

Bacterial RTX Toxins Allow Acute ATP Release from Human Erythrocytes Directly through the Toxin Pore*

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Background: Hemolysis induced by the two RTX toxins HlyA and LtxA depends on ATP receptor activation.

Results: HlyA and LtxA result in ATP release from human erythrocytes, which was unrelated to the main suggested ATP release pathway pannexin 1.

Conclusion: ATP is released through a toxin pore.

Significance: Adds new insights to the mechanism of these toxins.

ATP is as an extracellular signaling molecule able to amplify the cell lysis inflicted by certain bacterial toxins including the two RTX toxins α -hemolysin (HlyA) from *Escherichia coli* and leukotoxin A (LtxA) from *Aggregatibacter actinomycetemcomitans*. Inhibition of P2X receptors completely blocks the RTX toxin-induced hemolysis over a larger concentration range. It is, however, at present not known how the ATP that provides the amplification is released from the attacked cells. Here we show that both HlyA and LtxA trigger acute release of ATP from human erythrocytes that preceded and were not caused by cell lysis. This early ATP release did not occur via previously described ATP-release pathways in the erythrocyte. Both HlyA and LtxA were capable of triggering ATP release in the presence of the pannexin 1 blockers carbenoxolone and probenecid, and the HlyA-induced ATP release was found to be similar in erythrocytes from pannexin 1 wild type and knock-out mice. Moreover, the voltage-dependent anion channel antagonist TRO19622 had no effect on ATP release by either of the toxins. Finally, we showed that both HlyA and LtxA were able to release ATP from ATP-loaded lipid (1-palmitoyl-2-oleoyl-phosphatidylcholine) vesicles devoid of any erythrocyte channels or transporters. Again we were able to show that this happened in a non-lytic fashion, using calcein-containing vesicles as controls. These data show that both toxins incorporate into lipid vesicles and allow ATP to be released. We suggest that both toxins cause acute ATP release by letting ATP pass the toxin pores in both human erythrocytes and artificial membranes.

ATP is the main energy molecule for metabolic and enzymatic reactions for all cells. ATP, however, is also a well recognized signaling molecule for numerous physiological functions including regulation of secretion and absorption in epithelia,

pain perception, immune responses, such as host-pathogen interaction, interleukin release, chemotaxis, and control of the vascular diameter (1). Regulated, non-lytic release of ATP therefore occurs in many cell types. ATP can be released either via exocytosis of ATP-containing vesicles or via channels or transporters in the cell membrane (2–6). Human erythrocytes, like all other mammalian erythrocytes, lack vesicles, which indicate that release of ATP in these cells must be via channels or transporters in the plasma membrane. The first report of regulated ATP release (non-lytic) from erythrocytes showed release upon exposure to a reduced oxygen tension (7) and it was later shown that ATP led to dilation of the vasculature following P2 receptor stimulation of the endothelial cells (8). Subsequently, several studies have reported ATP release from erythrocytes after hypotonic stress (9), mechanical deformations (10), by β -adrenergic agonists (11, 12) and prostacyclin (PGI₂) analogs (13, 14).

Several ATP exit pathways have been suggested for non-lytic ATP release from erythrocytes including the cystic fibrosis transmembrane conductance regulator (CFTR)² (10, 15), pannexin 1 (9, 16), and recently, the voltage-dependent anion channel (VDAC) (14). Hence, hypotonic stress and low O₂ tension-induced ATP release from human erythrocytes were described to depend on pannexin 1 (9, 16). CFTR has been suggested to be necessary for ATP release upon mechanical deformation (10) as well as low O₂ and prostacyclin stimulation (16). It is, however, now recognized that CFTR is more likely to regulate other channels and not being directly involved in ATP release itself (17–21). In line with this, a recent study showed that CFTR is involved in ATP release induced by both low O₂ and prostacyclin stimulation but the exit pathway appeared to be pannexin 1 in the case of low O₂ tension (16) and VDAC for prostacyclin-mediated ATP release (14). Regarding β -adrenergic stimulation, neither pannexin 1 nor VDAC seem to mediate the ATP release (14) suggesting that yet another exit conduit may exist.

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² The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; POPC, 1-palmitoyl-2-oleoyl-phosphatidylcholine; HBS, HEPES-buffered saline; ATPe, extracellular ATP; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid.

A recent study did, however, show a correlation between β -adrenergic ATP release and pannexin 1 activation (22). Thus, ATP release from erythrocytes is well documented but the nature of the conduit pathway is not uniform and seems to depend on the initial stimulus.

We have shown previously that several pore-forming proteins, including bacterial cytolysins and pores formed after complement activation require extracellular ATP and P2 receptor activation to inflict full lysis of human erythrocytes (23–26). This implies that ATP must be present extracellularly in sufficient amounts after toxin insertion to activate P2 receptors in the erythrocyte membrane. The most likely explanation is that the cytolysins trigger ATP release from the erythrocytes, but it cannot be excluded that the constitutive release of ATP that occurs from most cells would be sufficient to support the hemolytic process. In the present study, we primarily wished to clarify whether ATP is actually released from erythrocytes via a non-lytic mechanism when exposed to bacterial cytolysins and, if so, to investigate the conduit pathway. To this end, we used artificial vesicles made of the phospholipid 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), pannexin 1 transgenic mice, antagonists against pannexin 1, and the VDAC. We focused on two bacterial toxins, HlyA from *Escherichia coli* and LtxA from *Aggregatibacter actinomycetemcomitans*, which we have previously shown to induce an ATP-dependent lysis of human erythrocytes. The results indicate that ATP is released through the toxin pore itself upon insertion into the plasma membrane. This release of ATP, as an early event in the hemolytic process, is likely to be the source for the following amplification that leads to cytolysis.

EXPERIMENTAL PROCEDURES

Chemicals and Solutions—Hepes-buffered saline (HBS) at 288 mosmol contained 132.0 mM Na⁺, 126.9 mM Cl⁻, 5.3 mM K⁺, 1.8 mM Ca²⁺, 0.8 mM Mg²⁺, 0.8 mM SO₄²⁻, 14.0 mM Hepes, and 5.6 mM glucose, pH 7.4, at 37 °C.

The antimicrobial peptide novicidin was a generous gift from Novozymes A/S (Denmark), *N,N*-dimethylformamide, Triton X-100, carbenoxolone, probenecid, sucrose, and calcein were from Sigma. TRO19622 was from Tocris Bioscience (UK), the ATP determining assay kit was from Invitrogen (Denmark), human hemoglobin ELISA kit from AH Diagnostics (Denmark), and POPC lipids from Avanti Polar Lipids (Alabaster, AL). Novicidin, carbenoxolone, and probenecid were dissolved in HBS. TRO19622 was dissolved in *N,N*-dimethylformamide to a stock concentration of 50 mM and stored at -20 °C until use.

Isolation of Erythrocytes—Blood was collected into 5-ml EDTA-containing tubes by venipuncture of healthy human volunteers after their written consent. The blood cells were washed three times in 0.9% NaCl (twice at 1162 × *g*, 3 min, 4 °C and once at 581 × *g*, 2 min, 4 °C) and the upper buffy coat containing the white blood cells was removed. The isolated erythrocytes were washed once in HBS (1162 × *g*, 3 min at 4 °C), diluted in HBS to a 2.5% (v/v) erythrocyte suspension (corresponding to ~125 × 10⁶ cells ml⁻¹), which was kept at 4 °C. A fresh blood sample was taken at the day of the experiment. The

sampling procedure of human blood was approved by the Danish Scientific Ethics Committee (M201100217).

