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Clostridium perfringens Epsilon-Toxin Acts on MDCK Cells by Forming a Large Membrane Complex

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Epsilon-toxin is produced by *Clostridium perfringens* types B and D and is responsible for a rapidly fatal enterotoxemia in animals, which is characterized by edema in several organs due to an increase in blood vessel permeability. The Madin-Darby canine kidney (MDCK) cell line has been found to be susceptible to epsilon-toxin (D. W. Payne, E. D. Williamson, H. Havard, N. Modi, and J. Brown, FEMS Microbiol. Lett. 116:161–168, 1994). Here we present evidence that epsilon-toxin cytotoxic activity is correlated with the formation of a large membrane complex (about 155 kDa) and efflux of intracellular K^+ without entry of the toxin into the cytosol. Epsilon-toxin induced swelling, blebbing, and lysis of MDCK cells. Iodolabeled epsilon-toxin bound specifically to MDCK cell membranes at 4 and 37°C and was associated with a large complex (about 155 kDa). The binding of epsilon-toxin to the cell surface was corroborated by immunofluorescence staining. The complex formed at 37°C was more stable than that formed at 4°C, since it was not dissociated by 5% sodium dodecyl sulfate and boiling.

Epsilon-toxin is produced by *Clostridium perfringens* types B and D and is responsible for a rapidly fatal enterotoxemia in sheep and other animals which causes heavy economic losses (24). It is synthesized as a relatively inactive prototoxin (296 amino acids) which is converted to a highly active mature protein by proteolytic removal of a basic N-terminal peptide (13 amino acids) (3, 16).

Epsilon-toxin is lethal and dermonecrotic. It has been reported to increase intestinal permeability (4), to cause kidney damage (9), to elevate blood pressure (27, 39), and to cause contraction of isolated rat ileum (40). A basic property of epsilon-toxin is that it increases vascular permeability. The toxin binds to vascular endothelial cells and causes severe vascular damage and edema in various organs (brain, heart, lung, and kidney) (5, 9). It was reported that the major pathological changes caused by enterotoxemia appear to occur in the brain (12). Moreover, it was shown that labeled toxin specifically accumulates in the brains of mice after intravenous injection and that the lethal activity of the toxin depends on its specific binding in the brain, probably to a sialoglycoprotein (28, 29). Certain amino acids of epsilon-toxin, such as histidine, tryptophan, and aspartic or glutamic acid have been found to be essential for its biological activity (35–38).

Recently, it has been found that epsilon-toxin has 20 and 27% identity with Mtx2 and Mtx3 toxins, respectively, of *Bacillus sphaericus* (22, 43) and 26.5% identity with the open reading frame c53 of *Bacillus thuringiensis*. However, the mechanism of action of these mosquitocidal toxins is unknown.

The Madin-Darby canine kidney (MDCK) cell line was found to be susceptible to epsilon-toxin. The alteration of cell viability determined by the conversion of 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazolyl)-3-(4-sulfophenyl) tetrazolium to a water-soluble formazan by the mitochondrial cytochrome systems or by the neutral red assay (an indicator of lysosomal integrity) was correlated with the lethal activity of epsilon-toxin in mice (21, 30). The cytotoxic effects were found to be very rapid (2.5 min) and potentiated by EDTA (21). The morphological effects of epsilon-toxin on cells included a condensation of the nucleus and a progressive swelling of the cells (13). The permeability of polarized MDCK cell monolayer monitored with [^{14}C]mannitol was increased in the presence of epsilon-toxin (13). The mechanism of action of epsilon-toxin is unknown, and no enzymatic activity has been attributed to it.

To investigate its biological activity, we explored the interaction of iodolabeled toxin with MDCK cells. We report here that the cytotoxic effects of epsilon-toxin are correlated with the formation of a membrane complex of about 155 kDa and efflux of intracellular K^+ without entry of the toxin into the cytosol.

MATERIALS AND METHODS

Epsilon-toxin. *C. perfringens* type D strain NCTC2062 was grown in TGY broth culture containing Trypticase (30 g/liter), yeast extract (20 g/liter), glucose (5 g/liter), and cysteine-HCl (0.5 g/liter) (pH 7.2) under anaerobic conditions at 37°C for 18 h.

The purification of epsilon-toxin was carried out by a modification of the method described by Bhown and Habeeb (3). The overnight culture supernatant was precipitated by 70% ammonium sulfate. The collected pellet was dialyzed against 10 mM Tris-HCl (pH 8) and chromatographed on a DEAE Sephadex CL6B ion-exchange column (Pharmacia) equilibrated with the same buffer. The epsilon prototoxin was recovered in the flowthrough and was concentrated. The column was eluted with 0.1 M NaCl in the same buffer. The eluate was dialyzed against 10 mM Tris-HCl (pH 7.5) and was chromatographed on a DEAE Sephadex CL6B column equilibrated with the same buffer. The flowthrough containing the epsilon-toxin was concentrated. The purified proteins were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Fig. 1).

Cell culture. MDCK and Vero cells were grown in Dulbecco modified Eagle medium (DMEM) supplemented with 2 mM glutamine and 10% fetal calf serum for MDCK cells or 5% fetal calf serum for Vero cells at 37°C in a 5% CO_2 incubator.

For membrane preparation, cells were grown in petri plates (10-cm diameter) to confluency. The monolayers were washed twice with phosphate-buffered saline (PBS) and detached from their support with a rubber policeman. The cells were resuspended in 150 μl of 10 mM Tris-HCl (pH 7.2)–50% sucrose containing 10 μM leupeptin and 1 μM pepstatin. Harvested cells were incubated for 10 min at room temperature and lysed by three cycles of freezing and thawing. Centrif-

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ugation at 900 × *g* for 10 min at 4°C removed the nuclei. The supernatant was centrifuged at 20,000 × *g* for 30 min. The pellet constituted the crude membrane preparation, and the supernatant constituted the soluble cytoplasmic fraction.

