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Interaction of *Clostridium perfringens* epsilon-toxin with biological and model membranes: A putative protein receptor in cells

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ABSTRACT

Epsilon-toxin (ETX) is a powerful toxin produced by some strains of *Clostridium perfringens* (classified as types B and D) that is responsible for enterotoxemia in animals. ETX forms pores through the plasma membrane of eukaryotic cells, consisting of a β-barrel of 14 amphipathic β-strands. ETX shows a high specificity for certain cell lines, of which Madin–Darby canine kidney (MDCK) is the first sensitive cell line identified and the most studied one. The aim of this study was to establish the role of lipids in the toxicity caused by ETX and the correlation of its activity in model and biological membranes. In MDCK cells, using cell counting and confocal microscopy, we have observed that the toxin causes cell death mediated by toxin binding to plasma membrane. Moreover, ETX binds and permeabilizes the membranes of giant plasma membrane vesicles (GPMV). However, little effect is observed on protein-free vesicles. The data suggest the essential role of a protein receptor for the toxin in cell membranes.

1. Introduction

Epsilon-toxin (ETX) is a pore-forming protein produced by toxinotypes B and D of *Clostridium perfringens* [1]. This aerotolerant, anaerobic, widespread bacteria is responsible for a certain number of diseases caused by different toxins. Among them, ETX is one of the most potent toxins known, for this reason, it is considered a potential biological weapon and is classified as a category B biological agent, although very few natural diseases have been reported in humans [2,3]. However, in animals, it is responsible for enterotoxemia, especially in sheep causing an important damage to the world economy, since these animals occupy a premier place in the livestock [4]. ETX belongs to the heptameric β-pore-forming toxin family that includes aerolysin and *C. septicum* alpha-toxin. Despite low sequence identity (14%), the general structure of the toxin is related to the pore-forming toxin aerolysin produced by *Aeromonas* species, although epsilon-toxin is much more potent by 100-fold than aerolysin. These toxins are characterized by the formation of a pore through the plasma membrane of eukaryotic cells, consisting of a β-barrel of 14 amphipatic β-strands [5].

ETX is synthesized during exponential growth of *C. perfringens* as a protoxin with a very low activity. Epsilon-protoxin (EPTX) is composed of 296 amino acids (32.9 kDa) and is activated by proteolytic cleavage by different proteases depending on the site of activation [6]. Trypsin cleaves 13 amino acids from the N-terminal and 22 from the C-terminal end, the result is a protein with a low toxicity in mice with an LD_{50} of 320 ng·kg^{-1}. The most active form of epsilon-toxin is obtained by a combination of trypsin and chymotrypsin that cleave 13 amino acids from the N-terminal and 29 from the C-terminal domains, obtaining a shorter protein and an LD_{50} of 50–65 ng·kg^{-1}. Moreover, lambda-protease cuts in 10 residues from the N-terminal and 29 from the C-terminal domain giving rise to an activity with LD_{50} = 110 ng·kg^{-1} [6].

The effect of ETX on membrane models is not clear. It has been described that the toxin is able to cause carboxyfluorescein leakage in multilamellar vesicles (MLV) and to insert in monolayers in a manner dependent on the toxin doses and the fluidity of the lipid [7]. Moreover, also the ability of ETX to permeabilize "black lipid" bilayers was studied, with the conclusion that the toxin causes permeability preferentially to anions in bilayers composed of different lipids [8]. Regarding the cellular effects, ETX shows a high specificity for a few cell lines, e.g., Madin–Darby canine kidney (MDCK), the first sensitive cell line identified and the most studied one, G-402 (human renal leiomyoblastoma cell line) or mpkCCDc14 (Mouse Cortical Collecting Duct cell line). Some cells susceptible of ETX attack *in vivo* are not sensitive if isolated and grown *in vitro*; indeed, kidney cell lines from ETX-susceptible animal species like lamb and cattle are resistant to the toxin, suggesting that an ETX receptor in primary cells is lost in cultured cell lines [9]. However, the canine cells used in the present study remain ETX-sensitive in secondary cultures. The mechanism proposed for ETX cytotoxicity follows different steps. In the first step, the toxin binds a specific receptor localized in the plasma membrane. The toxin appears to be localized as a heptamer in detergent-resistant domains (DRM) isolated from MDCK cells, so that a receptor could be localized in cholesterol-rich domains [10]. The second step of cytotoxicity consists of the heptamerization of the toxin and formation of the pore. This step is prevented by depletion of cholesterol by methyl-β-cyclodextrin that inactivates the binding of ETX to plasma membrane and the pore formation [11]. Different

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morphological changes caused by ETX have been described in the literature, in the first phase of intoxication a marked swelling is observed, followed by vacuolization, mitochondria disappearance, blebbing and membrane disruption [12].

In the present work, we have studied and compared the effect of ETX in membrane models and cells, to improve our understanding of the role of proteins and lipids in the interaction of ETX with plasma membrane. ETX shows no effect on vesicles of pure lipid composition, but it partitions into the membrane of giant plasma membrane vesicles formed by blebbing of MDCK cells, suggesting that a protein receptor is involved in the cytolytic process.

