Pore-forming epsilon toxin causes membrane permeabilization and rapid ATP depletion-mediated cell death in renal collecting duct cells

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Chassin C, Bens M, de Barry J, Courjaret R, Bossu JL, Cluzeaud F, Ben Mkaddem S, Gibert M, Poulain B, Popoff MR, Vandewalle A. Pore-forming epsilon toxin causes membrane permeabilization and rapid ATP depletion-mediated cell death in renal collecting duct cells. Am J Physiol Renal Physiol 293: F927–F937, 2007. First published June 13, 2007; doi:10.1152/ajprenal.00199.2007.—Clostridium perfringens epsilon toxin (ET) is a potent pore-forming cytotoxin causing fatal enterotoxemia in livestock. ET accumulates in distal-collecting duct cells and exhibits severe kidney damage, including selective degeneration of distal-collecting duct cells and that ET-GFP-infected mice exhibit severe kidney damage, including selective degeneration of the distal nephron. It has been suggested that renal distal-collecting duct cells may possess a specific receptor for ET. ET has been shown to bind to detergent-resistant membrane (DRM) microdomains (or lipid rafts) and to oligomerize to form an heptameric complex in rat synaptosomal and renal Madin-Darby canine kidney (MDCK) cell membranes (28, 29, 34). The toxin can also assemble and form nonspecific diffusion channels when inserted into artificial lipid bilayers (35). The resolution of the crystal structure of ET has revealed that the toxin forms a β-barrel pore exhibiting some structural similarities with aerolysin, another pore-forming toxin (22). Binding to specific membrane receptor(s) remains an absolute prerequisite for toxin toxicity in living cells (22). However, among the many cell lines tested, only MDCK and human renal leiomyoblastoma G-402 cells have been found to retain susceptibility to ET in vitro (34). In MDCK cells, the toxin has been shown to bind specifically to plasma membranes but not to intracellular organelles (34, 36). ET also induces rapid cell death without altering tight junction permeability, the actin cytoskeleton network, or endocytotic processes (36). With regard to the pathophysiological action of ET, two major issues remain to be clarified. First, the relevance to physiology of the insights gained from analyzing the effects of ET on MDCK cells is disputable because these cells have lost the main characteristic features and specific ion transport properties of the distal-collecting duct cells from which they are derived. Second, ET displays a lethal activity (in mice, the LD₅₀ is 100 ng/kg), which is 100-fold lower than that of the structurally related pore-forming toxins such as aerolysin (26). This raised the question of the cause-to-effect link between the membrane permeabilization and induction of death caused by ET.

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In this study, we analyzed the effects of ET on ion diffusion processes and the induction of cell death in a highly differentiated murine renal cortical collecting duct principal (CCD) cell line, mpkCCDc14 (4), which has retained the main characteristics of the parental CCDs from which they were derived (5). We show that the binding and oligomerization of ET in DRMs of mpkCCDc14 cells stimulated the electrogenic absorption of Na+ and induced a sustained rise in intracellular Ca2+. Furthermore, ET induced a rapid depletion of cellular ATP content, the permeabilization of mitochondrial membranes, and the mitochondrial-nuclear translocation of apoptosis-inducing factor (AIF), a potent, caspase-independent cell death effector (10, 11, 43). However, ET did not induce nuclear fragmentation, although it did trigger rapid cell necrosis. Rather unexpectedly, DRM disruption by methyl-β-cyclodextrin (MβCD), which impairs the membrane effects of ET, had no impact on the rapid fall in cell ATP and subsequent cell death caused by ET. These results indicate that ET, the most potent clodstridial toxin after botulinum and tetanus neurotoxins, induces rapid cell necrosis of renal collecting duct cells, which by the way of a mechanism that differs from that reported for many other pore-forming toxin and appears uncoupled to the membrane alterations.

MATERIALS AND METHODS

Materials. ET was purified from an overnight culture of C. Perfringens type D strain NCT2062 as described by Petit et al. (34). The purity of ET and epsilon toxin prototoxin (EPT; >90%) was checked by SDS-PAGE electrophoresis. Iodination of the purified toxin and prototoxin was performed as described (34). Experiments were carried out on mpkCCDc14 cells grown in DMEM:Ham’s F-12, 1:1 vol/vol, supplemented with hormones and 2% fetal calf serum at 37°C in 5% CO2-95% air atmosphere, as previously described (4). Cell viability was assayed by the MTT dye assay as described (34). When required, nuclei were stained with Hoechst H33342 (Sigma, St. Louis, MO).

Immunofluorescence studies. Imaging studies were conducted on living cells incubated with 10–7 M Alexa 488-tagged ET, either alone or with 100 nM Mitotracker Deep Red 633 (Molecular Probes, Eugene, OR). Indirect immunofluorescence was also performed on cells fixed with ice-cold methanol, using a purified rabbit anti-AIF polyclonal antibody (1:100 in PBS/0.3% Triton X-100, Cell Signaling Technology, Danvers, MA) and goat anti-rabbit-CY3-conjugated IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) as the secondary antibody. DNA was stained with SYTOX Green (1:500 in PBS/0.3% Triton X-100 by rotary shaking at 4°C for 30 min) at 4°C. Pelleted cells were resuspended in 250 μl PBS, containing a protease inhibitor cocktail and PMSF. The cell suspension was then homogenized and centrifuged (900 g, 10 min) at 4°C. The procedure was repeated once on the resulting pellet. The lysed supernatants were pooled and then centrifuged (19,000 g, 50 min) at 4°C. The resulting pellet was resuspended in 150 μl sucrose buffer containing protease inhibitor cocktail and PMSF. Pelleted membranes were resuspended in 25 μl Laemmli buffer and submitted to SDS-PAGE followed by autoradiography.

