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Anthrax Edema Toxin Requires Influx of Calcium for Inducing Cyclic AMP Toxicity in Target Cells

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The anthrax edema toxin comprises two proteins: protective antigen and edema factor. Anthrax protective antigen binds to the receptors on the surface of target cells and facilitates the entry of edema factor into these target cells. Edema factor (EF) is an adenylate cyclase that catalyzes the synthesis of cyclic AMP (cAMP) in the cytosol of the host cells. In this study, we examined the requirement of extracellular calcium for anthrax edema toxin-induced toxicity in host cells. The cAMP response generated by edema toxin was analyzed in a variety of cells, including CHO, macrophage-like RAW264.7, human neutrophils, and human lymphocytes. Our investigations reveal that after EF reaches the cell cytosol, a rapid influx of calcium is triggered in the host cell that has a pivotal role in determining the cAMP response of the affected cells. Although the cAMP response generated by edema toxin in different cell types varied in intensity and in the time of initiation, the influx of calcium invariably preceded cAMP accumulation. Agents that blocked the uptake of calcium also inhibited edema toxin-induced accumulation of cAMP in the host cells. This is the first report that demonstrates that edema toxin induces accumulation of cAMP in lymphocytes. By accumulating cAMP, a potent inhibitor of immune cell function, edema toxin may actually be poisoning the immune system and thus facilitating the survival of the bacteria in the host.

Anthrax, which is primarily a zoonotic disease, is transmissible from animals to humans. The causative agent of anthrax, *Bacillus anthracis*, is a gram-positive, spore-forming bacterium that produces a three-component exotoxin called the anthrax toxin complex (22). The three components of this toxin complex are protective antigen (PA [83 kDa]), edema factor (EF [89 kDa]), and lethal factor (LF [90 kDa]). Individually, all of the three proteins are nontoxic. However, the combination of PA and LF, called the lethal toxin, causes death in experimental animals (30), whereas the combination of PA and EF, known as the edema toxin, induces an increase in the intracellular cyclic AMP (cAMP) levels in the susceptible cells (20) and elicits skin edema upon subcutaneous injection (31). By increasing the intracellular cAMP concentrations in neutrophils, anthrax edema toxin inhibits phagocytosis and blocks both particulate as well as phorbol myristate acetate-induced chemiluminescence (25). Anthrax edema toxin can differentially regulate lipopolysaccharide-induced production of tumor necrosis factor alpha and interleukin-6 by increasing the intracellular cAMP levels in monocytes (16).

Both LF and EF require PA for their entry into target cells. During intoxication of the target cells, PA binds to the receptors on the cells surface (6). It gets cleaved by cell surface proteases, such as furin (18), to release an N-terminal 20-kDa fragment, PA_{20}, from the cell surface, thereby exposing a high-affinity site on the 63-kDa fragment, PA_{63}, still bound to the receptor. PA_{63} then binds to the catalytic components, EF or LF. The entire complex undergoes receptor-mediated endocytosis. The acidification of the endosome (13) results in the insertion of PA_{63} into the endosomal membrane (24) and the translocation of EF and LF into the cytosol of target cells (14), where they exert their toxic effects.

EF is an adenylate cyclase. After gaining access to the cell cytoplasm, it gets activated by calmodulin to catalyze the synthesis of cAMP in the host cells (20). Studies on the enzymatic activity of EF demonstrated that EF has a high catalytic activity with a V_{max} of 1.2 mmol cAMP/min/mg of protein (21). The adenylate cyclase activity of EF is very sensitive to concentrations of calcium, showing optimal activity at 0.2 mM and inhibition at higher concentrations of calcium. Activation of EF by calmodulin is calcium dependent. In the absence or presence of 50 μM calcium, the concentrations of calmodulin giving half-maximal activity are 5 μM and 2 nM, respectively (21).

The free-calcium concentration in nonexcitable cells is kept very low (usually 10 to 100 nM). The concentration of calcium outside the cell is approximately 2.0 mM (12). Is the basal concentration of free calcium in cell cytosol enough for calmodulin-dependent activation of EF? Or does EF, like many other calcium- and calmodulin-dependent adenylate cyclases (15, 33), requires the influx of calcium for optimal activation? To address these questions in the present study, we examined the requirement of extracellular calcium for anthrax edema toxin-induced accumulation of cAMP in host cells. The cAMP response generated by edema toxin was analyzed in a variety of cells, including CHO, cultured macrophages RAW264.7, and human immune effector cells, namely, neutrophils and lymphocytes, that may be physiological targets of anthrax edema toxin. Although the response generated by edema toxin in these cells varied in intensity and in the time of initiation, the dependence on extracellular calcium was a common feature. The data presented here demonstrate that, after the translocation of edema factor into the cell cytosol, increased influx of calcium is triggered in the host cell which plays a critical role in determining

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the ensuing cAMP response. The absence of calcium or the presence of calcium channel antagonists in the extracellular medium prevented the cAMP accumulation by edema toxin in the host cells. To the best of our knowledge, this is the first report demonstrating edema toxin-induced accumulation of cAMP in lymphocytes. By accumulating cAMP, edema toxin may be disrupting bactericidal functions of immune effector cells and disabling the host defense mechanism.

MATERIALS AND METHODS

Purification of edema factor. The procedures followed for the expression and purification of EF have been described in detail in a previous publication (19). Briefly, the construct, pPN-EF (containing full-length structural gene of edema factor under the control of T5 promoter), was transformed into *Escherichia coli* SG13009 cells. The cells were grown to an optical density at 600 nm of 0.8, induced, and later harvested. The cells were lysed and EF was purified to homogeneity using a two-step procedure involving Ni-nitrilotriacetic acid (NTA) metal-chelate affinity chromatography and SP-Sepharose cation-exchange chromatography.

Purification of protective antigen. PA was expressed and purified according to the procedures described previously (2). Briefly, *E. coli* BL21(DE3) cells harboring the plasmid pMS1 were induced with IPTG (isopropyl-β-D-thiogalactopyranoside) and later harvested. The periplasmic fraction was isolated, and the protein was purified to homogeneity by ResourceQ ion-exchange and phenyl-Sepharose hydrophobic interaction chromatography.

Cell culture. Macrophage-like cell line RAW264.7 was maintained in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum, penicillin (100 U/ml), and streptomycin (100 μg/ml). The Chinese hamster ovary (CHO) cell line was maintained in Eagle minimal essential medium (EMEM) supplemented with nonessential amino acids, 25 mM HEPES (pH 7.4), penicillin (100 U/ml), streptomycin (100 μg/ml), and 10% heat-inactivated fetal calf serum. Calcium-free EMEM (Gibco) or calcium-free Hanks balanced salt solution (HBSS; Gibco) was used for cell culture assays that were performed in calcium-free medium.

Isolation of lymphocytes and neutrophils from human blood. Human lymphocytes were obtained by sedimentation of heparinized whole blood on Ficoll-Hypaque density gradient, as detailed previously (10). The procedure for isolation of neutrophils from heparanized blood involves sequential sedimentation in dextran, density centrifugation in Ficoll-Hypaque, and lysis of contaminating red blood cells by osmotic shock, as described previously (10).

Adenylate cyclase activity of edema toxin in target cells. To determine the cAMP response generated by edema toxin in cells, the cells were treated with various concentrations of edema toxin in EMEM medium (with or without calcium) at 37°C. After incubation of the cells with the toxin for the indicated period of time, the media was removed, and the cells were lysed with lysis buffer (supplied with Biotrak cAMP EIA kit) at 37°C for 10 min. The cell debris was removed by centrifugation. The supernatant was collected and was processed for the enzyme-linked immunosorbent assay-based determination of intracellular cAMP concentrations by using the nonradioactive Biotrak cAMP EIA Kit (Amersham Pharmacia).

