Epsilon toxin is a potent toxin produced by Clostridium perfringens types B and D, which are responsible for a rapidly fatal enterotoxemia in animals. One of the main properties of epsilon toxin is the production of edema. We have previously found that epsilon toxin causes a rapid swelling of Madin-Darby canine kidney cells and that the toxin does not enter the cytosol and remains associated with the cell membrane by forming a large complex (Petit, L., Gibert, M., Gillet, D., Laurent-Winter, C., Boquet, P., and Popoff, M. R. (1997) J. Bacteriol. 179, 6480–6487). Here, we report that epsilon toxin induced in Madin-Darby canine kidney cells a rapid decrease of intracellular K\(^+\), and an increase of Cl\(^-\) and Na\(^+\), whereas the increase of Ca\(^{2+}\) occurred later. The entry of propidium iodide that was correlated with the formation of a large membrane pore. In artificial lipid bilayers, epsilon toxin caused current steps with a single-channel conductance of 60 pS in 100 mM KCl, which represented general diffusion pores. The channels were slightly selective for anions, but cations could also penetrate. Epsilon toxin formed wide and water-filled channels permeable to hydrophilic solutes up to a molecular mass of at least 1 kDa, which probably represents the basic mechanism of toxin action on target cells.

Epsilon toxin is synthesized by Clostridium perfringens types B and D. This toxin is the main virulence factor of C. perfringens type D, which is responsible for enterotoxemia in sheep, goat, and more rarely in cattle. Enterotoxemia is a rapidly fatal disease that causes important economical losses through the world.

Overgrowth of C. perfringens type D in the intestine of susceptible animals is accompanied by the release of large amounts of epsilon toxin, which is absorbed through the intestinal mucosa and diffuses to all the organs by the blood circulation. The main biological action of epsilon toxin is the production of edema. In experimental animals, epsilon toxin elevates the blood pressure, increases the vascular permeability, and causes edema and congestion in various organs including lungs and kidneys. Necrosis of the kidneys is also observed in lambs that have died from enterotoxemia (1, 2). Epsilon toxin is able to cross the blood-brain barrier and accumulates in the brain (3, 4). The terminal phase of enterotoxemia is characterized by neurological disorders (opiathotonus, convulsions, and agonal struggling). Epsilon toxin increases the permeability of the brain vasculature and causes perivascular edema, which is probably responsible for neuronal damage and neurological disorders (5, 6). In addition, epsilon toxin could directly interact with hippocampus neurons leading to an excessive release of glutamate (7, 8).

Epsilon toxin is cytotoxic for MDCK\(^1\) cells and at a lesser extent for the human leiomyoblastoma (G-402) cells (11–13). We have previously found that epsilon toxin induces swelling, blebbing, and lysis of MDCK cells and that the toxin does not enter the cytosol and remains associated with the MDCK cell membrane by forming a large complex (14). The cytotoxic activity was correlated with the formation of a large membrane complex and an efflux of K\(^+\) (14, 15). In addition, epsilon toxin causes a rapid decrease of the transepithelial resistance of MDCK cell monolayers.\(^2\)

Here, we report that in MDCK cells, epsilon toxin causes a rapid decrease of intracellular K\(^+\), a rapid increase of Cl\(^-\) and Na\(^+\), and a slower increase of Ca\(^{2+}\). The entry of propidium iodide (PI) into treated cells indicates that epsilon toxin forms large membrane pores. Investigation with artificial lipid bilayers shows that epsilon toxin elicits the formation of non-selective general diffusion channels permeable to molecules up to 1 kDa.

**EXPERIMENTAL PROCEDURES**

**Materials**—Epsilon toxin was purified as previously described (14). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and propidium iodide were from Sigma. The fluorescent probes CD222, DMEM, Dulbecco’s modified Eagle’s medium; MQAE, N-(ethoxycarbon-ylmethyl)-6-methoxyquinolium bromide; PI, propidium iodide; MOPS, 4-morpholinepropanesulfonic acid; PEG, polyethylene glycol.

\(^1\) The abbreviations used are: MDCK, Madin-Darby canine kidney; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco’s modified Eagle’s medium; MQAE, N-(ethoxycarbonyl)-6-methoxyquinolium bromide; PI, propidium iodide; MOPS, 4-morpholinepropanesulfonic acid; PEG, polyethylene glycol.

\(^2\) Manuscript in preparation.
sodium green, calcium green, and MQAE were from Molecular Probes.

