# α-Hemolysin from *Escherichia coli* uses endogenous amplification through P2X receptor activation to induce hemolysis

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***Escherichia coli*** **is the dominant facultative bacterium in the normal intestinal flora.** ***E. coli*** **is, however, also responsible for the majority of serious extraintestinal infections. There are distinct serotypical differences between facultative and invasive** ***E. coli*** **strains. Invasive strains frequently produce virulence factors such as α-hemolysin (HlyA), which causes hemolysis by forming pores in the erythrocyte membrane. The present study reveals that this pore formation triggers purinergic receptor activation to mediate the full hemolytic action. Non-selective ATP-receptor (P2) antagonists (PPADS, suramin) and ATP scavengers (apyrase, hexokinase) concentration dependently inhibited HlyA-induced lysis of equine, murine, and human erythrocytes. The pattern of responsiveness to more selective P2-antagonists implies that both $P2X_1$ and $P2X_7$ receptors are involved in HlyA-induced hemolysis in all three species. In addition, our results also propose a role for the pore protein pannexin1 in HlyA-induced hemolysis, as non-selective inhibitors of this channel significantly reduced hemolysis in the three species. In conclusion, activation of P2X receptors and possibly also pannexins augment hemolysis induced by the bacterial toxin, HlyA. These findings potentially have clinical perspectives as P2 antagonists may ameliorate symptoms during sepsis with hemolytic bacteria.**

alpha-hemolysin | *E. coli* | erythrocytes | hemolysis | P2X

The dominant facultative intestinal bacterium *Escherichia coli* (*E. coli*) frequently induces serious extraintestinal infections as neonatal meningitis, peritonitis, Gram-negative bacteriemia, and urinary infections including pyelonephritis (1, 2). There are, however, distinct serotypical differences between the facultative *E. coli* and the ones that invade the tissue and cause infection. The invasive *E. coli* strains frequently produce virulence factors such as the exotoxin α-hemolysin (HlyA) (1, 3). The frequency by which hemolytic *E. coli* strains can be isolated from patient samples increases with the severity of disease (1).

HlyA is a 107 kDa (4) protein that induces hemolysis by creating ≈2-nm-wide pores in the erythrocyte membrane. These pores are thought to increase the permeability and thereby produce cell swelling, which finally ruptures the erythrocyte. Thus, increasing the osmolality of the extracellular solution with cell-impermeate sugars inhibits the HlyA-induced hemolysis completely (5). If HlyA-induced hemolysis is merely a consequence of inserting non-selective pores into the plasma membrane of red blood cells, it is puzzling that the sensitivity to HlyA varies among species (6). This feature is not unique to HlyA, as the sensitivity to other pore-formers such as α-toxin from *Staphylococcus aureus* also shows great interspecies variability (7). Regarding *S. aureus*, the interspecies variation was explained through differences in expression levels of a specific receptor for α-toxin (8). This option has also been suggested for HlyA-induced hemolysis (9) but is not yet generally accepted (10).

In the present study, we investigate the possibility that HlyA requires P2-receptor activation to produce hemolysis. P2X receptors are ligand-gated cation channels activated by extracellular ATP. To date, seven subtypes of P2X receptors have been identified and are referred to as $P2X_{1-7}$. All P2X receptors are permeable to small monovalent cations and some have significant calcium permeability (11). Here we show that human, murine, and equine erythrocytes use a combination of $P2X_1$ and $P2X_7$ receptor activation for full HlyA-induced hemolysis to occur. This is particularly interesting, as prolonged stimulation of $P2X_7$ receptors are known to increase the plasma membrane permeability to an extent that eventually leads to lysis of certain cells (12). In macrophages it has been shown that pannexin1, a recently discovered pore-forming protein, is required for this increment in permeability (12, 13). Our data also support a role for pannexin channels in addition to P2X channels in HlyA-induced hemolysis.

## Results

**HlyA-Induced Hemolysis Requires Activation of Purinergic Receptors.** Supernatant from the α-hemolysin (HlyA)–producing *E. coli*–strain ARD6 lyses equine, human, and murine erythrocytes (Fig. 1). Figure 1 shows the HlyA-induced hemolysis as a function of time. Time-lapse experiments with murine and human erythrocytes attached to coverslips revealed that HlyA-induced hemolysis is a sequential process. Within the first 20 minutes, HlyA induced crenation of the red blood cells as a result of cell shrinkage, followed by a gradual volume increase and finally lysis of the cells (Figs. 1*A* and 1*B*, Movie S1). This sequential shrinkage and swelling also applies at the single-cell level. Thus, it is not different populations of red blood cells that either shrink or swell but, rather, that a single erythrocyte first shrinks and then swells as a consequence of HlyA application. The erythrocyte suspension (1.25%) was incubated with dilute *E. coli* supernatant (50 $\mu l \cdot ml^{-1}$). Erythrocytes from the three tested species showed marked difference in the responsiveness to HlyA (Fig. 1*C*) with the lowest sensitivity to HlyA in human erythrocytes. In all of the following experiments, the amount of added *E. coli* supernatant was adjusted to produce ≈50% hemolysis after 60 minutes' incubation.