Cytotoxicity assay. MDCK cells were grown to confluency 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) at 37°C in a 5% CO_2 incubator. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test was performed as described previously (14, 25). Fifty microliters of a freshly prepared solution of MTT (5 mg/ml; Sigma, Paris, France) in sterile PBS was added to each well. The plates were incubated at 37°C in 5% CO_2 for 3 h. Then 100 μl of lysis buffer (20% SDS, 50% dimethylformamide [pH 4.7]) was added to each well. After an overnight incubation at 37°C, the optical densities at 540 nm were measured with a Titer Tech 96-well multiscanner (Labsystems Multiskan Bichromatic).

Light microscopy and immunofluorescence. For light microscopy study, MDCK cells were grown on coverslips and incubated with epsilon-toxin for various periods of time as indicated. Such cultures and untreated control were examined in a phase-contrast microscope and photographed.

For immunofluorescence, the MDCK cells were grown on polycarbonate filters (Costar Transwell; pore size, 0.4 μm; diameter, 6.5 mm). The cells were seeded at a density of 7×10^4 per filter and used for experiments 5 days later. Immunofluorescence experiments were performed as described previously (11). Polyclonal antibodies purified against purified epsilon-toxin were raised in a rabbit as previously described (32), were purified by immunoaffinity, and were used at 15 μg/ml. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibodies (15 μg/ml; Vector Laboratories) were used as immunoconjugates. Actin cytoskeleton was visualized with FITC-phalloidin (1 μg/ml; Sigma).

Iodination of epsilon-toxin. Iodination was performed by incubating 5 μg of purified toxin or prototoxin in 20 μl of 0.3 M phosphate buffer (pH 6.8) containing 250 μCi of ^{125}I (17 Ci/mg; Dupont NEN, Les Ulis, France) for 15 min at room temperature in an Eppendorf tube coated with 50 μg of Iodogen (Pierce, Paris, France) according to the manufacturer's recommendation. Free ^{125}I was removed from the labeled protein by filtration on a Sephadex G-25 column equilibrated with 10 mM Tris-HCl (pH 7.8)–0.15 M NaCl–1% bovine serum albumin. The radioactive specific activity of the ^{125}I-epsilon-toxin or prototoxin was 1 to 2 mCi/mg of protein. Iodolabeled epsilon-toxin did not show a significant decrease in cytotoxicity on MDCK cells.

Binding of ^{125}I-labeled epsilon-toxin to cells and membrane preparations. MDCK and Vero cells were grown to confluency in six-well plates. ^{125}I-labeled epsilon-toxin or prototoxin (0.15 to 0.02 μg, representing 2×10^7 to 3×10^6 cpm) was added to 2×10^6 cells (13 to 2 ng of toxin/μg of total membrane protein) in 0.5 ml of DMEM with 5% fetal calf serum. After incubation at 4 or 37°C for appropriate periods of time as indicated in the figure legends, the cells were washed twice with 1 ml of PBS, pelleted, fractionated into membrane and cytoplasmic fractions as indicated above, and analyzed by electrophoresis.

Binding of ^{125}I-epsilon-toxin to membrane preparations was performed by incubating radiolabeled epsilon-toxin (0.07 μg) with 30 μg of total protein of membrane preparation in 50 μl (3 ng of toxin/μg of total protein) of DMEM or PBS without or with $CaCl_2$ for 30 min at 4 or 37°C. The membranes were washed twice with DMEM or PBS and resuspended in 30 μl of PBS. Five microliters of Laemmli sample buffer was added, and the samples were electrophoresed.

SDS-PAGE. SDS (0.1%)-PAGE (10 or 7% polyacrylamide gels) was performed as previously described (19). Samples were diluted with Laemmli sample buffer containing 0.3% 2-mercaptoethanol and 0.7% SDS (final concentrations) and boiled for 3 min prior loading on gel unless otherwise specified. Gels were stained with Coomassie brilliant blue R-250, dried, and autoradiographed.

Measurement of K^+ efflux. Cell monolayers in six-well plates containing 0.5 ml of culture medium were incubated with or without 13 ng of epsilon-toxin/μg of total membrane protein for 60 min at 37°C. The cells were washed twice with 20 mM HEPES–150 mM NaCl (pH 7.2) and lysed with 0.1 ml of 0.5% Triton X-100. The cell suspensions were diluted with 0.4 ml of H_2O, and the K^+ concentrations were determined by using a K^+-specific electrode and Beckman Synchron SX4.

RESULTS

Morphological alterations of cells induced by epsilon-toxin. MDCK cells treated with purified epsilon-toxin (Fig. 1) showed a marked swelling in the first phase of intoxication (Fig. 2B). Subsequently, one or several large blebs surrounded each cell (Fig. 2C to F). The blebs could be detached from the cells. They appeared transparent, containing no cell organelle. In the final phase, cell debris probably resulting from membrane disruption was observed.

The actin cytoskeleton visualized by FITC-phalloidin was unmodified and showed a pattern similar to that of untreated cells (data not shown).

Cytotoxicity does not require entry of epsilon-toxin into the cytosol. Agents able to block endocytosis mediated by acidic vesicles such as chloroquine, monensin, and an inhibitor of vesicle ATPase (bafilomycin A1) at concentrations known to be active (8) did not prevent the cytotoxic effects of epsilon-toxin (data not shown).

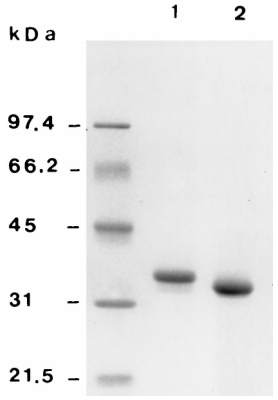

FIG. 1. SDS (0.1%)-PAGE (10% polyacrylamide gel; Coomassie blue stained) analysis of purified epsilon-prototoxin (2.8 μg; lane 1) and epsilon-toxin (2.8 μg; lane 2). The positions of molecular mass standards are shown on the left.

