

## Role of the Lipopolysaccharide-CD14 Complex for the Activity of Hemolysin from Uropathogenic *Escherichia coli*<sup>∇</sup>

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**Bacterial pathogens produce a variety of exotoxins, which often become associated with the bacterial outer membrane component lipopolysaccharide (LPS) during their secretion. LPS is a potent proinflammatory mediator; however, it is not known whether LPS contributes to cell signaling induced by those microbial components to which it is attached. This is partly due to the common view that LPS present in bacterial component preparations is an experimental artifact. The *Escherichia coli* exotoxin hemolysin (Hly) is a known inducer of proinflammatory signaling in epithelial cells, and the signal transduction pathway involves fluctuation of the intracellular-Ca<sup>2+</sup> concentration. Since LPS is known to interact with Hly, we investigated whether it is required as a cofactor for the activity of Hly. We found that the LPS/Hly complex exploits the CD14/LPS-binding protein recognition system to bring Hly to the cell membrane, where intracellular-Ca<sup>2+</sup> signaling is initiated via specific activation of the small GTPase RhoA. Hly-induced Ca<sup>2+</sup> signaling was found to occur independently of the LPS receptor TLR4, suggesting that the role of LPS/CD14 is to deliver Hly to the cell membrane. In contrast, the cytolytic effect triggered by exposure of cells to high Hly concentrations occurs independently of LPS/CD14. Collectively, our data reveal a novel molecular mechanism for toxin delivery in bacterial pathogenesis, where LPS-associated microbial compounds are targeted to the host cell membrane as a consequence of their association with LPS.**

The exotoxin hemolysin (Hly) produced by uropathogenic *Escherichia coli* (UPEC) is an important virulence factor in extraintestinal infections, such as urinary tract infections, newborn meningitis, bacteremia, and septicemia (33). Hly exerts a cytolytic effect when present in high concentrations, while low, sublytic concentrations have been demonstrated to induce intracellular-Ca<sup>2+</sup> signaling in epithelial cells, leading to proinflammatory responses, such as production of interleukin-6 (IL-6) and IL-8 (47). Hly is secreted from bacteria via the type I secretion pathway (26) and is found associated with bacterial outer membranes, in supernatants, and in outer membrane vesicles (5, 34). Hly forms high-molecular-weight complexes with lipopolysaccharide (LPS), and this interaction has been suggested to prevent aggregation and degradation of Hly as well as to promote an active conformation of the toxin (6, 12, 15). Whether there is also a molecular function of LPS in the Ca<sup>2+</sup> signaling and cytolytic activities of Hly remains unknown.

Detailed information regarding the signal transduction pathway elicited by LPS does exist, however. LPS is a large amphipathic molecule composed of the acylated, hydrophobic lipid A component, the LPS core, and the O-antigen polysaccharide. Host recognition of LPS is a multistep process initiated by the binding of LPS by the LPS-binding protein (LBP). This interaction enhances the binding of LPS to the glycosylphosphati-

dylinositol-linked receptor CD14, whose protein structure contains an NH<sub>2</sub> terminal hydrophobic pocket where lipid A binds (25). CD14 subsequently presents LPS to the TLR4/MD-2 receptor complex, which initiates the intracellular signaling cascade. The number and nature of lipid A's acyl chains are major determinants of the potency of LPS in eliciting TLR4-dependent host responses, a dynamic feature which is important for inflammation (3, 20).

In the current study, the biological relevance of LPS to the dual activities of Hly was studied. We found that LPS is essential for Hly-induced Ca<sup>2+</sup> oscillations and that this signal is dependent on CD14 and LBP, both known for their involvement in the recruitment of LPS to the eukaryotic membrane. Interestingly, the Hly-induced signaling occurs independently of the LPS receptor TLR4. To increase our understanding of the mechanism by which the Hly-induced Ca<sup>2+</sup> signaling occurs, the involvement of small GTPases was investigated. Small GTPases transmit signals from cell surface receptors via several signaling pathways. We found that activation of the small GTPase RhoA, but not Rac and Cdc42, was required for Ca<sup>2+</sup> signaling. In addition, our data revealed that the cytolytic activities of Hly occur independently of the LPS/LBP/CD14 complex, suggesting that cytolysis is induced via a mechanism different from that used for induction of Ca<sup>2+</sup> oscillations.

### MATERIALS AND METHODS

**Bacterial strains and plasmids.** Bacterial strains are listed in Table 1. The Hly operon (*hlyCABD*) from the fully sequenced human and rat pyelonephritogenic *E. coli* strain CFT073 (American Type Culture Collection [ATCC] no. 700928) was amplified together with an approximately 1.3-kbp-long 5' flanking region,

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TABLE 1. *E. coli* strains used in this study