We generally use filtered *E. coli* (ARD6) supernatant to induce hemolysis unless otherwise stated. This approach was chosen to ensure that our results would also apply *in vivo* where HlyA is released from *E. coli* together with various other components. When choosing this approach, we did, however, have to verify that the hemolysis induced by HlyA-producing *E. coli* could in fact be ascribed to HlyA. Therefore, we purified HlyA from our ARD6-culture. After purification, a suspension of the purified HlyA was separated on a 5–15% sodium dodecyl sulfate (SDS) gel. A single 100-kDa band appeared after Coomassie R staining, and mass

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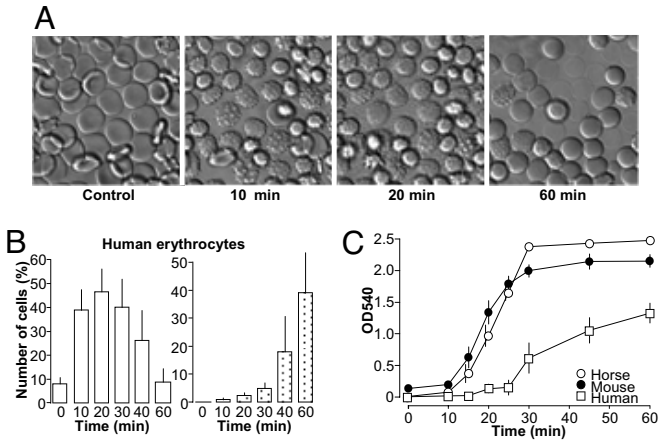


**Fig. 1.** $\alpha$-Hemolysin–induced hemolysis in equine, murine and human erythrocytes. (*A*) Effect of $\alpha$-hemolysin containing *E. coli* (ARD6, serotype OK:K13:H1) supernatant on human erythrocytes attached to a coverslip after 10, 20, and 60 minutes' incubation at 37 °C (see also Movie S1). (*B*) Summarized data. The total amount of crenated erythrocytes (open columns) and lysed erythrocytes (dotted columns) over time analyzed in image sequences collected over 60 minutes, at 0.1 Hz ($n$ = 8 human). (*C*) The overall hemolysis is shown as an increase in optical density at 540 nm (OD540) reflecting the hemoglobin concentration in the solution. The erythrocytes were incubated with *E. coli* supernatant (50 $\mu$l·ml$^{-1}$) from 0 to 60 minutes. $n$ = 5, 7, and 6 for equine, murine, and human, respectively.

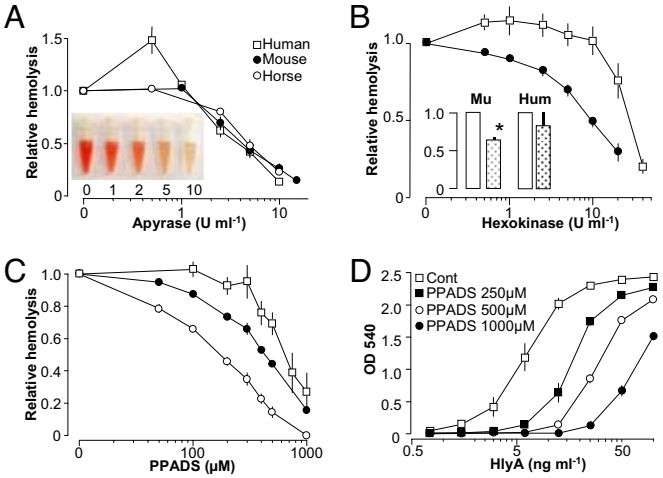


**Fig. 2.** HlyA-induced hemolysis of erythrocytes is inhibited by ectoATPases and purinergic antagonist. *E. coli* supernatant (60 minutes) induces hemolysis of human (square), murine (filled circles), and equine (open circles) erythrocytes. (*A*) Concentration–response curves for the ATP scavenger apyrase. *Inset* shows a representative picture of supernatant from murine erythrocytes subjected to HlyA in the presence of 0, 1, 2, 5 or 10 U ml$^{-1}$ apyrase. (*B*) Effect of hexokinase on the HlyA-induced lysis of human, murine, and equine erythrocytes; *inset* shows the effect of hexokinase (10 U ml$^{-1}$) on hemolysis induced by purified HlyA in murine and human erythrocytes). (*C*) Effect of the non-selective P2 receptor antagonist PPADS on HlyA-induced lysis of erythrocytes from all three species. (*D*) Concentration–response relationship of PPADS at various concentration of purified HlyA in human erythrocytes. Hemolysis was measured as OD540. Values are mean ± SEM; $n$ = 5–13.

