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Calcium Is Required for the Expression of Anthrax Lethal Toxin Activity in the Macrophagelike Cell Line J774A.1

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Anthrax lethal toxin, which consists of two separate proteins, protective antigen (M_r, 82,700) and lethal factor (M_r, approximately 83,000), is cytotoxic to the macrophagelike cell line J774A.1. Removal of calcium from the culture medium protected cells against the action of lethal toxin. Calcium depletion during the binding phase of intoxication afforded only partial protection. Further analysis showed that calcium removal caused some inhibition of protective antigen binding but that it had minimal effect on proteolytic conversion of protective antigen to the active 63-kilodalton fragment and that it had no effect on lethal factor binding. Cells to which lethal toxin had bound in the presence of calcium were protected when transferred to calcium-depleted culture medium, indicating a role for calcium at a postbinding stage. When ammonium chloride is present with lethal toxin, toxin accumulates in intracellular vesicles. Calcium-free medium protected these cells upon removal of the amine block, suggesting that calcium is also required at a step after internalization of lethal toxin. Calcium channel blockers inhibited $^{45}Ca^{2+}$ uptake and protected cells against cytotoxicity. Calmodulin inhibitors also protected against the action of lethal toxin, suggesting involvement of calmodulin at a step during intoxication. We conclude that calcium is required at several steps in the intoxication of cells by anthrax lethal toxin.

Many of the most potent bacterial protein toxins act by enzymatic inactivation of essential intracellular macromolecules. These toxins appear to share a common mechanism for gaining entry to the cytosol of target cells. This mechanism involves binding to a specific cell membrane receptor, followed by endocytosis, and translocation across a vesicular membrane to interact with the target molecule in the cytosol (7, 33, 35).

Anthrax toxin has long been thought to be an important virulence factor contributing to the pathogenesis of anthrax. The anthrax toxin is actually composed of two separate protein exotoxins, which we designate edema toxin and lethal toxin (12). Both toxins conform to the general model characteristic of many protein toxins in that they possess a B (binding) moiety and an A (enzymatically active) moiety (13). The anthrax toxins differ from most toxins in that the B and A components are separate, noncovalently linked proteins, and they are unique in that both toxins share a single B protein, the protective antigen (PA; 82.7 kilodaltons [kDa]). Thus, the edema toxin consists of PA acting with edema factor (EF; 88.8 kDa), while the lethal toxin consists of PA and lethal factor (LF; approximately 83 kDa) (26; A. M. Friedlander, *in* C. Saelinger (ed.), *Trafficking of Bacterial Toxins*, in press). As indicated by the fact that the B and A functions are located on separate proteins, each of the three protein components is inactive alone. Edema toxin produces edema in the skin of experimental animals, consistent with the demonstration that EF is a calcium- and calmodulin-dependent adenylate cyclase (25). The lethal toxin causes death in certain experimental animals and lyses mouse macrophages; a low-intravesicular-pH environment is necessary for its action (12). Macrophages appear to be uniquely susceptible to this toxin; LF concentrations of 1 to 10 ng/ml cause lysis (12).

The current evidence suggests that PA binds to a high-affinity receptor on the cell membrane, where it is proteolytically cleaved to a protein of approximately 63 kDa, thereby creating a binding site for LF or EF. The toxin appears to be internalized by receptor-mediated endocytosis and to pass through an acidic compartment to reach the cytosol (27; Friedlander, in press). By analogy to EF and other bacterial toxins, LF is assumed to act enzymatically, but the putative activity has not been identified, and the molecular events leading to cell death remain unknown.

It is well known that transport of molecules across biological membranes often has specific ion requirements (4, 18, 41, 53). More recently, calcium has been shown to be involved in the entry and action of several bacterial protein toxins, including diphtheria toxin (40), *Pseudomonas* exotoxin A (10), pseudomonal cytotoxin (42), shiga toxin (39), staphylococcal alpha-toxin (49), and *Clostridium perfringens* enterotoxins (19), as well as the plant protein toxins abrin and modeccin (40). In an attempt to elucidate the molecular mechanism of action of anthrax lethal toxin, we have studied the requirement for calcium in the cytotoxic process. Data demonstrate that calcium is required for maximum toxin binding and that an influx of calcium is required for cell killing by the lethal toxin. The process of intoxication may also require participation of calmodulin, because antagonists of this protein protected cells against the lethal effect of toxin.

MATERIALS AND METHODS

Abbreviations. The abbreviations used in this paper are as follows: DMEM, Dulbecco modified Eagle medium; EF, edema factor; EMEM, Eagle minimum essential medium with Earles balanced salts solution; HBSS, Hanks balanced salt solution; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; kDa, kilodalton; LDH, lactate acid dehydrogenase; LF, lethal factor; PA, protective antigen; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel

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electrophoresis; TFP, trifluoperazine; W-7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide.