Preparation of mpkCCDc14 cell membranes and electrophoresis. Cell pellets were scraped off, rinsed in ice-cold PBS, and centrifuged (300 g, 5 min) at 4°C. Pelleted cells were resuspended and incubated for 10 min at 4°C in 500 μl of sucrose buffer (250 mM sucrose, 3 mM imidazole, 1 mM EDTA, pH 7.2) containing a protease inhibitor cocktail (Complete, Roche, France) and 1 mM PMSF. The cell suspension was then homogenized and centrifuged (900 g, 10 min) at 4°C. The procedure was repeated once on the resulting pellet. The lysed supernatants were then centrifuged (19,000 g, 50 min) at 4°C. The resulting pellet was resuspended in 100 μl sucrose buffer containing protease inhibitor cocktail and PMSF. Pelleted membranes were resuspended in 25 μl Laemmli buffer and submitted to SDS-PAGE followed by autoradiography.

Preparation of detergent-insoluble membrane fractions and flotation centrifugation on sucrose gradient. Cell membranes were incubated in PBS containing protease inhibitors 1 mM PMSF and 1% Triton X-100 by rotary shaking at 4°C for 30 min. Triton X-100-insoluble pellets, corresponding to DRMs, were resuspended in 200 μl Triton X-100-free PBS. Supernatant proteins were precipitated by adding 700 μl of 62.5 mM Tris·HCl (pH 6.8), 2% wt/vol SDS, 10% glycerol, and 50 mM DTT and sonicated for 20 s at 4°C. Cell lysates were incubated in 200 μl PBS containing a protease inhibitor cocktail and used to detect the corresponding antigens. Protein phosphorylation was analyzed using antibodies against phosphorylated AMPK (1:500) and β-actin (1:1,000, Sigma) were used to detect the corresponding antigens. Protein phosphorylation was analyzed using antibodies against phosphorylated AMPK (1:500) and β-actin (1:1,000, Sigma) were used to detect the corresponding antigens. Protein phosphorylation was analyzed using antibodies against phosphorylated AMPK (1:500) and β-actin (1:1,000, Sigma) were used to detect the corresponding antigens.
for 30 min by rotary shaking. The lysates were brought to 40% sucrose in a final volume of 500 μl in polyallomer tubes (Beckman Coulter, Fullerton, CA) and overlaid with 3.5 ml of 30% sucrose in PBS, followed by 1 ml of 5% sucrose in PBS. The gradients were centrifuged at 150,000 g for 18 h at 4°C. Fractions (500 μl) were collected from the top of the tubes and then precipitated as described above and centrifuged (19,000 g, 30 min) at 4°C. Resuspended pellets in 25 μl Laemmli were boiled for 5 min and run on a 10% SDS-PAGE. Membranes were then subjected to Western blotting using an anti-caveolin-1 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA).

Treatment with PI-PLC. Confluent cells transfected or not with a plasmid encoding for the decay accelerating factor (DAF), a GPI-anchored protein, fused to GFP, kindly provided by P. Bocquet and A. Galmiche (15), were preincubated for 2 h with 10 μg/ml cycloheximide to inhibit protein neosynthesis and 6 U/ml of phosphatidylinositol-specific phospholipase C (PI-PLC; Sigma) in DM medium at 37°C (1, 30). mpkCCDcl4 cells were also preincubated with cycloheximide and PI-PLC, as described above, and then with unlabeled ET (10⁻⁷ M) for 60 min at 37°C or with purified C. septicum alpha toxin (10⁻⁶ M) for 3 h at 37°C. Triton-insoluble membrane fractions were then prepared and subjected to Western blotting. The blots were revealed using immunopurified rabbit antibodies directed against ET or alpha toxin (37).

Electrophysiological studies. The transepithelial electrical resistance (Rₑ), potential (PD), and short-circuit current (Iₛ) were measured on confluent mpkCCDcl4 cells grown on Transwell filters (0.4-μm pore size, Corning Costar, Cambridge, MA) as described (4). Iₛ recordings were also performed on sets of confluent cells in which the apical Na⁺-containing hormonal-free medium (HFM) was replaced by a Na⁺-free solution (in mM): 156 N-methyl-D-glucamine, 4 KCl, 0.7 MgCl₂, 0.4 MgSO₄, 1.05 CaCl₂, 20 glucose, 8 HEPES, pH 7.4, while the basal side of the filter was bathed with Na⁺-free solution (in mM): 132 K⁺, 4 N-methyl-D-glucamine, 4 MgCl₂, 2 NaCl, 10 HEPES/KOH, 2 MgATP, and 0.5 GTP. Cells were voltage-clamped to a steady-state holding potential of −60 mV, and the whole cell current was monitored throughout the experiment and sampled at 10 kHz (Axopatch 200A amplifier, Digidata 1322A and pClamp8 suite from Axon Instruments, Foster City, CA). When required, the transmembrane resistance was −60 mV was determined by the square voltage step method under the current clamp mode. Except when ET was added, cell cultures were superfused continuously using an extracellular solution containing (in mM) 130 NaCl, 2.7 KCl, 5 CaCl₂, 0.5 MgCl₂, 8 HEPES/Tris, 5.6 glucose, and 0.1% BSA (pH 7.4).