Recently, it has been found that endocytosis in MDCK is blocked at 97% by incubation of cells with 0.25 M sucrose and 30 mM benzyl alcohol (10). Epsilon-toxin showed the same cytotoxic activity on MDCK incubated with or without 0.25 M sucrose and 30 mM benzyl alcohol (Fig. 3A). In contrast, diphtheria toxin, which is known to enter into the cells through endocytosis to exert its cytotoxic activity (18), exhibited less cytotoxic activity in these conditions compared to the control without sucrose and benzyl alcohol (Fig. 3A).

An additional experiment to test the influence of endocytosis on epsilon-toxin cytotoxicity was performed with *C. perfringens* iota-toxin. The actin cytoskeleton is required for endocytosis (34), and iota-toxin ADP-ribosylates the actin monomers and disrupts the actin filaments (31). Pretreatment of MDCK with iota-toxin (10^{-7} M) for 5 h at 37°C did not prevent the intoxication by epsilon-toxin, whereas diphtheria toxin activity was decreased (Fig. 3B).

Immunofluorescence with polyclonal antibodies against epsilon-toxin was used in order to localize epsilon-toxin on polarized MDCK cells. As shown in Fig. 4, a peripheral labeling of cells was observed throughout the intoxication time course when epsilon-toxin was applied either on the basolateral or the apical side. However, cytotoxicity assayed by the MTT test was more rapid and more intense when the toxin was added on the apical side (Fig. 5B). In addition, immunolabeling of cells incubated with epsilon-toxin on the apical side showed cell alterations (enlargement and cell lysis) comparable to that observed with phase-contrast microscopic observations (Fig. 2). In all cases, fluorescence was not detected inside the cells, which strongly suggests that epsilon-toxin remained on the cell surface during intoxication and that it acts preferentially on the apical side of MDCK.

Efflux of intracellular K^+ induced by epsilon-toxin. To test if epsilon-toxin alters the membrane permeability, we determined the intracellular K^+ concentration. Figure 6 shows that 13 ng of epsilon-toxin/μg of total membrane protein induced an 80% reduction of the intracellular K^+ content after 60 min of incubation. Under the conditions applied, a 65% reduction of cell viability, as measured by the MTT test, was observed (Fig. 7C). This finding indicated that the K^+ efflux induced by epsilon-toxin was correlated with its cytotoxic effect.