Cell culture. J774A.1, a macrophagelike cell line, was obtained from the American Type Culture Collection (Rockville, Md.) and maintained in DMEM containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). For cytotoxicity experiments, 10^5 cells were plated in 1 ml of medium in a 2-cm^2, 24-well plastic tissue culture plate and grown for 2 days to approximately 80 to 90% confluence. Cell culture supplies were obtained from GIBCO Laboratories (Grand Island, N.Y.). Calcium-free EMEM was analyzed by atomic adsorption spectroscopy and found to contain 56 μM total calcium. Fetal bovine serum treated with Chelex to deplete calcium (residual amount of calcium, 88 μM) was kindly provided by Ulrike Lichti of the National Cancer Institute, National Institutes of Health, Bethesda, Md.

Chemicals and reagents. PA and LF were purified from *Bacillus anthracis* culture supernatants as described by Leppla (26). Biochemicals were obtained from Sigma Chemical Co. (St. Louis, Mo.). Nitrendipine was a gift from Heather Weber, University of Southern California, Los Angeles. Trypsin was obtained from Worthington Diagnostics (Freehold, N.J.).

The proteolytically activated PA fragment of approximately 63 kDa was prepared by trypsin activation of PA. Briefly, PA (18 mg) was incubated with 6 U of trypsin in a total volume of 9 ml of HEPES buffer, containing 10 mM $CaCl_2$ and 5 mM EDTA (pH 7.5), for 30 min at room temperature. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride. The 63-kDa fragment was separated from the 20-kDa fragment on a Mono Q column (Pharmacia Fine Chemicals, Piscataway, N.J.). The fractions containing the 63-kDa fragment were pooled and dialyzed against 0.01 M CHES buffer (2-[*N*-cyclohexylamino]-ethanesulfonic acid), pH 9.0.

Cytotoxicity assays. Cytotoxicity in response to anthrax lethal toxin was determined on the basis of the amount of LDH in control or toxin-treated cell monolayers, as described previously (12). LDH in control monolayers varied from 265 to 315 mU per well in different experiments. In some experiments, cytotoxicity was also determined by trypan blue exclusion (0.05%) or protein synthesis inhibition. The assay for protein synthesis was as described by Draper and Simon (6), except that cells were exposed for 15 min to [^{35}S]methionine (1,000 Ci/mmol) in methionine-free medium. The data from representative experiments are presented as the percent of controls, determined by using the means of triplicate samples, which differed by less than 10%. Each experiment was repeated more than twice.

Radioiodination of PA and LF. PA (90 μg) was radioiodinated with 1 mCi of [^{125}I]Bolton-Hunter reagent (2,000 Ci/mmol), as described by the manufacturer, except that the reaction was carried out in a 55-μl volume in 0.1 M HEPES buffer, pH 8.0, at room temperature for 40 min. The labeled PA was purified by passage over a Sephadex G-25 column (Pharmacia) equilibrated with 0.25% gelatin in phosphate-buffered saline and was stored in phosphate-buffered saline containing 1% bovine serum albumin. Specific activity was 1×10^7 to 1.5×10^7 cpm/μg, and the labeled PA retained 90 to 100% of its biological activity as assessed on the basis of macrophage cytotoxicity.

LF was radioiodinated by reacting 40 μg of LF with 1 μg of chloramine T and 1 mCi of Na^{125}I (2 Ci/mmol) in 40 μl of 0.1 M phosphate buffer, pH 7.0, at 4°C for 5 min. The reaction was stopped by the addition of sodium metabisulfite (2 μg in 10 μl), and LF was purified on a Sephadex G-25 column presaturated with bovine serum albumin. The specific activity obtained was about 1.5×10^7 to 2.0×10^7 cpm/μg, and the labeled LF retained 50 to 90% of its biological activity.

Binding studies. J774A.1 cells in 12-well plates were washed twice with cold HBSS for 5 min each time and then placed on ice. The medium was replaced with a saturating concentration (A. Friedlander et al., manuscript in preparation) of [^{125}I]PA (1 μg/ml) in cold binding medium (EMEM without sodium bicarbonate containing 1% bovine serum albumin and 25 mM HEPES, pH 7.4). After incubation for 3 h, cells were washed four times with cold HBSS to remove unbound PA and dissolved in 0.1 N NaOH, and radioactivity was counted. For LF binding, cells were first incubated with nonradioactive PA (1 μg/ml) for 16 h at 4°C to saturate the receptors. Cells were then washed four times to remove unbound PA and reincubated with a saturating concentration (Friedlander et al., in preparation) of [^{125}I]LF (1 μg/ml) in binding medium for 5 h at 4°C. Cells were then washed four times with cold HBSS to remove unbound LF and dissolved in 0.1 N NaOH, and radioactivity was counted. Data are presented as specific binding, defined as the difference between the mean binding of triplicate samples in the absence or presence of a 100-fold molar excess of nonradioactive ligand. The binding of triplicate samples varied by less than 10% of the mean. Nonspecific binding in the presence of nonradioactive PA or LF was about 10%.