**Cell Culture—**MDCK cells were grown in DMEM supplemented with 10% fetal calf serum at 37 °C in a 5% CO₂ incubator. For cytotoxicity and propidium assays, MDCK cells were grown to confluence in 96-well plates. The monolayers were washed once in DMEM and incubated with serial dilutions of epsilon toxin in DMEM (100 μl final volume in each well). The viability test using MTT was performed as described previously (14).

**Intracellular Ion Assays—**MDCK cells were grown in 6-well plates in DMEM supplemented with 10% fetal calf serum until confluence. After two washings with DMEM without serum, the cells were incubated with epsilon toxin (10⁻⁸ M) in DMEM without serum (0.5 ml per well) at 37 °C in a CO₂ incubator. At the indicated times, the cells were washed with 25 mM MOPS (pH 7) containing 150 mM glucose (MOPS-glucose) and lysed with 1% Triton X-100 (125 μl per well). Each fluorescent probe was added to 45 μl of lysed cells to a final concentration of 1 μM for CD222 (K⁺ assay) or 10 μM for sodium green, calcium green, and MQAE (Cl⁻ assay). The probes were measured in a spectrofluorimeter (Fluoroskan II; Labsystems) with the following filters: excitation 380 nm and emission 475 nm for CD222, excitation 355 nm and emission 538 nm for sodium green and calcium green, and excitation 355 nm and emission 475 nm for MQAE. The fluorescence obtained by washing MDCK cells with MOPS-glucose and lysing with 1% (w/v) Triton X-100 addition was considered as the 0% base line, and the fluorescence of MDCK cells incubated with serum-free DMEM containing 0.2% (w/v) Triton X-100 for 5 min at 37 °C in the absence of epsilon toxin and then washed with MOPS-glucose and lysed with 1% (w/v) Triton X-100 was considered as an activity of 100%. The data were expressed as percent of fluorescence quench.

**Propidium Iodide Influx—**For assay of PI entry, MDCK cells were grown on 96-well plates until confluency. PI (5 μg/ml) was added in the culture medium, together with epsilon toxin. At the indicated times, the plates were read with a spectrofluorimeter (Fluoroskan II; excitation 540 nm and emission 620 nm). The results were expressed as the percentage of fluorescence obtained by treatment with Triton X-100 (0.2%) for 30 min at 37 °C.

**Lipid Bilayer Experiments—**Black lipid bilayer membranes were formed as has been described previously (16) from a 1% solution of diphytanylated phosphatidylincholine, phosphatidylserine, and asolectin (Avanti Polar Lipids, Alabaster, AL) in N-decane. The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole with a surface area of 0.3 to 0.5 mm². The lipid bilayer membranes were formed by painting onto the hole solutions of the different lipids. The aqueous salt solutions (Merek) were used unbuffered and had a pH value around 6, if not indicated otherwise. Native and activated C. perfringens epsilon toxin were added from concentrated stock solutions to the aqueous phase bathing membranes in the black state. The temperature was kept at 20 °C throughout. The single-channel recordings were performed using a pair of Ag/AgCl electrodes with salt bridges switched in series with a voltage source and a highly sensitive current amplifier. The amplified signal was monitored with a storage oscilloscope, and the reconstitution of channels in the black lipid membrane was recorded with a strip chart recorder. Zero-current membrane potential measurements were performed by establishing a salt gradient across membranes containing 100 to 1000 epsilon toxin channels as they have been described earlier (17).

**RESULTS**

**Epsilon Toxin Induces in MDCK Cells an Early Loss of K⁺, an Entry of Na⁺ and Cl⁻, and a Later Influx of Ca²⁺—**We have previously reported that epsilon toxin causes a rapid swelling of MDCK cells and a subsequent loss of viability as monitored by the MTT test (14). Because epsilon toxin interacts with the cell membrane and has apparently no intracellular activity, its cytotoxicity and propidium assays have been evidenced in MDCK cells damaged by epsilon toxin (14, 15). To define more precisely the membrane permeability changes during the intoxication process of epsilon toxin, we analyzed the kinetics of intracellular cations (K⁺, Na⁺, and Ca²⁺) and anion (Cl⁻) versus the cytotoxic activity determined by the MTT test. Fig. 1 shows that intracellular K⁺ decreased rapidly. The 50% loss of K⁺ was observed within the first 5 min, whereas the 50% decrease of cell viability was recorded after 30 min of incubation with 10⁻⁸ M of epsilon toxin. At this time, more than 90% of intracellular K⁺ was lost. The intracellular concentrations of the other cations, Na⁺ and Ca²⁺, which are low in control cells, increased significantly in MDCK cells intoxicated with epsilon toxin (Figs. 1 and 2). The Na⁺ entry was rapid (50% increase of intracellular Na⁺ during 8 min) although slightly later than the loss of K⁺, but that of the divalent cation Ca²⁺ was delayed (50% increase of intracellular Ca²⁺ during 26 min). The increase of the intracellular Cl⁻ concentration was as rapid as the decrease of K⁺ (50% decrease during 5 min). This demonstrates that the intracellular concentrations of K⁺, Na⁺, Ca²⁺, and Cl⁻ changed dramatically in MDCK cells treated with epsilon toxin. The early intracellular concentrations changes concerned that of the monovalent ions K⁺, Cl⁻ and slightly delayed for Na⁺, whereas that of the divalent cation Ca²⁺ was slower.