Radioiodination of PA and EF. A total of 100 μg of the protein was allowed to react with 1 mCi of $Na^{125}I$ (specific activity, 17.4 Ci/mg) and 1 μg of Chloramine T in 40 μl of 0.1 M phosphate buffer (pH 7.0). After 1 min, the reaction was stopped by the addition of 5 μg of sodium metabisulphite. The labeled protein was separated from free radioactivity on a Sephadex G-25 column that was presaturated with 0.1 M phosphate buffer (pH 7.0) containing 1% bovine serum albumin (BSA). The specific activity was 1.2×10^7 cpm/μg for EF and 1.9×10^7 cpm/μg for PA.

Calcium uptake. To study the uptake of calcium (3), CHO cells were plated in 24-well plates and treated with edema toxin (12 nM PA, along with 1.1 nM EF) in EMEM containing 1 mM nonradioactive calcium (with or without the calcium channel antagonists). $^{45}CaCl_2$ (5 μCi/ml) was supplemented to successive wells after every 10 min. Incubation of the cells with $^{45}Ca^{2+}$ was allowed for 10 min in each well. The cells were then washed four times with HBSS and dissolved in 0.1 N NaOH. Radioactive counts associated with the cells were measured by scintillation counting to determine the uptake of $^{45}Ca^{2+}$ by the cells within 10 min of incubation with $^{45}CaCl_2$. The data has been expressed as percentage $^{45}Ca^{2+}$ uptake over control.

Binding of PA to the cell surface receptors. CHO cells were plated in 24-well plates and were grown to confluence. Before the start of the experiment, the cells were washed with cold calcium-free HBSS. Radioiodinated PA (12 nM) was then added to these cells in the presence or absence of calcium. After 30-min of incubation at 4°C, the cells were washed again to remove unbound protein. The cells were then solubilized with 0.1 N NaOH. Radioactive counts associated with the cells were measured to determine the amount of PA that bound to the cells in the presence or absence of calcium. To calculate the specific binding of PA, the difference between the mean binding of triplicate samples in the absence or the presence of a 100-fold molar excess of nonradioactive PA was taken.

To determine the effect of calcium channel antagonists on the binding of PA to the cell surface receptors, the cells were incubated with radioiodinated PA in cold medium containing calcium and 100 μM antagonist. The cells were later washed and solubilized, and the radioactivity associated with the cells was measured as detailed above.

Proteolytic activation of PA. CHO cells were allowed to grow to confluence in 24-well plates. The cells were washed, and radioiodinated PA (12 nM) was allowed to bind to the cells in the presence of calcium at 4°C. After 30 min, the cells were washed three times with cold calcium-free medium to remove unbound protein. The cells were then incubated, at 4°C, in the presence of calcium (with or without calcium channel antagonists) or in the absence of calcium to allow for the proteolytic cleavage of PA. After 90 min of incubation, the cells were lysed with sodium dodecyl sulfate (SDS) lysis buffer. The cell lysate was resolved on a SDS–12% polyacrylamide gel electrophoresis (PAGE). The gel was later dried and exposed to X-ray film. The band (corresponding to the 63-kDa fragment of PA) was cut from the gel, and the associated radioactivity was measured to determine the amount of PA that converts to the 63-kDa fragment in the presence or absence of calcium.

Oligomerization of PA and its insertion. CHO cells plated in 24-well plates were grown to confluence. The cells were washed with cold medium and radioiodinated PA (12 nM) was added to these cells in EMEM containing calcium. After incubation of the cells for an hour at 4°C, the cells were washed with cold calcium-free medium to remove unbound protein. The cells were then treated with fresh medium (20 mM morpholineethanesulfonic acid (MES)-Tris, 145 mM NaCl; pH 5.0) with or without calcium, for 1 min at 37°C. The cells were exposed to 1 mg of pronase E/ml for 10 min at 37°C, washed with HBSS, and then were lysed with SDS lysis buffer. The samples were subjected to SDS-PAGE on a 4 to 12% gradient gel. The gels were later dried and exposed to X-ray film. To compare oligomerization-insertion of PA in the absence or presence of calcium, the corresponding bands were cut from the gels, and the associated radioactivity was measured. To study the effect of calcium channel antagonists on the oligomerization of PA, the same procedure was followed except that the cells were treated with low-pH buffer in the presence of calcium and a 100 μM concentration of the respective antagonists.

Binding of EF to receptor-bound PA. Nicked PA was produced by treating 1 μM PA with 1 μg of trypsin/ml for 45 min at 37°C. Trypsinization of PA was stopped by adding 10 μg of soybean trypsin inhibitor/ml. CHO cells plated in 24-well plates were cooled and incubated with 16 nM nicked PA in calcium-containing EMEM for 20 min at 4°C. Unbound protein was removed by washing the cells with cold calcium-free medium. Fresh medium containing radioiodinated EF (11 nM) was then added to the cells in the presence or absence of calcium. After incubation of the cells for 45 min at 4°C, they were again washed and then solubilized with 0.1 N NaOH. Radioactive counts associated with the cells were measured and were corrected for nonspecific binding to determine the specific binding of EF to receptor-bound PA. To determine the effect of various calcium-channel antagonists on the binding of EF to the receptor-bound PA, the above-mentioned procedure was followed except that the incubation of EF was allowed in the presence of 100 μM antagonist in calcium-containing medium.

Translocation of EF. CHO cells were plated in 24-well plates and were grown to confluence. The cells were washed with cold medium and were then allowed to bind to 16 nM nicked-PA in calcium-containing EMEM. After incubation of the cells for an hour at 4°C, they were again washed to remove unbound protein. CHO cells were then incubated with 11 nM radioiodinated EF for an hour at 4°C. After the cells were washed with calcium-free HBSS, they were exposed to low-pH buffer, in the presence of calcium (with or without calcium channel inhibitors) or in the absence of calcium, for 1 min at 37°C. The cells were then exposed to pronase E (1 mg/ml) for 10 min at 37°C, and then pronase inhibitors were added. The cells were washed again and then solubilized in 0.1 N NaOH. Radioactive counts associated with the cells were measured to compare translocation of EF in the presence or absence of calcium.

Delivery of EF into the cell cytosol via pinosomes. CHO cells plated in 24-well plates, were washed with hypertonic medium (HBSS medium [with or without calcium] containing 0.5 M sucrose, 10% polyethylene glycol [average M_r, 1,000], and 1% BSA), before the addition of 100 nM EF to the cells in the same medium. After incubation of the cells for 9 min at 37°C, they were washed with hypotonic

medium (prepared after diluting 60 ml of HBSS medium with 40 ml of water) and then incubated at 37°C for 2.5 min. The cells were washed with calcium-free medium and incubated in medium with or without calcium. Intracellular cAMP concentrations of the cells were determined after incubation for the indicated periods of time. For studying the uptake of calcium in these cells, $^{45}CaCl_2$ (5 μCi/ml) was included in the incubation medium, and the uptake of calcium by the cells was measured as described previously.

Fura-2AM-based assay for calcium influx. The Fura-2AM assay has been described in detail elsewhere (11). Briefly, the cultured cells or the cells isolated from human blood were washed with HBSS by centrifugation at 700 × *g* for 1 min. The cells were then resuspended in HBSS containing 1 mM $CaCl_2$ and 1% BSA and were divided into aliquots. After incubation for 30 min, individual aliquots of the cells were treated with 3 μM Fura-2AM and 0.25 mM sulfinpyrazone (to retard transport of Fura-2AM out of the cells). Fura-2AM and sulfinpyrazone were added as 1,000-fold-concentrated stock solution in dimethyl sulfoxide, and incubation was allowed for 30 min at 37°C in dark. Extracellular dye was removed by washing the cells twice with HBSS at 700 × *g* for 1 min. The cells were resuspended in HBSS containing 1 or 3 mM $CaCl_2$ and then used for the assay.