Strain	Relevant characteristics for this study	Source or reference
CFT073	O6:K2:H1, smooth LPS	48
W3110	K-12, rough LPS	24
ARD20	W3110 pANN202-812, Hly <sup>+</sup> , Amp <sup>r</sup>	47
ARD21	W3110 pANN202-812B, Hly <sup>+</sup> , HlyC <sup>-</sup> , Amp <sup>r</sup>	47
ARD31	W3110 pLT404, Hly <sup>+</sup> , Chl <sup>r</sup>	This study
ARD32	W3110 $\Delta$ <i>msbB</i> (MLK1067) pGNH404, penta-acylated LPS, Hly <sup>+</sup> , Chl <sup>r</sup> , Amp <sup>r</sup>	24 and this study
WAM 1824	CL633 pSF4000, K-12, rough LPS, Hly <sup>+</sup> , Chl <sup>r</sup>	13, 49

using oligonucleotides Hly<sub>forw</sub>-XbaI (TTATCTAGAGGGTACTGGGAAGAC CAGGGTTA) and Hly<sub>rev</sub>-KpnI (ATAGGTACCTTAACGCTCATGTAAACT TTCTGTT). The resulting 8.6-kbp-long PCR product was digested by XbaI/KpnI and ligated into pUC18, generating pGNH404 (ampicillin resistant [Amp<sup>r</sup>]), or into the chloramphenicol-resistant (Chl<sup>r</sup>) pUC18 derivative pPCR-Script Cam SK(+) (Stratagene, Sweden), generating pLT404. In addition, Hly expression plasmids pANN202-812 (the wild-type [wt] *hly* operon from pHly152, Amp<sup>r</sup>) (47), pANN202-812B (with an insertion of TT at position 421 in *hlyC* of pANN202-812, producing Hly lacking acyl chains; Amp<sup>r</sup>) (31, 47), and pSF4000 (the *hly* operon from strain J96, Chl<sup>r</sup>) (49) were used. Bacteria were grown in Luria-Bertani medium (37°C) and supplemented with 20 µg/ml chloramphenicol or 100 µg/ml ampicillin as indicated.

**Toxin preparations.** To ensure that natural interactions between Hly and other bacterial components remained, Hly-containing supernatants from stationary overnight cultures were used. Hly was prepared fresh for each experiment, and lytic activity was measured in sheep blood assays (9), where the Hly concentration resulting in 60% hemolysis of a 2.5% erythrocyte suspension was defined as 1,000 hemolytic units (HU).

A highly purified preparation of Hly was prepared from *E. coli* strain WAM 1824 (CL633 pSF4000). Hly was concentrated by acid-ethanol precipitation from mid-log-phase culture supernatants, denatured by boiling with 1% sodium dodecyl sulfate (SDS), and separated by SDS gel electrophoresis. The Hly polypeptide was eluted from excised gel fragments and dialyzed, before being dissolved in 0.9% NaCl containing 1 mM CaCl<sub>2</sub>. As an internal control for noxious SDS levels in the end product, proHlyA (the inactive, nonacylated, extracellular proform of Hly that is produced in the absence of HlyC) was purified in parallel. The LPS-free proHlyA preparation showed no lytic or cytotoxic activity even at high concentrations. The details of this purification method will be described elsewhere (S. Pellett and R. A. Welch, unpublished data). The *Limulus* amoebocyte lysate assay (BioWhittaker, Walkersville, MD) was used to measure endotoxic units (EU), showing that the purified Hly preparation contained at least 10<sup>5</sup>-fold less LPS (<10<sup>-2</sup> EU/ml) than culture supernatants (6.4 × 10<sup>3</sup> EU/ml). The concentration of toxin was monitored by comparison of the results for Coomassie blue staining of the HlyA band in SDS-polyacrylamide gel electrophoresis gels to known amounts of beta-galactosidase as previously described (6).

**Cell lines and primary cell preparation.** The human bladder carcinoma cell line T24 (ATCC HTB-4) was grown on glass coverslips to 40 to 60% confluence as previously described (4), while primary renal epithelial cells (proximal tubule cells [PTC]) were prepared from 20-day-old Sprague-Dawley rats (47). All cells were serum starved (1%) in the absence of antibiotics 12 to 18 h prior to experiments (47). Animal studies were approved by Stockholms Norra Djurför-söksetiska Nämnd.

**Single-cell Ca<sup>2+</sup> recording.** Cells were incubated with 2 µM Fura-2/AM (acetylmethyl ester; Molecular Probes) in P buffer (100 mM NaCl, 4 mM KCl, 20 mM HEPES, 25 mM NaHCO<sub>3</sub>, 1 mM CaCl<sub>2</sub>, 1.2 mM MgCl<sub>2</sub>, 1 mM NaH<sub>2</sub>PO<sub>4</sub>·H<sub>2</sub>O, and 10 mM D-glucose) for 1 h before ratiometric imaging was performed. Ratiometric imaging was performed at 37°C using a heated chamber (Warner Instruments), mounted on a Nikon TS-100 microscope using a 40×/0.75-numerical-aperture Plan Fluor objective. Emission fluorescence was collected via a PTI image intensifier connected to a PTI Photometrics Coolsnap-cooled charge-coupled-device camera and analyzed using acquisition software from PTI (ImageMaster 3). Clusters containing 15 to 35 cells were chosen in each experiment. Cells were excited at 340 nm and 380 nm every 60 s, and emission was collected at 510 nm. Data shown represent single-cell traces where the ratio, presented in arbitrary units, reflects the change in intracellular-Ca<sup>2+</sup> concentration. Sublytic (130- to 200-HU) and lytic (1,500- to 2,000-HU) amounts of Hly were used as specified. Where indicated, cells were pretreated with monoclonal antibodies (1 h, 37°C) directed toward CD14 (BIG-7 [Biometec, Germany] and MEM18 [EXBIO, Czech Republic]), LBP (BIG-412; Biometec, Germany), or

isogenic immunoglobulin G control antibody (Biometec, Germany) or with 5 mM methyl-β-cyclodextrin (45 min, 37°C; Sigma, Sweden). Where stated, Hly was preincubated on ice with 5 U/ml polymyxin B (PmB) (30 min; Sigma, Sweden), 10 µg/ml penta-acylated LPS<sub>W3110</sub>  $\Delta$ *msbB* (4) (15 min), 6 µg/ml rhCD14 from a cell culture supernatant of p-POL-DHFR-hCD14-transfected CHO-cells (15 min; Biometec, Germany), or a corresponding control supernatant (15 min; Biometec, Germany) (mock treatment).