Intracellular Ca²⁺ measurements. [Ca²⁺], recordings were performed on cells grown on glass coverslips and loaded with fura-2-AM (2 μM for 20 min). Cells were placed on an inverted epifluorescence microscope (Axiovert, Zeiss, Germany) and continuously superfused (1 ml/min) with Krebs solution containing 5 mM glucose and 10 mM HEPES buffer (pH 7.2). They were illuminated alternately at 350 and 380 nm and image pairs were recorded at 520 nm every 10 s for 20 min. The ratio of the fluorescence intensities (Ex 350 nm/Ex 380 nm) was calculated on a pixel basis for each image pair. The mean value of the fluorescence ratio was normalized for each region of interest, and the changes in [Ca²⁺], were estimated without further calibration.

Data presentation and analysis. Results are given as means ± SE from (n) separate experiments. Statistical analyses were done using a two-tailed Student’s t-test. P values <0.05 were considered significant.

RESULTS

ET binds to plasma membranes of living mpkCCDcl4 cells. Imaging studies revealed that Alexa 488-tagged ET (10⁻⁸ M, 30 min) binds to apical and basolateral membranes of mpkCCDcl4 cells grown on filters (Fig. 1A). Alexa 488-tagged ET labeling remained restricted to the apical or basal cell surface membrane domain directly exposed to ET, with no fluorescent label being detected on the opposite basal or apical cell membrane domain (Fig. 1A). Alexa 488-tagged ET binding was prevented by preincubating the mpkCCDcl4 cells with an excess of unlabeled EPT (not shown), suggest-

Fig. 1. Epsilon toxin (ET) forms large insoluble complexes in mpkCCDcl4 cell membranes. A: confocal micrographs of cells grown on filters and incubated with 10⁻⁸ M Alexa 488-tagged ET for 30 min added either on the apical (left) or basal side (right) of the filters. ap, Apical; ba, basal. Scale bar = 10 μm. B and C: SDS-PAGE and autoradiography of cell membrane preparations (10 μg per lane) from confluent cells grown on filters incubated with 125I-ET (10⁻⁸ M; B) or with cell membranes at 37°C with 125I-epsilon prototoxin (EPT; lane 1), 125I-ET (lane 2), 125I-ET plus 10-fold excess (lane 3) or 10-fold excess (lane 4) of unlabeled EPT (C). D: SDS-PAGE and autoradiography of whole cell membranes (lane 1), Triton X-100-insoluble (lane 2), and -soluble (lane 3) membrane extracts incubated with 125I-ET. E: sucrose gradient fractionation of Triton X-100-insoluble membrane extracts from cells incubated with 125I-ET (10⁻⁸ M, 60 min). The 30-kDa ET monomers and 220-kDa ET complexes were recovered in the same fractions (lanes 2–5) containing the detergent-resistant membrane (DRM) marker caveolin-1. F: no 220-kDa labeled band was detected in Triton-insoluble (lanes 1 and 3) and Triton-soluble (lanes 2 and 4) fractions prepared from cells incubated with 10 mM methyl-β-cyclodextrin (MβCD) or 0.4% saponin and then with 125I-ET.
ing that EPT and ET may share a common membrane receptor. Additional imaging studies with organelle-specific dyes also confirmed that the toxin did not bind to any intracellular organelles (not shown).

ET forms large insoluble complexes in the detergent-resistant plasma membrane microdomains of filter-grown mpkCCD\textsubscript{cl4} cells. Previous studies suggested that the formation of membrane pores by ET is correlated to the appearance of toxin heptameric complexes (28, 34). We investigated the ability of ET to form multimeric complexes in both apical and basolateral membranes of mpkCCD\textsubscript{cl4} cells. SDS-PAGE analysis of lysed cell layers and quantification studies (not shown) revealed that \textsuperscript{125}I-labeled 220-kDa complexes were \textasciitilde 1.5-fold more abundant in the apical than in the basolateral membranes (Fig. 1B). \textsuperscript{125}I-ET, but not \textsuperscript{125}I-EPT, formed large insoluble complexes in purified membrane preparations (Fig. 1C, lanes 1 and 2). The formation of \textsuperscript{125}I-ET complexes was not altered when membranes were incubated with 10^{-8} M \textsuperscript{125}I-ET plus 10^{-8} M unlabeled EPT but was inhibited when membranes were incubated with a 10-fold excess of unlabeled EPT (Fig. 1C, lanes 3–4). When \textsuperscript{125}I-ET-labeled cells were lysed with Triton X-100, the \textsuperscript{125}I 220-kDa band was mainly recovered in the Triton X-100-insoluble fraction, and virtually none of it in the Triton X-100-soluble fraction (Fig. 1D, lanes 1–3). SDS-PAGE analysis of Triton X-100-insoluble fraction from mpkCCD\textsubscript{cl4} cells incubated with \textsuperscript{125}I-ET on the apical side of the filters and fractionated by flotation-centrifugation revealed that monomeric \textsuperscript{125}I-ET and \textsuperscript{125}I 220-kDa complexes were recovered in fractions enriched with the DRM marker caveolin-1 (Fig. 1E). These results indicate that ET forms complexes in DRMs, mainly on the apical side of renal mpkCCD\textsubscript{cl4} cells. Cholesterol is essential for maintaining the integrity of DRMs (6). Pretreating mpkCCD\textsubscript{cl4} cell membranes with M\textsubscript{Chol} from membranes (21), or with saponin (0.4\% for 60 min at 4^\circ C), a cholesterol-sequestering drug (41), completely prevented the formation of \textsuperscript{125}I 220-kDa complexes in both the Triton X-100-soluble and -insoluble fractions (Fig. 1F). However, live mpkCCD\textsubscript{cl4} cells treated with M\textsubscript{Chol} did exhibit some Alexa 488-tagged ET labeling; this was much weaker than that of M\textsubscript{Chol}-untreated cells, although some irregular, more intense patches were detectable (not shown), suggesting that ET binding can occur in noncholesterol-enriched areas of membrane.