RESULTS AND DISCUSSION

Requirement of extracellular calcium for anthrax edema toxin-induced cAMP accumulation in CHO cells. Anthrax edema toxin elicits a dose-dependent cAMP response in CHO cells. The intracellular cAMP levels of CHO cells increased more than 100-fold within 2 h of treatment with anthrax edema toxin (10 nM concentrations each of PA and EF). However, when the cells were treated with edema toxin in calcium-free medium, the toxin failed to generate cAMP response in CHO cells. In the absence of calcium, even higher doses of edema toxin (as high as 100 nM) failed to elevate intracellular cAMP levels in CHO cells (Fig. 1). The cAMP response of CHO cells to anthrax edema toxin was also abolished when the cells were treated with edema toxin in the presence of calcium chelator, EGTA (shown later as part of Fig. 3B). These results suggested that extracellular calcium was necessary for causing anthrax edema toxin-induced toxicity in CHO cells.

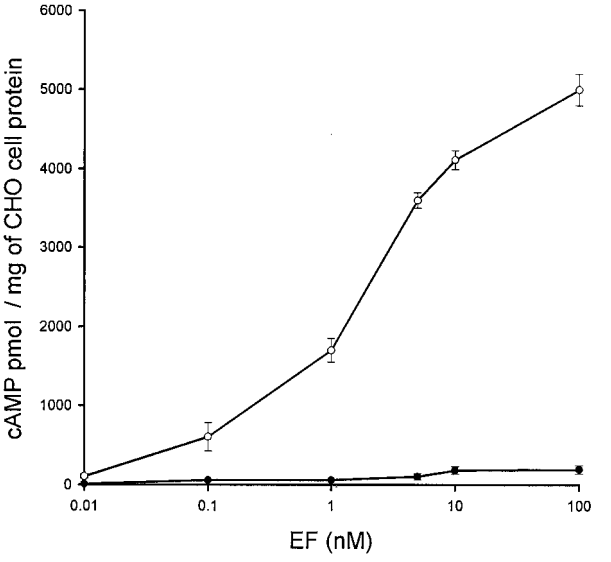

FIG. 1. Requirement of extracellular calcium for anthrax edema toxin-induced toxicity in CHO cells. CHO cells plated in 96-well plates were washed with calcium-free medium and then incubated with toxin (12 nM PA, along with the indicated concentration of EF) in calcium-free medium (●) or in medium containing 2 mM calcium (○). After 2 h of incubation, the intracellular cAMP concentration was measured. The basal level of cAMP in the untreated cells was 40 pmol/mg of cell protein (in presence of calcium) and 38 pmol/mg of cell protein (in absence of calcium). Cells treated with PA or EF alone maintained basal levels of cAMP, both in the presence or in the absence of calcium. The total protein content of the cells was 7 μg per well. All of the values are mean ± the standard deviation (SD) of three experiments done in triplicate.

To understand the relationship between extracellular calcium and anthrax edema toxin-induced cAMP response of CHO cells, the cells were treated with edema toxin in calcium-free media that had been supplemented with known concentrations of calcium chloride. It was observed that the cAMP response generated by edema toxin in CHO cells was dependent on calcium concentration in the incubation medium (Fig. 2).

Identification of calcium-requiring steps during intoxication of cells by anthrax edema toxin. During intoxication of target cells by anthrax toxin, PA, the binding moiety of the toxin, binds to cell surface receptors and gets proteolytically activated to oligomerize and bind to the catalytic moieties LF and EF. It was observed that the depletion of calcium from the extracellular medium marginally affected the binding and the nicking of PA on the cell surface (Table 1). However, the oligomerization of PA or its binding to EF were not affected by the absence of calcium in the medium (Table 1).

Subsequent to the binding of the toxin to the cell surface, the toxin is taken up by the cells. The edema toxin (a 12 nM concentration of PA, along with 6 nM EF) was allowed to bind to the cell surface in calcium-containing media at 4°C. The cells were then washed with calcium-free media to remove unbound toxin and were reincubated in medium with or without calcium for 40 min, at 37°C, to allow the uptake of the toxin by the cells. The cAMP response induced by the toxin in the treated cells was then determined. It was observed that when the uptake of edema toxin was allowed in the absence of calcium, the cAMP response induced by the toxin in CHO cells was markedly reduced (76 ± 5 pmol of cAMP/mg of CHO cell protein) compared to the response generated by the toxin in the presence of extracellular calcium (704 ± 10 pmol of cAMP/mg of CHO cell protein). These results clearly demonstrate that anthrax edema toxin requires extracellular calcium at a stage subsequent to its binding.

Anthrax edema toxin bound to the cells is internalized by endocytosis. Acidification of the endosome is required for the translocation of edema factor into the cytosol. To determine whether extracellular calcium was required at a step subsequent to endocytosis of the toxin, CHO cells were preincubated with 10 mM NH_4Cl and were treated with the toxin in the presence of calcium. In presence of NH_4Cl, the cells take up the toxin; however, the toxin is not translocated to cytosol and remains localized in the endosome (13). After incubation of the cells for an hour, they were washed with calcium-free medium to remove calcium, unbound toxin, and NH_4Cl. The cells were then incubated in medium with or without calcium. cAMP accumulation by edema factor was considerably reduced in cells that were incubated in calcium-free medium (59 ± 5 pmol of cAMP formed per mg of CHO cell protein [in 90 min]) after the removal of the amine block, in contrast to those that were incubated in calcium-containing medium (2,100 ± 20

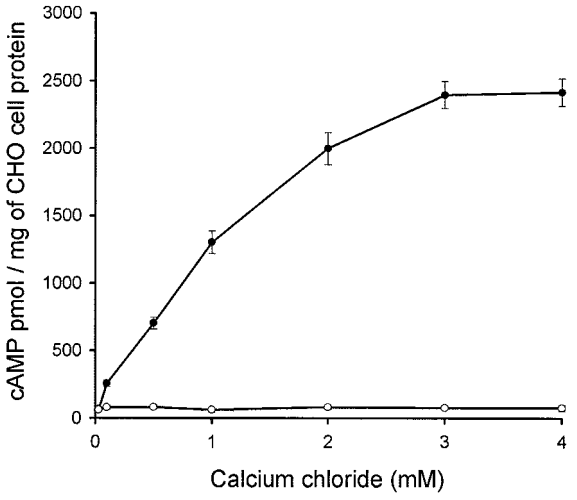

FIG. 2. Dependence of anthrax edema toxin-induced cAMP response of CHO cells on extracellular calcium concentration. CHO cells were grown to confluence. They were washed with calcium-free media. The cells were then treated with edema toxin (1 nM EF, along with 12 nM PA [●]) in medium containing known concentration of calcium. Control cells were not treated with toxin (○) but were incubated with medium containing known concentration of calcium. Intracellular cAMP concentrations were determined after 2 h of toxin treatment. The total protein content of the cells was 7 μg per well. All values are mean ± the SD of three different experiments done in triplicate.

pmol of cAMP formed per mg of CHO cell protein [in 90 min]). These results demonstrate that extracellular calcium is required for the expression of anthrax edema toxin toxicity at a stage subsequent to its internalization by endocytosis.

