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Evidence for *Pseudomonas* Exotoxin A Receptors on Plasma Membrane of Toxin-Sensitive LM Fibroblasts

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***Pseudomonas* exotoxin A enters mouse LM fibroblasts by receptor-mediated endocytosis and ultimately causes cell death. Here we present evidence for the existence of a specific receptor for the toxin. Toxin association with LM cells at 18 and 37°C, but not at 4°C, was highly specific. At 37°C, the association increased with time, reaching a steady state by 5 h. Binding to paraformaldehyde-fixed cells at 37°C was saturable (K_d = 5.4 nM), was reversible, and indicated ca. 100,000 binding sites per cell. It is believed that receptor-bound toxin is responsible for cell death. Once the kinetics of toxin entry were described, we examined the effect of reduced temperatures on the intracellular processing of toxin and thus its expression. Toxin-induced inhibition of protein synthesis was minimal at temperatures below 20°C. This was seen even though at 20°C sufficient toxin was internalized to kill cells, and toxin enzyme activity was maximal. Internalization of ^{125}I-labeled toxin, but not of ^{125}I-labeled horseradish peroxidase (marker of fluid-phase endocytosis), became rate limiting at 20°C or below. These data suggest that reduced temperatures block a step in the receptor-mediated endocytic pathway essential for the expression of *Pseudomonas* toxin activity.**

It is widely recognized that internalization of certain physiologically important ligands such as growth factors and peptide hormones takes place by a specialized endocytic process known as receptor-mediated endocytosis (reviewed in references 6 and 22). In addition, it is becoming clear that a number of potentially harmful agents, including viruses and bacterial toxins, are also internalized by receptor-mediated endocytosis (RME) (3, 5, 8, 14, 23, 28). A ligand that is internalized by RME first binds to specific receptors on the cell surface. The receptor-ligand complexes then cluster over specialized clathrin-coated regions of the membrane, and internalization of the complex is initiated. Recently we demonstrated that, in toxin-sensitive cells (mouse fibroblasts), pseudomonas exotoxin A (PE) is internalized through coated pits, and also obtained evidence that RME of PE is a prerequisite for expression of the biological activity of the toxin (5, 16). PE exerts toxicity by its enzymatic inactivation of cytoplasmic elongation factor 2 (9). PE is elaborated by *Pseudomonas aeruginosa* as a proenzyme, and intoxication of mammalian cells can be viewed as a multistep process involving (i) binding of toxin to specific receptors on the cell surface, (ii) internalization through coated areas, (iii) intracellular trafficking and processing of proenzyme to an enzyme active form, (iv) escape of the enzyme active polypeptide from membrane-limiting cytoplasmic vesicles, and (v) ultimately, ADP ribosylation of cytoplasmic elongation factor 2. The final step in the process has been fully described (9). Internalization via clathrin-coated areas of the membrane is suggested by electron microscopy (5, 16) and subcellular fractionation techniques (M. D. Manhart, C. B. Saelinger, and R. E. Morris, J. Cell Biol. **95**:434a). In this report, we present evidence that mouse LM fibroblasts possess receptors specific for PE and, secondly, that expression of PE toxicity and RME are concomitantly inhibited at temperatures below 20°C.

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MATERIALS AND METHODS

Cells. Mouse LM fibroblasts (ATCC CCL 1.2 LM) were maintained as monolayers in McCoy medium containing 5% heat-inactivated fetal calf serum and antibiotics (TCF). For experimental use, cells were seeded in 24-well culture plates at a concentration of 5×10^5 cells per ml and were incubated overnight before use. Where indicated, monolayers were fixed with 1% paraformaldehyde–phosphate-buffered saline (pH 7.4) for 30 min at 4°C. Fixed cells were washed in phosphate-buffered saline (pH 7.4) twice and in TCF three times and were reincubated (37°C) in TCF before use.

Iodination of PE. PE was purified from bacterial culture filtrates as described previously (11) and was iodinated with Bolton-Hunter (1) reagent (*N*-succinimidyl 3-(4-hydroxy, 5-[^{125}I]iodophenyl propionate) (Amersham Corp., Arlington Heights, Ill.). Before use, the reagent, cooled in ice, was dried against the walls of a reaction vial with a stream of dry nitrogen. Immediately after drying, 100 μg of PE in 100 μl of 0.1 M borate buffer (pH 7.4) was added to the vial and stirred on ice for 30 min. To stop the reaction, 100 μl of 0.2 M glycine in borate buffer was added, and the solution was mixed for 5 min. Iodinated protein was separated from unreacted ester by chromatography in a PD-10 column (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated in phosphate-buffered saline containing 0.2% gelatin. Two well-defined peaks of radioactivity were resolved. The protein-rich fractions were pooled, diluted to 10 μg/ml in TCF, divided into portions, and used immediately or frozen at −70°C. We found that 45 to 55% of the ester was incorporated into protein, and the specific activity of the ^{125}I- labeled PE was 2×10^6 to 6×10^6 cpm/μg. Preliminary experiments determined that both the molar ratio of PE to ester (between 1.0 and 2.5) and the pH of the reaction (7.4) were critical for the generation of a labeled probe with maximal specific binding and toxicity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and isoelectric focusing showed that ^{125}I-radioactivity migrated as a single band coincident with the migration of native PE.