Pannexin 1 Knock-out Mice—The pannexin 1 knock-out mouse was created from ES cell clone (EPD0309_3_B01) obtained from the the National Center for Research Resources-NIH (NCRR-NIH)-supported KOMP Repository and generated by the CSD consortium for the NIH funded Knock-out Mouse Project (KOMP, University of California). Pannexin 1 knock-out and wild type littermates were obtained by heterozygous breeding at the Department of Biomedicine, Aarhus University, according to Danish animal welfare regulations. Animals were anesthetized by isoflurane, decapitated, and blood was collected in EDTA-containing tubes and prepared as described above for human blood.

RTX Toxins—*E. coli* α -hemolysin (HlyA) was purified as described previously (27) (to a concentration ranging between 1.0 and 2.5 mg ml⁻¹). The hemolytic effect of the preparation can be ascribed to HlyA alone (26) and lipopolysaccharides (LPS) isolated from two *E. coli* stains (O55:B5 and O111:B4) either alone or in combination with extracellular ATP at increasing concentrations did not cause any detectable cell lysis by optical density measurements (data not shown). LPS-free leukotoxin A (LtxA) from *A. actinomycetemcomitans* was purified as previously described (28) to a stock concentration of 10–15 mg ml⁻¹.

For experiments on erythrocytes both toxins were diluted in HBS to produce either 10 or 50% hemolysis in a 1.25% erythrocyte suspension after 60 min at 37 °C at a swirl of 220 rpm. These concentrations are referred to as EC₁₀ or EC₅₀ in the following. For HlyA, EC₁₀ and EC₅₀ were 5 and 25 ng ml⁻¹, respectively. For LtxA, EC₁₀ and EC₅₀ were 2 and 10 μ g ml⁻¹, respectively.

For experiments on vesicles we used different concentrations for each toxin. For HlyA the concentrations were 5, 10, 15, 20, and 25 ng ml⁻¹. For LtxA the concentrations were 2, 5, 10, and 20 μ g ml⁻¹.

Vesicles—Large unilamellar (200 nm) POPC vesicles were packed with either ATP or calcein. The vesicles were prepared by re-suspending 10 mg of POPC in either calcein (50 mM in HBS) or ATP (50 mM in HBS). The final intracellular concentration was 338 mosM, as HBS had an osmolarity of 288 mM. The suspension was then subjected to 10 rounds of freezing in liquid N₂ and thawing in a water bath at room temperature before extrusion 10 times through 200-nm filters in a mini-extruder (Avanti Polar Lipids). Non-trapped ATP or calcein was removed by desalting the samples over a 10-ml gel filtration column (PD-10, GE Healthcare) and collecting the fraction eluting at 2.5–4 ml, which contained the largest concentration of ATP- or calcein-containing vesicles.

For both vesicle samples, HBS was used as the extravesicular solution. The extravesicular HBS was supplemented with 70 mM sucrose to reach a final extracellular concentration of 358 mosM. The extracellular milieu was made slightly hyperosmotic to decrease risk of osmotic swelling of the vesicles when they were subjected to the toxins. The vesicles were kept at 4 °C and used within 2 days. These preparations were diluted 20 times for ATPe and calcein fluorescence experiments.

ATP Release from Human Erythrocytes Induced by RTX Toxins

Extracellular ATP (ATPe) Measurements—ATPe was measured by an ATP-determination assay using firefly luciferase, which catalyzes the oxidation of luciferin in the presence of ATP and produces luminescence. The luminescence signal was recorded on a plate reader (Mithras LB 940, Berthold Technologies, Bad Wildbad, Germany). Two experimental procedures were used to measure ATPe, *i.e.* real-time and off-line.

Real-time luminescence was performed on vesicles loaded with ATP. The vesicles were diluted in HBS supplemented with 70 mM sucrose in 96-well plates. RTX toxins, vehicles, novocidin, or Triton X-100 were added at time 0 and ATP was measured every 5 min for 60 min.

Off-line luminescence was performed on human and murine erythrocytes. The suspensions of erythrocytes were incubated with RTX toxins in the presence or absence of antagonists (carbenoxolone, probenecid, or TRO19622) for 0, 5, and 10 min to a final erythrocyte concentration of 1.25% (containing $\sim 62.5 \times 10^6$ cells ml^{-1}). The suspension was then centrifuged ($1162 \times g$, 3 min at 4 °C) and the supernatant was divided into two fractions for either ATPe or hemoglobin measurements. For ATPe the supernatant was heated (100 °C, 30 s) to inhibit potential ecto-ATPase activity and placed on ice until measurements were conducted. For hemoglobin measurements the fraction of supernatant was placed on ice without boiling the sample. This protocol was chosen for erythrocytes instead of real-time measurements to be able to conduct free hemoglobin measurements in parallel on the supernatant.

ATPe levels for human and murine erythrocytes are expressed as nanomole of ATP/ 14×10^6 cells. For vesicles ATPe levels are normalized to the total ATP content of the vesicles after subjection to Triton X-100. All measurements were performed at room temperature and a standard curve based on known ATP concentrations was constructed.

Real-time luminescence was conducted on one series of experiments on human erythrocytes (Fig. 1). Here human erythrocytes were subjected to HlyA for 10 min. In this series of experiments the exact ATP levels are unknown as a standard curve was not run for each time points. Values are therefore given as luminescence values normalized to time 0.

Extracellular Hemoglobin Measurements—Free hemoglobin levels were measured to assure that ATP was released via a non-lytic mechanism. This was only performed on human erythrocytes and was done in parallel to ATPe measurements as described above. Human hemoglobin was measured by an immunoreactivity assay according to the manufacturer's guidelines (GenWay Biotech, San Diego, CA).

Calcein Release Measured by Fluorescence—All fluorescence measurements were conducted on a plate reader (Mithras LB 940). Release of calcein from the vesicles and the subsequent increase in fluorescence was monitored by excitation at 488 nm and recording emission at 515 nm every 10 min up to 60 min. The vesicles were diluted 20-fold in HBS, which lead to staying within the measuring range of the reader after adding 1% of Triton X-100. For each recording, RTX toxins, vehicles, novocidin, or Triton X-100 were injected and emission was followed for 60 min. The release of calcein was calculated according to Equation 1 (29),

$$\text{Dye leakage (\%)} = 100 \times (F - F_0)/(F_t - F_0) \quad (\text{Eq. 1})$$

where F is the fluorescence intensity achieved by RTX toxins or novocidin, and F_0 and F_t are fluorescence intensities without RTX toxins or novocidin and with the addition of Triton X-100, respectively.

Circular Dichroism Spectroscopy (CD)—A 10 mg ml^{-1} solution of POPC was titrated against HlyA and LtxA. The 200-nm lipid vesicles composed of POPC in HBS were prepared as described previously but without loading them with either ATP or calcein. Changes in the secondary structure of HlyA and LtxA in the presence of POPC were determined using a J810 spectropolarimeter (Jasco Spectroscopic Co., Hachioji, Japan) at 25 °C. Wavelength scans were recorded between 250 and 195 nm at 100 nm min^{-1} with a data pitch of 0.2 nm, a bandwidth of 2 nm, and an accumulation of 6 spectra using a 0.1-cm quartz cuvette (Hellma, Müllheim, Germany).

Fluorescence Spectroscopy—Fluorescence spectroscopy was done using a fluorescence spectrophotometer (Varian Cary, Agilent Technologies) exciting at 290 nm and recording the emission from 300 and 400 nm using a slit width of 2.5 nm for both of the monochromators, with the cuvette holder thermostated at 25 °C. HlyA or LtxA at 25 $\mu\text{g ml}^{-1}$ in HBS was titrated against POPC vesicles. The emission intensity was normalized according to dilution of the protein sample.

