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Mechanism of Leukotriene Generation in Polymorphonuclear Leukocytes by Staphylococcal Alpha-Toxin

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The effects of staphylococcal alpha-toxin on arachidonic acid metabolism in rabbit polymorphonuclear leukocytes (PMNs) were investigated and compared with those of the ionophore A23187 and the chemotactic tripeptide formylmethionyl-leucyl-phenylalanine (fMLP). Sublytic amounts of alpha-toxin stimulated the release of leukotriene B4 (LTB4) in PMNs in a dose-dependent manner. The toxin was several times more potent than fMLP but was not as effective as the ionophore. Preincubation of the toxin with neutralizing antibodies abolished the effect. Extracellular calcium was strictly required for eliciting LTB4 generation. Verapamil, a calcium channel blocker, inhibited fMLP-mediated LTB4 generation but had no effect on alpha-toxin- or A23187-exposed PMNs. Agents such as trifluoperazine and *N*-6(aminohexyl)-5-chloro-1-naphthalene sulfonamid that interfered with calmodulin activity, however, inhibited LTB4 generation in all cases. One minute after the addition of alpha-toxin, PMNs exhibited a severalfold enhancement in passive permeability to $^{45}Ca^{2+}$. In addition, these cells became permeable to sucrose but not to inulin or dextran. The influx pattern was consistent with the previous observation that alpha-toxin creates discrete transmembrane channels in erythrocytes with an effective internal diameter of 2 to 3 nm. The results suggest that alpha-toxin triggers the arachidonic acid pathway in PMNs by facilitating calcium influx into the cells, possibly via transmembrane toxin pores that serve as calcium gates. Generation of arachidonic acid metabolites in PMNs by sublytic amounts of alpha-toxin may represent an important cellular reaction that generally occurs during infections with *Staphylococcus aureus*.

Staphylococcal alpha-toxin is considered to be one of the most important pathogenetic factors in staphylococcal infections (23, 27). The toxin is secreted as a water-soluble 3S protein that, on binding to biological target membranes, oligomerizes to form amphiphilic ring-shaped hexamers (2, 4, 17). These become partially embedded within the lipid bilayer to create aqueous transmembrane pores with an effective diameter of 2 to 3 nm (3, 17). Although the toxin attacks virtually any mammalian cell, great variations in the susceptibility of different cell species toward the cytolytic action of the toxin have been noted (27).

In this study we investigated the effects of subcytolytic doses of alpha-toxin on lipid metabolism in rabbit polymorphonuclear leukocytes (PMNs). This study was of interest because confrontation of PMNs with alpha-toxin probably occurs very frequently during infections with *Staphylococcus aureus*, and the interaction of sublytic doses of alpha-toxin with these cellular targets has not been previously studied in any detail. We report here that the toxin induces a dose- and time-dependent generation of leukotriene B4 (LTB4) in rabbit PMNs. Results of experiments involving measurements of calcium uptake in the cells, in conjunction with studies on the effects of various inhibitors, indicate that alpha-toxin initiates stimulus-response coupling by allowing passive calcium flux from the extracellular medium into the cells. LTB4, an important product of arachidonic acid metabolism in PMNs (7, 8), is a very potent stimulator of granulocyte chemotaxis and adherence (16, 29). Formation of leukotrienes by PMNs following their interaction with this or other pore-forming toxins (4, 36, 37) therefore could represent a physiologically important cellular response that occurs at sites of bacterial infections.

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MATERIALS AND METHODS

Preparation of cells. PMNs were obtained from rabbit peritoneal lavage fluid, as described by Borgeat and Samuelsson (6). Cells were washed twice in Hanks balanced salt solution (HBSS with 0.5 mM Ca^{2+}, without magnesium, and supplemented with 25 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer). Erythrocytes were removd by hypotonic lysis. The differential cell count indicated that 92% of the cells were PMNs, 2% were eosinophils, 1% was lymphocytes, and 5% were monocytes. Cells were kept in RPMI 1640 medium–20% fetal calf serum for 90 min. They were then washed three times in HBSS (400 × *g* for 10 min), and 5×10^6 or 2×10^7 PMNs were suspended in 500 μl of HBSS. At the end of the experiment, the supernatant and the cells were processed for different determinations.