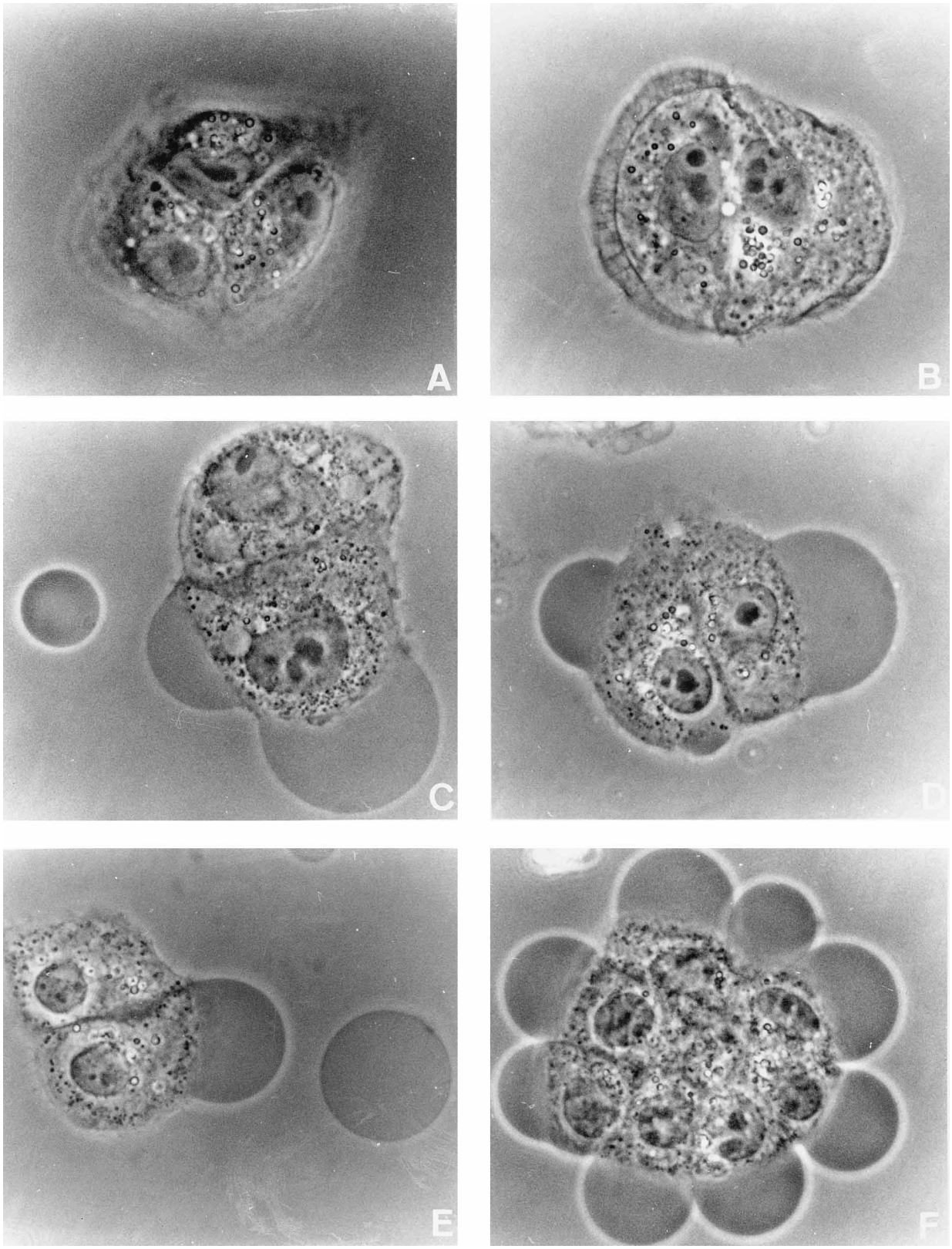

FIG. 2. Morphological changes of MDCK cells induced by epsilon-toxin. The cells were exposed to epsilon-toxin (50 to 375 ng/ml) for 60 min at 37°C in a 5% CO_2 incubator and were observed under phase contrast (magnification, ×100). (A) Control cells; (B to F) cells exposed to epsilon-toxin at 50 ng/ml (B), 120 ng/ml (C and D), 210 ng/ml (E), and 375 ng/ml (F). Swelling is observed in panel B, and blebbing is observed in panels C to F.

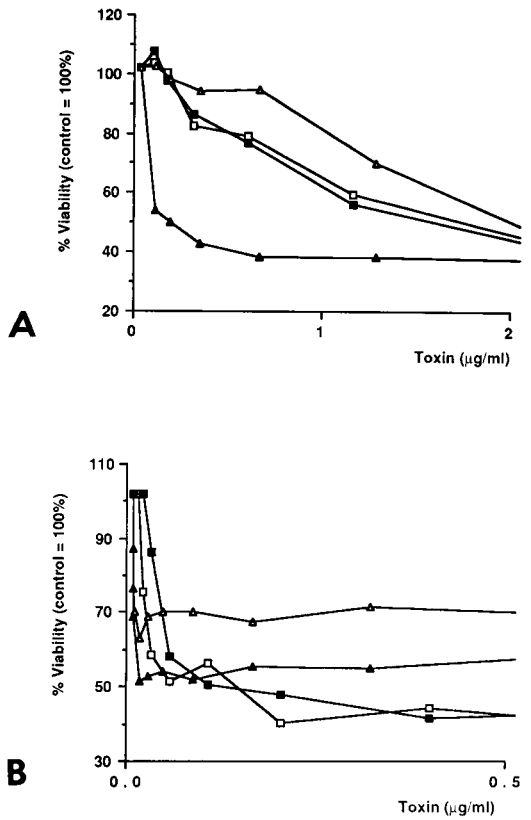

FIG. 3. Influence of endocytosis on epsilon-toxin and diphtheria toxin cytotoxic activity. (A) MDCK cells were preincubated with DMEM–5% fetal calf serum in the absence (■ and ▲) or presence (□ and △) of 0.25 M sucrose for 30 min at 37°C. Epsilon-toxin (■ and □) and diphtheria toxin (▲ and △) were added, and incubation was prolonged for 30 min at 37°C in the absence (■ and ▲) or presence (□ and △) of 0.25 M sucrose and 30 mM benzyl alcohol. Then the cells were washed and incubated in DMEM containing anti-epsilon-toxin or anti-diphtheria toxin antibodies for 5 h for epsilon-toxin-treated cells and 24 h for diphtheria toxin-treated cells. Cytotoxicity was measured by the MTT assay. (B) MDCK cells were preincubated in the absence (■ and ▲) or presence (□ and △) of *C. perfringens* iota-toxin (10^{-7} M) for 5 h at 37°C. Epsilon-toxin (■ and □) and diphtheria toxin (▲ and △) were added, and cells were incubated for 5 h and overnight, respectively, at 37°C.

Epsilon-toxin forms a large complex on MDCK cells. To characterize the interaction of epsilon-toxin with cells, ^{125}I-labeled epsilon-toxin was incubated with MDCK cells or Vero cells in DMEM containing 5% fetal calf serum. The membrane and cytoplasmic fractions were analyzed by SDS-PAGE and autoradiography. MDCK cell membrane preparations showed the presence of epsilon-toxin migrating both at 34 kDa, which is the expected size of monomeric epsilon-toxin, and at about 155 kDa (Fig. 8). This result suggests the formation of a large complex which was clearly detectable after 30 min of incubation and even more so at 60 min. The cytoplasmic fraction contained only small amounts of monomeric epsilon-toxin but no large complex. The material migrating at 34 kDa could represent noncomplexed, specifically bound ^{125}I-epsilon-toxin with a cell component or dissociation of the complex during electrophoresis. In contrast, Vero cells, which are insensitive to epsilon-toxin, did not show any binding of epsilon-toxin with either the membrane or the cytoplasmic preparation (Fig. 8).

The large membrane complex was dependent on the concentration of epsilon-toxin incubated with MDCK cells (Fig. 7A) and on the incubation time (Fig. 7B). The earliest detectable formation of the large membrane complex at 37°C occurred as soon as 15 to 20 min after addition of 13 ng of ^{125}I-labeled epsilon toxin/μg of protein (Fig. 7B) and corresponded to 10 to 20% of cytotoxicity as measured by the MTT test (Fig. 7C). Fifty percent of complex formation and cytotoxicity occurred at 30 to 40 min (Fig. 7B and C). After 2 h of incubation, the cells were detached from the support, and the signal corresponding to the large membrane complex had approximately the same intensity as at 1 h of incubation (data not shown). These results showed a correlation between the kinetic of the formation of the large membrane complex and that of cytotoxicity. In addition, the purified prototoxin (Fig. 1), which was not cytotoxic (Fig. 7C), did not induce the formation of a large membrane complex (Fig. 7D).

When epsilon-toxin (13 ng/μg of protein) was applied to the apical side of polarized MDCK cells, the kinetics of cytotoxicity and complex formation (Fig. 5) were similar to those observed with MDCK cells grown on a plastic support. However, when the basolateral side of MDCK cells was exposed to the toxin, the cytotoxicity and complex formation were reduced (Fig. 5). This result indicates that epsilon-toxin preferentially interacted with the apical side of MDCK cells.

