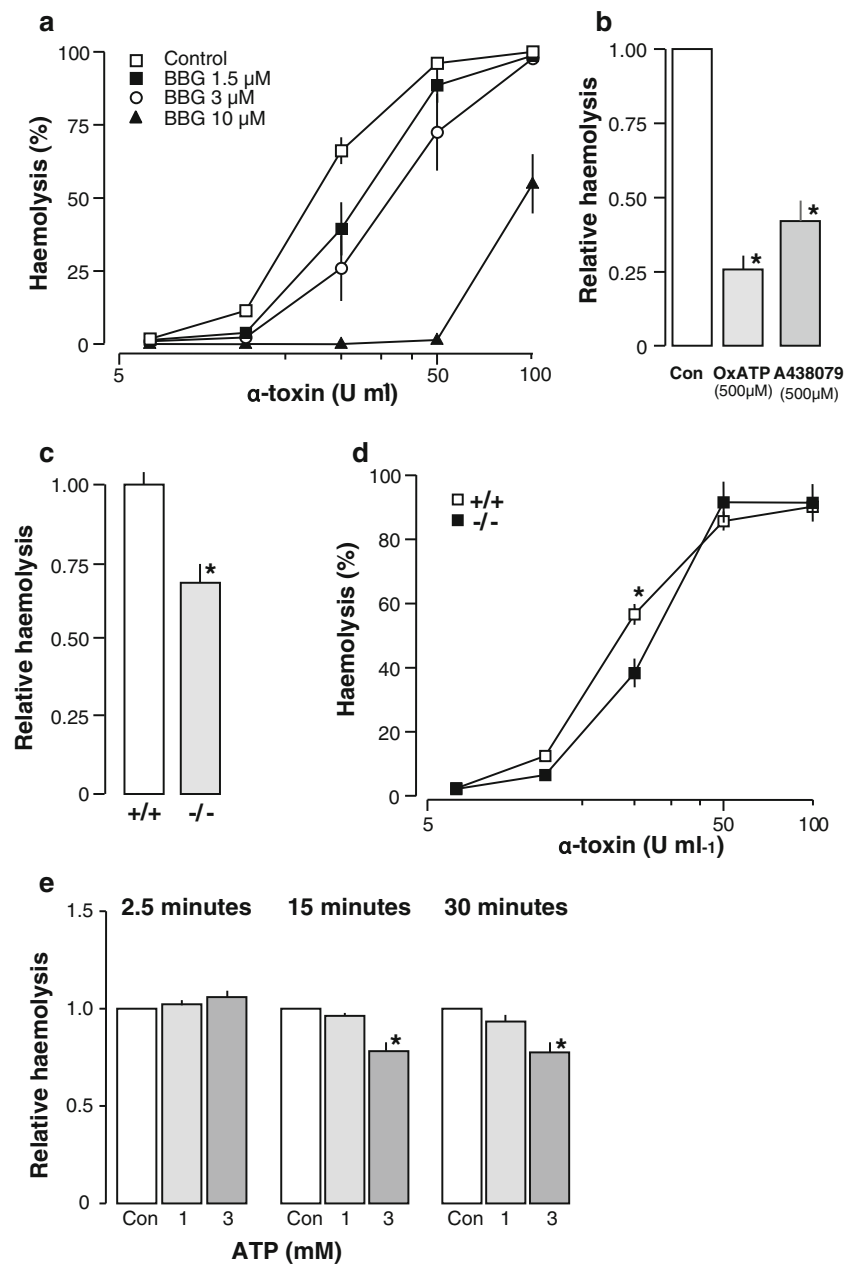
The HlyA-induced haemolysis was suggested to rely on secondary activation of pannexin 1 channels. This conclusion was based on the inhibitory effect of non-selective blockers of pannexin channels on HlyA-induced haemolysis. To our surprise, we found that both carbenoxolone and mefloquine at concentrations that produced complete inhibition of HlyA-induced haemolysis only showed minor effects on α-toxin-induced haemolysis. Figure 5 shows these subtle effects of the nonselective pannexin blockers on α-toxin-induced haemolysis of murine and equine erythrocytes.