**Dominant-negative RhoA.** The enhanced green fluorescent protein (EGFP)-tagged dominant-negative RhoAN19 (pEGFP-RhoAN19) expression vector was constructed by amplification of the RhoAN19 sequence from the pEXVRhoAN19 plasmid (36), using primers RhoA\_BamHI\_F (ATTCGGA TCCATGGCTGCCATCCGGAAGAAAC) and RhoA\_XbaI\_R (CGATCTA GATCACAAGACAAGGCAACCAGA). The PCR fragment was cloned into the BamHI and XbaI restriction sites of the pEGFP-C1 expression vector (BD Biosciences). pEGFP-RhoAN19 was transfected into PTC using Lipofectamine 2000 (Invitrogen).

**Affinity precipitation of GTP-Rho, GTP-Rac, and GTP-Cdc42.** GTP-Rac and GTP-Cdc42 bind the p21-binding domain of p21-activated kinase, and GTP-Rho binds the Rho-binding domain (RBD) of the effector protein rothekin. The glutathione *S*-transferase (GST)-RBD and GST-p21-binding domain (PDB) proteins were purified as previously described (17). Cytosolic lysates of PTC exposed to sublytic concentrations of Hly for the indicated times or untreated were prepared in ice-cold buffer and cleared by centrifugation (13,000 rpm, 4°C). They were further incubated with the GST-RBD or GST-PDB protein coupled to glutathione Sepharose (4°C, 45 min), washed, and resuspended in SDS-polyacrylamide gel electrophoresis sample buffer (28) for Western blot analysis using antibodies anti-RhoA (sc179), anti-Cdc42 (sc87) (Santa Cruz Biotechnology), and anti-Rac (clone 102) (Transduction Laboratories, Lexington, KY). The total GTPase content of the cytosolic lysate was similarly analyzed.

## RESULTS

**Role of LPS in the dual, concentration-dependent activities of Hly.** When analyzing the concentration-dependent effects of the UPEC virulence factor Hly, epithelial cells from the urinary tract are commonly used (27, 29, 47). When these cells are exposed to a high dose of the toxin, the cytolytic response can be observed as a rapid and sustained increase of Ca<sup>2+</sup>, while a low dose induces an oscillating intracellular-Ca<sup>2+</sup> signal, leading to proinflammatory responses. Both scenarios can be monitored at a single-cell level, using ratiometric imaging based on the Ca<sup>2+</sup>-binding properties of the fluorophore Fura-2/AM. A typical oscillatory fluctuation of intracellular-Ca<sup>2+</sup> concentration is shown by the pseudocoloration of a cell in Fig. 1A. Alternatively, semicontinuous Ca<sup>2+</sup> recording can be represented in a graph format. Since this communicates a more detailed representation of Ca<sup>2+</sup> fluctuations and enables comparison of Ca<sup>2+</sup> recordings from individual cells, the graph format was chosen for presentation of data throughout this report.

When cells are exposed to Hly, the toxin is naturally complexed with bacterial membrane components. To investigate whether these are of importance for the activity of Hly, a highly

