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Receptor-Mediated Entry of *Pseudomonas* Toxin: Methylamine Blocks Clustering Step

R. E. MORRIS,* M. D. MANHART, AND C. B. SAELINGER

University of Cincinnati College of Medicine, Department of Microbiology and Molecular Genetics, Cincinnati, Ohio 45267

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Clustering of ligands into coated regions of the plasma membrane is an early step in receptor-mediated endocytosis. The association of *Pseudomonas* exotoxin A (PE) with mouse LM fibroblasts was visualized by using biotinyl-PE and avidin-gold. Movement of PE into coated regions occurred within 30 s of warming monolayers to 37°C. This clustering was stopped by the primary amines methylamine and ammonium chloride but was not altered by the tertiary amine chloroquine. Toxin internalization was rapid, with a half-time of approximately 5 min. Although primary amines stopped clustering, they did not alter the rate of toxin internalization; they did alter the route followed after entry. We have shown previously that methylamine protects cells from the lethal action of PE. Here we suggest that methylamine protects, at least in part, by blocking clustering, and that receptor-mediated endocytosis is required for efficient expression of PE toxicity.

Receptor-mediated endocytosis (RME) is a process whereby ligands, including polypeptide hormones, serum proteins, and some viruses bound to specific receptors, are internalized via clathrin-coated pits on the plasma membrane (9, 11, 13, 23). We have previously presented evidence suggesting that *Pseudomonas* exotoxin A (PE) enters mouse LM fibroblasts by RME (6, 7). Here we demonstrate that clustering of receptor-bound PE occurs within 30 s of warming monolayers to 37°C. In addition, we provide ultrastructural evidence that the primary amines methylamine and ammonium chloride, but not the tertiary amine chloroquine or the ionophore monensin, inhibit the clustering event and thereby prevent expression of PE toxicity.

MATERIALS AND METHODS

Toxin. PE was purified by the method of Leppla (10) with minor modifications; purified toxin moved as a single major band with one minor component in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Toxin which was iodinated by the method of Bolton and Hunter (2) had a specific activity of 2×10^6 to 6×10^6 cpm/μg of protein and retained 80 to 95% of its biological activity. Biotinyl-PE was prepared by the method of Bayer et al. (1) with biotin *N*-hydroxysuccinimide ester (Calbiochem-Behring, La Jolla, Calif.). The molar ratio of activated biotin to PE was 5:1. When tested in an LM cell system for inhibition of protein synthesis (6), biotinyl-PE retained 90% of its biological activity.

Electron microscopy. Egg white avidin (E-Y Labs, San Mateo, Calif.) was succinylated and adsorbed onto the surface of 5.2 nm gold as described by Horisberger (8). The number of toxin-binding sites on the LM cell surface as determined with the 5.2-nm gold sols preparation was similar to that determined previously (6, 7) with horse spleen ferritin as the electron-dense marker and with an immunological bridging technique.

Mouse LM cell fibroblasts (ATCC CCL 1.2 LM, a derivative of L929 cells) were maintained as monolayers in McCoy 5A medium (GIBCO Laboratories, Grand Island, N.Y.) containing 5% heat-inactivated fetal calf serum (Reheis, Kankakee, Ill.), penicillin (200 U/ml), and streptomycin (0.2 mg/ml). For experimental use, cells were seeded in Leighton tubes (5×10^5 cells per tube) and grown overnight to approximately 80% confluency. Before use, monolayers were washed extensively and then incubated at 37°C for 90 min in Hanks balanced salt solution (HBSS) to exhaust exogenous biotin. Monolayers were cooled to 4°C and incubated in the cold with biotinyl-PE (100 ng/ml) for 30 min. After three washes with HBSS, avidin-gold (5.2 nm) was added (30 min, 4°C), and the cells were washed three times with cold HBSS and fixed immediately or incubated at 37°C in 3 ml of warm HBSS. At indicated times, monolayers were washed with cold phosphate-buffered saline (pH 7.4) and fixed with cold 0.5% glutaraldehyde in phosphate-buffered saline. Monolayers were fixed, dehydrated, and embedded as previously described (6, 7). All experiments were performed at least three times.