ET does not bind to GPI-anchored proteins. Previous studies demonstrated that two other pore-forming toxins, areolysin and \textit{C. septicum} alpha toxin, which share structural similarities with ET, bind to glycosyl phosphatidylinositol (GPI)-anchored protein receptors (1, 16). DRMs microdomains are highly enriched in GPI-anchored proteins (6). This raises the question of whether the putative receptor for ET and EPT is bound to the plasma membrane via a GPI anchor. GPI-anchored proteins can be removed from the cell surface using PI-PLC (23). Rather unexpectedly, after PI-PLC pretreatment with Alexa 488-tagged ET, the labeling of mpkCCD\textsubscript{cl4} cells was the same as that of untreated cells (Fig. 2A). The efficiency of the PI-PLC treatment was checked by using mpkCCD\textsubscript{cl4} cells transfected with a plasmid encoding for the GPI-anchored tag DAF-GFP. GFP fluorescence was detected in the perinuclear region, in cytoplasmic patches and delineated the plasma membranes of untreated cells, whereas the DAF-GFP tag was removed from the plasma membrane following PI-PLC treatment (Fig. 2B). We then investigated the relationship between GPI-anchored proteins and the formation of 220-kDa complexes. The formation of insoluble \textsuperscript{125}I-labeled complexes by ET was then compared with that induced by \textit{C. septicum} alpha toxin, which forms oligomers in DRMs (17) in the same Triton-insoluble membrane preparation of mpkCCD\textsubscript{cl4} cells pretreated or not with PI-PLC. As expected, the insoluble complex formed by the alpha toxin was almost undetectable after PI-PLC treatment, whereas the 220-kDa complexes formed by ET were unaffected (Fig. 2C). In addition, when tested with MTT, ET retained the same level of cytotoxic activity in mpkCCD\textsubscript{cl4} cells treated with PI-PLC as in un-
basal side induced a parallel and dose-dependent decrease in mV). Adding increasing concentrations of ET to the apical or membrane side of filter-grown mpkCCDcl4 cells.

ET causes rapid membrane permeabilization in mpkCCDcl4 cells. Confluent cells grown on filters formed tight epithelia with high transepithelial resistance ($R_T > 3,000 \, \Omega \cdot \text{cm}^2$) and negative transepithelial potential ($P_D$ ranging from $-60$ to $-80 \, \text{mV}$). Adding increasing concentrations of ET to the apical or basal side induced a parallel and dose-dependent decrease in both $R_T$ and $P_D$ (Fig. 3A). EPT did not affect $R_T$ or $P_D$ when it was added to the basal side of the cells (Fig. 3B). In contrast, the apical addition of high concentrations of EPT ($>10^{-9} \, \text{M}$) induced significant decreases in $R_T$ and $P_D$ (Fig. 3B). This suggested that inactive EPT was cleaved by protease(s) to form active ET. mpkCCD_{cl4} cells synthesize a serine protease, mCAP1 (46), which stimulates epithelial Na$^+$ channel (ENaC) activity, the rate-limiting step for Na$^+$ entry into collecting duct principal cells. This finding led us to test whether aprotinin, an inhibitor of serine proteases, could prevent the apical proteolytic cleavage of EPT caused by serine proteases. Pre-incubating cells for 18 h with 1 mg/ml aprotinin on the apical side significantly decreased $P_D$, an effect that reflects inhibition of the stimulatory action of serine protease on ENaC activity (46), but it also protected mpkCCD_{cl4} cells against the membrane depolarization caused by subsequent apical addition of EPT (Fig. 3C).

ET stimulates Na$^+$ absorption when added to the apical membrane side of filter-grown mpkCCD_{cl4} cells. The impact of the presence of large ET complexes in plasma membranes on electrogentic ion transport was studied using the short-circuit current ($I_{sc}$) method. Apical addition of $10^{-9} \, \text{M}$ ET caused a twofold increase in $I_{sc}$ within 20 min (Fig. 4A). In contrast, the depolarization of the basolateral membrane produced by adding ET to the basal side of the cells induced a rapid decline in $I_{sc}$ (Fig. 4A). These effects on $I_{sc}$ were completely abolished by apical or basal preincubation of the cells with a 100-fold excess of anti-ET antiserum on the same side as ET (Fig. 4B), whereas applying anti-ET antiserum to the opposite side did not impair the rise in $I_{sc}$ caused by ET (data not shown). Apical addition of the potent ENaC inhibitor amiloride ($10^{-5} \, \text{M}$) did not prevent the increase in $I_{sc}$ caused by subsequently adding ET to the apical side of the cells (not shown). This suggests that ET induced the influx of Na$^+$ through cation nonspecific pores. This view is supported by two other observations. 1) The blockade of Na$^+$-$K^+$-ATPase by incubating cells with basal ouabain ($5 \times 10^{-5} \, \text{M}$) blunted the rise in $I_{sc}$ caused by the subsequent apical addition of ET (Fig. 4C). 2) When the apical Na$^+$-rich HFM was replaced by Na$^+$-free medium (HFM medium), $I_{sc}$ decreased significantly and the apical addition of ET did not significantly increase $I_{sc}$: $23.3 \pm 1.7 \, \mu \text{A/cm}^2$ ($n = 7$) vs. HFM medium + ET: $38.9 \pm 3.8 \, \mu \text{A/cm}^2$ ($n = 7$), $P < 0.01$; apical Na$^+$-free medium: $6.2 \pm 0.8 \, \mu \text{A/cm}^2$ ($n = 6$) vs. apical Na$^+$-free medium + ET: $7.9 \pm 1.1 \, \mu \text{A/cm}^2$ ($n = 6$).

