[Ca$^{2+}$]$_i$ Oscillations and IL-6 Release Induced by α-Hemolysin from Escherichia coli Require P2 Receptor Activation in Renal Epithelia*

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Background: The virulence factor HlyA elicits [Ca$^{2+}$]$_i$ oscillations in renal epithelial cells. 

Results: These oscillations and following IL-6 release are reduced by inhibition or lack of P2Y$_2$ receptors.

Conclusion: The effects of HlyA in renal epithelial cells are mediated by P2Y$_2$ receptors.

Significance: ATP release and P2Y$_2$ receptor activation are essential parts of the early interaction between Escherichia coli and the renal epithelium.

Urinary tract infections are commonly caused by α-hemolysin (HlyA)-producing Escherichia coli. In erythrocytes, the cytotoxic effect of HlyA is strongly amplified by P2X receptors, which are activated by extracellular ATP released from the cytosol of the erythrocytes. In renal epithelia, HlyA causes reversible [Ca$^{2+}$]$_i$ oscillations, which trigger interleukin-6 (IL-6) and IL-8 release. We speculate that this effect is caused by HlyA-induced ATP release from the epithelial cells and successive P2 receptor activation. Here, we demonstrate that HlyA-induced [Ca$^{2+}$]$_i$ oscillations in renal epithelium were completely prevented by scavenging extracellular ATP. In accordance, HlyA was unable to induce any [Ca$^{2+}$]$_i$ oscillations in 132-1N1 cells, which lack P2R completely. After transfecting these cells with the hP2Y$_2$ receptor, HlyA readily triggered [Ca$^{2+}$]$_i$ oscillations, which were abolished by P2 receptor antagonists. Moreover, HlyA-induced [Ca$^{2+}$]$_i$ oscillations were markedly reduced in medullary thick ascending limbs isolated from P2Y$_2$ receptor-deficient mice compared with wild type. Interestingly, the following HlyA-induced IL-6 release was absent in P2Y$_2$ receptor-deficient mice. This suggests that HlyA induces ATP release from renal epithelia, which via P2Y$_2$ receptors is the main mediator of HlyA-induced [Ca$^{2+}$]$_i$ oscillations and IL-6 release. This supports the notion that ATP signaling occurs early during bacterial infection and is a key player in the further inflammatory response.

Escherichia coli is the dominant facultative bacterium in the normal intestinal flora but is also notorious for causing urinary tract infections. In severe cases, these may ascend and result in pyelonephritis (1), which potentially can cause scarring and renal insufficiency. The invasive E. coli strains are serotypically distinct from the facultative strains and frequently produce virulence factors such as the exotoxin α-hemolysin (HlyA)$^2$ (2, 3). HlyA belongs to the Repeat-in-Toxin (RTX) family, of which some are able to form pores in cell membranes, rendering the cells permeable to ions and water and ultimately lyse them (for review, see Ref. 4). Recently, we discovered that the HlyA-induced lysis of erythrocytes is amplified by ATP release and subsequent P2X receptor activation (5, 6), because either inhibition of the P2X receptors or scavenging of extracellular ATP markedly reduced the HlyA-induced hemolysis (5). This cellular amplification system was found to be equally relevant in hemolysis induced by other cytolysins such as α-toxin from Staphylococcus aureus (7), leukotoxin A from Aggregatibacter actinomycetemcomitans (8) and complement activation (9). Interestingly, the size of the created pore by bacterial toxins is critical for whether or not cell lysis is amplified by ATP (10). The authors show that bacterial pores too narrow to allow passage of ATP do not exhibit P2X receptor-dependent amplification of cell lysis (10). In support of this, we have shown that HlyA-induced ATP release is likely to occur directly through the HlyA-pore itself (11). This is exceedingly interesting because it implies that ATP will be released from any cell attacked by HlyA and thus, P2 receptors will be activated in an auto- and paracrine fashion directly subsequent to membrane insertion of HlyA. Moreover, an immediate consequence would be that the effect of HlyA would follow the P2 receptor expression pattern, which is one of the key elements in the current study.

In proximal tubule cells from rats, HlyA causes distinct, reversible oscillations in the intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) (12). In this study, these HlyA-induced [Ca$^{2+}$]$_i$ oscillations were suggested to require G-protein activation and to be essential for a successive release of IL-6 and IL-8 from the epithelium (12). We have previously demonstrated that spontaneous [Ca$^{2+}$]$_i$ oscillations in renal epithelia result from constitutive ATP release from the cells followed by P2 receptor activation (13). Based on the results in erythrocytes, we specu-

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2 The abbreviations used are: HlyA, α-hemolysin; MDCK, Madin-Darby canine kidney cells; mTAL, medullary thick ascending limb; ISOM, inner stripe of outer medulla; KC, keratinocyte chemoattractant.
lated that the HlyA-induced \([Ca^{2+}]\), oscillations in renal epithelia could result from ATP release, followed by auto- and para-crine signaling. This notion is supported by several studies, which show that extracellular ATP is a keen trigger of IL-6/IL-8 release from other cell types (14–18).

Here, we show that HlyA-induced \([Ca^{2+}]\), oscillations both in renal epithelial cells in culture (Madin-Darby canine kidney (MDCK) cells) and isolated medullary thick ascending limb (mTAL) from mice require P2 receptor activation. In MDCK cells and mTAL isolated from \(P2Y_2^{+/+}\) mice, the HlyA-induced \([Ca^{2+}]\), oscillations were markedly lowered by scavenging of extracellular ATP by apyrase. In murine mTAL, the HlyA-induced \([Ca^{2+}]\), oscillations were significantly less pronounced in \(P2Y_2^{-/-}\) mice compared with control. These data were substantiated in biosensor cells that do not express any type of P2 receptors. We demonstrate that HlyA-induced \([Ca^{2+}]\), oscillations could only be inflicted when these cells had been transfected with hP2Y2 receptors and thus, the HlyA effect completely depends on P2 receptor expression. Moreover, HlyA readily inflicted IL-6 release from the renal inner stripe of outer medulla (ISOM) from \(P2Y_2^{+/+}\) mice, whereas this response was completely absent in \(P2Y_2^{-/-}\) mice. These data pinpoint extracellular ATP as an early signaling molecule in the epithelial response to bacterial virulence factors. Thus, ATP-mediated P2 receptor signaling is likely to be a crucial player in the following inflammatory response.

