Supporting Information

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Materials and Methods

Preparations of Erythrocytes. Blood samples were collected in ethylenediaminetetraacetic acid (EDTA)–containing tubes, washed three times (1000 × *g*, 5 minutes, 4 °C) in Ca^{2+}-free saline solution, and the upper "white coat" layer removed. Defibrinated equine blood was purchased from Statens Serum Institut (Copenhagen, Denmark). Human blood was collected from seven healthy volunteers. Murine blood was obtained from C57BL/6 and BALB/c mice of either sex after the mice were killed by cervical dislocation. The experiments were approved by the Danish National Committee on Biomedical Research Ethics.

Mice. The $P2X_7$ *knock out* mice ($P2X_7^{-/-}$) mice on BALB/c background were compared with wild-type ($P2X_7^{+/+}$) littermates obtained from heterozogyte breeding. The $P2X_7^{-/-}$ mice were viable, with no apparent abnormalities. They were bred in-house according to the Danish law on research animal use. The $P2X_7^{-/-}$ mice were originally developed by Pfizer (New York, NY) and backcrossed into a BALB/c background.

Preparation of Bacteria and Toxins. The human uropathogenic *Escherichia coli* (*E. coli*) strain ARD6 (serotype OK:K13:H1, Statens Serum Institut), was streaked and grown on Luria-Bertani (LB) agar plates over night at 37 °C. One colony was transferred to 4 ml LB medium (Sigma, Schnelldorf, Germany) and cultured overnight to the late exponential growth phase (measured as the optical density at 600 nm), where production of α-hemolysin (HlyA) is maximal (≈14 hours, 37 °C; data not shown). The overnight culture was centrifuged at 11,500 *g* for 10 minutes at 4 °C; the supernatant was sterile filtered (filter pore size, 0.2 μm; Sartorius Biotech, Göttingen, Germany) and maintained at 4 °C until use. Fresh HlyA culture was prepared each day. The *E. coli* strain D2103 (Statens Serum Institut) was used as control for the HlyA-producing strain ARD6. Purified *Staphylococcus aureus* α-toxin was purchased from Sigma.

Purification of *E. coli* HlyA. HlyA was partially purified according to the method described by Bhakdi *et al.* (5). Briefly, 1 ml of *E. coli* (ARD6) culture was transferred to 250 ml prewarmed LB medium (10 mmol/l $CaCl_2$, 37 °C) under a constant swirl of 250 rpm. The supernatant was harvested in the exponential growth phase as described above. Afterward, glycerol was added (final amount of 3%, vol/vol), supplemented with 50 g polyethylene glycol (PEG4000, Sigma) and incubated 1 hour at 4 °C. The suspension was centrifuged at 35,000 *g* for 20 minutes at 4 °C, and the pellet (containing HlyA) was dissolved in 2 ml Ca^{2+} containing saline and sterile filtered. The sample was transferred to Amicon Ultra centrifugation devices to elute proteins less than 50 kDa or 100 kDa (Millipore, Billerica, MA). The hemolytic activity of every preparation (see below) was measured immediately after the purification. The sample was kept at −20 °C until use. A single protein band appeared after Coomassie R staining of an SDS electrophoretic gel, and HlyA content of this band was confirmed by MALDI-TOF mass spectroscopy (Reflex IV; Bruker, Billerica, MA). Purified toxin with high efficiency was generously provided by Dr. Sucharit Bhakdi (University of Mainz) as a gift.

Measurements of Hemolytic Activity. The red blood cells were suspended (2.5%) in Ca^{2+}-containing salt solution. The hemolytic activity was measured spectrophotometrically (Ultraspec III, LKB Biochrom, Cambridge, UK) at 540 nm. The red blood cell suspension was mixed 1:1 with diluted *E. coli* supernatant (50 $\mu l \cdot ml^{-1}$) with or without agonists or antagonists and incubated up to 120 minutes at 37 °C under constant swirl (250 rpm). The tubes were then centrifuged at 1000 *g* for 5 minutes at 4 °C, and the OD_{540} of the supernatants were determined as a measure of released hemoglobin. Complete hemolysis resulted in an increase in OD_{540} value from 0–0.1 to ≈2.5.