Proteolytic cleavage of PA on the cell surface. Cells were incubated with radioactive PA, as described above, for 3 h. Unbound PA was then removed by washing the cells four times with cold HBSS, and the cells were reincubated for 16 h at 4°C to allow proteolytic cleavage of PA to the 63-kDa fragment to occur on the cell surface. The cells were then washed three times with cold HBSS, heated at 100°C for 5 min in 5% glycerol–1% SDS–100 mM dithiothreitol–0.01% bromophenol blue, and analyzed by SDS-PAGE on 10% polyacrylamide gels (24). Gels were stained with Coomassie blue, dried, and exposed to film. The labeled bands at 83 and 63 kDa were excised, and radioactivity was counted.

Calcium uptake. For calcium uptake studies, the medium overlaying the cells was replaced with DMEM containing 10% fetal bovine serum with $^{45}CaCl_2$ (10 μCi/ml; 17 mCi/mg of calcium) and incubated for 10 min. Cells were then washed four times with HBSS and dissolved in 0.1 N NaOH, and radioactivity was counted.

RESULTS

Calcium requirement for cell killing by lethal toxin. Initial experiments in this laboratory (A. Friedlander, unpublished results) revealed that the mouse macrophagelike cell line J774A.1 is sensitive to the acute cytolytic effects of lethal toxin, as had been shown for mouse peritoneal macrophages (12). The J774A.1 cell line was used in all experiments in this report. Cells incubated for 3 h in medium containing lethal toxin showed dose-dependent cytotoxicity, as measured on the basis of the loss of LDH, whereas cells washed three times with calcium-free medium prior to toxin addition were not affected (Fig. 1). Complete protection was observed, even at PA and LF concentrations of 10 μg/ml each (data not shown), which was 100-fold higher than the concentration needed to kill >80% of J774A.1 cells in calcium-containing medium in 3 h. Protection was also achieved by washing cells with 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid] or H-EDTA (*N*-hydrox-

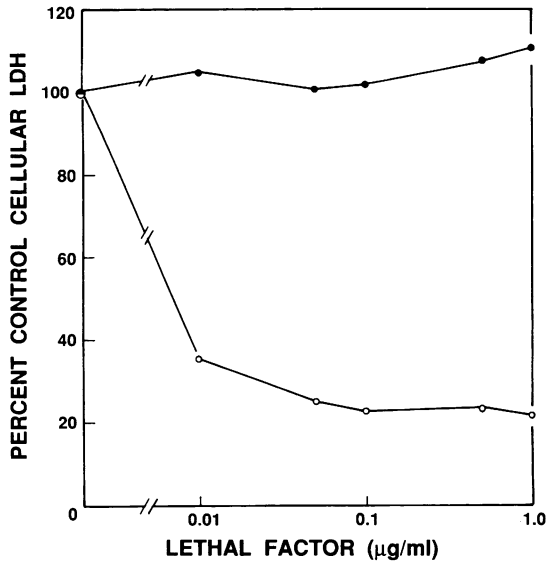

FIG. 1. Requirement for calcium for expression of anthrax lethal toxin cytotoxicity. J774A.1 cells were washed three times with EMEM without calcium. Cells were then exposed to lethal toxin (100 ng of PA per ml plus various amounts of LF) in EMEM containing 10% Chelex-treated fetal bovine serum with (○) or without (●) 1 mM $CaCl_2$. After 3 h at 37°C, cellular LDH was measured. There was no significant difference between controls, which consisted of cells incubated in medium with or without calcium in the absence of toxin.

yethylethylenediaminetriacetic acid). When calcium-free medium was supplemented with known concentrations of calcium (Fig. 2), toxicity was restored to 50% of the control level at 0.05 mM calcium chloride and to 100% of the control