**Experimental Procedures**

*Cells and Tissue—* Type 1 MDCK cells (passage 56–69) from the American Type Culture Collection (LGC Standards, Boras, Sweden) and 132-1N1 astrocytoma cells were grown in a monolayer on 25-mm diameter coverslips (VWR, Denmark) or in 96-well plates in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (Gibco, Invitrogen, Taastrup, Denmark), 2 mM glutamine and 1 unit/ml of penicillin and 100 μg/ml of streptomycin but without riboflavin and phenol red as previously described (19). The hP2Y2-transfected 132-1N1 astrocytoma cells were kindly provided by Robert Nicholas (Chapel Hill, NC) and grown in DMEM with 440 μg/ml of geneticin.

mTALs were isolated by manual dissection from ISOM from \(P2Y_2^{+/+}\) and \(P2Y_2^{-/-}\) mice (3–6 weeks) of either sex. The mice were on a B6D2/SV129 background, generously provided by Dr. B. H. Koller, University of North Carolina, NC. Animals were kept and handled according to the Danish animal welfare regulation.

*HlyA from E. coli—* HlyA was purified from the E. coli strain ARD6 (O6:K13:H1) as described (20) and used in a concentration (EC_{50}) that produces 50% hemolysis of human erythrocytes (1.25% v/v, 60 min at 37°C). This was determined as hemoglobin release measured as A_{540} nm of the supernatant in PowerWave Microplate Spectrophotometer, Biotek Instruments, Winooski, VT.

*Live Cell Imaging—* Cell cultures and mTAL were loaded with 5 μM fluo 4-AM and mounted in a semi-open perfusion chamber (modified RC-21BRFS, Warner Instruments, Hamden, CT). The cultured cells were imaged at 1 Hz on an inverted microscope (TE-2000, Nikon) with ×60/1.4 NA planApo objective (Nikon), a monochromator (488 nm, Visitech International, Sunderland, UK), and emission collected >520 nm by an intensified SVGA CCD camera and imaging software (Quanticell 2000/Image Pro, VisiTech). The mTALs were imaged at 1 Hz by ×40/1.35 NA planapo objective (Olympus), a monochromator (488 nm, Polychrome V, TILL-Photonic, Munich, Germany), and emission was collected by an iCCD-camera (sensicam qe, PCO, Kelheim, Germany) and software Live Acquisition (TILL-Photonic, Munich, Germany). All experiments were carried out at 37°C, pH 7.4, and fluorescence intensity was expressed relative to baseline. Regions of interest were placed over each of the cultured cells or side-by-side along the entire mTAL length and analyzed in Igor Pro (Wavemetrics, Lake Oswego, OR).

*Cytokine Measurements—* ISOM was dissected and incubated for 6 h at 37°C and 5% CO2 in DMEM in the presence or absence of HlyA (EC_{50}). Samples of the supernatant were frozen immediately (~20°C, storage <10 days). Cytokines were measured by a mouse IL-6 solid phase sandwich ELISA kit from BD Bioscience and a mouse keratinocyte chemoattractant (KC) solid phase sandwich ELISA kit from RayBiotech, Inc. (Norcross, GA) according to the manufacturer’s protocols. The amount of IL-6 and KC was determined by absorbance at 450 and 570 nm in a plate reader (PowerWave, Biotek Instruments, Winooski, VT).

*ATP Measurements—* MDCK cells grown in 96-well plates were incubated for 1 h in HEPES-buffered solution at 37°C. ATP release was determined by luciferin/luciferase (Invitrogen) in the presence or absence of HlyA (EC_{50}) with slight modifications to the instruction. All experiments included a standard row and samples and standards were read in a Mithras LB940 Multimode Reader (Berthold Technologies, Bad Wildbad, Germany).

*Solutions and Chemicals—* Probenecid, ATP-2',3'-dialdehyde (oxidized ATP), ATP, and apyrase were purchased from Sigma. Fluo 4-AM was from Invitrogen and MRS2179 was purchased from Tocris (Bristol, UK). YB-074 was kindly provided by Prof. Christa Müller (University of Bonn, Germany).

For solutions, see Table 1.

**Statistical Analysis—** Data are presented as mean ± S.E. mean. The n value indicates number of mice or cell culture preparations. Data were tested for normal distribution by Kolmogorov-Smirnov test. Statistical significance was determined by Student’s t test, Mann-Whitney-Wilcoxon test, or Wilcoxon matched pairs test.

