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Essential Role of Calcium in Cellular Internalization of *Pseudomonas* Toxin

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Pseudomonas exotoxin (PE) has been shown previously to enter mouse LM cells by receptor-mediated endocytosis, to block protein synthesis, and to cause cell death. The requirement for the divalent cation calcium in the binding and internalization of PE was examined. Biochemical studies showed that depletion of extracellular calcium with ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*'-tetra-acetic acid protected cells from the action of PE when the chelator was present during the internalization step. Extracellular calcium was not required for binding. We observed with immunoelectron microscopy that, in the cold, toxin bound to cell surfaces equally well in the presence or absence of chelator. In the presence of chelator, toxin was not cleared from the cell surface when cells were warmed to 37°C. Replenishment of calcium (2 mM $CaCl_2$), however, allowed normal rapid clearance of PE to occur. We suggest that internalization, but not binding, of PE by LM cells requires extracellular calcium.

Several protein toxins of both bacterial and eucaryotic origins exert their cytotoxic effects in the cytoplasm of susceptible mammalian cells. The manner by which the active toxin or biologically active subunits reach their targets remains unexplained. *Pseudomonas* exotoxin A (PE) is an extremely potent toxin, able to inhibit protein synthesis in sensitive cells when added in nanogram amounts; as such, it constitutes a useful probe to study cellular functions such as protein entry and processing.

Receptor-mediated endocytosis (RME) is a process by which cells bind and internalize important extracellular ligands, including certain hormones and serum proteins (6). During RME, there is evidence that ligands bind to receptors on the cell surface, move laterally into clathrin-coated regions, and from there are interiorized in endocytic vesicles. Recently, Davies and coworkers (4, 11) reported that the first step in internalization of α-2-macroglobulin, i.e., clustering of ligand-receptor complex into coated pit areas, requires extracellular calcium and can be inhibited by primary alkylamines. They also present evidence (4) that the calcium-dependent protein cross-linking enzyme transglutaminase is involved in internalization of ligands by RME. Inhibitors such as dansylcadaverine, bacitracin, and tolbutamide block both transglutaminase activity and clustering and internalization of α-2-macroglobulin. Thus, they hypothesize that protein cross-linking is required for RME.

The pathway of PE internalization from cell surface to cytoplasm has not been elucidated. We have reported previously (5) that *Pseudomonas* toxin enters cells by RME. PE appears to bind specifically to mouse fibroblasts and is rapidly cleared from the cell surface by internalization. Little or no toxin remains on the cell surface 30 min after binding, and uptake of toxin is coupled to receptor clustering over coated pit regions of the membrane (5). Alkylamines do not affect toxin binding but do inhibit toxin internalization and effectively neutralize expression of toxicity (5). Here we present evidence that extracellular calcium is required for expression of PE activity on mouse LM cells and that depletion of extracellular calcium prevents internalization of toxin but does not alter toxin binding to the cell surface.

MATERIALS AND METHODS

Toxin and antitoxin. *Pseudomonas* toxin was prepared by the method of Leppla (8). The toxin produces a single major band and one trace band on sodium dodecyl sulfate-acrylamide gel electrophoresis; the mean lethal dose for mice is 0.25 μg given intraperitoneally. Antitoxin was raised in rabbits against a purified toxin preparation, and the immunoglobulin G (IgG) fraction was obtained.

Cells. Mouse LM cell fibroblasts (ATCC CCL 1.2 LM, a derivative of L-929 cells) were maintained as monolayers in McCoy 5A medium (GIBCO Laboratories, Grand Island, N.Y.) containing 5% fetal calf serum (Rheheis, Kankakee, Ill.), streptomycin (0.2 mg/ml), and penicillin (200 U/ml). McCoy 5A medium includes 9×10^{-4} M calcium. For experimental purposes, cells were seeded at a concentration of 5×10^5 cells per ml and grown overnight to 70 to 80% confluency before use.