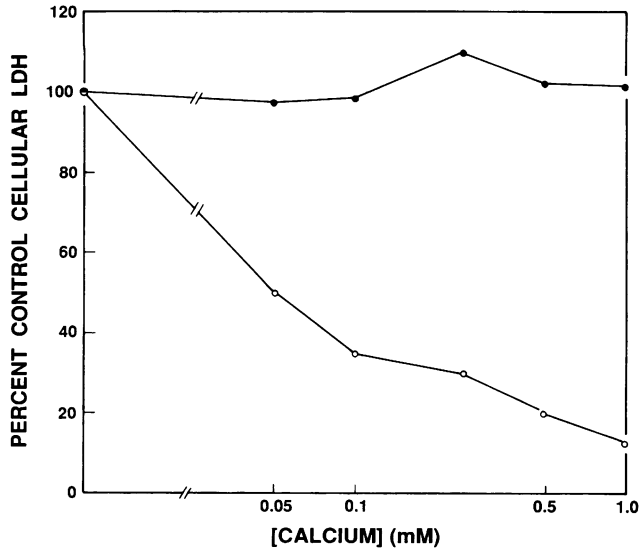

FIG. 2. Calcium concentration required for expression of anthrax lethal toxin cytotoxicity. J774A.1 cells were washed three times with EMEM without calcium. Cells were then incubated with (○) or without (●) lethal toxin (100 ng of PA per ml and 100 ng of LF per ml) in EMEM with 10% Chelex-treated fetal bovine serum in the presence of various concentrations of $CaCl_2$. Cellular LDH was measured after incubation of cells for 3 h at 37°C. The control consisted of cells incubated without calcium in the absence of toxin.

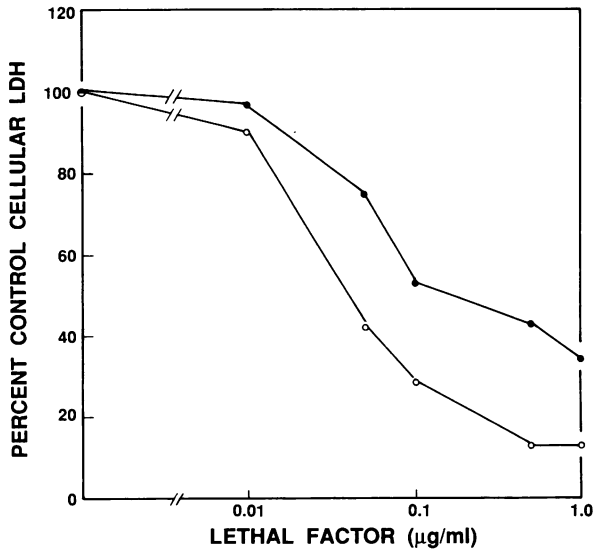

FIG. 3. Effect of calcium depletion during binding of anthrax lethal toxin. J774A.1 cells were washed three times with EMEM without calcium and exposed to lethal toxin (1 μg of PA per ml and various amounts of LF) in the presence (○) or absence (●) of 1 mM $CaCl_2$ in EMEM containing 10% Chelex-treated fetal bovine serum at 37°C for 1 h. After being washed to remove unbound toxin, cells were reincubated with DMEM with 10% fetal bovine serum for 2 h and LDH was measured.

level at 1 mM calcium chloride. Calcium depletion was similarly found to protect against lethal toxin when toxicity was measured by trypan blue exclusion or protein synthesis inhibition (data not shown).

Effect of calcium on interaction of PA and LF with cell surface receptor. To determine whether the decreased toxicity in the absence of extracellular calcium resulted from decreased toxin binding, we incubated cells with toxin in the presence or absence of calcium for 1 h to allow toxin to bind. Unbound toxin was then washed away, cells were reincubated in the presence of calcium, and toxicity was measured 2 h later. Binding of toxin in the absence of calcium afforded only partial protection against cytotoxicity (Fig. 3). To more clearly understand at which stage calcium depletion exerts its effect, we measured the binding of [^{125}I]PA to the cells at 4°C. Table 1 indicates that there was approximately 50% less binding of [^{125}I]PA in the absence of extracellular calcium. Further studies will be necessary to determine whether this is due to a change in the affinity or number of the receptors.

Following binding of PA to the cell receptor, the next step in the intoxication process is proteolytic cleavage of PA to a 63-kDa protein with the creation of a binding site for LF. To test whether calcium depletion inhibited proteolytic processing of PA, we bound [^{125}I]PA to the cell surface for 3 h in the presence of calcium at 4°C to avoid any effect of calcium depletion on binding. Cells were transferred to calcium-free medium and incubated for 16 h at 4°C to allow PA to be nicked. Analysis by SDS-PAGE showed only a slight inhibition of conversion of PA to the 63-kDa fragment in the absence of calcium. Proteolytic conversion was 52 and 42% in the presence and absence of calcium, respectively.

The next step in the action of toxin is the binding of LF to the 63-kDa PA fragment bound on the cell surface. To determine the effect of calcium depletion on LF binding, PA was allowed to bind and be nicked on the cell surface in the presence of calcium during overnight incubation at 4°C.

