

Listeriolysin O affects barrier function and induces chloride secretion in HT-29/B6 colon epithelial cells

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¹Institute of Clinical Physiology and ²Department of Gastroenterology, Charité Berlin, Germany; ³Institute of Medical Microbiology, Justus Liebig University, Giessen, Germany, and ⁵Helmholtz Centre for Infection Research, Braunschweig, Germany; and ⁴Division of Medical Engineering/Biotechnology, University of Applied Sciences, Jena, Germany

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Richter JF, Gitter AH, Günzel D, Weiss S, Mohamed W, Chakraborty T, Fromm M, Schulzke JD. Listeriolysin O affects barrier function and induces chloride secretion in HT-29/B6 colon epithelial cells. *Am J Physiol Gastrointest Liver Physiol* 296: G1350–G1359, 2009. First published April 16, 2009; doi:10.1152/ajpgi.00040.2009.—*Listeria monocytogenes* is a food-borne pathogen, which is able to induce diarrhea when residing in the intestine. We studied the effect of listeriolysin O (LLO), an extracellular virulence factor of *L. monocytogenes*, on intestinal transport and barrier function in monolayers of HT-29/B6 human colon cells using the Ussing technique to understand the pathomechanisms involved. Mucosal addition of LLO, but not a LLO mutant, induced a dose- and pH-dependent increase in short-circuit current (I_{SC}). Sodium and chloride tracer flux and DIDS sensitivity studies revealed that I_{SC} was mainly due to electrogenic chloride secretion. Barrier function was impaired by LLO, as assessed by transepithelial resistance (R') and mannitol flux measurements. Intracellular signal transduction occurred through Ca^{2+} release from intracellular stores and PKC activation. In conclusion, listeriolysin induces chloride secretion and perturbs epithelial barrier function, thus potentially contributing to *Listeria*-induced diarrhea.

listeria; diarrhea; transepithelial resistance; HT-29/B6 cells; calcium

VECTORIAL ION MOVEMENT THROUGH a subset of apical channels in the intestinal epithelium produces osmotic gradients that drive net fluid movement into the lumen. Regulation of anion secretion by intracellular second messengers is considered to be the main determinant for epithelial fluid secretion. Hence, from a general pathophysiological point of view, enteric pathogens may exert diarrheal effects through different mechanisms (24), for example, by damaging the epithelial barrier leading to leak-flux of solutes into the intestinal lumen (25) or inducing active fluid secretion through activation by second messengers such as cyclic nucleotides (6) often accompanied by an increase in free intracellular calcium concentration [Ca^{2+}]_i (36, 51).

Listeria monocytogenes is a gram-positive enteric pathogen. It may cause life-threatening diseases such as meningitis, meningoencephalitis, septicemia, or severe gastroenteritis (45). An infection may occur after consumption of *Listeria*-contaminated food, e.g., soft cheese or raw milk. Ingested *Listeria* are believed to invade small bowel enterocytes via an E-cadherin-dependent mechanism (12), but living bacteria are also shed via the feces (17) and are therefore present throughout the GI tract. Once infected, both humans and animals produce antibodies against the major *Listeria* virulence factor listeriolysin

O (LLO) (5, 14, 37), even in cases of noninvasive mild illness with diarrhea (17). Although sporadic gastroenteritis due to *L. monocytogenes* may be uncommon (44), several outbreaks of food-borne gastroenteritis are known to have been caused by *L. monocytogenes*, with diarrhea as the most prevalent symptom (2, 17, 30, 39, 45). LLO is an extracellularly secreted 56-kDa protein and a member of the cholesterol-dependent cytolysin family. The toxin's function for the infectious cycle of the bacterium is well understood. It plays an essential role in the escape mechanism from phagosomes as avirulent mutant strains lacking the *hly* gene are incapable of escaping from phagosomes and are therefore killed by host phagocytes (16). There is accumulated evidence to suggest that, besides its phagosome-lysing potential, LLO acts as a multifunctional virulence factor exerting various effects on host cells. These are 1) activation of the MAP kinase signaling pathway (50), 2) production of host signaling molecules such as inositol trisphosphate and diacylglycerol (48), 3) expression of cell adhesion molecules in infected endothelial cells (35), 4) NF- κ B activation (32) and 5) induction of mucus exocytosis in intestinal cells (15). These cellular responses are Ca^{2+} dependent and therefore regulated by [Ca^{2+}]_i (6). It has also been demonstrated that there is influx of calcium from the extracellular environment through LLO-induced pores (42) and a recent study proposed an LLO-mediated release of Ca^{2+} from intracellular stores in mast cells (28). Furthermore, spontaneous aggregation of lipid rafts on host cell membranes has been described to induce additional signaling cascades within the host cell (27).

Although a large amount of data is available on individual *Listeria* virulence factors acting during systemic infection, the underlying mechanisms of diarrhea largely remain unexplored. Listeriolysin is, however, a likely candidate for the diarrheic effect. Hence, we decided to investigate effects of purified listeriolysin on intestinal transport and barrier function using the human intestinal model epithelium HT-29/B6. These cells represent a stable, highly differentiated subclone derived from wild-type HT-29 cells by glucose deprivation (34). When grown on permeable supports, they form polarized epithelia with intact tight junctions, the key determinant of barrier function. In addition, HT-29/B6 cells are capable of producing vectorial Cl^- and mucin secretion (21, 34). Thus, this highly differentiated cell line possesses characteristic features of native intestinal epithelia necessary for maintenance of intestinal barrier function, as well as for the regulation of intestinal ion transport. For these reasons, HT-29/B6 cells have been used as model epithelium for mechanistic investigations of altered

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intestinal ion secretion and barrier function in several previous studies (9, 20, 21, 22, 34).

MATERIALS AND METHODS

Cell culture techniques. HT-29/B6 cells (34) of the 33rd–38th passage were seeded on Millicell-PCF (poly-carbonate-filter) cell culture inserts (Millipore, Eschborn, Germany) and cultured under standard conditions (pH 7.3; 37°C; 95% O₂ and 5% CO₂) in Petri dishes that contained RPMI (Roswell Memorial Park Institute, Buffalo, NY) medium with *N*-acetyl-L-alanine-L-glutamine and 2.0 g/l NaHCO₃ (FG1215, Biochrom, Berlin, Germany), supplemented with 10% FCS, 100 units/ml penicillin and 100 µg/ml streptomycin (Biochrom, Berlin, Germany). Transepithelial electrical resistance (*R'*) measurements were used to test for confluence. After 7–10 days of cultivation, confluent monolayers with a *R'* between 300 and 600 Ω·cm² were selected for the experiments.