ET induces an inward current and a rise in $[\text{Ca}^{2+}]_i$. Adding ET ($10^{-7} \, \text{M}$) to mpkCCD_{cl4} cells grown on glass, maintained under voltage clamping at a holding potential of $-60 \, \text{mV}$, induced an inward current which developed in a time-dependent manner parallel to a decrease in the cell membrane resistance (Fig. 4D and inset). The whole cell current change

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**Fig. 3.** Dissociated effects of ET and EPT on apical and basolateral cell membrane depolarization. A and B: transepithelial electrical resistance ($R_T$) and potential ($P_D$) were measured on confluent mpkCCD_{cl4} cells grown on filters and incubated without (○) or with (●) ET or EPT ($10^{-10}$ to $10^{-7} \, \text{M}$), which were added to the apical or basal side of the cell layers. C: $R_T$ and $P_D$ were measured on confluent cells grown on filters and sequentially incubated without (● Aprotinin) or with (+ Aprotinin) aprotinin for 18 h at 37°C and then without (○) or with (●) $10^{-8} \, \text{M}$ EPT for 60 min at 37°C. Aprotinin and EPT were both added to the apical medium bathing cells. Values are means ± SE from 4 to 6 separate experiments.
induced by ET, measured as $\Delta I$ (i.e., current recorded holding current), appeared after 1 to 3 min of toxin exposure and increased as a function of time: it had an amplitude of $-267.6 \pm 65.2$ pA ($n = 8$) after 10 min of ET application. These findings are consistent with the fact that ET inserts into membranes and forms nonspecific cationic pores (35). Imaging studies on living fura-2-loaded mpkCCD$\text{c}_{14}$ cells also showed that ET induced a sustained rise in $[Ca^{2+}]_i$, performed on cells incubated without ($\circ$) or with a $\times 100$-fold excess of anti-$\varepsilon$T antiserum ($\bullet$) for 30 min and then with $10^{-9}$ M ET (arrows). Anti-$\varepsilon$T antiserum and ET were both added on the same side (apical or basolateral) of the cell layers. $B$: $I_{sc}$ recordings performed before (○) and after basal addition of ouabain ($5 \times 10^{-3}$ M; □) and subsequent apical addition of ET (εT, $10^{-9}$ M, ●). Values are means ± SE from 4 to 6 experiments. $D$: representative recordings of membrane potential intensities, are means ± SE from 4 to 6 experiments. The increase in fluorescence ratio indicates a rise in $[Ca^{2+}]_i$.

$\varepsilon$T induces rapid ATP depletion and mitochondrial permeabilization. Adding the toxic to the apical side of the cells caused a rapid dose- and time-dependent decrease in cell viability at concentrations of ET above $10^{-9}$ M (Fig. 5A), and a concomitant decrease in cellular ATP content, with an almost complete depletion of cellular ATP 30 min after adding concentrations of ET of more than $10^{-9}$ M (Fig. 5B). Only a small fraction of total cellular ATP was recovered in the supernatant after incubating for 60 (not shown) or 90 min with either $10^{-9}$ M ET (3.6 ± 1.5%, $n = 5$) or $10^{-7}$ M ET (10.4 ± 4.5%, $n = 5$). Furthermore, adding extracellular ATP (5 or 10 mM) to the culture medium did not protect exposed cells against the loss in cell viability caused by the toxin (not shown).

