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# Role of $Ca^{2+}$ and $Mg^{2+}$ in Some Human Neutrophil Functions as Indicated by Ionophore A23187

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Studies with the divalent cation ionophore A23187 suggest that both $Ca^{2+}$ and $Mg^{2+}$ ion influx play a role in human peripheral blood neutrophil function. Degranulation of neutrophils occurred at ionophore concentrations of $10^{-5}$ M and was $Ca^{2+}$ but not $Mg^{2+}$ dependent. Modulation of neutrophil chemotaxis was enhanced optimally by $10^{-7}$ M ionophore and was both $Ca^{2+}$ and $Mg^{2+}$ dependent. Concentrations of ionophore as low as $10^{-12}$ M seemed to sensitize the cells to a concentration of phorbol myristate acetate which by itself was not chemotactic. These findings also indicate that factors other than $Ca^{2+}$ or increases in cyclic nucleotides are important to initiation and modulation of neutrophil function.

The essential functions of the neutrophil are to seek and destroy. These functions involve complex processes such as cell adherence, chemotaxis, phagocytosis, and degranulation. A relative requirement for $Ca^{2+}$ and/or $Mg^{2+}$ has been demonstrated in many of these neutrophil functions (1-4). Recent discovery of an ionophore A23187 for divalent cations $Ca^{2+}$ and $Mg^{2+}$ suggested its use in studying the role of an influx of $Ca^{2+}$ or $Mg^{2+}$ in some of the functions of neutrophils (17; R. L. Hamill, M. Gorman, R. M. Gale, C. E. Higgins, and M. H. Haehn, Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 12th, Atlantic City, N. J., Abstr. 65, p. 32, 1972). $Ca^{2+}$ or $Mg^{2+}$ moves into or out of the cell in the direction of the concentration gradient. Thus in the presence of very low external concentrations of $Ca^{2+}$ or $Mg^{2+}$ and A23187, any function that could depend on influx of $Ca^{2+}$ or $Mg^{2+}$ might be inhibited.

This ionophore has been shown to produce physiological responses such as histamine release from mast cells (8) that are dependent on calcium but not magnesium influx. Moreover, calcium has been proposed as a necessary component in second messenger action, i.e., cyclic nucleotide effects intracellularly (10, 16). Both chemotaxis of neutrophils and degranulation of these leukocytes have been demonstrated to be modulated by cyclic nucleotides (6, 23). In the present study we have asked questions as to whether or not ionophore A23187 could (i) serve as a chemotactic or degranulation stimulus or (ii) modulate chemotaxis or degranulation. We report that A23187 alone is an effective stimulus for degranulation but not for chemotaxis (under the conditions of our experiments) whereas it is capable of modulating the chemotactic response. The combination of nonchemotactic concentrations of phorbol myristate acetate (PMA) and ionophore can serve as a chemotactic attractant. The responses of degranulation are calcium but not magnesium dependent, whereas effects on chemotaxis of neutrophils seem to require both ions.

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## MATERIALS AND METHODS

**Preparation of leukocytes.** The neutrophils used in this study were obtained from healthy human donors. Cells for degranulation experiments were obtained by sedimenting whole blood with 6% dextran (10 ml of whole blood to 2 ml of dextran). Buffy coat was then sedimented with 2 volumes of 0.87% ammonium chloride to lyse erythrocytes. The neutrophils were then counted in a hemacytometer and resuspended to $6 \times 10^7$ neutrophils/ml in Hanks balanced salt solution (Grand Island Biological Co., Grand Island, N.Y.) without phenol red (HBSS). The neutrophils were diluted to $3 \times 10^7$ cells/ml with the indicated agents and incubated at 37 C for 30 min in all experiments. The cells were sedimented and the supernate was decanted. The cell pellet was resuspended in 0.5 ml of 0.2 M sucrose to which 0.01 ml of 10% Triton X-100 was added. The suspension was quick frozen and thawed seven times. The supernatant and cell pellets were then assayed for enzyme activity.

**Enzyme assays.** Lysozyme was measured according to the method of Shugar (18), glucose-6-phosphate dehydrogenase according to the method of Lohr and Waller (14), and β-glucuronidase according to the method of Talalay et al. (20). Glucose-6-phosphate dehydrogenase and lactic dehydrogenase

determinations are equally sensitive indicators of cell damage.