Electrophysiological measurements. HT-29/B6 cells were mounted into modified Ussing chambers. These were driven by an eight-channel computer-controlled voltage clamp device, as previously described (34). The bathing solution contained (in mmol/l): 113.6 NaCl, 2.4 Na₂HPO₄, 0.6 NaH₂PO₄, 21 NaHCO₃, 5.4 KCl, 1.2 CaCl₂, 1 MgSO₄, 10 D(+)-glucose. The solution was gassed and mixed using a bubble lift (95% O₂-5% CO₂, pH 7.4). Adjustment to pH 6.5 was done on the apical side only and was achieved by reducing the concentration of NaHCO₃ to 4 mM (substituted with NaCl). This made it possible to use essentially the same buffer solution and carbogen gas lift for the Ussing chamber experiments. To obtain a bicarbonate-free solution, NaHCO₃ was replaced by NaCl and buffered using 10 mM MES [2-(*N*-morpholino)ethanesulfonic acid]. This solution was then gassed with pure oxygen in the Ussing chambers. The temperature was kept at a constant temperature level at 37°C by means of a temperature-controlled water jacket. Each side of the monolayer was perfused with 5 ml of bathing solution and BASE of a final concentration of 0.1% and, to prevent foaming, 0.0002% sab simplex (Parke-Davies, Karlsruhe, Germany) was routinely added. Short-circuit current (*I*_{SC} in µA·cm⁻²) and transepithelial resistance (*R'* in Ω·cm²) were continuously recorded. The resistance of the bathing solution and the electrode offsets were determined and subtracted from the raw data before each experiment. *I*_{SC} was converted to monovalent cation flux equivalents and is given in µmol·h⁻¹·cm⁻². Bidirectional isotope fluxes of ³⁶Cl⁻ and ²²Na⁺ were measured using the method of Schultz and Zalusky (47). Na⁺ and Cl⁻ fluxes were determined by adding simultaneously ²²Na⁺ and ³⁶Cl⁻ to either the mucosal or serosal side. After 30 min of stabilization, two 10 min-periods were performed before and one after the addition of LLO when the induced *I*_{SC} response was at its maximum. Isotopes were determined by sequential counting in a 1480 WIZARD 3'' gamma counter (Perkin-Elmer, Abtsteinach, Germany) and a TRICARB 2100 TR liquid scintillation counter (Packard, Frankfurt, Germany). Tissues were matched for conductance for calculation of net fluxes. The residual flux (*J*_R) was calculated from the difference in *I*_{SC} and the net Na⁺ and Cl⁻ fluxes: $J_R = I_{SC} - (J_{Na} - J_{Cl})$. ³H-mannitol fluxes were conducted from the mucosal to the serosal side. Flux samples were taken in 10-min intervals as described above.

Cytotoxicity assay. As a monitor of cell disruption, the lactate dehydrogenase (LDH) release from the cells was measured (38). Briefly, LDH content in the mucosal bathing solution of controls and of LLO-treated cells in Ussing chambers was determined at indicated time points. After detergent extraction with 2% Triton X-100 for 20 min, the total LDH content of the residual cells was measured. Then, the percentage of LDH released into the supernatant could be calculated.

Measurement of intracellular Ca²⁺. Measurements of [Ca²⁺]_i were performed using the Ca²⁺-sensitive dye FURA-2AM (Sigma, Deisenhofen, Germany) based on a method described by Grynkiewicz et al. (29). Cells were cultured on coverslips under the same conditions as on filter supports. Before each experiment, semiconfluent

cells were incubated in control Ringer solution (in mmol/l: 151 Na⁺, 5 K⁺, 1.7 Ca²⁺, 0.9 Mg²⁺, 158.4 Cl⁻, 0.9 SO₄²⁻, 1 H₂PO₄⁻, 10 HEPES, and 2 glucose; pH 7.4) with 10 mM FURA-2AM for 30 min at room temperature. The dye was loaded by diffusion and intracellular cleavage of FURA-2AM to FURA-2. The coverslip was then placed into a perfusion chamber on the stage of an inverted microscope. Cells were perfused with control solution for at least 30 min to wash out extracellular dye before measurement started. Agents were injected into the perfusion reservoir. The excitation light was generated by a xenon lamp (Osram XPO 75 W/2, Munich, Germany) and filtered by two rotating filters (*F* = 6/s) at 340 and 380 nm. Relative fluorescence of the dye after excitation was registered at 510 nm by means of a photomultiplier (Hamamatsu 928 SF, Hamamatsu Photonics, Hamamatsu, Japan) with consequent signal detection with an EPC-9 patch-clamp amplifier (HEKA Electronics, Lamprecht, Germany). For data storage and data processing, the TIDA for Windows software was used. A change in the 340/380 nm fluorescence ratio represents a relative change in [Ca²⁺]_i.

Chemicals. H-8, *N*-[2-(methylamino)ethyl]-5-isoquinoline sulfonamide hydrochloride, was obtained from Biotrend Chemikalien (Cologne, Germany), ionomycin was purchased from Fluka (Buchs, Switzerland), chelerythrine from RBI (Natick, MA), DIDS (disodium 4,4'-diisothiocyanatostilbene-2,2'-disulfonate), theophylline, caffeine, forskolin, thapsigargin, and BAPTA-AM [1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis(acetoxymethyl) ester] from Sigma (Deisenhofen, Germany). Forskolin and thapsigargin were added to the serosal compartment, ionomycin and DIDS to the mucosal compartment. All of the other substances were added to both sides of the epithelium. To exclude direct interaction between inhibitors and the toxin, chelerythrine (50 µM) and thapsigargin (5 µM) were premixed with listeriolysin (5 µg/ml) in 1 ml of BSA-containing bathing solution, incubated for 5 min at 37°C, and then applied to the mucosal bathing solution, which added up to final concentrations of 10 µM of chelerythrine, 1 µM of thapsigargin, and 1 µg/ml of LLO. No differences were observed in the resulting *I*_{SC} response. Cell monolayers were loaded with BAPTA by preincubating the filter supports for 1 h in 50 µM of BAPTA acetoxymethyl ester (BAPTA-AM).

Expression and purification of listeriolysin O. Listeriolysin O (LLO) was expressed and purified from the wild-type *L. innocua* 6a strain, as previously described (18) with some modifications. An overnight bacterial culture grown at 37°C in brain-heart infusion broth was used to inoculate one liter of the chemically defined minimal medium (41). Following 48 h of incubation at 30°C, bacteria were removed by centrifugation, and the supernatant fluid was concentrated to 50 ml using a Millipore filtration apparatus with a cut-off point of 10 kDa. The crude supernatant was then batch-absorbed with Q-Sepharose (Pharmacia, Freiburg, Germany) and pre-equilibrated with loading buffer (50 mM NaH₂PO₄, pH 6.2) for 60 min. The nonabsorbed fraction was centrifuged and desalted by transferring through a super loop to a HiPrep 26/10 desalting column (Pharmacia, Freiburg, Germany), where loading buffer (50 mM NaH₂PO₄, pH 6.2) was used to elute the desalted fraction. This fraction was subsequently filtered through a Millipore filter (0.22 µm) and loaded onto a Resource-S column previously equilibrated with 50 mM NaH₂PO₄, pH 6.2. The pure toxin eluted reproducibly from the column at 0.21 to 0.28 M NaCl using elution buffer (50 mM NaH₂PO₄ 1 M NaCl). Fractions were collected and individually tested for hemolytic activity. Serial dilutions of toxin fractions were incubated with erythrocytes in PBS at pH 5.7 for 1 h at 37°C, briefly centrifuged, and the absorbance was measured at 414 nm. Lytic activity was calculated in relation to values obtained by using deionized water as the lytic agent. Protein desalting and purification processes were carried out using the high-performance chromatography system ÄKTA explorer and UNICORN control system (Pharmacia, Freiburg, Germany). LLO has shown a high purity on SDS-PAGE as it appeared as a single band of 58 kDa, which was recognized with LLO-specific monoclonal anti-