Acidification of the endosome causes conformational changes in PA, resulting in its insertion into the endosomal membrane, thereby forming channels for the translocation of catalytic moieties to the cytosol. The oligomerization and/or insertion of PA and the translocation of EF to the cell cytosol were unaffected by the absence of calcium in the extracellular medium (Table 1). This suggested that anthrax edema toxin required extracellular calcium at a stage subsequent to the translocation of EF into the cytosol of the target cells. To confirm this, PA-mediated delivery of EF was bypassed, and EF was directly introduced into the cytosol of CHO cells via the pinosomes. It was observed that the depletion of calcium from the extracellular medium did not affect the delivery of EF to the cell cytosol via the pinosomes (Table 2). However, the ensuing cAMP response was markedly reduced in these cells in comparison to the cells that were incubated in calcium-containing media (Table 2). These results confirmed that EF requires extracellular calcium after reaching the cell cytosol to generate an optimal cAMP response in the target cells.

Time course of $^{45}Ca^{2+}$ uptake versus time course of cAMP accumulation. To define temporally the calcium requirement of toxin-treated cells, we measured the influx of calcium as a function of time in toxin-treated cells. A dramatic rise in the influx of calcium was recorded within 10 min of toxin treatment. Furthermore, it was observed that the intracellular cAMP levels in the toxin-treated CHO cells started rising only after 10 min of toxin treatment. Thus, on a time scale it was confirmed that the influx of calcium precedes cAMP accumulation in the toxin-treated cells (Fig. 3A). Untreated cells, or cells treated with EF alone, did not show any significant increase in influx of $^{45}Ca^{2+}$ or any rise in the intracellular cAMP concentrations.

To study $^{45}Ca^{2+}$ uptake in PA-treated cells, PA was allowed to bind to cell surface receptors, get proteolytically activated and oligomerize in the presence of $^{45}Ca^{2+}$ at 4°C. It was observed that there was no increase in the uptake of $^{45}Ca^{2+}$ in CHO cells when PA was allowed to bind to the receptors, get proteolytically activated, and oligomerize on the cell surface (Table 1). This suggests that binding, nicking, or oligomerization of PA on the cell surface does not cause the influx of calcium that occurs in toxin-treated cells. To probe further, EF was allowed to bind to receptor-bound PA at 4°C, and $^{45}Ca^{2+}$ uptake by the cells was then measured. It was observed that the binding of EF to receptor-bound PA does not cause increased influx of calcium in the treated cells. To allow translocation of bound EF, the cells were exposed to low pH for a minute, and then $^{45}Ca^{2+}$ uptake was measured (Table 1). It was observed that increased influx of calcium was initiated after the translocation of EF into the cell cytosol.

Similar results were obtained in another experiment in which CHO cells were treated with edema toxin in the presence of NH_4Cl (to localize the toxin within the endosome). The cells were later washed (to remove amine block) and were incubated in medium supplemented with 5 μCi of $^{45}CaCl_2$ (to study influx of calcium after the translocation of EF into the cell cytosol)/ml. It was observed that, 25 min after the amine block was removed, an increased influx of calcium (i.e., a 656% ± 33% increase in calcium uptake compared to the control cells) occurred in toxin-treated cells. No influx of calcium was recorded in control cells that were not treated with toxin. These results suggested that the influx of calcium in toxin-treated cells occurs only after EF reaches the cell cytosol. To further confirm this, EF was directly introduced into the cytosol (via the pinosomes). Within 10 min of EF delivery, a rapid influx of calcium occurred in the target cells (Table 2).

On the other hand, when CHO cells were treated with EF along with a translocation-defective mutant of PA, Phe427Ala (1, 28), no increase in the influx of calcium was observed (Fig. 3A). The intracellular cAMP levels also did not rise in these cells. The Phe427Ala mutant of PA binds to cell surface receptors, gets proteolytically activated, and binds to LF/EF but is unable to translocate them to the cytosol (28). Taken together, these results led us to the conclusion that influx of calcium is triggered only after the translocation of edema factor into the cytosol. Moreover, the time profiles of calcium influx and cAMP accumulation show that calcium influx precedes cAMP accumulation in the toxin treated cells.

Effect of calcium-channel antagonists on edema toxin-induced accumulation of cAMP. The observation that the influx of calcium invariably preceded the rise in cAMP levels, induced by edema toxin in the host cells, raised a pertinent question: Is influx of calcium, a prerequisite for cAMP accumulation? We reasoned that if influx of calcium necessary for edema toxin-induced cAMP accumulation, then it should be possible to inhibit cAMP production by blocking the uptake of calcium. To investigate this, we analyzed the effect of agents that block uptake of calcium on anthrax edema toxin-induced cAMP accumulation in CHO cells. The nonpermeable calcium

TABLE 1. Radioactivity associated with CHO cells

Stage[b]	Radioactivity associated with CHO cells after treatment with ^{125}I-labeled PA or EF[a] (mean cpm ± SD):								$^{45}Ca^{2+}$ influx (% over control)
	In the presence of calcium	In the absence of calcium	In the presence of calcium and 100 μM antagonist						
			Nifedipine	Diltiazem	Dantrolene	Flunarizem	Verapamil	$LaCl_3$	
Binding of ^{125}I-PA	46,381 ± 931	40,101 ± 925	44,736 ± 836	46,527 ± 768	41,727 ± 822	40,890 ± 901	42,114 ± 845	42,364 ± 999	12 ± 2
Nicking of ^{125}I-PA	36,688 ± 734	31,032 ± 724	37,204 ± 834	34,740 ± 756	35,606 ± 829	30,180 ± 778	38,193 ± 834	32,101 ± 787	14 ± 3
Oligomerization and/or insertion of ^{125}I-PA	34,168 ± 697	33,756 ± 826	32,192 ± 787	30,600 ± 835	29,160 ± 834	31,624 ± 813	31,287 ± 733	31,456 ± 739	22 ± 2
Binding of ^{125}I-EF	16,499 ± 538	16,588 ± 642	15,955 ± 639	16,984 ± 624	16,127 ± 595	15,068 ± 676	16,239 ± 757	15,164 ± 596	19 ± 3
Translocation of ^{125}I-EF	4,934 ± 206	4,880 ± 211	4,379 ± 221	4,608 ± 198	4,818 ± 151	4,511 ± 232	4,923 ± 221	4,687 ± 173	625 ± 31

[a] The data presented are from triplicate samples from three different experiments. The cells were preincubated with a 100 μM concentration of antagonist for 15 min prior to the start of the experiment.
[b] For details of the experiments, see Materials and Methods.

chelator, EGTA, abolished calcium influx in toxin-treated cells and attenuated the cAMP response (Fig. 3B). La^{3+}, which competes with calcium for several calcium channels without being transported across the plasma membrane, was able to completely prevent the entry of calcium and also the ensuing cAMP response in toxin-treated cells (Fig. 3B).

Voltage-gated channels are most typically expressed in excitable cells. However, several reports have suggested the presence of L- and T-type channels in nonexcitable cells (such as lymphocytes and fibroblasts) as well (8, 23). To investigate the involvement of these channels in mediating calcium influx in toxin-treated cells, we used the selective calcium antagonists nifedipine, verapamil, diltiazem, and flunarizine. Nifedipine, a dihydropyridine antagonist of L-type calcium channels, effectively blocked calcium influx in toxin-treated CHO cells and reduced cAMP accumulation by sixfold (Fig. 3B). However, diltiazem, a benzothiazepine antagonist of L-type calcium, did not inhibit the entry of extracellular calcium and caused a slight decrease in the cAMP response of CHO cells to edema toxin (Fig. 3B). Slight inhibition of calcium influx and cAMP response of CHO cells was observed when the cells were treated with edema toxin in presence of verapamil, a papaverine antagonist of L-type channels, whereas flunarizine, a T-type channel antagonist, effectively blocked calcium influx in CHO cells and caused significant decrease in cAMP response of CHO cells to edema toxin (Fig. 3B). When CHO cells were treated with edema toxin in presence of dandrolene, which inhibits the release of calcium from intracellular stores, it was observed that both calcium influx and cAMP accumulation were slightly affected (Fig. 3B).