spectroscopy identified the band as HlyA (Fig. S1 *A* and *B*). As an additional control we used the supernatant from the *E. coli* strain D2103, a non-pathological laboratory strain of *E. coli* that does not produce HlyA. The supernatant from these bacteria did not induce hemolysis in human, murine, or equine erythrocytes (Fig. S1*D*). Furthermore, we compared our findings to HlyA kindly supplied by Prof. Sucharit Bhakdi, University of Mainz, Germany (with the activity of 10 ng·ml$^{-1}$ for ≈50% hemolysis, Fig. S2). In the following, when purified HlyA is mentioned, it is with reference to this preparation. During our initial tests of the biological activity of HlyA, we discovered that the ATP-scavenger apyrase completely inhibited HlyA-induced hemolysis of equine erythrocytes. This finding was truly surprising, as it implied extracellular ATP necessary for the hemolysis inflicted by HlyA-producing *E. coli*. As extracellular ATP is a signaling molecule that activates P2 receptors, our findings could suggest that the prevailing pore model for HlyA-induced hemolysis might be a simplification. Therefore, we tested the effect of ATP scavenging more thoroughly. We found that apyrase completely inhibited hemolysis of not only equine but also murine and human erythrocytes (Fig. 2*A*). In addition, hexokinase, which rapidly degrades adenosine triphosphate (ATP) to adenosine diphosphate (ADP), similarly reduced the HlyA-induced hemolysis in red blood cells of murine and human origins in a concentration-dependent manner (Fig. 2*B*). This finding was verified by purified HlyA (Fig. 2*B*, *inset*). It is worth noticing that, in human erythrocytes, both apyrase and hexokinase potentiated the HlyA-induced hemolysis at lower concentrations. The distinction might suggest a difference in P2 receptor expression pattern on the red blood cells between the species.

To validate the relevance of this finding, it was important to learn whether P2 receptor antagonists influenced the HlyA-induced hemolysis. The non-selective P2 receptor antagonist PPADS concentration-dependently decreased hemolysis induced by HlyA-producing *E. coli* in equine, murine, and human erythrocytes (Fig. 2*C*). The $EC_{50}$ value for PPADS was 520 $\mu$M, 400 $\mu$M, and 180 $\mu$M for human, murine, and equine erythrocytes, respectively. This finding was substantiated for the whole range of HlyA concentrations (Fig. 2*D*) tested in human erythrocytes exposed to purified HlyA. The concentration–response relationship was compatible with competitive antagonism, and it should be noted that the effect of even maximal toxin concentrations was reduced by the P2 receptor blocker. Thus, P2 receptor activation seems to be involved in HlyA-induced hemolysis. The non-selective P2 receptor antagonist suramin also concentration-dependently decreased HlyA-induced hemolysis in all three species (data not shown). In higher concentrations suramin does, however, cause dramatic erythrocyte shrinkage, and thus may not be suitable for evaluating P2 receptor implication in erythrocytes.

To evaluate whether the effect of the purinergic antagonist on hemolysis was merely a result of increased osmolality, we tested the effect of extracellular sucrose on the HlyA-induced hemolysis (data not shown). Sucrose (1 mM) only slightly decreased hemolysis (5.1% ± 1.7%), whereas 10 mM and 75 mM sucrose markedly decreased hemolysis (28.5% ± 5.0%, 82.8% ± 5.2%). Given that the concentrations of the antagonists and ATPases used in this study never exceeded 1 mM, the effect cannot be the result of increased osmolarity. Neither did our results reflect unselective binding between the antagonists and the toxin. This was tested in equine erythrocytes, which were preincubated with HlyA for 10–15 minutes at 37 °C or for 30 minutes at 4 °C, thoroughly washed, and re-suspended with or without the antagonists. Because HlyA is incorporated into the membrane during the preincubation, the erythrocytes proceeded to lysis in the absence of free HlyA. Fig. S2 shows that various pharmacological interventions reduced hemolysis after HlyA was prebound to the erythrocytes. The antagonists were, however, less efficient when added to washed erythrocytes in which the lytic process was already initiated.

**Which P2 Receptor(s) Is Involved in HlyA-Induced Hemolysis?** Erythrocytes express various types of P2 receptors. The P2 receptors that have been reported to be expressed in mature human erythrocytes include $P2Y_1$ (14), $P2Y_2$ (15), $P2Y_{13}$ (15), $P2X_1$ (15), and $P2X_7$ (16), whereas $P2Y_1$, $P2X_1$, $P2X_4$, and $P2X_7$ appear to be present in