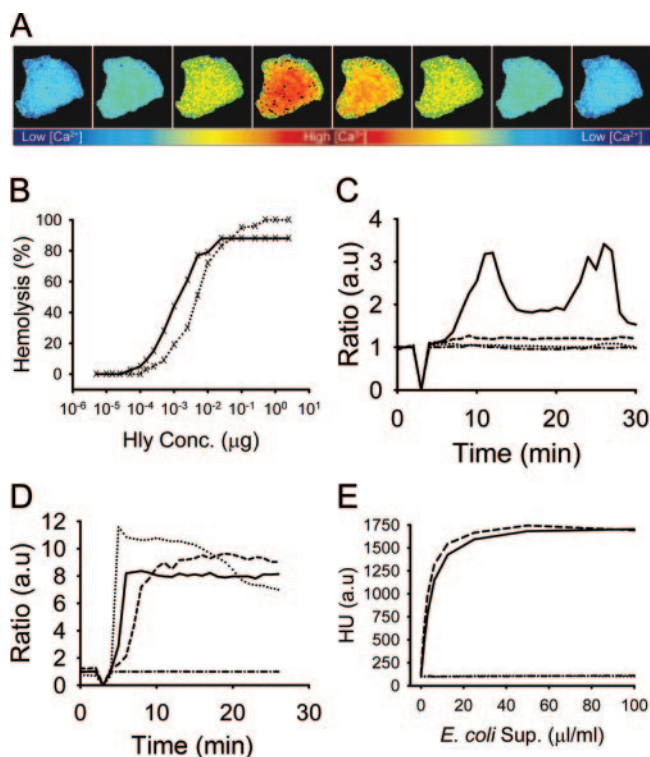


FIG. 1. LPS is required for Hly-induced Ca<sup>2+</sup> signaling but not for cytolysis. (A) Intracellular-Ca<sup>2+</sup> fluctuation visualized by ratiometric imaging in a single cell upon Hly stimulation. The pseudocolored cell shows the basal level of intracellular-Ca<sup>2+</sup> concentration as blue, while increases in intracellular Ca<sup>2+</sup> are depicted as yellow, red, and black. (B) Comparison of the hemolytic activity on sheep erythrocytes by LPS-depleted Hly (dotted line) and Hly-containing supernatant from the source strain (WAM 1824) (continuous line). (C and D) Single-cell recordings of intracellular Ca<sup>2+</sup> in PTC stimulated with a sublytic (130-HU) (C) or lytic (1,500-HU) (D) concentration of Hly-containing supernatant from ARD31 in the absence (continuous line) or presence (dashed line) of PmB. The dotted line represents cells stimulated with LPS-depleted Hly, while supernatant from strain W3110, which lacks Hly, serves as a negative control (dashed-dotted line). (E) Hemolysis of erythrocytes measured in HU upon stimulation with Hly-containing supernatant from ARD31 in the absence (continuous line) or presence (dashed line) of PmB or with non-Hly-containing control supernatant (W3110) with (dashed-dotted line) or without (dotted) PmB. A minimum of three independent experiments was performed, with 15 to 20 cells recorded in each experiment. The basal level of Ca<sup>2+</sup> in cells during the first 3 to 5 min of the recordings is shown; the addition of stimuli is indicated by closing the shutter for one time point (ratio = 0). a.u., arbitrary units; Conc., concentration; Sup., supernatant.

purified Hly preparation, depleted of LPS and other membrane components, was compared to supernatants from Hly-expressing bacterial cultures. The activities of Hly originating from different sources were possible to compare because HU, determined by contact-dependent hemolysis assays, were used to define the amount of stimuli to be used in each experiment, rather than the specific protein concentrations. Thus, the slight decrease in the hemolytic activity of the purified Hly preparation compared to that of the supernatant from which it was purified (Fig. 1B) is compensated for by the fact that the amounts used for stimulation of epithelial cells in the experiments described below were based on HU. The sublytic concentrations used in the experiments described throughout this

paper correspond to 130 to 200 HU, while the lytic concentrations of Hly correspond to 1,500 to 2,000 HU.

The effect of sublytic concentrations of purified Hly on primary rat PTC was compared to that of supernatant originating from strain ARD31. In addition to containing Hly, this supernatant contains LPS and other shedded membrane components. Interestingly, the purified Hly preparation was unable to induce any Ca<sup>2+</sup> response (Fig. 1C), while the supernatant originating from strain ARD31 rapidly induced an oscillating Ca<sup>2+</sup> signal (Fig. 1C). W3110, an isogenic strain which lacks the Hly-encoding plasmid, did not affect Ca<sup>2+</sup> homeostasis (Fig. 1C). This observation is in accordance with previously published data (47) and confirms that Hly is essential for the induction of Ca<sup>2+</sup> oscillations. A different scenario was revealed when lytic concentrations of the two Hly stimuli were used. Rapid, sustained increases in the intracellular-Ca<sup>2+</sup> concentration of PTC were observed when either the purified Hly preparation or the Hly-containing supernatant from ARD31 was used (Fig. 1D). A high, sustained concentration of intracellular Ca<sup>2+</sup> represents a prestage to cytolysis (47) and is eventually lethal for cells (7). Supernatant from W3110, lacking the Hly-encoding plasmid, did not induce any increase in Ca<sup>2+</sup> concentration (Fig. 1D). Collectively, this suggests that a cofactor required for induction of Ca<sup>2+</sup> signaling but not for cell lysis has been removed from the purified Hly preparation. One obvious candidate is LPS, which is reduced by a factor of 10<sup>5</sup> in the purified Hly preparation, as analyzed by *Limulus* assays.

**Components of the LPS recognition system are required for Hly-induced Ca<sup>2+</sup> oscillations.** LPS is a major component of the bacterial supernatant, and it is known to form a complex together with Hly (6, 12; Pellett and Welch, unpublished observations). To address whether there is a specific role for LPS in the activity of Hly, the Hly samples were preincubated with the LPS-binding polycationic peptide PmB. Treatment with PmB alters LPS interactions and neutralizes its immune-stimulatory effect (50). We found that preincubation of sublytic concentrations of Hly-containing supernatant from ARD31 with PmB abolished Hly-induced Ca<sup>2+</sup> signals in PTC (Fig. 1C). In contrast, PmB did not affect the lytic effect on these cells, as illustrated by the rapid and sustained elevation of Ca<sup>2+</sup> (Fig. 1D). Similarly, we found that PmB did not affect the lytic activity in a contact-dependent hemolysis assay on red blood cells (Fig. 1E). To exclude the possibility that PmB exerted any direct effect on erythrocyte viability, control experiments in which red blood cells were treated with supernatant originating from the isogenic strain W3110, thus lacking Hly, were performed. This supernatant showed no lytic effect on the red blood cells, either in the presence or in the absence of PmB (Fig. 1E). Collectively, these results strongly indicate that LPS is required as a cofactor for Hly-induced Ca<sup>2+</sup> oscillations, while the cytolytic activities of Hly occur independently of LPS.