Regarding video microscopy, the red blood cells were attached to coverslip by cell-tak (BD Biosciences, San Jose, CA; BBT-LifeScience, USA). The preparation was placed in an incubation chamber on an inverted microscope (Nikon TE2000, DK) at 37 °C. Time lapse recordings of the erythrocyte appearance in DIC (60X, NA1.2) were collected over 60 minutes after the addition of HlyA (sampling rate, 1 image every 10 seconds). The change in erythrocyte shape was compared with time controls without HlyA.

Immunoblotting. ***Preparation of Erythrocyte Membrane Fractions.*** Human and murine erythrocytes were isolated, lysed, and washed four times with 10 mmol/l Tris solution. After each wash, the suspension was spun at 16,000 *g* for 30 minutes at 4 °C and the hemoglobin-containing supernatant carefully removed. The final pellet was dissolved in Tris solution, separated by electrophoresis on 10% or 12% SDS gel, and blotted onto a PVDF membrane (Bio-Rad, Hercules, CA). Nonspecific binding was reduced by 5% skim milk powder in phosphate-buffered salt solution (PBS) overnight at 4 °C. The membranes were washed and incubated overnight at 4 °C with primary antibody (anti-$P2X_7$ or anti-$P2X_1$, Alomone, Jerusalem, Israel) diluted in PBS with 0.1% Tween20. Preadsoption controls were included for all antibodies with 1:1 peptide:antibody ratio. The membranes were washed thoroughly in PBS-Tween and incubated with peroxidase-conjugated anti-rabbit Ig antibody (DAKO, Glostrup, Denmark). Excess antibody was removed by extensive washing in PBS-Tween, and bound antibody was detected by an ECL chemiluminiscence kit (Amersham, Buckinghamshire, UK). Protein content was determined for purified HlyA as well as isolated erythrocyte membranes using a BCA Protein Assay Kit (Pierce, Rockford, IL).

Solutions and Materials. The Ca^{2+}-containing saline contained the following (in mmol/l): Na^+, 138.0; Cl^-, 132.9; K^+, 5.3; Ca^{2+}, 1.8; Mg^{2+}, 0.8; SO_4^{2-}, 0.8; HEPES, 14; glucose. 5.6, pH 7.4 at 37 °C. The Ca^{2+}-free saline contained (in mmol/l): Na^+, 150.0; Cl^-, 147.0; K^+, 5.0; Mg^{2+}, 1.0; HEPES, 10.0, pH 7.4 at 37 °C. The Tris solution for immunoblotting contained in (mmol/l): $C_4H_{11}NO_3 \cdot HCl$, 10.0. The PBS for immunoblotting contained (in mmol/l): Na^+, 283.2; Cl^-, 100.0; HPO_4^{2-}, 81.0; $H_2PO_4^-$, 21.5, pH 7.4 at 37 °C.

Apyrase, carbenoxolone, suramin, ATP-2′,3′-dialdehyde (oxidized ATP), KN-62, mefloquine, MRS2159, MRS2179, and hexokinase were obtained from Sigma. A 10-mmol/l quantity of glucose was added to hexokinase at concentrations greater than 10 U ml^{-1}. Brilliant blue G (BBG) was purchased from INC Biomedicals (Aurora, OH). MRS2211 was from Tocris Bioscience (Bristol, UK). All reagents were dissolved in distilled water and pH adjusted to 7.4 at 37 °C. KN-62 and mefloquine were dissolved in dimethyl sulfoxide (DMSO) to a final maximal concentration of 0.5% DMSO. Control experiments for DMSO effects alone were included.

