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Effect of Staphylococcal Alpha-Toxin on Intracellular Ca^{2+} in Polymorphonuclear Leukocytes

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Staphylococcal alpha-toxin, a channel-forming protein, stimulates leukotriene B4 formation in rabbit polymorphonuclear leukocytes (PMN) (N. Suttorp, W. Seeger, J. Zucker-Reimann, L. Roka, and S. Bhakdi, Infect. Immun. 55:104–110, 1987). The concept was advanced that transmembrane toxin pores act as Ca^{2+} gates allowing passive Ca^{2+} influx into the cell, thus initiating stimulus response coupling. A critical step in this hypothesis is the demonstration of an increase in the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$). $[Ca^{2+}]_i$ and membrane-associated Ca^{2+} were therefore monitored in quin-2- or chlorotetracycline-loaded PMN exposed to alpha-toxin. The effects of the Ca^{2+} ionophore ionomycin and the chemotactic tripeptide formylmethionyl-leucylphenylalanine (fMLP) were studied in parallel. All stimuli increased $[Ca^{2+}]_i$ in dose- and time-dependent manner. In the presence of an EDTA excess there was a decrease of $[Ca^{2+}]_i$ due to an efflux of Ca^{2+} in alpha-toxin- and ionomycin-treated cells, while addition of fMLP still induced an increase of $[Ca^{2+}]_i$. In the presence of verapamil, a Ca^{2+} channel blocker, $[Ca^{2+}]_i$ was reduced after stimulation with fMLP but not with alpha-toxin or ionomycin. Addition of fMLP and ionomycin but not of alpha-toxin to PMN resulted in a rapid and substantial mobilization of membrane-associated Ca^{2+}. The collective data demonstrate that exposure of PMN to staphylococcal alpha-toxin results in an increase in $[Ca^{2+}]_i$ which is due to an influx of extracellular Ca^{2+} and not to a mobilization of intracellularly stored Ca^{2+}. The concept of initiating stimulus response coupling by Ca^{2+} influx through transmembrane pores may be generally applicable to other channel-forming cytolysins.

The alpha-toxin is regarded as an important factor of *Staphylococcus aureus* pathogenicity (3, 18, 21). The toxin is secreted as a water-soluble 3S protein and oligomerizes in target membranes to form amphiphilic ring-shaped hexamers (5, 7–9, 13, 17, 26). These hexamers become partially embedded within the lipid bilayer to create aqueous transmembrane pores with an effective diameter of 2 to 3 nm (7, 13). In previous studies we demonstrated that the alpha-toxin stimulated arachidonate metabolism in isolated rabbit lungs (27), in cultured endothelial cells (31), and rabbit polymorphonuclear leukocytes (PMN) (34).

Results of experiments involving measurements of the influx and efflux of differently sized markers, in conjunction with studies on the effects of various inhibitors, led to the hypothesis that transmembrane toxin pores act as Ca^{2+} gates, allowing passive Ca^{2+} influx into the cell, initiating stimulus response coupling in the absence of overt cytolysis (34).

This model could be challenged by several alternative explanations: for example, the toxin-induced leukotriene formation might be induced by a poorly characterized membrane perturbation that results in mobilization of membrane-associated Ca^{2+}. Moreover, it may be argued that the amount of Ca^{2+} entering the cell through toxin pores is too low to initiate cellular responses or that the Ca^{2+}-extruding capacity of PMN is high enough to maintain the resting level of the cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) despite an influx of Ca^{2+} through toxin pores.

In order to characterize intracellular Ca^{2+} homeostasis more precisely, $[Ca^{2+}]_i$ and the membrane-associated Ca^{2+} were monitored in alpha-toxin-exposed PMN by fluorescence techniques. Data were compared with those obtained when PMN were exposed to two well-characterized stimuli, the Ca^{2+} ionophore ionomycin and the chemotactic tripeptide formylmethionylleucylphenylalanine (fMLP) (24, 29, 36).

(A part of this study constituted the M.D. thesis for E. H.)

MATERIALS AND METHODS

Materials. Quin-2 (free acid), quin-2/AM (tetraacetoxymethylester), and ionomycin were purchased from Calbiochem-Behring, Frankfurt, Federal Republic of Germany (FRG). The chemotactic tripeptide fMLP and chlorotetracycline (CTC) were from Sigma Chemical Co., Munich, FRG, and verapamil was a gift of Knoll AG, Lubwigshafen, FRG. *Staphylococcus aureus* alpha-toxin was prepared as described previously (7, 13) and kindly provided by S. Bhakdi, University of Giessen.