This finding prompted us to analyze whether the Hly-induced Ca<sup>2+</sup> response requires intersecting signaling pathways initiated by the interaction between LPS and its signaling receptor TLR4. Molecular characterization of TLR4 signaling in epithelial cells originates mainly from studies using different uroepithelial cell lines (2, 21, 40). In T24 cells, it was shown that wt LPS, expressing hexa-acylated lipid A, rapidly induces

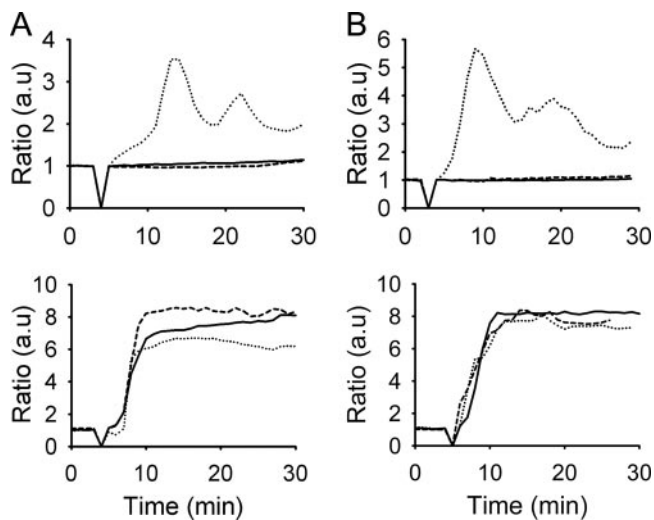


FIG. 2. Hly-induced  $\text{Ca}^{2+}$  signaling requires LBP and CD14 but not TLR4. Ratiometric single-cell recording of intracellular  $\text{Ca}^{2+}$  in TLR4-expressing T24 cells stimulated with sublytic (170- to 200-HU) (A and B, upper panels) or lytic (1,600- to 2,000-HU) (A and B, lower panels) concentrations of Hly-containing supernatant from ARD32. For panel A, cells were preincubated for 60 min with the anti-CD14 antibody BIG-7 (continuous line), the anti-LBP antibody BIG-412 (dashed line), or the isotypic immunoglobulin G control antibody (dotted line). For panel B, the Hly stimuli was preincubated with  $\text{LPS}_{\text{W3110 } \Delta\text{msbB}}$  (continuous line), rhCD14 (dashed line) or a mock treatment supernatant (dotted line) for 30 min prior to addition to the cells. A minimum of three independent experiments was performed, and 15 to 35 cells were recorded in each experiment. a.u., arbitrary units.

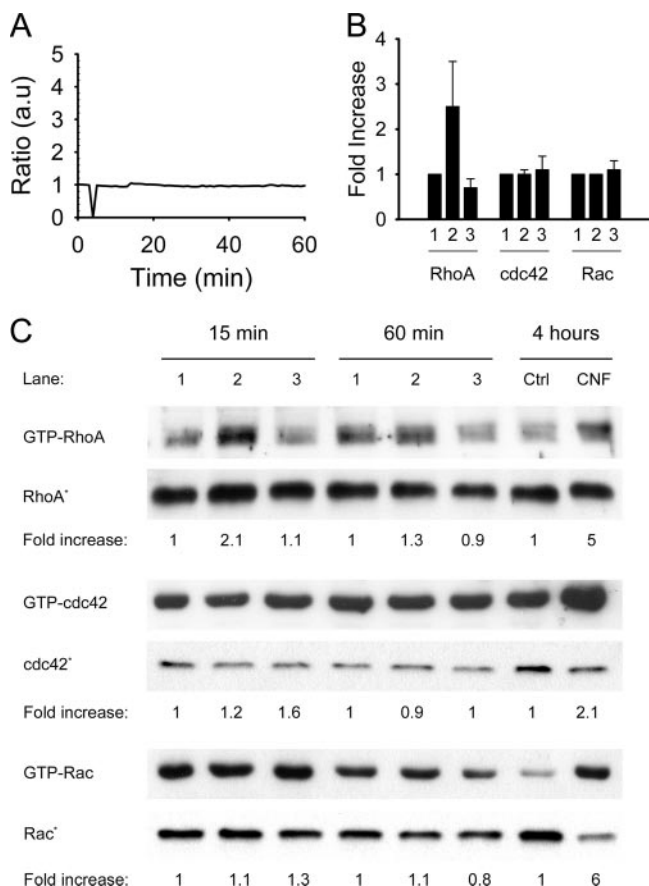
TLR4-dependent proinflammatory responses, while underacylated LPS, exemplified by penta-acylated LPS from the *E. coli* strain W3110  $\Delta\text{msbB}$ , was found to be an insufficient activator (4, 24). This effect was recently ascribed to an altered interaction between penta-acylated LPS and TLR4 (44). Knowing that penta-acylated LPS is unable to induce TLR4 signaling, we introduced a plasmid encoding Hly into strain W3110  $\Delta\text{msbB}$  (ARD32). Supernatant from this strain (containing Hly, penta-acylated LPS, and other membrane components) was used to investigate whether activation of TLR4 is required for the onset of Hly-induced  $\text{Ca}^{2+}$  oscillations. We found that sublytic concentrations of Hly induced  $\text{Ca}^{2+}$  oscillations in T24 cells (Fig. 2A, upper panel). This response was similar to those seen in T24 cells and PTC when the cells were stimulated with Hly-containing supernatant from the isogenic strain ARD31, expressing hexa-acylated LPS (Fig. 1B and data not shown). This finding suggests that TLR4 signaling is dispensable for the activity of Hly. This is also supported by previously published data showing that Hly induces  $\text{Ca}^{2+}$  oscillations in the TLR4-negative epithelial cell line A498 (4, 47).