Toxicity of labeled PE. To assess the biological activity of

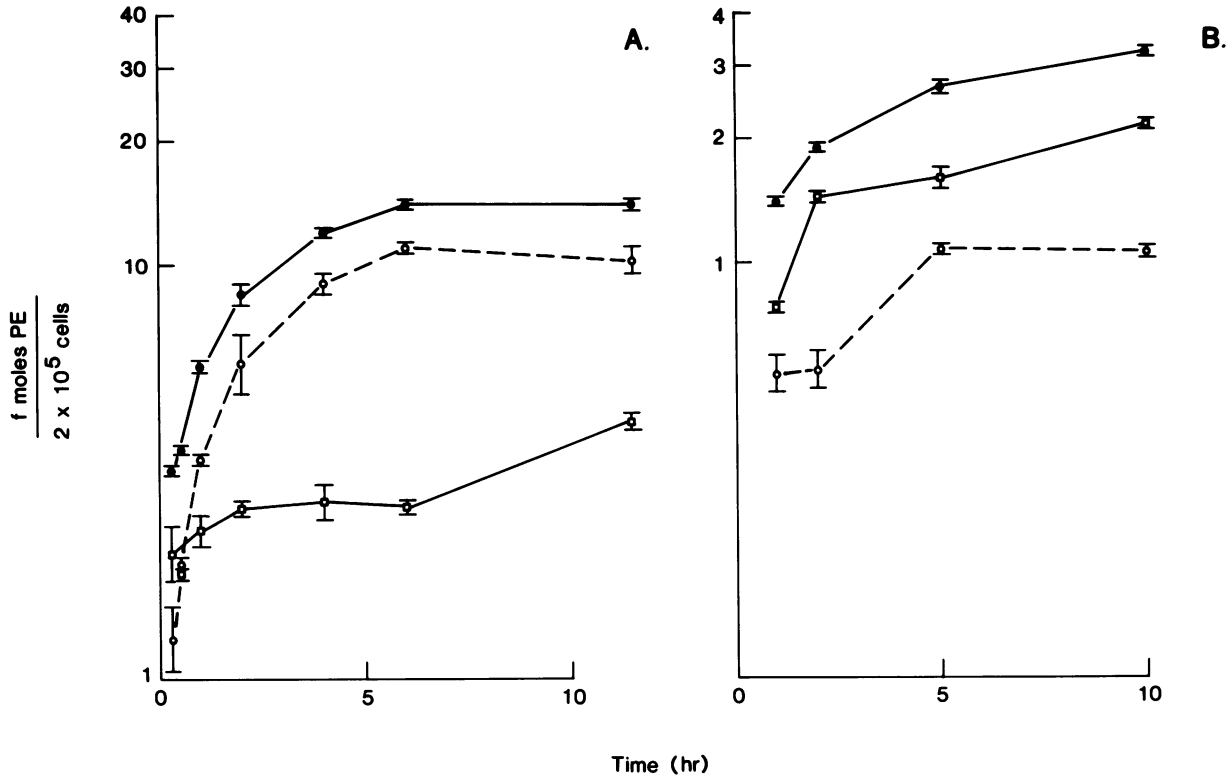

FIG. 1. Kinetics of association of ^{125}I-labeled PE with LM cells maintained at 18°C (A) and 4°C (B). Cell monolayers were incubated with 500 ng of ^{125}I-labeled PE per ml in the absence (●) or presence (□) of 10 μg of unlabeled toxin. At the times indicated, cell-associated radioactivity was determined. Specific binding (○) was determined by subtracting nonspecific (□) from total (●) binding. Points are means of triplicate samples ± one standard deviation.

PE derivatized with Bolton-Hunter reagent, nonradioiodinated ester (Pierce Chemical Co., Rockford, Ill.) was reacted with PE under conditions identical to those used to iodinate. The "sham-labeled" toxin showed a UV absorbance spectrum (at 320 to 260 nm) identical to that of ^{125}I-labeled PE, indicating similar levels of incorporation of hydroxyphenyl propionate. The sham-labeled toxin was compared with native toxin in an LM cell cytotoxicity assay (5). Sham-labeled PE had 80 to 90% of the biological activity of native toxin (data not shown).

Toxin binding assay. ^{125}I-labeled PE, diluted in TCF, was added to LM cell monolayers with or without 200-fold excess unlabeled toxin. After incubation at the temperatures and for the times specified, monolayers were washed four times with TCF, solubilized in 0.1 M NaOH, and counted in a Packard 5120 gamma counter. Protein was determined by the method of Lowry et al. (12). Each point is the mean of triplicate samples plus or minus a standard deviation, and experiments were repeated a minimum of three times.