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**TABLE 1**

| Composition of solutions | mTAL | mTAL (high Ca^{2+}) | MDCK | 132-1N1 |
|--------------------------|------|---------------------|------|---------|
| Na^+ (mM)                | 133.5| 127.5               | 132.0| 138.0   |
| K^+ (mM)                 | 5.3  | 5.3                 | 5.3  | 5.3     |
| Cl^- (mM)                | 125.8| 128.8               | 126.9| 132.9   |
| Ca^{2+} (mM)             | 1.0  | 5.0                 | 1.8  | 1.8     |
| Mg^{2+} (mM)             | 0.8  | 0.8                 | 0.8  | 0.8     |
| SO_4^2- (mM)             | 0.8  | 0.8                 | 0.8  | 0.8     |
| Glucose (mM)             | 5.6  | 5.6                 | 5.6  | 5.6     |
| HEPES (mM)               | 14.0 | 14.0                | 14.0 | 14.0    |
| Probenecid (mM)          | 5.0  | 5.0                 | 5.0  | 5.0     |
| Final measured osmolality (mosmol liter^-1) | 288 | 288                 | 293  | 293     |
HlyA Trigger P2R Activation in Renal Epithelia

FIGURE 1. MDCK cells increase [Ca\textsuperscript{2+}]\textsubscript{i} oscillatory activity after addition of HlyA. a, original representative trace showing the small number of spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} oscillations during baseline and (b) the increased activity in [Ca\textsuperscript{2+}]\textsubscript{i} oscillations after 5 min preincubation with HlyA in a concentration that causes 50% hemolysis in erythrocytes (EC\textsubscript{50}). c, the rise in fluorescence intensity after addition of ATP (100 \muM) 5 min after washout of HlyA. d, summarized data showing the number of oscillations of 100 cells\textsuperscript{-1} s\textsuperscript{-1} during baseline and 5 min after addition of HlyA (EC\textsubscript{50}). e, mean rise in intensity after addition of ATP (100 \muM). Values are mean ± S.E. (n = 18). Asterisk indicates statistical significance (p < 0.05).

Results

HlyA Induces [Ca\textsuperscript{2+}]\textsubscript{i} Oscillations in Cell Culture—HlyA is known to inflict reversible [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in proximal tubule cells from rat (12). Our data shows that HlyA similarly induces reversible [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in MDCK cells, a cell line isolated from canine distal tubules (fig. 1). Fig. 1a shows the spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} oscillations previously reported for this cell type (13). Five minutes after addition of HlyA (EC\textsubscript{50}), there was a significant increase in [Ca\textsuperscript{2+}]\textsubscript{i} oscillatory activity (Fig. 1, b and d). The HlyA-induced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations were reversible and the cells were still able to respond with an increase in [Ca\textsuperscript{2+}]\textsubscript{i} when exposed to ATP after washout of HlyA (Fig. 1, c and e). Please note that none of the cells showed a sudden drop in fluo 4-fluorescence upon exposure to HlyA, and thus do not lyse during the observation period. Scavenging of extracellular ATP with apyrase (10 units ml\textsuperscript{-1}, Fig. 2, a–e) abolished the HlyA-induced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in MDCK cells, suggesting involvement of purinergic signaling. Because scavenging of ATP markedly reduces the cellular response to HlyA, we anticipate that HlyA may trigger ATP release from MDCK cells. Fig. 2f shows that ATP release from MDCK cells increased immediately after addition of HlyA (EC\textsubscript{50}). However, it does not become statistically significantly different from control before 12 min incubation because of the mechanically induced ATP release that occurs upon addition of HlyA or vehicle. The release of ATP stayed elevated throughout the observation period.

If HlyA-induced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations are a consequence of ATP release and P2 receptor signaling it should depend on P2 receptor expression. Renal epithelial cells generally express a large variety of different P2 receptors (21, 22). The unspecific P2 receptor antagonists (PPADS and suramin) completely abolished the HlyA-induced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in MDCK cells (data not shown). To completely block all P2 receptor subtypes, the concentration of P2 receptor antagonists has to be substantial and thus, potentially cause unspecific effects. Therefore, we tested the effect of HlyA on ATP-biosensor cells. The 132-1N1 astrocytoma cell line does not express any P2 receptor subtypes and are thus, ideal control cells for potential P2 mediated signaling. In native 132-1N1 cells, addition of HlyA only caused discrete increases in [Ca\textsuperscript{2+}]\textsubscript{i} which could not be detected over baseline (Fig. 3, a, b, and g). The lacking effect was not a result of the inability of these cells to create receptor-mediated [Ca\textsuperscript{2+}]\textsubscript{i} increases in general, because the muscarine receptor agonist, carbachol (100 \muM), caused a sharp rise in [Ca\textsuperscript{2+}]\textsubscript{i}, in native 132-1N1 cells (Fig. 3, c and j). Note the complete lack of effect of ATP (100 \muM), which complies with a total absence of P2 receptors in these cells. The 132-1N1 cells, which are stably transfected with the hP2Y\textsubscript{12} receptor, show spontaneous [Ca\textsuperscript{2+}]\textsubscript{i} oscillations (Fig. 3d), which markedly increase after addition of HlyA (Fig. 3, e and h). The HlyA-induced [Ca\textsuperscript{2+}]\textsubscript{i} oscillations in hP2Y\textsubscript{12}-transfected 132-1N1 cells could be inhibited by the nonselective P2 receptor antagonist suramin (Fig. 3i). These results show that [Ca\textsuperscript{2+}]\textsubscript{i} oscillations induced by HlyA require P2 receptor activation in both MDCK and 132-1N1 cells.

HlyA-induced [Ca\textsuperscript{2+}]\textsubscript{i} Oscillations in Freshly Isolated mTAL Requires P2 Receptor Activation—To investigate the effect of HlyA in native renal epithelia, we used isolated murine mTAL, which has been extensively investigated with regard to its functional P2 receptor expression (23). mTAL isolated from

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P2Y2+/+ mice showed spontaneous [Ca2+]i oscillations with a mean value of 8.31 ± 1.33 oscillations 100 μm−1 min−1 with an average amplitude of 0.17 ± 0.08 arbitrary units (n = 19 in total). Please note that separate control experiments were done for each set of experiments and the values given are the average of all control experiments on mTAL from P2Y2 for each set of experiments and the values given are the average total). Please note that separate control experiments were done summarized ATP response after washout of apyrase.

