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Pasteurella multocida toxin: Targeting mast cell secretory granules during kiss-and-run secretion

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ABSTRACT

Pasteurella multocida toxin (PMT), a virulence factor of the pathogenic Gram-negative bacterium *P. multocida*, is a 146 kDa protein belonging to the A–B class of toxins. Once inside a target cell, the A domain deamidates the α-subunit of heterotrimeric G-proteins, thereby activating downstream signaling cascades. However, little is known about how PMT selects and enters its cellular targets. We therefore studied PMT binding and uptake in porcine cultured intestinal mucosal explants to identify susceptible cells in the epithelium and underlying lamina propria. In comparison with *Vibrio cholera* B-subunit, a well-known enterotoxin taken up by receptor-mediated endocytosis, PMT binding to the epithelial brush border was scarce, and no uptake into enterocytes was detected by 2 h, implying that none of the glycolipids in the brush border are a functional receptor for PMT. However, in the lamina propria, PMT distinctly accumulated in the secretory granules of mast cells. This also occurred at 4 °C, ruling out endocytosis, but suggestive of uptake *via* pores that connect the granules to the cell surface. Mast cell granules are known to secrete their contents by a "kiss-and-run" mechanism, and we propose that PMT may exploit this secretory mechanism to gain entry into this particular cell type.

1. Introduction

Pasteurella multocida (PM), first shown by Louis Pasteur to be the causative agent of fowl cholera, is a Gram-negative bacterium that has since been shown to cause a number of different diseases in a wide range of species, including humans, and for the most part, the pathogenic mechanisms involved are poorly understood or unknown (Harper et al., 2006; Wilkie et al., 2012). The virulence factors include a capsule, lipopolysaccharides, and the *P. multocida* toxin (PMT). The latter is characterized as a mitogenic dermonecrotic toxin and known to cause atrophic rhinitis in pigs, a syndrome including facial distortion, turbinate bone destruction and retarded growth (Wilkie et al., 2012; Orth and Aktories, 2012; Horiguchi, 2012).

PMT is a 1285 amino acids long, 146-kDa single-chain multifunctional toxin harboring two domains: a biologically active A domain in the C-terminal part, and a B domain for receptor-binding and membrane translocation in the N-terminal end. Elucidation of the crystal structure of the C-terminal has revealed three domains, C1, C2 and C3, of which the latter harbors a catalytic deamidase activity, acting on the α-subunit of heterotrimeric G proteins and thereby activating downstream signaling cascades (Aktories and Barbieri, 2005; Hoffmann and Schmidt, 2004; Orth and Aktories, 2012; Wilson and Ho, 2012). In contrast, little is known regarding the receptor-binding and translocation domains, the precise boundaries of which have yet to be defined, and moreover, identification of cellular receptors for PMT has been elusive (Orth and Aktories, 2012; Wilson and Ho, 2012). Whereas earlier reports suggested gangliosides GM_1, GM_2, and GM_3 (Dudet et al., 1996; Pettit et al., 1993), a more recent study has implicated other membrane lipids, such as sphingomyelin, phosphatidylcholine and possibly a protein co-receptor, in PMT-binding to the surface of cells (Brothers et al., 2011).

In the present work, we studied the binding and uptake of PMT in organ cultured porcine intestinal mucosal explants, a model system previously used in our studies of other toxins, such as *Vibrio cholera* toxin B-subunit (CTB) (Hansen et al., 2005), *Staphylococcus aureus* A- and B enterotoxins (Danielsen et al., 2013), and okadaic acid (Danielsen et al., 2014). In comparison with CTB, studied in parallel incubations, PMT bound very poorly to the epithelial surface, and no detectable toxin was taken up by the enterocytes despite the high content of glycolipids in the brush border membrane. In contrast, PMT targeted a distinct population of granular cells in the

Abbreviations: PMT, *Pasteurella multocida* toxin; CTB, *V. cholera* toxin B-subunit; SDS/PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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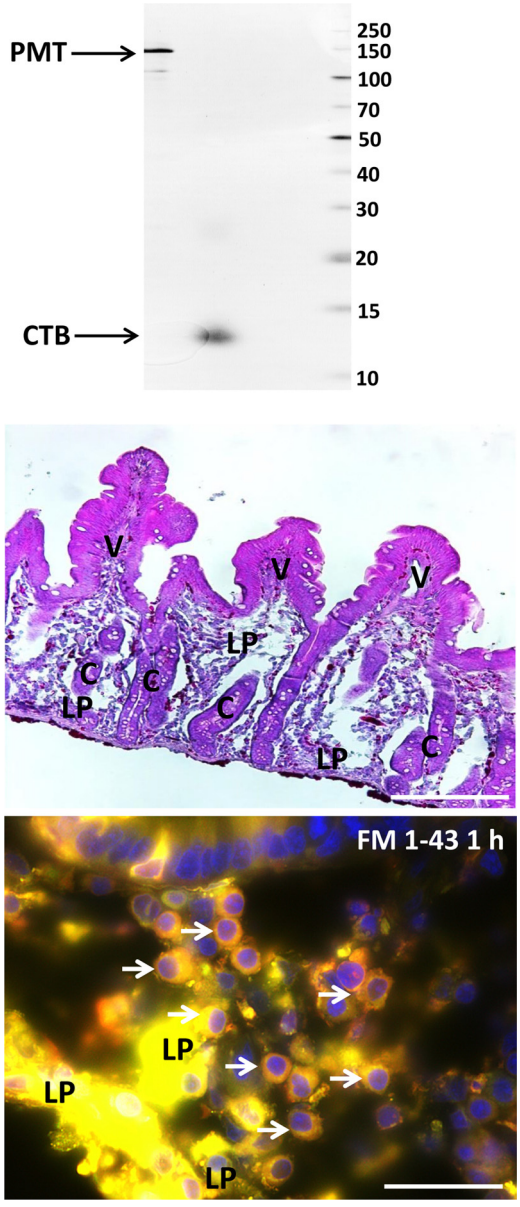