This discrepancy primed us to look further into the haemolytic process. The haemolysis is easily observed in the microscope, where α-toxin from *S. aureus* triggers an initial swelling, which eventually leads to lysis of the erythrocytes. Figure 6a shows the morphology of murine erythrocytes when exposed to α-toxin in a concentration that produced 90% haemolysis within 20 min. In contrast to HlyA [38], α-toxin-induced haemolysis is not preceded by a significant degree of shrinkage. Reducing the toxin concentration to slow down the haemolytic process does not change this pattern. Figure 6b shows the summarised data of three to seven experiments where the toxin concentration was reduced to produce 50% haemolysis after 30 min. In this situation, there was only a slight hint of crenation before the erythrocytes swelled and lysed when exposed to α-toxin.
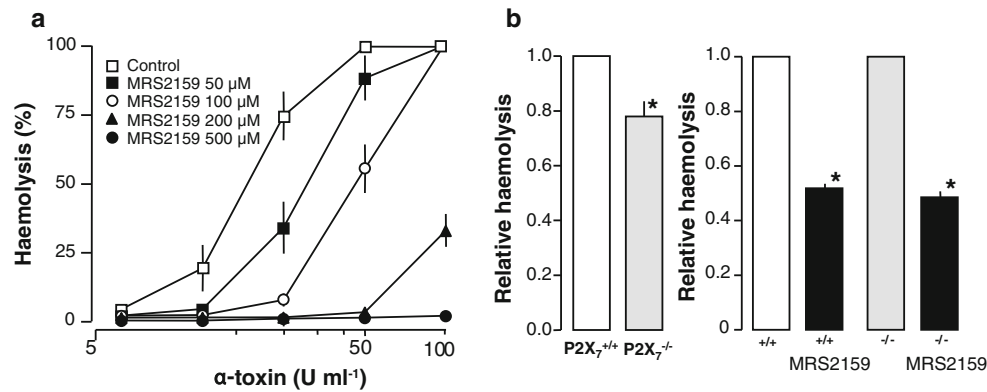
a

b

Fig. 4 **a** Effect of the $P2X_1$ antagonists (MRS2159) on α-toxin-induced haemolysis in murine erythrocytes. The erythrocytes were incubated with various concentrations of α-toxin and increasing concentrations of MRS2159 (mean±SEM, n=6–7). **b** Effect of $P2X_1$ blockade on α-toxin-induced lysis of $P2X_{7+/+}$ and $P2X_{7-/-}$ erythrocytes. The *left panel* show the haemolysis in erythrocytes from $P2X_{7+/+}$ and $P2X_{7-/-}$ mice (GSK) incubated with α-toxin (~10 U ml^{-1}) for 60 min, confirm-

ing the marginally lower haemolysis of in erythrocytes from $P2X_{7-/-}$ mice. The *right panel* shows the effect of the $P2X_1$ antagonist MRS2159 (50 μM) on the normalised haemolysis in erythrocytes form $P2X_{7+/+}$ and $P2X_{7-/-}$ mice. All values are normalised and presented as mean±SEM, n=6 animals of each genotype. *Asterisk* denotes a statistically significant difference from the control

The early volume reduction observed when erythrocytes are exposed to HlyA is triggered by increments in $[Ca^{2+}]_i$, which activates Ca^{2+}-sensitive K^+ and Cl^- channels [37]. Thus, a plausible explanation for the lack of shrinkage after α-toxin exposure could be a lower Ca^{2+} permeability of α-toxin compared to HlyA. This has previous been suggested from a comparative study of α-toxin- and HlyA-induced $^{45}Ca^{2+}$ flux [40]. Here, we determined the kinetics of α-toxin- and HlyA increments of $[Ca^{2+}]_i$ (Fig. 7). Figure 7a shows that α-toxin triggers significant increments of $[Ca^{2+}]_i$ in single erythrocytes within few minutes after α-toxin is added. Comparing this to the HlyA-induced changes in $[Ca^{2+}]_i$ (Fig. 7b), the α-toxin-triggered changes in $[Ca^{2+}]_i$ were slow relative to the