TABLE 1. Effect of calcium depletion and TFP on binding of PA and LF to J774A.1 cells

Medium	Specific binding[a] of:	
	PA[b]	LF[c]
Control medium	15,398 ± 304	14,859 ± 197
Medium without Ca^{2+}	7,806 ± 498	14,472 ± 141
Medium with TFP (25 μM)	5,527 ± 90	ND

[a] Difference, in mean counts per minute (± standard error), of triplicate samples bound with or without a 100-fold molar excess of nonradioactive ligand.

[b] J774A.1 cells were preincubated with TFP for 1 h at 37°C. In the case of calcium-depleted medium, cells were washed three times with EMEM without calcium. Cells were cooled and incubated with [^{125}I]PA (1 μg/ml) in the respective medium for 3 h.

[c] PA (1 μg/ml) was first allowed to bind for 16 h in the presence of calcium at 4°C. Cells were then washed three times with EMEM without calcium, [^{125}I]LF (1 μg/ml) was added with or without calcium, and cells were incubated for 3 h. ND, Not determined.

[^{125}I]LF was then added, and binding was measured in the presence or absence of calcium. Results presented in Table 1 show that calcium had no effect on the binding of LF.

Temporal analysis of calcium-dependent steps. The results presented above demonstrate that the complete protection afforded by calcium depletion over a 100-fold concentration range is not the result of impaired binding of toxin. These results suggest that calcium also plays a major role in a step subsequent to binding. This view was supported by experiments showing that cells incubated with PA and LF could still be protected from killing by removal of calcium up to 60 min after the addition of toxin (data not shown).

To identify more clearly the calcium-requiring step(s) involved in intoxication, we analyzed the role of calcium in the steps subsequent to binding and nicking. In the initial experiments of this type, we used cells with the 63-kDa PA fragment and LF prebound at 4°C in calcium-containing medium. Unbound toxin was then washed away, and cells were reincubated in medium with or without calcium at 37°C. As seen in Fig. 4, calcium depletion completely protected cells against lethal toxin. Since calcium depletion did not decrease LF binding (Table 1) or release cell-bound LF (unpublished data), it follows that calcium is needed at a step subsequent to toxin binding.

Effect of calcium depletion after lethal toxin internalization. After binding to cells, toxin is internalized and passes through an acidic intracellular compartment, as shown by the ability of amines to inhibit cytotoxicity (12). To further define temporally the calcium-requiring step(s), we asked whether calcium is required at a stage subsequent to the amine-sensitive step. Cells were preincubated with 10 mM NH_4Cl and exposed to toxin in calcium-containing medium for 90 min. Under these conditions, toxin is located mainly intracellularly (12). These cells were then washed with calcium-free medium to remove NH_4Cl, unbound toxin, and calcium and were reincubated in medium with or without calcium. We observed that, even under these conditions, removal of calcium protected cells from lethal toxin, whereas cells died when calcium was present (Fig. 5). Thus, extracellular calcium was required at a stage after the amine-sensitive step.

Effect of calcium channel blockers on sensitivity of J774A.1 cells to anthrax lethal toxin. The plasma membrane of eucaryotic cells maintains a low cytosolic calcium ion concentration, 10^{-6} to 10^{-8} M (9, 37), despite a very high extracellular calcium concentration, 10^{-3} M. This calcium ion gradient is maintained by energy-dependent pumping of

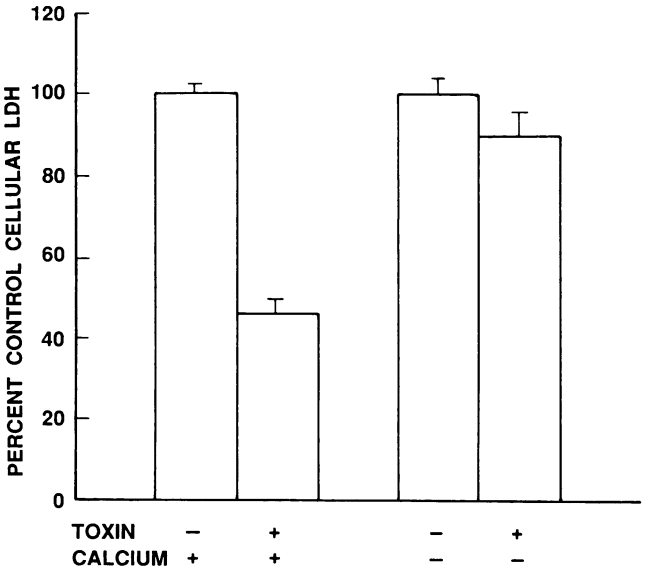

FIG. 4. Effect of calcium depletion, after toxin binding, on the cytotoxicity of anthrax lethal toxin. J774A.1 cells cooled by washing twice with cold EMEM were exposed to the 63-kDa PA fragment plus LF (each at 5 μg/ml) in binding medium for 3 h at 4°C. Cells were then washed three times with cold EMEM without calcium and incubated at 37°C for 3 h in the presence or absence of 1 mM $CaCl_2$ in EMEM with 10% Chelex-treated fetal bovine serum. Cellular LDH was then assayed. There was no difference between controls, which consisted of cells cultured under similar conditions in the absence of toxin that were reincubated in medium with or without calcium. Data are presented as the means ± standard error of triplicate samples.