Hemolytic Assay—Erythrocytes were suspended (final concentration of 1.25%) in HBS. HlyA or LtxA were added in 96-well plates with or without antagonists (carbenoxolone, probenecid, or TRO19622) and placed in an incubation chamber for 60 min at 37 °C under a constant swirl of 220 rpm. After centrifugation ($1162 \times g$, 3 min at 4 °C), the absorbance at 540 nm of the supernatant was measured and related to a sample where complete hemolysis was induced by hypoosmotic shock. Absorbance was measured in a plate reader (PowerWave Microplate Spectrophotometer, Biotek Instruments, Winooski, VT).

Purification of Membrane Proteins from Murine Erythrocytes—Erythrocytes from pannexin 1 wild type and knock-out mice were isolated as described above and were washed repeatedly in 10 mM Tris-HCl containing protease inhibitor (Roche Applied Science) and then frozen (−20 °C). After thawing the samples were again washed in 10 mM Tris-HCl containing protease inhibitor before the protein concentration was measured. Protein determination was performed using Pierce® BCA™ Protein Assay Kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol.

SDS-PAGE and Immunoblotting—Protein samples of ~ 10 μg (corrected according to densitometry of the Coomassie Brilliant Blue) were loaded on two identical 7.5% Mini-PROTEAN® Precast gels (Bio-Rad) and electrophoresis was performed for 1–1.5 h at 100 V at room temperature. Precision Plus Protein™ WesternC™ Standards (Bio-Rad) was used as a marker for the protein size. One gel was used for Coomassie Brilliant Blue staining and the other gel was blotted onto ethanol (Osmonics Inc.)-activated PVDF-PLUS membranes (pore size 0.45 μm , Micron Separations Inc., Westborough, MA) at 4 °C for 1 h at 100 V. Blocking of the membrane was performed for 1 h at room temperature with 1% Blotting Grade Blocker

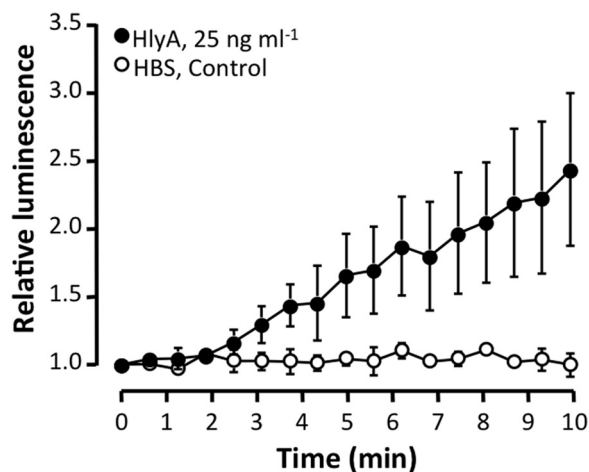


FIGURE 1. *E. coli* α -hemolysin induces ATP release from human erythrocytes. ATP release from human erythrocytes induced by HlyA from 0 to 10 min of incubation. ATP values are shown as luminescence normalized to time 0 in the presence of HlyA (black symbols) or vehicle (HBS, white symbols) ($n = 5$).

Nonfat dry milk (Bio-Rad) in 0.1 M PBS with 0.5% Tween 20 (PBS-T), before overnight incubation at 4 °C with primary antibody. Both the primary antibody (1:1,000 anti-pannexin 1 antibody (EPR5556, rabbit) Abcam®, Cambridge, UK) and the secondary antibodies (1:2,000 polyclonal goat anti-rabbit immunoglobulins/HRP-conjugated (DakoCytomation Denmark A/S, Glostrup, DK) for the primary antibody and 1:10,000 StrepTactin-HRP conjugate (Bio-Rad) for the ladder) were prepared in PBS-T. The membranes were incubated for 2 h at room temperature with the secondary antibodies before washing and subsequent addition of ECL reagents (5 min at room temperature, Bio-Rad) to obtain a chemiluminescent photo of the membrane.

Statistics—Data are presented as mean \pm S.E. The n -value indicates the number of animals or human individuals. The data were tested for normality by Kolmogorov-Smirnov test. Significant differences were determined by paired or unpaired Student's t test. A p value less than 0.05 was considered statistically significant.

RESULTS

RTX Toxins Induce ATP Release from Human Erythrocytes

Previously, we showed that hemolysis induced by the two RTX toxins HlyA from *E. coli* and LtxA from *A. actinomycetemcomitans* depends on activation of P2X receptor and pannexin 1 channels (24, 26). This was evident as various purinergic antagonists and pannexin 1 blockers as well as ATP degrading enzymes markedly inhibited hemolysis induced by both toxins. The most straightforward explanation is that ATP is released as an early event in the hemolytic process and amplifies the hemolytic process. As such, early ATP release has not been demonstrated. The extracellular ATP that triggers the P2 receptor activation could in principle also reflect the continuous, ongoing ATP release that can be detected in most living cells. Here, we investigate whether the toxins do cause a measurable ATP release to the extracellular milieu.

By the firefly luciferase assay, we found that HlyA induces ATP release from human erythrocytes within 2–3 min of incu-

bation. These data are presented in Fig. 1, which illustrates the ATP released from human erythrocytes subjected to HlyA (25 ng ml⁻¹) over time for 10 min. In principle, this ATP release could reflect release of the intracellular content of lysed erythrocytes. However, from our previous studies we know that HlyA does not lead to immediate lysis of the erythrocytes. Initially, HlyA causes striking erythrocyte shrinkage, which is maximal at about 20 min after exposure to HlyA at EC₅₀. This was documented both by time-lapse microscopy and flow cytometry (26, 30). In this study, we confirmed that the erythrocytes did not show any detectable lysis within the first 10 min of incubation. In parallel to the luminescence experiments, the erythrocytes were observed by time-lapse microscopy. Lysed erythrocytes are easily detected using differential interference contrast microscopy as formation of empty ghost membranes results in marked reduction of contrast. No lysed cells were detected within the time period (data not shown). Similar experiments were published previously (26, 30).

To substantiate non-lytic ATP release by RTX toxins, we chose parallel measurements of ATP by luminescence and an ELISA-based detection of hemoglobin release with a higher sensitivity than the regular optical density measurements of hemolysis. Both toxins induced a significant ATP release from human erythrocytes under circumstances where we could not detect hemoglobin release over baseline (Fig. 2, A–D).

Fig. 2C shows that addition of HlyA, and addition of LtxA at the EC₁₀ concentration, did not result in detectable hemoglobin release for up to 10 min. Addition of LtxA at the high concentration (EC₅₀), however, resulted in significantly increased free hemoglobin but only after 10 min of incubation (Fig. 2D), indicating that a limited level of hemolysis must have occurred.

We also measured the OD₅₄₀ of the supernatant from all experiments and found no significant change in optical density for either of the toxins (HlyA, 10 min, 0 versus 25 ng ml⁻¹: 0.0332 \pm 0.0017 versus 0.0337 \pm 0.0018, $n = 8$, and for LtxA, 10 min, 0 versus 10 μ g ml⁻¹: 0.0343 \pm 0.0018 versus 0.0345 \pm 0.0022, $n = 6$), which suggests that hemolysis was absent or below the limit of detection by OD₅₄₀ measurements.