Toxin internalization assay. A trypsin release assay was used to determine the extent of PE internalization. Monolayers were incubated with ^{125}I-labeled PE as described in the figure legends, rinsed three times with cold Hanks balanced salts solution, and incubated for 30 min at 4°C with 1.0 ml of trypsin (2% in Hanks balanced salt solution). Detached cells were immediately transferred to 1.5-ml polypropylene centrifuge tubes and spun for 2 min in a microfuge; pellets were washed twice in McCoy medium–25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid)–2% fetal calf serum, and the supernatants and pellets were counted. This treatment released >80% of surface-bound toxin. Preliminary experiments showed that protein synthesis was not inhibited (i.e., control cell levels) in cells exposed to 500 ng of PE per ml (30 min, 4°C), treated with trypsin as described above, and then incubated for 5 h at 37°C.

Electron microscopy. Direct visualization of toxin on the ultrastructural level was carried out by using biotinyl-PE and avidin-gold (electron dense marker; 5.2 nm). Preparation of reagents and protocol were as described previously (18).

Protein synthesis. Inhibition of protein synthesis was used as a measure of PE biological activity (5). Protein synthesis was measured by determining the amount of [^{3}H]leucine incorporated into trichloroacetic acid-insoluble material during a 30-min pulse at 37°C.

RESULTS

Demonstration of a PE toxin receptor on the surface of LM cells. The kinetics of ^{125}I-labeled PE binding by LM cells at different temperatures was determined in an initial set of experiments (Fig. 1A). When cells were maintained at 18 to 37°C, toxin association with fibroblasts was specific and saturable. At 18°C, saturation was reached within 5 h (Fig. 1A), and the quantity of ^{125}I-labeled PE associated with cells decreased by 80% in the presence of unlabeled PE. Similar experiments conducted at 37°C gave identical results, except that saturation of toxin binding occurred more rapidly (1.5 to 2.5 h). Although these experiments demonstrate specific association of ^{125}I-labeled PE with LM cells, the data cannot be used as a quantitative estimate of toxin binding exclusively; toxin is internalized by the cells at 18 and 37°C and thus creates non-equilibrium conditions. That internalization of toxin occurs at both temperatures was demonstrated with a

trypsin release assay. More than half of the radioactivity associated with LM cells after 4 h at 18°C was resistant to release by trypsin, indicative of an intracellular location. Similarly, after 15 min at 37°C, 70% of the total cell-associated radioactivity was intracellular (data not shown).

A standard method used to prevent internalization of ligand during analysis of receptor-ligand interaction is to allow cells and ligand to interact at 4°C (2). However, when binding of ^{125}I-labeled PE to cells at 4°C was assessed, the specific binding of toxin was very low, ranging from a maximum of 40% to as low as 15% of the total cell-associated radioactivity (Fig. 1B).

Toxin binding experiments also were carried out at the ultrastructural level, using biotinyl-PE and avidin-gold as readily traced labels. We have shown previously (18) that biotinyl-PE binding is specific, i.e., receptor mediated (2). Binding at 4°C also was saturable at approximately 100 ng of biotinyl-PE per ml (sites per section at 10, 50, 100, 250, and 500 ng/ml were 6.8, 9.4, 15.5, 16.1, and 15.6, respectively). Therefore, morphological data provide evidence for a specific PE receptor on the surface of LM cells.

The extremely high level of nonspecific binding at 4°C did not permit definitive binding studies with ^{125}I-labeled PE on chilled cells. Therefore, as an alternative means to prevent internalization of receptor-bound toxin, cells were pretreated with paraformaldehyde (7, 25, 29) before exposure to toxin. Paraformaldehyde-treated cells, incubated with ^{125}I-labeled PE at 37°C, released 95% of the cell-associated radioactivity upon trypsin treatment, demonstrating a total inhibition of internalization. Thus, fixed LM cells were used to conduct a more extensive analysis of PE binding.

Initial experiments with paraformaldehyde-fixed cells established that ^{125}I-labeled PE binding reached equilibrium after 1.5 to 2.5 h at 37°C. Therefore, for all experiments thereafter, ^{125}I-labeled PE was prebound to cells for 3 h at 37°C before analysis. Binding isotherms calculated from data generated by incubating paraformaldehyde-treated cells with ^{125}I-labeled PE at concentrations between 0.025 and 5 μg/ml in the presence or absence of 200-fold excess unlabeled PE are plotted in Fig. 2. At a toxin concentration of less than 1 μg/ml, PE binding was highly specific (i.e., 60 to 80% of the total). At a higher toxin concentration, nonspecific toxin binding increased markedly and masked receptor-mediated binding. The data indicate that the specific component of binding approaches saturation at 5 μg/ml. At 10 μg of PE per ml, the specific component of binding cannot be detected, indicating complete saturation of receptor binding between 5 and 10 μg/ml (data not shown). Analysis of specific binding by the method of Scatchard (24) is consistent with the presence of a single class of receptors with an apparent K_d of 5.4 nM (Fig. 2, inset). Extrapolation of the line to the abscissa indicates that each LM cell possesses ca. 100,000 PE receptors on the plasma membrane. A K_d of 5.4 nM predicts that the receptor population will be half saturated at a PE concentration of 367 ng/ml.

