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Clostridium difficile Toxin B Causes Apoptosis in Epithelial Cells by Thrilling Mitochondria

*INVOLVEMENT OF ATP-SENSITIVE MITOCHONDRIAL POTASSIUM CHANNELS**

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Targeting to mitochondria is emerging as a common strategy that bacteria utilize to interact with these central executioners of apoptosis. Several lines of evidence have in fact indicated mitochondria as specific targets for bacterial protein toxins, regarded as the principal virulence factors of pathogenic bacteria. This work shows, for the first time, the ability of the *Clostridium difficile* toxin B (TcdB), a glucosyltransferase that inhibits the Rho GTPases, to impact mitochondria. In living cells, TcdB provokes an early hyperpolarization of mitochondria that follows a calcium-associated signaling pathway and precedes the final execution step of apoptosis (*i.e.* mitochondria depolarization). Importantly, in isolated mitochondria, the toxin can induce a calcium-dependent mitochondrial swelling, accompanied by the release of the proapoptogenic factor cytochrome *c*. This is consistent with a mitochondrial targeting that does not require the Rho-inhibiting activity of the toxin. Of interest, the mitochondrial ATP-sensitive potassium channels are also involved in the apoptotic response to TcdB and appear to be crucial for the cell death execution phase, as demonstrated by using specific modulators of these channels. To our knowledge, the involvement of these mitochondrial channels in the ability of a bacterial toxin to control cell fate is a hitherto unreported finding.

Today, it is widely accepted that bacterial pathogens can manipulate the eukaryotic machinery to suit their own needs, frequently interfering with pathways controlling apoptotic cell death (reviewed in Ref. 1). Apoptosis occurs, depending on the stimuli, via two major pathways that converge on caspase 3 activation and are initiated by death receptors or alternatively by mitochondria (reviewed in Ref. 2). Bacteria can exploit both apoptotic pathways, often by producing protein toxins that mediate a long range cross-talk with host cells. In recent years, the targeting of mitochondrial membranes is emerging as a widespread strategy employed by bacterial pathogens in controlling the host cell destiny (reviewed in Ref. 3). The direct targeting of mitochondria, in fact, can allow bacteria to bypass upstream checkpoints of cell death, thus straightforwardly handling one of the central executioners of apoptosis. In this context, mitochondria have very recently been indicated as a key target for toxin activity, especially for certain pore-forming toxins that besides acting at the level of the eukaryotic cell membranes also organize pores in mitochondrial membranes (4–7). This allows *in fine* a leakage of proapoptotic factors, such as cytochrome *c*, in the cytosol and the consequent stimulation of apoptosis. The direct targeting of mitochondria, however, has been reported also for toxins devoid of pore-forming activity, such as those belonging to the large clostridial toxin family (reviewed in Ref. 8) and in particular for the lethal toxin from *Clostridium sordellii* (LT)[4] (9) and toxin A from *Clostridium difficile* (TcdA) (10). The exact mechanism by which the large clostridial toxins perturb the mitochondrial functionality, however, remains still to be defined.

C. difficile toxin B (TcdB) is another large clostridial toxin, largely accepted as a proapoptotic factor (11, 12). It is produced, together with TcdA, by pathogenic strains of *C. difficile*, recognized as the principal cause of antibiotic-associated pseudomembranous colitis (reviewed in Ref. 13). TcdA and TcdB are large protein toxins (TcdB, 269 kDa; TcdA, 308 kDa) that encompass three functional domains: the receptor binding domain, the intermediate part responsible for membrane translocation, and the N-terminal part harboring the glucosyltransferase activity. Both toxins are internalized by receptor-mediated endocytosis and require passage through an acidic compartment for activation (13). Once in the cytoplasm, they transfer from UDP-glucose a glucose moiety on the threonine 35/37 residue of Rho, Rac, and Cdc42, thus inactivating these GTPases (14). Rho, Rac, and Cdc42 are important regulatory proteins of mammalian cells that control many cellular processes (reviewed in Ref. 15), including the assembly/disassembly of the cell cytoskeleton. Cells exposed to these toxins lose

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[4] The abbreviations used are: LT, *C. sordelli* lethal toxin; mK_{ATP} channel, ATP-sensitive mitochondrial potassium channel; 5HD, 5-hydroxydecanoic acid; TcdA, *C. difficile* toxin A; TcdB, *C. difficile* toxin B; WT, wild-type; rec, recombinant; cyt, cytochorome; FCCP, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone; mAb, monoclonal antibody; MMP, mitochondrial membrane potential; MMHP, mitochondrial membrane hyperpolarization; JC-1, 5-5′,6-6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide; TMRM, tetramethylrhodamine ester; OLM, oligomycin; PI, propidium iodide; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NterTcdB, recombinant N-terminal domain of TcdB; NterLT82, recombinant N-terminal domain of *C. sordellii* LT82; fmk, fluoromethyl ketone; FITC, fluorescein isothiocyanate.

their ability to maintain a proper cytoskeleton architecture, undergoing cell retraction and rounding (8). These two potent cytotoxins differ in certain aspects, TcdB being more powerful, at least on cultured cells, than TcdA (13).

This work, however, has entirely been focused on TcdB and on its possible interaction with mitochondria. Our results disclose the ability of TcdB to provoke an early hyperpolarization of mitochondria in intact cells that follows a contained rise of cytosolic calcium and precedes the final execution step of apoptosis. Notably, the toxin can induce the swelling of isolated mitochondria in a calcium-dependent fashion, and this effect does not require the enzymatic activity of the toxin. Moreover, we have also shown that the apoptotic response to TcdB involves the mitochondrial ATP-sensitive potassium channels (mK_{ATP} channels), which appear to be crucial for the cell death execution phase. These mitochondrial channels, detected in various types of cells, have been reported to play a key role in the protection against different insults, including those leading to apoptosis (16). To our knowledge, this finding is hitherto uncharted for a bacterial protein toxin.

EXPERIMENTAL PROCEDURES

Cell Cultures—The human epithelial HEp-2 cells were grown in minimal essential medium supplemented with 10% (v/v) fetal calf serum, 50 units/ml penicillin, and 50 μg/ml streptomycin in a humidified atmosphere with 5% (v/v) CO_2 at 37 °C. For all experiments, 5×10^5 cells were seeded into 3-cm diameter Petri dishes. Twenty-four hours after seeding, cells were treated as specified (see below).

Toxins—TcdB was prepared and purified according to previously published methods (17). Recombinant TcdB (rec TcdB, cloned in the PQE 30 plasmid vector with a His_6 tag at the N terminus and expressed into the XL1 blue *Escherichia coli*) and *Helicobacter pylori* VacA were generous gifts from P. Boquet (Nice). VacA was activated following the procedure previously reported (4). The N-terminal domain (amino acids 1–546) of TcdB was prepared as follows. DNA encoding for the 546 N-terminal amino acids was amplified from *C. difficile* strain VPI10463 and cloned into pET28a vector. The recombinant N-terminal domain was produced in *E. coli* strain BL21 DE3 and purified on a cobalt column (Talon; Clontech), according to the manufacturer's recommendations. To test the enzymatic activity of the TcdB N-terminal domain, *in vitro* glucosylation of recombinant glutathione *S*-transferase-Rac (1 μg) was carried out in 50 mM triethanolamine, pH 7.5, containing 2 μl of UDP-[^{14}C]glucose (286.2 mCi/mmol; PerkinElmer Life Sciences), 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.3 mM GDP, and variable concentrations of recombinant N-terminal domain of TcdB (NterTcdB) or *C. sordellii* LT82 (NterLT82). The reaction was performed for 1 h at 37 °C and stopped by adding sample buffer followed by boiling for 3 min. Samples were then electrophoresed on 12% SDS-PAGE and autoradiographed.

Treatments—Before starting the experiments, we performed TcdB titration in living cells and tested three different concentrations among those causing an evident cell retraction (the morphological response that precedes the rounding) within 1 h of incubation. For all of the experiments, we have then chosen the concentration of 3 ng/ml toxin B (corresponding to 10^{-11} M) that induced cell retraction in the whole population within 30 min. HEp-2 cells were treated with 3 ng/ml wild-type (WT) TcdB or rec TcdB for different time lengths (1, 3, 6, 18, and 32 h). Isolated mitochondria (see below for the preparation) were challenged with (i) WT TcdB (1.5, 3, or 6 ng/ml), (ii) rec TcdB (3 ng/ml), (iii) NterTcdB (1.5, 3, 4.5, or 6 ng/ml), and (iv) VacA (5 μg/ml). It is important to underline that, except when compared with rec TcdB, the WT TcdB will be always referred to simply as TcdB.

For experiments with inhibitors, cells were pretreated for 30 min with the following compounds before TcdB administration: (i) carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP; 40 nM) (Sigma), an uncoupler of respiratory chain; (ii) oligomycin (OLM; 1 μM) (Sigma), an inhibitor of ATP synthase known to increase mitochondrial membrane potential (MMP); (iii) diazoxide or pinacidil (both 10 μM; Sigma), activators of mK_{ATP} channels; (iv) 5-hydroxydecanoic acid (5HD; 10 μM) (Sigma), which blocks the mK_{ATP} channel; and (v) monensin (10 μM) (Sigma), a lysosomotropic agent that alters the endosomal pH. Cells treated with each drug (FCCP, OLM, diazoxide, pinacidil, 5HD, or monensin) alone were considered as controls. For caspase inhibitor experiments, cells were pretreated with a 30 μM concentration of the specific inhibitor for 2 h before TcdB exposure. In particular, the following inhibitors were used: IETD-fmk for caspase 8, LEHD-fmk for caspase 9, and DEVD-fmk for caspase 3. As a negative control, we used HEp-2 cells treated with a heat-inactivated (98 °C for 10 min) TcdB. In this case, all of the toxin-induced effects were abolished.