Determination of LTB4, leukotrienes C4 and D4, and LTB4 metabolites. The procedure for the determination of LTB4, leukotrienes C4 and D4, and LTB4 metabolites has been described previously (35). Briefly, cells were placed on ice for 5 min at the end of the experiment and then centrifuged for 2 min at 8,000 × *g*. The supernatants were applied to 1-ml

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octadecyl solid-phase extraction columns (Analytichem, Harbor City, Calif.) that had been preconditioned with two column volumes of methanol and then with two column volumes of ice-cold water. The eluates were saved for determination of lactate dehydrogenase (LDH) activity and potassium. Leukotrienes were eluted with two successive washes of 0.5 ml of ice-cold methanol. Samples were dried by in vacuo evaporation, reconstituted in 50 μl of methanol, and stored at −30°C. The recovery of LTB4 for the extraction procedure was 77.4 ± 2.2% (n = 13). Samples were analyzed by reversed-phase high-pressure liquid chromatography (HPLC) as described previously (35), with methanol-water-acetic acid (68:32:0.16; vol/vol) used as a solvent system. Leukotrienes were identified by using synthetic leukotriene standards (gift of J. Rokach, Merck Frosst, Dorval, Quebec, Canada). The lowest detectable quantity was 1 ng for the various leukotrienes (35).

Radioimmunoassay for LTB4. Eluates from the extraction columns collected 0.5 min before to 0.5 min after the LTB4 standard retention time were dried under reduced pressure. The recovery for this step was 84 ± 2% (n = 3). LTB4 was determined in these samples by a radioimmunoassay with anti-LTB4 antibodies obtained from J. Salmon (Wellcome Research, Beckenham, United Kingdom). Details of the LTB4 radioimmunoassay regarding the detection limits and cross-reactivities of the antibody have been described previously (35). There was a correlation of 0.99 between LTB4 determined by HPLC and radioimmunoassay.

Marker flux studies. PMNs (5×10^6) were incubated in HBSS containing 0.5 mM Ca^{2+} with one of the stimuli for various time periods. The cells were then placed on ice and 1 μCi of $^{45}Ca^{2+}$ or 2.5 μCi of [^{3}H]sucrose, [^{3}H]inulin, or [^{3}H]dextran (New England Nuclear Corp., Dreieich, Federal Republic of Germany) were added, and cells were kept on ice for 15 minutes. Then, PMNs were washed once in calcium-free HBSS, and the pellets were solubilized by the addition of 100 μg of melittin per ml for 15 min (21) and processed for determination of radioactivity.

Protocol for stimulation and inhibition of cells. PMNs were stimulated with 0.05 to 20 μM (usually 4 μM) A23187 (a calcium ionophore; Calbiochem-Behring, Frankfurt, Federal Republic of Germany), 0.001 to 10 μM (usually 0.1 μM) formylmethionyl-leucyl-phenylalanine (fMLP; Sigma Chemical Co., Munich, Federal Republic of Germany) or 5 to 100 μg (0.156 to 3.12 μM) of alpha-toxin per ml. In some experiments cells were preincubated with verapamil (Knoll AG, Ludwigshafen, Federal Republic of Germany), *N*-6(aminohexyl)-5-chloro-1-naphthalene sulfonamid (W7; Rikkaken Co., Nagoya, Japan), or trifluoperazine (Roehm Pharma, Weiterstadt, Federal Republic of Germany). There was no independent effect of the solvent 0.1% dimethyl sulfoxide.

Alpha-toxin and alpha-toxin antiserum were prepared as described previously (17). LDH activity was determined photometrically, and potassium was determined by flame photometry (Standard Methode zur Bestimmung der Aktivität der Laktatdehydrogenase (E.C. 1.1.1.27). Z. Klin. Chem. Klin. Biochem. **10**:188–189, 1972).

Statistical methods. Data were analyzed by one-way analysis of variance for unbalanced data, by simple regression, and by a two-tailed Student's t test for unpaired samples (14).