prompt rise caused by HlyA. These observations from single-cell studies were verified in a larger population of erythrocytes by flow cytometry. Figure 7c shows histograms of cellular fluo 4 fluorescence intensities (single experiments) at various times after α-toxin and HlyA exposure. The concentration of HlyA and α-toxin was adjusted to give 50% lysis after 60 min. Both haemolysins significantly increased the fluo 4 fluorescence over time. The summary of the kinetic differences of α-toxin and HlyA-induced $[Ca^{2+}]_i$ increases is depicted in Fig. 7d. Again, the α-toxin-induced $[Ca^{2+}]_i$ changes developed considerably slower than the HlyA-induced changes (Fig. 7d, n=3). We tested whether, this slowly developing increase in fluo 4 fluorescence actually reports an increase in $[Ca^{2+}]_i$ induced by α-toxin. Firstly, chelating extracellular Ca^{2+} with EGTA resulted in a reduction of $[Ca^{2+}]_i$ to baseline values (suppl. Fig. 1a). Secondly, we confirm the previous findings from $K_{Ca}3.1$-deficient mice known to have a higher degree of α-toxin-induced haemolysis compared to controls [14]. We similarly found that inhibitors of $K_{Ca}3.1$ (TRAM-34 and clotrimazole) markedly potentiate the α-toxin-induced haemolysis (suppl. Fig. 1b), which is consistent with the preceding increase in $[Ca^{2+}]_i$ to trigger channel activation.
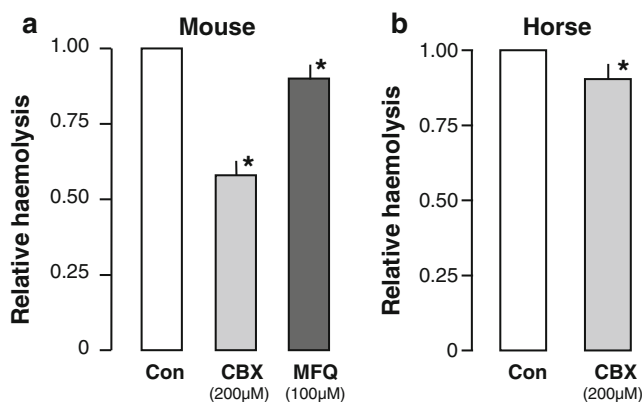
a **Mouse**

b **Horse**

Fig. 5 α-Toxin-induced haemolysis of murine and equine erythrocytes is reduced by pannexin 1 antagonists. **a** The haemolysis was reduced by carbenoxolone (200 μM) and mefloquine (100 μM) in murine erythrocytes (n=4 animals). **b** Carbenoxolone (200 μM) only had a minor effect on the haemolysis induced by α-toxin in equine erythrocytes (n=4 animals). The haemolysis was induced with ~10 U ml^{-1} α-toxin adjusted to give 50% haemolysis after 60 min at 37°C

Discussion

α-Toxin from *S. aureus* is a pore-forming exotoxin known to produce cell damage in numerous cell types and has been extensively used as a tool for cell permeabilisation to gain access to the cell interior [8]. Here, we show that α-toxin from *S. aureus* takes advantage of purinergic signalling to inflict haemolysis. P2 receptor-mediated potentiation of the cytotoxic effect of membrane pores is not unique to α-