bodies in immunoblot assay. Protein concentration was determined using a standard assay (10). For a second set of experiments recombinant listeriolysin was purchased from Diatheva (Fano, Italy), which yielded the same results. Mutants LLO S484 and SAA were generated via overlap extension PCR using primers: S484 forward 5'-CGCTAAA-GAATCCACTGGTTTAG and its complementary oligonucleotide as reverse primer and SAA forward 5'-CGCTAAAGAATCCACTGG-TTTAGCTTGGGAAGCGGCGAGAACGGTAA and its complementary oligonucleotide as the reverse primer. Briefly, two fragments of the 3' part of LLO between the EcoRI and BamHI sites were amplified using the mutagenesis primers and outside primers, including either the EcoRI or the BamHI site. Fragments were combined and amplified using the outside primers. After cloning into pBluescript, the fragments were complemented with a PCR product containing the five regions of LLO, including its promoter as a Sall-EcoRI fragment. Subcloning, expression, purification, and functional testing was carried out as described for WT LLO (18). Both mutants were confirmed by DNA sequencing.

Statistics. The results are given as means \pm SE. The data were tested for differences by means of the unpaired two-tailed *t*-test with Bonferroni correction for multiple testing. Inhibitor data were normalized on respective control responses within the same experiment and compared by one-sample *t*-testing against 100%. $P < 0.05$ was considered significant.

RESULTS

Monolayers with stable transepithelial resistance (R^t) values after 5–6 days of cultivation were mounted into Ussing chambers under voltage-clamp conditions for use in subsequent experiments.

Effect of listeriolysin on electrophysiological properties of HT-29/B6 monolayers. The addition of 2 $\mu\text{g/ml}$ ($= 35 \text{ nM}$) of listeriolysin O (LLO) to the mucosal bath of an Ussing chamber elicited a transient increase in short-circuit current (I_{SC}) from 0.15 ± 0.01 to $1.00 \pm 0.21 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($\Delta I_{SC} = 0.85 \pm 0.21 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$) (Fig. 1A), while R^t was transiently decreased from $550 \pm 40 \Omega\cdot\text{cm}^2$ to $206 \pm 24 \Omega\cdot\text{cm}^2$ ($n = 6$) (Fig. 1B). For comparison, stimulation with 10 μM of serosal forskolin, a standard secretagogue acting via adenylate-cyclase activation, raised the short-circuit current to a maximum level of $1.66 \pm 0.02 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$. The LLO-induced I_{SC} peaked 3.9 \pm 0.2 min after addition of the toxin, whereas a maximum drop in R^t was noted after 6.9 \pm 0.3 min ($P < 0.001$). The I_{SC} and R^t peak responses to LLO were dose-dependent (Fig. 2). The effect of LLO on I_{SC} and R^t in HT-29/B6 monolayers was found repeatedly inducible and almost fully reversible at low concentrations, e.g., at 1 $\mu\text{g/ml}$ (Fig. 3), but only partly reversible at higher concentrations such as 8 $\mu\text{g/ml}$ (data not shown). When a LLO-containing solution, which had previously produced a transient I_{SC} response in HT-29/B6 monolayers, was transferred to a second HT-29/B6 epithelium immediately after the I_{SC} effect had ceased, no response could be observed. Transferring LLO-containing solution while the I_{SC} response was still present in the donor chamber did elicit a response, though smaller in magnitude. LLO showed little effect when applied to serosal bathing solution: 2 $\mu\text{g/ml}$ changed I_{SC} by $4.7 \pm 0.4 \text{ nmol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($n = 4$) or 2% of the mucosal response. To test for absorption of LLO to the filter support, we covered the mucosal surface of the monolayers with an identical filter membrane. Subsequently, the I_{SC} response with LLO applied to a filter-covered mucosal side decreased to a mere 7.4% of the original response. Therefore, it is reasonable to conclude that the cell culture filter also significantly blocked the access

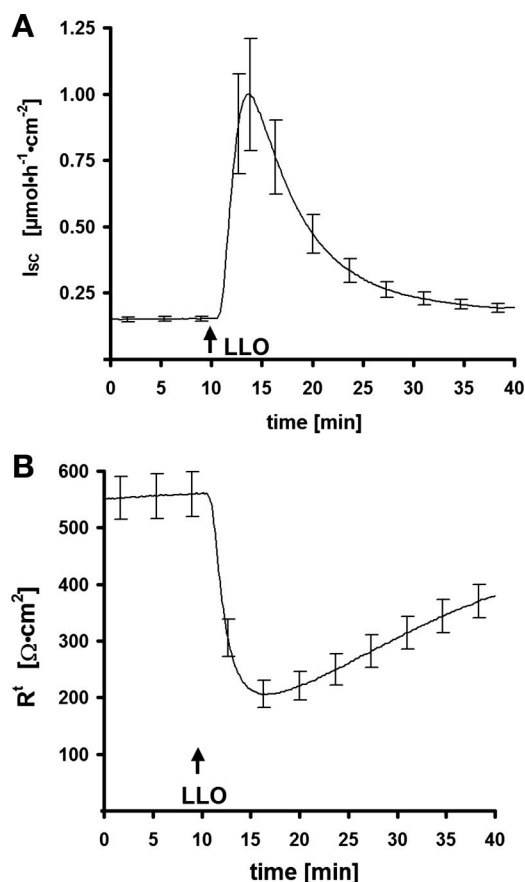


Fig. 1. Effect of listeriolysin O (LLO) on electrical parameters. A: time graph of short-circuit current (I_{SC}). B: transepithelial resistance (R^t) of confluent monolayers of HT-29/B6 cells after LLO challenge. After an equilibration period, LLO (2 $\mu\text{g/ml}$) was added to the mucosal bathing solution (arrows indicate time of addition). Graphs represent means \pm SE ($n = 6$).

of listeriolysin to the epithelium when applied to the serosal bath. Specificity of the LLO effect was tested using mutant toxin variants. On application of mutant LLO, the I_{SC} response entirely failed when working with nonlytic SAA variant ($0.95 \pm 0.24\%$ of control I_{SC} , $n = 4$, $P < 0.01$) and decreased when applying S484 variant with a residual lytic potency of about 25% (11) ($28.94 \pm 8.72\%$ of control I_{SC} , $n = 4$, $P < 0.01$) (Fig. 4A). Because the effects of LLO are assumed to be mediated by its lytic potency toward cellular membranes and increase with lower pH values (46), we varied the mucosal bathing solution in acidity. A reduction to pH 6.5 in the mucosal bathing solution almost tripled the peak I_{SC} response to LLO ($276.7 \pm 45.5\%$ of control I_{SC} , $n = 5$, $P < 0.05$), while the shape of the response was uninfluenced. At the same time, lower pH values had no effect on a caffeine- (10 mM) induced I_{SC} response. This indicates that the cellular secretory response to calcium-mobilizing agents remained unaffected [1.84 ± 0.26 at pH 6.5 vs. $2.02 \pm 0.13 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ at pH 7.4, $n = 3$, nonsignificant (ns)].