erythroid progenitor cells (17). To test which of these purinergic receptors participates in the HlyA-induced hemolysis, we addressed the receptors in question individually. As the $P2Y_1$ receptor is implicated in sorbitol-induced hemolysis of plasmodium-infected human and murine erythrocytes (14), we tested whether this receptor was responsible for the HlyA-induced hemolysis. The $P2Y_1$ receptor antagonist MRS2179 did not affect the HlyA-induced hemolysis (Fig. S3*A*) at concentrations (up to 500 $\mu$M) beyond what was needed to inhibit hemolysis in *Plasmodium berghei*–infected erythrocytes (14). As there are no specific antagonists for $P2Y_2$ receptors, we examined the effect of HlyA in transgenic mice. The HlyA-induced hemolysis was similar in erythrocytes from $P2Y_2^{-/-}$ and $P2Y_2^{+/+}$ mice (Fig. S3*B*). In the case of the $P2Y_{13}$ we tested the antagonist MRS2211, which has been reported to display some selectivity toward the $P2Y_{13}$ receptor (18). MRS2211 decreased HlyA-induced hemolysis significantly in human and murine erythrocytes (Fig. S3*C*). This finding contradicts our results with hexokinase (degrading ATP to ADP), which should stimulate rather than inhibit the ADP-sensitive $P2Y_{13}$ receptor. Therefore, hexokinase and MRS2211 should give opposing results if the $P2Y_{13}$ receptor is involved. As this is not the case, the $P2Y_{13}$ receptor is an unlikely candidate for the P2 receptor involved in HlyA-induced hemolysis. We cannot exclude the possibility that the inhibition produced by MRS2211 is mediated through another P2 receptor.

In principle, this leaves only the P2X receptors to be considered. Fig. 3*A* shows that the non-selective blocker of P2X receptors Evans blue potently reduced the HlyA-induced hemolysis, suggesting that a P2X-receptor is involved in this hemolysis. Of the P2X-receptors expressed in erythrocytes, we regarded the $P2X_7$ as the most likely mediator of HlyA-induced hemolysis for the following reasons. The $P2X_7$ receptors are known to undergo a transition to a greater permeability state, which eventually leads to lysis in certain cells (12). The $P2X_7$ receptor has been reported to interact with the channel protein pannexin1 (12), and the complex creates a sizeable pore permeable to larger molecules such as ethidium bromide (13). Pannexin1 is expressed in human red blood cells (19) and has recently been suggested as the ATP release channel in erythrocytes (20). To test whether $P2X_7$ receptors participate in HlyA-induced hemolysis, we used antagonists with relative selectivity for $P2X_7$: Brilliant Blue G (BBG), ATP-2′,3′-dialdehyde (OxATP), and KN-62 (21). All antagonists concentration-dependently decreased hemolysis in equine, murine, and human erythrocytes (Fig. 3). Equine and human erythrocytes were more sensitive to all of the tested substances compared with murine erythrocytes. In this context, it should be mentioned that the murine $P2X_7$ receptor is known to be less sensitive to KN-62 compared with the human receptor (22). The protection against hemolysis by P2X receptor antagonism was again substantiated for the whole concentration range of purified HlyA in human erythrocytes using BBG as an example of a $P2X_7$ antagonist (Fig. 3*D*). Again the antagonist shows a substantial effect on HlyA-induced hemolysis even under HlyA concentrations that produced maximal hemolysis. The inhibition of hemolysis by OxATP was verified using purified HlyA in murine and human erythrocytes (Fig. 3*E*, *inset*). The novel selective, competitive $P2X_7$ receptor antagonist A438079 reduced the hemolysis in human erythrocytes, but was less efficient in murine erythrocytes (Fig. 3*F*). Immunoblots of plasma membrane-fractions for the $P2X_7$ receptor confirm that human and murine erythrocytes express a protein of relevant size (66 kDa, Fig. 3*G*, and in full in Fig. S4*B*). It will require further investigation to fully establish the relative contribution of P2X receptors in the HlyA-induced hemolysis. With our current tools, we cannot exclude the possibility of contributions from other P2X receptors in the HlyA-induced hemolysis in any of the species studied.

Fig. S4*A* shows the HlyA-induced hemolysis in murine ($P2X_7^{+/+}$ and $P2X_7^{-/-}$) erythrocytes. The murine erythrocytes show a similar degree of hemolysis in response to HlyA irrespective of their