RESULTS

Generation of LTB4 in PMNs by staphylococcal alpha-toxin. Alpha-toxin was an effective stimulator of LTB4 generation in rabbit PMN (Fig. 1A). Preincubation of alpha-toxin (but not of A23187 or fMLP, the other two stimuli used) with neutralizing antibodies abolished the effect (data not shown). The effectiveness of alpha-toxin in eliciting LTB4 release from PMNs ranged between that of fMLP and A23187; i.e., 1.56 μM alpha-toxin and 0.1 μM fMLP induced the release of 30.85 ± 3.58 and 5.86 ± 0.47 ng of LTB4 in 2×10^7 PMNs, respectively. In the presence of 10 μM A23187, the most potent stimulus, 55.22 ± 6.22 ng of LTB4, was released from 5×10^6 PMNs. A concentration of 1.26 μM alpha-toxin resulted in a continuous LTB4 release over 30 min in PMNs, whereas the LTB4 generation peaked at 5 min in the presence of A23187 or fMLP (Fig. 1B).

The omega-hydroxy and omega-carboxy metabolites of LTB4 amounted to a maximum of 3% of the LTB4 released by PMNs following either stimulus. None of the stimuli tested induced a measurable release of peptidoleukotrienes.

Exposure of PMNs to 0.156 to 1.56 μM alpha-toxin resulted in only a moderate release of LDH from the target cells after 30 min. At alpha-toxin concentrations of 0.156, 0.312, 0.625, 0.936, and 1.56 μM, LDH releases of 7.4, 10.8, 19.0, 19.0, 22.6%, respectively, were measured (standard error [SE], <3%; n = 7 for each concentration). A concentration of 3.12 μM alpha-toxin induced overt cell damage, as indicated by LDH release of 43.1 ± 9.5%. In the presence of the ionophore or fMLP, LDH release was less than 3%. Potassium release amounted to 49.6 ± 3.7% in PMNs exposed to 1.56 μM alpha-toxin for 30 min (n = 6), to 8.9 ± 3.6% in PMNs exposed to 0.5 μM fMLP for 5 min (n = 4), and 18.7 ± 4.4% in PMNs exposed to 10 μM A23187 for 15 min (n = 3).

Dependence of the LTB4 generation by alpha-toxin on extracellular calcium and effects of calmodulin and calcium channel antagonists. When 2×10^7 PMNs were incubated in calcium-free buffer with 1.56 μM alpha-toxin, a release of only 1.07 ng of LTB4 was measured. The addition of 0.01, 0.05, and 0.1 mM Ca^{2+} to the medium caused an increase in LTB4 release to 7.3 ± 0.5, 21.5 ± 2, and 30.5 ± 4.75 ng of LTB4 (n = 4 for each concentration), respectively. In the absence of calcium no LTB4 was found in supernatants of PMNs stimulated with A23187. Under these conditions LTB4 released from fMLP-stimulated PMNs ranged between 0.5 and 1 ng.

The addition of Ca^{2+} to PMNs following preincubation of the cells with alpha-toxin, fMLP, or A23187 in calcium-free buffer resulted in the immediate generation of LTB4, while continuation of calcium-free conditions did not enhance LTB4 release (data not shown).

To more specifically delineate the role of calcium in the stimulation of LTB4 generation by alpha-toxin, the effects of trifluoperazin (a calcium-calmodulin antagonist [32]), W7 (a calmodulin antagonist [19]), and verapamil (a calcium channel blocker [22]) were tested and compared with the responses elicited by A23187 and fMLP (Fig. 2). Concentrations of 10 to 50 μM trifluoperazine and 50 to 100 μM W7 substantially reduced alpha-toxin-, A23187-, and fMLP-induced LTB4 release in PMNs. In contrast, 50 to 100 μM verapamil inhibited LTB4 generation only in fMLP- but not in A23187- or alpha-toxin-stimulated PMNs (Fig. 2). In these studies no cytotoxic effect of the inhibitors was noted, as judged by LDH release. Moreover, the generation of LTB4 on stimulation with 20 μM exogenous arachidonate was not different in PMNs incubated with trifluoperazine (up to 50 μM), with W7 (up to 100 μM) or with the solvent; i.e., arachidonate bypassed the inhibition by trifluoperazine or W7.