Protein synthesis studies. Inhibition of protein syn-

thesis was used as the index of toxin activity. In brief, LM cell monolayers in 24-well culture dishes were incubated with toxin (100 ng/ml) at 4°C for 30 min, washed with tissue culture medium to remove unbound toxin, and reincubated at 37°C for 5 h to allow the expression of toxicity. This amount of toxin under these conditions routinely gives 75 to 85% reduction of protein synthesis. Protein synthesis was measured by determining [^{3}H]leucine incorporation into trichloroacetic acid-precipitable material during a 30-min pulse period as described previously (12). Protein was measured by the method of Lowry et al. (9). The inhibition of protein synthesis was determined by comparing the incorporation level of [^{3}H]leucine in toxin-treated cells with that in control cells. All experiments were carried out two to four times in triplicate, and results are expressed as counts per minute per microgram of protein ± one standard deviation.

Immunoelectron microscopy. Precooled cell monolayers grown in Leighton tubes were incubated with toxin (100 ng/ml) at 4°C for 30 min and then reincubated in toxin-free medium for 30 min at 37°C. The cells were washed three times with cold phosphate-buffered saline (PBS) and immediately fixed with 0.5% glutaraldehyde in PBS. To visualize toxin, we incubated fixed cells with rabbit antitoxin (IgG) prepared against PE, followed by treatment with goat anti-rabbit IgG and, lastly, with horse spleen ferritin to which rabbit IgG had been chemically conjugated. All immunohistochemical reagents were adsorbed three times with LM cells before use. Samples were prepared for viewing and scored for cell surface distribution of toxin as described previously (5, 14, 15). Control samples in which each of the immunohistochemical steps was omitted were included in each experiment. An occasional molecule was observed in nonspecific association with the membrane in these specimens.

RESULTS

We reported previously that PE enters mouse LM fibroblasts by RME (5). Since extracellular calcium has been shown to be required for binding (7) or for receptor-mediated internalization (4) of several protein ligands or both, the requirement for this ion in the binding, internalization, and subsequent expression of PE activity was examined. First, the ability of the divalent cation chelator ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid (EGTA) to block PE toxicity was examined. LM cell monolayers were preincubated with EGTA and then allowed to bind PE in the cold and in the presence of chelator. After unbound toxin was removed, monolayers were reincubated for 5 h at 37°C in medium with chelator, and protein synthesis was measured. EGTA (2 mM) fully protected cells from PE; i.e., protein synthesis was normal in these cells in contrast to an 86% reduction in monolayers exposed to toxin in the absence of chelator (data not presented).

Receptor-mediated internalization of ligand involves binding of ligand to a receptor on the cell surface, clustering of receptors, and subsequent internalization of the receptor-ligand complex. Several experiments were carried out to determine at what stage in this process calcium is required. Monolayers were incubated with PE for 30 min at 4°C in tissue culture medium in the absence of chelator. Unbound toxin was removed, and the monolayers were reincubated at 37°C in medium containing EGTA. Data (Table 1) showed that the depletion of calcium after toxin binding occurred reduced toxicity significantly. However, in the absence of preincubation with chelator, nontoxic levels of EGTA (up to 5 mM) did not provide full protection to LM cells. A further experiment was designed to evaluate the effect of having chelator present only during toxin binding. Since the concentration of toxin used in most experiments (100 ng/ml) caused a high level of inhibition of protein synthesis which might have precluded detection of small amounts of decreased binding, cell monolayers were incubated with toxin (10 to 100 ng/ml) in the presence or absence of EGTA, unbound toxin was removed, and monolayers were reincubated in chelator-free medium. At all concentrations of toxin tested, similar levels of toxicity were seen whether binding occurred in the presence or absence of chelator. (Fig. 1). Thus, the results of these two sets of experiments suggest that depletion of extracellular calcium does not affect ligand-receptor binding, but that calcium is required for a subsequent step in the intoxication process.

To determine whether extracellular calcium is required during toxin entry or after toxin internalization, we determined the ability of chelator to protect cells when added to monolayers at

TABLE 1. Protection afforded by addition of chelator after *Pseudomonas* toxin is bound to LM cells[a]

Cells	EGTA concn (mM)	Protein synthesis	
		Counts per minute/microgram	% Inhibition
Toxin treated	0	25.7 ± 9.1	84
	1.0	62.6 ± 6.5	63
	2.0	119.0 ± 14.5	36
	3.5	127.6 ± 8.7	33
	5.0	135.4 ± 10.1	37
Control	0	160.2 ± 31.2	
	1.0	169.8 ± 8.6	
	2.0	187.4 ± 11.0	
	3.5	189.6 ± 9.7	
	5.0	216.0 ± 18.9	

[a] Toxin (100 ng/ml) was incubated with cell monolayers in medium without EGTA for 30 min at 4°C (binding step); unbound toxin was removed, and monolayers were reincubated for 5 h at 37°C in toxin-free medium containing EGTA.

