CHANGES IN IONIC MOVEMENTS ACROSS RABBIT POLYMORPHONUCLEAR LEUKOCYTE MEMBRANES DURING LYSOSOMAL ENZYME RELEASE

Possible Ionic Basis for Lysosomal Enzyme Release

P. H. NACCACHE, H. J. SHOWELL, E. L. BECKER, and R. I. SHA'AFI

From the Departments of Physiology and Pathology, University of Connecticut Health Center, Farmington, Connecticut 06032

ABSTRACT

Changes in the movements of Na^+, K^+, and Ca^{+2} across rabbit neutrophils under conditions of lysosomal enzyme release have been studied. We have found that in the presence of cytochalasin B, the chemotactic factor formyl methionyl leucyl phenylalanine (FMLP) induces within 30 s large enhancements in the influxes of both $^{22}Na^+$ and $^{45}Ca^{+2}$ and an increase in the cellular pool of exchangeable calcium. The magnitude of the changes induced by cytochalasin B and FMLP exceeds that induced by FMLP or cytochalasin B alone, and cannot be explained on the basis of an additive effect of the two agents. However, these compounds either separately or together produce much smaller enhancements in ^{45}Ca efflux. The divalent cation ionophore A23187 also produces a rapid and large increase in the influxes of both ^{22}Na and $^{45}Ca^{+2}$ in the presence and absence of cytochalasin B. We have also found an excellent correlation between calcium influx and lysosomal enzyme release. ^{42}K influx is not significantly affected by any of these compounds. On the other hand, a large and rapid increase of ^{42}K efflux is observed under conditions which give rise to lysosomal enzyme release. A flow diagram of the events that are thought to accompany the stimulation of polymorphonuclear leukocytes (PMNs) by chemotactic or degranulating stimuli is presented.

KEY WORDS   ions · leukocyte · enzyme release · chemotoxin

Alterations in the plasma membrane potential and/or the concentration of free intracellular calcium have been implicated in the mechanisms of stimulus-secretion coupling in a number of cell types (9, 24). There is some indirect evidence which suggests that similar mechanisms may be involved in lysosomal enzyme secretion by polymorphonuclear leukocytes (PMNs). For example,

neutrophil degranulation can be induced by most of the factors that are known to enhance the locomotion of the PMNs (3). In addition, the calcium ionophore A23187 has been shown to be a potent secretagogue for PMNs in the presence but not in the absence of extracellular Ca^{+2} (15, 28).

The role of monovalent cations in enzyme release from PMNs is, as yet, not well understood. It has been recently shown that lysosomal enzyme release from PMNs induced either by the synthetic

chemotactic factor formyl methionyl leucyl phenylalanine (FMLP), or by A23187, a Ca^{+2} ionophore, is significantly enhanced by extracellular K^+ (27, 28).

Recently, the movements of Ca^{+2}, Na^+, and K^+ across the plasma membranes of human and rabbit neutrophils have been studied and it has been found that the transport of these ions is significantly altered in the presence of chemotactic factors (7, 8, 11, 22). The purpose of these studies is to examine the changes in the movements of Ca^{+2}, Na^+, and K^+ across the plasma membranes of rabbit peritoneal PMNs under conditions which lead to lysosomal enzyme release.

MATERIALS AND METHODS

General Procedure

Polymorphonuclear leukocytes were obtained from white albino rabbits (2-3 kg). PMNs were collected from peritoneal exudates 12-14 h after the intraperitoneal injection of 400-500 ml of sterile 0.1% glycogen in isotonic saline (4). They were then washed once and resuspended in Hanks' balanced salt solution (HBSS), the millimolar composition of which was as follows: NaCl, 124; KCl, 4; Na_2HPO_4, 0.64; KH_2PO_4, 0.66; $CaCl_2$, 0.5; $MgSO_4$, 0.74; $NaHCO_3$, 15.2; Tris (hydroxymethyl) aminomethane, 10.0, pH 7.3. Glucose (1 mg/ml) and crystalline bovine serum albumin (1 mg/ml) were added to HBSS. When not specified, the cells were suspended in this Hanks' balanced salt solution for both flux studies and lysosomal enzyme measurement. This somewhat low concentration of extracellular calcium ion was used in routine studies to maximize the specific activity of Ca^{45} and still have a significant amount of release.

The experiments were carried out at a cell concentration of 10^7/ml and at 37°C. Cell viability and nonspecific release were assessed by the amount of lactic dehydrogenase (LDH) recovered in the supernates from the cell suspensions. In all of the experiments to be reported, LDH release was negligible (<4% of the total). The cell suspensions were divided into two batches: one batch was used for enzyme release studies and the other for flux studies.