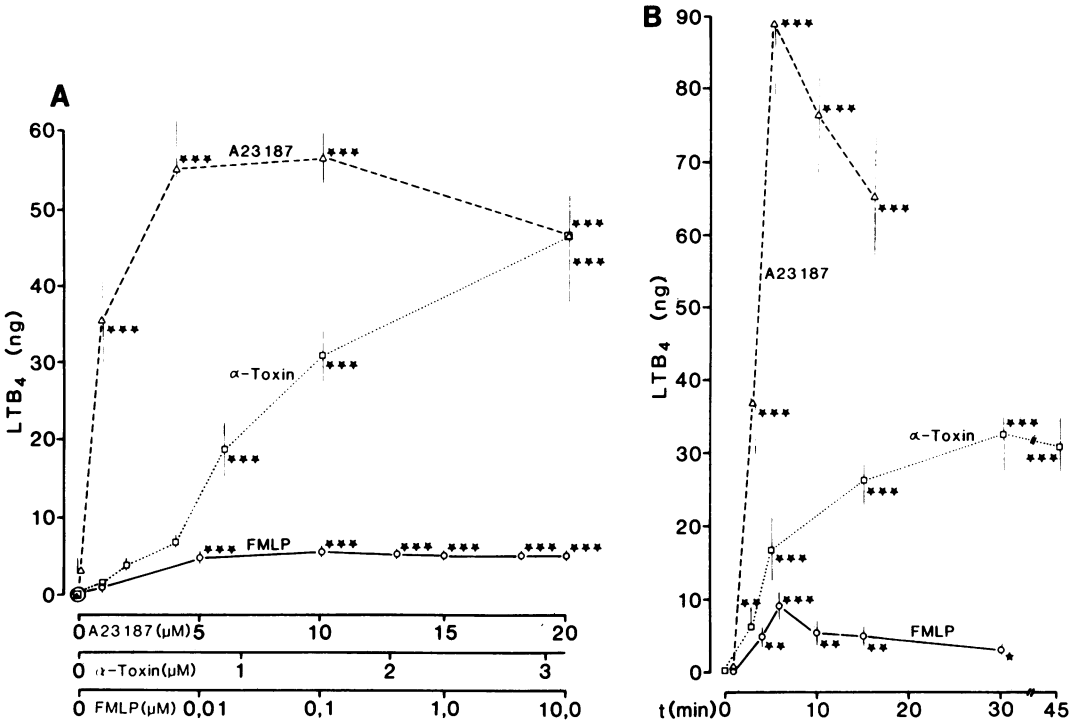

FIG. 1. (A) Dose-dependent generation of LTB4 in rabbit PMNs by staphylococcal alpha-toxin, A23187, and fMLP. A total of 2×10^7 cells were incubated with alpha-toxin for 30 min and with fMLP for 5 min. A total of 5×10^6 PMNs were exposed to A23187 for 15 min. The experiments were terminated by centrifugation at 8,000 × *g* for 20 s. The concentration of LTB4 was determined in the supernatant by HPLC. Each data point is the mean ± SE of 9, 5, or 4 experiments each with alpha-toxin, A23187, or fMLP, respectively. (B) Time course of the generation of LTB4 by staphylococcal alpha-toxin-, A23187-, and fMLP-stimulated PMNs. A total of 2×10^7 cells (5×10^6 cells for A23187) were stimulated with 1.56 μM alpha-toxin, 10 μM A23187, or 0.1 μM fMLP for various time periods. Samples were then processed for determination of LTB4. Data are presented as means ± SE of six experiments with alpha-toxin or A23187 and of three experiments with fMLP. Data were analyzed by a one-way analysis of variance. Symbols, *, $P < 0.05$, **, $P < 0.01$; ***, $P < 0.001$.