Although hexa-acylated and penta-acylated LPS interact differently with TLR4, they do not differ in their interactions with the elements acting upstream of the receptor. Both LPS molecules form complexes with components involved in the recruitment of LPS to the eukaryotic cell membrane, e.g., LBP and CD14 (44). There is a possibility that the role of LPS in the induction of  $\text{Ca}^{2+}$  oscillations by Hly may be related to an analogous mechanism for the recruitment of the Hly-LPS complexes to the target cells. To test this hypothesis, T24 cells were

stimulated in the presence of two antibodies inhibiting the interactions between LPS and CD14: BIG-7, which binds to amino acids 9 to 13 and 39 to 44, and MEM18, which binds to amino acids 39 to 44 or amino acids 57 to 64, in the LPS-binding pocket of CD14 (25). Both antibodies completely abrogated the oscillatory  $\text{Ca}^{2+}$  response to sublytic concentrations of Hly (in supernatant from ARD32) (Fig. 2A, upper panel [for BIG-7], and data not shown [for MEM-18]), while the isotype-matched control antibody did not affect the  $\text{Ca}^{2+}$  response (Fig. 2A, upper panel). Hly stimulation of cells in the presence of antibody BIG-412, which blocks LBP-mediated presentation of LPS to CD14, also prevented the induction of  $\text{Ca}^{2+}$  oscillations (Fig. 2A, upper panel). Thus, Hly utilizes the well-established LPS/CD14 interaction to target the cell membrane. To further test the hypothesis that LPS acts as a bridging molecule between Hly and CD14, we preincubated a sublytic concentration of Hly supernatant with excess LPS (10  $\mu\text{g}/\text{ml}$ ) prior to stimulation of the T24 cells, assuming that this would out-compete the interaction between Hly and CD14. We found that this treatment completely abrogated  $\text{Ca}^{2+}$  oscillations (Fig. 2B, upper panel). As predicted, addition of excess recombinant human CD14 (6  $\mu\text{g}/\text{ml}$ ) also prevented  $\text{Ca}^{2+}$  signaling (Fig. 2B, upper panel), while mock-treated cells (see Materials and Methods for details) responded with oscillations (Fig. 2B, upper panel). However, none of these treatments influenced the cytolytic activity of the toxin on epithelial cells (Fig. 2A and B, lower panels), verifying that the cytolytic activity of Hly occurs via a separate mechanism that differs from the LPS/LBP/CD14-dependent, Hly-induced  $\text{Ca}^{2+}$  oscillations.

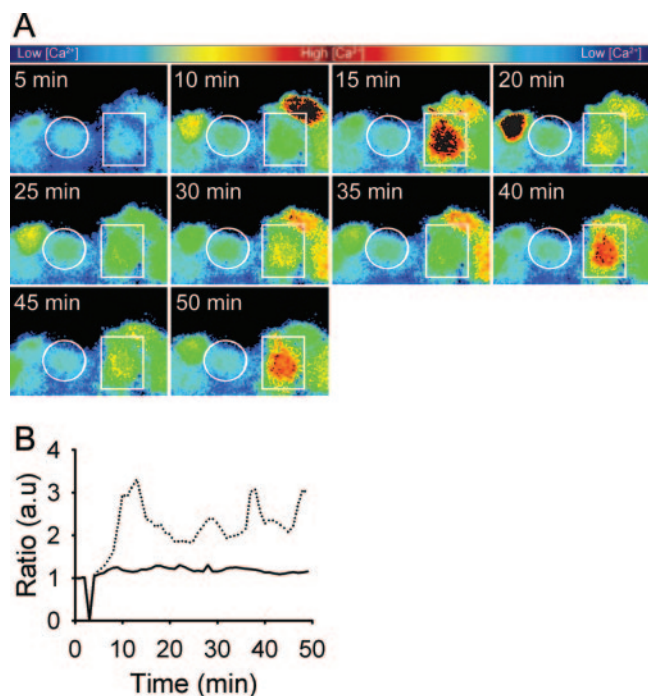
**Hly-induced  $\text{Ca}^{2+}$  signaling requires activation of the small GTPase RhoA.** Numerous signaling pathways in eukaryotic cells originate from receptors located in distinct cholesterol-rich membrane compartments called lipid rafts. These compartments recruit and concentrate receptors and signaling molecules, such as CD14. Lipid rafts were recently shown to be crucial for LPS signaling in monocytes and CHO cells (45). Signal components, such as small GTPases of the Rho subfamily (RhoA, Rac, and Cdc42), which may be recruited to lipid rafts, are well-known regulators of the actin cytoskeleton (11). In addition, recent data suggest that RhoA also transduces signals upon cell stimulation, involving PIP2, PLC $\gamma$ 2, and  $\text{Ca}^{2+}$  mobilization (39). To address whether lipid rafts and associated signaling components are required for Hly-triggered  $\text{Ca}^{2+}$  oscillations, experiments were performed with the isogenic strains ARD20 (wt Hly) and ARD21 (Hly $^{-}$ ). The culture supernatants from these strains have very similar compositions, though ARD20 expresses active Hly, while ARD21 expresses a nonhemolytic proform (31, 47). In addition, the Hly $^{-}$  mutant is unable to induce intracellular- $\text{Ca}^{2+}$  oscillations in PTC (47). To investigate whether lipid raft structures are involved in mediating Hly-induced  $\text{Ca}^{2+}$  oscillation, PTC were treated with the cholesterol-depleting agent methyl- $\beta$ -cyclodextrin (5 mM) prior to stimulation with sublytic concentrations of Hly from ARD20. We found that this treatment completely abrogated  $\text{Ca}^{2+}$  oscillations (Fig. 3A), which suggests that intact lipid rafts are a prerequisite for Hly-induced signaling.

Affinity-binding assays were performed to investigate whether activation of any of the small GTPases RhoA, Rac, and Cdc42 is required to mediate Hly-induced  $\text{Ca}^{2+}$  signaling.