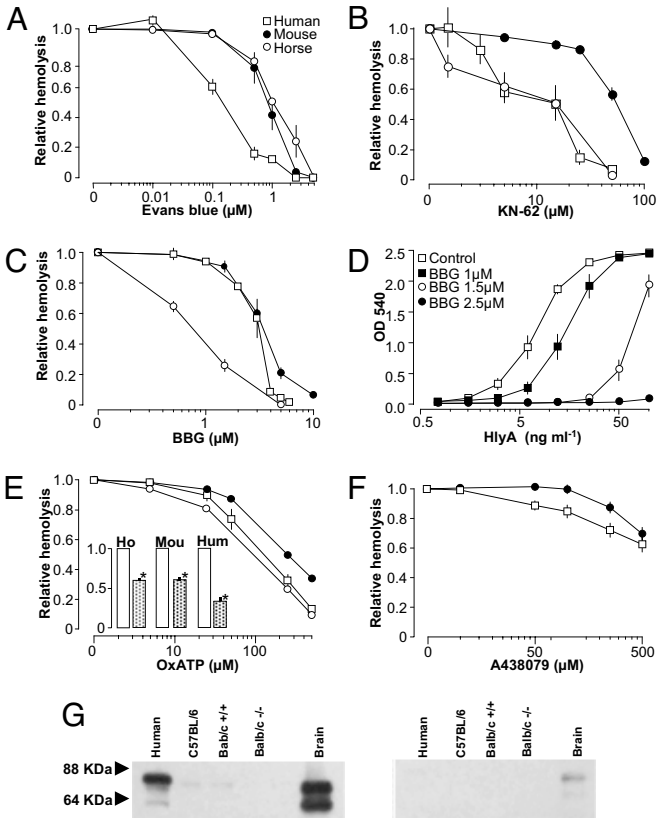


**Fig. 3.** HlyA-induced hemolysis is inhibited by $P2X_7$ receptor antagonists. HlyA-induced hemolysis in human (squares), mouse (filled circles), and horse (open circles). Hemolysis induced by HlyA-producing *E. coli* was reduced by increasing concentrations of (*A*) Evans Blue, (*B*) KN-62, and (*C*) Brilliant Blue G (BBG). (*D*) Concentration-dependent effect of BBG at various concentrations of purified HlyA. ATP-2′,3′-dialdehyde (OxATP) (*E*) likewise reduced the hemolysis induced by HlyA-producing *E. coli* and by the purified toxin (*inset*, OxATP, 500 $\mu$M). (*F*) The selective $P2X_7$ antagonist A438079 showed an effect mainly on human erythrocytes. Values are mean ± SEM, $n$ = 5–13. (*G*) Immunoblots with a C-terminal antibody directed against $P2X_7$ receptor (dilution 1:200). *Left panel* show a similar blot with peptide preadsorption.

genotype. The $P2X_7^{-/-}$ mice and $P2X_7^{+/+}$ mice were originally generated by Pfizer and were backcrossed into BALB/c background. We did not detect any discrepancies between the sensitivity to HlyA-induced hemolysis in erythrocytes isolated from BALB/c and C57BL/6 mice (data not shown), even though the C57BL/6-strain is known to have a genetic variation in the C terminus of the $P2X_7$ receptor (23). These data are consistent with the miniscule effect of A438079 on murine erythrocytes and the low protein expression of the $P2X_7$ receptor in murine erythrocytes (Figs. 3*F* and 3*G*).

These results imply that there is at least one additional P2 receptor involved in the HlyA-induced hemolysis in murine erythrocytes. As the $P2X_1$ and $P2X_7$ share similar inhibitor profiles for BBG, KN-62 and OxATP (24), we tested the $P2X_1$ antagonists MRS2159 and NF449. MRS2159 concentration-dependently inhibited hemolysis in erythrocytes from horse ($EC_{50}$:150 $\mu$M) and mouse ($EC_{50}$: ≈250 $\mu$M). Human erythrocytes were relatively insensitive to the antagonist, but at a concentration above 250 $\mu$M, we did see a small and statistically significant reduction (Fig. 4*A*). This effect was much more pronounced if purified HlyA was used (Fig. 4*C*). This implies that there might be differences in the cellular response in respect to whether they are subjected HlyA in a pure form or in combination with other *E. coli* constituents. NF449 concentration-dependently inhibits the HlyA-induced hemolysis in

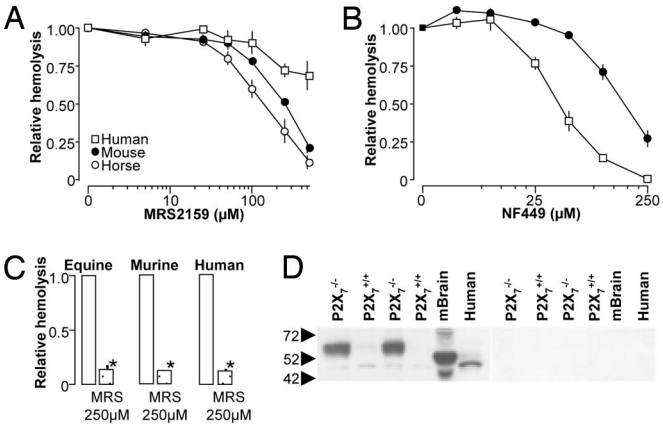


**Fig. 4.** Effect of the $P2X_1$ antagonists (MRS2159 and NF449) on HlyA-induced hemolysis in equine, murine, and human erythrocytes. (*A*) Erythrocytes were incubated with HlyA-containing *E. coli* supernatant and increasing concentrations of MRS2159 (mean ± SEM, $n = 7–8$). (*B*) Erythrocytes were incubated with purified HlyA and increasing concentrations of NF449 (mean ± SEM, $n = 5–6$). (*C*) Effect of 250 $\mu$M MRS2159 in hemolysis induced by purified HlyA. (*D*) Immunoblotting with an antibody directed against $P2X_1$ receptor (diluted 1:200); *right panel* shows a parallel blot with peptide preadsorption. Protein isolation and immunoblotting were repeated three times, with similar results.