FIGURE 2. HlyA-induced [Ca2+]i oscillations in MDCK cells are inhibited by scavenging of ATP released by the epithelial cells. a, representative trace showing baseline [Ca2+]i oscillations in the presence of apyrase (10 units ml−1). b, [Ca2+]i oscillations after application of HlyA (EC50) in the presence of apyrase (10 U ml−1). c, the response to ATP after 5 min washout of apyrase. d, the summarized data with the ATP scavenger apyrase (10 units ml−1, n = 10). e, the summarized ATP response after washout of apyrase. f, the relative release of ATP from control MDCK cells without HlyA, and from MDCK cells with HlyA in a concentration that causes 50% hemolysis in erythrocytes (n = 18). Values are mean ± S.E. Asterisk indicates statistical significance (p < 0.05).

Because murine mTAL also expresses P2X6 (24) and possibly P2Y1 receptors (23), we tested the effect of YB-074, which is a P2Y6 and P2X1-3 receptor antagonist (25) and MRS2179, an agonist with preference for P2Y1 receptors. YB-074 (100 μM) did not reduce the [Ca2+]i oscillations in P2Y2−/− mTAL further (p = 0.64) at a concentration that was shown to completely block ATP-induced transport inhibition in mTAL (24). These data indicate that P2X receptors are not the main receptors involved in HlyA-induced [Ca2+]i oscillations in murine mTAL.

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tor is unlikely to be involved in the generation of these oscillations.

HlyA-induced Release of IL-6 and IL-8—Uhlén et al. (12) has shown that HlyA-induced [Ca\(^{2+}\)]\(_i\) oscillations trigger the release of IL-6 and IL-8 from rat proximal tubule cells. Because ATP is involved in the transcription and release of IL-6 from respiratory epithelia (14), we speculated that HlyA-induced ATP release may stimulate release of cytokines from renal epithelia. After incubation for 6 h, the baseline release of IL-6 from ISOM isolated from P2Y2\(^+/+\) mice was 47.0 ± 9.9 pg ml\(^{-1}\) (mg of tissue)\(^{-1}\) and HlyA increased the release to 101.4 ± 23.3 pg ml\(^{-1}\) (mg of tissue)\(^{-1}\) (Fig. 6a, \(p = 0.028\)). In ISOM from P2Y2\(^{-/-}\) mice, the baseline IL-6 release was 31.47 ± 4.0 pg ml\(^{-1}\) (mg of tissue)\(^{-1}\). Addition of HlyA did not result in a statistically significant increase in IL-6 release in ISOM from P2Y2\(^{-/-}\) mice (51.37 ± 9.3 pg ml\(^{-1}\) (mg of tissue)\(^{-1}\), \(p = 0.132\)). Moreover, the HlyA-induced IL-6 release was statistically significantly lower in ISOM from P2Y2\(^{-/-}\) compared with P2Y2\(^{+/+}\) (\(p = 0.043\)). These results show that IL-6 is released from murine ISOM in response to HlyA and that the release requires P2Y2 receptor activation. Mice do not express IL-8. Instead KC is accepted as the mouse homologue to human IL-8 because it binds to the IL-8 type 2 receptor (26–28). As shown in Fig. 6b, HlyA releases KC in both P2Y2\(^+/+\) and P2Y2\(^{-/-}\) and there is no difference in the released amount (\(p = 0.45\)). Surprisingly, we observed a small but statistically significant difference in baseline KC release between P2Y2\(^+/+\) and P2Y2\(^{-/-}\) (Fig. 6b). These results show that HlyA-induced KC release from murine ISOM in contrast to IL-6 does not involve P2Y2 receptors.

Discussion

Urinary tract infections are frequently inflicted by the Gram-negative bacterium *E. coli* (29) and strains that produce the virulence factor HlyA are more prone to inflict severe cases such as pyelonephritis (for review see Refs. 1 and 30). Uhlén et al. (12) found that HlyA from *E. coli* supernatant induces [Ca\(^{2+}\)]\(_i\) oscillations in rat proximal tubule cells followed by IL-6 and IL-8 release. This potentially suggests that HlyA enables the renal
epithelial cells to initiate an immunological response before the bacteria have crossed the epithelial barrier.

The current study considers the possibility that HlyA-induced [Ca\(^{2+}\)] oscillations are mediated by ATP released from the renal epithelial cells followed by P2 receptor activation. This notion is based on the abundance of ATP-sensitive P2 receptors expressed in virtually all types of renal epithelial cells (for overview see Ref. 21) and that these receptors specifically have been shown to be responsible for spontaneous [Ca\(^{2+}\)] oscillations observed in renal epithelia (13). Moreover, there is substantial evidence that extracellular ATP induces IL-6 and IL-8 release from other tissues (14–18). Interestingly, it was previously shown that IL-6 and IL-8 concentrations are higher in urine from children with febrile urinary tract infection compared with asymptomatic bacteriuria and that bacteria isolated from febrile children were more likely to produce virulence factors including HlyA (31). As mentioned, we have previously established that ATP release and P2X receptor activation is important for the cytolytic effect of HlyA in erythrocytes. Recently, we have shown that ATP may pass directly through the HlyA pore (11). This finding was somewhat surprising because previous studies on the biophysical properties of HlyA, as a pore, suggest it to be cation selective (32, 33) and not immediately favor passage of negative charged ATP. Nevertheless, we found that HlyA insertion induced non-lytic ATP release from artificial phospholipid vesicles, which is consistent with HlyA either passing through the pore itself or slip through between the membrane and protein at sites of HlyA insertion (11). That said, it is currently not known how HlyA induces ATP release from renal epithelia. One could speculate that ATP leaves through the HlyA pore, but ATP may equally as well be released through one of the established ATP release pathways in renal

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**FIGURE 4.** [Ca\(^{2+}\)] oscillations induced by HlyA in murine mTAL are inhibited by the ATP scavenger apyrase. a, pseudocolour images of fluo-4-AM-loaded mTAL before and after addition of HlyA. The arrows show areas of increases in [Ca\(^{2+}\)] in response to HlyA application. b, original representative trace showing [Ca\(^{2+}\)] oscillatory activity after addition of HlyA (EC\(_{50}\)). c, trace showing the rise in [Ca\(^{2+}\)] in response to ATP (10 μM) added to test the viability of the tubule at the beginning of experiments. d, summarized data showing oscillations (100 μM min\(^{-1}\)) after addition of HlyA (EC\(_{50}\)) in P2Y\(_{2}\) mTAL (n = 6) and in the presence of apyrase (10 units ml\(^{-1}\), n = 5). Values are mean ± S.E. Asterisks indicate statistical significance (p < 0.05).