Cell Death Assays—Quantitative evaluation of apoptosis was performed by using the following flow cytometry methods: (i) double staining using an FITC-conjugated annexin V/propidium iodide (PI) apoptosis detection kit (Eppendorf, Milan, Italy), which allows discrimination between early apoptotic, late apoptotic, and necrotic cells, and (ii) evaluation of DNA fragmentation in ethanol-fixed cells using PI (Sigma).

Activation of Caspases in Living Cells—The activation state of caspase 8, 9, and 3 was evaluated by using the CaspGLOW fluorescein active caspase staining kit (MBL, Woburn, MA). This kit provides a sensitive means for detecting activated caspases in living cells. The assay utilizes specific caspase inhibitors (IETD-fmk for caspase 8, LEHD-fmk for caspase 9, and DEVD-fmk for caspase 3) conjugated to FITC as the fluorescent marker. These inhibitors are cell-permeant and nontoxic and irreversibly bind to the caspase active form. The FITC label allows detection of activated caspases in apoptotic cells directly by flow cytometry. Control and treated HEp-2 cells were incubated with FITC-IETD, FITC-LEHD-fmk, and FITC-DEVD-fmk for 1 h at 37 °C following the manufacturer's instructions. Samples were thereafter washed three times and immediately analyzed on a cytometer by using the FL-1 channel. Two additional experimental controls were also considered: (i) samples prepared by pretreating cells with specific caspase 8, caspase 9, or caspase 3 inhibitor before TcdB administration and (ii) unlabeled HEp-2 cells (negative control).

MMP in Living Cells—The MMP of controls and treated-HEp-2 cells was studied by using a 5–5′,6–6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazol-carbocyanine iodide (JC-1;

Molecular Probes, Inc., Eugene, OR) probe. In line with this method, living cells were stained with 10 μM JC-1, as previously described (18). Tetramethylrhodamine ester (TMRM; 1 μM) (Molecular Probes) (red fluorescence) was also used to confirm data obtained by JC-1.

Single Cell Assay for $[Ca^{2+}]_i$ Recording—Optical fluorimetric recordings with fura-2/AM were used to evaluate the intracellular calcium concentration ($[Ca^{2+}]_i$). Fura-2/AM stock solutions were obtained by adding 50 μg of Fura-2/AM to 50 μl of 75% Me_2SO plus 25% pluronic acid. Cells were bathed for 60 min at room temperature with 5 μl of stock solution diluted in 1 ml of extracellular solution (125 mM NaCl, 1 mM KCl, 5 mM $CaCl_2$, 1 mM $MgCl_2$, 8 mM glucose, and 20 mM HEPES, pH 7.35) for a final Fura concentration of 5 μM. This solution was then removed and replaced with extracellular solution, and the dishes were quickly placed on the microscope stage. To measure fluorescence changes, a Hamamatsu (Shizouka, Japan) Argus 50 computerized analysis system was used, recording every 12 s the ratio between the values of light intensity at 340- and 380-nm stimulation. The basal level of $[Ca^{2+}]_i$ was estimated as ~70 nM using the calibration standard kit (Molecular Probes), equivalent to a ratio value of about 0.7. Thapsigargin (100 nM; Alomone Laboratories) was used to evaluate the role played by internal stores in the fast increase of $[Ca^{2+}]_i$.

Immunofluorescence-intensified Video Microscopy—After 1 and 18 h of TcdB incubation, control and treated cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature and then permeabilized with 0.5% Triton X-100 in PBS for 5 min at room temperature. After washing in the same buffer, samples were incubated at 37 °C for 1 h with monoclonal antibodies (mAbs) against mitochondria (Chemicon International, Inc.) and with polyclonal antibodies, prepared as previously described (19), specific for LT and cross-reacting with TcdB. After washing, samples were double-labeled with anti-mouse Alexa Fluor 594 (Molecular Probes) and anti-rabbit Alexa Fluor 488 (Molecular Probes). Following extensive washings, samples were mounted with glycerol/PBS (2:1) and observed with a Olympus BX51 fluorescence microscope. Images were captured by a color chilled 3CCD camera (Hamamatsu, Japan), and normalization and background subtraction were performed for each image. Figures were obtained by the OPTILAB (Graftek) software for image analysis.

Preparation of Isolated Mitochondria—HEp-2 cells grown in monolayer were harvested by a solution containing 0.25% (w/v) trypsin and 0.02% (w/v) EDTA, in calcium- and magnesium-free PBS and collected by centrifugation. After three washings in PBS, cells were resuspended in Homo-buffer (10 mM Hepes, pH 7.4, 1 mM EGTA, 0.1 M sucrose, 5% bovine serum albumin, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor mixture (Roche Applied Science) and maintained for 10 min on ice. After this time, cells were homogenized with about 100 strokes of a Teflon homogenizer with a B-type pestle as previously reported (20) for 10 min at 4 °C to remove intact cells and nuclei, and the supernatants were further centrifuged at 10,000 × *g* at 4 °C for 10 min to precipitate the heavy membrane fractions (enriched in mitochondria). These fractions were then purified by standard differential centrifugation. The mitochondrial pellet obtained was resuspended in swelling buffer containing 0.1 M sucrose, 0.5 M sodium succinate, 50 mM EGTA at pH 7.4, 1 M phosphoric acid (H_3PO_4), 0.5 M MOPS, and 2 mM rotenone, kept on ice, and used within 2 h from the preparation. Protein content in the mitochondrial preparation was determined by a spectrophotometric method using bovine serum albumin as a standard. The purity of mitochondria preparation was assessed by Western blot, checking subunit I of cytochrome *c* oxidase (mAb by Chemicon International). The main problem in obtaining a very purified mitochondria preparation is represented by the possible contamination with other intracellular organelles, such as vesicles from the endolysosomal compartment and Golgi apparatus. Thus, before performing the swelling experiments, we tested the purity of our mitochondrial preparation by flow cytometry after staining with mAbs specific to endolysosomal compartment or Golgi vesicle antigens, Rab-5 and GM130, respectively (both from Santa Cruz Biotechnology). Samples were incubated at 4 °C for 1 h, and, after washing, they were labeled with anti-mouse Alexa Fluor 488 (Molecular Probes). After a 45-min incubation at 4 °C, samples were washed and immediately analyzed by a cytometer. As negative and positive controls, we used purified mitochondria incubated with mouse IgG1 immunoglobulin or with mAb to VDAC-1 (Santa Cruz Biotechnology), respectively, followed by anti-mouse Alexa Fluor 488.

Swelling Induction in Isolated Mitochondria—Mitochondria (0.5 mg of protein/ml) were resuspended in swelling buffer at the final volume of 1.5 ml. As a general rule, the reagents (toxins and drugs) under investigation were added 5 min after the recording had initiated. Total recording time was 25 min. A useful positive control for these experiments consisted of the addition of 300 μM Ca^{2+}, a Ca^{2+} concentration that opens the protein transition pore. This causes amplitude swelling that is accompanied by a decrease of $\Delta\Psi$ and an increase of the outer membrane permeability, leading to the release of proteins (*i.e.* cytochrome c (cyt *c*)) that are normally stored in the intramembrane space. The $\Delta\Psi$ of isolated mitochondria can be quantified by multiple methods. Here, we used a cytofluorimetric analysis after mitochondria staining with 1 μM tetramethylrhodamine/methyl ester/perchlorate (TMRM; Molecular Probes). By this method, the incorporation of dye TMRM was measured in the FL3 channel: low levels of TMRM incorporation (revealed by a decrease of red fluorescence) indicated a low $\Delta\Psi$ (21). We herein tested the effects produced on mitochondria $\Delta\Psi$ by (i) WT TcdB, (ii) rec TcdB, (iii) NterTcdB, and (iv) VacA, either in the presence or absence of 10 μM Ca^{2+}. These measurements were also performed on mitochondria preincubated with diazoxide or FCCP. In parallel, the supernatants of swelling reactions, carried out in the absence of TMRM, were examined by a commercial enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN) for the cyt *c* detection.

Flow Cytometry Analysis of Isolated Mitochondria—All samples were analyzed with a FACScan cytometer (BD Biosciences) equipped with a 488-nm argon laser. To exclude debris, during analyses, samples were gated based on light scattering properties in the side scattering and forward scattering modes. The red fluorescence emission (due to TMRM dye) of untreated mitochondria was put up in correspondence with the 10^2 chan-