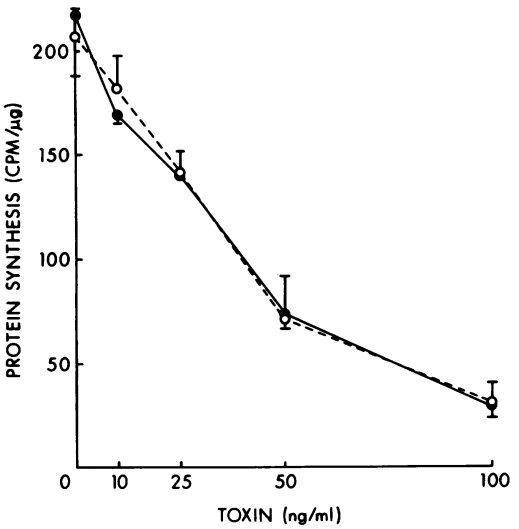

FIG. 1. Chelator does not block initial association of PE with LM cells. LM cell monolayers were incubated with several concentrations of PE for 30 min at 4°C in the presence (○) or absence (●) of 2 mM EGTA. The medium was removed, and monolayers were reincubated for 5 h at 37°C in medium not containing EGTA before measurement of protein synthesis. The error bars represent one standard deviation of triplicate samples.

intervals after removal of unbound toxin (Fig. 2). PE was incubated with cells for 30 min at 4°C, and the cells were washed and reincubated in fresh culture medium at 37°C. At indicated times, medium containing EGTA was added to the monolayers, and after 5 h, cellular protein synthesis was assayed. As seen before, the addition of EGTA immediately after binding of toxin provided maximum protection (30% inhibition of protein synthesis versus 87% if chelator step was not included). The ability to protect cells by adding EGTA after removing unbound toxin was rapidly lost. Chelation of extracellular calcium at 2.5 min or later provided reduced protection relative to cells washed with medium not containing EGTA (approximately 47 and 70% reduction in protein synthesis in presence and absence of chelator after 2.5, 5, or 10 min). Therefore, we suggest that extracellular calcium is required for an early step in the internalization process. Experiments were performed to confirm more precisely the role of calcium in the expression of toxicity. Data presented in Fig. 3 show that the addition of calcium to medium depleted with EGTA allowed full expression of PE toxicity. Cell monolayers were incubated with toxin for 30 min at 4°C, washed, and reincubated at 37°C in fresh culture medium containing EGTA (2 mM) and various concentrations of $CaCl_2$ (Fig. 3A). The addition of 2 mM $CaCl_2$ totally overcame the protection afforded by the chelator, and protein synthesis was inhibited by 84%. Similarly, cells were incubated with toxin and EGTA, washed, and reincubated in PBS containing $CaCl_2$ (Fig. 3B). Again, replenishment of calcium (1.0 mM) allowed full expression of toxicity. The addition of higher concentrations of calcium did not potentiate the action of the toxin nor was the combination of 2 mM EGTA and 2 mM $CaCl_2$ toxic to the cells (data not shown).

Similar experiments were performed with magnesium as the divalent cation. In contrast to the results obtained with $CaCl_2$, adding $MgCl_2$ after binding did not allow expression of toxin activity. At all concentrations of the magnesium salt used (0.1 to 2 mM), only minimal inhibition of protein synthesis was observed (data not presented).

Immunoelectron microscopy. Electron microscopic studies were carried out to corroborate the biochemical studies. PE was allowed to bind in the cold and cells were washed and reincubated for 30 min at 37°C. Horse spleen ferritin was used to visualize surface toxin. Similar ferritin labeling was observed whether EGTA was present throughout the entire experimentation period (11.2 sites per section), present during binding and entry (14.6 sites), or present during entry only (12.3 sites) (data not shown). This level was comparable to that observed on cells maintained at 4°C (12.2 sites per section).