Fig. 1. PMT, CTB, and mucosal explants. Top image: SDS/PAGE of ~10 μg of PMT and CTB. Both toxin preparations showed polypeptides of the expected size (146 kDa and 12 kDa, respectively) with little or no detectable impurities. (A cocktail of molecular weight markers are shown in the right lane.) Center image: Hematoxylin–eosin staining of a section of an intestinal mucosal explant. The explants are cultured villus-side upwards (V), with crypts (C) and lamina propria (LP) freely exposed to the medium. Bar: 200 μm. Bottom image: The lamina propria of a mucosal explant cultured for 1 h in the presence of the lipophilic dye FM 1–43 as described in Section 2. The dye stains the tissue intensively, and all cells are labeled. Bar: 20 μm.

lamina propria, identified as mast cells by their granular content of tryptase. Similarly to Alexa hydrazide, a small fluorescent polar tracer, PMT was taken up into the mast cell granules also at 4 °C, ruling out endocytosis. Instead, PMT may enter *via* a pore, transiently connecting the secretory granule with the cell membrane during its "kiss-and-run" cycle of secretion.

2. Materials and methods

2.1. Materials

Pasteurella multocida toxin (PMT) was obtained as a lyophilized solid from Calbiochem Merck Millipore (www.merckmillipore.com), Alexa 488/594-conjugated *V. cholera* toxin B subunit (CTB), Alexa 488 hydrazide, Alexa 488 protein labeling kit, a fixable form of FM 1–43 (N-(3-triethylammoniumpropyl)-4-(4-(dibutylamino)styryl)pyridinium dibromide), lysotracker, Alexa 488/594-conjugated secondary antibodies, YO-PRO-1 and propidium iodide (PI) membrane permeability/dead cell apoptosis kit, and ProLong antifade reagent with DAPI from Molecular Probes/Life Technologies (www.lifetechnologies.com), a rabbit antibody to tryptase from Biorbyt (www.biorbyt.com), a rabbit antibody to Major Basic Protein (MBP) from Abcam (www.abcam.com), and PageRuler™ Broad Range Unstained Protein Ladder from Thermo Scientific (www.thermoscientific.com).

2.2. Animals

All animal experimentation in Denmark is subject to ethical evaluation by the Ministry of Justice's Council for Animal Experimentation. The present work was performed under license 2012-15-2934-00077.

Segments of jejunum, taken about 2 m from the pylorus of overnight-fasted, post-weaned pigs, were surgically removed from the anaesthetized animals by licensed staff at the Department of Experimental Medicine, the Panum Institute, University of Copenhagen. After obtaining the intestinal segments, the animals were sacrificed by an injection with pentobarbital/lidocaine (1 mg/kg bodyweight).

2.3. Organ culture of mucosal explants

Jejunal segments of approximately 20 cm in length were quickly removed from the animals and cooled in ice-cold RPMI medium. Within 10 min, mucosal explants (~0.1 g) were excised with a scalpel and cultured mucosal side upwards on stainless grids placed in organ culture dishes in 1 ml of RPMI medium at 37 °C or 4 °C for periods of 15 min to 2 h, essentially as described previously (Danielsen et al., 1982). When present, Alexa 488-conjugated PMT and Alexa 488/595-conjugated CTB were added to a final concentration of 5 μg/ml. FM 1–43 was used at a concentration of 10 μg/ml, Alexa 488 hydrazide at 20 μg/ml, and lysotracker at 10 μM.

Apoptotic cells were visualized by incubation of mucosal explants on ice for 30 min in the presence of YO-PRO-1 (5 μl/ml) and propidium iodide (5 μl/ml). After culture, the explants were quickly rinsed in fresh medium and immersed in fixative at 4 °C.

2.4. SDS/PAGE

SDS/PAGE in 15% gels was performed essentially as described by Laemmli (Laemmli, 1970). After electrotransfer onto Immobilon membranes, protein was visualized by staining with Coomassie brilliant blue.

2.5. Preparation of Alexa 488-conjugated PMT

Alexa 488-conjugated PMT was prepared for use in fluorescence microscopy. The conjugation was performed using a commercial conjugation kit essentially according to the protocol supplied by the manufacturer. Briefly, 50 μg of lyophilized PMT was dissolved in 100 μl H_2O. 11 μl 0.1 M $NaHCO_3$, pH 9.0, was added, and the solution transferred to a reaction tube with Alexa 488 reactive dye. After incubation for 1 h at room temperature with magnetic stirring in the dark, unconjugated dye was removed by extensive dialysis against PBS. The Alexa 488-conjugated PMT was aliquotted and stored in the dark at −20 °C until use.