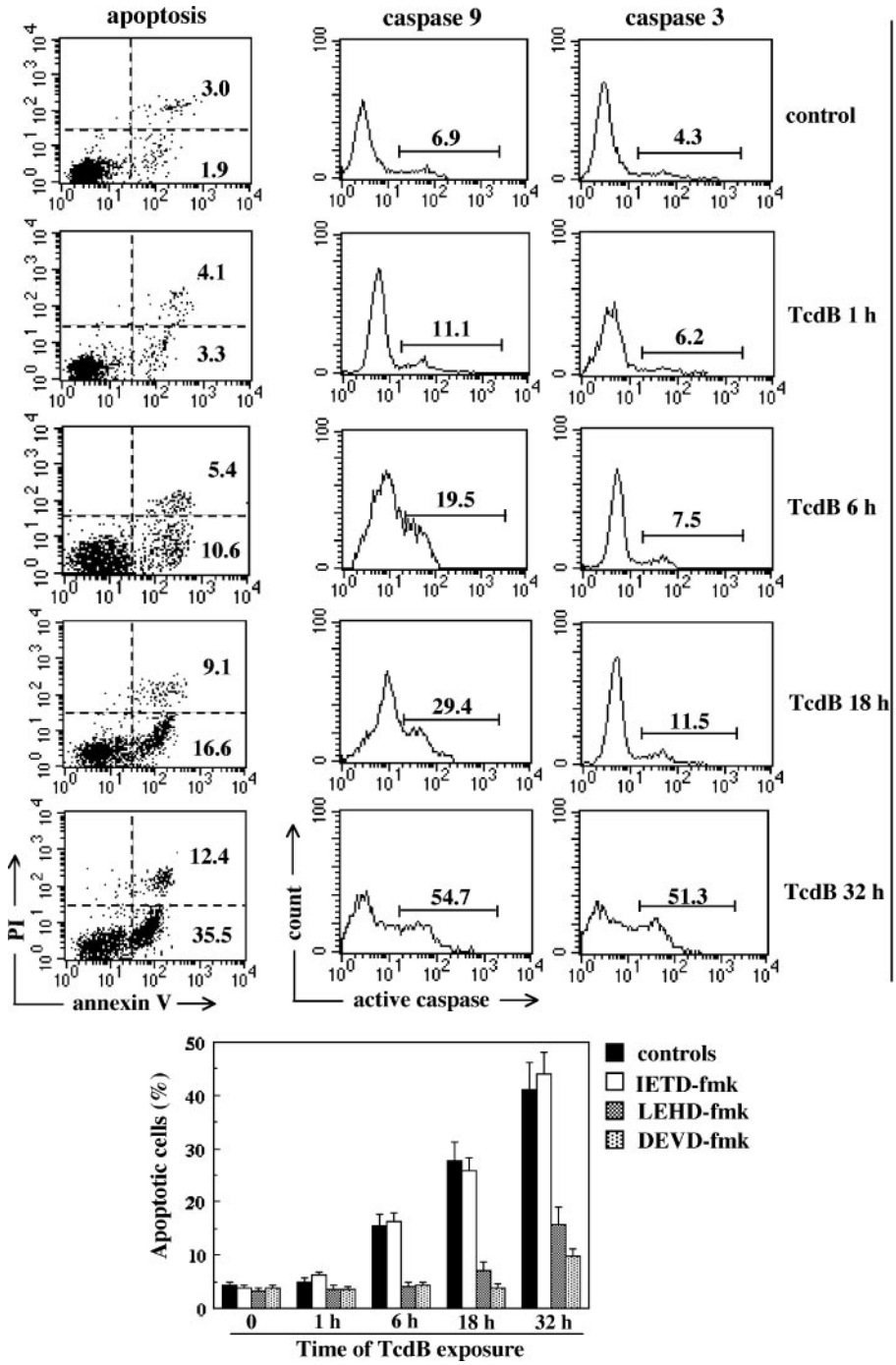

FIGURE 1. **TcdB-induced apoptosis is mediated by caspases 9 and 3.** *A*, biparametric flow cytometry analysis of apoptotic HEp-2 cells after double staining with annexin V-FITC/PI (*left column*). In the *top* and *bottom right quadrants* of each plot, annexin V/PI double positive cells and annexin V single positive cells are represented, respectively. In the *middle* and *right columns*, the analysis of the activation state of caspases 9 and 3 in intact living cells, obtained by using the CaspGLOW fluorescein active caspase staining kit, is reported. *Numbers* on each plot refer to the percentage of cells containing these caspases in their active form. *First row*, control; *second row*, TcdB, 1 h; *third row*, TcdB, 6 h; *fourth row*, TcdB, 18 h; *last row*, TcdB, 32 h. Results obtained in a representative experiment are reported. *B*, graph showing the amount of apoptosis in HEp-2 cells pretreated with IETD-fmk (caspase 8 inhibitor), LEHD-fmk (caspase 9 inhibitor), or DEVD-fmk (caspase 3 inhibitor) before TcdB intoxication. Mean ± S.D. of the percentages of annexin V-positive cells obtained in four different experiments are shown. Statistical analyses indicate a significant ($p < 0.01$) decrease of proapoptotic activity of TcdB in cells pretreated with LEHD-fmk and DEVD-fmk but not with IETD-fmk.

nels and considered as basal emission. Basal emission was recorded for 5 min, and after this time reagents to be tested were added, and the effect was monitored for the following 25 min. Dot plots of red fluorescence emission as a function of the time, obtained in each condition we used, were statistically analyzed by using CellQuest software for a Macintosh computer (BD) in order to determine the percentage of mitochondria with depolarized membrane.

Assay for Cytochrome c Release—Release of cyt *c* was evaluated by a sensitive and specific immunoassay, using a commercial enzyme-linked immunosorbent assay kit (R&D Systems), according to the manufacturer's instructions. The light emitted was quantified by using a microtiter plate reader at 405 nm. Cyt *c* concentration was expressed as ng/ml.

RESULTS

Apoptosis Induced by C. difficile Toxin B Is Mediated by Caspases 9 and 3—Although several lines of evidence have pointed to TcdB as a proapoptotic factor (11, 12), the actual mechanism that leads to cell death is not yet precisely defined. Hence, to address this question, we first assayed which of the two main apoptotic pathways was involved. First, we conducted dose-dependent experiments by using different concentrations of TcdB (1.5, 3, and 6 ng/ml). The results obtained (data not shown) indicated that the minimum dose of the toxin able to induce some apoptotic effects within 24 h was represented by 3 ng/ml. In fact, 1.5 ng/ml toxin was completely ineffective in living HEp-2 cells. In light of this, we performed specific time course experiments by using 3 ng/ml TcdB. The time-dependent effects of the toxin in terms of cell death and caspase activation are reported in Fig. 1. After a 1-h incubation of cells with TcdB, the toxin induced the activation of caspase 9 in a low percentage of cells (Fig. 1, *middle column*, 11 ± 1% *versus* 6 ± 9% in the control). Importantly, neither a significant activation of caspases 8 (not shown) and 3 (*right column*; 6 ± 2% *versus* 4 ± 3% in the control) nor cell death, in terms of AV-positive cells (*left column*), were detectable (*right column*). After 6 h of TcdB intoxication, a significant ($p <$ 0.01) increase in the percentage of either cells positive to

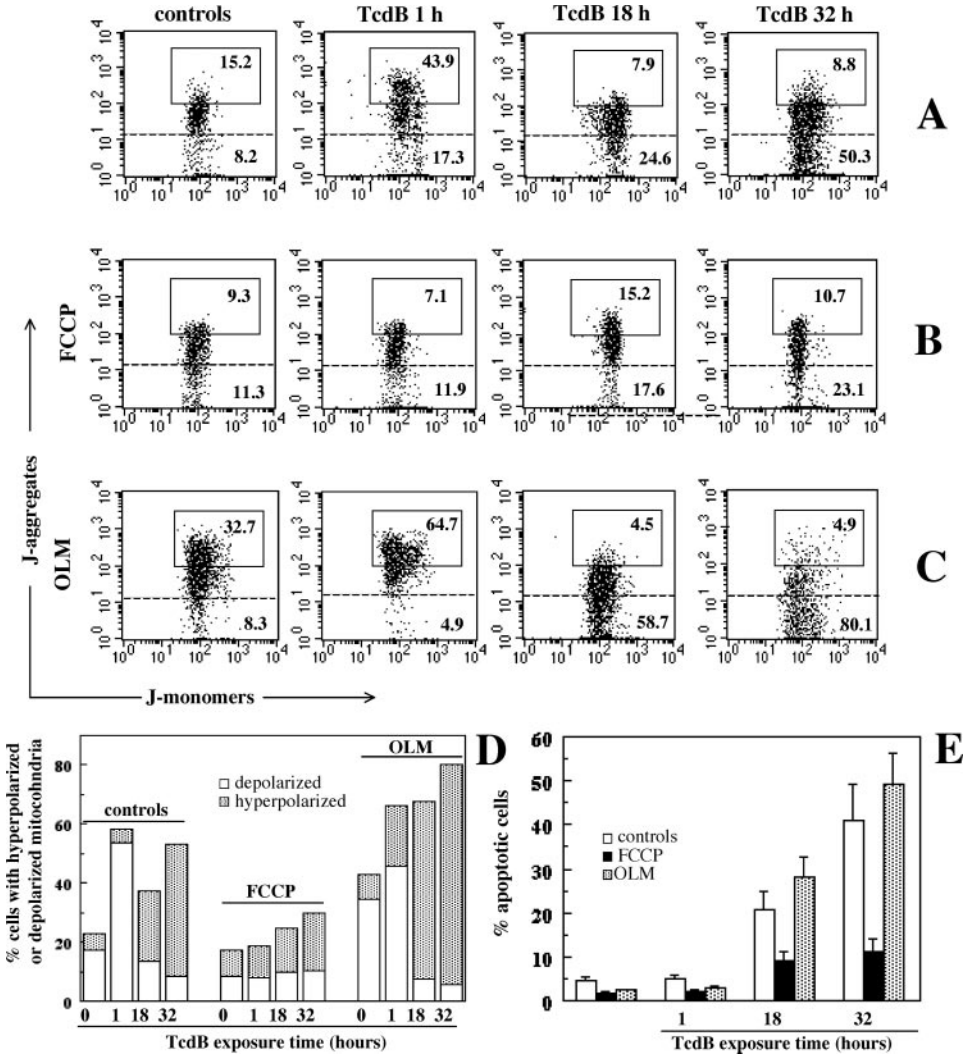

FIGURE 2. **Role of mitochondrial membrane potential in TcdB-induced apoptosis in living cells.** *A–C*, biparametric flow cytometry analysis of MMP after staining with JC-1 in HEp-2 cells treated with TcdB for 1 h (*second column*), 18 h (*third column*), and 32 h (*fourth column*) in the absence (*A*) or presence of FCCP (*B*) or OLM (*C*). The *numbers* reported in the *boxed area* represent the percentages of cells with hyperpolarized mitochondria. In the area *under* the *dashed line*, the percentage of cells with depolarized mitochondria is reported. Results obtained in a representative experiment are shown. *D*, mean ± S.D. of the percentages of cells with hyperpolarized or depolarized mitochondria obtained from four different experiments. Statistical analyses indicate a significant ($p < 0.01$) decrease of cells with either hyperpolarized or depolarized mitochondria in cells pretreated with FCCP before TcdB administration with respect to cells pretreated with OLM or treated with TcdB alone. *E*, mean ± S.D. of the percentages of annexin V-positive cells obtained in four different experiments. Statistical analyses indicate a significant ($p < 0.01$) decrease of proapoptotic activity of TcdB in cells pretreated with a low dose of FCCP.