Fig. 6 α-Toxin-induced haemolysis of murine erythrocytes. **a** The effect of α-toxin on murine erythrocytes attached to a coverslip after 5-, 10- and 20-min incubation at 37°C. **b** The summarised data from murine erythrocytes subjected to a concentration of the toxin adjusted to produce 50% haemolysis after 30 min. *Left* part of the figure shows the number of crenated cells and the *right* indicates the number of lysed cells at the given time point. Values are given as mean ±SEM, $n=3$–7
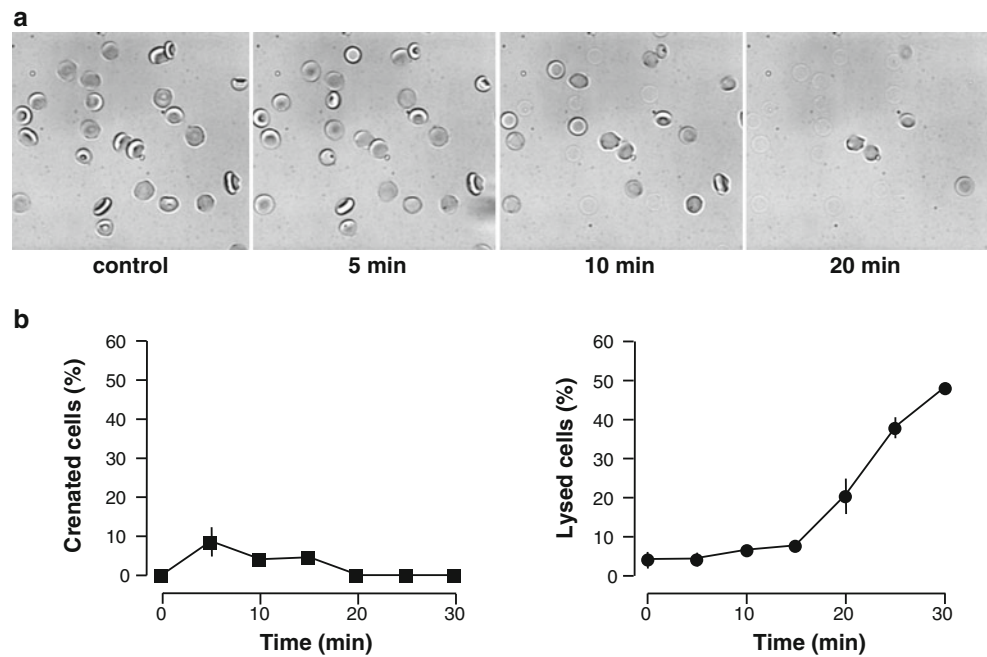
toxin. A substantial part of the haemolysis observed after application of α-haemolysin from *E. coli* (HlyA) can be ascribed to ATP release and subsequent P2 receptor stimulation [38]. Inhibition of P2 receptors and scavenging of extracellular ATP were able to abolish the HlyA-induced haemolysis. The further analysis of various antagonist revealed that the relevant P2 receptors are mainly the $P2X_7$ receptor in human and the $P2X_1$ receptor in murine erythrocytes. Here, we are able to show that very similar to HlyA-induced haemolysis, α-toxin also requires P2 receptor activation to elicit lysis. The α-toxin-induced haemolysis was nearly completely inhibited by the non-selective P2 receptor antagonist PPADS and by scavenging extracellular ATP with hexokinase. In this context, please note that apyrase potentiated rather than inhibited the α-toxin-induced haemolysis. We cannot provide any plausible explanation for the finding but can only mention that similar effects can be observed by adding serum albumin [11]. Administration of ATP together with α-toxin markedly potentiated the haemolysis early in the lytic process. This finding supports the notion that P2 receptors are involved in amplification of α-toxin induced haemolysis. In the later stages of lysis (after 15 and 30 min), however, ATP caused a substantial inhibition of the haemolysis. This may reflect either desensitisation of the P2X receptors or an inhibitory effect of ATP on pannexin channels [30].

The importance of P2-recptors in α-toxin-induced haemolysis was further substantiated by the anti-haemolytic effect of more selective P2 receptor blockers. Comparable to HlyA-induced haemolysis, inhibitors of the $P2X_7$ and the $P2X_1$ channels significantly reduced the α-toxin-induced haemolysis in murine erythrocytes. In addition, erythrocytes

from $P2X_{7-/-}$ mice showed reduced haemolysis (~25%) compared to controls at concentrations of α-toxin, which caused 50% lysis after 60 min.