2. Materials and methods

2.1. Materials

Egg sphingomyelin (SM), cholesterol (Ch), diacylglycerol-3-ethyl phosphocholine (ethyl PC), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC), phosphatidylinositol-4-phosphate (PiP), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) dipalmitoleoylphosphatidylcholine (DPolPC), dilinoleoylphosphatidylcholine (DLolPC), cardiolipin (heart, bovine) (CL) and the lipophilic fluorescent probe lissamine rhodamine phosphatidylethanolamine (Rho-PE) were supplied by Avanti Polar Lipids (Alabaster, AL, USA). Egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE) egg phosphatidylglycerol (PG) liver phosphatidylinositol (PI) and spinal cord phosphatidylserine (PS) were purchased from Lipid Products (South Nutfied, UK). 8-Aminonaphtalene-1,3,6-trisulfonic acid sodium salt (ANTS) and p-xylenebis(pyridinium) bromide (DPX) were supplied by Molecular Probes, Inc. (Eugene, OR). Trypsin-coated agarose beads, isopropyl beta-D-thiogalactopyranoside (IPTG) and propidium iodide were supplied by Sigma-Aldrich (Madrid, Spain). Hoechst 33342 was purchased from Life-Technologies (Carlsbad, CA, USA). A ganglioside natural mixture composed of GM1, GD1a, GD1Tb and GD1b (GS) was supplied by Bachem (Heidelberg, Germany). Glutathione Sepharose 4B was supplied by GE Healthcare (Little Chalfont, UK). Protease inhibitors were purchased from Hoffmann-La Roche (Basel, Switzerland).

2.2. Methods

2.2.1. Epsilon-toxin expression and purification

Epsilon-protoxin (EPTX) and epsilon-protoxin-GFP (EPTX-GFP) were produced and purified as recombinant fusion proteins consisting of GST-EPTX and GST-EPTX-GFP in a pGEX 4T1 expression vector. The plasmids were kindly provided by J. Blasi (Barcelona, Spain). The purification of the toxins was performed following a published protocol [13]. Briefly, either EPTX or EPTX-GFP were induced overnight with 0.4 mM isopropyl beta-D-thiogalactopyranoside (IPTG) at room temperature in 250 ml LB medium culture supplemented with ampicillin. Cells were pelleted and resuspended in cold PBS (supplemented with protease inhibitors), frozen in liquid nitrogen, thawed and sonicated. Then the homogenate was centrifuged at 15,000*g* for 20 min. The obtained supernatant was incubated with 0.75 ml glutathione Sepharose 4B for 2 h at 4 °C. After two washes with PBS, the recombinant proteins were eluted by thrombin cleavage in PBS containing 2.5 mM $CaCl_2$. When required, EPTX and EPTX-GFP were cut and activated incubating with trypsin-coated agarose beads for 1 h at room temperature.

2.2.2. Large unilamellar vesicle (LUV) preparation and permeabilization assay

Vesicle efflux was measured with the ANTS:DPX system [14] using a described method [15,16]. Briefly, the desired amounts of lipid stocks dissolved in chloroform:methanol solution (2:1 v/v) were mixed in the required proportions, and the solvent was evaporated to dryness under a stream of nitrogen. Traces of solvent were removed by leaving the samples under high vacuum for at least 2 h. The samples were hydrated in 12.5 mM ANTS, 45 mM DPX, 75 mM NaCl and 2.5 mM HEPES, pH 7.4. LUV (100 nm diameter) were prepared following the extrusion method [17]. The vesicles were extruded using Nuclepore filters (0.1 μm pore diameter), at a temperature above the transition temperature of the lipid mixtures, and the vesicle suspension was passed through a PD-10 desalting column to remove non-entrapped ANTS and DPX. A previously adjusted isosmotic buffer solution was used for this process (75 mM NaCl, 2.5 mM HEPES, pH 7.4), and the lipid concentration was measured in terms of lipid phosphorous [18]. ETX was added to 0.05 mM vesicles in a stirred quartz cuvette, and leakage was followed in terms of ANTS fluorescence in a Microbeam PTI spectrofluorimeter (Barcelona, Spain) using a cutoff filter (470 nm) placed between the sample and the emission monochromator and setting the ANTS emission at 520 nm and the excitation at 355 nm. The percentage leakage induced by the membrane-perturbing agent was calculated by the equation Leakage (%) $= (F_i - F_0) / (F_{max} - F_0) \times 100$, where F_0 is baseline leakage and corresponds to the fluorescence of the vesicles at time 0, F_i is the fluorescence after a certain period of incubation with the peptide and F_{max} is the maximum leakage that corresponds to the fluorescence value obtained after addition of Triton X-100.

2.2.3. Cell culture

Madin–Darby canine kidney epithelial cells (MDCK) were grown at 37 °C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin-glutamine.

2.2.4. Measurement of apoptotic and necrotic nuclei

Cell nuclei were stained with Hoechst 33342 or propidium iodide (PrI) to distinguish between apoptotic and necrotic nuclei by epifluorescence microscopy. This assay was performed as described [19]. Briefly, after treatment with ETX, MDCK cells were incubated 15 min with 1 μM Hoechst and 1.5 μM propidium iodide (PrI). The plate was next moved to an epifluorescence microscope to obtain images of stained nuclei. To differentiate apoptotic from normal and necrotic nuclei, the images were treated using Image J software (National Institutes of Health, Bethesda, MD, USA).