calcium from the cytosol to mitochondria, endoplasmic reticulum, and the extracellular medium (9, 37). The requirement of extracellular calcium for expression of lethal toxin activity, demonstrated in Fig. 1, could be due to the necessity for uncontrolled calcium influx, which has been suggested to be a final common mediator of cell death (8, 36, 43). Therefore, we measured the influx of $^{45}Ca^{2+}$ as a function of time after the addition of lethal toxin. As shown in Fig. 6A, there was a dramatic rise in the influx of calcium beginning at 90 min, before any evidence of cell death (Fig. 6B). Next, we examined the effect of the calcium channel blockers verapamil (14) and nitrendipine (3) on lethal toxin activity. In initial experiments, we determined that a verapamil or nitrendipine concentration of 100 μM was required to inhibit $^{45}Ca^{2+}$ uptake in normal or lethal-toxin-treated cells. Preincubation of cells with this concentration of verapamil protected them against lethal toxin cytotoxicity (Fig. 6B). Verapamil was found to protect cells against PA and LF concentrations of 1 μg/ml each (highest concentrations tested; data not presented). Similar results were obtained with nitrendipine. These observations suggest that calcium ion influx is required to kill cells. In agreement with these results, Co^{2+} and La^{3+}, which also block the calcium channel (11, 15, 50, 54), protected cells from lethal toxin when they were present at concentrations of 5 and 10 mM, respectively (data not shown).

Protective effect of calmodulin inhibitors. The requirement for calcium in the action of the anthrax lethal toxin could be due to the involvement of calmodulin (32). To study this possibility, we examined the effect of the calmodulin inhibitors, TFP (29) and the aminonaphthol derivative W-7 (16, 22), on the lethal toxin activity. The presence of 25 μM TFP

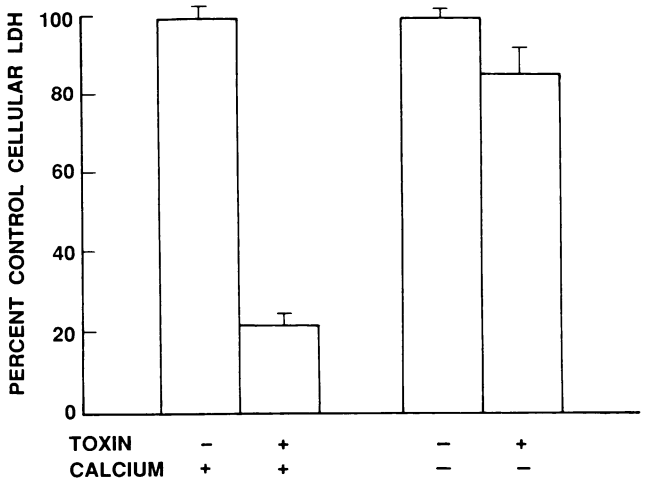

FIG. 5. Effect of calcium depletion (after the amine-inhibitable step) on the cytotoxicity of anthrax lethal toxin. J774A.1 cells were preincubated with 10 mM NH_4Cl for 30 min at 37°C. Lethal toxin (1 μg/ml each of PA and LF) was added, and cells were incubated for an additional 90 min. At this time, cells were washed three times with EMEM without calcium and reincubated, in the absence of NH_4Cl, with or without 1 mM $CaCl_2$ for 2 h. Cellular LDH was then assayed. There was no difference between the LDH values of the controls, which consisted of cells preincubated with NH_4Cl that were not exposed to toxin before reincubation in medium with or without calcium. Data are presented as the means ± standard error of triplicate samples.