annexin V (*left column*, *third row*) or with active caspase 9 (*middle column*, *third row*) was evident. By contrast, a small, but not significant ($p > 0.05$ *versus* control cells) increase in caspase 3 activation was observed at this time point (*right column*, *third row*). After 18 h, the percentage of cells with active caspase 9 (29 ± 4%) and active caspase 3 (11 ± 5%) significantly increased (Fig. 1*A*), whereas caspase 8 failed to reveal any activation state (not shown). It is noteworthy that at this time, almost 20% of cells showed apoptotic features (Fig. 1, 18 h, *left panel*). Prolonging TcdB exposure time up to 32 h, a further increase of caspase 9 (54 ± 7%; *middle panel*) and caspase 3 activation (51 ± 3%; *right panel*), together with a significant augmentation in the percentage of apoptotic cells, was observed (more than 40%; *left panel*). Importantly, caspase 8, which defines the receptor-mediated pathway, was also activated in cells after a 32-h exposure to TcdB. The fact that the observed TcdB-induced caspase 9 activation preceded that of caspase 8, allowed us to hypothesize that the toxin may trigger apoptosis in HEp-2 cells via an effect, either direct or indirect, on mitochondria. To verify this assumption, we performed experiments by using specific caspase inhibitors. Results reported in Fig. 1*B* clearly show that pretreatment with LEHD-fmk (a caspase 9 inhibitor) or DEVD-fmk (a caspase 3 inhibitor) significantly ($p < 0.01$) prevented the TcdB-induced apoptosis. By contrast, IETD-fmk (a caspase 8 inhibitor) was unable to protect HEp-2 cells from the effects induced by the toxin.

These data suggest that TcdB can induce apoptosis via the intrinsic pathway, thus ascribing an ancillary role to caspase 8 in TcdB-induced cell death in our experimental system.

Role of Mitochondrial Membrane Potential in TcdB-induced Apoptosis in Living Cells—Therefore, going backward into the pathway controlled by caspase 9, we measured the MMP during time course experiments (0, 1, 18, and 32 h of TcdB treatment) using the JC-1 probe. JC-1, a very sensitive reagent largely used to evaluate MMP changes occurring in apoptosis, allows the measurement of both the earlier events (*i.e.* hyperpolarization) (22) and later events (*i.e.* depolarization) (18) state of mitochondria. Strikingly, after 1 h of TcdB incubation (Fig. 2*A*, *second panel*), a peculiar change occurred in toxin-treated cells, represented by the mitochondrial membrane hyperpolarization (MMHP) (43 ± 9% *versus* 15 ± 2% in control cells). This increase of MMP was followed, as a late event, by the characteristic drop of MMP (typical of cell apoptosis) after 18 and 32 h (Fig. 2*A*, *third* and *fourth panels*, respectively). To explore the possible causal link between MMHP and apoptosis in our experimental system, we used two different "mitochondriotropic" agents able to modulate MMP. Pretreatment with a noncytotoxic dose (40 nM) of FCCP, which causes the mitochondrial proton gradient dissipation (23) and abolishes $\Delta\Psi$ increase (24), led to a significant reduction of TcdB-induced MMP changes (Fig. 2, *B* and *D*). In fact, FCCP inhibited the MMHP found in cells exposed to TcdB for 1 h (Fig. 2*B*, 7.1%), and, consequently, also the drop of MMP

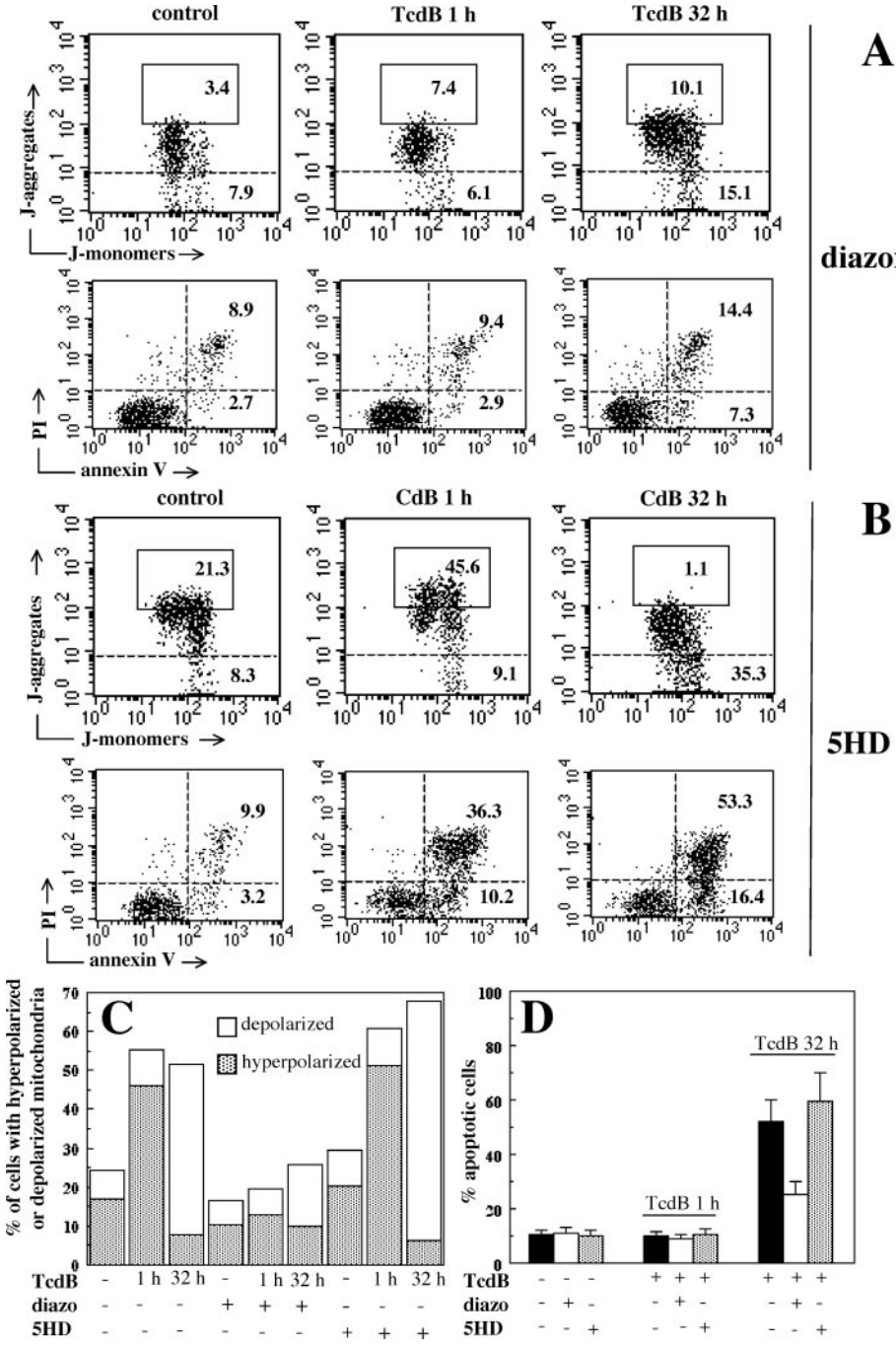

FIGURE 3. **Implication of mitochondrial potassium channels in TcdB-induced apoptosis in living cells.** *A* and *B*, biparametric flow cytometry analysis, after staining of living HEp-2 cells with JC-1 or annexin V/PI, of TcdB-induced effects in terms of MMP (*first row*) and apoptosis (*second row*) in the presence of diazoxide (*diazo*) (*A*) or 5HD (*B*). The *numbers* reported in the *boxed area* (*first row* of *A* and *B*) represent the percentages of cells with hyperpolarized mitochondria. In the area *under* the *dashed line* of the same *panels*, the percentage of cells with depolarized mitochondria is reported. In the *second row* of *A* and *B*, in the *top* and *bottom right quadrants* of each plot, annexin V/PI double positive cells and annexin V single positive cells are represented, respectively. *C*, results obtained in a representative experiment are shown. *D*, mean ± S.D. of the percentages of cells with hyperpolarized or depolarized mitochondria obtained from four independent experiments. Statistical analyses indicate a significant ($p < 0.01$) decrease of cells with either hyperpolarized or depolarized mitochondria in cells pretreated with diazoxide before TcdB administration with respect to cells pretreated with 5HD or incubated with TcdB alone.

induced by a longer exposure to TcdB was prevented (Fig. 2, *B* and *D*). Accordingly, FCCP significantly ($p < 0.01$) inhibited TcdB-induced apoptosis (Fig. 2*E*). By contrast, a low dose of OLM (1 μM), a specific inhibitor of the F_0-ATPase that allows the conversion of the proton gradient into ATP (25), induced (after 1 h) a significant MMHP *per se* (32.7% *versus* 15.2% in control) (Fig. 2, *C* and *D*) that was further significantly increased by its association with TcdB (64.7% *versus* 43.9% in cells treated with TcdB given alone) (Fig. 2, *C* and *D*). Prolonging the incubation time with TcdB up to 18 and 32 h, cells pretreated with OLM showed a high percentage of depolarized mitochondria (Fig. 2, *C* and *D*), and in parallel, a significantly higher ($p < 0.01$) percentage of cells underwent apoptosis (Fig. 2*E*).

In summary, FCCP was able to inhibit both the early (hyperpolarization) and the late (depolarization) TcdB-induced mitochondrial effects and to consequently prevent apoptosis. By contrast, OLM, apparently because of its hyperpolarizing effect on the mitochondrial membrane, bolsters apoptosis in cells challenged with the toxin. Taken together, these findings point to the MMHP as a prerequisite for TcdB-induced apoptosis.