Listeriolysin-induced Cl^- secretion. The increase in I_{SC} suggested activation of anion secretion in response to LLO. Electrogenic Cl^- secretion occurring in response to several secretagogues was previously demonstrated in HT-29/B6 monolayers, whereas no evidence was found to suggest an induction of sodium absorption in this cell clone (34, 20). The

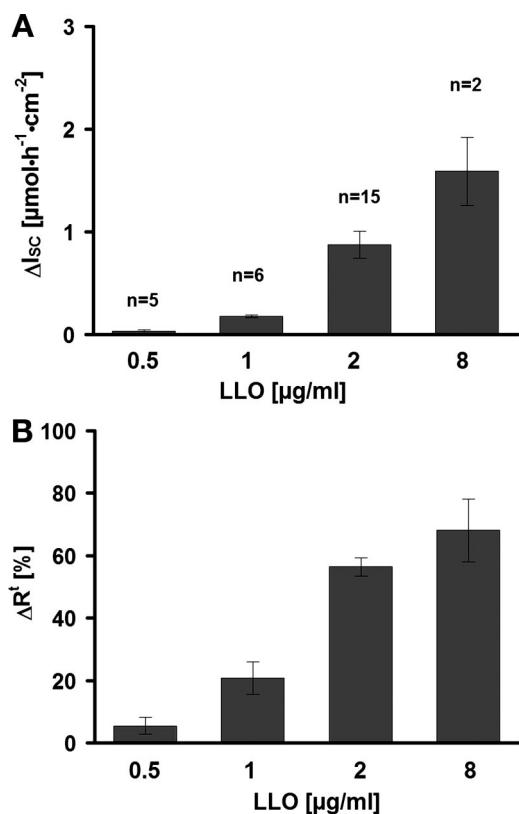


Fig. 2. Dose-response to LLO. Dose-response curve (peak responses) of LLO-induced absolute changes in short-circuit current (I_{SC}) (A) and corresponding relative changes in transepithelial resistance (R') (B). Bars represent different LLO concentrations as indicated. Values are given as means \pm SE (the number of monolayers for each concentration is shown at bottom line of A).

ionic basis of the LLO-induced I_{SC} was identified through unidirectional Na^+ and Cl^- fluxes. Unidirectional Na^+ fluxes were not significantly increased after the addition of 5 $\mu\text{g/ml}$ of LLO. However, LLO did increase the serosal-to-mucosal and the net Cl^- flux, thus indicating active electrogenic chloride secretion, whereas the net Na^+ flux (active sodium absorption) and the residual flux (usually assumed to reflect active bicarbonate secretion) remained unchanged (Table 1). Numerically, there was a good correlation between ΔI_{SC} ($0.46 \pm 0.05 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$) and chloride net flux in secretory direction ($-0.39 \pm 0.18 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$) supporting the conclusion that the LLO-induced I_{SC} was mainly due to electrogenic chloride secretion. Chloride secretion was not caused by leakage from LLO pores, since 1 mM lanthanum, a blocker of LLO pore-induced conductance (8), did not affect the LLO-induced I_{SC} response ($97.4 \pm 26.3\%$ of control I_{SC} , $n = 6$, ns). To characterize the contribution by apical chloride channels, DIDS (4,4'-diisothiocyanostilbene-2,2'-disulfonic acid), a blocker of calcium-activated chloride channels, was applied. It inhibited the LLO-induced I_{SC} response ($22.6 \pm 9.0\%$ of control I_{SC} , $n = 4$, $P < 0.01$) (Fig. 4A) and blocked the effect of LLO completely when added in HCO_3^- -free solution ($3.2 \pm 0.1\%$ of control I_{SC} , $n = 3$, $P < 0.01$). DIDS also immediately blocked the I_{SC} response when added to the mucosal bathing solution shortly after LLO (Fig. 4B).

Listeriolysin affects epithelial barrier. An activation of short-circuit current (I_{SC}) is always accompanied by a drop in

transepithelial resistance (R') due to the opening of conductive transport sites (e.g., ion channels). Thus, the question arose whether the observed decrease in R' seen in response to LLO was merely the result of the I_{SC} increase or due to an additional barrier effect. Evidence in favor of an additional barrier effect was 1) the different time graphs of the R' and I_{SC} peak responses to LLO (Fig. 1) and 2) the incomplete reversibility of the R' decrease at higher LLO concentrations when I_{SC} response had already ceased again.

To clarify this, a direct comparison to another secretagogue, the adenylate cyclase activator forskolin, was made. Compared with LLO, induction of an I_{SC} of similar magnitude by forskolin [$1.21 \pm 0.09 \mu\text{mol}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$ ($n = 3$)] led to a clearly smaller reduction of R' ($\Delta R'_{\text{Forskolin}} = 96 \pm 5 \Omega\cdot\text{cm}^2$ vs. $\Delta R'_{\text{LLO}} = 240 \pm 15 \Omega\cdot\text{cm}^2$, $P < 0.01$; initial R' values were not different between both groups) (Fig. 5). Exposure of HT-29/B6 monolayers to 5 $\mu\text{g/ml}$ of LLO led to a significant increase in permeability for mannitol, as indicated by the

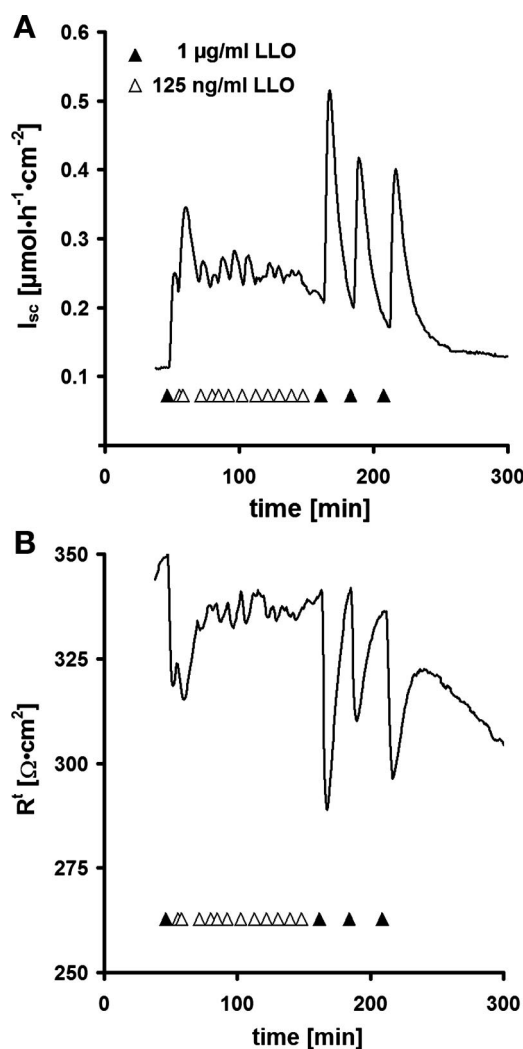


Fig. 3. Repetition of the LLO effect. Short-circuit current (I_{SC}) (A) and transepithelial resistance (R') responses to LLO (B) were repeatedly inducible and reversible. LLO in concentrations of 125 ng/ml or 1 $\mu\text{g/ml}$ was repeatedly added to the mucosal compartment of HT-29/B6 monolayers as indicated by triangles. A representative experiment is shown (out of 3 yielding the same result).