Furthermore, the potentiating effect of ATP in the early phase of α-toxin-induced haemolysis was not observed in erythrocytes from $P2X_{7-/-}$ mice. Note, however, that ATP still reduced haemolysis in the late phases of α-toxin-induced haemolysis, which emphasises that this effect of ATP is not mediated via the $P2X_7$ receptor. Taken together, the data are consistent with a P2-receptor-dependent amplification of the α-toxin-induced lysis, which in murine erythrocytes is caused by $P2X_1$ and $P2X_7$ receptor activation.

The involvement of P2 receptor activation in α-toxin-induced haemolysis are supported by previous early observations obtained before the purinergic signalling was fully established as a significant auto- and paracrine signalling system. Both the haemolytic [41] and the lethal [1] effect of α-toxin from *S. aureus* was found to be inhibited by the P2 receptor antagonist suramin in rabbit and mice, respectively. We have verified this effect for both HlyA and α-toxin induced lysis of human and mice erythrocyte (data not shown). Similarly, suramin also inhibited the haemolysis induced by other types of pore-induced haemolysis such as complement activation in erythrocytes from sheep [12]. Suramin does, however, in itself inflict severe crenation of the erythrocytes at higher concentrations [38] and thus, the reduction of haemolysis may not necessarily be attributed to P2 receptor inhibition but could be a consequence of an opposing volume reduction of the red blood cells. In the light of the observations presented here, inhibition of P2 receptors is a
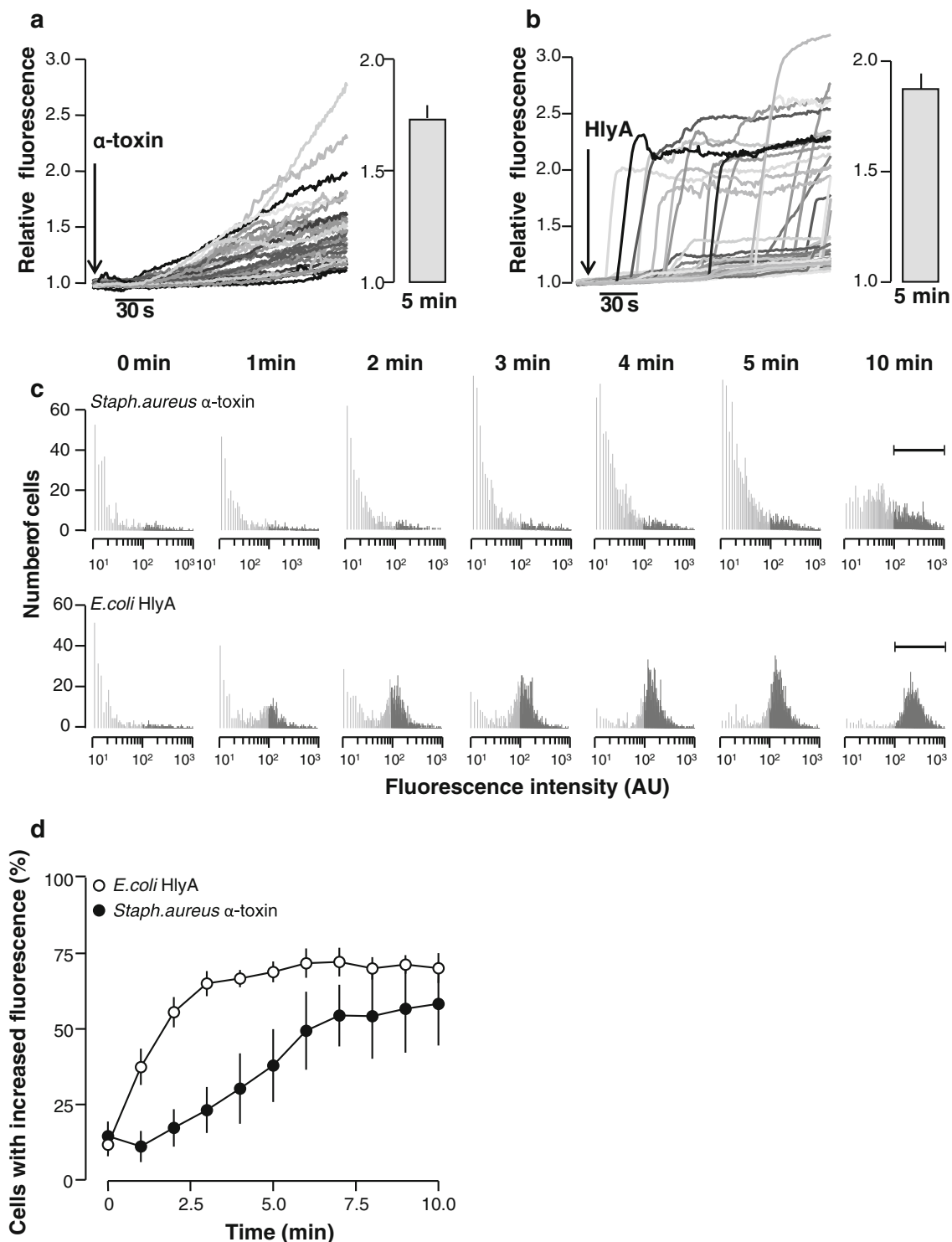
Fig. 7 α-Toxin-induced increments in [Ca]ᵢ. **a** The effect of α-toxin on the [Ca]ᵢ in single, murine erythrocytes on coverslips loaded with fluo 4-AM (5 μM) for 60 min at 37°C. *Left panel* is a representative trace and the *right* shows the summarised data after 5 min (mean± SEM, *n*=3 animals). **b** The single fluo 4-loaded murine erythrocytes in response to HlyA. *Left panel* is a representative trace and the *right*