Implication of mK_{ATP} Channels in TcdB-induced Apoptosis in Living Cells—It has recently been reported that an increased MMP could be associated with a block of mK_{ATP} channels in neuron mitochondria (26). To investigate whether this could occur also in epithelial cells treated with TcdB, we analyzed both the early and late mitochondrial effects induced by the toxin, in the presence of (i) diazoxide, an opener of mK_{ATP} channels; (ii) 5HD, a mK_{ATP} channel inhibitor; or (iii) a combination of both drugs. This last set of experiments was performed to verify the specificity of the action of diazoxide on mK_{ATP} channels (27). Fig. 3 reports one representative experiment of the effects of the mK_{ATP} channel activator diazoxide or of the blocker 5HD on the two distinct mitochondrial phases (hyperpolarization and depolarization) observed in HEp-2 cells in response to TcdB. As shown in Fig. 3*A*, pretreatment with diazoxide significantly ($p < 0.01$) prevented MMHP (early phase) induced by a 1-h treatment with TcdB (Fig. 3*A*). In fact, the percentage of cells with hyperpolarized mitochondria var-

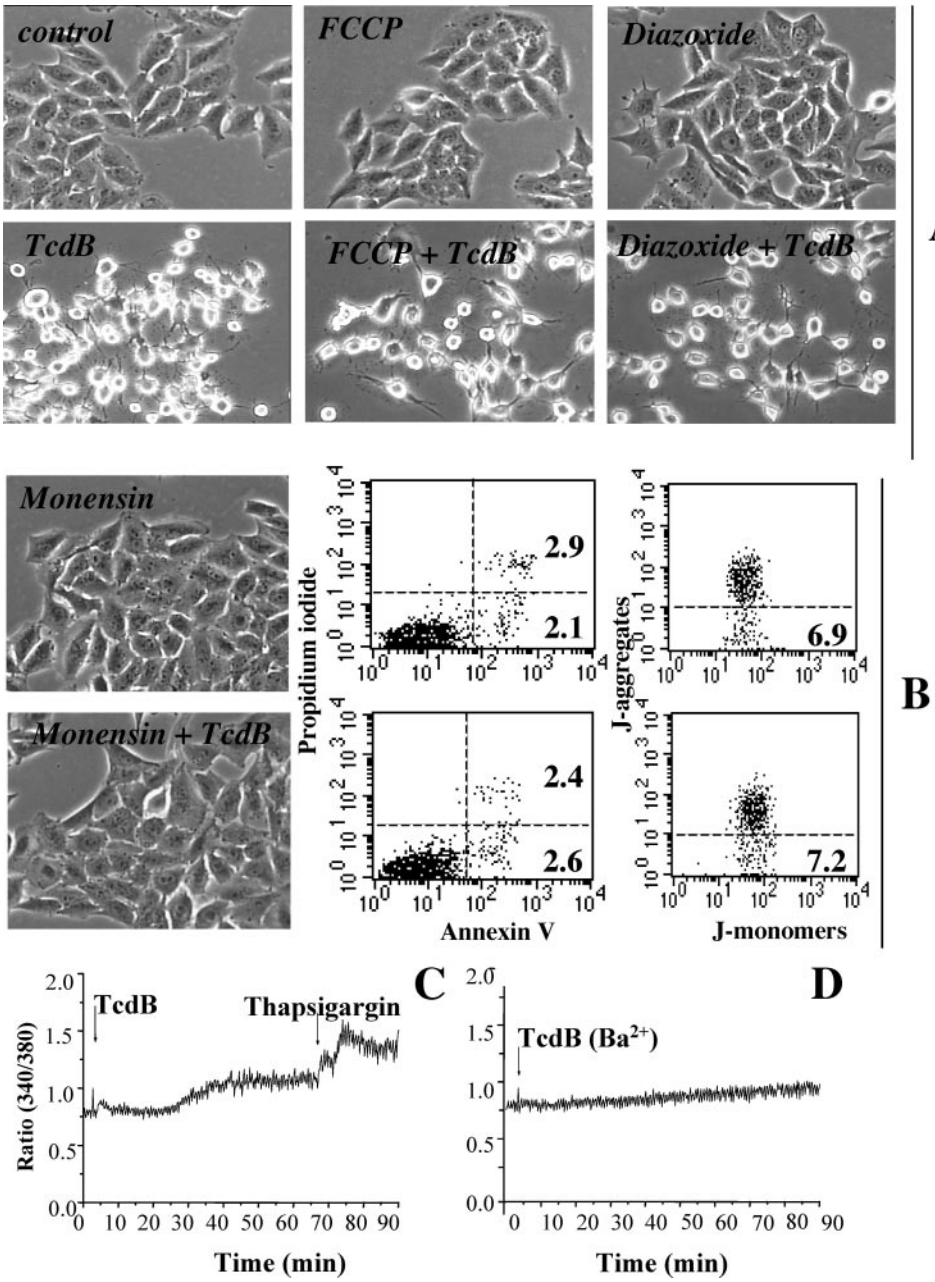

FIGURE 4. **Influence of different inhibitors on the TcdB-induced effects in living cells.** *A*, phase-contrast analysis of HEp-2 control cells (*first row*, *left panel*), cells treated with TcdB for 1 h (*second row*, *left panel*), FCCP-treated cells (*first row*, *middle panel*), cells pretreated with FCCP 1 h before TcdB exposure (*second row*, *middle panel*), cells treated with diazoxide (*first row*, *right panel*), and cells pretreated with diazoxide before TcdB intoxication (*second row*, *right panel*). *B*, phase-contrast analysis (*left column*) and biparametric flow cytometry analysis of living cells pretreated with monensin 1 h before TcdB intoxication and stained with annexin V/PI (*middle column*) or JC-1 (*right column*). In the *first row*, HEp-2 cells treated with monensin alone, considered as experimental control, are shown. In the *middle column*, numbers reported in the *top* and *bottom right quadrants* of each plot represent annexin V/PI double positive cells and annexin V single positive cells, respectively. In the *right column*, the *numbers* in the area *under* the *dashed line* indicate the percentage of cells with depolarized mitochondria. Results obtained in a representative experiment are shown. Magnification, ×1,000. *C* and *D*, Ca^{2+} evaluations in living cells. *C*, after 3 min of control recording, the application of TcdB (*first arrow*) does not significantly modify $[Ca^{2+}]_i$ within the successive 30–35 min. After that time frame, intracellular calcium begins to progressively rise until a ratio value of about 1.2, this occurring within 45 min after the addition of the toxin. The successive application of thapsigargin (*second arrow*) after stabilization of $[Ca^{2+}]_i$ induces a fast increase of $[Ca^{2+}]_i$, which reaches a ratio value of about 1.5. *D*, to support the role of extracellular calcium in the TcdB-induced effects, cells are bathed in a medium in which Ca^{2+} is replaced by Ba^{2+} before the addition of TcdB (*arrow*). In these experiments, the cytosolic calcium level remains constant after exposition to the toxin. Two measurements representative of at least 40 are shown.

ied from 43.9 in TcdB-treated samples (Fig. 2*A*) to 7.4 when TcdB was added after diazoxide preexposure (Fig. 3*A*). It is noteworthy that, comparing the control untreated cells with those treated with diazoxide, it was possible to observe a reduction of the percentage of cells with hyperpolarized mitochondria in the presence of this mK_{ATP} channel opener (3.4% *versus* 15.2% in the control). The protective effect of diazoxide at the mitochondrial level was also detectable after 32 h of TcdB exposure (compare Fig. 3*A* with Fig. 2*A*). In fact, when the mK_{ATP} channels were maintained opened by diazoxide, the depolarization of mitochondrial membrane (late phase) induced by TcdB was reduced about 3.5-fold (Fig. 2*A*, 50.9% of cells with depolarized mitochondria with respect to 15.1% (Fig. 3A)). In Fig. 3*C*, a mean of the results obtained from four independent experiments is shown. As expected, diazoxide was also able to inhibit the final event induced by the toxin (*i.e.* cell death) (Fig. 3, *A*, *second row*, and *D*). Similar results were gained by using pinacidil, an alternative activator of mK_{ATP} channels (data not shown). The specificity of these effects was also confirmed by using 5HD alone (Fig. 3*B*). In fact, this mK_{ATP} channel blocker, not cytotoxic *per se*, was unable to significantly prevent the effect of TcdB at the mitochondrial level (neither hyperpolarization nor depolarization), and hence apoptotic cell death normally occurred (compare Fig. 3*D* with Fig. 2*E*). In order to prove that the observed effects were specifically due to the activity of diazoxide on mK_{ATP} channels, 5HD was also employed in association with diazoxide or pinacidil. In these experimental conditions, 5HD completely prevented the protection exerted by these mK_{ATP} channel activators both on TcdB-induced MMP alterations and cell death. These data are strongly suggestive for a direct involvement of these channels in the pathway followed by TcdB to trigger apoptosis.