**Method of chemotaxis.** Cells for chemotaxis were obtained from buffy coat after allowing 30 $cm^3$ of whole heparinized blood to settle at 1 g at room temperature for 1 to 2 h. The buffy coat was diluted in medium 199 with Hanks salts (Grand Island Biological Co.), and neutrophils were enumerated in a hemacytometer and spun onto membrane filters (5-$\mu$m pore size; Millipore Corp., Bedford, Mass.) using a cytocentrifuge (Shandon Southern Instruments, Sewickly, Pa.). The filters were then placed into a modified Boyden chamber (Neuroprobe Corp., Bethesda, Md.). Agents were then added to either the cell side or the opposite or attractant side of the chamber, and the assays were performed as previously described (6).

**Calcium and magnesium ions.** In these experiments no attempt was made to remove calcium or magnesium ion from the cells by chelating agents. The cells were resuspended in medium 199 (for chemotaxis) and in Hanks salts to which calcium or magnesium salts were not added. Under experimental conditions measurements of calcium and magnesium concentrations were made by atomic absorption spectrometer (Jarrell Ash 810), which indicated a concentration of 5 $\mu$M calcium and 6 $\mu$M magnesium in the medium on the cell side of the filter and 43 $\mu$M calcium and 33 $\mu$M magnesium in the medium on the attractant side of the filter 30 s after the start of the chemotaxis experiment. At the midpoint of the chemotaxis assay, concentrations of calcium and magnesium were 23 $\mu$M and 15 $\mu$M in the medium on the cell side and 48 $\mu$M and 33 $\mu$M in the medium on the attractant side of the filter.

Similarly, under experimental conditions calcium and magnesium concentrations were measured in the degranulation experiments at 5 and 30 min after the addition of HBSS (to which calcium and magnesium salts were not added) to neutrophils washed two times in HBSS without calcium or magnesium. Under these conditions the calcium and magnesium concentrations were 76 $\mu$M and 33 $\mu$M in the medium at the termination of the experiment at 30 min.

The concentration of calcium and magnesium ion in complete HBSS is 1.26 mM $Ca^{2+}$ and 0.81 mM $Mg^{2+}$. Concentrations of these salts in complete medium 199 used for chemotaxis measurements were identical since Hanks salts were used in this medium.

**Agents.** Zymosan (Nutritional Biochemical Corp., Cleveland, Ohio) was used to treat the leukocyte donors' serum to provide a stimulus for enzyme release, and cytochalasin B (Aldrich Chemical Corp., Milwaukee, Wis.) was preincubated with the neutrophils (5 $\mu$g/ml) 15 min at 37 C as outlined by Goldstein et al. (11). PMA was obtained as a chromatographically pure compound with a molecular weight (determined by mass spectrometer) of 616 from Consolidated Midland Corp., Brewster, N.Y. A23187, ionophore, was the generous gift of Otto K. Behrens, Eli Lilly Research Laboratories, Indianapolis, Ind.

Stock solutions of PMA and A23187 were prepared in spectral grade dimethyl sulfoxide and stored in the dark at −70 C or below. Dilutions from stock solutions resulted in percentages of dimethyl sulfoxide of 0.1% or less in each experiment. At this concentration there is no assayable effect of this solvent on either neutrophil function or enzyme determinations.

## RESULTS

**Degranulation of neutrophils.** The upper portion of Table 1 demonstrates the effects of A23187 in the absence of other agents on the degranulation of neutrophils. Note that the effective concentration was above $10^{-7}$ M. Like some agents which cause external degranulation, enzymes of both special and azurophil granules were released whereas the cytoplasmic enzyme glucose-6-phosphate dehydrogenase was not. In the absence of external

TABLE 1. *Effects of ionophore A23187 alone on degranulation*[a]

| Experimental conditions | Lysozyme | β-Glucuronidase |
|---|---|---|
| Control (no additions) | 5.6 | 3.7 |
| Complete HBSS + ionophore ($10^{-5}$ M) | 46.4 (40.8)[b] | 20.1 (16.4)[b] |
| Complete HBSS + ionophore ($10^{-6}$ M) | 27.4 (21.8) | 9.8 (6.1) |
| Complete HBSS + ionophore ($10^{-7}$ M) | 7.5 (1.9) | 5.8 (2.1) |
| Control (no additions) | 5.4 | 4.2 |
| Complete HBSS + ionophore ($10^{-5}$ M) | 23.0 (17.6)[c] | 19.5 (15.3)[c] |
| HBSS without calcium + ionophore ($10^{-5}$ M) | 7.4 (2.0) | 6.9 (2.7) |
| HBSS without magnesium + ionophore ($10^{-5}$ M) | 22.0 (16.6) | 28.1 (23.9) |
| HBSS without calcium or magnesium + ionophore ($10^{-5}$ M) | 7.6 (2.2) | 5.7 (1.5) |

[a] Glucose-6-phosphate dehydrogenase activity was not detected in the supernate and remained associated with the cell pellet in every case.