To test these calculated values experimentally, competition experiments were conducted. Fixed LM cells were incubated with several concentrations of unlabeled toxin before the addition of a nonsaturating concentration of ^{125}I-labeled PE (Fig. 3). The concentration of unlabeled toxin that reduces binding of ^{125}I-labeled PE to 50% of maximum is an approximation of the calculated K_d. The midpoint of the curve is approximately 150 ng/ml (2 nM), which is in

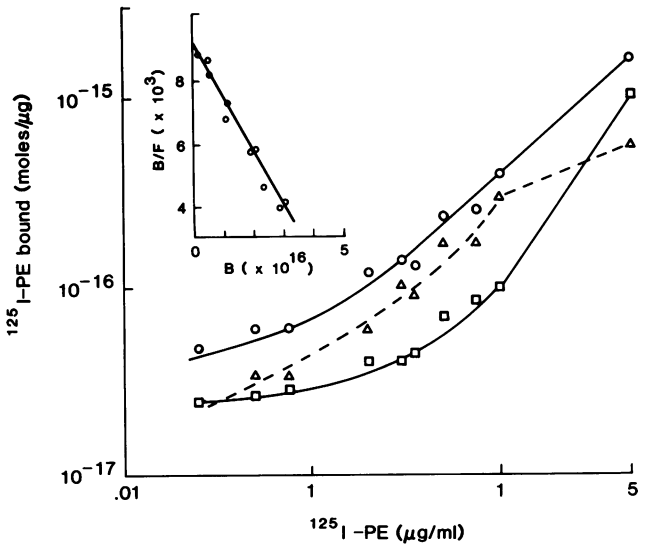

FIG. 2. Binding of ^{125}I-labeled PE to paraformaldehyde-fixed cells at 37°C. Paraformaldehyde-treated cells were incubated with ^{125}I-labeled PE (0.025 to 5 μg/ml) in the absence (○) or presence (□) of 200-fold excess unlabeled PE for 3 h at 37°C. Binding was measured as described in the text. Specific binding (△). Values are means of triplicate samples. Inset: Scatchard plot analysis of specific binding. Bound counts (B), expressed as nanomoles of PE per μg of cell protein, are plotted on the abscissa and bound/free (B/F) are plotted on the ordinate. The line, fitted by linear regression, has a correlation coefficient of 0.988.

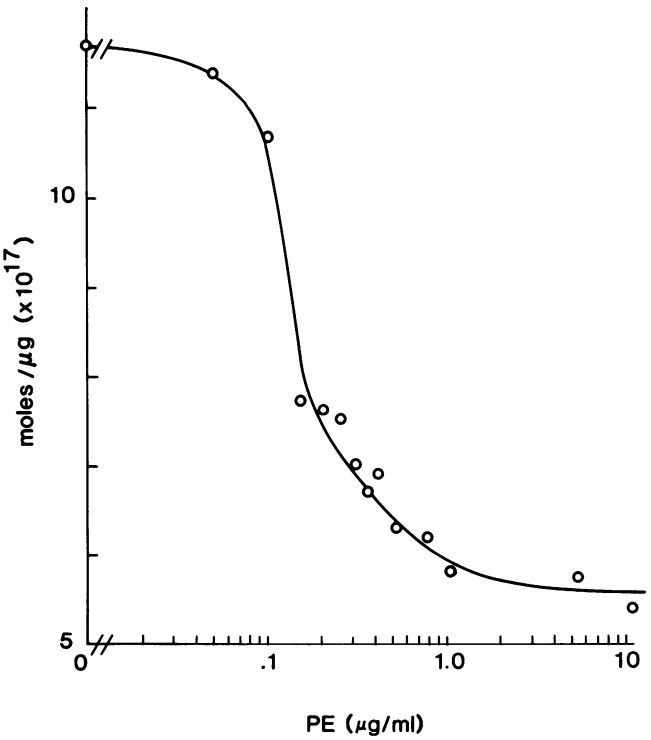

FIG. 3. Inhibition of ^{125}I-labeled PE binding with native PE. Paraformaldehyde-fixed cell monolayers were incubated with increasing concentrations of PE. After 15 min at 37°C, ^{125}I-labeled PE was added to the monolayers at a concentration of 0.05 μg/ml, and they were reincubated for 3 h at 37°C. Cells were then washed four times with TCF, and the amount of ^{125}I-labeled PE bound was determined.

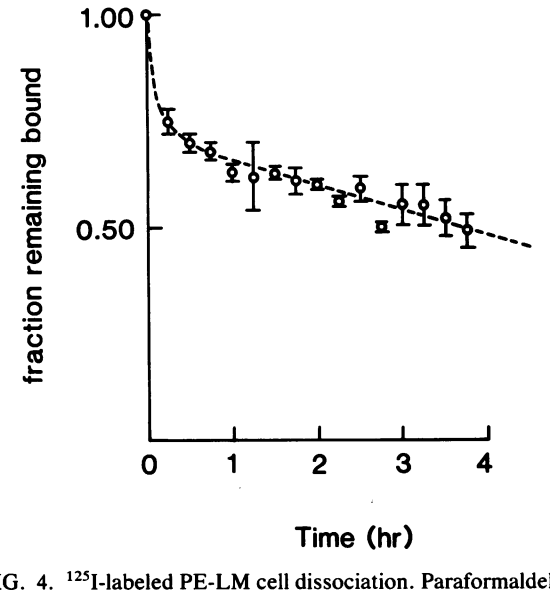

FIG. 4. ^{125}I-labeled PE-LM cell dissociation. Paraformaldehyde-fixed cells were incubated with 500 ng of ^{125}I-labeled PE for 3 h at 37°C. Monolayers were washed three times with TCF and reincubated in TCF containing 100 μg of unlabeled PE per ml (time zero). The fraction of bound toxin remaining cell associated was determined at the times indicated. The experiment was repeated three times. Specific binding represented 80% of the total in the experiment shown. Points are means of triplicate samples ± standard deviation.