**FIGURE 5.** The effect of HlyA in P2Y\(_{2}\) mTAL and the effect of P2 receptor antagonist on [Ca\(^{2+}\)] oscillatory activity after addition of HlyA. a, bar graph showing the summarized [Ca\(^{2+}\)] oscillations (100 μM min\(^{-1}\)) after addition of HlyA (EC\(_{50}\)) in P2Y\(_{2}\) mTAL (n = 13) and P2Y\(_{2}\) mTAL (n = 10). b, bar graph showing mean change (100 μM min\(^{-1}\)) in [Ca\(^{2+}\)] oscillatory activity after addition of HlyA (EC\(_{50}\)) in P2Y\(_{2}\) KO and P2Y\(_{2}\) WT and in the presence of the irreversible non-selective P2X receptors antagonist oxidized ATP (50 μM) (n = 11) or the P2Y\(_{2}\) receptor antagonist YB-074 (100 μM) (n = 5). Values are mean ± S.E. Asterisks indicate a p value < 0.05 compared with WT.
epithelia; vesicular release (34) or through connexin hemichannels (35).

Here, we confirm that HlyA induces [Ca^{2+}], oscillations in renal epithelial cells. Similar to the previously reported effect in rat proximal tubular cells (12), HlyA-induced oscillations were also observed in MDCK cells and isolated murine mTAL. These HlyA-induced [Ca^{2+}], oscillations were previously shown to be inhibitable by IP3 receptor blockage (12) and thus, require release of Ca^{2+} from intracellular Ca^{2+} stores. This finding potentially argues for G protein activation in response to HlyA, which is certainly supported by ATP-mediated P2 receptor activation. It must, however, be noted that other groups support the view that [Ca^{2+}], events are caused by the HlyA-pore itself without any kind of subsequent signaling (36). To test the concept of HlyA-induced P2Y receptor activation, we used the same types of renal epithelia, in which we previously characterized the ATP and P2 receptor dependence of spontaneous [Ca^{2+}], oscillations: MDCK cells and murine mTAL. We show that HlyA (EC_{50}) accentuates the [Ca^{2+}], oscillations observed in cultured MDCK cells. At this concentration, the effect of HlyA did not cause cell lysis in either of the preparations (measured as immediate drop of fluo 4 fluorescence). Moreover, the cells readily showed a brisk increase in [Ca^{2+}], in response to extracellular ATP after washout of HlyA, a response that serves as a further viability control of the cells. The HlyA-induced [Ca^{2+}], oscillations in MDCK cells were significantly reduced by P2 receptor antagonists (data not shown) and scavenging of extracellular ATP. This finding was substantiated in 132-1N1 cells, whose only cell line presently known to be completely absent of P2 receptors. In these cells, which hardly show any spontaneous [Ca^{2+}], oscillations (13), HlyA only inflicted an occasional few increases in [Ca^{2+}], which could not be detected over baseline in the summarized data. However, when 132-1N1 cells were transfected with the hP2Y_2 receptor, the baseline oscillatory activity increased significantly as previously demonstrated (13) and now HlyA readily triggered a vivid [Ca^{2+}], oscillatory activity. These data are consistent with the hypothesis that P2 receptor activation is a player in HlyA-induced [Ca^{2+}], oscillations in general.

To test this in intact renal tissue, we used murine mTAL, where the P2 receptor expression has been functionally characterized (23). The P2Y_2 receptor is the predominant receptor expressed on the luminal and basolateral membrane in mTAL (23). We found the HlyA-induced [Ca^{2+}], oscillations to be substantially lower in mTAL isolated from P2Y_2^{−/−} mice compared with P2Y_2^{+/−}. However, opposed to 132-1N1 cells, where HlyA did not show any significant effect in the absence of P2Y_2 receptors, there is still a significant HlyA-induced [Ca^{2+}], oscillatory activity in mTAL from P2Y_2^{−/−} mice. However, the HlyA-induced [Ca^{2+}], oscillations were completely abolished in P2Y_2^{+/+} mTAL in the presence of apyrase, which indicates that the remaining oscillations in P2Y_2^{−/−}/mTAL is mediated through other P2 receptors expressed in this segment (P2Y_1/P2Y_6 and P2X_1/P2X_4/P2X_5) (24). The P2X receptor antagonist oxidized ATP was unable to reduce the HlyA-induced [Ca^{2+}], oscillations further in mTAL isolated from P2Y_2^{−/−} mice. This means that the P2X receptors are not responsible for the remaining [Ca^{2+}], oscillations. Moreover, the P2Y_4/P2X_{1−3} receptor antagonist YB-074 (25) and the specific P2Y_4 receptor antagonist MRS2179 did not affect the HlyA-induced [Ca^{2+}], oscillations in mTAL isolated from P2Y_2^{−/−} mice. Thus, the P2Y_1/P2Y_4 receptors are not responsible for the remaining [Ca^{2+}], oscillations observed in P2Y_2^{−/−} mTAL. In addition, the mTAL has been found to produce mRNA for the P2Y_2 receptor, whereas P2X receptors apparently are not involved in the HlyA-induced effects. In erythrocytes, however, P2X receptor antagonists completely block HlyA-induced hemolysis, whereas P2Y receptors do not play a role in HlyA-induced effects in these cells (5).