[b] Numbers in parentheses are the enzyme activity released minus the control.

[c] Values of percentage of enzyme released are the average of duplicates which vary less than 10%. This is one of four experiments which show similar results. Calcium and magnesium ion concentrations in the external medium in these experiments are indicated in Materials and Methods.

calcium ion as seen in the lower portion of Table 1, ionophore did not affect enzyme release. These data indicate that the ionophore requires the presence of calcium ion in the medium to cause enzyme release. Medium lacking only magnesium ion is as effective as complete medium in producing the response of the cells to ionophore.

The experiments in Table 2 demonstrate that the ionophore is similar to and approximately additive with other stimuli to degranulation. Addition of ionophore $10^{-5}$ M or zymosan-treated serum (from the same donor as the neutrophils) combined with cytochalasin B gave approximately equal effects on degranulation. When added together, the release of activity of β-glucuronidase was approximately the sum of each stimulus separately, although that of lysozyme is less. As noted in the lower portion of Table 2, addition of cyclic 3',5'-guanosine monophosphate (cyclic GMP) enhanced enzyme activity released when ionophore was the only primary stimulus. These experiments demonstrate that the ionophore stimulus to degranulation is similar in many ways to other degranulation stimuli both in responsiveness and in ability to be modulated by a cyclic nucleotide (13, 22).

**Leukocyte chemotaxis.** Ionophore acted as a modulator of chemotaxis most effectively at a concentration of $10^{-7}$ M (Table 3), but was ineffective at concentrations an order of magnitude higher and markedly diminished in its enhancement at the lower concentration. It can be seen, however, in the lower portion of Table 3 that the ionophore's modulating effect required both calcium and magnesium ions. Each ion by itself was ineffective.

Ionophore alone was ineffective as a stimulus to initiate chemotaxis when placed on the attractant side of the membrane filter in concentrations from $10^{-5}$ to $10^{-14}$ M (experiments not shown). However, if combined with PMA at a concentration that is not chemotactic (in this case 2 mg/ml) (Table 4), the combination of agents was capable of serving as a chemotactic attractant. In experiments not shown here it has been demonstrated that when both agents were placed on the cell side of the filter with medium 199 on the attractant side there was no more random movement than when medium 199 was used on both sides. If A23187 was placed on cell side and PMA (2 ng/ml) on the attractant side, the effect was the same as that seen with both agents on the attractant side.

Since PMA has been shown to be weakly chemotactic (6), in addition to its ability to increase the concentration of intracellular cyclic GMP in other cell types (8) and in neutrophils

TABLE 2. *Effects of ionophore A23187 plus other stimuli on degranulation*[a]

| Experimental conditions | Total enzyme activity released (%)[b] | |
|---|---|---|
| | Lysozyme | β-Glucuronidase |
| HBSS + ionophore ($10^{-5}$ M) | 29.3 | 14.4 |
| HBSS + ionophore ($10^{-7}$ M) | 7.9 | 0.2 |
| HBSS + ZTS + CB | 25.7 | 14.5 |
| HBSS + ZTS + ionophore ($10^{-5}$ M) | 38.7 | 35.9 |
| HBSS + ZTS + CB + ionophore ($10^{-7}$ M) | 29.2 | 14.6 |
| HBSS + ionophore ($10^{-5}$ M) | 13.3 | 11.9 |
| HBSS + ionophore ($10^{-5}$ M) + cGMP ($10^{-7}$ M) | 21.7 | 18.5 |
| HBSS + ionophore $10^{-5}$ M + cGMP ($10^{-8}$ M) | 11.9 | 11.9 |
| HBSS + cGMP $10^{-8}$ M | 0.9 | 0.9 |

[a] ZTS is zymosan-treated serum; CB is cytochalasin B. Glucose-6-phosphate dehydrogenase activity was not detected in the supernate and remained associated with the cell pellet in every case. Other conditions are as in Table 1.