good agreement with the K_d calculated from the Scatchard analysis.

The association of PE with fixed LM cells was shown to be reversible. Monolayers were incubated with ^{125}I-labeled PE until equilibrium was reached; they were then washed and reincubated in the presence of 100 μg of unlabeled toxin. At various times thereafter, the fraction of ^{125}I-labeled PE remaining bound to cells was determined. ^{125}I-labeled PE initially dissociated very rapidly during the first 20 min, but thereafter, a slower linear dissociation of toxin was seen (Fig. 4). Approximately half of the bound label was displaced within 3 h; these dissociation kinetics were observed consistently in three experiments.

Temperature-dependent expression of toxicity. Having established the presence of a PE binding moiety on LM cells, we examined the effect of temperature on uptake and expression of PE. Internalizations of ^{125}I-labeled PE and ^{125}I-labeled horseradish peroxidase (HRP) were compared. This afforded a comparison of a ligand (PE) internalized by RME (16) with one (HRP) internalized by fluid-phase endocytosis (26). Recently, Straus (27) showed that L-cells (the parent cell line for LM cells) do not possess mannose-specific binding sites for HRP.

Internalization of PE (Fig. 5) and HRP (Fig. 6) was linear with time. The rate of internalization of PE decreased moderately at temperatures between 37 and 24°C but slowed dramatically below 24°C. In contrast, HRP exhibited a gradual decrease in the rate of internalization at all temperatures between 37 and 4°C. Arrhenius plots of the data (Fig. 5 and 6, insets) show that HRP was internalized by a process with an energy of activation (E_a) of 19.6 kilocalories (kcal) (82.1 kJ) per mol and a temperature coefficient (Q_{10}) of 2.3. This is in excellent agreement with values reported by Steinman et al. (26). It is also in good agreement with the values reported by us for internalization of another bacterial toxin, diphtheria toxin, by a resistant mouse cell line (17). Unlike HRP internalization, PE internalization is complex since two distinct values for E_a can be calculated. Between 37 and 23°C, the E_a is 16.9 kcal (70.7 kJ) per mol and Q_{10} is 2.5. Below 16°C, the E_a is 49.1 kcal (205.6 kJ) per mol and Q_{10} is 22.9. This biphasic pattern of internalization has been reported for asialo-orosomucoid, another ligand internalized by RME (30). The inflection point on the Arrhenius plot is ca. 20°C. The precise meaning of E_a in our experiments is not clear since the value represents a summation of several complex events comprising the endocytic process. Nevertheless, one may conclude from the data that the two distinct activation energy values obtained for PE entry into LM cells reflect the fact that one or more critical steps in RME become rate limiting near 20°C. That HRP does not exhibit a similarly complex Arrhenius plot suggests that the rate-limiting step at 20°C is not shared for substances internalized exclusively by fluid-phase endocytosis.

It was subsequently observed that the rate-limiting step of RME influences the expression of toxicity of PE internalized by mouse fibroblasts. Since PE toxicity is expressed only after endocytosis (i.e., in the cytoplasm), the level of inhibition of protein synthesis caused by PE internalized at various temperatures was measured as a means of discerning differences in route of toxin uptake by LM cells. PE toxicity abruptly diminished at temperatures near 20°C (Fig. 7). The concentration of PE required to cause 50% inhibition of protein synthesis was more than fivefold greater at 20°C than at 26°C. ADP ribosylation activity when wheat germ was used as substrate was similar at temperatures between 18 and 30°C (data not shown). Thus, the minimum toxicity expressed below 20°C is not due to an inhibition of enzyme activity at these temperatures.

DISCUSSION

We have shown previously (5, 16) that PE enters mouse fibroblasts after clustering into coated areas on the membrane and moves within endosomes to the Golgi region and ultimately to lysosomes. Based on toxin entry by this pathway, it was concluded that PE is internalized by toxin-sensitive cells via RME. Such a pathway of entry necessitates the existence of a cell surface receptor (2).