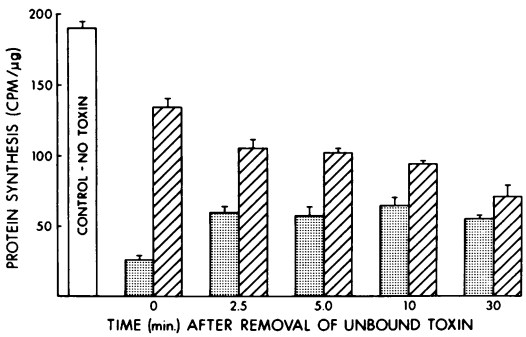

FIG. 2. Depletion of extracellular calcium blocks early step in toxicity. Mouse LM fibroblast monolayers were incubated with toxin (100 ng/ml) for 30 min at 4°C. Cells were washed thoroughly to remove unbound toxin and were reincubated in fresh toxin-free medium at 37°C. At indicated times, the medium was aspirated and replaced with medium containing 2 mM EGTA (cross-hatch) or medium without EGTA (stippling). Protein synthesis was measured 5 h after the removal of unbound toxin. The open bar represents cells incubated in medium with EGTA but without toxin for the entire experiment. The error bars represent one standard deviation of triplicate samples.

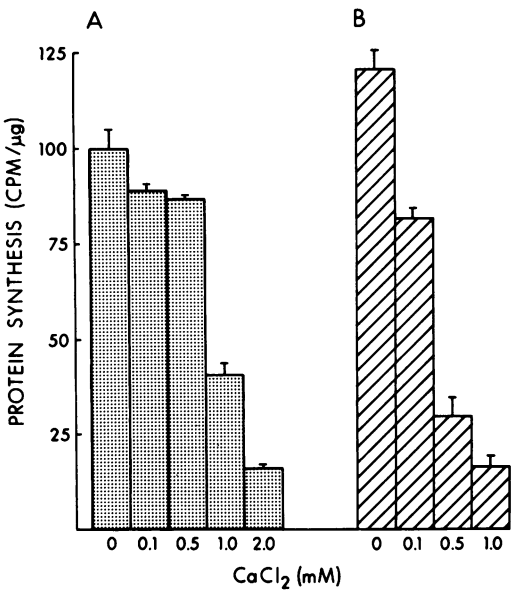

FIG. 3. Expression of *Pseudomonas* toxin activity in the presence of calcium. (A) PE(100 ng/ml) in complete tissue culture medium (TCM) was added to cells for 30 min at 4°C. Unbound toxin was removed, and cells were reincubated at 37°C in TCM-EGTA (2 mM) plus the indicated concentrations of $CaCl_2$ for 5 h. Cells were then assayed for protein synthesis. (B) PE (100 ng/ml) in TCM-EGTA (2 mM) was added to cells for 30 min at 4°C. Unbound toxin was removed, and cells were reincubated at 37°C in PBS plus the indicated concentrations of $CaCl_2$ for 5 h and then assayed for protein synthesis. The error bars represent one standard deviation of triplicate samples.

When cells were incubated with toxin in tissue culture medium containing EGTA and reincubated in toxin-free medium containing EGTA and increasing concentrations of calcium, toxin was cleared from the cell surface (Table 2). As in the biochemical studies, the addition of 2 mM calcium totally overcame the EGTA block, and normal levels of internalization were seen. In the presence of the low calcium concentration (0.1 mM), a reduction of approximately 30% in the quantity of surface-localized ferritin was noted. Magnesium did not substitute for calcium in the entry process, since toxin was not internalized in the presence of $MgCl_2$. These observations suggest that the depletion of extracellular calcium does not alter the binding of toxin to LM cells but does block internalization of bound toxin, and they are in agreement with our observations utilizing the inhibition of protein synthesis to measure biological expression of PE.

A representative LM cell is shown in Fig. 4. Note that the ferritin grains clustered exclusively within the coated pit region of the cell membrane.

DISCUSSION

We have shown previously (5) that the internalization of PE by mouse fibroblasts exhibits the essential characteristics of RME. A combined biochemical-electron microscopic approach has been exploited to analyze the role of extracellular calcium in the initial interactions of PE with LM cells. The entry of toxin was monitored both by assaying the inhibition of protein synthesis and by visualizing the clearance of toxin from the cell surface. The data generated by these two approaches support the hypothesis that the expression of toxicity requires free extracellular calcium. A similar requirement for calcium for the activity of *Clostridium perfringens* enterotoxin on Vero and HeLa cells was reported by Matsuda and Sugimoto (10).