[b] Values expressed as activity remaining in supernate after enzyme activity released from untreated controls is subtracted.

TABLE 3. *Effects of ionophore A23187 alone on chemotaxis*

| Additions (cell side of filter) | % BF[a] control |
|---|---|
| $10^{-6}$ M ionophore | 119 |
| $10^{-7}$ M ionophore | 446 |
| $10^{-8}$ M ionophore | 192 |
| Complete HBSS + ionophore ($10^{-7}$ M) | 540 |
| HBSS without $Ca^{2+}$ | 106 |
| HBSS without $Ca^{2+}$ + ionophore ($10^{-7}$ M) | 152 |
| HBSS without $Mg^{2+}$ | 97 |
| HBSS without $Mg^{2+}$ + ionophore ($10^{-7}$ M) | 115 |
| HBSS without $Ca^{2+}$ and $Mg^{2+}$ | 85 |
| HBSS without $Ca^{2+}$, $Mg^{2+}$ + ionophore ($10^{-7}$ M) | 107 |

[a] Bacterial factor from *Escherichia coli* (9) is on the attractant side throughout this experiment. Bacterial factor is suspended in medium 199 with Hanks salts to which $Ca^{2+}$ and $Mg^{2+}$ have been added or, as indicated under "Additions" where these ions were absent, from the media on the attractant side. The results expressed are the mean of triplicate experiments in which there is 10% standard error of the mean. Variation of less than ±20% from the bacterial factor controls are not significant.

TABLE 4. *Effects of ionophore A23187 plus PMA on chemotaxis*

| Additions to attractant side of filter[a] | % BF control |
|---|---|
| Bacterial factor (*E. coli*) | 100 |
| PMA[b] | 0 |
| Ionophore ($10^{-12}$ M) | 0 |
| PMA + ionophore ($10^{-8}$ M) | 15 |
| PMA + ionophore ($10^{-9}$ M) | 4 |
| PMA + ionophore ($10^{-10}$ M) | 7 |
| PMA + ionophore ($10^{-11}$ M) | 25 (20)[c] |
| PMA + ionophore ($5 \times 10^{-12}$ M) | 35 |
| PMA + ionophore ($10^{-12}$ M) | 43 (54, 50, 67)[c] |
| PMA + ionophore ($10^{-13}$ M) | 27 |
| PMA + ionophore ($10^{-14}$ M) | 21 |

[a] Addition of PMA to the cell side of the filter and ionophore to the attractant side resulted in essentially no random movement of cells to the attractant side of the filter, i.e., the same number of cells that would find their way to this side of the filter when only Medium 199 was on the attractant side of the filter. However, with PMA alone on the attractant side and ionophore on the cell side the results were essentially similar to those shown here.

[b] PMA concentration throughout experiment is 2 ng/ml.

[c] Numbers in parentheses indicate other experiments in which the combination of 2 ng of PMA per ml and the indicated concentration of ionophore produced a similar response.

(Goldberg, Haddox, Hill and Estensen, unpublished observations), we also examined other agents that could be expected to increase intracellular cyclic GMP and were not chemotactic. Acetylcholine ($10^{-5}$ M) which will increase cyclic GMP up to sixfold in neutrophils (Goldberg, Haddox, Hill, and Estensen, unpublished observations; 22), phenylephrine ($10^{-4}$ M), and prostaglandin $F_2\alpha$ ($10^{-7}$ M), when combined with ionophore and placed on the attractant side of the filter ($10^{-5}$ to $10^{-14}$ M), were not chemotactic. This result indicates that whereas very low concentrations of PMA and ionophore had a chemotactic effect, this was not associated only with the property of PMA to enhance cyclic GMP accumulation intracellularly, since other agents that elevate intracellular cyclic GMP did not act as chemotactic agents in combination with ionophore.

## DISCUSSION

We have demonstrated that in two of the essential functions of neutrophils, degranulation and modulation of chemotaxis, ionophore has an effect. First ionophore functions as degranulating agent at $10^{-6}$ and $10^{-5}$ M in the presence of external calcium but not in its absence. This response seems to be similar to another degranulating stimulus, zymosan-treated serum or complement (11). Second, ionophore at lower concentrations requires both calcium and magnesium ions in the bathing media to have an effect on chemotaxis. In combination with a nonchemotactic concentration of PMA, but not other agents capable of increasing intracellular cyclic GMP, ionophore has been shown to increase chemotactic response.