Parallel studies with these calcium channel antagonists demonstrated that these inhibitors did not affect the binding, nicking, and oligomerization of PA or the binding and internalization of EF into the cytosol of these cells (Table 1). It was thus deduced that these inhibitors directly affected the calcium influx that was triggered after the translocation of EF into the host cells. The reduced level of cAMP in the toxin- and antagonist-treated cells may reflect the inability of edema factor to generate an optimal cAMP response in the presence of calcium channel blockers or may be a consequence of phosphodiesterase activity. To rule out the latter possibility, we repeated these experiments in presence of phosphodiesterase inhibitor, 3-isobutyl-1-methyl-xanthine (IBMX). No alteration in inhibition of cAMP production, in antagonist-treated cells, was observed (data not shown). Thus, it was deduced that the inhibition of cAMP accumulation in antagonist-treated cells was specifically because of inhibition of adenylate cyclase activity of edema factor, in these cells. Taken together, these results suggest that the influx of extracellular calcium is necessary for edema toxin induced-cAMP accumulation in the host cells.

CHO cells are quite sensitive to anthrax edema toxin. The intracellular cAMP levels of CHO cells increase as much as 200-fold upon stimulation with an optimal concentration of anthrax edema toxin. Thus, these cells provide an excellent system for the study of the biochemical events that follow toxin

TABLE 2. cAMP response generated by EF after entry into the cell cytosol via the pinosomes[a]

Time of incubation (min)	Radioactivity associated with the CHO cells after ^{125}I-EF was delivered to the cells (mean cpm ± SD):		cAMP accumulation (mean pmol/mg of CHO cell protein ± SD):	
	In the presence of calcium	In the absence of calcium	In the presence of calcium	In the absence of calcium
5	66,452 ± 1,145	62,960 ± 1436	1,874 ± 78	289 ± 11
30	36,348 ± 987	35,481 ± 932	1,030 ± 45	104 ± 7
60	5,624 ± 249	5,243 ± 193	137 ± 10	69 ± 4

[a] For details of the experiments, see Materials and Methods.

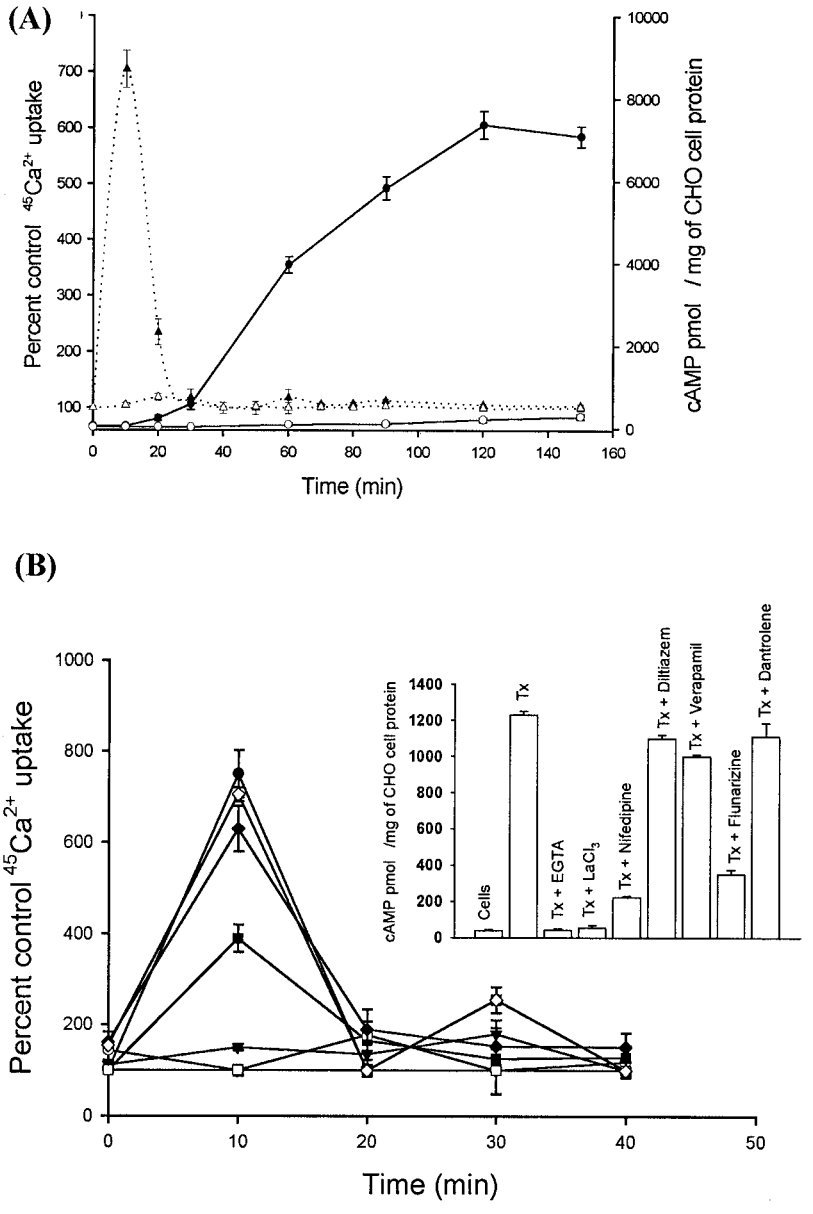

FIG. 3. $^{45}Ca^{2+}$ uptake and cAMP accumulation in toxin-treated CHO cells in the absence or the presence of antagonists. (A) The CHO cells were treated with 11 nM wild-type EF plus 12 nM wild-type PA (solid symbols) or with 11 nM wild-type EF plus 12 nM translocation-defective PA (open symbols) in calcium-containing medium. At the indicated times, the cAMP accumulation (solid line) and $^{45}Ca^{2+}$ influx (dotted line) were determined, in parallel experiments. $^{45}Ca^{2+}$ uptake data are presented as the percent uptake of $^{45}Ca^{2+}$ in the treated cells relative to that observed in the control cells (that were not treated with the toxin). $^{45}Ca^{2+}$ uptake in the control cells was 4,250 ± 45 cpm per 10 min per well of CHO cells. cAMP levels in the control cells did not vary with time and was equal to 40 ± 3 pmol/mg of CHO cell protein. The total protein content of the cells was 7 μg per well. The data are mean ± the SD of three different experiments done in triplicates. (B) The CHO cells were treated with a 100 μM concentration of antagonist before additiona of the wild-type edema toxin to the cells. $^{45}Ca^{2+}$ influx was determined over a period of 40 min for cells treated with toxin in absence of any inhibitor (●) or in the presence of EGTA (○), $LaCl_3$ (▼), nifedipine (▽), verapamil (■), flunarizine (□), dantrolene (♦), or diltiazem (◇). In a parallel experiment, cAMP accumulation was determined after 40 min in these cells (data are presented as a bar diagram). The values presented here are the means ± the SD of data collected from three different experiments performed in triplicate.

internalization. Having evaluated the role of extracellular calcium in inducing anthrax edema toxin-mediated cAMP response in CHO cells, we extended our study to other cell types that may serve as physiological host of edema toxin during infection.