The present study also confirms that HlyA triggers release of IL-6 and IL-8 from renal tissue. In murine ISOM, where the main components are thick ascending limbs, the baseline IL-6 release was similar in tissue isolated from P2Y_2^{−/−} and P2Y_2^{+/+} mice. Interestingly, the HlyA-induced IL-6 release was completely absent in ISOM isolated from P2Y_2^{−/−} mice. This finding strongly suggests that HlyA-induced ATP release and subsequent P2Y_2 receptor activation are required for the IL-6 secretion in ISOM. Interestingly, ATP is known to release and subsequent P2Y_2 receptor activation are required for the IL-6 secretion in ISOM. Interestingly, ATP is known to
induce release of IL-6 in a P2Y<sub>2</sub> receptor-dependent fashion from human airway epithelia (14) and from the human renal epithelial cell line A498 (38). Because release of IL-6 is important for clearance of the bacterial infection and IL-6-deficient mice have higher mortality in response to E. coli-induced pyelonephritis (39), it would be interesting to investigate whether the same is true for P2Y<sub>2</sub><sup>-/-</sup> mice. We could also show that HlyA readily inflicted release of KC, the murine IL-8 homologue (26–28). Surprisingly, the HlyA-induced KC release did not require P2Y<sub>2</sub> receptor activation in murine ISOM. This is not immediately consistent with the clear P2Y receptor dependence of the HlyA-induced IL-8 release in cultured human uroepithelia (40). It must, however, be stressed that KC only is a homolog of IL-8 and its release may not be regulated completely similarly to the human IL-8. Moreover, there are still remaining [Ca<sup>2+</sup>], oscillations in P2Y<sub>2</sub><sup>-/-</sup> mice and if the KC release is slightly more Ca<sup>2+</sup> sensitive than the IL-6 release, it could potentially be unaffected by the lack of P2Y<sub>2</sub> receptors.

Our current findings support an important role of ATP-mediated auto- and paracrine signaling in the first encounter of renal epithelial cells with uropathogenic E. coli. One could speculate that extracellular ATP may act as a potential defense mechanism against bacteria. Extracellular ATP is a potent inhibitor of renal epithelial transport (26, 41, 42) and could thereby increase the washout of intratubular bacteria. Moreover, the current data are consistent with ATP as the mediator of HlyA-induced IL-6 release and thus, potentially essential to immune system-mediated eradication of the infection. On this note, it is interesting to speculate that the P2Y<sub>2</sub> receptor is required for an adequate immune response in renal epithelia.

The critical point is: why is HlyA a virulence factor in terms of ascending urinary tract infections? Generally, hemolysins have been suggested to provide the iron important to sustain bacterial growth, because chelation of iron significantly hampers the growth of E. coli (43). Iron is recognized as an essential nutrient for many of the key steps in the development of any pathogen in its host (44). Hemolysis has been speculated to be an important physiological source of iron for E. coli, because the iron-transport system of the bacterium use heme and hemoglobin and not transferrin and lactoferrin (45). This certainly is one potential way for HlyA to enhance bacterial growth in vivo and thus, be a virulence factor. It does, however, not necessarily explain why this increases the chance of the bacterium to advance into the kidney. Here interaction with the epithelial barrier is likely to take precedence. In this context, it is interesting that both E. coli (46) and aerolysin from Aeromonas hydrophila have been shown to modify tight junctions (47) and several cytokines including IL-6 have indeed been shown to modulate the expression pattern of occludins and claudins as well as modulate tight junctional function in certain epithelia/endothelia (48, 49). Thus, it is likely that HlyA and the following ATP-dependent cytokine release may interfere with the barrier function of the epithelia and thus, its ability to keep the bacteria from invasion.

It is still not known what the functional phenotype of the P2Y<sub>2</sub> receptor is in respect to ascending urinary tract infections. Our presented data points to ATP release and signaling as one of the very early events when an epithelial barrier is exposed to HlyA. Therefore, these data support that interference with purinergic signaling may significantly affect the pathogenesis of urinary tract infections.