AMP-activated protein kinase (AMPK) is a ubiquitous, heterotrimeric, metabolic-sensing Ser/Thr kinase that is activated by any stress that depletes cellular ATP (18). We next investigated the possible mechanisms responsible for the rapid cell death caused by ET. At all the concentrations tested ($10^{-10}$ to $10^{-7}$ M for 60 min), the toxin failed to induce large-scale DNA fragmentation (Fig. 6A), and no induction of the apoptotic caspase 3 was detected in ET-treated cells (not shown). Moreover, preincubating the cells with 20 M VAD.fmk, a wide-spectrum caspase inhibitor, did not protect the cells against the rapid loss in cell viability caused by the toxin (not shown). As shown in Fig. 6B, ET stimulated the expression of Bcl-2-associated protein X (Bax),
which is known to induce mitochondrial membrane permeabilization and cytoplasmic release of cytochrome c (25, 32). These findings led us to test whether ET also induced nuclear translocation of AIF (43), a mitochondrial caspase-independent cell death effector that has been shown to induce both chromatin condensation and DNA fragmentation (10, 11). Western blot analysis revealed that AIF was present in mitochondrial-enriched fractions from untreated and ET-treated mpkCCD
d cells, but not in the cytosol (see Fig. 5D). Immunofluorescence studies demonstrated that AIF had translocated from the mitochondria into the nuclei of cells incubated with increasing concentrations of ET (Fig. 6, C and D). Little or no AIF staining was seen in the untreated cells (Fig. 6C, left), whereas AIF aggregates could be detected in multiple nuclei after incubating for 30–45 min with 10⁻⁸ M ET (Fig. 6C, right). In addition, the pan-caspase inhibitor Z-VAD.fm (20 μM, 30 min) did not prevent the mitochondrial-nuclear translocation of AIF caused by 10⁻⁸ M ET (Fig. 6D). Imaging studies also revealed that ET induced a concentration-dependent manner a marked reduction in the size of nuclei (Fig. 6E). Overall, these findings strongly suggest that ET may promote oncosis (cell necrosis) as a result of a rapid ATP depletion process associated with mitochondrial-nuclear translocation of AIF.

_**Extraction of cholesterol from DRMs impedes ion diffusion, but does not prevent ATP depletion and subsequent cell death caused by ET.**_ The cause-to-effect linkage between membrane permeabilization and cell death induction has been established for many pore-forming toxins. Thus we tested whether cholesterol-affecting drugs, which impair ET oligomerization in DRMs, could protect cells against the toxin. Apical addition of MβCD (1 and 10 mM, 60 min) produced reduction in the rapid fall in \( R_T \) and \( P_C \) caused by the subsequent apical addition of 10⁻⁸ M ET (Fig. 7A). Preincubating the cells with MβCD also inhibited the rise in \( \Delta \psi_m \) mitochondrial potential analyzed by flow cytometry on cells incubated without (−) or with (+) ET. E: electron micrographs of confluent mpkCCD
d cells incubated without (−ET) or with (+ET) 10⁻⁸ M ET for 30 min at 37°C. Bar = 10 μm. Note the integrity of tight junctional complexes and the presence of focal areas of altered mitochondria (arrowheads). F: representative illustrations of \( \Delta \psi_m \) mitochondrial potential analyzed by flow cytometry on cells incubated without (−) or with (+) ET 10⁻⁸ M ET for 30 min at 37°C. In this experiment, 38% of the cells exhibited a loss of \( \Delta \psi_m \). G: confocal micrograph showing the absence of colocalization between the Mitotracker Red (red) and Alexa 488-tagged ET (green).
inhibitor of calpains, ALLN (25 μM, 30 min), used alone or with 5 mM MβCD, also failed to prevent cell death caused by the toxin (not shown).

**DISCUSSION**

ET is an extraordinarily potent pore-forming toxin, the lethal dose of which in mice is 100-fold lower than that of structurally related, pore-forming toxins such as aerolysin (26). Here, we show that the immortalized mpkCCD<sub>cl4</sub> cells (4) remained highly sensitive to ET. ET preferentially binds to the apical membranes of cells grown on filters, and oligomerizes there, and does diffuse intracellularly. We also show that the inactive EPT can be processed to form fully active ET by proteases present in the apical plasma membrane, but not in basal plasma membrane. These findings are of physiological relevance, since the kidney is actively involved in the elimination of ET and in the mouse kidney ET accumulates particularly on the luminal (i.e., apical) side of the distal collecting duct, where it attacks the cells (42). Binding studies strongly suggest that ET and EPT bind to the same mpkCCD<sub>cl4</sub> cell membrane receptor. The number of receptors for ET appears to be greater in the apical than basolateral membranes of mpkCCD<sub>cl4</sub> cells. A similar asymmetric in vitro distribution of toxin receptors has been already reported for other pore-forming toxins: iota toxin (38), *Vibrio cholerae* RTX (14), and aerolysin (3). So far, the nature of the ET receptor(s) is still unknown. Many clostridial toxins, such as *C. tetani* toxin (30), *C. septicum* alpha toxin, and aerolysin produced by *Aeromonas hydrophilia*, which display many similarities with ET (1, 12, 16), specifically bind to GPI-anchored proteins. However, PI-PLC, which removes most GPI-anchored proteins (23) and prevents binding and subsequent biological activities of the toxins listed above, does not prevent <sup>125</sup>I-ET binding or the subsequent formation of large <sup>125</sup>I complexes in mpkCCD<sub>cl4</sub> cell membranes. These findings indicate that the receptor for ET is not a GPI-anchored protein receptor. This is consistent with structural studies showing that, despite ET displays remarkable overall structural similarity with aerolysin, its domain II which is involved in the interaction with GPI anchors (24) differs widely from that of aerolysin (9). Preliminary studies using an ET overlay procedure similar to that used by Abrami et al. (1) revealed that ET predominantly bound to a protein with an apparent molecular weight of 42 kDa (not shown). This binding was prevented by incubating membranes with an identified 37-kDa protein previously isolated from MDCK cells using affinity purification with ET linked to Sepharose beads (34). The fact that cholesterol sequestration does not fully abolish the binding of ET-Alexa488 to living mpkCCD<sub>cl4</sub> cells suggests that the receptors for ET may be nonexclusively localized in DRMs.