A minimum of 10 sections per sample were scored stained. The biotinyl-PE–avidin-gold complexes will be designated PE-gold. Each siting was scored as to its location (surface, periphery, or intracytoplasmic), number of gold grains, and place of residence (noncoated surface, coated pit, coated vesicle, or noncoated intracellular vesicle). A distinction between surface and periphery was made because gold seen in the

periphery, i.e., the area circumscribed by 500 nm within the cytoplasm, may reside on the cell surface but appear to be intracellular because of the plane of sectioning. If the siting was intracytoplasmic, the size, electron density, and (if known) type of organelle were recorded. Sections for photography were stained sequentially with uranyl acetate and Reynolds lead citrate.

RESULTS

In a previous report (6), we noted that ferritin-tagged PE, which was initially diffusely distributed on the plasma membrane, moved to coated regions with warming, but no attempt was made to quantitate this phenomenon. The use of PE-gold allowed us to reexamine this observation with more thorough quantitation and to monitor the effects of amines on toxin clustering and internalization. Initial experiments in which biotinyl-PE was incubated with cells in the cold in the presence or absence of 200-fold excess native PE established that binding was specific (data not shown).

The early stages in the interactions of LM cells and toxin were followed on the ultrastructural level; the kinetics of internalization from a representative experiment are shown in Fig. 1. At 4°C, PE-gold was randomly distributed on the LM cell surface with only 2 to 5% of the total sitings in coated regions (based on five experiments). Immediately upon warming to 37°C, PE-gold moved to coated regions, with maximum clustering occurring within 30 s; at this time, between 18 and 24% of surface-bound toxin (five separate experiments) was in coated regions (Fig. 2A and B). Within 5 min of the warming of the monolayers to 37°C, 50% of the PE-gold was located intracellularly in small or intermediate-sized vesicles usually containing 5 to 10 gold grains. Morphological observations were corroborated by following the rapid shift of ^{125}I-labeled PE from a trypsin-releasable (surface-bound) to a trypsin-resistant (intracellular) pool (Fig. 3). Within 5 min at 37°C, 60% of the cell-associated ^{125}I-labeled PE was no longer susceptible to release by trypsin treatment, suggesting that toxin was rapidly interiorized. From these data, a half-time of internalization of approximately 5 min was determined. Between 10 to 15 min after warming, more than 50% of the total PE-gold was located in the vicinity of the Golgi cisternae in vesicles which appeared devoid of clathrin and typically contained 10 to 30 gold grains per vesicle (Fig. 2C). The increase in gold grains per vesicle as a function of time is indicative of toxin concentration within the cells. Within 30 min at 37°C, the majority of the PE-gold was located in larger vesicles that usually contained amorphous material (Fig. 2D). This is consistent with the morphology of secondary lysosomes (4).

Methylamine stops clustering. After the kinetics of toxin clustering and internalization were established, we evaluated the effect of several compounds on these processes. Methylamine, ammonium chloride, and chloroquine are lysosomotropic agents which raise intracellular pH and interfere with receptor recycling (14, 18, 19). The carboxylic ionophore monensin inhibits the secretion of several secretory proteins and the transport of membrane glycoproteins (17) but does not alter the clustering of cell surface receptors. In our studies, the lysosomotropic agents and the ionophore protected LM cells from the action of PE (6). Data presented here show that methylamine and ammonium chloride prevent or significantly reduce movement of receptor-bound PE into coated regions (Fig. 4). Normal clustering, however, proceeds in the presence of chloroquine or monensin. Even though the initial movement of toxin to coated regions was blocked by the primary amines, the total internalization (measured morphologically) was not altered; at 10 min, 38, 43, and 48% of total PE-gold sitings were intracellular in the presence of HBSS, methylamine, and chloroquine, respectively. In addition, in preliminary