Data Analysis and Statistics. Data are presented as mean ± SEM. The *n* value indicates the number of trials for each drug. For the experiments on $P2X_7^{-/-}$ vs. $P2X_7^{+/+}$ murine erythrocytes, *n* denotes the number of animals. 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 post test) for multiple comparisons as appropriate. In both cases, a *P* value less than 0.05 was considered statistically significant.

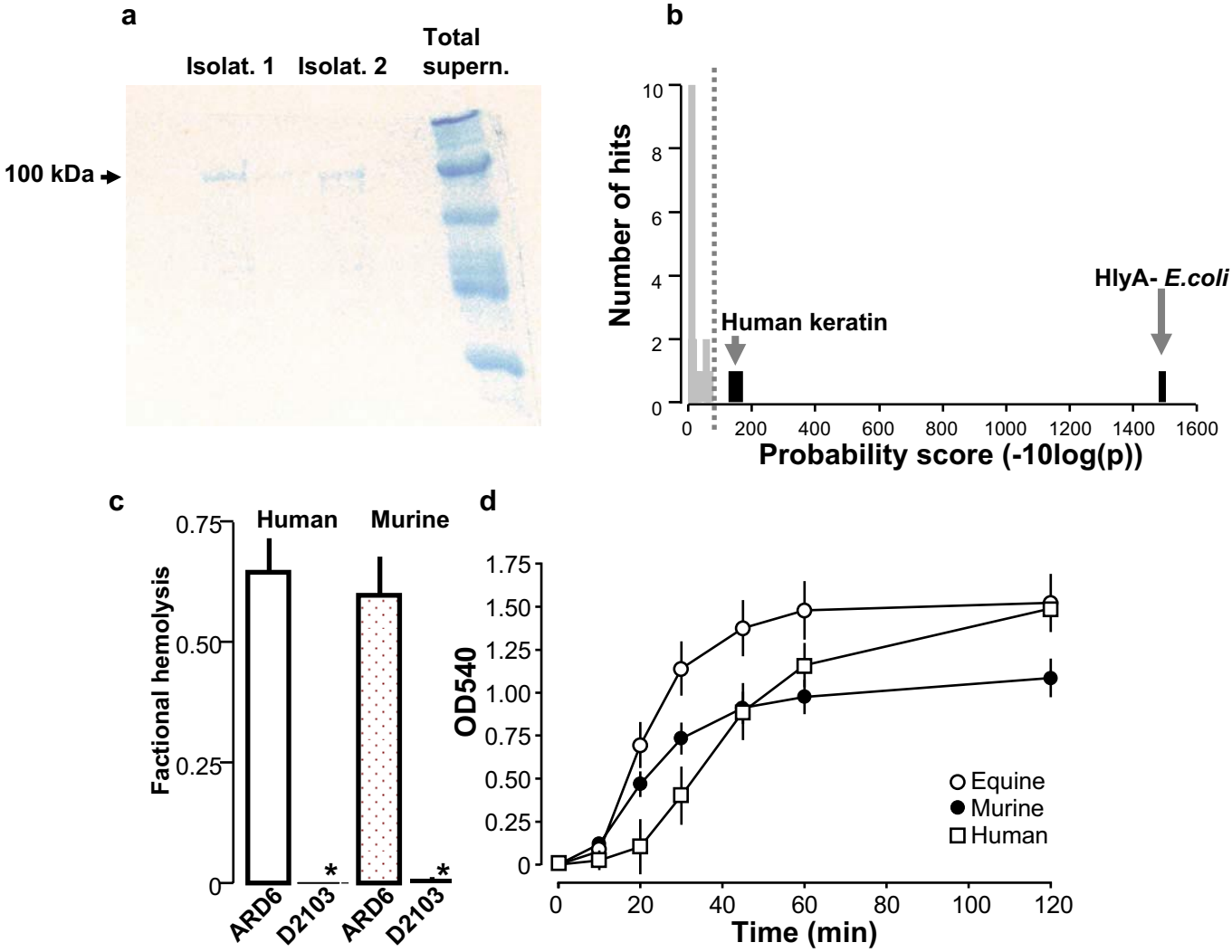


Fig. S1. Purified HlyA produces hemolysis. HlyA was purified by the procedure given in Materials and Methods. (*A*) Coomassie gel of two different purifications. (*B*) Peptide summary report from the mass spectroscopy. (*C*) Lysis of human ($n = 7$) and murine ($n = 8$) erythrocytes induced by the 100 and 50 $\mu l \cdot ml^{-1}$ supernatant, respectively from either the HlyA-producing *E. coli* strain ARD6 or D2103, which does not produce HlyA. (*D*) Time curve for the purified toxin provided by Sucharit Bhakdi, University of Mainz. CBX, carbenoxolone; ox, oxATP; Sur, suramine.