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**Fig. 6. Effects of ET on DNA fragmentation and AIF distribution.** A: nuclear DNA was analyzed by agarose gel electrophoresis from untreated mpkCCD<sub>cl4</sub> cells (0) and cells exposed to increasing concentrations of ET for 60 min. B: Western blot analyses of Bax and β-actin, used as the internal control, in cells without (0) or with <sup>10</sup>-8 M ET treatment for various times. C: confocal micrographs of cells dual-stained with an anti-AIF antibody (red) and SYTOX green DNA dye (green). In contrast to untreated cells (left), nuclear AIF staining was detected in multiple nuclei (arrowheads) after incubating for 45 min with <sup>10</sup>-8 M ET (right). Bars = 5 μm. D: quantitative analysis of the time course of AIF staining in nuclei from cells incubated with <sup>10</sup>-8 M ET for various times or with Z-VAD.fmk (20 μM, 30 min) and then with <sup>10</sup>-8 M ET for an additional 60 min. Values are means ± SE from 25 to 35 individual cell recordings from 2 separate experiments. *P < 0.05 vs. time 0 value. E: confluent cells incubated in the absence (none) or presence of various concentrations of ET for 60 min were stained with the nuclear dye Hoechst H33342 (10 μg/ml). ET induced a dose-dependent reduction in the size of nuclei (arrowheads).
The formation of the toxin heptamer (28, 29, 34) induces membrane permeabilization, which is prevented by the removal of cholesterol by MβCD. This suggests that cholesterol-rich lipid raft microdomains certainly act as concentration platforms favoring oligomerization of ET (2). Binding and oligomerization of ET to MDCK cells grown on a solid support induce rapid decrease of intracellular K+ and a concomitant increase in Cl−, Na+, and Ca2+ (34). Here, we show that ET triggers the electrogenic entry of ENaC-independent Na+ driven by the basolateral Na+/K+-ATPase when the toxin is applied to the apical side of mpkCCD14 cells grown on filters. The fact that ET had no effect on INa under apical Na+-free conditions suggests that the toxin induces a cation-permeable pore in the apical membrane of collecting duct cells, which allows the massive entry of Na+ into mpkCCD14 cells. Accordingly, the following possible scenario can be proposed for the first phase of the disease: circulating ET and inactive EPT in the bloodstream are filtered by the glomeruli, thus exposing ET to the distal collecting duct cells to relatively high amounts of the active toxin. Moreover, the proteases present at the apical surface of the collecting duct cells (i.e., the luminal membrane) may convert the inactive prototoxin into the fully active toxin. ET pores formed in the apical membrane cause a massive intracellular influx of Na+ and are then extruded by basolateral Na+/K+-ATPase, which is responsible for an ensuing sudden increase in Na+ absorption. This hypothesis could explain, at least in part, the sudden rise in blood pressure observed in intoxicated animals (38, 44, 45).

ET also induces a rapid rise in [Ca2+], which is correlated to the fall in membrane resistance and induction of sustained inward ionic current. Hence, it is conceivable that the Ca2+ ions flow into the cells through the toxin-induced membrane pores. Indeed, ET induces an efflux of K+, sustained influxes of Ca2+ and Na+, and the entry of small molecules into MDCK cells, which would be consistent with the formation of a rather large pore in the cell membrane (35). The possibility that ET may bind and directly activate endogenous Ca2+ conductance is rather unlikely, because the inhibition of heptameric complex formation by cholesterol-affecting drugs protects mpkCCD14 cells against a sudden rise in [Ca2+], and, furthermore, Ca2+ channels are not known to be associated with DRM. However, we cannot exclude the possibility that ET-induced depolarization of mpkCCD14 cells could trigger the opening of endogenous voltage-dependent Ca2+ channels.

Fig. 7. Lipid raft disruption by MβCD reduces ion diffusion caused by ET in mpkCCD14 cells. A: R0 and P0 were measured on mpkCCD14 cells incubated without or with MβCD (1 or 10 mM, 60 min) and then with ET (ɛT, 10−8 M, 60 min). B: Ion currents were performed on cells preincubated without (◼) or with 1 mM MβCD alone (●) and then with 10−9 M ɛT alone (■) or MβCD plus ɛT (●). Both MβCD and ɛT were added to the apical side of the cells. C: time lapse recordings of [Ca2+]i changes were performed on cells loaded with fura-2-AM (2 mM for 20 min). Recordings were performed on untreated cells (none), cells incubated with 1 mM MβCD alone, or with 10−7 M ɛT or MβCD plus 10−7 M ɛT. D: whole cell patch-clamp experiments in voltage clamp mode (HP = −60 mV) showing that the ionic inward current induced by 10−7 M ɛT (●) is strongly reduced when cells were treated for 15 min with 5 mM MβCD before adding ET (○). Values are means ± SE from 5–7 separate experiments. *P < 0.05 between groups.

Fig. 8. Lipid raft disruption by MβCD does not impair the depletion in ATP and subsequent oncotic caused by ET in mpkCCD14 cells. A and B: cells were preincubated or not with 5 mM MβCD and then with ET (ɛT) for an additional 45 min. No differences were observed in the expression (A) or in the quantitative analysis of AIF nuclear staining (B). Values are means ± SE of 43–73 individual cell recordings from 3 separate experiments. C: cellular ATP content in cells incubated with 1 mM MβCD alone (●), ɛT alone (■), ɛT plus 1 mM MβCD (○), or ɛT plus 10 mM MβCD (□) for 90 min at 37°C. D: time course of cell viability assessed by the MTT test on confluent mpkCCD14 cells incubated with 10−8 M ɛT alone (●), ɛT plus 1 mM MβCD (○), or ɛT plus 10 mM MβCD (□). Values are means ± SE from 3 separate experiments.
ther experiments are needed to test this latter possibility, and to find out whether the release of Ca\(^{2+}\) from intracellular Ca\(^{2+}\) stores contributes to the observed increase in [Ca\(^{2+}\)].