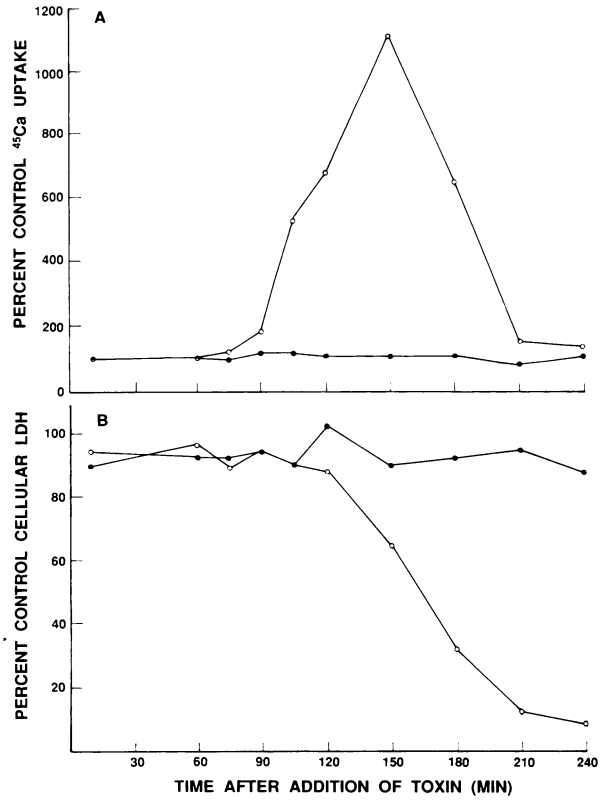

FIG. 6. Effect of verapamil on the time course of calcium influx and cytotoxicity induced by anthrax lethal toxin. J774A.1 cells were preincubated with (●) or without (○) 100 μM verapamil for 1 h. Lethal toxin (PA and LF, each at 100 ng/ml) was added, and at the indicated times ^{45}Ca influx (A) and LDH release (B) were measured in parallel experiments. ^{45}Ca uptake (in counts per minute per well per 10 min) in control cells incubated without toxin was 754 ± 33 and 1,151 ± 62 cpm/10 min per well in cells treated with and without verapamil, respectively.

or 40 μM W-7 had a strong protective effect against anthrax lethal toxin (Fig. 7). Preincubation with TFP reduced PA binding by 64% (Table 1) but had no effect if added only during binding, without preincubation. Preincubation with TFP had no effect on PA nicking (data not shown).

Effects of protease inhibitors. The requirement for extracellular calcium in the expression of lethal toxin activity, together with the requirement for proteolytic activation of PA, suggested that calcium-dependent proteases may be involved. Therefore, we measured the protective activity of leupeptin and 1,10-*O*-phenanthroline, known inhibitors of calcium-dependent proteases (21). No protection against lethal toxin was afforded by preincubating cells for 1 h with 1 mM leupeptin or 1,10-*O*-phenanthroline; concentrations greater than 1 mM were toxic (data not shown). These results imply that calcium-dependent proteases do not play a role in the expression of lethal toxin activity.

DISCUSSION

Data presented in this paper provide evidence that calcium plays a significant role in the expression of lethal toxin activity. Anthrax lethal toxin was not able to kill the murine macrophagelike cell line J774A.1 when calcium was omitted from the culture medium. Magnesium was not able to substitute for calcium (data not presented). Calcium has previously been shown to be required for the action of diphtheria toxin (40), *Pseudomonas* exotoxin A (10), pseudomonal leukocidin (17), shiga toxin (39), and the plant toxins abrin, modeccin, and ricin.

Throughout this study, cytotoxicity was measured on the basis of the release of cytoplasmic LDH. The possibility existed that calcium depletion could interfere with cellular LDH release without actually inhibiting cell death. However, other markers of cell viability, including protein synthesis and trypan blue exclusion, confirmed that cells exposed to lethal toxin in calcium-free medium were fully protected against killing.

We found that calcium depletion had only a modest effect on the binding of toxin (Fig. 3, Table 1), similar to what has been reported for diphtheria toxin (40). Calcium depletion inhibited proteolytic activation of PA only minimally, and binding of LF was not affected at all. Thus, the effect of calcium depletion appears to be predominantly at a postbinding step of intoxication.

When calcium was depleted after binding of PA and LF, cells were completely protected against killing (Fig. 4). Cells preincubated with NH_4Cl internalize toxin, which accumulates intracellularly without causing cell death (12). Upon removal of the amine block, calcium depletion still totally protected these cells from killing (Fig. 5). These experiments suggest a role for calcium at a stage following binding and internalization of the toxin.

Lethal toxin was found to cause a large influx of $^{45}Ca^{2+}$ into cells; this influx was totally blocked by pretreatment with 100 μM verapamil (Fig. 6A). The same concentration of verapamil also protected against anthrax lethal toxin cytotoxicity (Fig. 6B). Similarly, verapamil has been reported to protect cells against abrin, modeccin, and, to some extent, ricin, but it has no protective effect against diphtheria toxin (40). Verapamil has also been shown to enhance the cyto-