2.2.5. Flow cytometry

Cell viability was assayed using flow cytometry as described [20] with some modifications. MDCK cells were incubated with ETX at different concentrations. After toxin treatment cells were detached with 1× citric saline buffer (135 mM KCl, 15 mM sodium citrate, pH 7.3) or 0.25% trypsin-EDTA, centrifuged at 2,000*g* for 5 min and washed twice in PBS. Finally 0.5 μM propidium iodide was added, and cells were analyzed with a BD Facscalibur Cell Quest flow cytometer (BD, Franklin Lakes, NJ U.S.). The data were treated using the WinMDI 2.9 free software.

2.2.6. Lipid extraction from MDCK cells

MDCK cells were grown to confluency in 10,175 cc flasks. Cells were detached with a manual cell scraper and centrifuged at 1,500 rpm 5 min. The pellet was diluted with 10 volumes of hypo-osmotic lysis buffer (1.2 mM acetic acid, 4 mM $MgSO_4$, pH 3.2) and incubated for 15 min in ice with stirring. Lysed cells were centrifuged at 31,000*g* for 15 min at 4 °C and washed twice with the same buffer. Lipids were extracted adding 250 μl of 0.6 M perchloric acid to the same volume of pelleted cells and centrifuging at 14,000*g* for 15 min. The obtained pellet was resuspended in 2.5 ml cold chloroform/methanol (2:1, [v:v]) and incubated for 30 min at room temperature with stirring. In the next step, 5 ml of cold 0.1 N HCl was added, and after mixing, the sample was centrifuged at 1,700*g* for 20 min. Two phases were so obtained, an upper phase

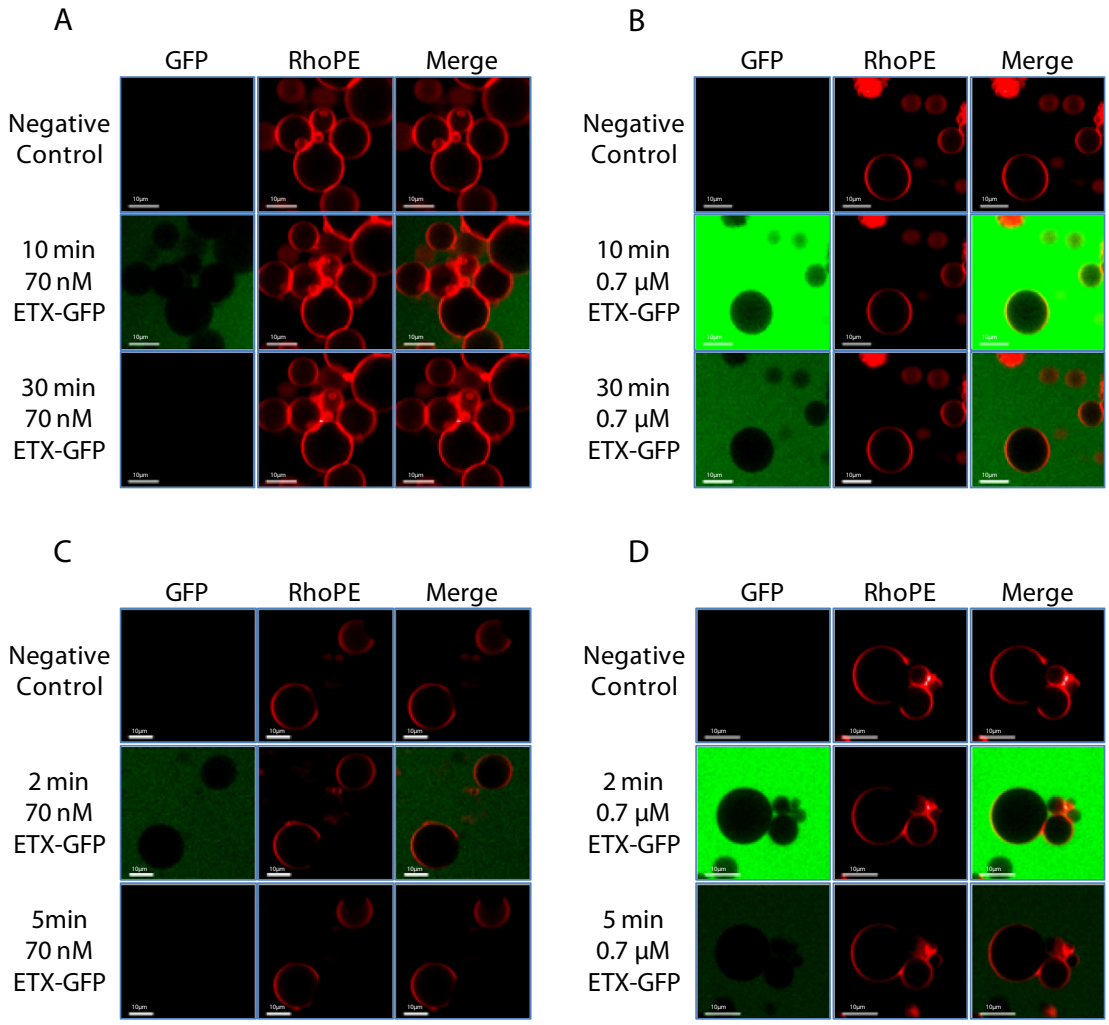

Fig. 1. Confocal images of GUV composed of (A,B) DOPC and (C,D) DOPC:SM:Ch 2:1:1, incubated with different concentrations of ETX-GFP. Bar: 10 µm.

("aqueous phase") that was discarded and a lower phase ("chloroformic phase") that was collected.