To rule out the possibility that either the uncoupler FCCP or the mK_{ATP} channel opener diazoxide could aspecifically interfere with the pathway intoxication of TcdB, we analyzed by phase-contrast microscopy the response to TcdB in cells pretreated

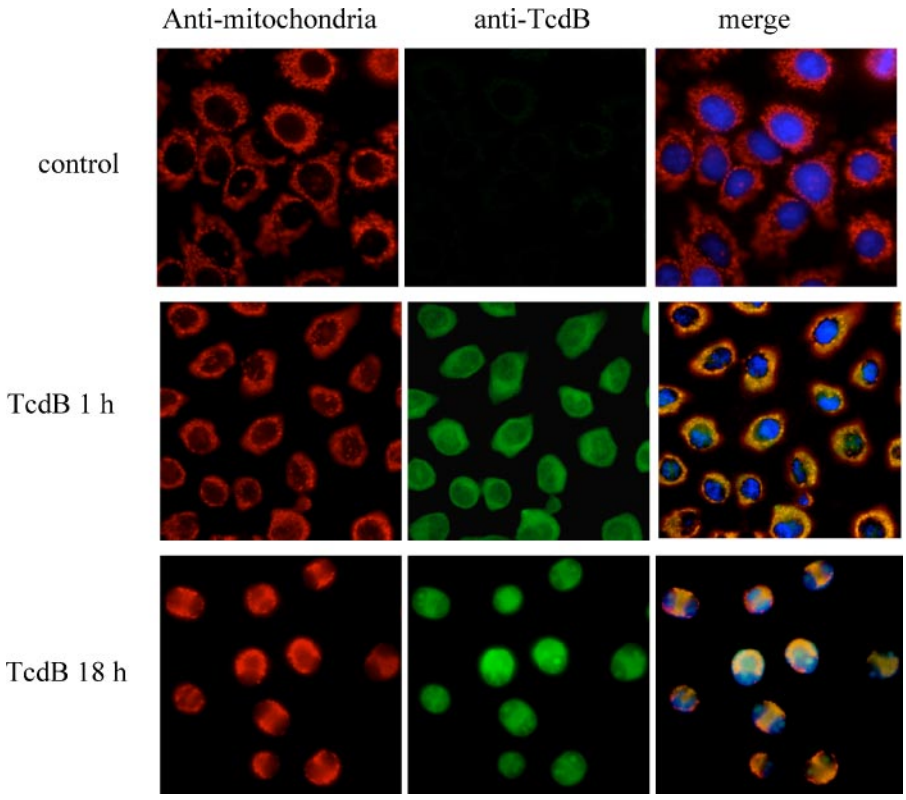

FIGURE 5. **TcdB co-localizes with mitochondria in living cells.** Control HEp-2 cells (*first row*) and cells treated with TcdB (3 ng/ml) for 1 h (*second row*) or for 18 h (*third row*) are shown. Fluorescence micrographs are shown of cells after triple immunostaining with rabbit polyclonal antibodies that recognize TcdB (*green*, *middle column*), with mouse monoclonal antibodies against mitochondria (*red*, *left column*), and with the nuclear staining Hoechst. TcdB is clearly detectable in the cytosol, in close association with mitochondria (see the *yellow fluorescence* in the *right column*, *merge images*). Magnification, ×1,200.

with FCCP or diazoxide. We observed that, also in the presence of FCCP, cells exposed to TcdB underwent the typical morphological signs of intoxication (*e.g.* retraction of the cell body followed by the rounding up) (Fig. 4*A*, *second row*, *middle panel*; compare with cells treated with TcdB alone in the *second row*, *left panel*). Similar results were obtained when HEp-2 cells were pretreated with diazoxide (Fig. 4*A*, *second row*, *right panel*). These data prove the activity of the toxin also in cells pretreated with the drugs and confirm that FCCP and diazoxide did not induce *per se* any significant alteration of the cell morphology (Fig. 4*A*, *first row*, *central* and *right panel*, respectively; compare with control untreated cells in the *first row*, *left panel*). Moreover, as stated in the Introduction, TcdB-induced morphological changes occur after the internalization and activation of the toxin in the endosomal vesicles (13). Thus, we wanted to verify whether the apoptotic response was also dependent on the endocytic process and intravesicular activation of the toxin or could simply go after the external application of the toxin. To address this question, TcdB endosomal activation was blocked by using monensin, a lysosomotropic agent that alters the endosomal pH (28). As shown in Fig. 4*B*, pretreatment with 10 μM monensin prevented not only the TcdB-provoked cell retraction and rounding up (*left column*, *bottom panel*) but also the mitochondrial alterations (*right column*, *bottom panel*; compare with Fig. 2*A*) and the consequent apoptosis (*central column*, *bottom panel*; compare with Fig. 1*A*). This demonstrates that the apoptotic response to TcdB goes after the classical pathway of internalization and endosomal activation of the toxin. Interestingly, we also found that TcdB can induce, following its internalization, a contained cytosolic calcium rise in intact cells. By using a single cell assay, we observed that the basal level of calcium concentration $[Ca^{2+}]_i$ that was about 0.7 (a representative experiment is shown in Fig. 4*C*; mean value of six independent experiments, 0.8 ± 0.1), corresponding to ~70 nM, increased after TcdB application. In fact, the $[Ca^{2+}]_i$ level augmented during the successive 30–35 min of challenge with the toxin, reaching a ratio value of about 1.2 within 45 min (Fig. 4*C*; mean value of six independent experiments, 1.3 ± 0.3, $p <$ 0.01 with respect to control cells at time 0). The calcium rise could be imputed to an extracellular calcium entry, since application of thapsigargin, a drug that causes the exit of calcium from internal stores, provoked a fast increase of $[Ca^{2+}]_i$ that reached a ratio value of about 1.5 (Fig. 4*C*; mean value of six independent experiments, 1.5 ± 0.3). In support of this finding, in cells maintained in a medium in which Ca^{2+} was replaced by Ba^{2+}, the cytosolic calcium level remained constant after TcdB addition (Fig. 4*D*). Altogether, these findings indicate that, once internalized, TcdB can provoke the entry of extracellular calcium in living cells.

TcdB Acts Directly on Mitochondria—We then asked whether the above reported effects of TcdB on mitochondria could result from a direct interaction of the toxin with mitochondria. To shed light on this point, we first investigated the intracellular localization of TcdB by double labeling immunofluorescence analyses in living cells. As shown in Fig. 5, TcdB and mitochondria appeared co-localized in the cytoplasm as early as after 1 h of challenge with the toxin (*second row*, see *merge* images). This remained unchanged until after 18 h of TcdB treatment, when the rounding up of the cells was completed. Obviously, the presence of TcdB in the close vicinity of mitochondria suggests but does not prove that the toxin impacts these organelles. To further explore this question, we challenged TcdB with mitochondria isolated from HEp-2 cells. The purity degree of our mitochondrial preparation was checked by flow cytometry to exclude the presence of remnants of other intracellular organelles, such as vesicles from the endolysosomal compartment and/or Golgi apparatus. Results obtained (Fig. 6*A*) show that the isolated mitochondria were completely negative either for the endolysosomal compartment antigen Rab-5 or for the GM130, a protein that characterizes Golgi apparatus, whereas they were strongly