Several limitations are inherent in the immunoelectron microscopic (IEM) technique used here. This technique is qualitative, not quantitative. The number of ferritin cores varied from one to >5 per conjugate, and therefore, scoring of toxin sites was dependent upon visualization of clusters of ferritin molecules. Thus, it can be difficult to determine whether a single or multiple antigenic site is being viewed.

Also, the immunohistochemical method allows detection only of surface-associated toxin. It does not allow detection of toxin which dissociates from the cell surface and is shed into the medium. PE which has been internalized cannot be detected by this method. To detect intracellular toxin, it is necessary either to use PE labeled directly with an electron-dense marker (e.g., horse spleen ferritin or colloidal gold) or to

TABLE 2. Calcium dependence of *Pseudomonas* toxin internalization by LM cells[a]

Internalization conditions	Ferritin labeling[b]
TCM (no EGTA) + 0 mM $CaCl_2$	2.2 ± 1.0
TCM[c] + 0 mM $CaCl_2$	9.2 ± 2.6
TCM[c] + 0.1 mM $CaCl_2$	6.7 ± 1.7
TCM[c] + 2.0 mM $CaCl_2$	3.5 ± 1.3
TCM[c] + 2.0 mM $MgCl_2$	10.9 ± 1.2
PBS + 0.1 mM $CaCl_2$	5.5 ± 1.7
PBS + 1.0 mM $CaCl_2$	3.6 ± 0.6
PBS + 1.0 mM $MgCl_2$	11.1 ± 1.5

[a] Toxin (100 ng/ml) was incubated with LM cell monolayers for 30 min at 4°C in EGTA-containing tissue culture medium; monolayers were washed and reincubated at 37°C for 30 min in complete medium or in PBS ± $CaCl_2$ or $MgCl_2$.

[b] Sites per section; minimum of 10 sections scored as described in text. Cells which were maintained at 4°C for 30 min in the presence of toxin and fixed with glutaraldehyde immediately had 12.2 ± 3.2 sites per section.

[c] TCM, Tissue culture medium; contains 2 mM EGTA.