2.2.7. GUV preparation

Giant unilamellar vesicles (GUV) were prepared using a published method [21]. Briefly, pure lipid stocks were prepared by diluting commercially available lipids in powder into a chloroform/methanol (2:1 v/v) organic solution to a 0. 2 mM concentration with 0.3 mol% Rho-PE. From this solution, 5 µl was spread onto two platinum-wire electrodes attached to a specially designed polytetrafluoroethyl (PFTE)-made circular wells, and solvent traces were removed in a high vacuum desiccator for 2 h. Then the circular wells were fitted into specific holes within a specially designed home-made chamber. The platinum wires were attached in direct contact with a glass cover slide with epoxy glue. The chamber was equilibrated for 15 min to the desired temperature (above the T_m of all lipids) using an incorporated water bath. The Pt-wires were covered with a GUV formation buffer (2.5 mM HEPES, 75 mM NaCl, pH 7.4), and the chamber was connected to a generator through the platinum electrodes following an electric field cycle: 6 min 0.22 V, 20 min 1.9 V and 90 min 5.3 V. At the end of the cycle, the electric field and water were both disconnected, and vesicles were left to equilibrate for 30 min. Finally, the chamber was moved to an inverted confocal fluorescence microscope (Nikon D-ECLIPSE C1, Nikon Inc., Melville, NY, USA) to visualize the vesicles attached to the platinum wire. Image treatment and quantification were performed using the software EZ-C1 3.20 (Nikon, Tokyo, Japan).

2.2.8. Formation of giant plasma membrane vesicles (GPMV)

GPMV were formed by blebbing of MDCK cells following a described method [22] with same modifications. Briefly, MDCK cells were grown to confluency and washed twice with GPMV buffer (2 mM $CaCl_2$, 10 mM HEPES, 0.15 M NaCl, pH 7.4). Flasks were then incubated with 1.5 ml of freshly prepared GPMV reagent (25 mM formaldehyde and 2 mM DTT in GPMV buffer) for 1 h at 37 °C with stirring. After incubation, GPMV were gently decanted, centrifuged at 14,000 rpm in a minispin and washed twice with GPMV buffer. They were then resuspended in 500 µl GPMV buffer and added to a µ-slide 8-well chamber previously incubated with polylysine. Vesicles were let to lay down for at least 3 h, and 2.5 µM Rho-PE was added to stain GPMV membranes. After 15 min incubation, vesicles were visualized with an inverted confocal fluorescence microscope (Nikon D-ECLIPSE C1, Nikon Inc., Melville, NY, USA). Image treatment and quantification were performed using the software EZ-C1 3.20 (Nikon Inc.).

3. Results

3.1. ETX and pure lipids

The capacity of ETX to permeabilize lipid bilayers was tested with LUV loaded with ANTS-DPX. PC, SM, Ch, ethyl PC, POPC, DOPC, DPolPC, DLolPC, CL, PE, PG, PS, PI, PiP and GS were used either alone or in various combinations to assay vesicle leakage induced by ETX. The results were