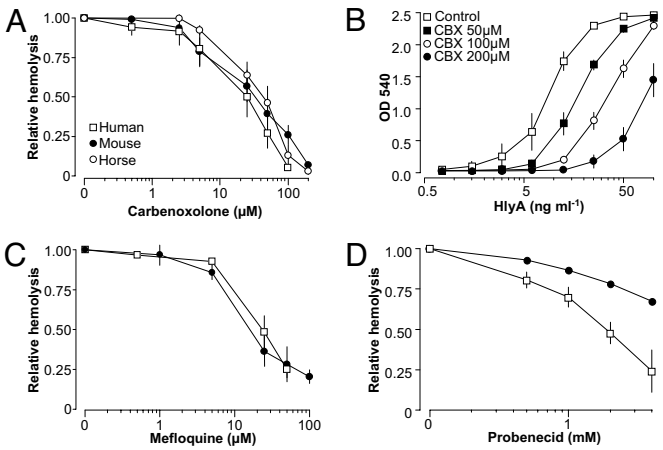


**Fig. 5.** HlyA-induced hemolysis of human, murine, and equine erythrocytes is inhibited by pannexin1 antagonists. Hemolysis was induced by HlyA-containing supernatant from *E. coli*. The hemolysis was concentration-dependently decreased by carbenoxolone (*A*), which also reduced the hemolysis induced by purified HlyA over a wide range of concentrations (*B*). Hemolysis induced by HlyA-producing *E. coli* was also reduced by mefloquine (*C*) and probenecid (*D*). Values are given as mean ± SEM; $n = 5–13$.

human (Fig. 4*B*). NF449 was much less efficient in murine erythrocytes in agreement with the murine $P2X_1$ receptor being relative resistant to this inhibitor (25). It should be emphasized that even though NF449 is a suramin derivative it did not provoke the same volume changes in erythrocytes as suramin. Immunoblots of the $P2X_1$ receptor are known to show up to 4 bands in various tissues; a 45 kDa non-glycosylated, a 60 kDa glycosylated and a 95/120 kDa band that might be the polymerized form of the receptor (26, 27). In our hands the $P2X_1$ receptor antibody consistently recognized a 45 KD band and a very weak 60 kDa band in blots of plasma membranes from murine and human erythrocytes (Fig. 4*D*). Interestingly, we found that the expression level 60 kDa band was much higher in the $P2X_7^{-/-}$ mice as compared to controls (similar in three preparations, Fig. 4*D*). In this immunoblot the protein levels are adjusted to avoid overloading of the bands from the $P2X_7^{-/-}$ mice, which leaves the 60 kDa band almost undetectable in the $P2X_7^{+/+}$ mice. This apparent up-regulation of the $P2X_1$ receptor might potentially conceal a hemolytic phenotype in the $P2X_7$ receptor–deficient mice. Taken together, these data support the hypothesis that both the $P2X_1$ and $P2X_7$ receptor are relevant for the HlyA-induced hemolysis. Our results point to significant interspecies variations, in which the $P2X_7$ receptor is more important for hemolysis in human erythrocytes.

**HlyA-Induced Hemolysis Is Prevented by Pannexin1 Antagonists.** Carbenoxolone (28), mefloquine and probenecid (30) have been used as antagonists with relative selectivity for pannexin1. Carbenoxolone significantly decreased the level of hemolysis in all three species with similar sensitivity (Fig. 5*A*). The effect of carbenoxolone was again tested for the whole range of HlyA concentrations (purified toxin, Fig. 5*B*), also showing sizeable effects under maximal HlyA concentrations. Mefloquine and probenecide were tested only in murine and humane erythrocytes. The $EC_{50}$ for mefloquine was 25 $\mu$M in human and 18 $\mu$M in murine erythrocytes. Probenecid inhibited hemolysis in human erythrocytes, with an $EC_{50}$ of 2 mM, but was less effective in murine erythocytes. Recently, the known $Cl^-$-channel antagonists NPPB and niflumic acid have been shown to inhibit pannexin channels as well (31). Both substances reduced the HlyA-induced hemolysis, with a substantially more pronounced effect on human erythrocytes (Fig. S5).

## Discussion

$\alpha$-Hemolysin from *E. coli* (HlyA) is known to lyse cells by forming pores in the plasma membrane (1, 5, 6). HlyA is able to permebilize both biological membranes and artificial lipid bilayers by inserting itself in an apparently receptor-independent manner (32). To our surprise, we discovered that this pore-formation triggers P2X receptor and pannexin channel activation in red blood cells of equine, murine, and human origins. This means that the HlyA-pores takes advantage of a specific cellular amplification system to inflict the full hemolytic response.