shows the summarised data after 5 min (mean±SEM, *n*=3 animals). **c** The fluo 4-loaded murine erythrocytes in suspension measures with flow cytometry. The figure shows the distribution of erythrocytes with different fluorescence intensities in a representative experiment before and after addition of α-toxin and HlyA. **d** The summarised results of cells with a fluorescence over 10^2 (*n*=3 animals for each toxin)

plausible explanation for the suramin effects mentioned above.

This notion is supported by other sporadic indications of the necessity for ATP release and P2 receptor activation for
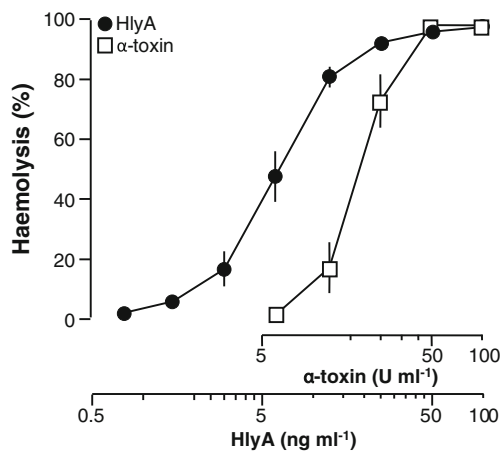
Fig. 8 Is a replot of the data shown in Fig. 3 (the control curve, α-toxin-induced haemolysis) against the control curve (HlyA-induced haemolysis) Fig. 2 published in [38]

α-toxin-induced cytotoxicity. α-Toxin is known to provoke significant reduction of the intracellular ATP concentration in various cell types such as monocytes [7, 42], jurkat cells [13] and human lung epithelial cells [7, 42]. This profound reduction is mostly caused by ATP release to the exterior. Accordingly, α-toxin has been shown to cause severe thrombocyte aggregation [2], a process intimately associated with P2Y$_{12}$ [17, 33], P2Y$_1$ [16, 19, 34] and P2X$_1$ [31] receptor activation.

Furthermore, the substance KN62, which is a known P2X$_7$ receptor antagonist, was previously proven to reduce

phospholipase D activity in synaptosomes in response to α-toxin [32]. Despite all these independent observations that point to an association between α-toxin insertion in plasma membranes and purinergic signalling, the present report is, to our knowledge, the first that shows a functional P2 receptor activation as consequence of α-toxin application.