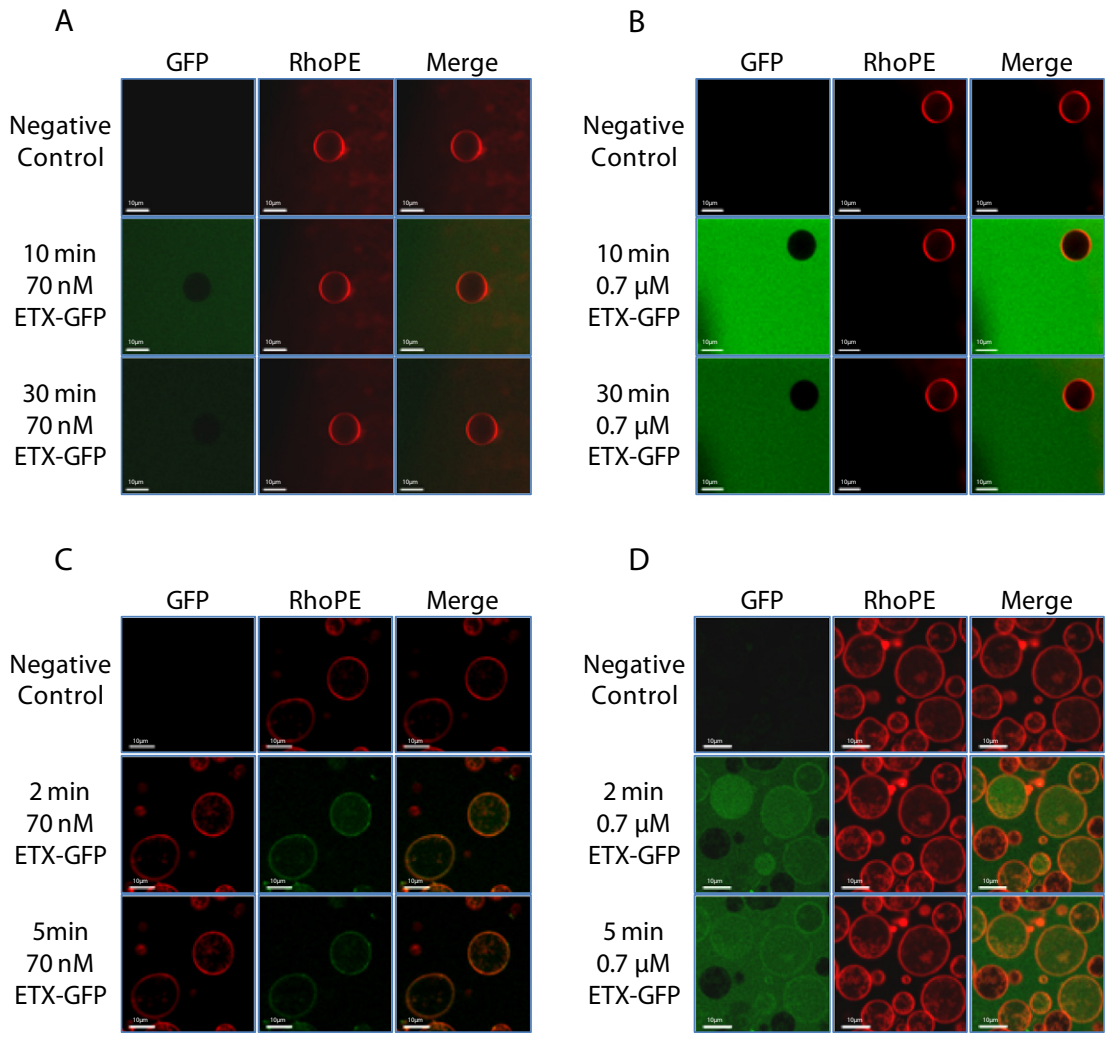

Fig. 2. Confocal images of (A,B) GUV composed of MDCK cells lipid extract and (C,D) GPMV formed by blebbing of MDCK cells, incubated with different concentrations of ETX-GFP. Bar: 10 μm.

essentially negative, with <5% leakage 30 min after toxin addition (results not shown). Only when the bilayers consisted of pure DOPC some release of aqueous contents (approx. 15% in 30 min) was detected.

3.2. Binding of ETX-GFP to GUV and GPMV membranes

Giant unilamellar vesicles (GUV) were formed to check the ability of ETX to interact with unilamellar lipidic vesicles visible with a confocal microscope, using Rho-PE to stain them and to differentiate liquid-ordered from disordered domains. To visualize the toxin, an ETX tagged with GFP was used (ETX-GFP). Since in the LUV studies only DOPC allowed some leakage, GUV of DOPC were formed. Moreover, to evaluate the influence of liquid-ordered domains on the activity of ETX, GUV composed of a ternary mixture of DOPC:SM:Ch (2:1:1 mol ratios) were also formed. This mixture was checked because of the results described in the literature that suggest localization of ETX in "raft" domains of MDCK [10]. No binding of ETX-GFP was detected to GUV of either pure DOPC or a DOPC:SM:Ch ternary mixture (Fig. 1).

To exclude a possible implication of some specific lipid, found in the toxin-sensitive cells, GUV composed of a lipid extract of MDCK cells were formed. Moreover, to evaluate the implication of a possible protein receptor GPMV obtained by blebbing of MDCK cells were formed. The basic difference between these two types of vesicles is probably in their protein content.

ETX-GFP did not bind membranes of GUV composed of the MDCK cells lipid extract (Fig.2A, B), confirming the inability of ETX-GFP to insert in bilayers composed of pure lipid. However, ETX-GFP did bind GPMV, i.e., giant vesicles consisting of MDCK plasma membranes obtained by blebbing. Moreover, in some vesicles the ETX-GFP appeared to have internalized through GPMV membrane (Fig 2C, D).

3.3. Treatment of MDCK cells with ETX-GFP

The plasma membrane of MDCK cells was stained with Rho-PE, to establish whether ETX-GFP co-localized with the cell membrane. The results shown in Fig. 3 indicate that ETX-GFP co-localized with plasma membrane at 70 nM already after 2 min incubation. Moreover, cells showed a vacuolization process after 5 min incubation and an extended necrosis after 15 min.

ETX-GFP caused MDCK permeabilization, allowing the entrance of PrI, confirming the necrotic effects checked for ETX by cell counting (Fig. 4). Also, the entrance of ETX-GFP was visible in both images after 10 min incubation.

3.3.1. Measurement of apoptotic and necrotic nuclei

The ability of ETX to cause cell death was checked in MDCK at different times and concentrations. This assay allows differentiating between apoptosis and necrosis using a morphologic approach. PrI is a nucleic acid stain used to count cells in multicolor fluorescent techniques.