The chemotactic factor FMLP was synthesized as previously described (25). A stock solution of this factor (1 mM) was made in dimethyl sulfoxide (DMSO) and used throughout this study. Subsequent dilutions were made before use in the appropriate buffers. Although no effects of DMSO could be observed at any of the dilutions used (<0.01% DMSO), the appropriate concentrations of DMSO were present in the controls. Radioisotopes (^{22}Na, ^{42}K, and ^{45}Ca) as chloride salts in water were all purchased from New England Nuclear (Boston, Mass.). Ouabain and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis,

Mo.) and all other reagents were analytical grade. The calcium ionophore A23187 was a generous gift of Dr. Robert Hamil of Eli Lilly and Co. (Indianapolis, Ind.) whom we wish to thank. Cytochalasin B was obtained from Aldrich Chemical Co., Inc. (Wilwaukee, Wis.) and made up as a stock solution at 5 mg/ml in DMSO. The silicone oil was purchased from Harwick Chemical Corp. (Cambridge, Mass.) (Versilube F50, density 1.05 g/ml). All the experiments were carried out in siliconized glassware and at 37°C.

Transport Studies

Fluxes were measured by a fast and reproducible technique which has been described in detail previously (22). The principle of this method is that at a preset time an aliquot of known volume (0.8 ml) of a cell suspension is layered on top of a 0.5-ml layer of silicone oil in 1.5-ml capacity microcentrifuge tubes. The separation of the cells from the suspending medium was accomplished by a single centrifugation (0.5 min) in an Eppendorf microcentrifuge (Brinkmann Instruments, Inc., Westbury, N. Y.) (maximum speed 11,000 rpm reached in <10 s). This procedure was found to provide a rapid and reproducible separation of the cells from the suspending medium. As it has been reported previously (22), it was found that practically no radioactivity could be found in the oil layer. The cell pellets and/or the supernates can then be analyzed for radioactivity. Whenever solubilization of the cell pellets was required, the supernate and the oil layer were aspirated by suction and the bottom of the tubes containing the cells were excised and transferred to test tubes to which 0.5 ml of 5% HNO_3 and 0.1% Triton X-100 was added. The cell pellets were then dislodged from the tube tips by forceful aspirations with Pasteur pipettes and vigorous mixing. The samples were incubated for 1 h at 37°C with occasional agitation and then centrifuged at 1,000 g for 10 min. Aliquots of the supernates were then sampled for radioactivity determination using Formula 963 (New England Nuclear) as a scintillant. This solubilization procedure was found to yield more consistent results than that previously described (22).

For the sake of convenience, the results are expressed as counts/pellet, and a pellet contains on the average 8 × 10^6 cells. The terms influx and efflux are used here only to denote inward and outward movement. Values of actual steady-state cation fluxes have already been published (22). In these studies the variation between duplicate samples for either Na^+ or K^+ fluxes is <10% whereas for Ca^{+2} the variation can be as high as 20%. The situation is quite different when comparing samples from different rabbits. The variation in this case can be as high as 50%. The results presented here are all from single experiments, and each experiment has been repeated at least three different times.

For efflux experiments, the cells were preincubated with the desired isotope for 30-45 min at 37°C. They were then centrifuged at 1,000 g in a refrigerated Sorvall

RC3 centrifuge (DuPont Instruments, Sorvall Opera-tions, Newtown, Conn.) for 2 min in 15-ml conical tubes. The supernate was aspirated and the pellet resuspended in 1 ml of the desired buffer containing no radioactivity and transferred to 1.5-ml Eppendorf microcentrifuge tubes with a Pasteur pipette. They were then centrifuged for about 5 s in an Eppendorf microcentrifuge, the supernate was removed and the pellet was gently resuspended in another milliliter of buffer. This was repeated three times and provided sufficient removal of the radioactive medium. The cells were then resuspended in the desired volume of thermally equilibrated buffer and divided into the number of flasks needed. The whole washing procedure was completed in <4 min. As reported in earlier studies, we generally found that >90% of the cells were viable (22, 25). For these efflux studies, the variation between duplicate samples is <5%. Furthermore, the extracellular compartment in such studies is considered infinitely larger than the intracellular compartment.

In all of the experiments in which the combined effects of cytochalasin B and the chemotactic factor or the ionophores were studied, cytochalasin B (5 μg/ml) was added first, followed about 1 min later by the isotope and the desired reagent. In preliminary studies, we have found that the effect of cytochalasin B in inducing release in rabbit PMNs collected from peritoneal exudates is very rapid (<1 min). In fact, release can be induced when both FMLP and cytochalasin B are added simultaneously. The same procedure was employed for the efflux experiments, except that the isotope was omitted. This protocol was established in an attempt to reproduce the same conditions used in the studies of lysosomal enzyme secretion (28).