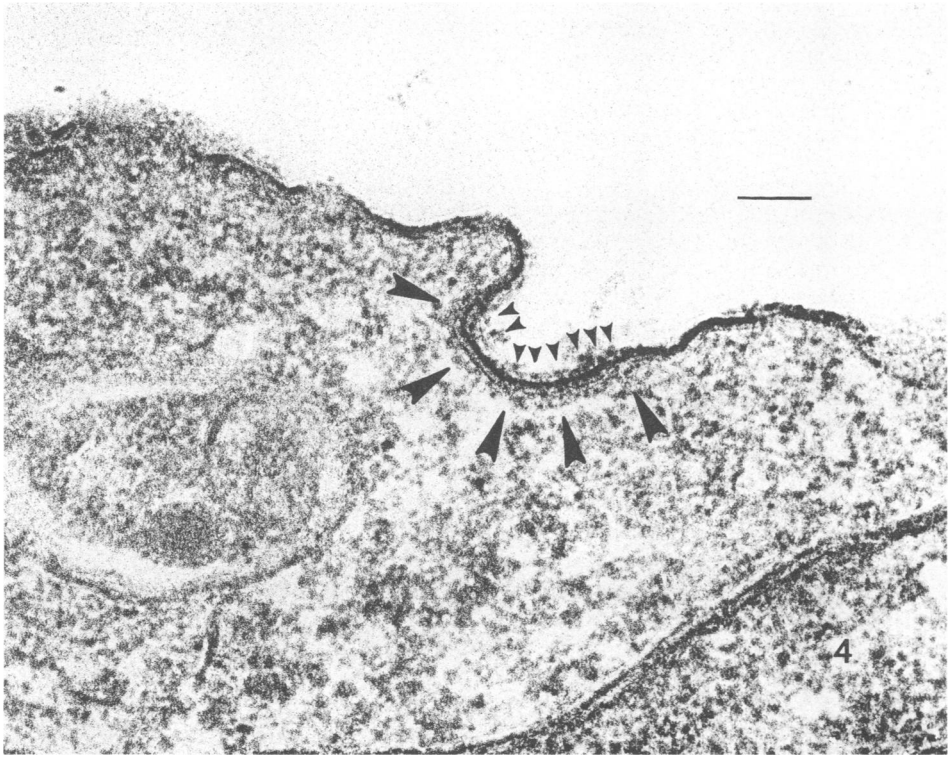

FIG. 4. Binding of *Pseudomonas* toxin to LM cell monolayers. Precooled LM cell monolayers were incubated with PE (100 ng/ml) for 30 min at 4°C. The monolayers were washed three times with warm, toxin-free medium and reincubated for 30 min at 37°C. Samples were stained with bismuth subnitrate to increase the electron density of ferritin (1). Bar, 100 nm. Note that the ferritin cores (toxin-binding sites) localized within a coated region of the cell membrane (small arrows). The coated region is identified by the bristle-like structures (large arrows), giving this region a thickened appearance.

render cells permeable to the immunohistochemical reagents (17). Using the latter method, we obtained preliminary evidence that toxin is in membrane-bound vesicles after 30 min of incubation at 37°C.

One problem in determining the fate of PE (or any other internalized protein toxin) is that only a very few molecules of toxin or enzyme-active fragments are required to inactivate cytoplasmic elongation factor 2 and thus kill the cell (16, 18). The question can always be raised as to whether one is dealing with the productive toxin molecules or with toxin that is ultimately degraded and does not participate in the lethal event. However, it is felt that a combined approach to this problem provides the best possibility for determining the fate of toxin.

Several mechanisms were considered to explain protection afforded by EGTA. It was considered that calcium might be required for toxin binding. Classical binding experiments with ^{125}I-labeled PE have not been included in this study for two reasons: to date we have not been able to obtain PE which retains full biological activity when labeled with greater then 1 to 2 molecules of ^{125}I per 10 toxin molecules, and binding studies carried out with available iodinated toxin preparations have shown only low levels of specific binding at 37°C and have not shown saturability at 4°C (unpublished data). This may be because the number of receptors for PE and LM cells are near the lower limits of detection, using classical binding assays with radiolabeled toxin. When measuring the number of receptors for diphtheria toxin, Middlebrook et al. (13) reported that the lower limit for detection is 5,000 receptors per cell (binding affinity of 10^9 liters of diphtheria toxin per mol). From IEM observations, we calculated that there are approximately 7,000 PE binding sites per LM cell. IEM studies showed similar numbers of PE molecules on the cell surface when the binding step was carried out in the presence or absence of chelator. In addition, there was equal expres-

sion of toxicity whether PE was allowed to bind in the presence or absence of calcium. Taken together, the data suggest that calcium is not required for toxin binding.

Our results suggest that internalization or at least the initiation of internalization occurs rapidly after binding. We have reported previously that the addition of specific antitoxin to LM cell monolayers 2.5 min after the addition of PE only partially blocks expression of toxicity (12). Similarly, the level of toxin on the cell surface as detected by IEM is reduced within 5 min with incubation at 37°C (5). We have shown here that within 2.5 min (Fig. 2) a calcium-dependent step in the intoxication process has begun. All data suggest that the initial steps in the internalization of PE by LM cells occur very rapidly. Similar findings have been reported for other ligands. Goldstein et al., (6) reported internalization and delivery of low-density lipoprotein to lysosomes within 10 min. Carpenter and Cohen (2) found that at 37°C, the half-life of bound epidermal growth factor on the cell surface of human fibroblasts is approximately 2 min. Because of the rapidity of the process, it is technically difficult to identify events involved in ligand internalization. Considerable work has been carried out by Pastan and colleagues to determine how the formation of ligand-receptor complexes is coupled to the process of internalization. They have proposed an obligatory role for a transglutaminase enzyme in mediating the internalization of several ligands (4). Transglutaminase is calcium dependent and is inhibited by primary amines (4). Although we cannot directly implicate transglutaminase as the mediator of PE internalization, we have provided circumstantial evidence that this enzyme or another calcium-dependent enzyme may play a significant role in the expression of toxicity. Expression of PE toxicity is inhibited by various primary amines (5, 12) and, as this report shows, is also calcium dependent.