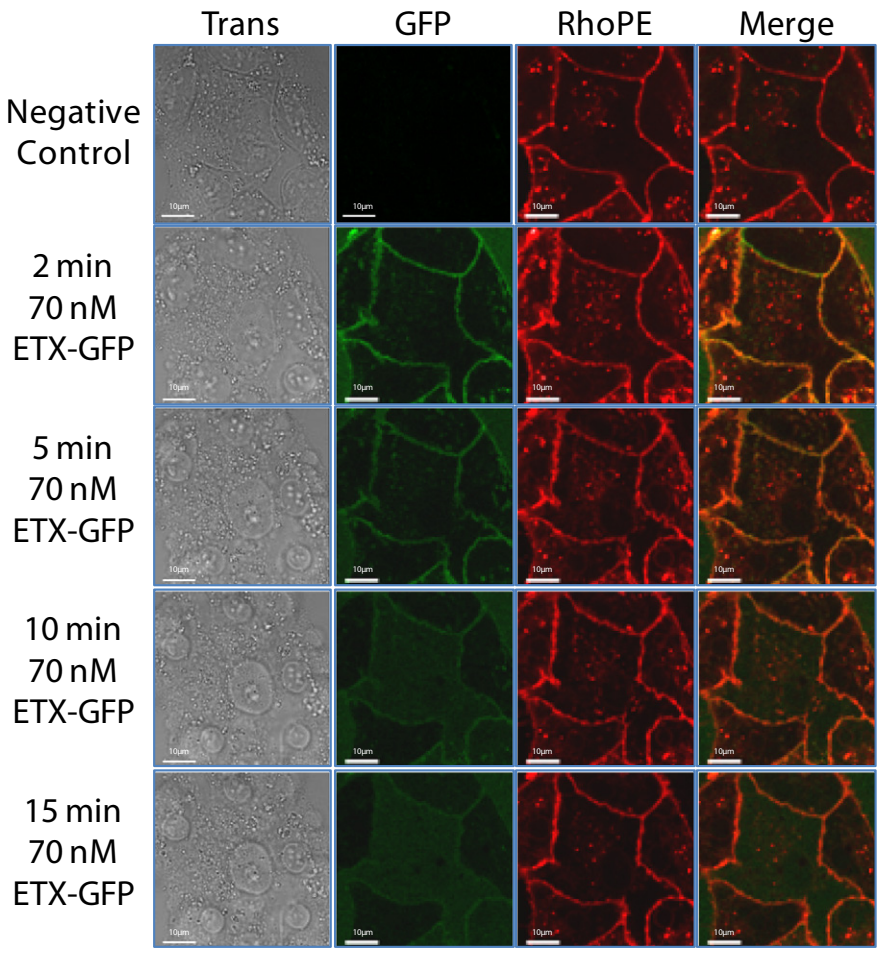

Fig. 3. Transmitted light and confocal images of MDCK cells treated with 70 nM ETX-GFP and stained with Rho-PE to stain plasma membrane.

Once the dye is bound to nucleic acids, its fluorescence is enhanced by 20- to 30-fold. Since PrI is membrane impermeant to viable cells, it is used to detect necrotic cells in culture [23]. However, cells in advanced states of apoptosis undergo morphological changes, especially in nuclei as chromatin condensation (pyknosis) and, at later stages, nuclei fragmentation (karyorrhexis). Pyknotic and fragmented nuclei were counted staining cell nuclei with Hoechst 33342 [24].

Fig. 5 shows that ETX has caused necrotic effects with time and dose-dependence. Moreover, no clear increase in apoptotic nuclei was detected with concentration or time. ETX has also shown the ability to kill MDCK cells at concentrations in the nanomolar order confirming it to be one of the most potent bacterial toxins described in the literature [3].

ETX does not exhibit any toxic activity in other cell lines checked, such as epithelial cells (HeLa human cervix adenocarcinoma cells) intestinal cells (CaCo2 colorectal adenocarcinoma cells), MEF (mouse embryonic fibroblasts) or GM95 (mouse melanoma cells).

3.3.2. Cell viability of MDCK cells treated with trypsin and citric buffer

Flow cytometry was performed to evaluate cell viability of MDCK cells detached with either citric buffer and trypsin. It is known that trypsin hydrolizes a certain number of cell surface proteins and plasma membrane receptors that allow cell adhesion. Indeed trypsin is commonly used to detach cells grown on flasks and plates [25]. This experiment allows us to evaluate the ability of ETX to kill cells treated with citric buffer and cells treated with trypsin, that have possibly been deprived of the putative cell receptor necessary for ETX cytotoxicity.

Flow cytometry results obtained with MDCK cells treated with citric buffer showed an increase of necrosis from ~25% to ~70% when treated with 8 nM ETX and no significant effect at 2 nM (Fig. 6). However, MDCK cells treated with trypsin did not show a significant increase in necrosis when treated with 2 or 8 nM ETX. Moreover, an increase of basal cell death in cells detached with citric buffer was observed (Fig. 6).

4. Discussion

GFP-tagged ETX had shown no insertion in GUV bilayers composed of DOPC or of the DOPC:SM:Ch (2:1:1) ternary mixture at the different concentrations checked; however, the toxin did localized on the MDCK cell surface and caused cytotoxicity. The most interesting conclusion of this work could be derived from the inability of the toxin to insert in GUV composed of a lipid extract from MDCK cells, and the co-localization in GPMV formed from MDCK cells plasma membranes (Fig. 2). The differences between these vesicles are perhaps only in protein content. GPMV have maintained membrane proteins and perhaps a putative receptor that could be necessary for binding of ETX to plasma membrane cells, toxin oligomerization and finally pore formation. These results are reinforced by the toxin co-localization with MDCK plasma membrane stained with Rho-PE in cells (Fig. 3). Also in support of the receptor theory is the higher cytotoxicity obtained for MDCK detached with citric buffer than with trypsin. For this experiment we have used two different approaches to detach cells. In the first case, we have used citric buffer, commonly used in flow cytometry protocols [26,27]. This buffer allows to detach cells without proteases and it ensures the