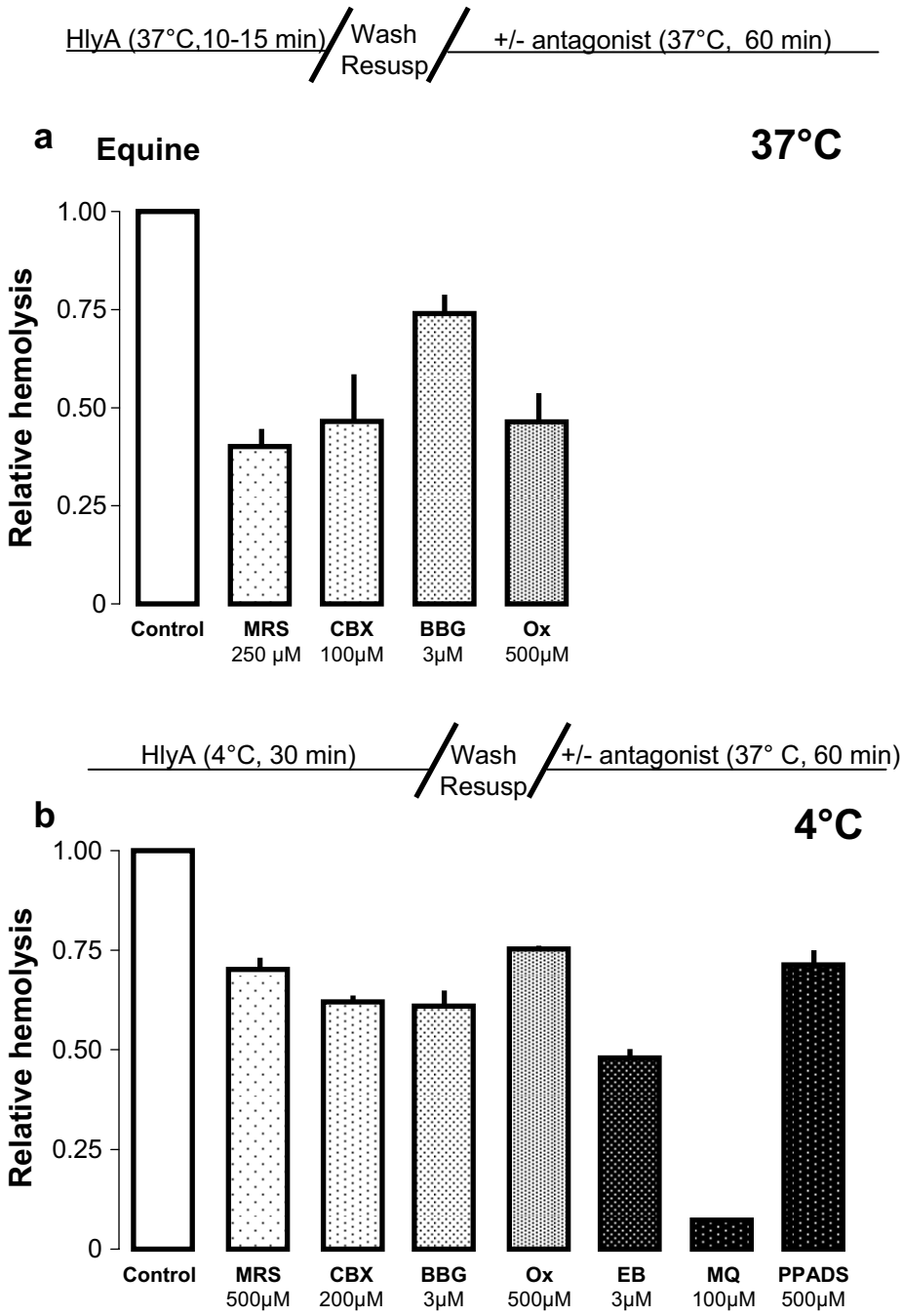


Fig. S2. Preincubation of HlyA in the absence of antagonists. (*A*). Erythrocytes were incubated 10–15 minutes with supernatant containing HlyA (adjusted to give ≈50% hemolysis) at 37 °C to prebind the hemolysin to the erythrocyte membranes. The cells were washed and re-suspended in saline solution containing the various antagonists. The reduction in hemolysis is expressed relative to hemolysis obtained in washed erythrocytes in the absence of antagonists. (*B*) Similar type of experiment. Only here was HlyA prebound to the erythrocytes 30 minutes at 4 °C. The number of preparations tested is three to eight for each antagonist. Asterisks indicate $P < 0.05$. BBG, brilliant blue G; CBX, carbenoxolone; EB, Evans blue; MRS, MRS2159; MQ, mefloquine; Ox, oxidized ATP.