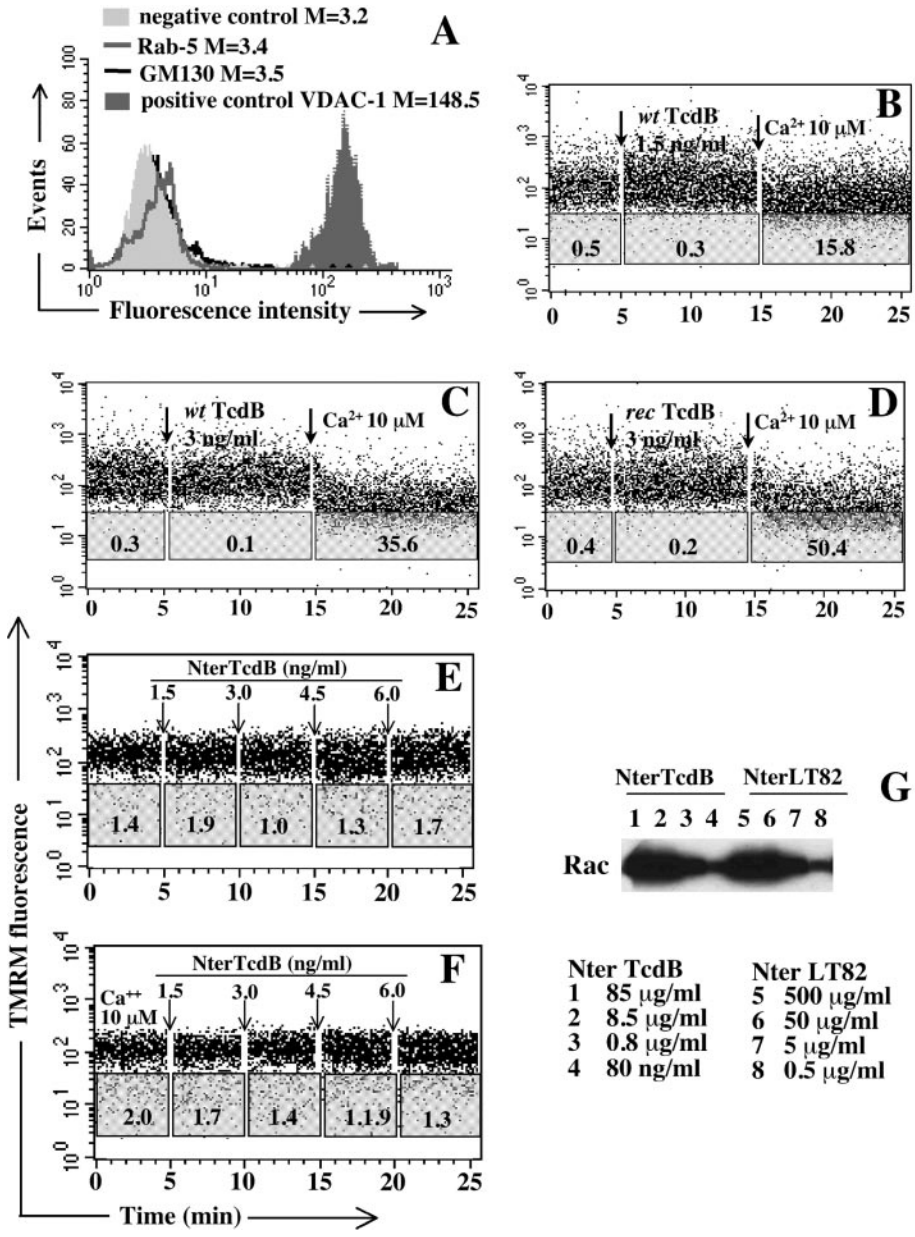

FIGURE 6. **TcdB causes a calcium-dependent swelling of isolated mitochondria.** *A*, cytofluorimetric analysis of mitochondria preparation after staining with mAb to Rab-5 (*deep gray empty histogram*), GM130 (*black empty histogram*), or VDAC-1 (*deep gray full histogram*) to test its degree of purity. The negative control (IgG1 plus anti-mouse Alexa 488) is represented by the *light gray full histogram*. The *numbers* represent the median values of fluorescence intensity histograms. Statistical analysis performed by Kolmogorov/Smirnov test indicated that, whereas VDAC-1 sample was significantly different ($p < 0.01$) from negative control, Rab-5 and GM130 samples were not ($p > 0.05$). Flow cytometric analyses show that isolated mitochondria are completely negative both for the endolysosomal compartment antigen Rab-5 and for the GM130, a protein that characterizes Golgi apparatus while appearing strongly positive for the mitochondria protein VDAC-1. *B–F*, cytofluorimetric analyses representing the swelling profile of the mitochondria isolated from HEp-2 cells monitored by means of variations in TMRM fluorescence as a function of time. The *numbers* in *gray areas* of each plot represent the percentage of mitochondria that underwent MMP decrease. Results obtained in a representative experiment are shown. *B–D*, TcdB induces a dose-dependent effect on isolated mitochondria. *B*, 1.5 ng/ml WT TcdB; *C*, 3 ng/ml WT TcdB. Note that 3 ng/ml rec TcdB (*D*) induces effects quantitatively comparable with those caused by WT TcdB. Importantly, TcdB (both WT and rec) carried on their effects only in the presence of 10 μM Ca^{2+}. *E* and *F*, the NterTcdB is completely ineffective on isolated mitochondria, irrespective of the concentration used (from 1.5 to 6 ng/ml), either in the absence (*E*) or in the presence (*F*) of 10 μM Ca^{2+}. *G*, it is worth noting that NterTcdB (on the *left*) retained the *in vitro* glucosylation activity on Rac at the different doses used, confirming the catalytic activity of this recombinant molecule. Different concentrations of NterLT82 (on the *right*) are used as positive controls.

positive for the mitochondria protein VDAC-1 (voltage-dependent anion channel). Moreover, in this set of experiments, in addition to different doses of the WT TcdB, we used a rec TcdB (prepared as described under "Experimental Procedures") to rule out the possibility that an unidentified contaminant could be the cause of the mitochondrial swelling observed after exposure to the wild-type toxin. Fig. 6 shows representative profiles of the mitochondrial swelling induced by 1.5 ng/ml WT TcdB (Fig. 6*B*), 3 ng/ml WT TcdB (Fig. 6*C*), or 3 ng/ml rec TcdB (Fig. 6*D*). It is noteworthy that challenge with 6 ng/ml WT TcdB caused a response similar to that induced by a 3-ng/ml concentration of the toxin (data not shown), and 1.5 ng/ml WT TcdB (Fig. 6*B*), a dose ineffective in living cells, could partially induce swelling in isolated mitochondria. Importantly, TcdB alone (both WT and rec) caused no effect on mitochondria if not followed by addition of 10 μM Ca^{2+}, in keeping with our observation (see above) that TcdB allows the entry of extracellular calcium and the consequent rise of its cytosolic level. We thus wondered if the TcdB molecule could contain a mitochondrial targeting sequence. To address this question, we analyzed the amino acid sequence of the toxin by a P-SORT program (29). This program predicted TcdB to be a mitochondrial protein and localized a possible cleavage site for mitochondrial presequence (NRKQL) in the N-terminal catalytic portion of the toxin. Hence, to verify whether the catalytic domain portion was also responsible for the mitochondrial swelling, we applied NterTcdB on purified mitochondria. This toxin fragment (amino acid residues 1–546) represents the catalytic domain of TcdB that is translocated into the cytosol of eukaryotic cells (30). Strikingly, this construct, although retaining the *in vitro* glucosylation activity on the Rac molecule (Fig. 6*G*), was completely ineffective on isolated mitochondria, irrespective of the concentration used (from 1.5 to 12 ng/ml), either in the presence or in the absence of 10 μM Ca^{2+} (Fig. 6, *E* and *F*). Fig. 6*G* shows the glucosylation of Rac (obtained by autoradiography, as

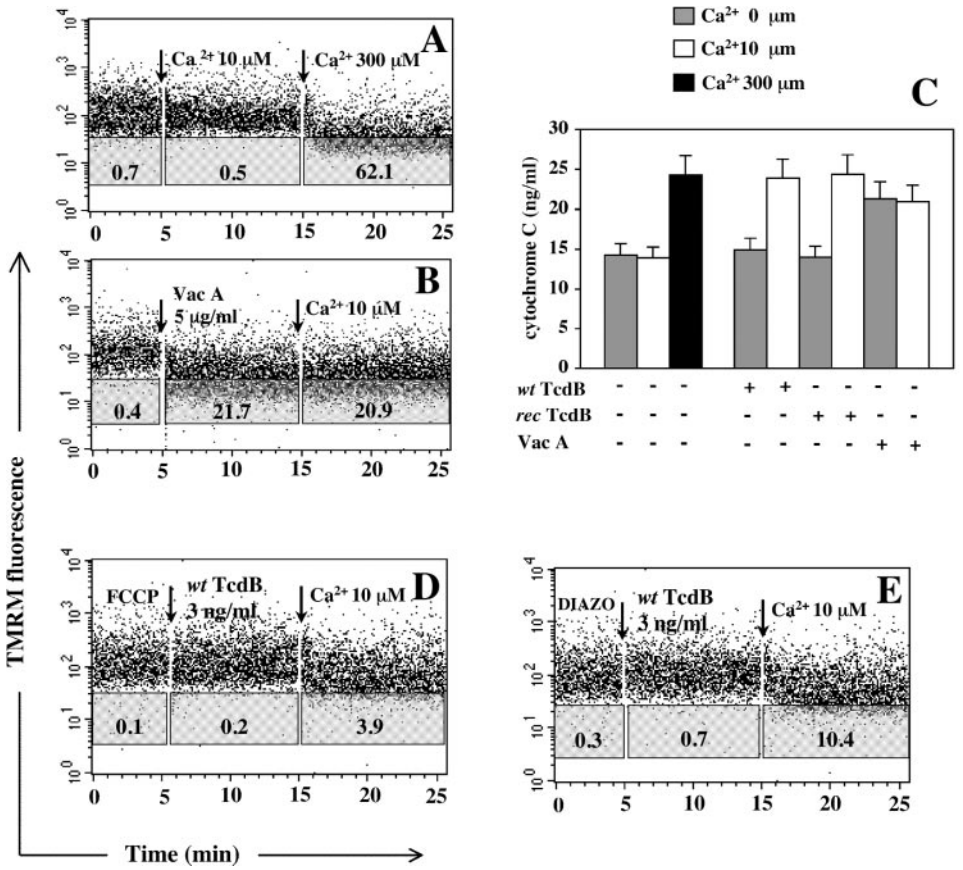

FIGURE 7. **Effects of TcdB on cytochrome C release and mK_{ATP} channels in isolated mitochondria.** *A*, *B*, *D*, and *E*, cytofluorimetric analyses representing the swelling profile of the mitochondria isolated from HEp-2 cells monitored by means of variations in TMRM fluorescence as a function of time. The *numbers* in the *gray areas* of each plot represent the percentage of mitochondria that underwent MMP decrease. Results obtained in a representative experiment are shown. *A*, 10 μM calcium does not induce *per se* any sign of mitochondrial swelling, whereas 300 μM calcium induces a rapid loss of mitochondrial membrane potential. *B*, the pore-forming VacA, used as positive control, induced rapid mitochondrial swelling without entailing a calcium rise. *C*, quantitative evaluation of cyt *c* release in the supernatant of isolated mitochondria as revealed by the enzyme-linked immunosorbent assay method. Note the role of Ca^{2+} and the absence of significant differences between WT and rec toxin. *D* and *E*, pretreatment of isolated mitochondria with FCCP (*D*) and diazoxide (*DIAZO*) (*E*) significantly prevents TcdB-induced Ca^{2+}-dependent mitochondrial swelling.

described under "Experimental Procedures") induced by different concentrations of NterTcdB and, as a positive control, the N-terminal catalytic portion of the *C. sordellii* toxin (NterLT82), which is known to act on the Rac molecule (9). All of this indicates that the N-terminal domain, although presumably involved in targeting mitochondria, is not the "very" toxin domain responsible for the effects observed at the mitochondrial level. Also, it is conceivable that a specific protein conformation could be required for TcdB-induced mitochondrial effects.

All in all, it appears that TcdB can directly target mitochondria and cause their swelling in a calcium-dependent fashion (*cf.* Fig. 6). Importantly, however, as shown in Fig. 7*A*, the addition of 10 μM calcium chloride did not induce *per se* any sign of mitochondrial swelling, although it was indispensable for the TcdB-induced mitochondrial response (Fig. 6, *B–D*). A change in calcium concentration, however, is not generally mandatory for mitochondrial swelling. In fact, the channel-forming toxin VacA induced swelling in a significant percentage of mitochondria without entailing a Ca^{2+} rise (Fig. 7*B*). On this basis, we also investigated the role of Ca^{2+} in modulating the ability of TcdB to induce the release of cyt *c* from mitochondria purified from HEp-2 cells. This was carried out by analyzing the supernatant of swelling experiments (before TMRM staining), by means of an enzyme-linked immunosorbent assay. The results obtained are reported in Fig. 7*C*. In particular, we observed that cyt *c* release was induced by both WT TcdB and rec TcdB, at a similar extent, only in the presence of 10 μM Ca^{2+}. This Ca^{2+} concentration was unable *per se* to cause cyt *c* release, which was instead provoked only by higher Ca^{2+} concentration (300 μM). Cyt *c* release, however, was not strictly calcium-dependent in our system, in keeping with what was reported above (see Fig. 7*B*). Finally, in favor of a direct targeting of mitochondria by TcdB, is also the observation that both FCCP and diazoxide pretreatment of isolated mitochondria significantly prevented TcdB-induced Ca^{2+}-dependent mitochondrial swelling (Fig. 7, *D* and *E*, respectively) and also reduced TcdB-induced cyt *c* release (FCCP, 13.2 ± 3.2 ng/ml; diazoxide, 14.4 ± 3.7 ng/ml). This was in accordance with what we found in intact cells (Fig. 3), demonstrating that the involvement of mK_{ATP} channels in TcdB-induced apoptosis does not require the toxin-dependent Rho inhibition as an intermediate step, thus further supporting our hypothesis of a direct targeting of mitochondria by TcdB.