Alpha-toxin enhancement of passive permeability of PMN for Ca^{2+}. The data presented above indicate that Ca^{2+} is the critical link between the action of alpha-toxin on PMN membranes and the cellular stimulation of the arachidonic acid cascade. The simplest explanation for this finding is that alpha-toxin pores that form in the target cell bilayer act as nonphysiological Ca^{2+} gates to permit the passive flux of ion from the extracellular medium into the cells. This hypothesis was tested as follows. PMNs were treated with fMLP, A23187, or alpha-toxin at 37°C for the indicated periods of time; and the passive permeability of these cells for Ca^{2+} was assessed by exposing them to $^{45}Ca^{2+}$ for 15 min at 0°C. There was an enhanced passive permeability of PMNs for $^{45}Ca^{2+}$ 1 min after exposure of the cells to alpha-toxin (Fig. 3). In contrast, no enhanced passive influx of $^{45}Ca^{2+}$ at 0°C was noted in fMLP- or A23187-stimulated PMNs, an observation that is consistent with the mode of action of these agents, which do not form transmembrane pores. To characterize the putative transmembrane pore created by alpha-toxin, PMNs were incubated with 0.781 μM alpha-toxin for 1 min and then placed on ice, and the passive influx of $^{45}Ca^{2+}$, [^{3}H]sucrose, [^{3}H]inulin, or [^{3}H]dextran was determined during a 15-min incubation at 0°C. Experiments (n = 5) were done simultaneously. Uptake of radioactivity in alpha-toxin-exposed PMNs was as follows: $^{45}Ca^{2+}$, 502 ± 75% (mean ± SE) of control; [^{3}H]sucrose, 287 ± 24% of control; [^{3}H]inulin, 132 ± 27% of control; [^{3}H]dextran, 112 ± 14% of control. Radioactivity taken up by control cells (n = 5) was 3,070 ± 349 cpm (mean ± SE) for Ca^{2+}, 1,800 ± 258 cpm for sucrose, 1,013 ± 241 cpm for inulin, and 669 ± 86 cpm for dextran. Data were analyzed by a two-tailed Student's t test for unpaired samples, taking the uptake of [^{3}H]dextran as a marker for nonspecific extracellular entrapment. There was a significant ($P < 0.001$) influx of small marker molecules ($^{45}Ca^{2+}$ and [^{3}H]sucrose; molecular weight, 324) but not large marker molecules ([^{3}H]inulin and [^{3}H]dextran; molecular weights, 5,000 and 58,000 respectively). These results were thus compatible with the previously reported (3, 17) effective internal diameter of 2 to 3 nm for the transmembrane pores formed by alpha-toxin.

DISCUSSION

S. aureus alpha-toxin induced LTB4 generation in rabbit PMNs. This observation was in accordance with recent reports on a growing list of bacterial toxins that exhibit this capacity (10–12, 34). There are, however, qualitative and quantitative differences in the amount of leukotrienes that is generated and the profile of leukotrienes that is formed. Thus, large amounts of LTB4 were registered in this study, while poor LTB4 but substantial leukotriene C4 and D4 formation in human PMNs was reported by others with *Escherichia coli* hemolysin, *Pseudomonas aeruginosa* cytotoxin, *Bacillus alvei* alveolysin, and alpha-toxin (10–12, 34). This might be related to the toxin used, the species of the target cells, the incubation conditions applied (e.g., addition of glutathione [10]), or the assays used (HPLC, thin-layer chromatography, bioassay [11, 12]).