Thus, ATP release and P2 receptor activation seems to be a general consequence for pore-forming bacterial toxins to induce lysis of erythrocytes. This suggests that ATP is released from the cells early in the process before there is substantial cell lysis. The mechanism by which ATP is initially released during toxin-induced haemolysis is not understood. It could in principle occur via the toxin pore or by activation of any of the channels previously suggested to conduct ATP in erythrocytes. As pannexin channels are put forward as an ATP release pathway in erythrocytes [25], it is interesting to know why α-toxin-induced haemolysis does not depend upon activation of pannexin channels to the same extend as for HlyA. Secondly, why does α-toxin not produce significant shrinkage of the erythrocytes as observed by HlyA. The answer to both questions is likely to be closely associated. The α-toxin is a heptameric structure that creates 1–1.5 nm wide pores in erythrocytes as determined in EM studies [15, 28], which has been confirmed in permeability studies (1.14 nm) [5, 27] and from the crystal structure (1.4 nm, in 1.8 Å resolution) [39]. This is tentatively narrower than the pore created by HlyA, which has been estimated ~1 nm to based on channel
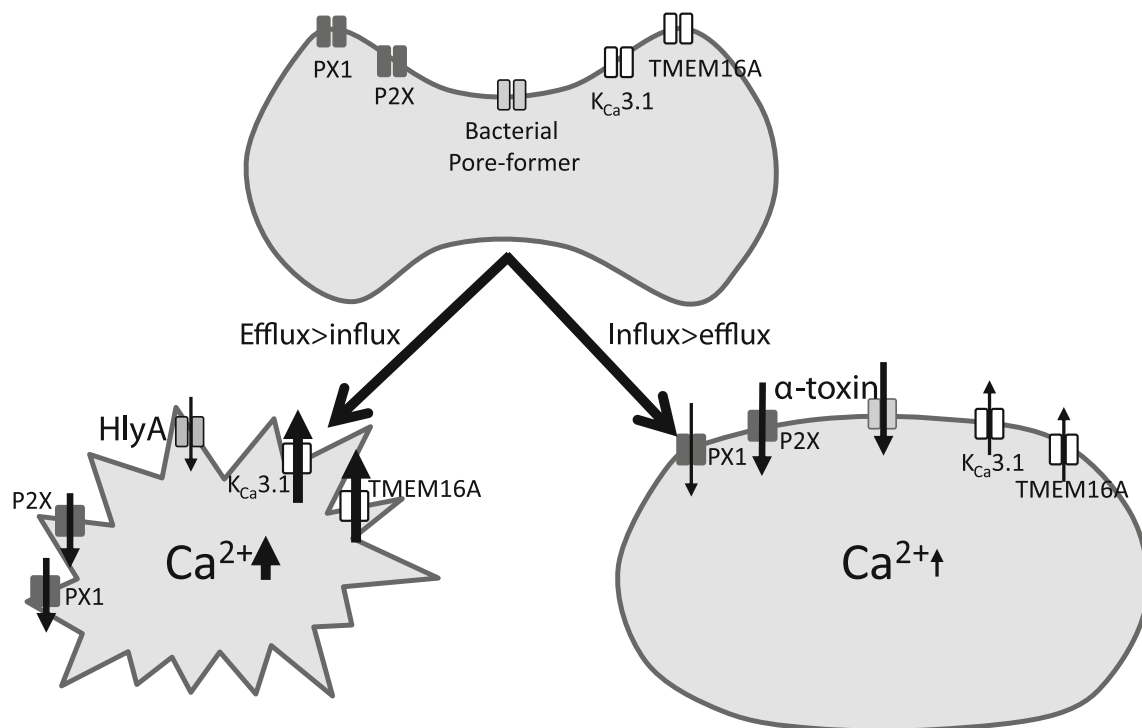