Cell preparation. PMN were obtained from rabbit peritoneal lavage fluid as described previously (34). Briefly, cells were washed twice in Hanks balanced salt solution (HBSS) with 0.5 mM Ca^{2+}, without Mg^{2+}, and supplemented with 25 mM HEPES buffer [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid]. Erythrocytes were removed by hypotonic lysis. The differential cell count indicated that 94% of the cells were PMN. Cells were kept in medium RPMI 1640 with 20% fetal calf serum for 90 min before experiments were initiated.

Measurement of cytosolic free Ca^{2+}. Quin-2 loading was performed as described (15, 37). Briefly, 5×10^7 PMN/ml were incubated in RPMI 1640 supplemented with 25 mM HEPES. Quin-2/AM was added from a 10 mM stock solution in dimethyl sulfoxide (DMSO) to a final concentration of 50 μM for 20 min at 37°C, and then cells were diluted fivefold with warm medium and incubation was continued for 40 min. Loading was monitored by checking the shift of the emission

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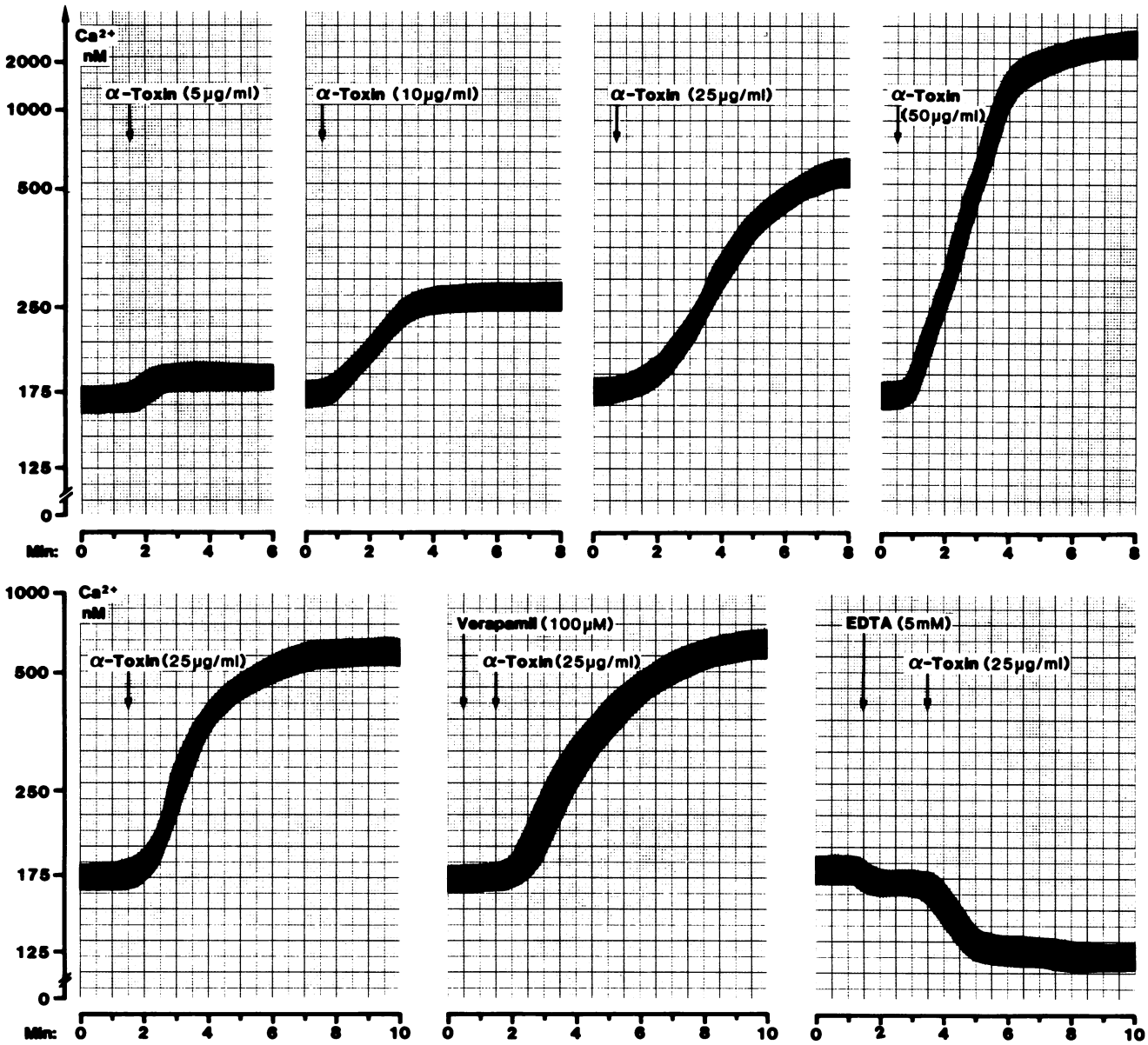