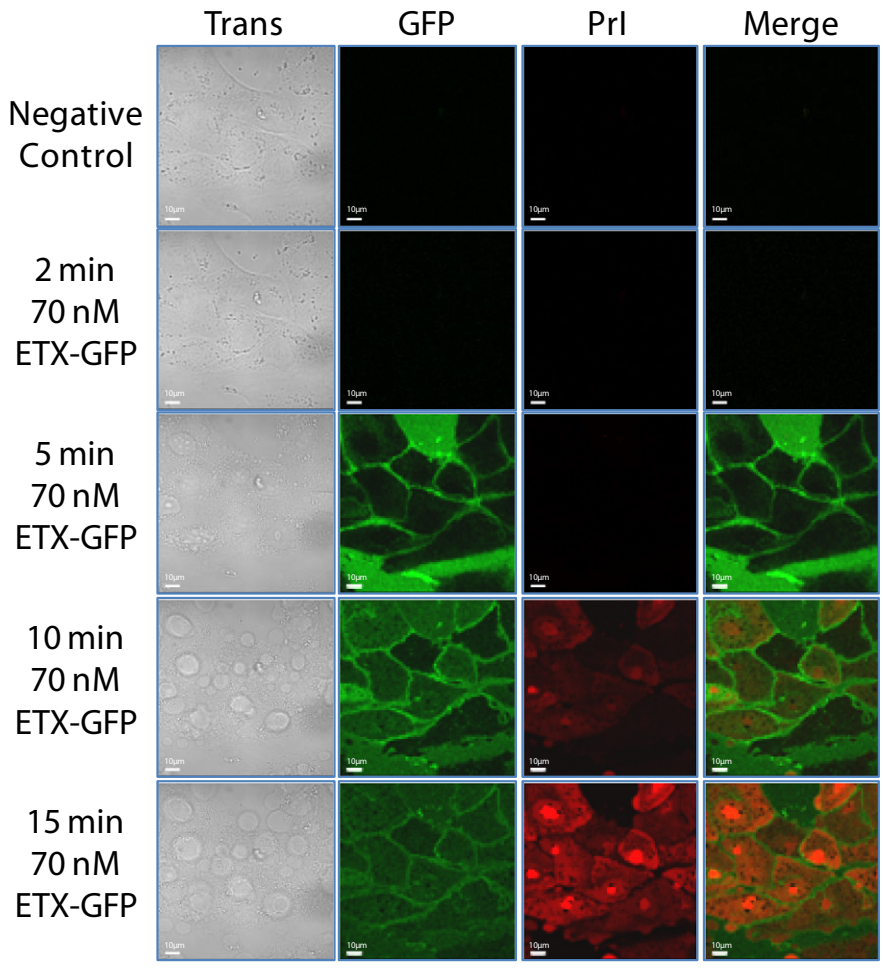

Fig. 4. Transmitted light and confocal images of MDCK cells treated with 70 nM ETX-GFP and stained with Propidium Iodide (PrI) to evaluate permeability and to mark necrotic cells. Bar: 10 μm.

maintenance of plasma membrane proteins. Trypsin is routinely used in cell biology to detach cells taking advantage of its ability to cleave the proteins binding the cultured cells to the dish [25]; moreover, it could also cleave proteins on the cell surface, including putative receptors [26]. In our results, the cell population detached with trypsin have shown no significant differences in cytotoxicity when treated with ETX at 2 and 8 nM with respect to the negative control. However, cells treated with citric buffer have shown an increase from ~25% to ~70% when treated with 8 nM ETX. The difference could be due to the presence of a cell receptor in citrate-treated cells that is cutoff by trypsin treatment. Note, however, that cells treated with citric buffer have shown a higher basal death, indicating a toxicity of this reagent to MDCK cells. These results are in agreement with the specificity of ETX for MDCK cells, previously described [28]. ETX has shown no activity in several cell lines different from MDCK cells, among them epithelial and intestinal cells that are known to be infected and killed by ETX in vivo [6] (data not shown).

Cell counting assays have allowed a general characterization of the cell death caused by ETX. The results show that the toxin caused a fast and extended necrosis at very low concentrations with no significant increase in apoptotic nuclei (pyknotic nuclei and karyorrhexis). ETX is known to be a pore-forming protein with structural similarity with aerolysin from *Aeromonas hydrophila*. Aerolysin induces leakage of 6-carboxyfluorescein (CF) in LUV [29]; however, in unpublished results, ETX has shown no activity in LUV not even in the presence of charged lipids, which are described in the literature to increase the permeabilization of vesicles after treatment with several other proteins [29–32]. Note in particular that aerolysin [29] can permeabilize pure lipid bilayers. Moreover, the presence of a liquid-ordered phase seems not to have any effect on ETX activity in vesicles, although the localization of ETX in "raft" domains has been described in the literature [10].

Also relevant in this contest is the increase in permeability for both GPMV and MDCK cells treated with ETX-GFP, allowing toxin internalization. In GPMV images, we can see an increase in green signal in the inner area of some vesicles (Fig. 2D). Moreover, using PrI (Fig. 4), we have seen that the internalization of this dye happened simultaneously with the internalization of ETX-GFP. These observations support the idea that the toxin enters through an unspecific increase in permeability rather than through a regulated transport system. This increase in permeability is preceded by a localization of ETX-GFP on the cell surface and could be a result of an advanced phase of cytotoxicity including formation of large pores.