Anthrax edema toxin requires influx of calcium for inducing cAMP toxicity in neutrophils and cultured macrophages. It has been previously demonstrated by O'Brien et al. (25) that anthrax edema toxin increases cAMP levels in neutrophils, inhibits phagocytosis, and blocks both particulate, as well as phorbol myristate acetate-induced chemiluminescence. Phagocytosis by macrophages is also inhibited when they are treated

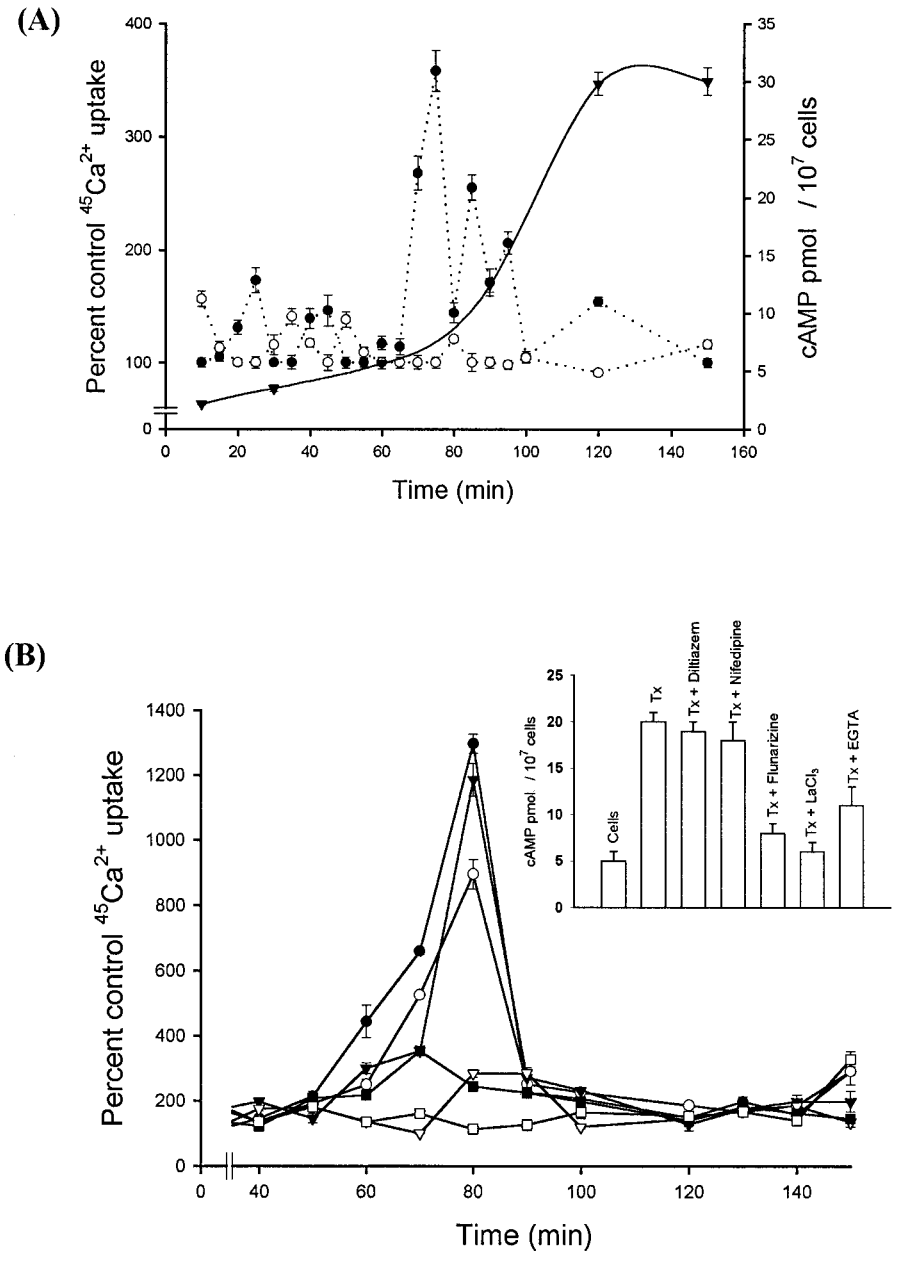

FIG. 4. $^{45}Ca^{2+}$ uptake and cAMP accumulation in edema toxin-treated neutrophils. (A) Neutrophils were isolated from the blood of healthy donors and were treated with edema toxin (12 nM PA, along with 1 nM EF) in calcium-containing medium. $^{45}Ca^{2+}$ uptake was measured in cells treated with edema toxin (●) and in cells treated with PA alone (○). In parallel, cAMP measurements were done. Cells that were not treated with toxin or cells treated with PA alone maintained 4 pmol of cAMP per 10^7 cells. The intracellular cAMP concentration in edema toxin-treated CHO cells is shown (▼). (B) Neutrophils were treated with a 100 μM concentration of the antagonist for 15 min before the addition of anthrax edema toxin. $^{45}Ca^{2+}$ uptake was measured in cells that were treated with toxin in absence of any inhibitor (●) or in the presence of nifedipine (○), diltiazem (▼), $LaCl_3$ (▽), flunarizine (■), or EGTA (□). cAMP measurements were done 90 min after toxin treatment (the results are presented as a bar diagram). None of the antagonist had any affect on cAMP levels of cells that were not treated with toxin. The values presented here are the means ± the SD of data collected from three different experiments done in triplicate.

with agents that increase their intracellular cAMP concentrations (26). Several other macrophage functions, including migration, spreading, and adhesion (7), superoxide production (32), and bacterial killing are also inhibited by these agents. Thus, edema factor, by increasing intracellular cAMP concentration in macrophages and neutrophils, might be suppressing the host phagocytic response and facilitating the survival and replication of the invading organism, *B. anthracis*.

Both neutrophils and the cultured macrophages, RAW264.7, respond poorly to anthrax edema toxin (Fig. 4A and 5A). It was observed that extracellular calcium was necessary for causing edema toxin-induced toxicity in these cells. It