The formation of the large membrane complex was observed in vitro when the MDCK cell membrane preparation was incubated with radiolabeled epsilon-toxin (Fig. 9). The association of radioactivity with the large membrane complex was completely blocked when ^{125}I-epsilon-toxin was tested in the presence of an excess of unlabeled epsilon-toxin (Fig. 9A) or neutralizing polyclonal or monoclonal antibodies against epsilon-toxin (Fig. 9B). This result strongly suggests that the formation of the large membrane complex was due to specific binding of epsilon-toxin to an MDCK cell membrane receptor.

Stability of the large membrane complex and divalent cation requirement. The stability of the large membrane complex was investigated with MDCK cell membranes incubated with radiolabeled epsilon-toxin at either 4 or 37°C. When binding was performed at 4°C, the large complex remained intact after addition of sample buffer with 0.7% (final concentration) SDS (Fig. 7D). However, no large complex was detected with boiled and unboiled samples supplemented with 5% (final concentration) SDS (Fig. 10A). In contrast, incubation of epsilon-toxin with cell membranes at 37°C led to a very stable large complex under these conditions (Fig. 10A).

No difference was observed in large complex formation when the membranes were incubated with ^{125}I-epsilon-toxin in PBS in the presence or absence of 1 mM $CaCl_2$ or EDTA, suggesting that divalent cations are not required (Fig. 10A).

Involvement of cell protein(s) in the large membrane complex. The large membrane complex was cleaved by proteinase K or trypsin, yielding a 74- or 144-kDa fragment, respectively (Fig. 10B). This could correspond to cleavage of epsilon-toxin or cell membrane protein(s) forming the complex. MDCK cell membranes treated with proteinase K prior to incubation with radiolabeled epsilon-toxin showed the formation of a complex of 95 kDa (data not shown). In contrast, pretreatment of cell membrane with phospholipase C (420 μg/mg of total protein), phosphatidylinositol phospholipase C (24 U/mg of total protein), or *N*-acetyl neuraminidase (4 U/mg of total protein) did not modify the size (about 155 kDa) of the complex formed with epsilon-toxin (data not shown). This finding indicates that epsilon-toxin associated with at least one membrane protein.

DISCUSSION

The cell morphological changes induced by epsilon-toxin consist of significant swelling, blebbing, and cell lysis. Similar

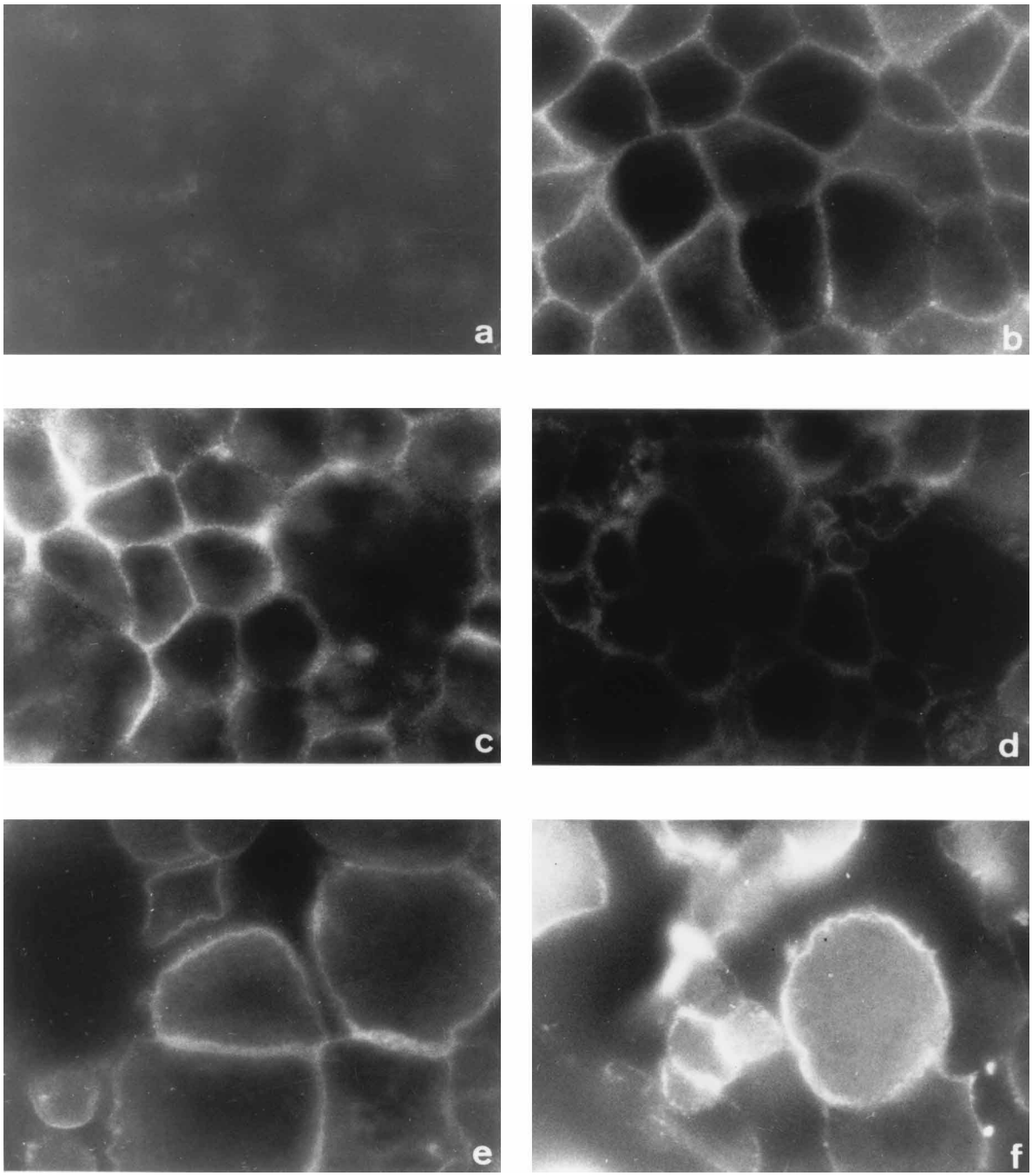

FIG. 4. Cell localization of epsilon-toxin on polarized MDCK cells. Cell localization of epsilon-toxin by immunofluorescence on control cells (a) and on cells treated with epsilon-toxin (1 μg/ml) (b to f) on the basolateral side for 30 (b) and 60 min (c) and on the apical side for 10 (d), 30 (e), and 60 (f) min. Cells were fixed and stained with antibodies against epsilon-toxin and FITC-labeled anti-rabbit antibody.