The requirement of a receptor protein on the target cell surface for bacterial toxins to exert their lytic role is not exceptional. It has been found for *Escherichia coli* alpha-hemolysin [33], Vac A toxin from *Helicobacter pylori* [34] and *Staphylococcus aureus* pore-forming toxins [35], among others. The nature of the receptor is not always known. *E. coli* alpha-hemolysin binds glycophorin to lyse red blood cells [33], and *H. pylori* Vac A binds receptor tyrosine phosphatase beta leading to cytoplasmic vacuolization of gastric epithelial cells [34]. No structural or functional pattern arises from the few examples of bacterial toxin receptors known. In the case of the proposed ETX receptor, little can be said, beyond the fact that it appears to be a trypsin-sensitive protein. A possible candidate identified by Ivie et al. [36] is the hepatitis A virus cellular receptor 1 (HAVCR1), which could contribute to the

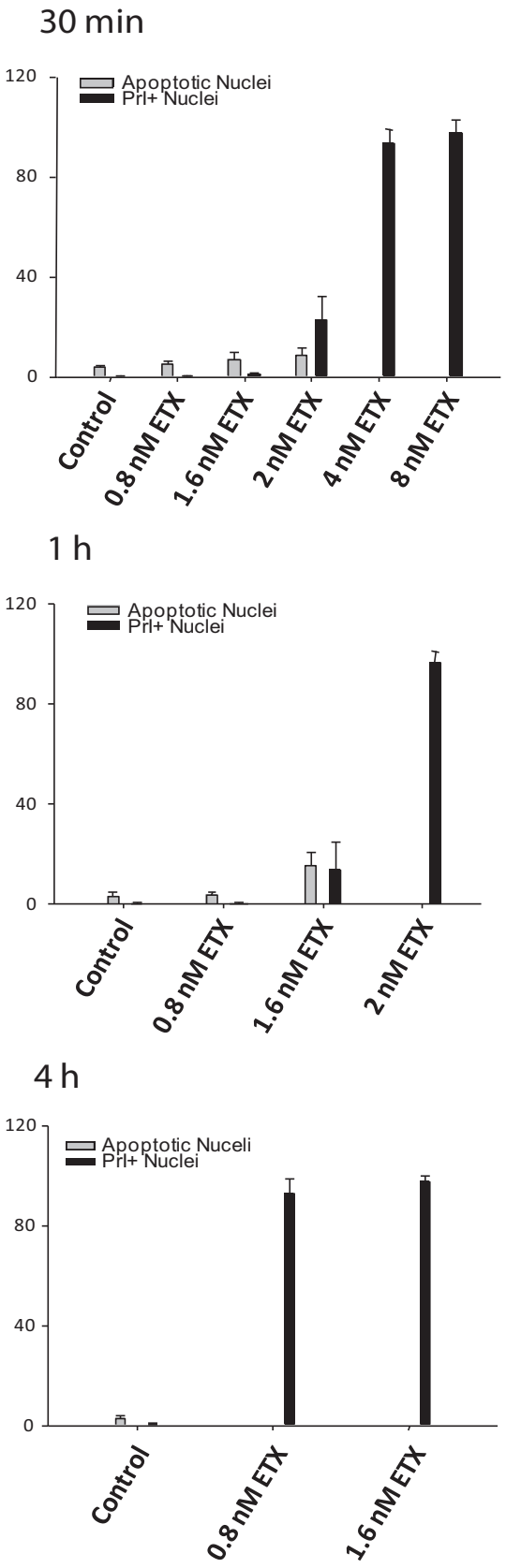

Fig. 5. Apoptotic and necrotic nuclei of MDCK cells treated with ETX (0.8 to 8 nM) after 30 min, 1 h and 4 h incubation.

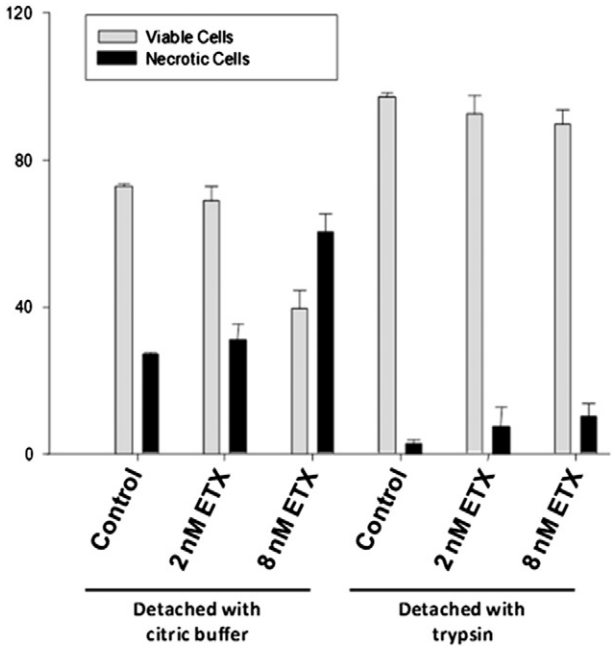

Fig. 6. Flow cytometry assay performed using propidium iodide (PrI) to stain necrotic cells was used to check the viability of MDCK cells treated with either citric buffer or trypsin.

cytotoxicity of the protein acting as a receptor or co-receptor in MDCK cells and the human kidney cell line ACHN.

In conclusion, the present study supports, with model membrane and cell biology studies, that ETX requires a cell receptor, probably a protein to bind plasma membrane and cause cell death. Moreover, the toxin causes necrosis accompanied by a vacuolization process. Also, ETX causes permeabilization in GMPV and MDCK cells.

Acknowledgments

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