**FIG. 3.** Hly-induced cell signaling occurs in lipid rafts and involves RhoA activation. (A) Ratiometric single-cell recording of intracellular  $Ca^{2+}$  in PTC reveals an abrogated  $Ca^{2+}$  response in cells treated with methyl- $\beta$ -cyclodextrin prior to stimulation with sublytic concentrations of Hly-containing supernatant from ARD20 (130 HU). Three independent experiments were performed, and 15 to 20 cells were recorded in each experiment. (B and C) Affinity precipitation of GTP-RhoA, GTP-Cdc42, and GTP-Rac from untreated cells (lane 1), cells exposed to sublytic concentrations of Hly-containing supernatants from ARD20 (wt Hly) (lane 2), or cells exposed to Hly-containing supernatant from ARD21 (inactive proform of Hly) (lane 3). Panel B shows a statistical overview of the means  $\pm$  standard deviations for three independent experiments, while panel C shows the details of one representative experiment. Cytotoxic necrotizing factor (CNF)-treated cells were used as a positive control (41). Values were normalized to the total amount of RhoA, Cdc42, and Rac present in the cytosolic extract (indicated as Rho\*, Cdc42\*, and Rac\*) and reported as "Fold increase," which represents the ratio between the optical density of the GTP-bound GTPase and that of the GTPase present in the cytosolic extract. a.u., arbitrary units.

Thus, an assay based on the ability of activated GTP-bound forms of the small GTPases to interact with the specific effector domains rhotekin (RhoA) and p21-activated kinase (Rac, Cdc42) was used. GTPase activation levels were analyzed in PTC stimulated with sublytic concentrations of Hly, which in parallel experiments were shown to elicit  $Ca^{2+}$  oscillations. As these were initiated a few minutes after Hly was added, activation of small GTPases was analyzed 15 and 60 min after the addition of Hly-containing culture supernatant. Cells stimulated for 15 min with ARD20 showed a two- to threefold increase in activated RhoA compared to untreated cells (Fig.



**FIG. 4.** Hly-induced cell signaling requires RhoA. Ratiometric single-cell recording of intracellular- $Ca^{2+}$  concentration in PTC stimulated with sublytic concentrations (130 HU) of Hly containing supernatant from ARD20. (A) Time series of images depicting the intracellular- $Ca^{2+}$  concentrations in pseudocolor according to the bar. The white circle indicates one cell transfected with dominant-negative RhoA (pEGFP-RhoN19). The boxed cell, as well as surrounding cells, is nontransfected. (B) Single-cell tracings of the circled cell (transfected with dominant-negative RhoA [pEGFP-RhoN19]) (continuous line) and the boxed nontransfected cell (dotted line). A minimum of three independent experiments was performed, and 15 to 20 cells were recorded in each experiment. a.u., arbitrary units.

3B, lanes 1 and 2, and C, top panel). Activation was specific for RhoA since no increase in GTP-bound Cdc42 or Rac could be detected (Fig. 3B and C, middle and lower panels). In control experiments, we found that none of the small GTPases were significantly activated in cells stimulated with supernatant from ARD21, containing the proform of Hly (Fig. 3B, lane 3, and C). This confirms that activation of RhoA occurs as a consequence of Hly stimulation and is not caused by unspecific stimulation by other bacterial-membrane-associated compounds.

To analyze whether activated RhoA also is functionally involved in mediating Hly-induced  $Ca^{2+}$  signaling, ratiometric  $Ca^{2+}$  imaging experiments in which PTC had been genetically engineered to express an EGFP-tagged dominant-negative mutant form of RhoA (pEGFP-RhoN19) were performed. These cells were identified in the microscope based on their expression of EGFP, while neighboring, nontransfected cells served as internal controls. When cells were exposed to sublytic doses of Hly-containing supernatant from ARD20, the dominant-negative form of RhoA was found to completely abrogate the  $Ca^{2+}$  response. One such cell is depicted as circled in Fig. 4A. In addition, the semicontinuous recording from the same experiment is represented by a continuous line in the graph presented in Fig. 4B. In contrast, the same stimulus was found to induce a prompt  $Ca^{2+}$  response in neighboring, nontrans-

ected cells within the same cell cluster (Fig. 4). Similarly,  $\text{Ca}^{2+}$  oscillations were also induced in cells transfected with the pEGFP-C1 vector control (data not shown). In summary, our data suggest that activation of RhoA is a prerequisite for mediating Hly-induced  $\text{Ca}^{2+}$  signaling.

## DISCUSSION

UPEC is the most common causative agent of urinary tract infections (16). During the course of infection, bacteria ascend into the urinary tract. Depending on the virulence factors expressed, bacteria will preferentially colonize the bladder, causing cystitis, or the kidney, resulting in pyelonephritis. In addition to the attachment organelle P pili, the exotoxin Hly is one of the virulence factors that are most frequently associated with pyelonephritogenic isolates of UPEC (16, 32). Functional studies of Hly activity have until recently almost exclusively been focused on the toxin's cytolytic activity. In these experiments, red blood cells are often used as targets, which may not reflect the full biological relevance of Hly. According to the natural route of infection, bacteria first interact with epithelial cells lining the urogenital tract before breakage of this barrier allows bacteria to reach into deeper tissues. Once they reach the renal interstitium, a massive inflammatory response which commonly results in the clearance of bacteria at the cost of irreversible scar formation is initiated (23, 32). On some occasions during the infection process, bacteria may come into contact with the vasculature, where both nucleated cells and erythrocytes may become targets for the cytolytic activity of Hly. However, at early stages of infection, when bacteria ascend through and colonize the urinary tract, Hly may act on the epithelial cells of the tubular linings, thereby directly or indirectly participating in the cross talk between the mucosal lining and inflammatory cells. An important role for Hly in the kinetics of inflammation-related tissue responses during the first 8 h of infection was recently shown using multiphoton-microscopy-based intravital imaging of infected rat cortical proximal tubules (32).