References
1. Johnson, J. R. (1991) Virulence factors in Escherichia coli urinary tract infection. Clin. Microbiol. Rev. 4, 80–128
2. Bhakdi, S., Mackman, N., Menestrina, G., Gray, L., Hugo, F., Seeger, W., and Holland, I. (1988) The hemolysin of Escherichia coli. Eur. J. Immunol. 18, 135–143
3. Cavalleri, S. J., Bohach, G. A., and Snyder, I. S. (1984) Escherichia coli a-hemolysin: characteristics and probable role in pathogenicity. Microbiol. Rev. 48, 326–343
4. Skals, M., and Praetorius, H. A. (2013) Mechanisms of cytokinin-induced cell damage: a role for auto- and paracrine signalling. Acta Physiol. (Oxf.) 209, 95–113
5. Skals, M., Jørgensen, N. R., Leipziger, J., and Praetorius, H. A. (2009) a-Hemolysin from Escherichia coli uses endogenous amplification through P2X receptor activation to induce hemolysis. Proc. Natl. Acad. Sci. U.S.A. 106, 4030–4035
6. Larsen, C. K., Skals, M., Wang, T., Cheema, M. U., Leipziger, J., and Praetorius, H. A. (2011) Python erythrocytes are resistant to a-hemolysin from Escherichia coli. J. Membr. Biol. 244, 131–140
7. Skals, M., Leipziger, J., and Praetorius, H. A. (2011) Haemolysis induced by a-toxin from Staphylococcus aureus requires P2X receptor activation. Pflugers Arch. 462, 669–679
8. Munksgaard, P. S., Vorup-Jensen, T., Reinholdt, J., Söderström, C., Poulsen, K., Leipziger, J., Praetorius, H. A., and Skals, M. (2012) Leukotoxin from Aggregatibacter actinomycetemcomitans causes shrinkage and P2X receptor-dependent lysis of human erythrocytes. Cell Microbiol. 14, 1904–1920
9. Hejl, I. L., Skals, M., Leipziger, J., and Praetorius, H. A. (2013) P2X receptor stimulation amplifies complement-induced haemolysis. Pflugers Arch. 465, 529–541
10. Masin, J., Fiser, R., Linhartova, I., Osicka, R., Bumba, L., Hewlett, E. L., Benz, R., and Sebo, P. (2013) Differences in purinergic amplification of osmotic cell lysis by the pore-forming RTX toxins Bordetella pertussis CyaA and Actinobacillus pleuropneumoniae ApvXa: the role of pore size. Infect. Immun. 81, 4571–4582
11. Skals, M., Bjaeldse, R. G., Reinholdt, J., Poulsen, K., Vad, B. S., Otzen, D. E., Leipziger, J., and Praetorius, H. A. (2014) Bacterial RTX toxins allow acute ATP release from human erythrocytes directly through the toxin pore. J. Biol. Chem. 289, 19098–19109
12. Uhlén, P., Laestadius, A., Jahnuikainen, T., Söderblom, T., Bäckhed, F., Celsi, G., Brismar, H., Normark, S., Aperia, A., and Richter-Dahlfors, A. (2000) a-Haemolysin of uropathogenic E. coli induces Ca<sup>2+</sup> oscillations in renal epithelial cells. Nature 405, 694–697
13. Geyti, C. S., Odgaard, E., Overgaard, M. T., Jensen, M. E., Leipziger, J., and Praetorius, H. A. (2008) Slow spontaneous [Ca<sup>2+</sup>] oscillations reflect nucleotide release from renal epithelia. Pflugers Arch. 455, 1105–1117
14. Douillet, C. D., Robinson, W. P., 3rd, Milano, P. M., Boucher, R. C., and Rich, P. B. (2006) Nucleotides induce IL-6 release from human airway epithelia via P2Y<sub>2</sub> and P2X<sub>3</sub> MAPK-dependent pathways. Ann. J. Physiol. Lung Cell Mol. Physiol. 291, L734–L746
15. Hanley, P. J., Musset, B., Renigunta, V., Limberg, S. H., Dalpke, A. H., Sus, R., Heeg, K. M., Preissig-Müller, R., and Daut, J. (2004) Extracellular ATP induces oscillations of intracellular Ca<sup>2+</sup> and membrane potential and promotes transepithelial IL-6 in macrophages. Proc. Natl. Acad. Sci. U.S.A. 101, 9479–9484
16. Ihara, H., Hirukawa, K., Goto, S., and Tomari, A. (2005) ATP-stimulated interleukin-6 synthesis through P2Y receptors on human osteoblasts. Biochem. Biophys. Res. Commun. 326, 329–334
17. Inoue, K., Hosoi, J., and Denda, M. (2007) Extracellular ATP has stimulatory effects on the expression and release of IL-6 via purinergic receptors in normal human epidermal keratinocytes. J. Invest. Dermatol. 127, 362–371
18. Yoshida, H., Kobayashi, D., Ohkubo, S., and Nakahata, N. (2006) ATP
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stimulates interleukin-6 production via P2Y receptors in human HaCaT keratinocytes. *Eur. J. Pharmacol.* **540**, 1–9

19. Praetorius, H. A., and Spring, K. R. (2001) Bending the MDCK cell primary cilium increases intracellular calcium. *J. Membr. Biol.* **184**, 71–79

20. Bhakdi, S., Mackman, N., Nicaud, J. M., and Holland, I. B. (1986) *Escherichia coli* hemolysin may damage target cell membranes by generating transmembrane pores. *Infect. Immun.* **52**, 63–69

21. Leipziger, J. (2003) Control of epithelial transport via luminal P2 receptors. *Am. J. Physiol. Renal Physiol.* **284**, F419–F432

22. Unwin, R. J., Bailey, M. A., and Burnstock, G. (2003) Purinergic signaling along the renal tubule: the current state of play. *News Physiol. Sci.* **18**, 237–241

23. Jensen, M. E., Odgaard, E., Christensen, M. H., Praetorius, H. A., and Leipziger, J. (2007) Flow-induced [Ca²⁺], increase depends on nucleotide release and subsequent purinergic signaling in the intact nephron. *J. Am. Soc. Nephrol.* **18**, 2062–2070

24. Marques, R. D., de Bruijn, P. I., Sorensen, M. V., Bleich, M., Praetorius, H. A., and Leipziger, J. (2012) Basolateral P2X receptors mediate inhibition of NaCl transport in mouse medullary thick ascending limb (mTLA). *Am. J. Physiol. Renal Physiol.* **302**, F487–F494

25. Bagi, Y., Hausmann, R., Rosefort, C., Rettinger, J., Schmalzing, G., and Müller, C. E. (2011) Discovery of potent competitive antagonists and positive modulators of the P2X2 receptor. *J. Med. Chem.* **54**, 817–830

26. Lee, J., Cacalano, G., Camerato, T., Toy, K., Moore, M. W., and Wood, W. I. (1995) Chemokine binding and activities mediated by the mouse IL-8 receptor. *J. Immunol.* **155**, 2158–2164

27. Chauffier, K., Laiguillon, M. C., Bougault, C., Gosset, M., Priam, S., Salvat, C., Mladenovic, Z., Nouriassat, G., Jacques, C., Houard, X., Berenbaum, F., and Sellam, J. (2012) Induction of the chemokine IL-8/Kc by the articular cartilage: possible influence on osteoarthritis. *Joint Bone Spine* **79**, 604–609