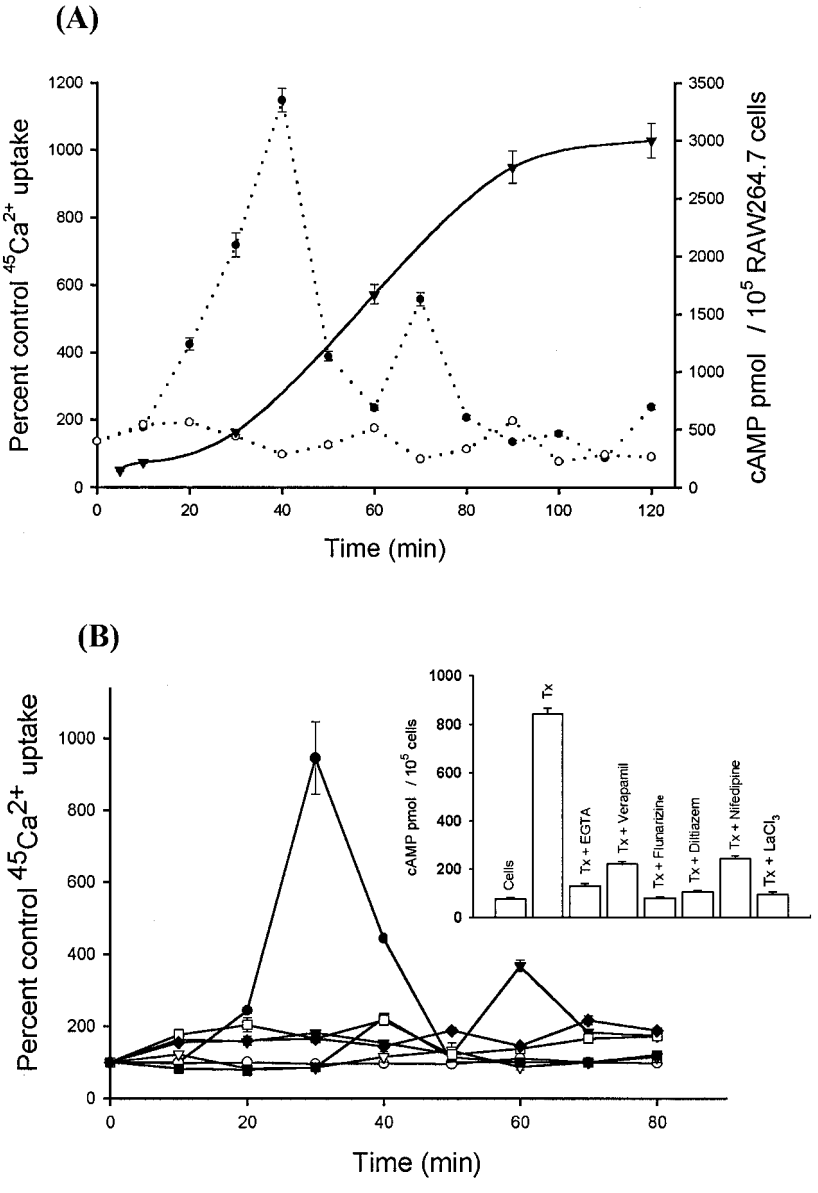

FIG. 5. $^{45}Ca^{2+}$ uptake and cAMP accumulation in edema toxin-treated RAW264.7 cells. (A) RAW264.7 cells were treated with edema toxin (12 nM PA, along with 1 nM EF) in calcium-containing medium. At the indicated times, $^{45}Ca^{2+}$ uptake was measured in toxin-treated cells (●) and also in the cells treated with PA alone (○). Cells that were not treated with toxin or those that were treated with PA alone maintained basal concentration of intracellular cAMP that did not change with time (95 ± 4 pmol/10^5 cells). The intracellular cAMP concentration in edema toxin-treated cells is shown (▼). (B) The cells were treated with a 100 μM concentration of antagonist for 15 min before addition of the toxin. The $^{45}Ca^{2+}$ influx was determined in cells treated with toxin in the absence of any inhibitor (●) or in the presence of EGTA (○), flunarizine (▼), nifedipine (▽), verapamil (■), diltiazem (□), or $LaCl_3$ (♦). After 2 h of incubation with the toxin, the intracellular cAMP level was determined in the RAW264.7 cells (the results are presented as a bar diagram). None of the antagonist had any affect on the cAMP levels of the control cells that were not treated with the toxin. The data presented here is mean ± the SD of three experiments.

was further observed that, in both the neutrophils and the cultured macrophages, treatment with anthrax edema toxin resulted in the increase in influx of extracellular calcium and that was soon followed by the rise in the intracellular cAMP levels (Fig. 4A and 5A).

To determine whether the increase in the influx of calcium essential for cAMP accumulation by edema toxin in these cells, we analyzed the cAMP response generated by edema toxin in these cells after blocking the influx of calcium. Calcium channel blockers nifedipine and diltiazem do not prevent the entry of calcium from the extracellular medium into the neutrophils, but they inhibit the movement of calcium between cytosol and the intracellular stores (27). We observed that the cAMP response generated by edema toxin in neutrophils was not affected by the presence of diltiazem or nifedipine in the medium (Fig. 4B). These results indicate that calcium release from intracellular stores has minor, if any, role in anthrax edema toxin-induced toxicity of cells. However, flunarizine,

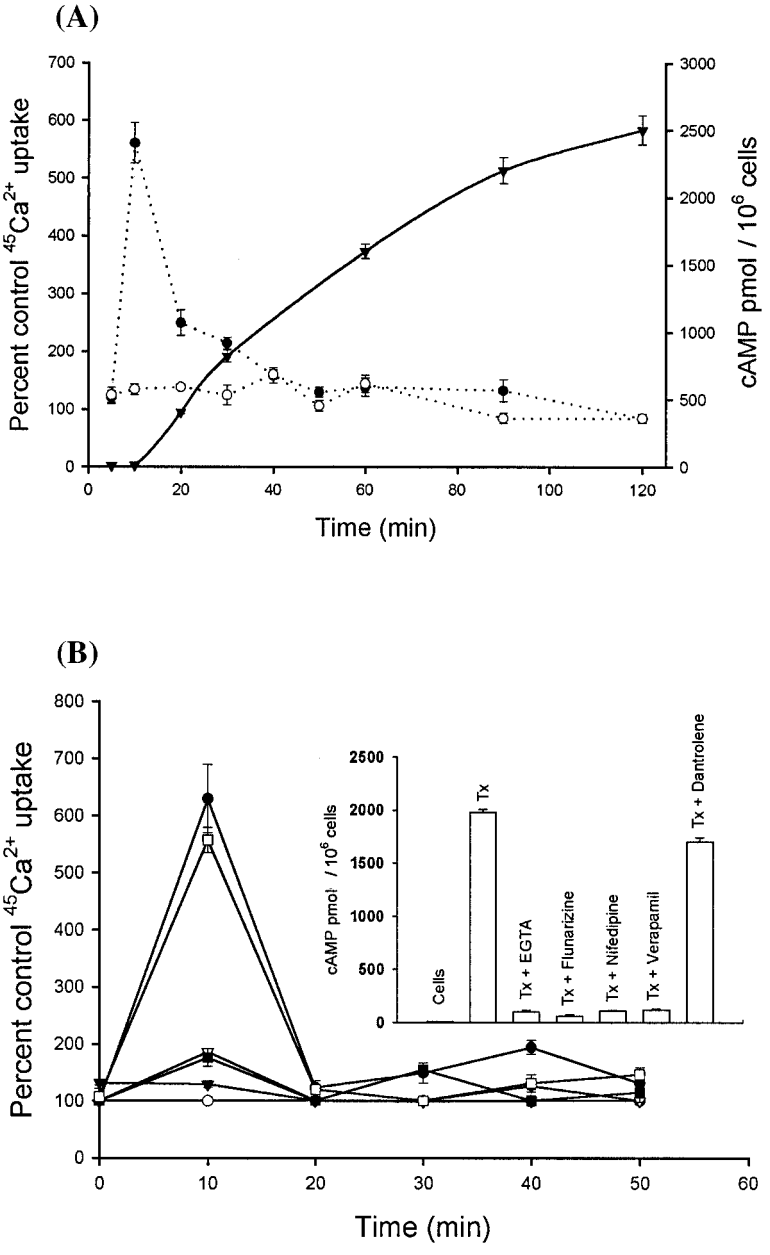

FIG. 6. $^{45}Ca^{2+}$ uptake and cAMP accumulation in toxin-treated lymphocytes. (A) Lymphocytes were isolated from the blood of healthy donors and were treated with toxin (12 nM of PA, along with 1 nM EF) in calcium-containing medium. $^{45}Ca^{2+}$ uptake was measured in edema toxin-treated cells (●) and in cells treated with PA alone (○). In parallel, cAMP measurements were done. Cells that were not treated with toxin or those that were treated with PA alone maintained 8 ±2 pmol of cAMP per 10^6 cells. Intracellular cAMP concentration in edema toxin-treated cells is shown (▼). (B) Lymphocytes were treated with a 100 μM concentration of antagonist for 15 min before addition of the toxin. $^{45}Ca^{2+}$ influx was measured in the toxin-treated cells and is presented as the percent increase $^{45}Ca^{2+}$ uptake over control for cells that were treated with toxin in absence of any inhibitor (●) or in the presence of EGTA (○), flunarizine (▼), nifedipine (▽), verapamil (■), or dantrolene (□). cAMP measurements were made 90 min after toxin treatment (the results are presented as a bar diagram). None of the antagonist had any affect on cAMP levels of cells that were not treated with toxin. The values represent the mean ± the SD of data from three different experiments.

lanthium chloride, and EGTA effectively blocked calcium influx in toxin-treated neutrophils and also attenuated the cAMP response (Fig. 4B). Similarly, in toxin-treated RAW264.7 cells, the influx of calcium was suppressed by verapamil, nifedipine, diltiazem, flunarizine, and EGTA and by the competing La^{3+} ions (Fig. 5B). These agents also inhibited anthrax edema toxin-mediated cAMP response of these cultured macrophages (Fig. 5B). From these results we infer that anthrax edema factor requires the uptake of extracellular calcium for generating optimal cAMP response in neutrophils and cultured macrophages.