Free Hemoglobin as Measure of Cell Lysis

To further substantiate the notion that RTX toxin can cause non-lytic ATP release from erythrocytes, we tried to calibrate the three assays, *i.e.* detecting hemoglobin by ELISA or OD₅₄₀ or detecting ATPe, in terms of cell lysis. Fig. 3 shows values for hemoglobin and ATPe for predetermined amounts of erythrocytes (measured by coulter counting) lysed with hypotonic stress. It appears that sensitivity of the hemoglobin ELISA was higher (easily detecting 62,500 lysed cells) than OD₅₄₀ measurements, which required 150,000 lysed cells (Fig. 3A). The data also show that if the HlyA-induced ATP release (Fig. 2A) had reflected cell lysis alone, one should have been able to detect the lysis with the ELISA-hemoglobin assay, contrary to our observation (Fig. 2C). However, one could potentially envision that the released ATP is a result of contamination of the erythrocyte preparation by other formed blood components. If they were more susceptible to the RTX toxins compared to erythrocytes, they would lyse before it is possible to detect an increase in extracellular hemoglobin. Only our microscopy

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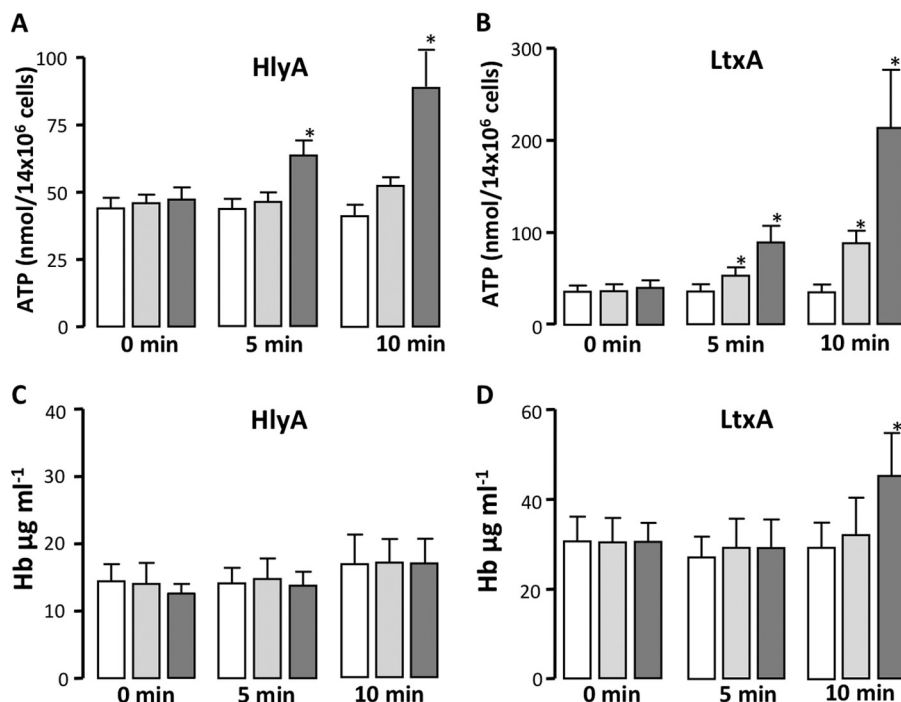


FIGURE 2. **ATP and hemoglobin release induced by HlyA and LtxA.** ATP release in human erythrocytes induced by HlyA (A) or LtxA (B) at 0 (white), EC₁₀ (light gray), and EC₅₀ (dark gray) after 0, 5, and 10 min of incubation ($n = 5-7$). Release of hemoglobin from human erythrocytes induced by HlyA (C) or LtxA (D) at 0 (white), EC₁₀ (light gray), and EC₅₀ (dark gray) after 0, 5, and 10 min of incubation ($n = 6-7$). *, denotes a significant difference from the control (white bar) within each time period.

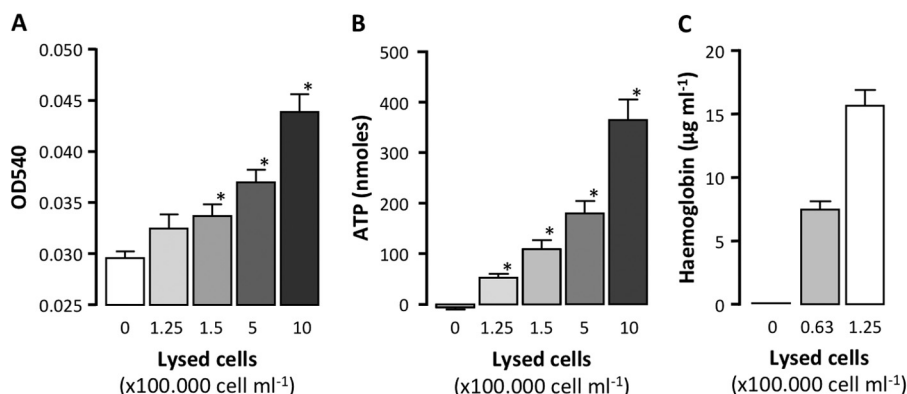


FIGURE 3. **Controls for ATPe and hemoglobin assays.** A, optical density at 540 nm measured in five samples of human erythrocytes lysed by hypotonic stress. The sample contained 0, 125,000, 250,000, 500,000, and 1,000,000 cells ml⁻¹ as counted by flow cytometry ($n = 13$). B, total ATPe levels in five samples of human erythrocytes lysed by hypotonic stress. The sample contained 0, 125,000, 250,000, 500,000, and 1,000,000 cells ($n = 8$). C, total hemoglobin levels in three samples of human erythrocytes lysed by hypotonic stress; containing 0, 62,500, or 125,000 cells ml⁻¹. The level of hemoglobin in a control sample without cells was $0.0029 \pm 0.00129 \mu\text{g ml}^{-1}$ ($n = 10$).

does not support the idea of substantial contamination. All of our preparations were submitted to microscopy and we never detected anything but erythrocytes in these preparations. Moreover, neither platelets nor leukocytes are known to be more susceptible to HlyA than erythrocytes (31, 32). Thus, we are convinced that the released ATP is extremely unlikely to result from cell lysis.

ATP Release Unaffected by Pannexin 1 Inhibition

The main intrinsic ATP exit pathway in erythrocytes has been suggested to be via pannexin 1 channels. Thus, we tested the effect of the pannexin 1 channel blocker carbenoxolone using a concentration (100 μM) known to completely block HlyA-induced hemolysis (26). We observed a minor effect of carbenoxolone on

ATPe levels after HlyA incubation (Fig. 4A) but no impact on ATP release induced by LtxA (Fig. 4B). As mentioned above, we have previously shown that carbenoxolone significantly decreases hemolysis induced by both toxins (24, 26). Here, we confirmed our previous findings (HlyA-induced hemolysis decreased from 51.2 ± 5.6 to $1.9 \pm 0.4\%$ in the presence of 100 μM carbenoxolone, $n = 5$; LtxA-induced hemolysis decreased from 47.4 ± 4.0 to $21.3 \pm 4.4\%$ in the presence of 100 μM carbenoxolone, $n = 7$). Because carbenoxolone completely inhibits lysis induced by HlyA, but only has a minor impact on ATP release supports the notion that ATP may be released also via non-lytic mechanisms.