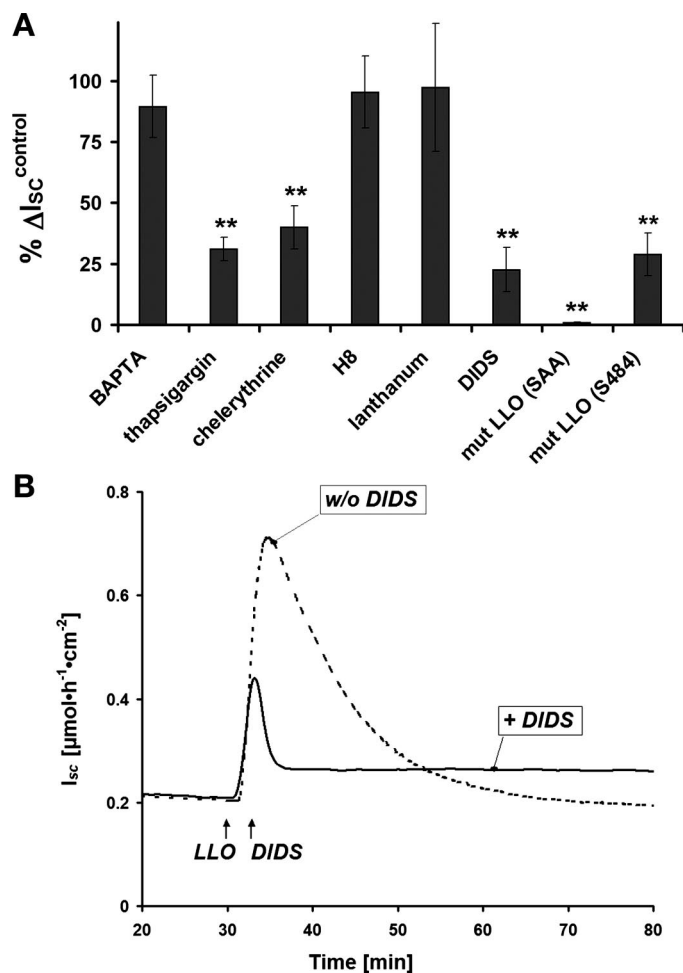


Fig. 4. Effect of inhibitors on LLO response. A: effect of different inhibitors on LLO-induced I_{sc} response is shown. BAPTA-AM (50 μ M) is a membrane-permeable Ca^{2+} chelator. Thapsigargin (1 μ M) depletes intracellular Ca^{2+} stores by inhibiting the intracellular Ca-ATPase. Chelerythrine (10 μ M) inhibits PKC, and H-8 (10 μ M) inhibits PKA. Lanthanum (1 mM) inhibits LLO-pore conductance and calcium channels. DIDS (500 μ M) is an inhibitor of calcium-activated chloride channels (CaCC), SAA and S484 are LLO-mutants attenuated (S484) and nonlytic (SAA) by amino acid substitution (see MATERIALS AND METHODS). The maximum I_{sc} response in the presence of the respective inhibitor was compared with monolayers pretreated with the carrier only (ΔI_{sc} set to 100%). Values are expressed as means \pm SE of six monolayers for each inhibitor; LLO mutants and DIDS were $n = 4$.

** $P < 0.01$. B: inhibitory effect of DIDS (500 μ M) is shown when added to the rising I_{sc} response to LLO. Graphs represent one out of three similar experiments.

mucosal-to-serosal tracer flux (Table 2). To test for LLO effects on viability of the cells, a lactate dehydrogenase (LDH) release assay was performed. Another measure of the viability status of secretory epithelia is their ability to respond to

Table 1. LLO effect on ion fluxes

	I_{sc}	J_{Na}^{ms}	J_{Na}^{sm}	J_{Na}^{Net}	J_{Cl}^{ms}	J_{Cl}^{sm}	J_{Cl}^{Net}	J_{Res}
Control	0.12 \pm 0.01	0.99 \pm 0.10	0.94 \pm 0.08	0.05 \pm 0.13	0.91 \pm 0.10	0.92 \pm 0.04	-0.01 \pm 0.11	0.06 \pm 0.12
LLO	0.57 \pm 0.04	1.37 \pm 0.11	1.26 \pm 0.16	0.11 \pm 0.22	1.18 \pm 0.10	1.57 \pm 0.12	-0.40 \pm 0.16	0.07 \pm 0.23
Δ	0.46 \pm 0.05	0.38 \pm 0.16	0.32 \pm 0.18	0.06 \pm 0.24	0.26 \pm 0.12	0.65 \pm 0.13	-0.39 \pm 0.18	0.00 \pm 0.23
P	<0.001	NS	NS	NS	NS	<0.001	<0.05	NS

Effect of listeriolysin (LLO) on unidirectional ^{22}Na and ^{36}Cl fluxes in HT-29/B6 monolayers. Data are given in μ mol·h⁻¹·cm⁻² and represent means \pm SE of 9 paired monolayers (matched for conductance). I_{sc} , short-circuit current; J_{Na} , unidirectional sodium flux; J_{Cl} , unidirectional chloride flux; ms, from mucosal to serosal; sm, from serosal to mucosal; Net, net flux ($J_i^{ms} - J_i^{sm}$); NS, not significantly different.

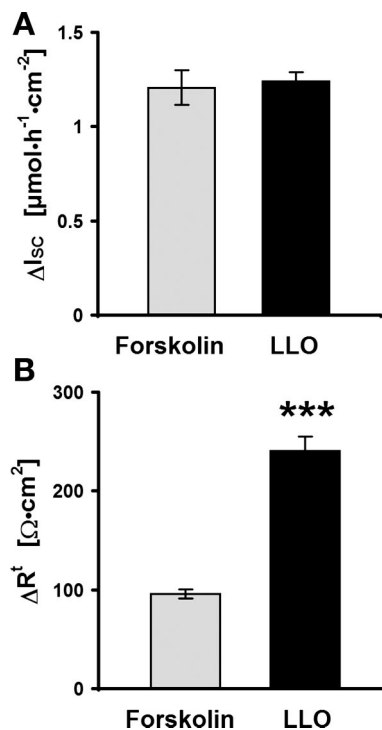


Fig. 5. Barrier effect of LLO. LLO- and forskolin-induced short-circuit current (A) and corresponding changes in transepithelial resistance (B) are presented. Bars represent peak responses and are given as means \pm SE ($n = 6$). *** $P < 0.001$ vs. forskolin.

secretagogues, such as the adenylate cyclase activator forskolin (20 μ M) or the phosphodiesterase inhibitor theophylline (10 mM). When a combination of these two secretagogues was applied, no difference was observed in control vs. 1 μ g/ml of LLO-preincubated monolayers (99.9 \pm 1.8% of control response, $n = 5$, ns), whereas preincubation with 8 μ g/ml of LLO decreased the subsequent secretory response to the secretagogues (83.6 \pm 4.1% of the control response, $n = 4$, $P < 0.05$) (Fig. 6, black bars). LDH release was increased only after stimulation with higher LLO concentrations such as 8 μ g/ml of LLO (9.0 \pm 1.3%, $n = 4$, $P < 0.01$) (Fig. 6, gray bars). After the addition of 5 μ g/ml of LLO LDH release increased, which was paralleled by changes in I_{sc} and R^t (Fig. 7, A–C). This indicates that the barrier effect of high LLO concentrations is also due to cell damage, e.g., caused by the lytic potential of LLO. Thus, in addition to inducing active chloride secretion, listeriolysin also influences epithelial barrier function of the intestinal epithelium.