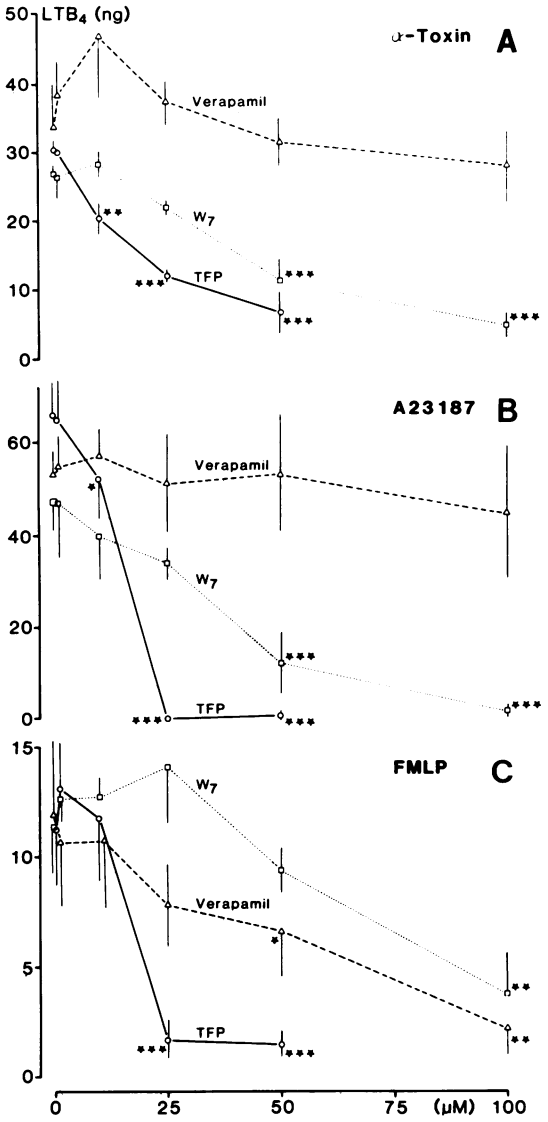

FIG. 2. Effects of the calmodulin antagonists trifluoperazine (TFP) and W7 and the calcium channel blocker verapamil on staphylococcal alpha-toxin- (A), A23187- (B), and fMLP- (C) induced LTB4 release from rabbit PMNs. A total of 2×10^7 cells (5×10^6 for A23187) were incubated simultaneously with 1.56 μM alpha-toxin, 10 μM A23187, or 0.1 μM fMLP and one of the respective inhibitors. Data presented are means ± SE of four separate experiments (trifluoperazine, W7, and verapamil; either stimulus). For determination of LTB4, statistical analysis, and symbols for statistical significance, see legend to Fig. 1. Trifluoperazine and W7 inhibited LTB4 release after either stimulus in a dose-dependent manner. Verapamil, however, blocked only fMLP-induced (but not alpha-toxin- and A23187-induced) LTB4 generation.

Because all previous studies on the toxin-induced formation of leukotrienes in PMNs were performed on a purely descriptive level (10–12, 34), an attempt to place the phenomenon within a conceptual framework appeared warranted.

A key event for triggering of the arachidonic acid pathway is an increase in intracellular free calcium (1, 15, 24, 25, 38). This evokes the activation of calcium-dependent phospholipases (39), probably via a calmodulin-mediated step (13, 28), with the subsequent liberation of membrane-bound arachidonic acid and the formation of cell-specific arachidonic acid metabolites (see Fig. 4). There are, at present, two accepted mechanisms for increasing intracellular free calcium levels. The first is a receptor-mediated pathway, as exemplified by the action of fMLP (5, 15, 25, 30, 33, 38). After binding of the ligand to its cell surface receptor, a sequence of events follows which includes an opening of receptor-operated calcium channels and mobilization of intracellularly stored calcium; the latter process may be linked to phosphatidylinositol metabolism (24–26, 38). The second mechanism is the translocation of extracellular calcium into the cells via a calcium ionophore such as A23187 (7, 31). On the basis of this and previous studies with pore-forming proteins (4, 36, 37), we propose a third mechanism for calcium-dependent triggering of the arachidonic acid cascade which envisages a passive flux of calcium ions through discrete transmembrane pores that are formed by alpha-toxin and other cytotoxic agents (Fig. 4). Evidence for this mechanism obtained in this study is summarized as follows. The alpha-toxin-induced generation of LTB4 was strictly dependent on the presence of extracellular calcium. Hence, stimulation of the arachidonic acid cascade was not noted when cells were treated with alpha-toxin in calcium-free buffer, and incubation in increasing concentrations of calcium restored the toxin-induced formation of LTB4 in a dose-dependent manner. In addition, PMNs exposed to alpha-toxin exhibited an en-