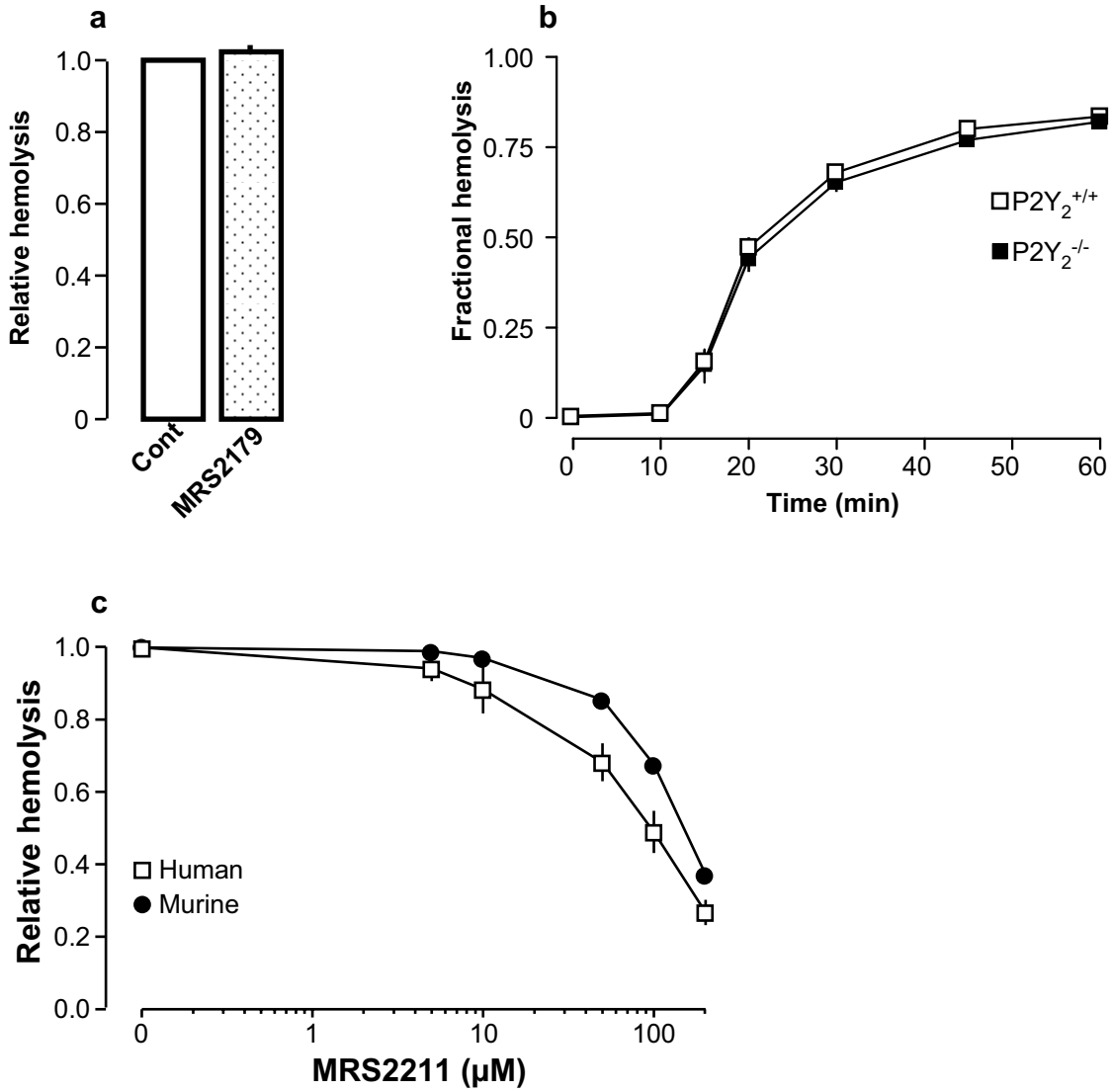


Fig. S3. HlyA-induced hemolysis and P2Y receptors. (a) Effect of the selective $P2Y_1$ receptor blocker, MRS 2179 (500 μmol/l) on the HlyA-induced hemolysis after 60 minutes of incubation of mouse erythrocytes ($n = 4$). (*B*) Time course of the HlyA-induced hemolysis in $P2Y_2^{+/+}$ and $P2Y_2^{-/-}$ respectively. (*C*) Effect of the $P2Y_{13}$ receptor blocker MRS2211 on the HlyA-induced hemolysis ($n = 6$).