Because of the insignificant effect of carbenoxolone on ATP release induced by HlyA we also tested probenecid, another

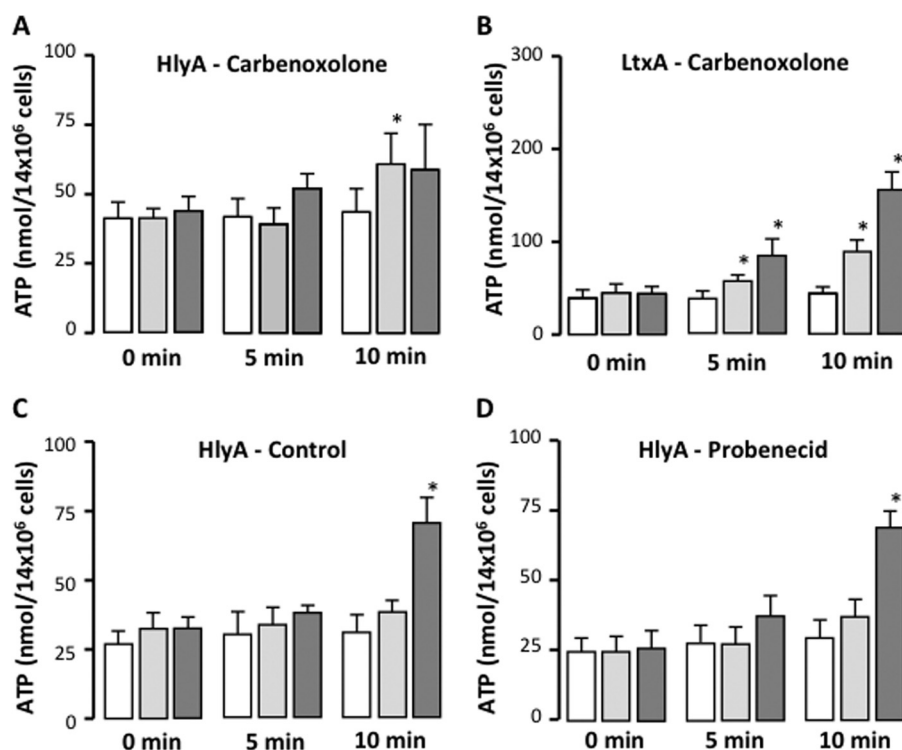


FIGURE 4. **ATP release induced by HlyA and LtxA is independent of pannexin 1 channel.** Top, ATP release in human erythrocytes induced by HlyA (A) or LtxA (B) in the presence of 100 μM carbenoxolone at 0 (white), EC₁₀ (light gray), and EC₅₀ (dark gray) after 0, 5, and 10 min of incubation ($n = 5-7$). The corresponding controls without carbenoxolone are shown in Fig. 2, A and B. Lower, ATP release in human erythrocytes induced by HlyA at 0 (white), EC₁₀ (light gray), and EC₅₀ (dark gray) after 0, 5, and 10 min of incubation during control (C) and in the presence of 1 mM probenecid (D) ($n = 5$).

pannexin 1 antagonist. Probenecid (1 mM) significantly reduced hemolysis from 46.8 ± 0.9 to $28.9 \pm 4.0\%$, $n = 5$, but had no effect on ATP release (Fig. 4, C and D). To further investigate a potential role of pannexin 1 in ATP release, erythrocytes from pannexin 1 knock-out and wild type mice were incubated with HlyA. Here we found no difference in ATP release (Fig. 5, A and B), despite a very apparent reduction of pannexin 1 expression in the erythrocyte membrane (Fig. 5C). LtxA was not tested on the pannexin 1-deficient mice as murine erythrocytes are insensitive to LtxA (33).

ATP Release Unaffected by VDAC Inhibition

Recent studies have found VDAC to be involved in prostacyclin receptor-mediated ATP release from human erythrocytes (14). To test whether VDAC was involved in ATP release in our system, we used the specific VDAC blocker TRO19622. Fig. 6 shows that TRO19622 (50 μM) did not significantly affect the ATP release induced by either HlyA or LtxA. Like in the case of pannexin channel inhibitors, we also tested for an effect of TRO19622 on hemolysis. We found that TRO19622 resulted in a significant decrease in HlyA-induced hemolysis (from 45.9 ± 6.4 to $28.4 \pm 5.1\%$, $n = 6$). Thus, because TRO19622 inhibits hemolysis but has a minor effect on ATP release, these data also suggest the occurrence of non-lytic ATP-release by HlyA. Our data do not support a role for VDAC in LtxA-induced ATP release or in LtxA-induced hemolysis as hemolysis was similar in the absence and presence of 50 μM TRO19622 (49.4 ± 4.4 and $48.1 \pm 4.2\%$ respectively, $n = 6$).

Validity of the Methods (Comparing ATPe Versus Human Hemoglobin Measurements)

Example 1—ATP release after HlyA at EC₅₀ after 10 min was 41.2 ± 3.8 to 88.7 ± 12.7 nM = 47.5 nM (data according to Fig. 2A). If all ATP came from hemolysis it would correspond to $\sim 125,000$ cells ml^{-1} according to Fig. 3B. This number of lysed cells would then correspond to ~ 15 $\mu\text{g ml}^{-1}$ hemoglobin (Fig. 3C). According to Fig. 2C there is no significant hemoglobin release after addition of HlyA EC₅₀ after 10 min.

Example 2—50 nM ATP release after LtxA addition corresponds to 10 min at EC₁₀ (Fig. 2B). Again this would correspond to 125,000 lysed cells ml^{-1} if all ATP came from lysis (Fig. 3B) and this would correspond to ~ 15 $\mu\text{g ml}^{-1}$ hemoglobin (Fig. 3C). In Fig. 2D LtxA releases ~ 15 $\mu\text{g ml}^{-1}$ hemoglobin after 10 min of EC₅₀, whereas no hemoglobin release was detected at EC₁₀. Therefore, hemolysis must have occurred at EC₅₀ but not at EC₁₀. However, the ATP release was above 150 nM (Fig. 2B) meaning that far from all ATP came from cell lysis after 10 min EC₅₀.

ATP-loaded Lipid Vesicles Release ATP in a Non-lytic Way in Response to Both RTX Toxins

So far, the experiments have not supported that HlyA and LtxA inflict ATP release through one of the described cellular pathways. Therefore, we speculated that ATP may simply exit through the toxin pore. To test this we made POPC vesicles containing either ATP or calcein. The calcein-containing vesicles were included to control for the possibility of ATP being detected extracellularly because of vesicle lysis. Calcein is a

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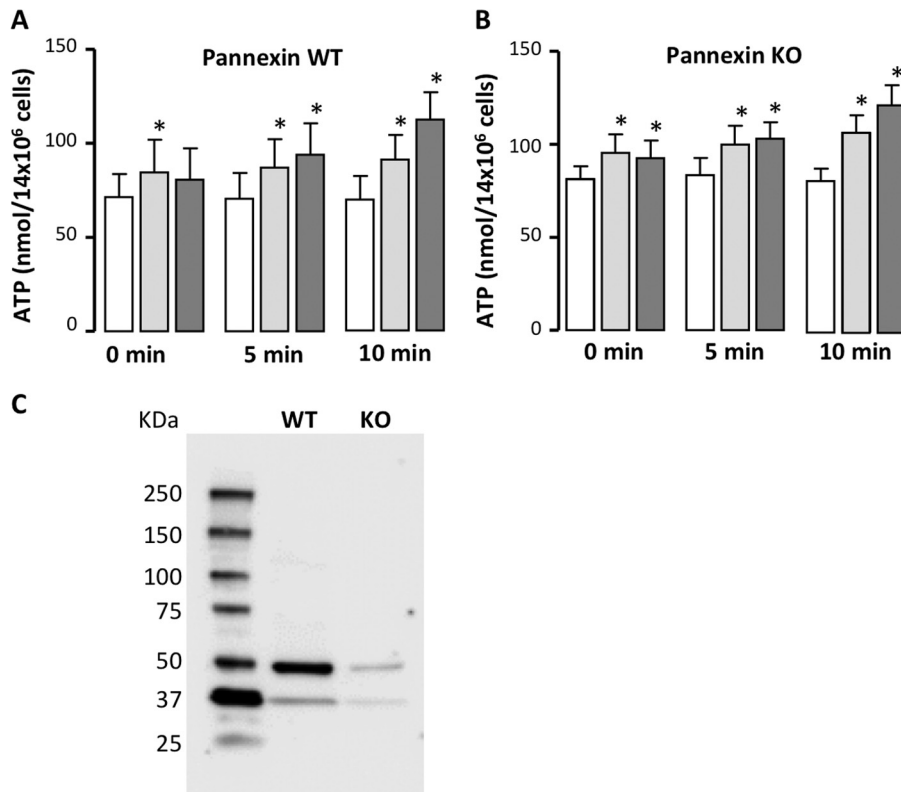