Pharmacological testing for intracellular messengers. Because PKC, PKA, and elevation of $[Ca^{2+}]_i$ are potent inducers

Table 2. LLO affects barrier function as indicated by an elevated mannitol flux

	I_{SC}	$J_{Mannitol}^{ms}$
Control	0.127 ± 0.007	0.042 ± 0.001
LLO	1.240 ± 0.049	0.060 ± 0.001
Δ	1.113 ± 0.050	0.018 ± 0.001
<i>P</i>	<0.001	<0.001

I_{SC} and 3H -mannitol flux ($J_{Mannitol}^{ms}$) from mucosal to serosal side were measured before and after LLO challenge. LLO (5 μ g/ml) exposure resulted in a 43% increase of the mucosal-to-serosal mannitol flux. Data are given in μ mol·h⁻¹·cm⁻² and represent means ± SE of 12 monolayers.

of active ion secretion in intestinal epithelia (4), we characterized the LLO-induced Cl⁻ secretion by means of respective inhibitors. The inhibitors used here had previously been tested with respect to their action on HT-29/B6 cells (22, 34). For quantitative comparison, the LLO-induced I_{SC} response after preincubation with the respective inhibitor was measured in parallel experiments with and without inhibitor. The PKC inhibitor chelerythrine (10 μ M) decreased the I_{SC} peak response to 42 ± 5% (P < 0.05; n = 6) of control values (Fig. 4A). Premixing chelerythrine with LLO did not affect LLO response (99.5 ± 5.4% of control I_{SC} ; mean ± SE, n = 4, ns; see MATERIALS AND METHODS). In contrast to chelerythrine, the PKA inhibitor H-8 (10 μ M) did not affect the LLO response (Fig. 4A).

Involvement of calcium signaling was investigated by two experimental approaches. First, changes in [Ca²⁺]_i were directly measured using FURA-2 and second, agents known to interfere with calcium signaling were tested for their capacity to inhibit the LLO response in FURA and in Ussing experiments. Additionally, FURA-2 measurements offered the possibility to examine LLO effects in Ca²⁺-free medium, which is not feasible in Ussing chambers because epithelia immediately lose barrier function in response to Ca²⁺-free conditions (as a result of E-cadherin disaggregation).

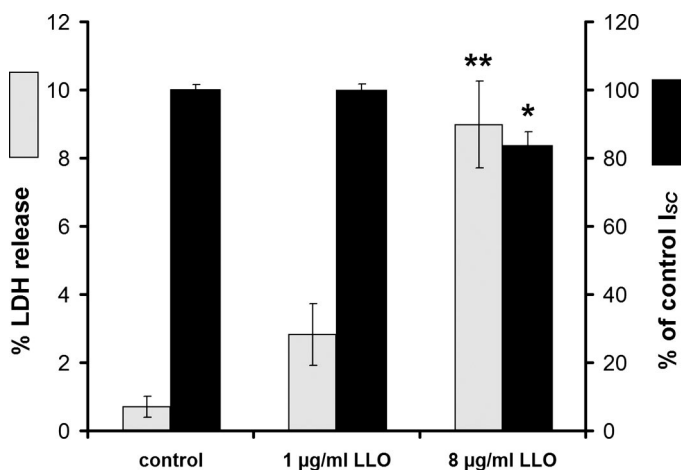


Fig. 6. LLO effect on HT-29/B6 viability. Monolayers were stimulated with 10 mM of theophylline and 20 μ M of forskolin after the LLO-induced I_{SC} response had ceased. Control represents monolayers without LLO prestimulation (black bars, right y-axis). Bars represent percent of control response at plateau phase of I_{SC} . Gray bars (left y-axis) indicate percentage of cellular LDH released into the mucosal bathing solution after addition of LLO (with LLO concentrations as indicated). Data are means ± SE (n = 5 for 1 μ g/ml of LLO and controls; n = 4 for 8 μ g/ml; * P < 0.05 and ** P < 0.01).

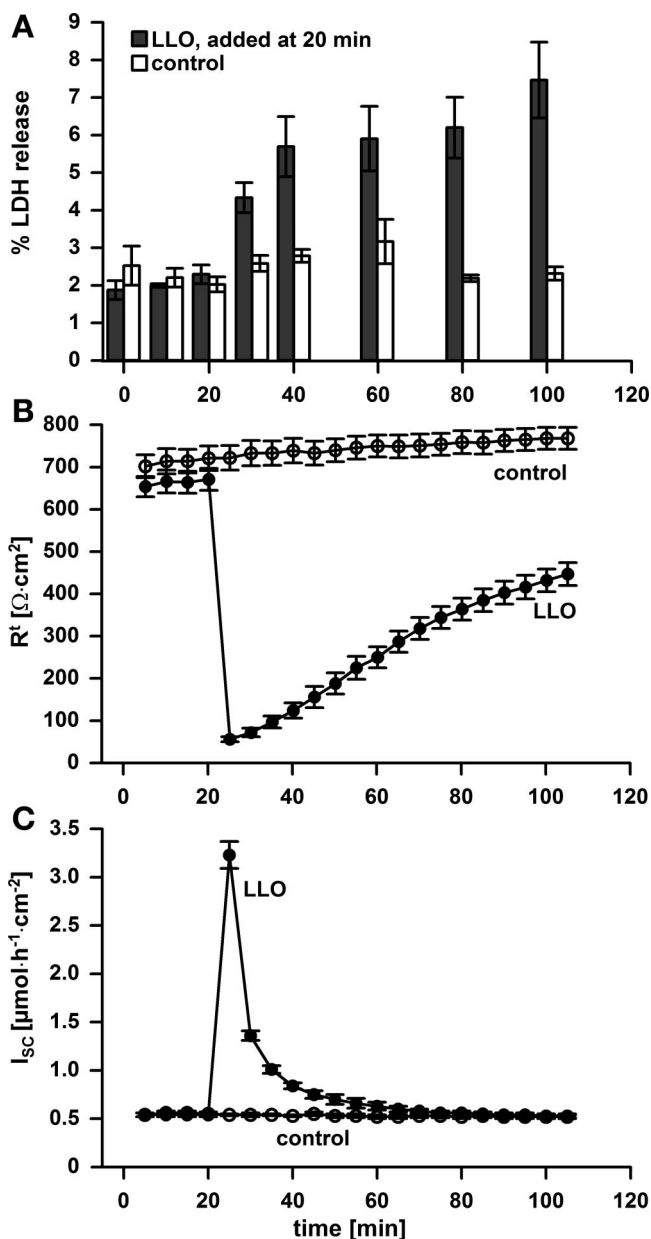


Fig. 7. LLO-dependent LDH release. LDH release into the mucosal bathing solution (A) was measured as a function of time before and after LLO stimulation. Monolayers were stimulated with 5 μ g/ml of LLO at 20 min after sampling for the LDH assay. Control represents monolayers without LLO. As corresponding electrical parameters of the LLO response I_{SC} (B) and R_t (C) are shown. Data are expressed as means ± SE (n = 4).