FIG. 1. Quin-2 responses of PMN exposed to staphylococcal alpha-toxin (5, 10, 25, or 50 μg/ml, top row) or alpha-toxin (25 μg/ml) plus verapamil or EDTA (bottom row). The traces shown are representative determinations performed on at least four occasions.

spectrum from that of quin-2/AM (430 nm) to that of quin-2 (492 nm). Just before use, a sample of the cell suspension was centrifuged (2,000 × *g*, 2 min) and resuspended in HBSS. Fluorescence measurements were performed in an Aminco-Bowman spectrophotofluorometer (Colora, Lorch, FRG). The cuvette was thermostated (37°C) and magnetically stirred. Excitation and emission wavelengths were 339 and 492 nm, respectively. The intracellular concentration of quin-2 was determined by comparing the Ca^{2+}-dependent fluorescence of unloaded cells treated with 0.1% Triton X-100 with the fluorescence of a standard solution of quin-2 (free acid) in the presence of unloaded cells, also treated with 0.1% Triton X-100; for this we used buffer conditions resembling the intracellular milieu (37). The intracellular quin-2 concentration was usually about 1.0 mM. The calibration of quin-2 fluorescence as a function of $[Ca^{2+}]_i$ was performed as described by Tsien et al. (37). At the end of the experiment, quin-2 was released from the cells by 0.1% Triton X-100, and the fluorescence in the presence of 1 mM Ca^{2+} (F_{max}) and 5 mM EDTA (F_{min}) was recorded. The intracellular Ca^{2+} concentration was calculated as follows: $[Ca^{2+}]_i = K_d(F\text{-}F_{min})/(F_{max}\text{-}F)$, where F is the fluorescence of the sample at a particular stage of the experiment and K_d is the effective dissociation constant of 115 nM Ca^{2+} binding to quin-2, the same as that used for lymphocytes on the assumption that intracellular pH and Mg^{2+} in neutrophils are not significantly different from those of lymphocytes. F_{max} and F_{min} were corrected for autofluorescence, which was determined on vehicle exposed PMN.

Usually 2 × 10^6 quin-2-loaded PMN in HBSS containing 0.5 mM Ca^{2+} were used in an experiment. After the baseline fluorescence had been determined, alpha-toxin, ionomycin, or fMLP was added to the cuvette. There was no independent effect of the vehicle DMSO (maximally 0.5%) on $[Ca^{2+}]_i$. At the end of the experiment, F_{max} and F_{min} were determined.