structural changes including swelling, disruption of the plasma membrane, and nuclear condensation have been reported by Hambrook et al. (13). The epsilon-toxin effects differ from those caused by toxins disorganizing the cytoskeleton such as *C. difficile* toxins, *C. novyi* alpha-toxin, and C2 toxin, which are characterized by cell retraction followed by rounding up with many cytoplasmic extensions still adherent to the support (26). *C. difficile* ToxA induces blebbing in some cell lines. However, the ToxA-mediated blebs are smaller and contain cell organelles (7) in contrast to that observed with epsilon-toxin.

The rapidity of the cytotoxic effects (15 to 20 min) and the alteration of cell morphology argue for a direct action of epsilon-toxin on the cell membrane. Similar observations were reported by Hambrook et al. (13). Toxins which act intracellularly usually enter into the cells by mean of low-pH endosomes or by vesicles routing through the Golgi apparatus. Agents which block the endocytic acidification (chloroquine, monensin, and bafilomycin A1) and which inhibit the internalization of many toxins into the cytosol were unable to prevent epsilon-toxin cytotoxicity. Certain toxins, such as cholera toxin

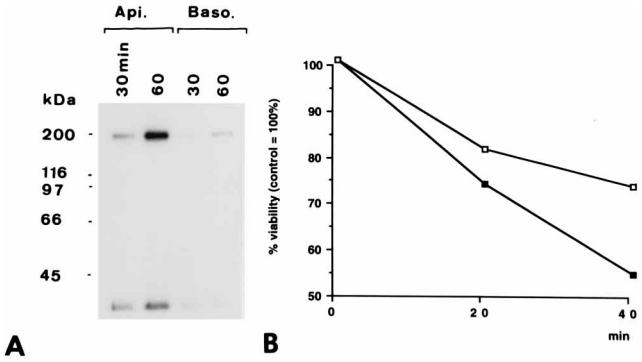

FIG. 5. Large-complex formation and cytotoxic activity of epsilon-toxin on polarized MDCK cells. (A) Large membrane complex formed with ^{125}I-labeled epsilon-toxin (13 ng/μg of total protein) applied on the apical (Api.) or basolateral (Baso.) side at 37°C for 30 and 60 min. Samples were analyzed by SDS (0.1%)-PAGE (7% polyacrylamide gel) and autoradiography. (B) Cytotoxicity of epsilon-toxin (13 ng/μg of total protein) applied on the basolateral (□) and apical (■) sides and measured by the MTT test.

and Shiga toxin, are internalized by receptor-mediated endocytosis and are routed through the Golgi apparatus, since they are blocked by brefeldin A, which is known to disrupt the Golgi stacks (6, 42). However, MDCK cells are resistant to brefeldin A (41), and thus the Golgi pathway cannot be explored in MDCK cells. Benzyl alcohol and sucrose, which were found to block endocytosis in MDCK cells (10), did not inhibit the epsilon-toxin cytotoxic activity, whereas diphtheria toxin effects were prevented. In addition, the actin disorganization by iota-toxin of the cytoskeleton, which seems to be required for membrane traffic (34), was unable to prevent the epsilon-toxin cytotoxicity. Recently, similar results were obtained by Lindsay (20). He showed that a reduction of endocytosis either by increasing Ca^{2+} and/or Mg^{2+} or in the presence of sodium azide did not modify the epsilon-toxin cytotoxicity. Immunofluorescence studies provide additional evidence that epsilon-toxin is localized on the cell surface during the intoxication process. Antibodies against epsilon-toxin showed a surface labeling of polarized MDCK cells incubated with epsilon-toxin when toxin was applied on the basolateral or the apical side. The cell morphological alterations were observed essentially when epsilon-toxin was applied on the apical side. Taken together, these data strongly suggest that epsilon-toxin remains associated to the cell membrane during the intoxication process and that it acts preferentially on the apical side of MDCK cells.

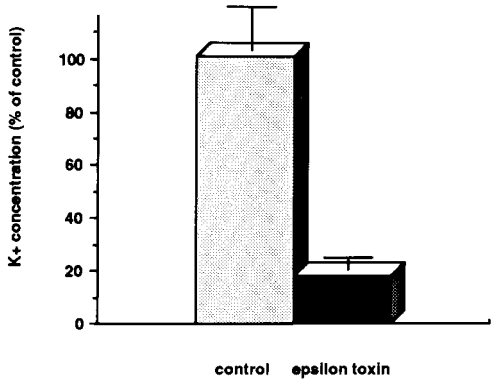

FIG. 6. Epsilon-toxin causes a significant reduction of the intracellular K^+ concentration. Relative K^+ concentrations were determined in untreated and toxin-treated MDCK cells after 60 min of incubation. Data are means of four determinations ± standard deviations (error bars).