FIGURE 5. **HlyA-induced ATP release is similar in erythrocytes from pannexin 1 knock-out and wild type.** ATP release in pannexin 1 wild type (A) and knock-out murine erythrocytes (B) induced by HlyA at 0 (white bar), EC₁₀ (light gray bar), and EC₅₀ (dark gray bar) after 0, 5 and 10 min of incubation. *n* = 9 for each genotype. *, denotes a significant difference from the control (white bar) within each time period. C, representative immunoblotting for pannexin 1. Erythrocyte membrane proteins from wild type (WT, left) and pannexin1 knock-out (KO, right) mice. The expected size of pannexin 1 is 48 kDa. The result is repeated in 2 wild type and 5 knock-out mice.

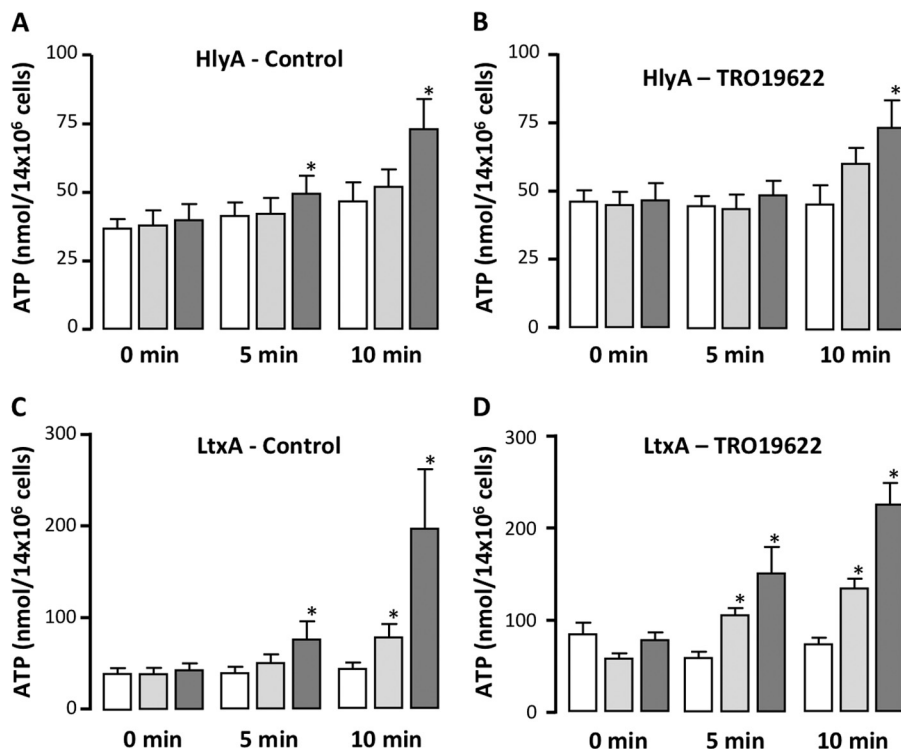


FIGURE 6. **ATP release induced by HlyA and LtxA is independent of VDAC.** ATP release in human erythrocytes induced by HlyA (top) or LtxA (bottom) at 0 (white), EC₁₀ (light gray), and EC₅₀ (dark gray) after 0, 5, and 10 min of incubation. ATP values are shown during control (left) and in the presence of TRO19622 (50 μ M, right) (*n* = 6–7). *, denotes a significant difference from the control (white bar) within each time period.

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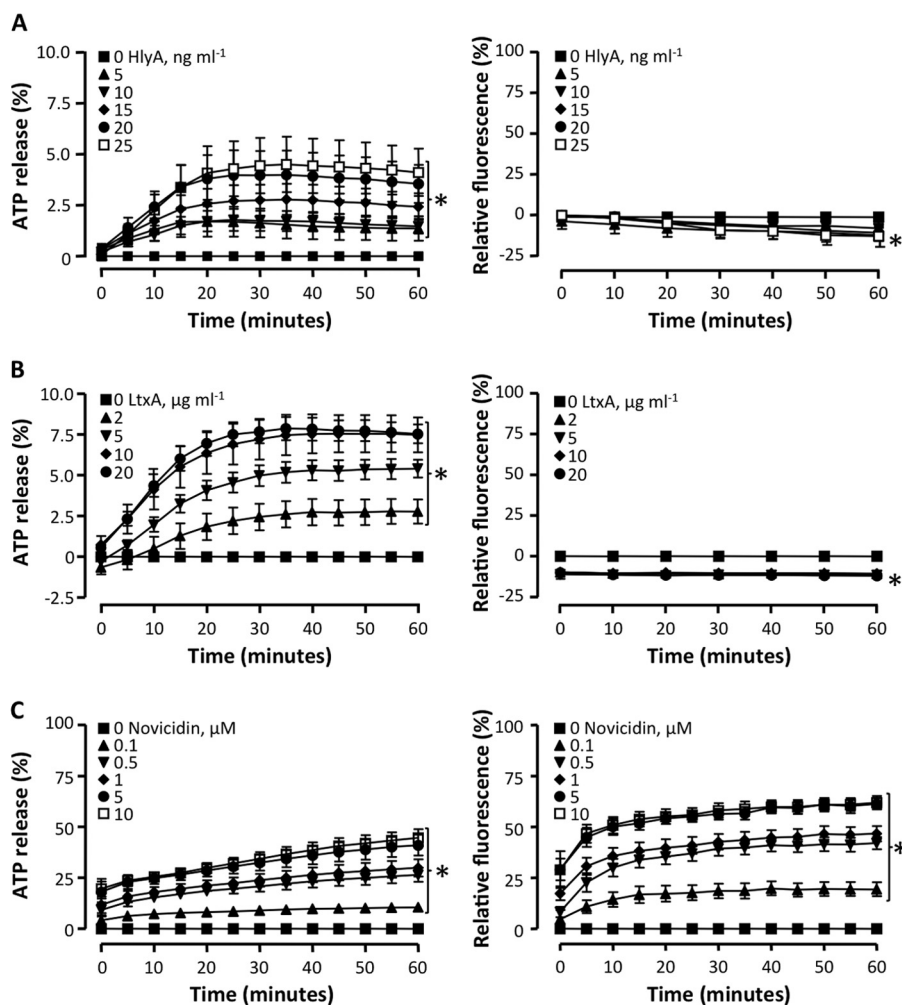


FIGURE 7. Release of ATP and calcein from artificial vesicles induced by HlyA, LtxA, and novicidin. Artificial vesicles (POPC) loaded with either 50 mM ATP (left panels) or 50 mM calcein (right panels) were subjected to HlyA (A), LtxA (B), or the peptide novicidin (C) at increasing concentrations and ATP and calcein fluorescence were measured every 5–10 min up to 60 min. All values are normalized to the total ATP or calcein content induced by Triton X-100, $n = 8–10$ (A and B) and $n = 6$ (C). *, denotes a statistical difference from the control curve (black squares).

fluorescent probe that is self-quenching at high concentrations. If the vesicles are lysed, the calcein will be diluted, giving rise to an increase in sample fluorescence (34).