It is generally assumed that a convincing demonstration of receptor function necessitates specificity, saturability, high affinity, and reversible ligand binding at 4°C. However, we were not able to satisfy all of these criteria in the PE-LM cell system. At reduced temperature (i.e., 4°C) only minimal specific PE binding by mouse fibroblasts was demonstrable, even after prolonged incubation periods. This fact has been verified by several other investigators (15; D. Fitzgerald, Ph.D. thesis, University of Cincinnati, Cincinnati, Ohio, 1982). Therefore, we conclude that the behavior of PE as a ligand at 4°C is aberrant. Since specificity of ligand binding at 4°C could not be demonstrated by the "classic" method of "cold" ligand competition, we resorted to two alternative methods to demonstrate specific receptor binding of PE, i.e., ultrastructural analysis utilizing biotinyl toxin-colloidal gold probes, and pretreatment of LM cell monolayers with paraformaldehyde. Fixation of cells with this aldehyde ablates internalization but does not significantly alter the specificity of ligand binding. Binding of ^{125}I-labeled mannose-bovine serum albumin conjugates to mannose receptors on the surface of alveolar macrophages is essentially identical in paraformaldehyde-treated and untreated cells (29). Similarly, paraformaldehyde fixation does not alter the magnitude of binding of epidermal growth factor to human cells (7, 25).

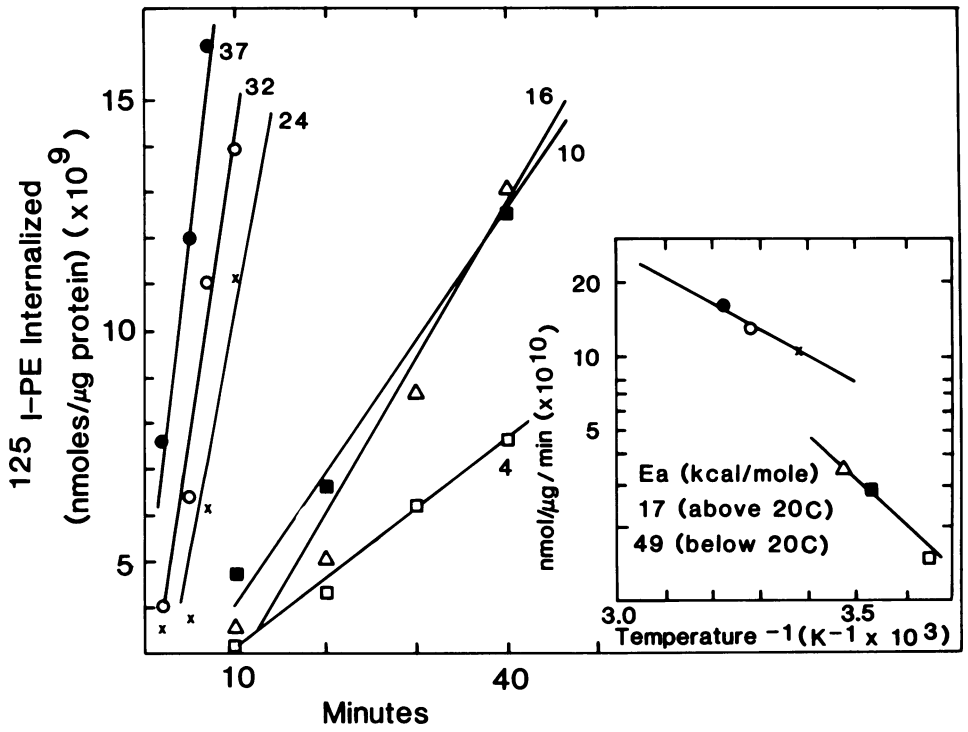

FIG. 5. Effect of temperature on ^{125}I-labeled PE internalization. LM cell monolayers, equilibrated to temperature, were exposed to ^{125}I-labeled PE (500 ng/ml). At the times indicated, monolayers were quickly washed with ice-cold Hanks balanced salt solution and exposed to trypsin (2% in Hanks balanced salt solution) for 30 min at 4°C to remove surface-bound toxin. Each point is the mean of triplicate samples. Lines were fitted by linear regression. Inset: The slopes of the lines at each temperature are plotted against the inverse of absolute temperature. Best fit lines, determined by linear regression, have correlation coefficients of −0.97 and −0.98 above and below 20°C, respectively. Symbols: ●, 37°C; ○, 32°C; x, 24°C; △, 16°C; ■, 10°C; □, 4°C.