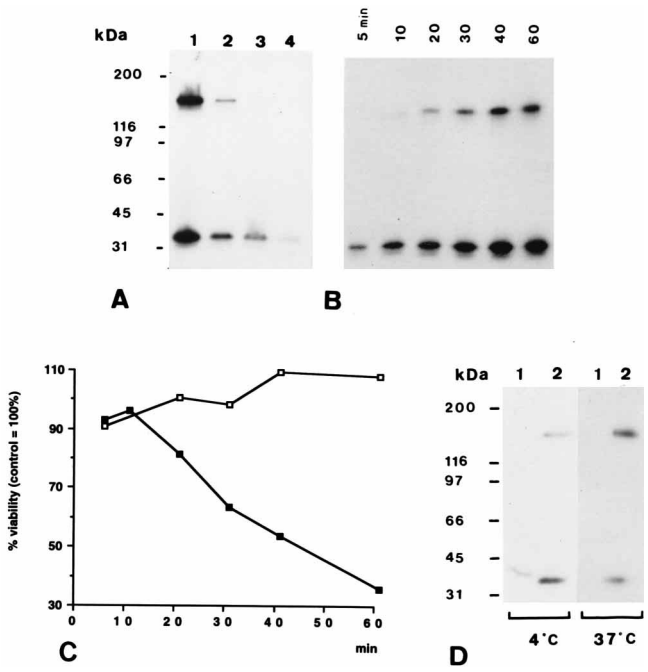

FIG. 7. Dose response (A) and kinetics (B) of large-membrane complex formation in MDCK cells. (A) MDCK cells were exposed to ^{125}I-labeled epsilon-toxin at 13 (lane 1), 7 (lane 2), 4 (lane 3), and 2 (lane 4) ng/μg of total protein for 1 h at 37°C. (B) MDCK cells were exposed with ^{125}I-labeled epsilon-toxin (13 ng/μg of total protein) for 5 to 60 min. The cells were harvested, and samples of the membrane preparations were analyzed by SDS (0.1%)-PAGE (7% polyacrylamide gel) and autoradiography. (C) MDCK cells were incubated with prototoxin (□) and epsilon-toxin (■) (13 ng/μg of total protein) for 5 to 60 min at 37°C. (D) Formation of a large membrane complex in MDCK cells with epsilon-toxin at 4 and 37°C but not with the prototoxin. MDCK cells were exposed to ^{125}I-labeled epsilon-prototoxin (13 ng/μg of total protein) (lane 1) or to ^{125}I-labeled toxin (13 ng/μg of total protein) (lane 2) at 4 and 37°C for 1 h. Samples of the membrane preparation were analyzed by SDS (0.1%)-PAGE (7% polyacrylamide gel) and autoradiographed.

The morphological modifications with enlargement of cell volume suggest that epsilon-toxin alters the membrane ion

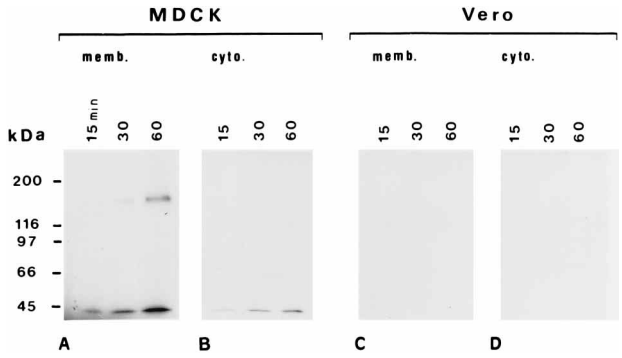

FIG. 8. Autoradiography showing large-complex formation analyzed by SDS (0.1%)-PAGE (7% polyacrylamide gel). MDCK cells and Vero cells were exposed to ^{125}I-labeled epsilon-toxin (13 ng/μg of total protein) at 37°C for 15, 30, and 60 min. The cells were harvested and fractionated in membrane (memb.) and cytoplasmic (cyto.) preparations as described in Materials and Methods. Samples of each fraction were electrophoresed and autoradiographed. Large-complex formation was observed only with membrane preparation from MDCK cells. Positions of molecular size markers are indicated on the left side.

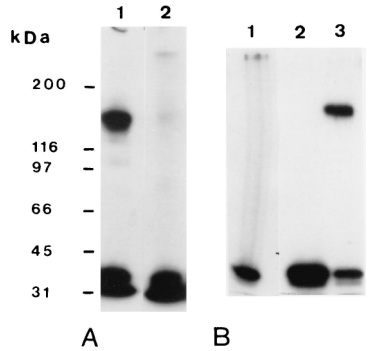

FIG. 9. Inhibition of radiolabeled large membrane complex formation in MDCK cells by polyclonal and monoclonal antibodies against epsilon-toxin and by unlabeled epsilon-toxin. (A) Cell membrane preparations were preincubated in the absence (lane 1) or presence (lane 2) of an excess of unlabeled epsilon-toxin for 5 min at 37°C and then incubated with ^{125}I-labeled epsilon-toxin (3 ng/μg of total protein) for 30 min at 37°C. (B) Cell membrane preparations were incubated with ^{125}I-labeled epsilon-toxin (3 ng/μg of total protein) in the absence (lane 3) or presence of polyclonal antibodies (1/100 final concentration; lane 1) or monoclonal antibody (Central Veterinary Laboratory, Weybridge, United Kingdom; 1/100 final concentration; lane 2) against epsilon-toxin for 30 min at 37°C. Samples were analyzed by SDS (0.1%)-PAGE (7% polyacrylamide gel) and autoradiography.

permeability. Indeed, our data show that epsilon-toxin induced a significant depletion of the intracellular K^+ content of MDCK cells. The interaction of epsilon-toxin with the cell membrane could result in an increase of K^+ permeability and perhaps of other ions.

Our findings with ^{125}I-labeled epsilon-toxin showed that the toxin, and not the prototoxin, can associate specifically with the membrane of sensitive MDCK cells, forming a large complex of about 155 kDa, but not with insensitive Vero cells. The formation of the large membrane complex is rapid (15 to 20 min) and is correlated with the appearance of the cytotoxicity. This finding suggests that the formation of the large membrane complex is essential for cytotoxicity.