**Epsilon Toxin Induced a Cell Permeability to Propidium Iodide—**The pore-forming toxins such as Staphylococcus aureus α-toxin recognizes a cell surface receptor, oligomerizes, and inserts into the membrane (18). At high concentration (>300 nM) α-toxin forms large pores permitting the entry of PI into cells (19). PI internalization was assayed in MDCK cells treated with epsilon toxin. Fig. 3 shows that the entry of PI correlated with the loss of cell viability measured by the MTT test. The 50% entry of PI matched the 50% increase of intracellular Ca²⁺ (26–27 min), and both are close to the 50% loss of cell viability (32 min) induced by epsilon toxin intoxication. This indicates that epsilon toxin induced the formation of large pores in MDCK cell membranes even at a low concentration (10⁻⁸ M) similar to α-toxin.

To evaluate the size of the pores caused by epsilon toxin, competition with uncharged compounds of various molecular mass were performed to block the channels induced by epsilon toxin and subsequently to inhibit the effects of the toxin on cell viability and entry of PI into MDCK cells. Such techniques have been used previously with various hemolysins, for example from Escherichia coli, Clostridium septicum, or S. aureus (20–22). Glucose (300 mM), sucrose (300 mM), and PEG300 (25 mM) impaired neither the cytophagic activity in the MTT test nor the entry of PI induced by epsilon toxin. PEG6000 but not PEG3350 (25 mM) blocked the entry of PI stimulated by epsilon toxin (data not shown). However, these data were not highly reliable, because PEG1000 (25 mM) and above were to some extent cytophagic for MDCK cells. In any case the results indicate that epsilon toxin forms large pores in MDCK cells at least...
2 nm in diameter based on the size of PEG1000 (23).

Activation Increases the Channel-forming Activity of C. perfringens Epsilon Toxin in Lipid Bilayer Membranes—The in vivo experiments indicated that epsilon toxin formed pores that were at least permeable to ions. Thus, it could be possible that epsilon toxin forms ion-permeable channels in membranes. In a first set of experimental conditions we studied the effect of the non-activated epsilon toxin on membranes formed from a variety of different lipids such as phosphatidylcholine, phosphatidylserine, and asolectin. In all cases, we were able to observe some low channel-forming activity, which means that only a small number of channels were formed even at very high concentrations of non-activated epsilon toxin. Substantially increased channel-forming activity was observed, however, when epsilon toxin was activated by trypsin treatment before its addition to the aqueous phase bathing lipid bilayer membranes. Again, we did not observe any lipid specificity. Approximately, the same membrane activity were observed for all lipids used in this study. The relation between membrane conductance and toxin concentration in the aqueous phase was linear, which suggests that there is no association-dissociation reaction between non-conducting monomers and conducting oligomers and that the conductive units are long-lived.

FIG. 4. Single-channel recording of a diphytanoyl phosphatidylethanolamine/N-decane membrane in the presence of activated epsilon toxin from C. perfringens. 10 min after the formation of the membrane 80 ng/ml of activated epsilon toxin was added to the aqueous phase on one side of the membrane. The aqueous phase contained 1 M KCl (pH 6). The applied membrane potential was 50 mV; T = 20 °C.