In an effort to reconstruct the molecular events for successful toxin entry, we have shown the necessity for extracellular calcium. We have also shown that calcium is required early in the process of toxin internalization. Therefore, we propose that calcium is needed as a cofactor for a component of the internalization system. It is possible that a membrane-bound or cytoplasmic transglutaminase is the essential component. However, without further confirmatory evidence, the role of this enzyme remains conjectural. Since calcium ions are known to play an essential role in a wide range of cellular functions, including endocytosis (3), it would be premature to suggest the exact mechanism of protection in the situation described here.

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LITERATURE CITED

1. **Ainsworth, S. K., and M. J. Karnovsky.** 1972. An ultrastructural staining method for enhancing the size and electron opacity of ferritin in thin sections. J. Histochem. Cytochem. **20**:225–229.
2. **Carpenter, G., and S. Cohen.** 1976. ^{125}I-labelled human epidermal growth factor. Binding, internalization and degradation in human fibroblasts. J. Cell. Biol. **71**:159–171.
3. **Cheung, W. Y.** 1980. Calmodulin plays a pivotal role in cellular regulation. Science **207**:19–27.
4. **Davies, P. J. A., D. R. Davies, A. Levitzki, F. R. Maxfield, P. Milhaud, M. C. Willingham, and I. Pastan.** 1980. Transglutaminase is essential in receptor mediated endocytosis of α_2-macroglobulin and polypeptide hormones. Nature (London) **283**:162–167.
5. **FitzGerald, D., R. E. Morris, and C. B. Saelinger.** 1980. Receptor-mediated internalization of Pseudomonas toxin by mouse fibroblasts. Cell **21**:867–873.
6. **Goldstein, J. L., R. G. W. Anderson, andd M. S. Brown.** 1979. Coated pits, coated vesicles and receptor mediated endocytosis. Nature (London) **279**:679–685.
7. **Kaplan, J.** 1981. Polypeptide-binding membrane receptors: analysis and classification. Science **212**:14–20.
8. **Leppla, S. H.** 1976. Large scale purification and characterization of exotoxin of *Pseudomonas aeruginosa*. Infect. Immun. **14**:1077–1086.
9. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
10. **Matsuda, M., and N. Sugimoto.** 1979. Calcium-independent and dependent steps in action of *Clostridium perfringens* enterotoxin on HeLa and VERO cells. Biochem. Biophys. Res. Commun. **91**:629–636.
11. **Maxfield, F., M. Willingham, P. Davies, and I. Pastan.** 1979. Amines inhibit the clustering of α_2-macroglobulin and EGF on the fibroblast cell surface. Nature (London) **277**:661–663.
12. **Michael, M., and C. B. Saelinger.** 1979. Toxicity of pseudomonas toxin for mouse LM cell fibroblasts. Curr. Microbiol. **2**:103–108.
13. **Middlebrook, J. L., R. B. Dorland, and S. H. Leppla.** 1978. Association of diphtheria toxin with Vero cells. J. Biol. Chem. **253**:7325–7330.
14. **Morris, R. E., and R. B. Fritz.** 1975. Ultrastructural visualization of virus-induced surface antigens: comparative studies of three immunohistochemical techniques. J. Histochem. Cytochem. **23**:855–862.
15. **Morris, R. E., and R. B. Fritz.** 1976. Expression of virion and tumor-associated antigens on the surface of chick embryo cells infected with strain MC29 avian leukosis virus. Cancer Res. **36**:414–419.
16. **Moynihan, M. R., and A. M. Pappenheimer.** 1981. Kinetics of adenosinediphosphoribosylation of elongation factor 2 in cells exposed to diphtheria toxin. Infect. Immun. **32**:575–582.
17. **Willingham, M. C., and S. Yamada.** 1979. Development of a new primary fixative for electron microscopic immunocytochemical localization of intracellular antigens in cultured cells. J. Histochem. Cytochem. **27**:947–960.
18. **Yamaizumi, M., E. Mekada, T. Uchida, and Y. Okada.** 1978. One molecule of diphtheria toxin fragment A introduced into the cell can kill the cell. Cell **15**:245–250.