**Purinergic Antagonists and EctoATPases.** This conclusion is based on the striking blocking effect on HlyA-induced hemolysis imposed by various inhibitors of purinergic signaling. The ATP scavenging enzymes apyrase and hexokinase continuously degrade extracellular ATP, which might otherwise stimulate P2 receptors. These enzymes almost abolished the HlyA-induced hemolysis in all three species that we tested, suggesting that ATP is being released in sufficient amounts to amplify the hemolytic process. We substantiated this finding by testing P2 receptor antagonists that were structurally different from apyrase and hexokinase. PPADS, a non-selective purinergic antagonist, like apyrase, entirely eliminates the HlyA-induced hemolysis in all three species. Taken together, these data imply a central role for P2 receptors in HlyA-induced hemolysis. In this context it is important to underscore that suramin (another non-selective P2 antagonist) has previously been shown to reduce both the lethal (33) and hemolytic actions (34) of $\alpha$-toxin from *S. aureus* in mice and to inhibit hemolysis induced by complement activation in red blood cells from sheep (35). We found that suramin did inhibit HlyA-induced hemolysis. Unfortunately, suramin inflicted fairly significant shape changes (crenation) in the erythrocytes, and therefore we are not confident that the effect of suramin on hemolysis results from P2 receptor inhibition.

This new idea of P2 receptor–dependent amplification of HlyA-induced hemolysis is supported by the obvious sequential events the process. Within the first 20 minutes, HlyA produced intense crenation of the red blood cells, which was likely a result of volume reduction. This was followed by a gradual swelling that eventually ruptured the cells. The crenation and swelling did not occur in different population of red blood cells but could be observed within a single red blood cell.

**Which P2 Receptors Mediate the HlyA-Induced Hemolysis?** HlyA-induced hemolysis is caused by swelling and rupture of the erythrocytes, which very likely result from formation of pores in the plasma membrane. Our results imply that HlyA triggers P2 receptor activation, which eventually leads to membrane rupture. The $P2X_7$ receptor was the most likely P2-receptor candidate in HlyA-induced hemolysis. The rationale is that the $P2X_7$ has been shown to dilate during continual ATP stimulation, creating a pore with very high conductivity (36, 37), a feature ascribed either to intrinsic characteristics of P2X receptors (38, 39) or to interaction with pannexin1 (12, 13).

This finding led us to test a series of antagonists with known selectivity toward the $P2X_7$ receptor. We were able to show that BBG, KN-62, and OxATP all drastically reduced the HlyA-induced hemolysis in all species. $P2X_7$ receptors have previously been shown to be expressed in human (11), rat (40), and canine erythrocytes (16, 41), and we confirmed $P2X_7$ receptor expression by immunoblotting in both human and murine erythrocytes.

In human erythrocytes, the $P2X_7$ receptor seems to be the main receptor involved in HlyA-induced hemolysis. However, as the new selective $P2X_7$ receptor antagonist A438079 only partially reduced the HlyA-induced hemolysis, and as $P2X_1$ receptor antagonists inhibited lysis of human erythrocytes in higher concentrations, we cannot exclude a contribution of $P2X_1$ receptor subtype in the response. Similarly, we are not able to exclude heteromers of P2X receptors, including $P2X_1/P2X_4$ (42) and $P2X_4/P2X_7$ (43).

The HlyA-induced hemolysis in murine erythrocytes was found to be less sensitive to $P2X_7$ receptor antagonists. This means that $P2X_7$ receptors play a lesser role in HlyA-induced hemolysis in murine erythrocytes. The HlyA-induced lysis of murine erythrocytes was, however, very sensitive to the $P2X_1$ receptor antagonist MRS2159. The other $P2X_1$ inhibitor, NF449, reduced hemolysis of murine erythrocytes only in high concentrations, consistent with the murine $P2X_1$ receptor being resistant to this suramin analogue (25). These data implicate the $P2X_1$ and $P2X_7$ receptors as the functionally relevant P2 receptors for HlyA-induced hemolysis in mice. This finding might very well be relevant for other pore-forming toxins belonging to the RTX-family (44).

**The Role of ATP in Hemolysis.** Regardless of the P2 receptor involvement in HlyA-induced hemolysis, ATP alone, even in high concentrations (1 mM, 24 hours), does not by itself induce hemolysis in any of the three species tested here (data not shown). Sluyter *et al.* reported a similar resistance to ATP in human erythrocytes (1 mM, 24 hours), whereas ATP exposure in canine erythrocytes led to a significant degree of hemolysis (41). This resistance to ATP in murine, equine, and human red blood cells is quite surprising in light of our current data. This means that ATP is required, but not sufficient, to induce hemolysis in most species. One possibility is that the hemolysis is a combination of at least two events, in which HlyA primes the red blood cells to become sensitive to ATP. Resent data imply that ATP itself inhibits pannexins (31, 45). Thus an alternative explanation is that ATP in high concentration prevents itself from being pro-hemolytic. Further studies are obviously needed to pinpoint the exact signal transduction pathway for the HlyA-induced hemolysis.