FURA-2 measurements revealed that LLO transiently elevated [Ca²⁺]_i with a shape and time response similar to that observed for the LLO-induced short-circuit current. [Ca²⁺]_i started to increase about 1.5 min after the addition of 1 μ g/ml of LLO (Fig. 8A). This increase was due to both intracellular Ca²⁺ release, as well as Ca²⁺ uptake. Release of intracellular Ca²⁺ was demonstrated in calcium-free medium, in which LLO still evoked a response, although this increase in [Ca²⁺]_i was much smaller (Fig. 8A). A similar response in calcium-free medium was observed with thapsigargin (200 nM), an inhibitor of the sarco-/endoplasmic reticulum calcium-ATPase (SERCA) (Fig. 8B). By blocking the calcium-ATPase, thapsigargin ele-

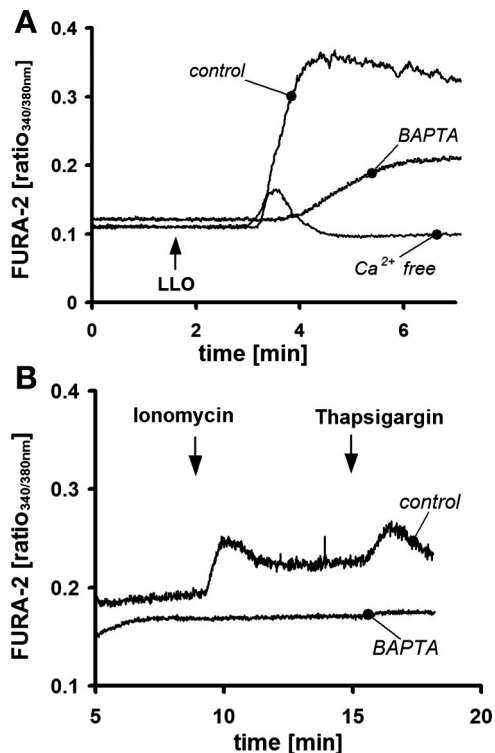


Fig. 8. LLO effect on intracellular calcium. LLO elevated $[Ca^{2+}]_i$, measured as fluorescence emission (as ratio of 340/380 nm excitation) of FURA-2-loaded HT-29/B6 cells. A: listeriolylin stimulation was partially inhibited in BAPTA-AM preincubated cells and displayed an altered time graph in Ca^{2+} -free solution (both compared with the effect in control cells). B: BAPTA preincubation inhibited both the ionomycin- (1.5 μ M) and the thapsigargin- (200 nM) induced $[Ca^{2+}]_i$ elevation. A typical experiment is shown which is representative of at least three independent experiments. Arrows mark time of addition of the respective agents.

vates cytosolic Ca^{2+} by inhibiting reuptake into intracellular storage compartments. The thapsigargin-induced $[Ca^{2+}]_i$ peak could only be triggered once, as could the LLO-induced Ca^{2+} increase in similar experiments in calcium-free medium (data not shown). Therefore, LLO induced a release of calcium from intracellular storage compartments, as well as an inflow of extracellular Ca^{2+} . Premixing thapsigargin with LLO did not affect LLO response ($99.2 \pm 19.0\%$ of control I_{SC} ; mean \pm SE, $n = 4$, ns; see MATERIALS AND METHODS).

The intracellular concentration of free Ca^{2+} can be buffered to low levels using BAPTA, a calcium-chelating agent. While BAPTA-loaded cells did not show an increase in $[Ca^{2+}]_i$ after Ca^{2+} inflow was triggered by the ionophore ionomycin (1.5 μ M) and a thapsigargin (200 nM)-induced increase in $[Ca^{2+}]_i$ was also blocked by BAPTA (Fig. 8B), the LLO-induced $[Ca^{2+}]_i$ elevation was only partially inhibited (Fig. 8A) in the same setup. Interestingly, in Ussing chamber experiments BAPTA was ineffective in blocking LLO- (Fig. 4A) and thapsigargin- (Fig. 9) -induced I_{SC} responses. However, BAPTA was effective in partially blocking a caffeine-induced I_{SC} response, which mirrors elevation of $[Ca^{2+}]_i$ caused by release from intracellular stores by activating the ryanodine receptor (40) and therefore proved the potential effectiveness of BAPTA also in the Ussing setup (Fig. 9). Taken together, these results indicate that LLO triggers BAPTA-insensitive Ca^{2+} signaling by release from thapsigargin-sensitive intracellular storage compartments.

DISCUSSION

There is accumulating evidence of *Listeria monocytogenes* causing diarrhea (45, 39, 30, 2, 17), which often precedes systemic infection. To understand the pathological processes, it is necessary to clarify how diarrheal effects are mediated and whether or not active intestinal ion and fluid secretion and/or an impaired epithelial barrier function may contribute. This study investigates some effects of listeriolylin O (LLO)—the *sine qua non* virulence factor that is involved in various cellular actions (31), in particular those related to systemic dissemination of the bacterium (53). Cholesterol-dependent cytolysins such as LLO affecting diverse signal pathways already at sublytical concentrations (27, 28, 32, 51, 53) have not been associated with diarrhea so far. Therefore, we focused on the functional effects of purified LLO on model intestinal epithelia in Ussing chambers, which allow for real-time observation of electrophysiological parameters indicative of ion secretion and epithelial barrier function (9, 20, 22, 34).

Listeriolylin induces Ca^{2+} -mediated active electrogenic chloride secretion. The secondary active chloride secretory system of epithelial cells involves several transporters acting in concert. Apical chloride efflux is considered to be the rate-limiting step and activation of PKA and/or elevation of $[Ca^{2+}]_i$ have been identified as stimulating pathways (4). Chelerythrine, an inhibitor of the calcium-dependent protein kinase (PKC), and thapsigargin, an inhibitor of intracellular Ca^{2+} -ATPase, were found to block LLO-induced chloride secretion, as opposed to the cyclic-nucleotide-dependent protein kinases (PKA)-inhibitor H-8. Accordingly, FURA-2 experiments directly showed that LLO causes an increase in intracellular Ca^{2+} concentration. The assumed involvement of intracellular signal transduction by Ca^{2+} is further supported by the transient and self-limiting I_{SC} response to LLO, which is typical of calcium-mediated chloride secretion (33) with PKC as an important mediator in calcium-driven cellular responses and believed to activate calcium-activated chloride channels (CaCC) (4, 26). As a piece of direct evidence, DIDS, a calcium-activated chloride channel blocker, inhibited the LLO response. The LLO effect depended on pore formation, since a