Becker and Showell (3) have demonstrated that chemotaxis is dependent in part on external concentrations of both magnesium and calcium. Our data are consistent with their findings but suggest additionally that calcium or magnesium ion influx is of central importance in chemotaxis. To support the notion that calcium ion influx is important, Smith and Ignarro (19) have recently reported that A23187 promotes association of calcium with neutrophils (which may represent influx or adsorption).

The foregoing conclusion is placed in question, however, since ionophore has actions other than changing ion influx. Ionophore is known to increase cyclic GMP levels (19). As we have demonstrated for chemotaxis (6) and as others have indicated for degranulation (17, 22), cyclic GMP itself and agents which promote cyclic GMP accumulation in neutrophils enhance both of these functions. The effects of ionophore therefore are as one would predict for an agent which can increase cyclic GMP. To complicate matters further, calcium ion seems to be necessary for function of both cyclic GMP (10) and cyclic 3',5'-adenosine monophosphate (16). Indeed, Goldstein et al. (12) have recently reported that increases in calcium ion concentration in the bathing media of neutrophils are under some conditions sufficient to cause release of lysozyme. Their data also would indicate that A23187, although enhancing lysozyme release, has little effect on release of β-glucuronidase, whereas both our data and those of Smith and Ignarro (19) indicate that β-glucuronidase is released in addition to lysozyme. For the latter discrepancy we can only offer the explanation that we used 15-fold more neutrophils than did Goldstein et al. (12). Goldstein et al. (12) further report that PMA may act to release lysozyme in the absence of external calcium, indicating that this ion may not always be required.

Gallin and Rosenthal (9) have presented evidence that in neutrophils exposed to chemotactic attractants, calcium ion "efflux" is increased. Gallin and Rosenthal's data are also consistent with rapid release of externally bound calcium ion (as they point out in their discussion) and may not actually represent flux across the plasma membrane. The sugges-

tion that efflux rather than influx represents an important feature of chemotaxis of neutrophils is at variance with our data, which suggest that influx is important.

The effects of higher concentrations of ionophore, i.e., $10^{-5}$ to $10^{-6}$ M for degranulation and $10^{-7}$ to $10^{-8}$ M for modulation of chemotaxis, are more easily understood than the effects of concentrations below $10^{-11}$ M. While our rationale for using these very low concentrations was to try to construct a chemotactic attractant by combining "simple" agents with some "known" action, we were unable to manufacture an attractant. Becker (2) also was unable to show a chemotactic effect of A23187 while showing an enhancement of spontaneous motility. There may be many reasons for this. One is the failure of such combinations of agents to form a concentration gradient. Another may be that PMA may have another property that makes it an effective chemotactic attractant. Since the experiments outlined in Table 4 work just as well with ionophore on the cell side and PMA on the attractant side, we believe that ionophore may simply provide a "sensitizing" function such as steroids seem to for actions involving receptoradenylcyclase complex (15).

In summary, we view the seeking and destructive functions of the neutrophil as follows: the neutrophil must be directed toward the target (initiation of chemoxtaxis), its journey modified by local conditions (modulation), and finally it must interact with the target (degranulation). Agents capable of inducing the latter function could be capable of all three, and indeed fragments of complement are both chemotactic (21) and capable of causing degranulation (11).

One could predict that lower concentrations of an agent would initiate, higher modulate, and highest degranulate neutrophils. Ionophore fits this prediction as does PMA (6, 21). In the case of each of these agents, the highest concentration degranulates neutrophils whereas a lower one modulates. In these experiments even lower concentrations of both agents initiate chemotaxis. Both cyclic GMP and calcium ion, then, seem to play a role in control of these neutrophil functions. Other factors could be necessary, however, since agents which can alter intracellular cyclic GMP alone or in combination with ionophore are not chemotactic attractants. There is, for instance a requirement for magnesium ion in addition to calcium ion in modulation of chemotaxis. Becker (2) has indicated that A23187 can stimulate spontaneous movement in the absence of calcium in the media. This may indicate that rabbit neutrophils are different than human neutrophils or that spontaneous motility is a different function than modulation of chemotaxis. Finally, each stimulus or each agent may act at a different portion of a series of causal or related events. Therefore, whereas ionophore requires calcium and/or magnesium, PMA (12) does not.

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