**HlyA Sensitivity.** A consequence of the above-mentioned finding is that the action of HlyA is likely to be determined by the P2 receptor expression pattern of the given tissue. This could explain the interspecies variation in the sensitivity to HlyA. As one example, our study confirms the previous finding that human erythrocytes are more resistant to HlyA than murine (5). One possible explanation is that the sensitivity to HlyA relates to the size of the erythrocytes. It has previously been shown that the osmotic resistance of erythrocytes is a function of their size, with smaller cells being more susceptible to hemolysis (46). As equine and murine erythrocytes are about half the size of human erythrocytes, we cannot exclude the possibility that the difference in HlyA-sensitivity is related to cell size. It should be emphasized that our results show slight variation with regard to whether hemolysis was induced by *E. coli* supernatant or purified toxin. There is, however, no specific pattern; some antagonist are more effective in supernatant-induced hemolysis (OxATP) and some when the purified toxin is used (BBG and MRS2159). The main point is that purinergic receptor inhibition is likely to protect against the cell damage inflicted by hemolytic *E. coli*.

**Interaction Between P2X and Pannexins.** Our data suggest that pannexin channels are involved in HlyA-induced hemolysis. Prolonged stimulation of $P2X_7$ primes the receptor to undergo a transition to a second permeability state (11–13). It has been suggested that interaction with the pore-protein pannexin1 is responsible for the observed pore enlargement (12, 13), which in turn can lead to cell lysis. In the present study three nonselective antagonists of pannexin channels, carbenoxolone (13), mefloquine (47), and probenecid (30), markedly decreased hemolysis in both murine and human red cells. Carbenoxolone and mefloquine also inhibit connexins; but this cross-reactivity has little significance, as they are unlikely to be expressed in red blood cells. In this regard it is worth mentioning that Brilliant Blue G and A438079 recently has been shown to reduced the conductance of *Xenopus* oocytes injected with pannexin 1 alone (45). The two $Cl^-$ channel blockers, NPPB and niflumic acid, were recently shown to reduce pannexin1 currents (niflumic acid to a lesser degree). Both substances also inhibited lysis of human erythrocytes but had little effect on murine erythrocytes. As various P2X receptor subtypes are involved in the HlyA-induced hemolysis, it is unlikely that $P2X_7$-pannexin1 complex is an absolute requirement for the hemolysis. Our results favor the conclusion that any P2X receptor would be able to trigger the suggested pannexin activation, possibly via a rise of intracellular $Ca^{2+}$ concentration. One can speculate that the toxin lyses only cells expressing P2X receptors and pannexins. In this context, it is noteworthy that $\alpha$-toxin (48) and HlyA (49) induce interleukin $\beta$1 (IL-1$\beta$) release from human monocytes, a process that, in human macrophages, is known to result from $P2X_7$ and pannexin activation (50). This could suggest that cells that express $P2X_7$ and pannexins respond to HlyA through activation of these proteins.

**Clinical Perspectives.** The question remains as to whether these findings are relevant in a clinical setting. Gram-negative sepsis is usually not associated with massive intravascular hemolysis. Sepsis is, however, in general followed by a reduced amount of red blood cells, partly as a result of hemolysis and partly because of eryptosis ("apoptosis" in erythrocytes) (51). It is known that pore-forming toxins activate human platelets (52), and that platelet ADP receptors contribute to the initiation of intravascular coagulation (29). Pore-forming bacterial toxins in the blood are likely to release ATP, which might in the end trigger thrombocyte aggregation. Thus, the cellular effects of hemolysins might add to the total clinical picture of sepsis. As both the purified toxin and the bacterial supernatant are antagonized with P2X and pannexin blockers, it is possible that inhibition of P2X receptors and pannexin channels also ameliorate cytotoxic effects of $\alpha$-hemolytic *E. coli in vivo*. Further studies are, however, needed to determine whether these speculations have any validity.

## Methods

**Preparations of Erythrocytes.** Equine blood was purchased from Statens Serum Institut (Copenhagen, Denmark). Human blood was collected from seven healthy volunteers. The experiments were approved by the Danish National Committee on Biomedical Research Ethics. Murine blood was obtained from C57BL/6 and

BALB/c mice after cervical dislocation. The $P2X_7$ *knock out* mice ($P2X_7^{-/-}$) mice were bred in house according to the Danish law on research animal use (see *SI Methods*).

**Preparation of Bacteria and Purification of *E. coli* HlyA.** HlyA was purified according to the method described by Bhakdi *et al.* (5). Hemolysis was measured spectrophotometrically in erythrocyte supernatant (Ultraspec III, LKB Biochrom, Cambridge, UK) as optical density at 540 nm (see SI Text).

**Immunoblotting.** Isolated plasma membrane proteins from human and murine erythrocytes were separated and blotted by standard procedures (primary antibodies, Alomone, Jerusalem, Israel and secondary peroxidase-conjugated antibody; DAKO, Glostrup, Denmark). Preadsoption controls were included for all antibodies with 1:1 peptide-antibody ratio (see also *SI Methods*).

**Solutions, Materials, Data Analysis, and Statistics.** Data are presented as mean ± SEM. The *n* value indicates number of trials for each drug. For the experiments on $P2X_7^{-/-}$ vs. $P2X_7^{+/+}$ mice erythrocytes, *n* equals number of animals. (For statistics, please see *SI Text*).

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