Anthrax edema toxin requires calcium influx for inducing toxicity in lymphocytes. The intracellular cAMP levels of lymphocytes undergo a striking increase after 10 min of treatment

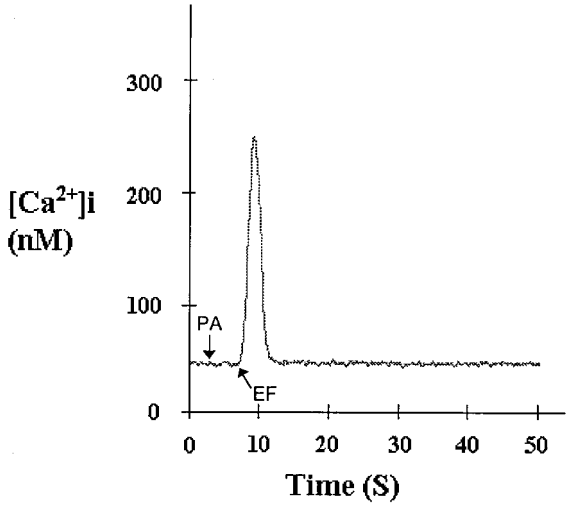

FIG. 7. Rise in cytosolic calcium observed upon treatment of lymphocytes with edema toxin. Lymphocytes were isolated from the blood of healthy individuals and were loaded with Fura-2AM as described in Materials and Methods. We added 12 nM PA to these cells in calcium-containing medium. Changes in cytosolic calcium were assessed by measuring the variation in the fluorescence of Fura-2AM-loaded cells. We then added 1 nM EF to these cells, and the variation in fluorescence was again recorded. Tracings are representative of three individual experiments with similar results.

of the lymphocytes with anthrax edema toxin (Fig. 6A). The accumulation of cAMP was dose and time dependent. To the best of our knowledge, it has never been reported previously that anthrax edema toxin can cause elevation in cAMP levels of lymphocytes. Indeed, such a massive elevation in the cAMP levels of lymphocytes can lead to an alteration in of critical immunoregulatory genes (4), apoptosis (9), decrease in T-cell proliferation (29), and decrease in immune response (5). As observed with other cell types, the cAMP response generated by anthrax edema toxin in lymphocytes was dependent on extracellular calcium. cAMP levels in lymphocytes increased from basal levels of 8 ± 1 pmol/10^7 cells to 11 ± 2 pmol/10^7 cells within 1 h of treatment with anthrax edema toxin (6 nM concentrations each of PA and EF) in the absence of extracellular calcium and to $1{,}200 \pm 45$ pmol/10^7 cells upon treatment with the toxin in calcium-containing medium. Experiments with radioactive calcium revealed that lymphocytes respond to treatment with edema toxin by increasing the influx of calcium within the first 10 min of toxin treatment (Fig. 6A). Furthermore, it was observed that the agents that inhibited calcium uptake also downregulated cAMP accumulation by edema factor in these cells (Fig. 6B).

For a more critical evaluation of the intracellular calcium levels, at the time of the edema toxin-induced increase in calcium influx, Fura-2AM dye was used to detect changes in intracellular calcium levels in the treated lymphocytes. It was observed that the treatment of lymphocytes with PA alone did not cause any increase in the basal levels of intracellular calcium (Fig. 7). This is in agreement with our other results that led us to the conclusion that the influx of calcium that occurs in the toxin-treated cells is not caused by PA. Upon addition of EF to the PA-treated lymphocytes, a massive increase in intracellular calcium concentration, $[Ca^{2+}]_i$, was recorded. The elevated levels of calcium persist in the cells just for a few seconds and then rapidly decline (Fig. 7). The EF-induced increase in $[Ca^{2+}]_i$ could be blocked by the addition of nonpermeable calcium chelator, EGTA, in the medium (data not shown). This suggested that the EF-induced increase in $[Ca^{2+}]_i$ was caused by a potent influx of extracellular calcium in the toxin-treated cells. Further studies on Fura-2AM-loaded lymphocytes demonstrated that when lymphocytes are treated with anthrax edema toxin (12 nM PA, along with 1 nM EF) in the presence of 1 mM extracellular calcium, the EF-induced influx of calcium causes $[Ca^{2+}]_i$ to rise 200 ± 11 nM (over the basal concentration). When the extracellular calcium concentration is increased to 3 mM, then the EF-induced influx of calcium causes $[Ca^{2+}]_i$ to increase upto 350 ± 19 nM. This suggested that the EF-induced increase in $[Ca^{2+}]_i$ was dependent on the extracellular calcium concentration.

The data presented here provide evidence that extracellular calcium plays a critical role in inducing anthrax edema toxin-mediated cAMP toxicity in the host cells. Depletion of calcium from the extracellular medium did not affect the PA-mediated delivery of EF into the target cells. However, after it reached the cell cytosol, EF required extracellular calcium for generating an optimal cAMP response in the host cells. It was further shown that after EF reached the cell cytosol, a rapid influx of calcium was triggered in the host cells. The surge of calcium ions was soon followed by an accumulation of cAMP in these cells. Agents that blocked calcium uptake, concomitantly inhibited edema toxin-induced accumulation of cAMP in these cells, suggesting that the influx of calcium was necessary for generating optimal cAMP response in the host cells. These calcium channel antagonists that block cAMP toxicity of anthrax edema toxin may be evaluated for their therapeutic potential against anthrax.

Several groups have shown that calcium- or calmodulin-dependent adenylate cyclases from sources as diverse as paramecium, *Drosophila melanogaster*, and mammalian brain (15, 33) are activated by influx of calcium. Indeed, studies on calmodulin show that transitory increase in calcium concentration was necessary for the interaction of calcium with calmodulin (17). The accompanying calcium-induced structural transitions in calmodulin coordinate the interaction of calmodulin with target enzymes and proteins, resulting in their activation (17). The fact that stimulus-response coupling mediated by calmodulin involves several steps suggests that different enzymes may be activated by different conformations of calmodulin and that stepwise changes exhibited by calmodulin at different calcium levels may be used to regulate different metabolic pathways. Thus, it is quite possible that the influx of calcium that is induced after the translocation of EF into the host cells facilitates interaction between EF and its eukaryotic activator, calmodulin. This hypothesis may also explain why the attenuation of calcium entry results in downregulation of edema toxin-mediated cAMP response.

Hitherto, most of the pathological effects associated with anthrax infection have been attributed to the lethal toxin. The contribution of the edema toxin to virulence and pathogenesis is not well understood. The data presented above show that, unlike lethal toxin, which primarily affects macrophages, edema factor affects different types of cells. By accumulating

cAMP in cells of the immune system, edema factor may actually be paralyzing host immune defense, thereby facilitating replication and survival of the invading bacterium. Indeed, anthrax edema factor provides an excellent example of how cleverly *B. anthracis* exploits the control system that normally operates as a negative modulator of immune cell functions.

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