Evidence supporting this hypothesis was reported some years ago when Hly was shown to exert dual, concentration-dependent activities on primary epithelial cells originating from the renal proximal tubules (29). While high toxin concentrations result in cell lysis, sublytic concentrations were shown to elicit oscillatory fluctuations of intracellular- $\text{Ca}^{2+}$  concentration, occurring with a periodicity of 12 min (47). The  $\text{Ca}^{2+}$ -signaling system is known to operate over a wide temporal range (8), and the slow, Hly-induced  $\text{Ca}^{2+}$  oscillation, in which the intracellular- $\text{Ca}^{2+}$  concentration differed within the physiological range of 0.1  $\mu\text{M}$  to 1.5  $\mu\text{M}$ , was found to contribute to the pathophysiology of infection by triggering the production of the proinflammatory cytokines IL-6 and IL-8 (47). Induction of similar oscillating  $\text{Ca}^{2+}$  responses has also been reported to be triggered by other pore-forming toxins. Listeriolysin O produced by *Listeria monocytogenes* elicits  $\text{Ca}^{2+}$  oscillations in HEK293 cells (37), while those oscillations induced by pneumolysin from *Streptococcus pneumoniae* activate the p38 mitogen-activated protein kinase in neuroblastoma cells (43). Thus, it seems that the induction of signal transduction cascades involving oscillating  $\text{Ca}^{2+}$  signals is a more wide-

spread activity of pore-forming toxins than previously appreciated.

Interestingly, proinflammatory effects of sublytic doses of Hly have also been reported in neutrophils, where the production of large quantities of lipoxygenase and superoxide is induced (10, 19). Although the details of the signals leading to these responses are unknown, it is interesting to note that the concept of sublytic doses of Hly evoking cellular reactions independently from transmembrane pore formation and cytolysis was hypothesized more than 15 years ago (10).

Hly has long been known to physically interact with LPS; however, the biological role of LPS in toxin activity is unclear (12, 15). Our experiments and previous results reveal that LPS at best plays a marginal or indirect role in the cytolytic activity of Hly, both in erythrocytes and in epithelial cells (6). In contrast, the ability of Hly to induce  $\text{Ca}^{2+}$  oscillations strictly requires LPS as a cofactor. Based on data presented in this report, we propose the following model: LPS acts as a carrier molecule that delivers Hly to CD14, which aids in directing this multimolecular complex to the cell membrane, where signaling is initiated. Alternatively, Hly may also directly interact with CD14, which has been shown to interact with other diacylated lipopeptides (42). It is unlikely, however, that CD14 functions as a signaling receptor, since this is a glycosylphosphatidylinositol-linked protein lacking an intracellular signaling domain (46). Although the nature of the signaling receptor is currently unknown, several candidate proteins have been suggested to act as receptors for both sublytic and lytic concentrations of Hly on nucleated cells and erythrocytes (14, 30). Others have suggested that the activity of Hly on target cells depends on the toxins' pore-forming capacities rather than receptor interactions (27). However, these results are difficult to comprehend in light of the fact that the same group has previously published contradictory findings relating to pore-independent signaling of low concentrations of the Hly toxin in nucleated cells (10).

Several bacterial toxins exert their cellular effects by initial interaction with lipid rafts, as exemplified by cholera toxin, anthrax, and listeriolysin (1, 18, 38). Our data suggest that this may also be true for Hly-induced cell signaling, although this must be addressed in further detail. CD14 has been shown to direct LPS to lipid rafts for induction of cell signaling (45), suggesting that the same may occur in CD14-mediated cell targeting of Hly. Due to their dynamic features, lipid rafts can modulate intracellular signal transduction cascades via spatial and temporal recruitment and compartmentalization of relevant signaling molecules. Indeed, we found that disruption of lipid raft integrity abolished  $\text{Ca}^{2+}$  signaling. RhoA has been shown to concentrate in lipid rafts, and its interaction with downstream effector molecules leads to intracellular signaling (35). Interestingly, we found that  $\text{Ca}^{2+}$  signaling required the recruitment of activated RhoA to the plasma membrane and that the introduction of dominant-negative RhoA completely abolished the  $\text{Ca}^{2+}$  oscillations. Further experiments will reveal whether RhoA mediates a direct or indirect link to L-type voltage-operated  $\text{Ca}^{2+}$  channels and the  $\text{IP}_3$  receptor, both known to be required for Hly-induced  $\text{Ca}^{2+}$  oscillations (47).

The novel function of the LPS recognition system presented in this study may represent a general mechanism for activation of alternate signal transduction events by other LPS-associated

microbial molecules. Whether such molecules are expressed from bacteria expressing smooth or rough LPS is irrelevant, since lipid A is reported as the major CD14-interacting part of LPS (25), and accordingly, CD14 is unable to discriminate between these different chemotypes (22). Similarly,  $\text{Ca}^{2+}$  oscillations are induced by Hly originating from strains expressing smooth, rough, and deep-rough LPS (47; data not shown). Thus, we suggest that the common view of LPS as a contaminant in bacterial protein preparations ought to be reevaluated, and LPS should be considered a potential virulence mechanism in targeting exotoxins to the correct membrane compartment.

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