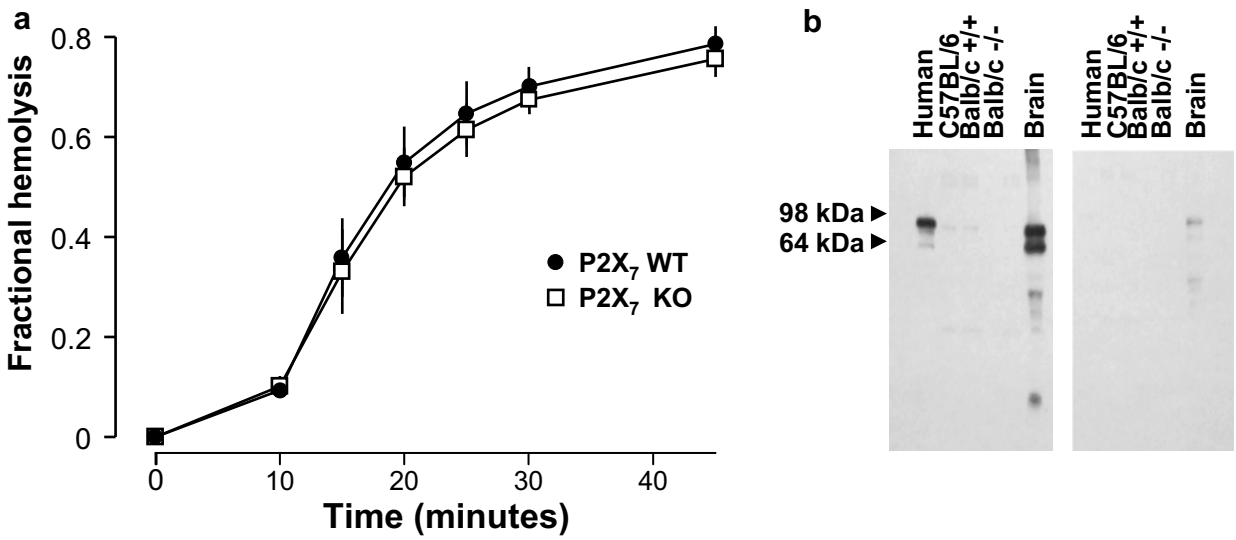


Fig. S4. HlyA-induced hemolysis in $P2X_7$ receptor–deficient mice. (*A*) Time curve for HlyA-induced hemolysis $P2X_7$ receptor deficient mice originally produced by Pfizer compared to wild-type controls (balb/c background, $n = 5$). (b) The full immunoblot shown of Fig. 3.

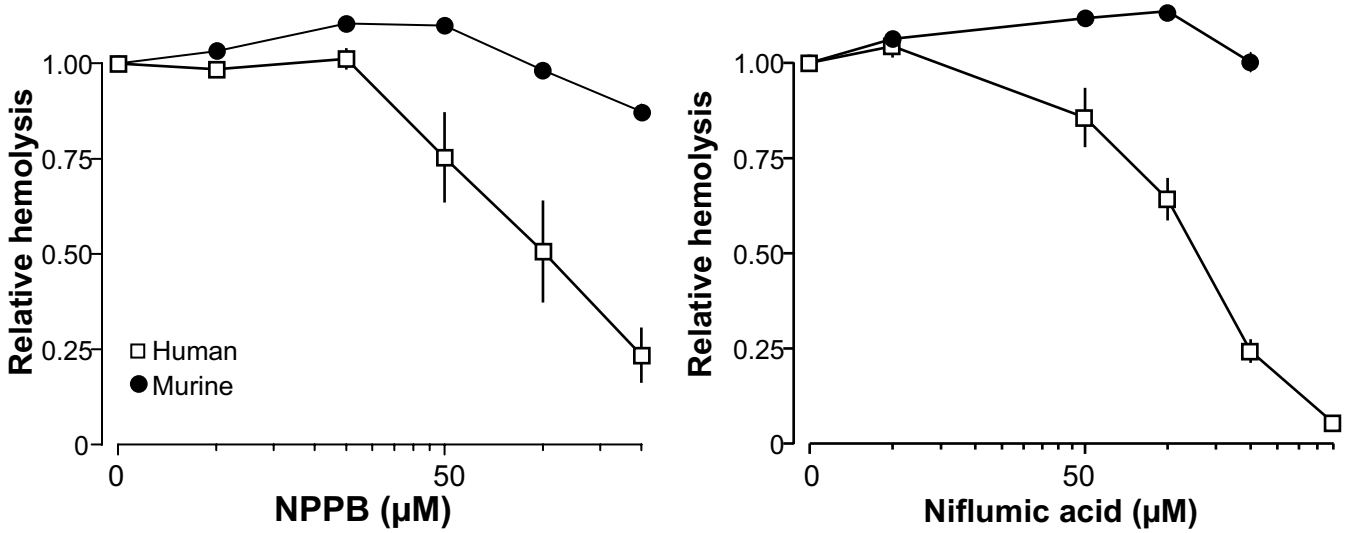
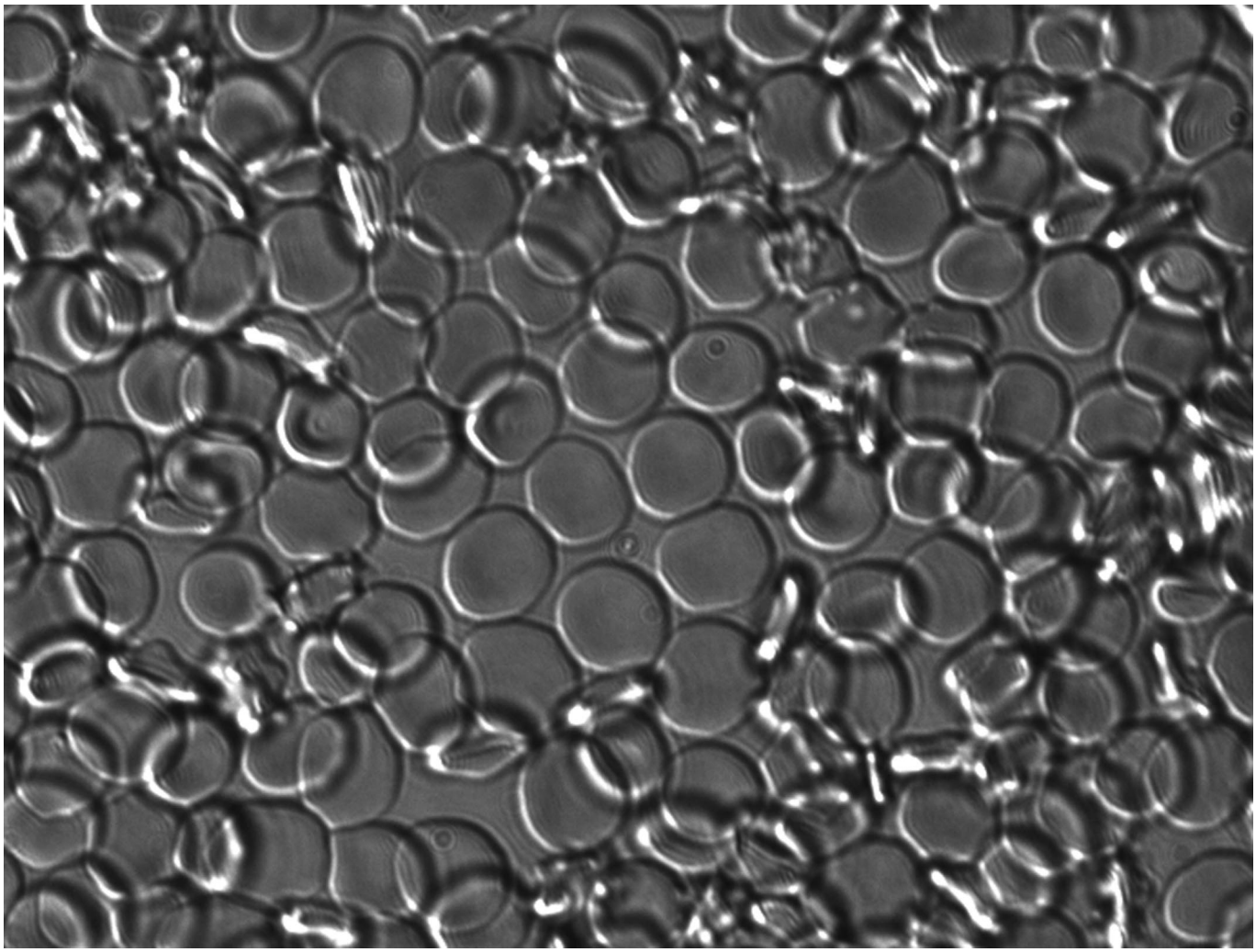


Fig. S5. HlyA-induced hemolysis of human and murine erythrocytes is inhibited by pannexin1 antagonists. Hemolysis was induced by HlyA-containing supernatant from *E. coli*. In addition to carbenoxolone, mefloquine, and probenecid, the hemolysis was concentration-dependently decreased by (*A*) NPPB and niflumic acid over a wide concentration range. Both substances that are known Cl^- channel antagonists have lately been shown to inhibit pannexin channels as well. Both substances had a much more pronounced effect on human erythrocytes. Values are given as mean ± SEM; $n = 5–6$.



Movie S1. Time lapse of human erythrocytes observed on an invertied microscope at 37°C for 60 minutes. *E. coli* supernatant 50 μl·ml^{-1} was added at time 0. Imaging rate was 10^{-4} Hz.

Movie S1 (AVI)