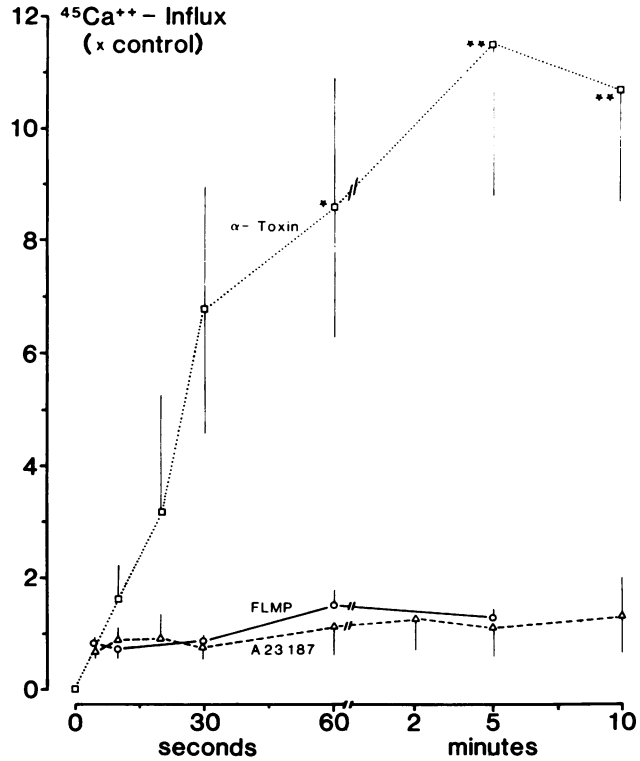

FIG. 3. Time course of passive $^{45}Ca^{2+}$ uptake at 0°C in alpha-toxin-, A23187-, and fMLP-exposed PMNs. Cells were incubated with 0.781 μM alpha-toxin, 4 μM A23187, or 10 nM fMLP for various time periods. Then cells were cooled to 0°C and exposed to 1 μCi of $^{45}Ca^{2+}$, and the passive influx of the isotope was determined. Data presented are means ± SE of four separate experiments (three experiments for A23187 and fMLP). Radioactivity associated with control cells was as follows ($n = 4$): at 10 s, 1,014 ± 248 (mean ± SE) cpm; at 1 min, 1,491 ± 323 cpm; at 5 min, 2,613 ± 443 cpm; at 15 min, 3,331 ± 558 cpm. For statistical analysis and symbols for statistical significance, see the legend to Fig. 1.

