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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