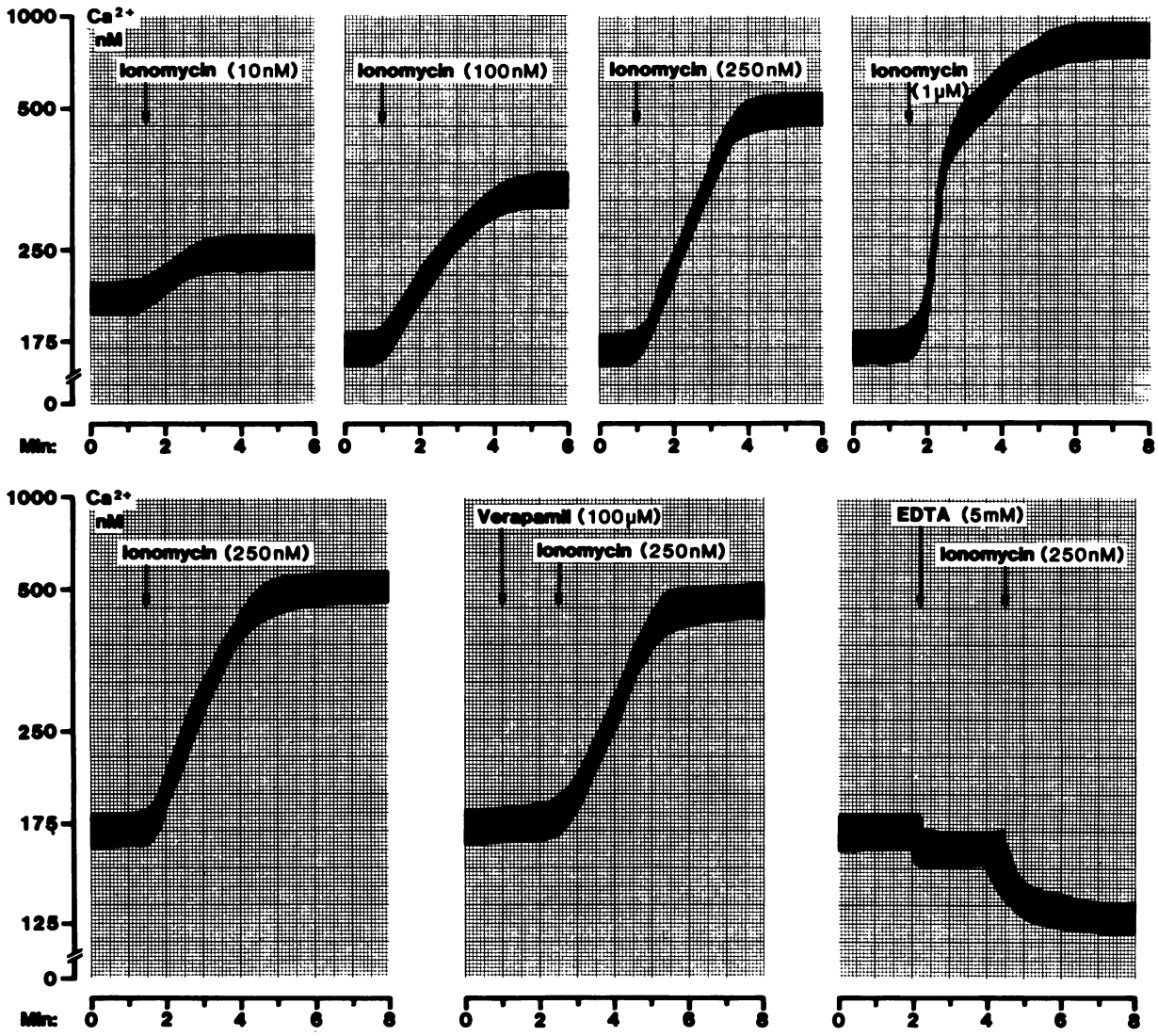

FIG. 2. Quin-2 responses of PMN exposed to the Ca^{2+} ionophore ionomycin (10,100, or 250 nM or 1 μM, top row) or 250 nM ionomycin plus verapamil or EDTA (bottom row). The traces shown are representative determinations performed on at least four occasions.

The resting level of $[Ca^{2+}]_i$ in rabbit PMN was in the range of 175 to 200 nM; this is slightly higher than that in human PMN, which has been reported to be 118 nM (2) and 143 nM (20), and which was 120 ± 10 nM in our hands (data not shown).