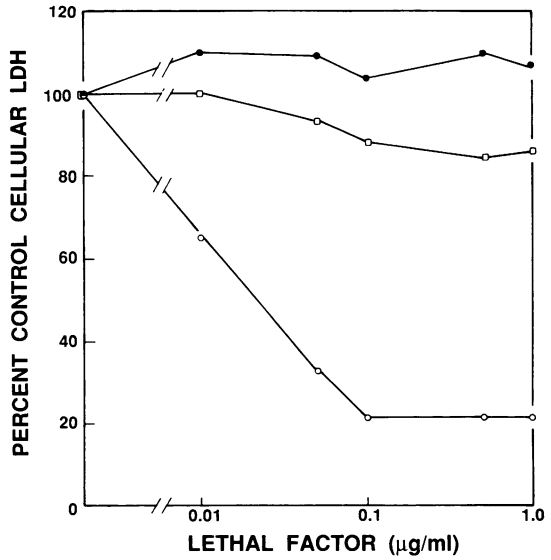

FIG. 7. Ability of TFP and W-7 to protect cells against anthrax lethal toxin. J774A.1 cells were preincubated in 25 μM TFP (●), 40 μM W-7 (□), or unamended medium (○) for 1 h at 37°C. Lethal toxin (PA [100 ng/ml] plus the indicated amount of LF) was added, and cells were incubated for 3 h at 37°C. Cellular LDH was then assayed. Controls consisted of cells incubated with TFP, W-7, or unamended media which were not exposed to toxin. There was no significant difference in the LDH values of controls treated with and without inhibitors.

toxic effect of a conjugate of epidermal growth factor and *Pseudomonas* exotoxin A, perhaps by blocking its degradation within cells (1, 2).

The protection against lethal toxin afforded by preventing the influx of calcium by either removal of extracellular calcium or use of calcium channel blockers is consistent with the hypothesis that an increase in the cytosolic calcium concentration due to the influx of extracellular calcium is the final mediator of cell death caused by different toxic substances and conditions (8, 36, 43). The mechanism by which an increased calcium concentration in the cytosol causes cell death has been postulated to include, under different conditions, activation of calcium-dependent enzymes, including endonucleases (5, 31), proteases (46–48), and phospholipases (30, 34). Determination of the relevance of these observations to lethal toxin will require further experimentation.

The inability of inhibitors of calcium-dependent proteases (leupeptin and 1,10-*O*-phenanthroline) to block lethal toxin action is consistent with our results, which show that calcium depletion had a minimal effect on protease nicking of cell-bound PA. Furthermore, it suggests that leupeptin-sensitive, intracellular proteases were not involved in any cell processing of PA and LF required for cytotoxicity or in the final mechanism of cell death, as has been implicated after calcium influx in some cell types (8, 36, 43, 46, 51).

Calcium may not be required directly for the expression of lethal toxin. Rather, calcium may be important for activating the calcium-binding protein, calmodulin. The present results do support an involvement of calmodulin because the known calmodulin inhibitors, TFP and W-7, protected J774A.1 cells against killing by anthrax lethal toxin. Because TFP was found to inhibit binding of PA to cell receptors, a possible mechanism may involve phosphorylation or dephosphorylation of proteins by calmodulin-dependent protein kinases (32, 44, 45), resulting in modification of the PA receptor so that it cannot bind toxin. Phosphorylation of the cytoplasmic domain of the epidermal growth factor receptor has been shown to sharply reduce the affinity of the receptor for its ligand (45). Depending on the cell type, TFP itself has been reported to decrease or have no effect on epidermal growth factor receptors (48). While our data show that TFP altered the binding of PA, it is certainly possible that TFP has additional effects on postbinding processes, as has been reported for other ligands. For example, TFP protects against modeccin while having no effect on its binding (40). In some cell types, calmodulin inhibitors increase sensitivity to epidermal growth factor and an epidermal growth factor-*Pseudomonas* exotoxin A conjugate, perhaps by delaying degradation of the ligands (23). Furthermore, TFP inhibits phagocytosis in J774A.1 cells (20). These studies, showing abnormalities in endosome or lysosome function, and additional reports of calmodulin involvement in the movement of endocytosed material to lysosomes (38, 52) suggest that calmodulin inhibitors could be responsible for interference with the processing and intracellular transport of anthrax toxin. Finally, calmodulin may be involved in the final cytolytic mechanism of lethal toxin, which remains obscure.

In conclusion, we report that calcium is involved in several stages in the intoxication of cells with anthrax lethal toxin. Calcium depletion inhibited somewhat the binding of PA to cell receptors, and calcium was necessary, at a stage after toxin binding and internalization, for the expression of cytotoxicity. Blocking of calcium influx by any means protected the cells. The inhibition by calmodulin inhibitors suggests that lethal factor may share with the adenylate cyclase component of anthrax edema toxin a requirement for calmodulin in the expression of its activity. Further studies are in progress to elucidate the exact mechanism of action of anthrax lethal toxin at the molecular level.

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