Fig. 9 Model of HlyA- and α-toxin-induced haemolysis

conductance [4] and around 2–3 nm in permeability studies [6]. Therefore, it seems counterintuitive that HlyA-induced haemolysis requires a significant activation of pannexin channels, whereas α-toxin does not. The final swelling and lysis of the erythrocytes result from a net ion influx. Thus, the Na^+ influx in magnitude must be significantly larger that the K^+ exit. This is supported by the finding that the α-toxin-induced ATP depletion and cell damage is completely inhibited by removal of extracellular Na^+ [20]. We have previously shown that the cell shrinkage after membrane insertion of HlyA is a consequence of significant increments in the $[Ca^{2+}]_i$ with subsequent activation of $K_{Ca}3.1$ and TMEM16A [37]. This is consistent with a study showing increased lysis in $K_{Ca}3.1$-deficient murine erythrocytes compared to controls when exposed to α-toxin [14]. The increment in $[Ca^{2+}]_i$ is a consequence of an increase Ca^{2+} influx over the plasma membrane, which happens prior to cell lysis [22]. Our present data show that the increment in $[Ca^{2+}]_i$-induced by α-toxin has a much slower kinetic than the case is for HlyA. These findings support previous data that α-toxin pores have a higher selectivity for monovalent cations as compared to HlyA [20, 40]. This implies that HlyA more effectively induces the Ca^{2+}-dependent K^+ and Cl^- conductances and thus inflict a volume reduction, whereas this is markedly less prominent after addition of α-toxin. The larger Ca^{2+} influx after HlyA exposure is likely to protect the cells from immediate lysis via the significant cell shrinkage. Indeed, low concentrations of HlyA protect erythrocytes against osmotic mediated lysis [21], which is in agreement with the HlyA-induced haemolysis being relatively more protracted. This is illustrated in Fig. 8, where the haemolysis induced by increasing concentrations of α-toxin (from Fig. 4) is replotted together with a similar curve for HlyA (from Fig. 2 in [38]). As one can appreciate, the α-toxin concentration range from negligible to full haemolysis covers one order of magnitude, whereas this requires two orders of magnitude in the case of HlyA-induced haemolysis.

Our data combining $[Ca^{2+}]_i$ measurements and transmitted light imaging suggest that α-toxin less effectively activates $K_{Ca}3.1$ and TMEM16A compared to HlyA. This may explain why one does not observe considerable volume reduction after α-toxin exposure. This is supported by the lower degree of potentiation in lysis of the $K_{Ca}3.1$ antagonists (TRAM-34 and clotrimazole, Suppl. Fig. 2) compared to prominent effects observed for HlyA-induced lysis [37]. The model, we suggest, for the volume changes induced by either of the haemolysins is shown in Fig. 9. Haemolysis is a result of a net influx of ions that exceeds the efflux. In this model, the Na^+ influx pathways consist of the toxin itself, P2X receptors and pannexin channels. The efflux pathways are constituted by $K_{Ca}3.1$ and TMEM16A. HlyA insertion leads to a prompt Ca^{2+} influx, which is a substantial trigger of the efflux pathway leading to cell shrinkage. In the case of α-toxin insertion, the Ca^{2+} influx occurs more gradually, which potentially cause less effective stimulation of the efflux pathways. In this situation the KCl efflux does not exceed the Na^+ influx through α-toxin and P2X channels the cell will not shrink and thus, be more susceptible to lysis.

What does that mean for the pannexin channel involvement? Firstly, if the KCl efflux is less after α-toxin insertion compared to HlyA, the influx through α-toxin and P2X channels may be sufficient to cause the lysis. Thus, activation of an additional influx pathway such as pannexin channel may potentiate haemolysis, without being an absolute requirement for the lysis to occur. Secondly, as Ca^{2+} in itself is a trigger for pannexin activation [26], a lesser overall Ca^{2+} influx during α-toxin-induced haemolysis may also explain the lesser degree of pannexin involvement.

Our results underscore that amplification of haemolysis induced by pore-forming bacterial toxin is not a curiosity found in HlyA-induced haemolysis but can be generalised to include α-toxin as well. Thus, it is very likely that this principle can be generalised to more pore formers and thereby open the door for general use of P2 receptor antagonist to counteract bacterial-induced cell damage.

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