Properties of the C. perfringens Epsilon Toxin Channel—Single-channel experiments were also performed with salts other than KCl to obtain some information on the size and selectivity of the epsilon toxin channel. The results are summarized in Table I. The replacement of chloride and potassium...
by the less mobile acetate and lithium ions had a considerable influence on the single-channel conductance. The influence of the replacement of chloride by acetate on the single-channel conductance was, however, more substantial (see Table I), which suggests that the channel formed by epsilon toxin in lipid bilayer membranes is at least slightly anion-selective. Table I shows also the average single-channel conductance, G, as a function of the KCl concentration in the aqueous phase. We observed a 1:1 relationship between conductance and KCl concentration, which would be expected for wide, water-filled channels similar to those formed by general diffusion porins of Gram-negative bacteria (24). This probably means that the channel formed by the C. perfringens epsilon toxin represents a general diffusion pore.

**Selectivity of the Epsilon Toxin Channel**—The selectivity of the epsilon toxin channel was measured in zero-current membrane potential measurements in the presence of salt gradients. After incorporation of a large number of channels in membranes bathed in 100 mM KCl, 5-fold salt gradients were established across the membranes by the addition of small amounts of concentrated KCl solution to one side of the membrane. In all cases, the more diluted side of the membrane became negative, which indicated preferential movement of anions through the epsilon toxin channel, i.e. it is anion-selective as was already suggested from the single-channel data (Table I). The zero-current membrane potential for a 5-fold KCl gradient was on average about −19 mV at the more diluted side (mean of four experiments). Analysis of the zero-current membrane potentials using the Goldman-Hodgkin-Katz equation (17) suggested that cations could also have a certain permeability through the epsilon toxin channel, because the ratio of the permeabilities $P_K$ and $P_{Cl}$ was 0.30 (± 0.02; see Table II). This result represents another indication that the C. perfringens epsilon toxin forms a general diffusion pore, because both anions and cations (albeit at smaller rate than the anions) could penetrate the channel, and their ratios reflect their mobility sequence.

**DISCUSSION**

**C. perfringens Epsilon Toxin Forms Pores Permeable to Ions and PI in MDCK Cells**—As previously reported, the in vivo experiments suggest that epsilon toxin forms a complex in the membranes of the MDCK cells and does not enter their cytoplasm. The formation of the complex of the activated toxin molecule results in the loss of ions from the cells and in cell death (14, 15, 26). These results suggest that C. perfringens epsilon toxin destroys the barrier function of the cytoplasmic membrane of MDCK cells but not of other cells, which probably do not contain a cell surface receptor for the toxin. Here, we show that epsilon toxin causes in MDCK cells a very early loss of $K^+$ and entry of Na$^+$ and $Cl^-$, whereas the intracellular concentration of the divalent cation Ca$^{2+}$ increased later similarly to the loss of cell viability monitored by the MTT test. Thus, epsilon toxin seems to form not very selective pores through the MDCK cell membrane permitting the flux of different ions. Differences in fluxes of monovalent and divalent cations have also been observed with other pore-forming toxins. As an example, staphylococcal $\alpha$-toxin at low concentrations creates in susceptible cells such as keratinocytes pores permeable to $K^+$ but not to Ca$^{2+}$, and at high concentrations a rapid flux of $K^+$ and Ca$^{2+}$ is observed. The heterogeneity of pore sizes remains unexplained (27). The pores induced by epsilon toxin were also permeable to PI. Inhibition of PI entry with PEG of different molecular mass indicated that the pores were large in size (at least 2 nm in diameter), but this is tentative, because high molecular mass PEG were cytotoxic for MDCK cells. The cell entry of PI induced by epsilon toxin matched the loss of cell viability. A good correlation was observed between the kinetics of PI entry and that of the MTT test. This raises the question whether the pores formed by epsilon toxin in MDCK cells are responsible alone for the loss of viability by release of ions and other essential molecules in the external medium. Thus, we cannot exclude the possibility that epsilon toxin may elicit additional cellular activities such as to trigger an intracellular signaling leading to cell death.

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**Table I**

| Salt        | $c$ (M) | $G$ (pS) | $n$ |
|-------------|---------|----------|-----|
| KCl         | 0.03    | 0.03     | 2   |
|             | 0.1     | 0.1      | 3   |
|             | 0.3     | 0.3      | 2   |
|             | 1.0     | 1.0      | 6   |
|             | 3.0     | 3.0      | 3   |
| LiCl        | 1.0     | 1.0      | 4   |
| K$^+$ acetate (pH 7.0) | 1.0 | 1.0 | 4 |