The large membrane complex induced by epsilon-toxin at 4°C is less stable than that induced at 37°C, since it is dissociated by 5% SDS and heating. This is consistent with the recognition of a specific receptor by the toxin, occurring at 4 or 37°C, and a maturation phase occurring only at 37°C and leading to the formation of a stable complex. The maturation step could correspond to the formation of a stronger linkage between the toxin and its receptor. This is possibly related to a molecular conformational change, to an insertion of the toxin bound to its receptor in the membrane, or to a combination of both mechanisms.

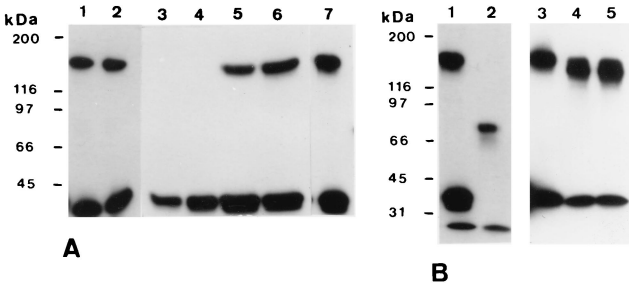

FIG. 10. Ca^{2+} requirement, heat stability, and cleavage by proteinase K and trypsin of the large membrane complex. (A) MDCK cell membrane preparations were incubated with ^{125}I-labeled epsilon-toxin (3 ng/μg of total protein) in PBS (lane 1) or in PBS containing 1 mM $CaCl_2$ (lane 2) or 2 mM EDTA (lane 7) for 30 min at 37°C. Cell membrane preparations were incubated in PBS with ^{125}I-labeled epsilon-toxin (3 ng/μg of total protein) for 30 min at 4°C (lanes 3 and 4) or at 37°C (lanes 5 and 6). Then the samples were supplemented with 5% (final concentration) SDS and were boiled for 5 min (lanes 3 and 5) or not boiled (lanes 4 and 6) prior to electrophoresis and autoradiography. (B) Cell membrane preparations were incubated with ^{125}I-labeled epsilon-toxin (3 ng/μg of total protein) for 30 min at 37°C and then with proteinase K (100 μg/mg of protein [lane 2]) or trypsin (330 [lane 4] or 165 [lane 5] μg/mg of protein) for 30 min at 37°C prior to electrophoresis and autoradiography. Undigested control samples are in lanes 1 and 3.

The epsilon-toxin action resembles to some extent that of *C. perfringens* enterotoxin (CPE). CPE is produced by certain *C. perfringens* strains and is responsible for food-borne intoxication (15). CPE is a 36-kDa protein which is cytotoxic for Vero and Caco-2 cells (44). The proposed CPE mechanism of action includes (i) binding of CPE to a 45- to 50-kDa membrane protein or to a 22-kDa protein receptor (17) of sensitive cells and formation of a small complex, (ii) insertion and/or conformational change of CPE within the small complex which becomes resistant to protease(s), (iii) large-complex formation by interaction of the small complex with a 70-kDa protein, and (iv) increase of membrane permeability for small molecules. Steps i and ii can occur at 4°C, but a temperature of 37°C is required for steps iii and iv. CPE large complex can alter the membrane permeability by directly forming pores or by another unidentified mechanism (23, 44, 45). In contrast to CPE, the large membrane complex induced by epsilon-toxin seems to form in only one step.

Toxins such as *Staphylococcus aureus* alpha-toxin modify the permeability of cell membranes (2). The alpha-toxin (33 kDa) binds to a specific receptor in the membrane of susceptible cells which could be a protein or a glycoprotein possibly coupled to a phospholipid. Membrane-bound monomers of alpha-toxin form heptamers, preferentially at 37°C, which generate nonselective pores permitting the leakage of molecules smaller than 1,000 to 4,000 Da (2). The alpha-toxin is also able to form pores in planar lipid bilayers (1). In contrast, epsilon-toxin was unable to form pores in large unilamellar vesicles constituted of egg phosphatidylcholine and phosphatidic acid (9:1) either at neutral or at acidic pH (data not shown). Moreover, no pore formation could be observed in attempts to incorporate the toxin in the membrane of lipid vesicles by using Triton X-100, *n*-octylglucoside, or sodium cholate as described by Rigaud et al. (33) (data not shown). This observation confirms that epsilon-toxin cannot interact with membranes through a simple spontaneous mechanism. In addition, no spontaneous aggregation of epsilon-toxin in solution was observed upon electrophoresis of radiolabeled epsilon-toxin.

It was reported that among 12 cell lines tested, only MDCK cells were sensitive to epsilon-toxin (30). In addition, we found that PC12 cells and intestinal I407, T84, and Caco-2 cells were insensitive (data not shown). The fact that epsilon-toxin is cytotoxic for a restricted number of cell lines and that the large membrane complex is observed with MDCK cells and not with insensitive cells such as Vero cells argues for the presence of a specific membrane receptor on sensitive cells. Pretreatment of MDCK cell membranes with proteinase K led to a reduced size of the complex induced by epsilon-toxin, which indicates that a cell membrane protein could correspond to the specific receptor or to a component of the epsilon-toxin complex. Using affinity purification with epsilon-toxin linked to Sepharose beads, a 37-kDa protein was isolated from MDCK cell membranes (data not shown). The function of this protein remains to be determined. The composition of the large membrane complex is still speculative and could correspond to the interaction of epsilon-toxin with a protein receptor and then to (i) oligomerization of the toxin, (ii) oligomerization of receptor, or (iii) binding to some other membrane component(s). The

large membrane complex induced by epsilon-toxin could lead to a pore formation and to a cell permeability alteration. The alteration of membrane K^+ permeability suggests that epsilon-toxin has a pore-forming activity. However, one cannot rule out the possibility of epsilon-toxin modifying a specific ion channel which could increase the cell membrane permeability. Further work is in progress to identify the precise mechanism of action of epsilon-toxin.

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