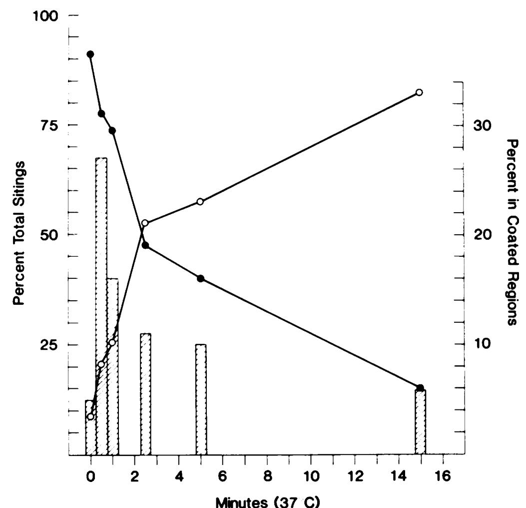

FIG. 1. Rapid clustering and internalization of PE. Precooled monolayers were incubated with biotinyl-PE (100 ng/ml) for 30 min at 4°C, washed with cold HBSS, incubated with avidin-gold for 30 min at 4°C, washed again with cold HBSS, and incubated at 37°C. At the indicated times, monolayers were washed with phosphate-buffered saline, fixed with 0.5% glutaraldehyde, and processed for electron microscopy. Symbols: ●, plasma membrane-associated sitings; ○, peripheral plus intracytoplasmic sitings (both expressed as percentages of total sitings); ▨, sitings in coated pits or vesicles (expressed as percentage of surface and peripheral sitings).