DISCUSSION

In the present work, we report the ability of the protein toxin TcdB to trigger apoptosis via the involvement of mitochondrial ATP-dependent potassium channels, an absolutely novel finding in the bacterial world. Importantly, we also demonstrated that TcdB impacts mitochondria and induces apoptosis by exploiting, as a crucial event, an early MMHP state, apparently dependent on the block of mK_{ATP} channels. MMHP is a mitochondrial state that has been indicated either as an early event in the apoptotic program (31, 32) or as a peculiar mitochondrial state influencing the commitment to cell death (32, 33) (*i.e.* sensitizing cells to mitochondria-mediated proapoptotic stimulations) (34, 35). Consistent with this, the early MMHP state provoked by TcdB was followed by the typical loss of mitochondrial membrane polarization, known to be associated with the apoptosis execution phase. The crucial role played by MMHP in TcdB-induced apoptosis was confirmed by the results obtained using FCCP and OLM. In fact, whereas TcdB-induced

MMHP and apoptosis were impaired by partially dissipating the mitochondrial proton gradient via treatment with low concentrations (20–40 nM) of the protonophore FCCP, pretreatment of cells with OLM, which prevents the transport of H^+ back into the matrix from the intramembrane space, reinforced the toxin-induced effects. Both FCCP and OLM act on the F_0-F_1 complex, but they produce different alterations in the mitochondrial polarization state; FCCP, like diazoxide, seems to regulate the mitochondrial homeostasis by preventing the MMHP, whereas OLM, which induced *per se* MMHP, sensitized epithelial cells to TcdB-induced early mitochondrial effects responsible for the subsequent apoptotic cascade. As concerns the mK_{ATP} channels, their involvement in TcdB activity was principally proved by the observation that their pharmacological activation by specific openers, such as diazoxide or pinacidil, significantly prevented either mitochondrial hyperpolarization or apoptosis, whereas the addition of the mK_{ATP} channel inhibitor 5HD restored the sensitivity to TcdB-induced effects. So far, the mK_{ATP} channels have been identified in the inner mitochondrial membrane of the liver (36), cardiac (37, 38), neural (39), and lymphoblastoid cells (40). Their opening was demonstrated to lead to an enhanced resistance to injuries caused by different stimulations, including those of apoptotic inducers (40–43). Hence, consistently, in TcdB-exposed epithelial cells, treatment with the mK_{ATP} channel opener diazoxide resulted in the maintenance of mitochondrial ionic homeostasis and mitochondrial membrane potential and in apoptosis hindering. This could parallel the results previously obtained in other experimental systems, such as ischemic brain injury protection (44) or cardioprotection (38). Intriguingly, a cardiotoxic effect of TcdB, both in primary cardiomyocytes and in zebrafish embryo, has very recently been reported by Hamm *et al.* (45).

We also provide herein evidence that TcdB caused a contained increase in the cytosolic calcium level that turned out to be indispensable to provoke the swelling of isolated mitochondria and the release of cytochrome *c*. Importantly, mK_{ATP} channel opener diazoxide blocked the mitochondrial response either in intact cells or in purified mitochondria. Although the relationship between mK_{ATP} activity and calcium remains unclear, it is accepted that Ca^{2+} is one of the factors that can either influence or in turn be influenced by mK_{ATP} channel activity (46). Activation of these channels, in fact, dampens mitochondrial calcium overload, at least in cardiomyocytes (47), and, on the other hand, an increased calcium concentration has been recently hypothesized to block the mK_{ATP} channel in Jurkat T cells (46). Noticeably, the calcium onset, indispensable for TcdB-induced effects, was absolutely dispensable for the swelling of isolated mitochondria caused by VacA, whose ability to provoke cytochrome *c* leakage seems to be dependent on its membrane channel-forming activity (4). Our results thus support the assumption that ion channels on the mitochondrial inner membrane influence cell function in specific ways that can be detrimental or beneficial to cell survival.

In order to gain a direct access to mitochondria, the entry of toxins into cells is obviously required. Some toxins, including TcdB, have to be internalized by endocytosis (8), whereas others require injection into cytosol via a type III secretion system (reviewed in Ref. 48) or, like VacA, can use a mechanism relying on toxin channel activity (reviewed in Ref. 49). Alternatively, vesicle-mediated traffic has been described to exist between cellular plasma/endosome membranes and mitochondria (50). Interestingly, a recent computer-based survey showed that up to 5% of the proteins encoded in the genome of *E. coli* have sequence features that could serve as mitochondrial targeting information (51). This frequent "predisposition" can account for the endosymbiotic origin of mitochondria (52), which, although becoming *bona fide* organelles of eukaryotic cells, maintained the basic mechanisms of bacterial protein transport henceforth used for exchanges between mitochondria and the eukaryotic cytosol (52) (reviewed in Ref. 53). Moreover, the similarity that has been evidenced between bacterial protein toxins and proteins that act as apoptosis regulators at the level of mitochondria (54) adds intriguing clues in regard to evolutionary considerations.

The targeting of mitochondrial membranes by bacterial toxins is becoming a powerful strategy to control cell viability (3). In fact, the list of toxins that might translocate to host cell mitochondria and cause apoptosis is exponentially increasing. The best studied example is the *H. pylori* VacA that has been demonstrated to localize to mitochondria both *in vitro* and in cells and to induce changes in mitochondrial membrane permeability that correlate the membrane channel activity of the toxin (11, 49, 55). Membrane-associated porins, such as *Neisseria gonorrhoeae* Por B (5) or *Staphylococcus aureus* α-toxin (56), also alter membrane permeability of isolated mitochondria. Moreover, mitochondria of mammalian cells can also be targeted by various virulence factors, including the *E. coli* Orf19 (57), the translocated intimin receptor of *E. coli* (58), and *Salmonella* SipB (59), although neither the modality by which this occurs nor the outcome of mitochondria targeting have been investigated in detail. In the group of large clostridial toxins, to which TcdB belongs, the first one reported to localize to mitochondria has been purified TcdA that induces early mitochondrial damages in intact cells, including cytochrome *c* release, ATP depletion, reactive oxygen production, and cell apoptosis (10, 60). It was also shown that TcdA could induce leakage of cytochrome *c* from isolated mitochondria (10), with a still undefined mechanism. More recently, the LT toxin from *C. sordellii* has been reported to localize to mitochondria, hence inducing cytochrome *c* release and apoptosis (9). However, the newest results obtained by using a recombinant TcdA showed the failure of such a recombinant toxin in provoking the swelling of isolated mitochondria, a response that was on the contrary achieved with the wild-type TcdA (61). The authors ascribed this discrepancy to peptidic or lipidic contaminants that were possibly present in the wild-type preparation, thus challenging the previous assumption of TcdA as a toxin directly targeting mitochondria. In the case of TcdB, the use of the recombinant protein as well as the observed co-localization of TcdB with mitochondria support an action of the toxin on these organelles. It should also be considered that TcdB enzymatically acts by inhibiting the Rho GTPases and that these regulatory proteins are critically involved in modulating apoptosis (1). As concerns the activity on isolated mitochondria, however, the glucosyltransferase moiety of the toxin failed to induce any sig-

nificant change (*i.e.* the swelling of these organelles). Accordingly, no apoptosis was detected. These results clearly highlight that a toxin domain different from the catalytic one may be responsible for the effects observed at the mitochondrial level. Indeed, the large molecular weight of TcdB leads to speculation that it could contain, in addition to its enzymatic activity, several other molecular activities, an aspect that is not unique among bacterial virulence factors (reviewed in Ref. 62). As an example, the IpaB protein of *Shigella* spp. is implicated in the internalization of the bacterium into the host intestinal cell but also possesses the ability to trigger apoptosis in macrophages by activating caspase 1 (63).

Hence, taking into account data from the literature (1, 11, 64) and from the present work, it appears that TcdB might induce apoptosis via three different pathways: (i) the toxin-inhibiting activity on Rho GTPases, which causes the breakdown of the actin cytoskeleton that in turn results in the dismantling of the cell, loss of anchorage from the substrate, and the subsequent morphological changes associated with apoptosis; (ii) the toxin enzymatic activity-dependent activation/inactivation of transcription factors involved in production of pro- or antiapoptotic proteins (in fact, by manipulating Rho GTPases, TcdB can modify the expression of apoptosis-related genes, some of them being regulated by transcription factors controlled either directly or indirectly by Rho proteins) (65); and (iii) the target of mitochondria that leads to the modulation of channels and the subsequent proapoptotic mitochondrial pathway activation. The results reported herein principally apply to the mitochondrial pathway and give the following hypothetical picture. Shortly after having gained access to the cytosol, TcdB interacts with mitochondria influencing the functional state of mK_{ATP} channels. This is associated with an increased cytosolic calcium concentration and with an alteration of mitochondrial membrane potential (*i.e.* inducing hyperpolarization). This series of events could play a key role in bolstering the execution of apoptotic cell death.

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