**Table II**

| Toxin               | Single-channel conductance in 1 M KCl | Zero-current membrane potential for 5-fold KCl gradient | Selectivity ratio $P_K/P_{Cl}$ |
|---------------------|---------------------------------------|---------------------------------------------------------|-------------------------------|
| Aerolysin           | 650 pS                                 | −24 mV                                                  | 0.21                          |
| $\alpha$-toxin      | 820 pS                                 | −22 mV                                                  | 0.25                          |
| $\varepsilon$-toxin | 550 pS                                 | −19 mV                                                  | 0.30                          |

$V_m$ is the electrical potential on the dilute side minus the potential of the concentrated side. The membranes were formed from diphytanoyl phosphatidylcholine/N-decane. The aqueous solutions were unbuffered and had a pH value of about 6. The permeability ratio $P_K/P_{Cl}$ was calculated using the Goldman-Hodgkin-Katz equation (17). The results for aerolysin and $\alpha$-toxin were taken from Ref. 31.
C. perfringens Epsilon Toxin Forms Channels in Lipid Bilayer Membranes—In agreement with the possible destruction of the barrier function of the cytoplasmic membrane of MDCK cells, we were able to identify channel formation in artificial lipid bilayer membranes. Essential for the formation was the trypsin-mediated activation of the protein. No obvious lipid specificity was detected in the experiments. Channels were formed with all lipids tested here. This result seems to represent a contradiction to receptor-mediated destruction of cells. However, it has to be kept in mind that many cytolytic bacterial toxins, such as the repeats in toxin toxins (28–30), α-toxin from S. aureus (31–33), and aerolysin from Aeromonas sobria (31, 34) form channels in lipid bilayer membranes without the need of receptors, whereas they all need a receptor for biological activity. Lipid bilayers have smooth surfaces without any surface structure including the surface-exposed carbohydrates of biological membranes, which means that the toxins can interact with the hydrocarbon core of the lipid bilayer and can insert without the help of receptors, although receptors may promote such an interaction (35).

It is noteworthy that the formation of channels in lipid bilayers mediated by C. perfringens epsilon toxin was not a rare event. The addition of 100 ng/ml of this protein to the aqueous phase bathing a diphytanoyl phosphatidylcholine/N-decanol membrane was able to increase the conductance of lipid bilayer membranes considerably, and more than 1000 channels were formed within about 20 to 30 min under these conditions in a membrane was able to increase the conductance of lipid bilayer membranes without the need of receptors, although receptors may promote such an interaction (35).

Properties of the C. perfringens Epsilon Toxin Channel in Lipid Bilayer Membranes—The epsilon toxin channel is presumably formed by protein oligomers with an apparent molecular mass of about 155 kDa when inserted into cell membrane (14). This makes about five times the molecular mass of a monomer. It is noteworthy that a common architecture of cytolytic toxins is the heptamer, which has been found for α-toxin from S. aureus (33), aerolysin from Aeromonas hydrophila (34), and the cytolytic toxin ClyA of E. coli (36, 37). If the epsilon toxin oligomer has a somewhat different mobility in SDS polyacrylamide gel electrophoresis than the monomer, it is also possible that the membrane channel is formed by a heptamer of the 30-kDa monomer. In such a case the membrane-spanning part of the epsilon toxin should be formed by β-strands, as it is the case at least for α-toxin and aerolysin (33, 34). In fact, secondary structure prediction performed with the primary structure of epsilon toxin (10) reveals several stretches in the protein that can possibly form membrane-spanning amphipathic β-strands.

Besides oligomer formation the epsilon toxin channel also shares some other features with those formed by aerolysin and α-toxin. All three channels are long-lasting channels with lifetimes in the range of minutes. They are all anion-selective channels caused probably by an excess of positively charged groups in or near the channel (see Ref. 31 and Table II). Nevertheless, all three toxin channels represent general diffusion pores, because they do not contain selectivity filters or binding sites for ions, as judged from the linear dependence of the single-channel conductance from the aqueous salt concentration observed for all three channels. This probably also means that the channels are wide and water-filled and are permeable to solutes up to a molecular mass of at least 1 kDa. Furthermore, the single-channel conductance itself is also very similar in all three systems as Table II clearly indicates. We did not find any similarity between the sequences of the three toxins. However, this does not represent a serious problem for our assumption, because aerolysin and α-toxin do also not show sequence similarities despite a similar architecture of the membrane channel.

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Clostridium perfringens Epsilon Toxin Induces a Rapid Change of Cell Membrane Permeability to Ions and Forms Channels in Artificial Lipid Bilayers
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