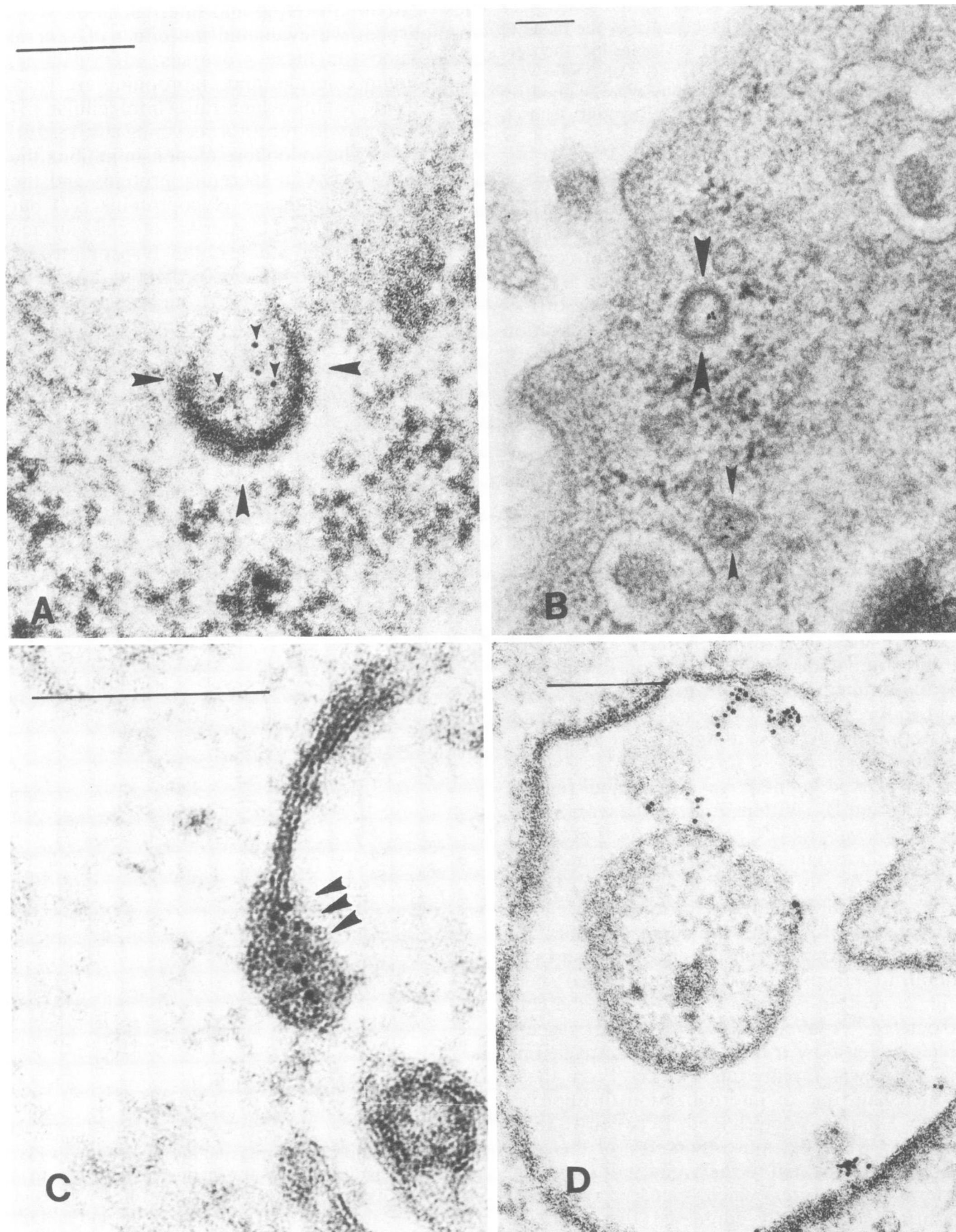

FIG. 2. Internalization of PE-gold after cold incubation (4°C) and warming to 37°C for 30 s (A), 1 min (B), 10 min (C), and 30 min (D). After 30 s (A), note the occurrence of PE-gold (small arrows) within a clathrin-coated vesicle (large arrows). After 1 min (B), note PE-gold in a coated vesicle (large arrows) and in noncoated vesicle (endosome, small arrows). After 10 min (C), PE-gold is in the Golgi region (arrows). After 30 min (D), the preponderance of PE-gold is seen within large cytoplasmic vesicles containing amorphous material; these vesicles are morphologically identified as lysosomes. Bar, 100 nm.

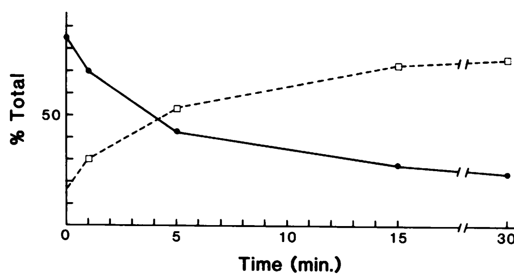

FIG. 3. Internalization of PE. LM cell monolayers were incubated with ^{125}I-labeled PE (500 ng/ml; specific activity, 9.2 × 10^6 cpm/μg of PE). At the indicated times, monolayers were rapidly cooled, washed with cold HBSS, and incubated 15 min at 4°C with 1 ml of 2.5% trypsin. The detached cells were transferred to 1.5-ml polypropylene centrifuge tubes and pelleted in a microfuge. Pellets were washed twice in McCoy medium containing 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid) and 2% fetal calf serum, and the radioactivity in the supernatants and pellets was counted. Symbols: ●, trypsin sensitive; □, trypsin resistant.

experiments the rates of internalization of ^{125}I-labeled PE by LM cells were similar in the presence and absence of methylamine.

Although overall internalization was similar, the routes of toxin entry were different in the presence of amines and in control cells. As reported by Ohkuma and Poole (12), methylamine induces the formation of very large electron-transluscent vacuoles. In the presence of methylamine, the majority of the PE-gold (88% of sitings) seen within LM cells after 10 min of warming was in these large vacuoles, and only rarely was toxin seen in vesicles adjacent to the Golgi region. In the presence of ammonium chloride, PE-gold was distributed in large electron-translucent vesicles (39%), small electron-dense vesicles (31%), lysosome-like vesicles (18%), and in unidentifiable vesicles (12%). A similar distribution of PE-gold within the cell occurred with the tertiary amine. In the presence of monensin, intracytoplasmic PE-gold was equally distributed in small and large endocytic vesicles after 10 min.

DISCUSSION

Eucaryotic cells possess two basic mechanisms for internalizing proteins from the external environment. One is a nonspecific endocytic mechanism not involving receptor-mediated binding and is termed fluid phase endocytosis. Uptake of substances by fluid phase endocytosis is time and concentration dependent, and internalized proteins appear to be delivered in vesicles directly to the lysosomal apparatus. The second mechanism for protein accumulation by cells is RME; this process necessitates specific binding of a protein ligand to the cell surface and is saturable, subject to competitive inhibition, and reversible (3). Recent evidence from several laboratories suggests that ligands internalized by RME are not delivered directly to lysosomes but rather move through a series of organelles en route to lysosomes. After binding to membrane receptors, ligands such as epidermal growth factor, low-density lipoprotein, alpha-2 macroglobulin, and β-galactosidase are internalized in specialized clathrin-coated pits, are transferred to endocytic vesicles (endosomes), and migrate to the *trans*-region of the Golgi complex and ultimately to lysosomes (21, 23, 24).