Two problems were encountered with the quin-2 registration in alpha-toxin-treated PMN. (i) There was some cytolysis in cells exposed to alpha-toxin; 10 min after the addition of 25, 10, and 5 μg of alpha-toxin per ml, PMN had released lactate dehydrogenase (LDH) as follows: 9.1 ± 2.7%, 6.8 ± 2.0%, and 3.4 ± 1.8% (mean ± SE, n = 3), respectively. (ii) The transmembrane toxin pores, which have been sized at about 2 nm diameter, allowed an efflux of quin-2 from the cytosol. Both facts resulted in a time-dependent appearance of small amounts of quin-2 in the extracellular space. In order to obtain an accurate estimate of $[Ca^{2+}]_i$ in alpha-toxin-exposed PMN, a second experimental protocol was therefore used which incorporated a quick wash of cells before $[Ca^{2+}]_i$ was determined. PMN were loaded with quin-2, washed, and exposed to different alpha-toxin concentrations in HBSS without Ca^{2+} and containing 0.2 mM EDTA. After 1, 2, 5, and 10 min, cells were spun for 20 s at 1,000 × g and resuspended in the same buffer. Samples were added to the cuvette, and after 5 s, when the baseline had been recorded, Ca^{2+} was added to give a final concentration of 2 mM. This procedure resulted in an immediate (within 5 s) and full quin-2 signal, the height of which was dependent on the alpha-toxin dose and the exposure time of the cells to this toxin. With this protocol, no quin-2 was detectable in the supernatant of alpha-toxin-exposed PMN. $[Ca^{2+}]_i$ data obtained in this way were qualitatively but not quantitatively identical to the data given in Fig. 1.

Determination of membrane-associated Ca^{2+}. The fluorescence loss of PMN loaded with CTC was used as a marker for mobilization of membrane-associated Ca^{2+} as described elsewhere (23, 30). PMN (10^7) were incubated in RPMI 1640 buffered with 25 mM HEPES in the presence of 100 μM CTC for 40 min at 37°C. Cells were washed twice and resuspended in HBSS, and 10^6 PMN in HBSS were added to a thermostated and magnetically stirred cuvette. Fluorescence was determined in an Aminco-Bowman flourometer at 390 and