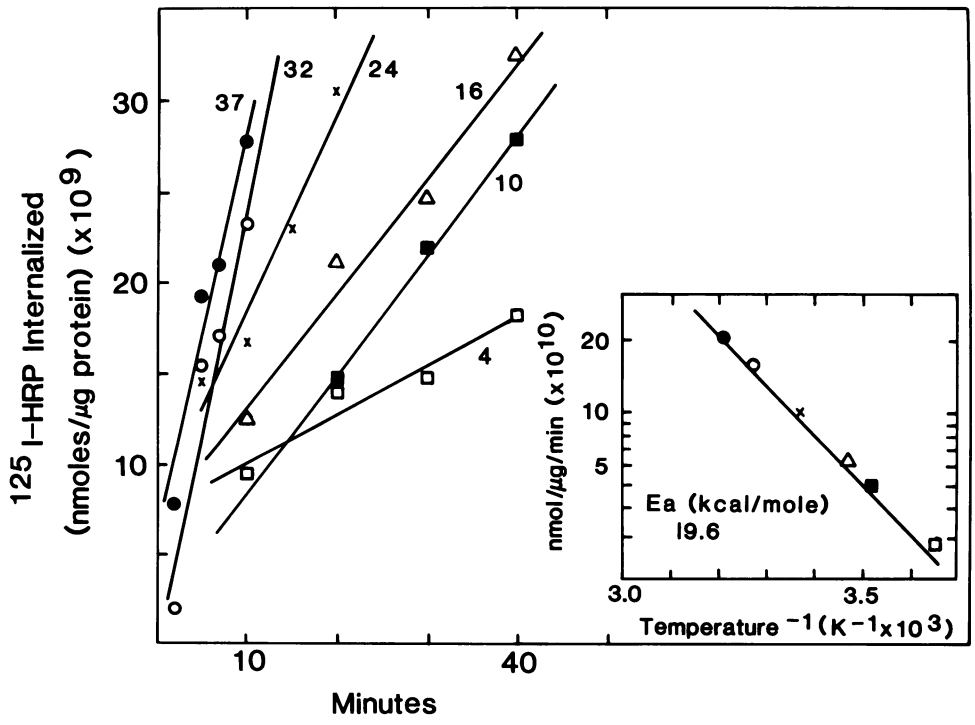

FIG. 6. Effect of temperature on ^{125}I-HRP internalization. HRP was iodinated by the method of Roth (21) to a specific activity of 4×10^7 cpm/μg. Cells, equilibrated to temperature, were exposed to ^{125}I-labeled HRP (25 μM) and treated as described in the legend to Fig. 5. Inset: Same as in Fig. 5. The correlation coefficient of the line is −0.96. Symbols: ●, 37°C; ○, 32°C; x, 24°C; △, 16°C; ■, 10°C; □, 4°C.

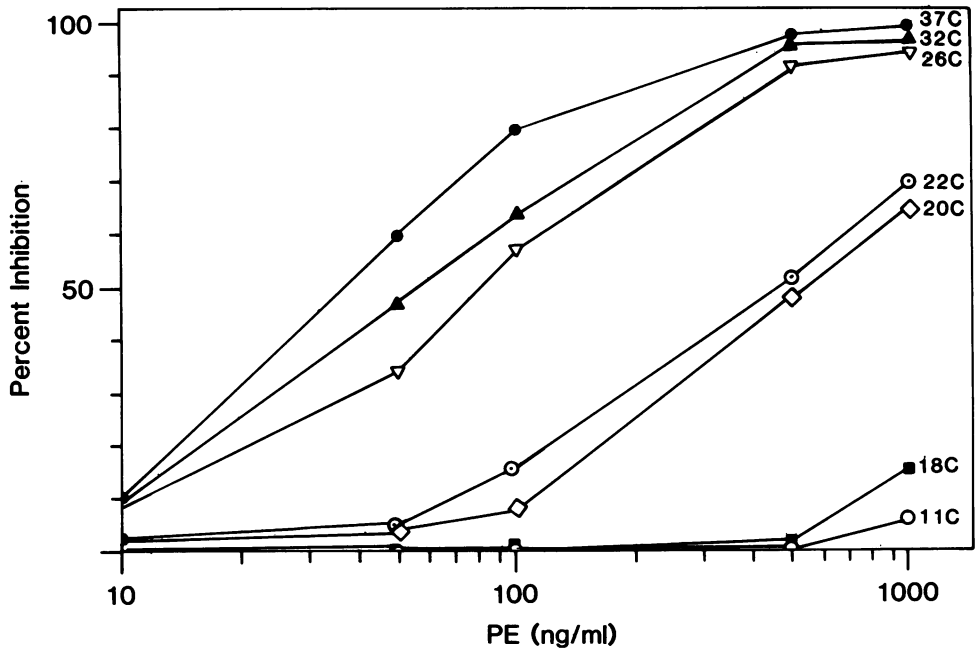

FIG. 7. Effect of temperature on expression of PE toxicity. PE was bound to cells at 4°C for 30 min, unbound toxin was removed by washing, and cells were warmed to temperature for 5 h. Cells were then pulsed for 30 min at 37°C. Percent inhibition was determined by comparing the counts per minute per microgram of protein in toxin-treated samples with that in control cells maintained at the same temperature. Symbols: ●, 37°C; ▲, 32°C; ▽, 26°C; ⊙, 22°C; ◇, 20°C; ■, 18°C; ○, 11°C

The data presented for fixed LM cells suggest that the criteria of bona fide receptor function (2) are satisfied when fixed cells are incubated with PE.

Furthermore, the ultrastructural studies lead to a similar conclusion by a completely different method of analysis. At 4°C, binding of biotinyl-PE to LM cells is saturable and can be blocked with excess native toxin (i.e., is competitive). In contrast to radiochemical assays, the electron microscopic method allows selectivity in quantitating toxin binding sites on the cell surface. Electron microscopy permitted discrimination of specific cell-bound toxin from nonspecifically trapped (by pseudopodia) or glass-associated toxin. The ultrastructural analysis is highly reproducible and routinely reveals a consistent number of toxin sites on the cell surface (18).