Importantly, our findings reveal that the mechanism by which ET induces rapid cell demise differs from that of other bacterial pore-forming toxins. Under many pathological conditions, apoptosis and necrosis often coexist (33). Interestingly, even though low concentrations of ET activate the apoptotic Bax, they do not induce nuclear cell fragmentation in mpkCCD\(_{14}\) cells, but, instead, they induce oncosis. This is reminiscent of a previous report, in which ET-dependent necrosis in MDCK cells was monitored by the entry of propidium iodide and found to correlate with cell mortality (36). This may be due to the fact that these renal collecting duct cells, which exhibit high metabolic and glycolytic activities, are highly susceptible to rapid ATP depletion and subsequent oncosis. The mechanism by which ET induces cell death clearly differs from that caused by chemical agents producing ATP depletion in many other pore-forming toxins. For example, the *C. perfringens* enterotoxin (CPE), which also causes a rapid increase in [Ca\(^{2+}\)], in intestinal-like CaCo-2 cells, induces DNA fragmentation and caspase- and calpain-dependent apoptosis or oncosis, at low and high doses, respectively (8). In sharp contrast, even at low concentrations ET failed to activate caspase 3 or to induce advanced DNA fragmentation. Consistent with the rapid fall in cellular ATP, ET binding to mpkCCD\(_{14}\) plasma membranes leads to the activation of AMPK, which is a sensitive indicator of reduced cellular energy status (19), and subsequent inhibition of many phosphorylation processes, including the phosphorylation of S6 ribosomal protein downstream of AMPK, which reflects inhibition of the TOR pathway, a major stimulus of protein synthesis (7). The ET effects also result in inducing permeabilization of mitochondrial membranes reflected by the release of cytochrome c into the cytoplasm and the stimulated expression of Bax (25, 32), leading to mitochondrial-nuclear translocation of AIF. AIF is a potent apoptogenic protein (43), which seems to be sufficient by itself to cause cell death in a variety of cell systems (39). In response to various stress effectors, AIF exits from the outer mitochondrial membrane and enters the nucleus, where it activates endonucleases that cause nuclear condensation and DNA degradation (10). Although ATP has been shown to be required for AIF-chromatin condensation in cell-free systems (43), nuclear redistribution of AIF can be observed even in a context of ATP depletion (11, 39). The results from imaging studies have identified abundant AIF immunostaining in nuclei from ET-treated cells, very similar to that reported in ATP-depleted OK cells (39). These findings suggest that the mitochondrial permeabilization caused by ET promotes the mitochondrial-nuclear translocation of AIF, homogeneous chromatin condensation and nuclear shrinkage, which are considered to be typical features of oncosis. However, in contrast with many other pore-forming toxins, ET failed to induce DNA fragmentation, suggesting that the energy-dependent processes required for the apoptotic program are rapidly dissipated following the rapid ATP depletion process.

Is the oncosis induced by the toxin directly linked to the increase in [Ca\(^{2+}\)], and/or to the concomitant increase in ion transport through the toxin pores formed in cell membranes? Moderate increase in [Ca\(^{2+}\)], or massive changes in [Ca\(^{2+}\)], secondary to the formation of large plasma membrane pores induced by low or high doses of CPE in intestinal-like Caco-2 cells have been shown to be responsible for calmodulin- and calpain-dependent apoptosis or oncosis, respectively (8). In the present study, calpain and calmodulin inhibitors did not prevent cell death induced by ET, albeit one cannot rule out the possibility that the formation of large-size ET channels (35) may facilitate the leakage of cytoplasmic molecules, thereby impairing the effect of the inhibitors tested. Moreover, even transient activation of calmodulin- and calpain-dependent apoptosis appears unlikely. Indeed, the removal of cholesterol by MβCD, which prevented ET oligomerization in lipid rafts and massively reduced Ca\(^{2+}\) and Na\(^{+}\) influx, did not prevent the sudden decrease in cellular ATP or the subsequent oncosis. Therefore, these findings suggest that a massive and sudden rise in [Ca\(^{2+}\)], is not an absolute prerequisite for the induction of cell necrosis by ET. The possibility that some wide cation channels unaffected by MβCD treatment could be sufficient to allow a modest rise in [Na\(^{+}\)] and [Ca\(^{2+}\)], leading to the induction of oncosis in renal collecting duct cells, cannot be firmly ruled out. Further studies will be needed to identify the mechanism by which the binding of ET to the plasma membrane of collecting duct cells can trigger cell ATP depletion and subsequent cell necrosis.

To conclude, the cytotoxic effects of ET in mpkCCD\(_{14}\) cells are dual and comprised of a pore-forming, cholesterol-dependent phase that occurs in DRMs, and an ATP depletion-induced oncosis that is almost completely resistant to the removal of cholesterol. The renal collecting duct mpkCCD\(_{14}\) cells therefore offer an attractive new cell system that could be used to analyze the cytotoxic action of ET, and to identify its membrane receptor.

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Epsilon toxin toxicity in collecting duct cells