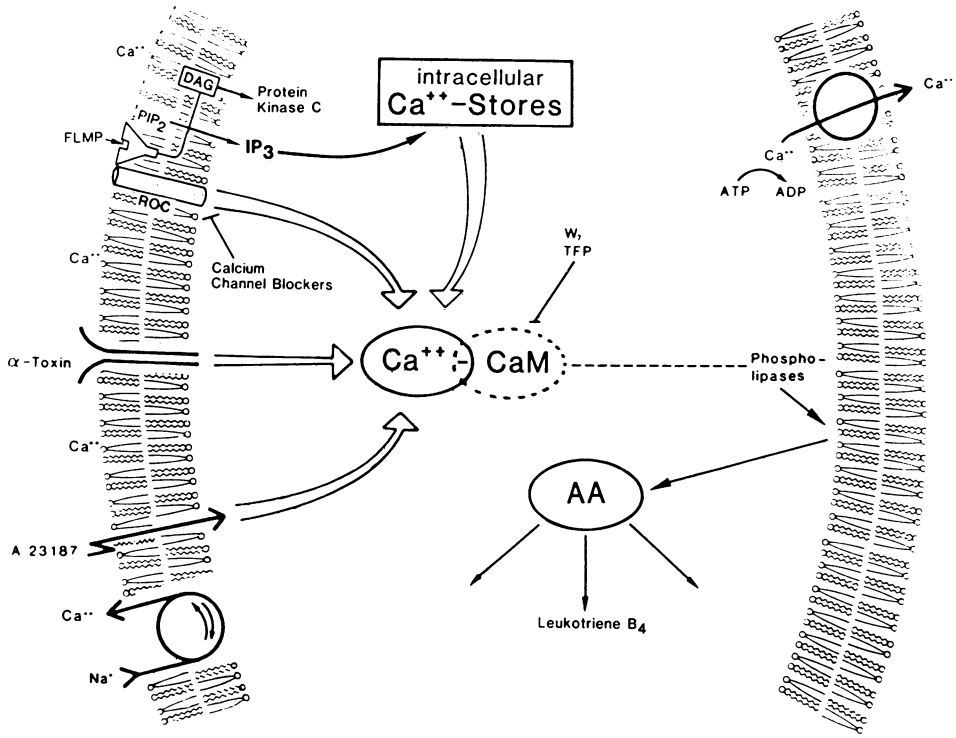

FIG. 4. Proposed mechanism of staphylococcal alpha-toxin-induced LTB4 generation in PMNs. Discrete transmembrane pores created by hexamerized alpha-toxin allowed calcium influx along the steep extra- to intracellular gradient. Increased intracellular calcium concentrations possibly activate calmodulin (CaM)-dependent, phospholipases with the subsequent release of arachidonic acid (AA) and the formation of leukotrienes. This initiation of a cellular response would be distinct from that of A23187 or the receptor-mediated stimulus fMLP. The calcium ionophore A23187 transports Ca^{2+} across a membrane along a calcium gradient; fMLP activates receptor-operated calcium channels (ROC) and initiates phosphatidylinositol metabolism (generation of 1,2-diacylglycerol [DAG] and inositol 1,4,5-triphosphate [IP_3] from phosphatidylinositol 4,5-bis-phosphate [PIP_2]).

hanced passive permeability for $^{45}Ca^{2+}$. Time course studies indicated that this effect preceded the generation of LTB4. Calcium influx into alpha-toxin-exposed cells was not selective, as indicated by the parallel, augmented influx of [^{3}H]sucrose. Larger marker molecules such as [^{3}H]inulin or [^{3}H]dextran were, in contrast, not taken up by the cells. This influx pattern was compatible with results of previous studies in which it was indicated that alpha-toxin created circumscribed, small pores in target cell bilayers with effective diameters of 2 to 3 nm (3, 17). The observed passive influx of calcium at 0°C into toxin-treated cells contrasted markedly with the actions of A23197 and fMLP. As expected, no passive calcium flux was registered for these two stimuli because they did not generate transmembrane pores.

LTB4 formation induced by alpha-toxin was not inhibited by verapamil, a blocker of physiological calcium channels (22). This supports the contention that calcium influx into the cells occurred via a nonphysiological pore. The effect of verapamil was also not observed in A23187-stimulated cells. This was expected because the calcium ionophore also bypasses the physiological calcium channels. In marked contrast, verapamil significantly reduced the generation of LTB4 in cells exposed to the receptor-mediated stimulus fMLP.

Calcium-calmodulin has been shown to be involved in the stimulation of phospholipases (13, 15, 28). The uniform inhibition of LTB4 generation in PMNs exposed to alpha-toxin, fMLP, or A23187 by the potent, albeit not entirely specific, calmodulin antagonists trifluoperazine and W7 (19, 32) were in accordance with the concept that the stimulatory action of all three agents indeed merged at the level of intracellular calcium.

This concept appears to be extendable to channel-forming proteins in general. Thus, endothelial cells treated with *P. aeruginosa* cytotoxin (37), staphylococcal alpha-toxin (36), or pore-forming C5b–C8 complement complexes (unpublished data) similarly liberated arachidonic acid metabolites in a calcium-dependent fashion.

The generation of LTB4 in PMNs induced by alpha-toxin may be of considerable biological importance. LTB4 possesses broad and potent biological activities, including chemotactic properties and augmentation of PMN adhesion to endothelial cells (20, 29). These activities result in positive feedback mechanisms that are probably important for recruitment and stimulation of PMNs at sites of infection. It is conceivable that such mechanisms are at least partially responsible for the previously described enhancing effect of alpha-toxin on the phagocytic activity of PMNs in vitro (18). The situation in vivo is likely to be very much more complex because arachidonic acid metabolites generated by other target cells, e.g., prostacyclin derived from endothelial cells (9), may partially counteract the effects of LTB4. The net effect of arachidonic acid pathway stimulation by alpha-toxin is thus probably dependent on the tissue that is involved. These considerations do not alter the basic recognition that the generation of LTB4 in PMNs induced by sublytic doses of alpha-toxin is probably a very widespread event in staphylococcal disease. Secondary phenomena trig-

gered by passive calcium influx through transmembrane pores formed by various cytolysins may be important in general in a variety of pathophysiological processes.

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