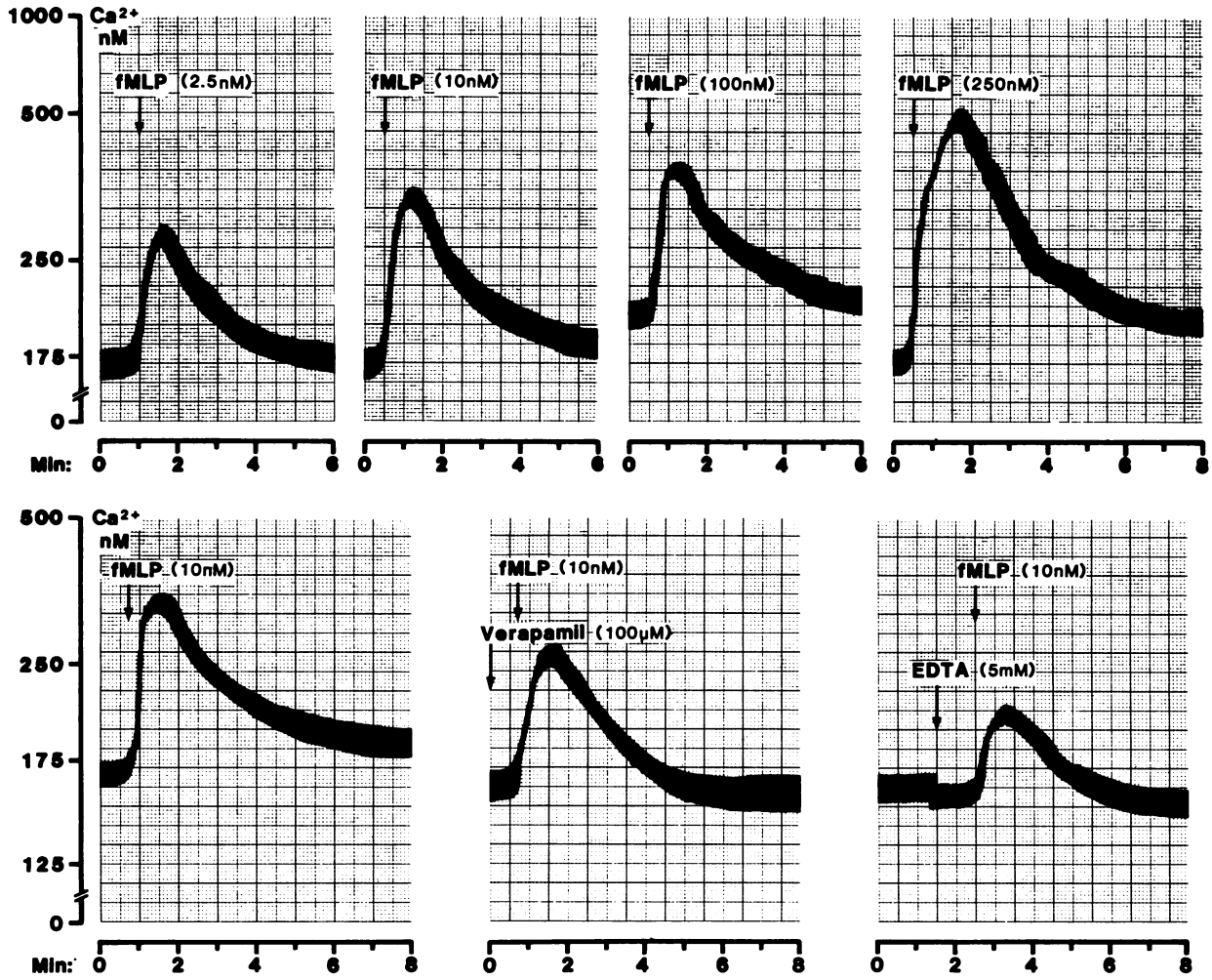

FIG. 3. Quin-2 responses of PMN exposed to the chemotactic tripeptide fMLP (2.5, 10, 100, or 250 nM, top row) or 10 nM fMLP plus verapamil or EDTA (bottom row). The traces shown are representative determinations performed on at least four occasions.

520 nm as excitation and emission wavelengths, respectively.

Statistical methods. Data were analyzed by a two-tailed Student's *t* test for unpaired samples (12).

RESULTS

Increase in $[Ca^{2+}]_i$ after exposure of PMN to staphylococcal alpha-toxin, ionomycin, and fMLP. Addition of 5 to 50 μg of alpha-toxin per ml to PMN resulted in a dose-dependent increase in $[Ca^{2+}]_i$ (Fig. 1, upper row). With the modified protocol (see Methods and Materials), $[Ca^{2+}]_i$ increased after 4 min of exposure to 5, 10, 25, and 50 μg of alpha-toxin per by 79.6 ± 24, 182 ± 39, 595 ± 93, and >1,000 nM (mean ± SE, n = 5), respectively.

The Ca^{2+} ionophore ionomycin in the range of 10 to 1,000 nM similarly increased $[Ca^{2+}]_i$ in PMN in a time- and dose-dependent manner (Fig. 2, upper row); 10, 100, 250, and 1,000 μM ionomycin resulted in an increase of $[Ca^{2+}]_i$ of 40 ± 4, 334 ± 19, 573 ± 120, and 653 ± 93 nM (mean ± SE, n = 4), respectively. Ionomycin instead of A23187 was used in the present study because of the autofluorescence of A23187.

The chemotactic tripeptide fMLP in the range of 2.5 to 250 nM dose- and time-dependently increased $[Ca^{2+}]_i$ in PMN (Fig. 3, upper row). fMLP concentrations of 2.5, 10, 100, and 250 nM resulted in an increase in $[Ca^{2+}]_i$ of 150 ± 13, 160 ± 23, 210 ± 31, and 243 ± 23 nM (mean ± SE, n = 8), respectively. In contrast to alpha-toxin and ionomycin, fMLP induced a peak of $[Ca^{2+}]_i$ which subsequently declined to almost basal $[Ca^{2+}]_i$ levels, suggesting that there is a self-limiting effect of fMLP on $[Ca^{2+}]_i$. There was no enhanced LDH release from ionophore- or fMLP-treated PMN.

Leukotriene B4 (LTB4) generation was not determined in this study. Compared with our previous data obtained for the same cell type (34), there was a close correlation between an increase of $[Ca^{2+}]_i$ and LTB4 formation with respect to the time as well as the doses of stimuli studied.