Adding HlyA or LtxA at increasing concentrations showed concentration-dependent ATP release from the vesicles with both toxins (Fig. 7, A and B, left panels). Please note that the rate of ATP release increase with the concentration of both RTX toxins. With time, the reaction reaches a steady state between ATP release from the vesicles and ATP degradation from the extravesicular luciferin/luciferase assay. Neither of the toxins resulted in calcein release from the vesicles. On the contrary, calcein fluorescence decreased (Fig. 7, A and B, right panels). This reduction in fluorescence was more prominent at higher concentrations of toxins, and is likely to reflect further quenching of the intravesicular calcein resulting from vesicular shrinkage. As mentioned previously the extravesicular solution is hyperosmotic. When the toxins are inserted into the vesicle membrane it becomes more water permeable, water will leave the vesicle through the pores and the vesicle will shrink. To verify our method, we used novicidin, an antimicrobial peptide known to destroy the vesicle membrane by forming transient pores and leakage from the vesicle, and at higher concentra-

tions the membrane completely disintegrates (35). When novicidin was added at 5 concentrations (0.1–10 μM) to the vesicles, we found a clear and simultaneous ATP and calcein release (Fig. 7C). Both luminescence (ATP) and fluorescence (calcein) were measured every 5 min up to 60 min. Here it can be noted that the reaction does not reach a steady state but that the ATP keeps on increasing as more and more vesicle lyse. These data support the ability of the method to distinguish conductive ATP release from vesicle lysis. When comparing the amount of ATP release induced by novicidin at 0.1 μM to that induced by the highest concentrations of toxins, the amount of ATP release is comparable. However, novicidin caused a clear calcein release, whereas no calcein release was detected for either HlyA or LtxA. Thus, it seems quite clear that both toxins are able to induce ATP release from these artificial membranes in a non-lytic fashion and we hypothesize that ATP exits via a pore induced by the toxins that excludes calcein release.

Discrete Structural Changes of HlyA and LtxA upon Binding to POPC Vesicles—We investigated the structural changes of the two toxins when binding to POPC vesicles by circular dichroism (CD) and fluorescence spectroscopy. The CD signal for LtxA in solution indicates a typical α -helical signature, with two

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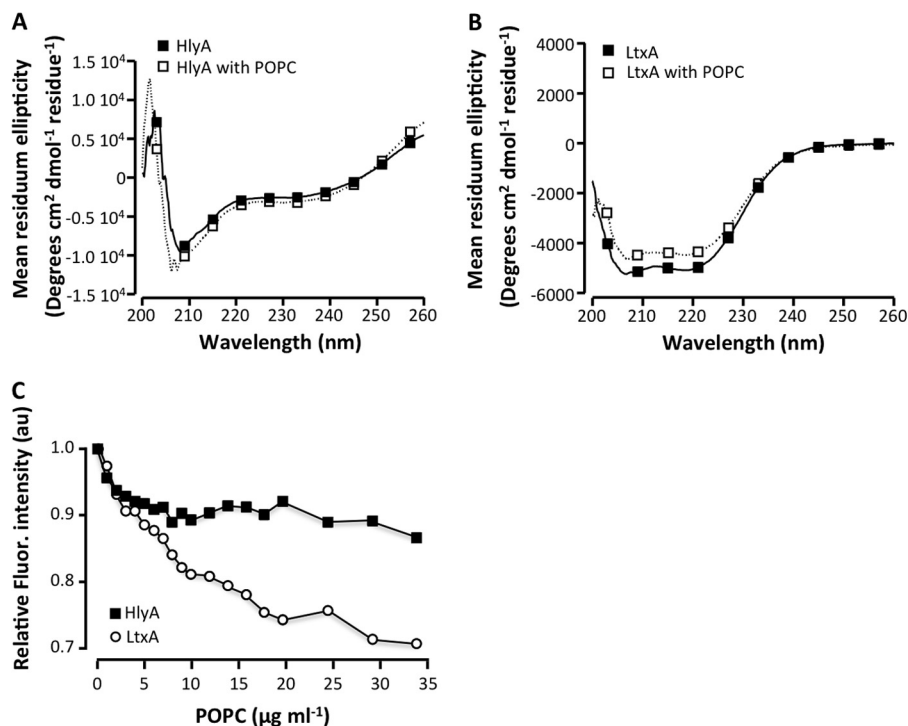


FIGURE 8. **Discrete structural changes of HlyA and LtxA upon binding to POPC vesicles.** Circular dichroism spectroscopy of HlyA (A) and LtxA (B) in the presence of POPC vesicles. Wavelength scans were recorded between 250 and 195 nm at 100 nm min⁻¹. Fluorescence spectroscopy for HlyA and LtxA (C) was done exciting at 290 nm and recording the emission from 300–400 nm. 0.025 mg ml⁻¹ HlyA or LtxA was dissolved in HBS, and titrated with POPC vesicles. The emission intensity was normalized according to dilution of the protein sample. All experiments were performed at 25 °C.

local minima at 208 and 222 nm (Fig. 8A). In a recent study of LtxA in diphenylcarbonyl chloride membranes (36), there were no strong indications of an increase in α -helix contents for LtxA upon lipid addition. Similarly, we observe very little structural change for LtxA in POPC vesicles. The CD signature for HlyA seems to indicate that this protein is more unstructured but again we do not observe any changes upon the addition of POPC (Fig. 8B).

Tryptophan emission spectra were recorded during titration with POPC vesicles. These spectra provide information about conformational changes in the vicinity of the tryptophan residues of HlyA and LtxA. Tryptophan fluorescence is due to four tryptophan residues in HlyA (Trp-431, -480, -579, -914) and five tryptophan residues in LtxA (Trp-116, -430, -478, -577, -901). For both proteins we observe a decrease in fluorescence intensity (Fig. 8C) and a very slight red shift when adding POPC vesicles indicating that the tryptophan residues become more solvent exposed when the toxins bind POPC vesicles. These results are in agreement with previously published results for LtxA and its structure in the presence of zwitterionic vesicles (phosphatidylcholine) (36). Regarding HlyA, however, Ostolaza and Goñi (37) reported a rise in tryptophan fluorescence in the presence of lipids vesicles (phosphatidylcholine).

DISCUSSION

In previous studies, we have shown that hemolysis inflicted by several pore-forming proteins such as bacterial cytolysins and the membrane attack complex rely on purinergic receptor activation (23–26). This was shown with a battery of P2 receptor antagonists and by scavenging ATP in the extracellular solution. Either of these perturbations caused significant reduction

of the cytolysin-induced hemolysis. In that light, it is reasonable to assume ATP to be released to the extracellular milieu during the process of hemolysis. The primary objective of the present study was therefore to identify whether the two RTX toxins, α -hemolysin (HlyA) from *E. coli* and leukotoxin A (LtxA) from *A. actinomycetemcomitans*, trigger ATP release from human erythrocytes early in the hemolytic process and, if so, to investigate the ATP release pathway.