The data presented here represent the first demonstration of a receptor for PE. Ray and Wu (20) reported binding of PE to CHO cells, but their data did not indicate whether binding is mediated by specific receptors. In the experiments described here, ^{125}I-labeled PE binding to paraformaldehyde-fixed LM cells is specific, and the addition of native PE displaces 80% of the bound radiolabeled ligand. Binding is reversible, since 50% of bound ^{125}I-labeled PE was displaced by unlabeled PE within 3 h (Fig. 4). Binding is also saturable. At PE concentrations above 1 μg/ml, the proportion of toxin specifically bound represents an ever-decreasing fraction of the total. Although the total amount of bound toxin increases when cells are incubated with concentrations of PE above 1 μg/ml, the fact that the specificity is reduced to less than 50% of the total suggests that the population of high-specificity receptors is saturated. This saturation is probably masked at high (non-physiological) concentrations, at which PE may bind to a second low-affinity population of receptor sites that are not saturated at a toxin concentration of 10 μg/ml. Such a conclusion is supported by competition studies showing that not all ^{125}I-labeled PE was displaced by native PE, even when added at a 1,000-fold excess. Lastly, the affinity of the receptor (2 to 5 nM) is in good agreement with the minimal concentrations of PE (1 to 2 nM) that are required for expression of toxicity. Given all of these facts, it is likely that receptor-bound toxin is responsible for cell death.

After the successful demonstration of receptor-mediated binding of PE and subsequent internalization of the complex by RME, reduced temperatures of incubation were used as a strategy to study intracellular processing and activation of toxin by LM cells. Low temperatures ablate expression of PE activity. This inhibition could be explained in several ways; these include (i) preventing internalization of enough toxin to kill cells, (ii) inhibiting activation of PE (acidification step), (iii) blocking escape of toxin into the cytosol, and (iv) reducing ADP ribosyltransferase activity. The fact that ADP ribosyltransferase activity is maximal in vitro between 18 and 30°C would rule against the last explanation. The shift in efficient expression of toxicity also cannot be explained by a failure to internalize a sufficient number of toxin molecules to stop protein synthesis. Incubation of cells with 100 ng of PE per ml for 2 min at 37°C leads to a 50% inhibition of protein synthesis (5). The internalization rates calculated from Fig. 5 indicate that equivalent amounts of PE are internalized in 2 min at 37°C and in 12 min at 10°C. Since sufficient toxin was internalized at all temperatures examined to inhibit protein synthesis, the absence of toxicity at lowered temperatures may be ascribed to the different modes of PE entry or routes of intracellular trafficking operative above and below 20°C. Above 20°C, PE is internalized and encounters an environment which facilitates toxin activation and escape of internalized toxin into the cell cytoplasm; therefore, toxicity is expressed. Below 20°C, PE internalization still proceeds but toxicity is minimal. It can

be hypothesized that low temperatures block one or more of the following: the clustering of receptors into coated regions, the acidification of endosomes, dissociation of ligand and receptor, and membrane fusion events. Preliminary experiments in our laboratory show that at 19°C, biotinyl-PE moves rapidly (30 to 60 s) into coated pits, but remains there for up to 10 min. Thus, clustering occurs at both 19 and 37°C, but the internalization process is delayed at the lower temperature. Dunn et al. (4) have shown that the degradation of asialofetuin in a perfused rat liver is blocked below 20°C; this is due to blockage of fusion of endosomes with lysosomes. Wolkoff et al. (31) have examined the processing of asialoglycoproteins by rat hepatocytes. Their data suggest that reduced temperatures (18°C) alter receptor-ligand dissociation and the delivery of ligand to lysosomes. Oka and Weigel (19) also found that the slow dissociation of intracellular receptor-ligand complexes and ligand degradation stop at 18°C. Marsh et al. (13) have shown that at 20°C, Semliki Forest virus is internalized by RME but is not delivered to lysosomes. In a more recent study involving fusion mutants of Semliki Forest virus, Kielian et al. (10) have shown that acidification occurs normally at 20°C in BHK cells.

Studies are under way in our laboratory to determine the step(s) in toxin internalization that is blocked at low temperatures. At present, we predict that escape from endosomes is reduced. Toxin accumulates in endosomes with increasing time at 37°C (16). The numerous membrane fusion events which occur might provide a chance for escape of active toxin molecules into the cytoplasm. Blockage of toxin-receptor dissociation or reduction of endosome-endosome fusion or both would reduce the chance of a random escape and result in reduced toxicity.

In summary, we have characterized the association of ^{125}I-labeled PE with LM cells and have shown that the interaction is mediated by a receptor. We calculate that each LM cell possesses ca. 100,000 high-affinity PE receptors with a K_d of 5.4 nM. In addition, there appears to be a still undetermined number of low-affinity toxin receptors on the surface of LM cells. We also have shown by direct comparison of RME and fluid-phase endocytosis that a temperature-dependent step unique to RME becomes severely rate limiting below 20°C. The biological activity of PE is also inhibited at the reduced temperature. The correlation between endocytic rate of toxin uptake and expression of its activity (i.e., inhibition of protein synthesis) shows that a normal step in toxin processing is blocked at reduced temperatures.

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