Effect of excess EDTA on stimulus-induced $[Ca^{2+}]_i$ in PMN. Addition of staphylococcal alpha-toxin or ionomycin to PMN in the presence of 5 mM EDTA (Ca^{2+} in HBSS was 0.5 mM) resulted in a decrease of $[Ca^{2+}]_i$, suggesting that either stimulus facilitated a passive movement of Ca^{2+} along its gradient (Fig. 1 and 2, right lower graphs). In contrast, $[Ca^{2+}]_i$ increased in EDTA-exposed PMN stimulated with fMLP, although the increase was less than that in the absence of EDTA. The peak increase of $[Ca^{2+}]_i$ after addition of 10 nM fMLP was 198.5 ± 18 nM and 59 ± 19 nM

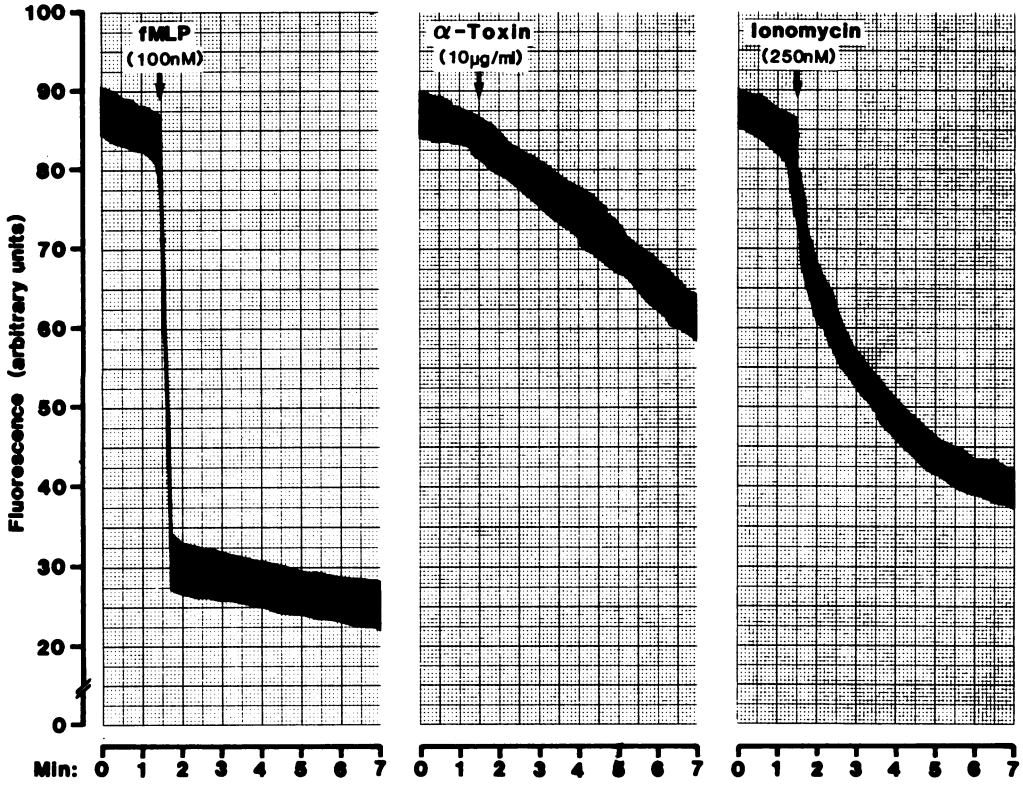

FIG. 4. Fluorescence responses of CTC-loaded PMN to fMLP, ionomycin, and staphylococcal alpha-toxin. The traces shown are representative determinations performed on at least four occasions.

(mean ± SE, $n = 4$) ($P < 0.05$) in the absence and in the presence of 5 mM EDTA, respectively (Fig. 3, lower row).

Effect of verapamil on stimulus-induced $[Ca^{2+}]_i$ in PMN. Verapamil (100 μM), a Ca^{2+} channel antagonist (35), had no effect on the alpha-toxin- or ionophore-induced increase in $[Ca^{2+}]_i$ (Fig. 1 and 2, middle lower graphs). It reduced, however, the peak of $[Ca^{2+}]_i$ after fMLP addition (Fig. 3, middle lower graph). The peak increase in $[Ca^{2+}]_i$ after addition of 10 nM fMLP was 183 ± 14 nM; after addition of 10 nM fMLP in the presence of 100 μM verapamil, it was 123 ± 16.7 (mean ± SE, $n = 9$) ($P < 0.05$). Diltiazem at 100 μM similarly reduced the fMLP-induced increase in $[Ca^{2+}]_i$ (data not shown).