We found that both toxins resulted in a significant ATP release within 10 min of incubation and that the ATP release could be detected in the apparent absence of cell lysis. Our previous pharmacological data strongly suggest that the pannexin 1 channel in erythrocytes is implicated in the hemolytic process by most pore formers. Therefore, it was remarkable that we could not find any support for pannexin 1 as an ATP release channel in response to either of the toxins. Although the pannexin channel blockers carbenoxolone and probenecid inhibited toxin-induced hemolysis, neither had an effect on toxin-induced ATP release. Accordingly, we observed similar HlyA-induced ATP release from erythrocytes isolated from pannexin 1 knock-out and wild type mice. The pannexin 1-deficient mice were only tested for HlyA, as murine erythrocytes are insensitive to LtxA (33).

The VDAC, suggested to be an ATP release channel in murine epithelial cells (5), was recently proposed to be involved in prostacyclin-mediated ATP release from human erythrocytes (14). Therefore we tested the effect of the substance TRO19622, which has been shown to inhibit VDAC. However, TRO19622 did not affect ATP release induced by either of the toxins. To examine the possible significance of other suggested

ATP release channels, *i.e.* the maxi-anion channels (2, 38–40) and CFTR (41, 42), we attempted to use DIDS as a non-selective anion channel blocker. However, this drug was incompatible with the ATP determining assay (data not shown). Regarding CFTR, this channel was initially suggested as an important ATP release channel. According to recent literature, however, it seems that CFTR in itself is not the ATP release channel but, instead, this transporter is involved in regulatory mechanisms leading to ATP release through other exit pathways (17–21). Our previous data has, however, left limited, if any, room for CFTR in the HlyA-induced hemolysis as we found similar HlyA-induced lysis in erythrocytes isolated from CFTR wild type and knock-out mice (26).

Because none of the described ATP release pathways seems to be involved in the early toxin-induced ATP release, we speculated that ATP may be released through the toxin pore itself. When considering this option, one has to acknowledge that true pore formation has been questioned for both toxins, in particular LtxA (36, 43). Nevertheless, it is well established that both HlyA and LtxA are membrane-damaging toxins (44–49) but conclusive details regarding their membrane interaction are unknown for both proteins as structural models are currently lacking. Several studies have suggested RTX toxins to damage cells by creating transmembrane pores in host cell membranes. For HlyA, this was suggested when the toxin was presented to an artificial planar bilayer composed of phosphatidylcholine. Here, a large conductance was detected using patch clamp (45, 50), and experiments using osmotic protectants predicted the pore size to be ~ 3 nm based on the protection against hemolysis by inulin (44). Soloaga *et al.* (51), however, presented evidence that HlyA does not permeate the membrane bilayer, but only inserts into the outer bilayer without creating a transmembrane pore. For LtxA there is also no conclusive evidence for pore formation. Whereas numerous studies have shown binding of HlyA to artificial membranes (37, 52–55) it is debated whether LtxA inserts into artificial bilayer membranes, unless a specific receptor is present. LtxA specifically targets human $\beta 2$ integrins, which are expressed particularly on monocytes, macrophages, and neutrophils and causes cell death often preceded by a cytokine response (56–58). However, LtxA is also able to lyse erythrocytes (24, 59), which do not express $\beta 2$ integrin (28, 60). This means that $\beta 2$ integrin is not absolutely required for membrane insertion of LtxA and therefore cannot be an indispensable receptor for LtxA-mediated effects. In this sense, a recent study showed that cholesterol significantly increases the chance of LtxA binding, and suggests that either $\beta 2$ integrin or cholesterol need to be present (61). Lear *et al.* (62) showed that LtxA does not insert into artificial membranes made of 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) or 1-palmitoyl-2-oleoyl-phosphatidylserine (POPS) and channel/pore activity was observed only if the lipid bilayer was broken and reformed in the presence of LtxA. This difference was explained by the lack of the $\beta 2$ integrin receptor in the artificial membranes. The ability of LtxA to form pores is supported only by a study on HL-60 promyelocytic leukemia cells. The conclusion was based on an osmotic protection against lysis by maltose and the pore was estimated to be ~ 0.96 nm corresponding to the diameter of this saccha-

ride (63). Patch clamp studies on HL-60 cells supported this by showing a large conductance and collapse of the membrane potential when LtxA was present (64). Recent data on T lymphocytes and artificial membranes argues against direct pore formation and suggest that the cytotoxicity of LtxA originates from membrane destabilization without the formation of transmembrane pores (36, 43). The latter studies showed membrane interaction with artificial membranes, and the conclusion from these studies was that LtxA binds via two steps: (i) membrane association and (ii) membrane destabilization, which occurs through a modification of the bilayer structure demonstrated by leakage of dye (calcein or 8-aminonaphthalene-1,3,6-trisulfonic acid/*p*-xylene-bis(*N*-pyridium bromide) (ANTS/DPX)). The authors suggest that these two events are independent as: 1) was seen with PC membranes and 2) was seen with phosphatidylethanol membranes. Because membrane destabilization leads to cell lysis, the destabilization must increase the membrane permeability for ions and water. In a cell physiological context this phenomenon can be considered to reflect formation of at least a transient pore or channel in the membrane.

In the present study, we used POPC membranes and found no evidence of calcein leakage for up to 60 min in the presence of up to $40 \mu\text{g ml}^{-1}$ LtxA, which according to Walters *et al.* (36) and Brown *et al.* (43) indicates that the membrane is still intact. At this concentration of toxin, we could, however, detect marked ATP release from the vesicles. Thus, the membrane must in this situation have an increased permeability for ATP irrespective of whether LTX forms a transmembrane pore. This could be taken as a circumstantial argument for pore formation because this is an easily detectable ATP release without any detectable calcein dequencing as a measure of vesicular lysis. The method of calcein dequencing is primarily designed to detect vesicular lysis and cannot completely exclude a small degree of calcein leakage from the vesicles. We have, however, performed numerous experiments on erythrocytes incubated with calcein for volume change measurements. We did not detect any calcein leakage from the cells after addition of either HlyA or LtxA before lysis occurred. Thus, we saw an initial shrinkage of the cells followed by a gradual swelling in the presence of both toxins (24, 30), and not until after the cell membrane had burst was it possible to detect calcein leakage. Calcein and ATP are both negatively charged and comparable in size, with calcein having a molecular mass of 622 Da and ATP of 508 Da. Nevertheless, our findings suggest an apparent preferentiality for letting ATP pass.

We have demonstrated that the two RTX toxins HlyA and LtxA inflict substantial ATP release as an early event in the hemolytic process. This ATP release cannot be ascribed to any known pathway for ATP release in the erythrocyte, which implies that ATP is released through the toxin pore itself. This notion was substantiated by studies on artificial lipid vesicles, which showed that membrane insertion of the bacterial toxins inflicted a substantial non-lytic ATP release. This leads us to propose that membrane insertion of bacterial toxins is closely associated with ATP release to the extracellular surface.

These findings may have significant implications for the general biological effects of bacterial pore formers. It implies that no matter what cell type is attacked by the toxin, ATP release

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will automatically follow. Because P2 receptors are widely distributed in mammalian cells this would entail that the purinergic signaling system will be activated in tissues exposed to cytolysin producing bacteria. This could potentially be relevant for the pathogenesis of *E. coli*-induced sepsis. It is well documented that ATP and importantly its degradation product ADP are potent activators of thrombocytes (65). Thus, HlyA could potentiate thrombocyte aggregation and pro-coagulation, which is a prerequisite for the microthrombosis that constitutes a hallmark of sepsis (66, 67) and worsen the overall prognosis (68).

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