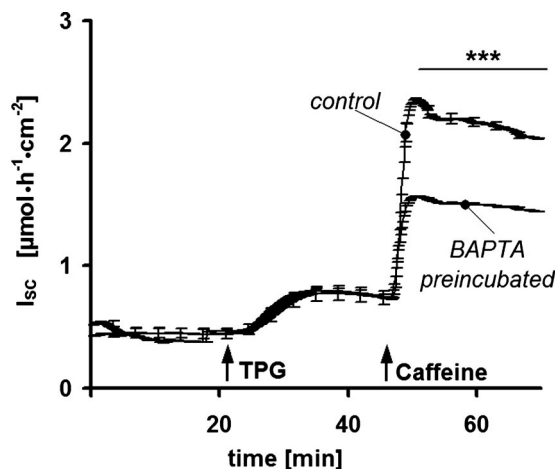


Fig. 9. Modulation of intracellular calcium in Ussing chambers. Recordings indicate I_{SC} . BAPTA (50 μ M) partially inhibited the caffeine- (1.5 mM), but not the thapsigargin- (1 μ M) induced I_{SC} response. All data are expressed as means \pm SE ($n = 3$). *** $P > 0.001$ vs. BAPTA.

nonlytic LLO-mutant failed to trigger an I_{SC} response. Also, the fact that reduced pH values with a likely increase in the lytic potency of LLO (46) did intensify the I_{SC} response provides further evidence for pore formation in this context. In addition, pore conductance at the plasma membrane should have been inhibited by La^{3+} (8). Therefore, it seems that pore formation is more likely to be relevant within the cell, probably affecting intracellular Ca^{2+} stores, which has been demonstrated for mast cells (28). This interpretation is also backed by the thapsigargin sensitivity of the LLO response.

Signaling through increased intracellular calcium concentration is expected to be blocked by calcium chelation, e.g., with BAPTA. However, BAPTA was ineffective in blocking LLO-induced chloride secretion in HT-29/B6 cells. Experimental shortcomings can be ruled out, since BAPTA did partially inhibit the LLO-stimulated increase in intracellular Ca^{2+} concentration in FURA-2 experiments and, likewise, partially inhibited a caffeine triggered I_{SC} response in Ussing experiments. Similar observations of BAPTA only partially inhibiting Ca^{2+} -mediated epithelial Cl^- secretion have been reported before (19), and it has been suggested that BAPTA may be locally "overcharged" by rapidly increasing Ca^{2+} concentrations in microdomains (7, 49). Furthermore, BAPTA-inaccessible sites were reported in muscle cells (52). Accordingly, thapsigargin inhibited a subsequent LLO-stimulation of I_{SC} but its own I_{SC} response was also insensitive to BAPTA. This could explain why preincubation of epithelial monolayers with BAPTA-AM was not effective in blocking the I_{SC} response to LLO, while preincubation with thapsigargin reduced this response. Whether the inflow of extracellular Ca^{2+} plays a role and is primary or secondary to its release from Ca^{2+} stores cannot be directly established with an Ussing chamber, since a depletion of extracellular calcium will open the tight junctional pathway ("calcium switch"), thus rendering I_{SC} measurements impossible. However, FURA-2 experiments revealed that both an inflow and a release of calcium from intracellular stores are triggered by LLO in HT-29/B6 cells, and both events are indistinguishable with regard to their time graphs. In line with our findings, Gekara et al. (28) used mast cells to demonstrate that, in addition to Ca^{2+} influx, LLO also triggers release of Ca^{2+} from intracellular stores, which was mediated by Ca^{2+} channel-dependent and -independent mechanisms. Altogether, we conclude that, in HT-29/B6 cells, LLO induces calcium release from intracellular stores, thereby activating a classical signaling pathway of epithelial chloride secretion.

Magnitude and time graph of LLO-induced chloride secretion. The dose-dependent increase in I_{SC} shows that the LLO-induced response is limited, at least at submaximal LLO concentrations, by its regulation via intracellular messengers rather than the number of membrane conductance sites (chloride channels). The fact that LLO-induced epithelial Cl^- secretion spontaneously declined toward its control value despite the continuous presence of LLO may suggest either an inactivation or desensitization of the target cell by LLO. Removal of membrane-associated LLO (pores), e.g., via endocytosis or membrane blebbing, will inactivate the agent and thus stop Cl^- secretion, if the remaining LLO molecules are incapable of further pore formation. Indeed, experiments in which LLO was reused (applied to a second monolayer) showed that the LLO solution was not effective anymore, whereas monolayers responded again to restimulation with fresh toxin. Therefore, the

spontaneous reversal in LLO-induced Cl^- secretion is likely to be due to inactivation of the toxin. However, some desensitization is common in intracellular Ca^{2+} signaling, and the transient I_{SC} response may well be a combination of both.

Diarrheagenic effects of listeriolysin. Our results demonstrate that listeriolysin is capable of inducing active chloride secretion and may significantly affect the barrier function of the epithelium at higher concentrations. It should be noted that factors such as 1) composition of luminal contents, 2) localization and production rates of the toxin, and 3) possible effects of immune cells or the enteral nervous system, are difficult to predict and not reflected by the in vitro system used in this study. However, it could clearly be demonstrated that listeriolysin O itself effectively elicited a diarrheic response in epithelial cells. We, furthermore, demonstrated a strong dependence of the LLO-induced I_{SC} response on pH of the surrounding milieu, which suggests a higher potential for diarrheic action in the acidic segments of the gastrointestinal tract like the proximal small intestine. Obviously, the adherence and invasion of *Listeria* (53) are no prerequisite for diarrheic effects, since they are inducible by a sterile-filtered LLO preparation. This is most likely caused by cell damage due to the lytic activity of LLO. However, we cannot exclude other mechanisms contributing to the barrier defect e.g., alterations in tight junction proteins.

In general, epidemiological data of food-borne outbreaks of gastroenteritis (2, 17, 30, 39, 45) support the idea of *Listeria* being a diarrheic agent, as do the few feeding studies on animals challenged with live *Listeria* (3, 23), although high doses of ingested bacteria appear to be necessary. Some information, which, at first sight, seems to be counter-indicative of a diarrheic potential is available from a human challenge study by Angelakopoulos et al. (1) using mutant *Listeria* strains of attenuated pathogenicity but not deficient in listeriolysin O. No signs of prominent diarrhea were found in that study. However, this constitutes no direct evidence against a diarrheic effect of wild-type strains of *Listeria*, which may colonize the GI tract more efficiently and release significantly higher amounts of LLO, while the colonic resorptive capacity might compensate for a more moderate ion and fluid secretion induced by the attenuated *Listeria* strain in the upper gastrointestinal tract.

The diarrheagenic effects of listeriolysin have pathophysiological implications, because induction of intestinal secretion allows pathogens to spread to new hosts. On the other hand, active intestinal secretion is a defense mechanism of the host preventing the entry of pathogens. Induction of intestinal fluid secretion is accomplished with listeriolysin by hijacking the enterocyte's calcium-signaling pathway and significantly impairing the epithelial barrier at high LLO concentrations. Hence, the present experimental data assign listeriolysin to be a potent diarrheagenic agent of *Listeria*.

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