Effect of stimuli on membrane-associated Ca^{2+}. Addition of alpha-toxin (10 μg/ml) to CTC-loaded PMN resulted in an insignificant decrease of membrane-associated Ca^{2+}. The slope of the registration was almost identical to the spontaneous decay of the CTC fluorescence. In the presence of 5, 10, and 50 μg of alpha-toxin per ml, the loss of fluorescence amounted to 2.1 ± 1.3, 2.6 ± 1.4, and 7.7 ± 1.7 arbitrary units (AU), respectively, within the first minute (mean ± SE, $n = 4$) (Fig. 4). fMLP, however, induced a substantial and very rapid mobilization of membrane-associated Ca^{2+}. In the presence of 10 and 100 μM fMLP, fluorescence dropped 48 ± 5 and 52 ± 4 AU, respectively, within the first minute (mean ± SE, $n = 5$). Ionomycin had an intermediate effect on mobilization of membrane-associated Ca^{2+}; 100 and 250 nM ionomycin induced a decrease in CTC fluorescence of 19 ± 3.5 and 27 ± 6.5 AU, respectively, within the first minute.

DISCUSSION

This study demonstrates that exposure of PMN to staphylococcal alpha-toxin results in an increase in $[Ca^{2+}]_i$. This effect is related to influx of extracellular Ca^{2+} and not to mobilization of intracellularly stored Ca^{2+}. Moreover, in the presence of an excess of extracellular EDTA, Ca^{2+} leaks out of the toxin-treated cell. A Ca^{2+} channel blocker does not affect the toxin-induced increase in $[Ca^{2+}]_i$. These observations support the contention that stimulation of arachidonate metabolism in staphylococcal alpha-toxin-exposed PMN is the consequence of passive Ca^{2+} flux from the extracellular medium into the cells through toxin pores (34).

The effects of staphylococcal alpha-toxin on $[Ca^{2+}]_i$ were compared with those of two well-characterized stimuli, the Ca^{2+} ionophore ionomycin and the receptor-mediated agonist fMLP (24, 29, 36). In the presence of a Ca^{2+} ionophore, Ca^{2+} gradients across membranes tend to dissipate (24). The data presented are in accordance with this notion. The current knowledge on intracellular Ca^{2+} homeostasis after fMLP treatment indicates that two events are initiated after receptor occupancy. First, phosphatidylinositol metabolism is stimulated, resulting in the formation of inositol 1, 4, 5-triphosphate (4). This compound potently mobilizes Ca^{2+} from intracellular stores (25). In addition, diacylglycerol is formed, which activates protein kinase C (19). Second, the permeability of the plasma membrane for Ca^{2+} is increased, possibly by opening Ca^{2+} channels (2). The observations of an increase in $Ca^{2+}]_i$ after fMLP addition in the presence of 5 mM extracellular EDTA and of a rapid and substantial drop in CTC fluorescence, an indicator of mobilization of membrane-associated Ca^{2+}, are in agreement with this notion. The reduction of $[Ca^{2+}]_i$ after fMLP (but not after alpha-toxin and ionomycin) addition in the presence of verapamil suggests that fMLP activates channels in PMN which contribute to an increase in $[Ca^{2+}]_i$. Interestingly, LT B4 formation was also reduced by verapamil in fMLP but not

in alpha-toxin or ionomycin-stimulated PMN (34). Interpretation of these data is complicated by the fact that little is known about receptor- or voltage-operated Ca^{2+} channels in PMN (14). In addition, a rather high concentration of verapamil was used that probably had many effects unrelated to inhibition of Ca^{2+} influx (11).

The concept of stimulus response coupling developed for staphylococcal alpha-toxin may be of significance, since this concept should be applicable to other channel-forming proteins. An increase in $[Ca^{2+}]_i$ in PMN and enhanced arachidonate metabolism were indeed noted in PMN and endothelial cells exposed to the terminal complement complexes C5b-8 and C5b-9 (22, 28, 33). In addition, endothelial cells generated large amounts of prostacyclin in the presence of *Pseudomonas aeruginosa* cytotoxin, another pore-forming cytolysin (32). It is probable that analogous stimulus response coupling following Ca^{2+} influx occurs in cells attacked by other pore formers, such as *Escherichia coli* hemolysin (6), streptolysin-O (10), and lymphocytolysin (16). In a related context, it is noteworthy that staphylococcal alpha-toxin is emerging as a useful tool for selective permeabilization of the plasma membrane, an approach that is being exploited to characterize minimal requirements for exocytosis (1).

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