28. Becker, M. D., O’Rourke, L. M., Blackman, W. S., Planck, S. R., and Rosenbaum, J. T. (2000) Reduced leukocyte migration, but normal rolling and arrest, in interleukin-8 receptor homologue knockout mice. *Invest. Ophthal. Vis. Sci.* **41**, 1812–1817

29. Foxman, B. (2010) The epidemiology of urinary tract infection. *Nat. Rev. Urol.* **7**, 653–660

30. Bien, J., Sokolova, O., and Bozko, P. (2012) Role of uropathogenic *Escherichia coli* virulence factors in development of urinary tract infection and kidney damage. *Int. J. Nephrol.* **2012**, 681473

31. Benson, M., Iodal, U., Agace, W., Hellström, M., Mårlid, S., Rosberg, S., Sjöström, M., Wettergren, B., Jönsson, S., and Svanborg, C. (1996) Interleukin (IL)-6 and IL-8 in children with febrile urinary tract infection and asymptomatic bacteriuria. *J. Infect. Dis.* **174**, 1080–1084

32. Benz, R., Schmid, A., Wagner, W., and Goebel, W. (1989) Pore formation by the *Escherichia coli* hemolysin: evidence for an association-dissociation equilibrium of the pore-forming aggregates. *Infect. Immun.* **57**, 887–895

33. Ropele, M., and Menestrina, G. (1989) Electrical properties and molecular architecture of the channel formed by *Escherichia coli* hemolysin in planar lipid membranes. *Biochim. Biophys. Acta* **985**, 9–18

34. Bjaelde, R. G., Arnaudtitter, S. S., Overgaard, M. T., Leipziger, J., and Praetorius, H. A. (2013) Renal epithelial cells can release ATP by vesicular fusion. *Front. Physiol.* **4**, 238

35. Sipos, A., Vargas, S. L., Toma, I., Hanner, F., Willecke, K., and Peti-Peterdi, J. (2009) Connexin 30 deficiency impairs renal tubular ATP release and pressure natriuresis. *J. Am. Soc. Nephrol.* **20**, 1724–1732

36. Koschinski, A., Repp, H., Unver, B., Dreyer, F., Brockmeier, D., Valeva, A., Bhakdi, S., and Walev, I. (2006) Why *Escherichia coli* α-hemolysin induces calcium oscillations in mammalian cells: the pore is on its own. *FASEB J.* **20**, 973–975

37. Bailey, M. A., Imbert-Teboul, M., Turner, C., Marsy, S., Srai, K., Burnstock, G., and Unwin, R. J. (2000) Axial distribution and characterization of basolateral P2Y receptors along the rat renal tubule. *Kidney Int.* **58**, 1893–1901

38. Kruse, R., Säve, S., and Persson, K. (2012) Adenosine triphosphate induced P2Y₂ receptor activation induces proinflammatory cytokine release in uroepithelial cells. *J. Urol.* **188**, 2419–2425

39. Khalil, A., Tullus, K., Barfai, T., Bakht, M., Jaremko, G., and Brauner, A. (2000) Renal cytokine responses in acute *Escherichia coli* pyelonephritis in IL-6-deficient mice. *Clin. Exp. Immunol.* **122**, 200–206

40. Säve, S., and Persson, K. (2010) Extracellular ATP and P2Y receptor activation induce a proinflammatory host response in the human urinary tract. *Infect. Immun.* **78**, 3609–3615

41. Lehrmann, H., Thomas, J., Kim, S. I., Jacobi, C., and Leipziger, J. (2002) Luminal P2Y2 receptor-mediated inhibition of Na⁺ absorption in isolated perfused mouse CCD. *J. Am. Soc. Nephrol.* **13**, 10–18

42. Kishore, B. K., Chou, C. L., and Knepper, M. A. (1995) Extracellular nucleotide receptor inhibits AVP-stimulated water permeability in inner medullary collecting duct. *Am. J. Physiol.* **269**, F863–F869

43. Bullen, J. J., Rogers, H. J., and Leigh, L. (1972) Iron-binding proteins in milk and resistance to *Escherichia coli* infection in infants. *Br. Med. J.* **1**, 69–75

44. Ratledge, C., and Dover, L. G. (2000) Iron metabolism in pathogenic bacteria. *Annu. Rev. Microbiol.* **54**, 881–941

45. Torres, A. G., and Payne, S. M. (1997) Haem iron-transport system in enterohaemorrhagic *Escherichia coli* O157:H7. *Mol. Microbiol.* **23**, 825–833

46. Wood, M. W., Breitschwerdt, E. B., Nordone, S. K., Linder, K. E., and Gookin, J. L. (2012) Uropathogenic *E. coli* promote a paracellular urothelial barrier defect characterized by altered tight junction integrity, epithelial cell sloughing and cytokine release. *J. Comp. Pathol.* **147**, 11–19

47. Bürker, R., Krug, S. M., Rosenthal, R., Günzel, D., Fromm, A., Zeitz, M., Chakraborty, T., Fromm, M., Epple, H. J., and Schulzke, J. D. (2011) AEROLYSIN from *Aeromonas hydrophila* perturbs tight junction integrity and cell lesion repair in intestinal epithelial HT-29/B6 cells. *J. Infect. Dis.* **204**, 1283–1292

48. Rochford, K. D., Collins, L. E., Murphy, R. P., and Cummins, P. M. (2014) Downregulation of blood-brain barrier phenotype by proinflammatory cytokines involves NADPH oxidase-dependent ROS generation: consequences for interendothelial adherens and tight junctions. *PloS One* **9**, e101815

49. Suzuki, T., Yoshinaga, N., and Tanabe, S. (2011) Interleukin-6 (IL-6) regulates claudin-2 expression and tight junction permeability in intestinal epithelium. *J. Biol. Chem.* **286**, 31263–31271