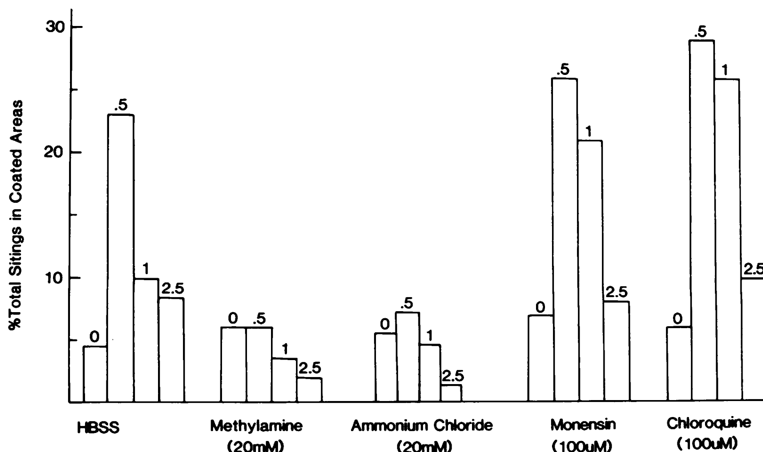

FIG. 4. Inhibition of clustering of PE into coated areas of the membrane. Monolayers were incubated with biotinyl-PE and avidin-gold as described in the legend to Fig. 1. Agents were present from 15 min before cooling to the end of the experimentation period. All agents were dissolved in HBSS except monensin, which was dissolved in ethanol and diluted in HBSS. Time of incubation at 37°C (in minutes) is shown above each bar.

As shown in this paper, PE enters cells in membrane-bound vesicles from which it must escape to interact with its cytoplasmic target, elongation factor 2. We envision two mechanisms by which this may occur. First, after entry of PE into endosomes or lysosomes, it may encounter a low-pH environment (14, 20). The acid pH may facilitate the insertion of the toxin molecule into the membrane of the organelle, resulting in the translocation of the intact toxin or enzymatic fragment. Such a mechanism has been proposed for another bacterial toxin, diphtheria toxin (5, 16), and several enveloped viruses (11, 22). Although the lysosome represents an ideal organelle for such an event, data from Sandvig and Olsnes (16) suggest that diphtheria toxin in lysosomes is very rapidly degraded by proteolysis and is not expressed. This observation suggests that toxin might enter the cytoplasm from an endosome or from a vesicle in the Golgi region. Nonlysosomal acidic compartments having a central role in RME have been demonstrated (15, 20) and could represent the site where active toxin enters the cytoplasm. Alternatively, if the movement of the toxin or toxin-receptor complex from one organelle to another occurs by organelle-organelle collision resulting in membrane fusion, it is possible that a few toxin molecules might escape during membrane coalescence. That organelle fusion occurs is supported by our observations that there is a direct correlation between the time after toxin internalization and the number of gold grains viewed per organelle, i.e., 1 to 5 grains after 1 min increasing to 30 to 50 grains after 30 min.

From the ultrastructural evidence presented here, we conclude that toxin rapidly enters LM cells through coated regions of the membrane after binding to specific PE receptors. It moves in endocytic vesicles to the Golgi region and then to lysosomes. A similar shuttling of epidermal growth factor from plasma membrane to the Golgi region has been described (24). Methylamine and ammonium chloride, but not chloroquine, prevent the clustering of toxin into coated regions. Despite the inhibition of clustering, toxin was internalized at a comparable rate but by a different route. Based on the morphological data, it is suggested that toxin internalization in the presence of methylamine is due to nonreceptor-mediated pinocytosis and not to RME, as normally occurs. Since the continued presence of primary amines prevents inhibition of protein synthesis by PE in toxin-sensitive mammalian cells, our data suggest that toxin internalized by generalized nonconcentrative pinocytosis is not expressed because it is delivered to lysosomes and degraded. We postulate that the alternate route of toxin traffic through the Golgi region is the normal pathway for expression of toxicity. Since we show in this report that methylamine blocks RME but not nonadsorptive pinocytosis, we suggest that PE delivered directly to the lysosomal apparatus is not toxic and that RME is obligatory for the expression of toxicity.

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