Lysosomal Enzyme Release

The procedure used for the measurement of lysosomal enzyme release in rabbit PMNs is the same as that described previously (27, 28). For the sake of simplicity, only the results concerning lysozyme release are presented whenever a direct comparison between the results obtained with tracers and enzyme release is necessary. Similar, if not identical, results were obtained with β-glucuronidase which was measured in every instance.

RESULTS

Effect of the Chemotactic Factor
FMLP and Cytochalasin B
on ^{45}Ca Movement

The chemotactic factors, FMLP and others, are able to induce lysosomal enzyme secretion in PMNs in suspension only if a suitable concentration of cytochalasin B is present (14, 16, 25, 27, 28). To see if a change in ^{45}Ca movement occurs in the presence of cytochalasin B, we have studied

the effect of 5×10^{-9} M FMLP on ^{45}Ca influxes across rabbit PMN membranes in the presence and absence of cytochalasin B. The results of a representative experiment are summarized in Fig. 1. As has been reported previously (22), FMLP produces a small but consistent (more than 10 experiments) increase in ^{45}Ca influx. In addition, cytochalasin B alone is able to produce a significant increase in ^{45}Ca influx (three experiments). An enhanced influx of calcium ions in the presence of cytochalasin B alone has been reported previously in human PMNs stimulated by C5a (11). However, the addition of FMLP to cells already incubated for 1 min with cytochalasin B elicits a large and a rapid enhancement of ^{45}Ca influx. This increase is significantly larger than the sum of the two increments induced by the two agents separately. It is interesting to point out that under the same experimental conditions, the kinetics of lysosomal enzyme release and Ca^{+2} influx are indistinguishable.

The observed increase in ^{45}Ca influx in the presence of FMLP and cytochalasin B is most likely the result of an increase in the inward
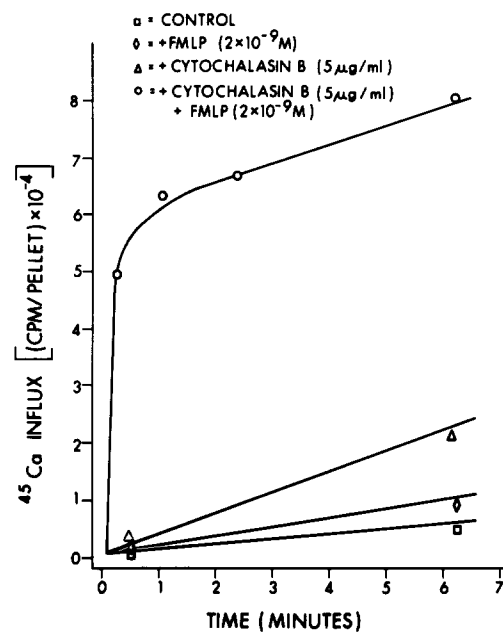

FIGURE 1 The time-course of ^{45}Ca influx across rabbit PMN membranes under various experimental conditions. Cytochalasin B (5 μg/ml) was added 1 min before the addition of FMLP and the isotope. Each point represents the average of duplicate determinations. The maximum variation between duplicate samples in this experiment was 10%. The suspending medium is HBSS.

permeability of the neutrophil membranes to calcium and an increase in the amount of intracellular exchangeable calcium. To test for an increase in intracellular exchangeable Ca^{+2} and to see if this increase can be related to enzyme release, the following sets of experiments were carried out. A known volume of cells at a final concentration of 10^7 cells/ml was added to an isotonic buffer solution containing ^{45}Ca. The suspension was allowed to equilibrate at 37°C for 45 min. This time was sufficient for the radioactivity to reach a steady-state (i.e., $\frac{dCPM}{dt} = 0$, where CPM is the counts in the cell/mg protein and t = time; for more details, see reference 22). At the end of the incubation period, cytochalasin B to give a final concentration of 5 μg/ml was added to the cell suspension, and aliquots were transferred to ice-cold test tubes containing the desired amounts of FMLP. Once the cells are transferred to zero degrees, they usually lose about 30% of their radioactivity. After this sudden initial loss in counts, the "steady-state" calcium level remains constant during the period of manipulation. The tubes are then transferred to 37°C and incubated for 5 min. This is sufficient time for release to take place. The tubes were then transferred to 0°C, sampled, and processed for lysosomal enzyme release and Ca^{45} uptake as described in Materials and Methods. We are well aware of the

difficulties in such manipulation, and this is the reason the results are expressed in terms of relative fluxes. On the other hand, this procedure was necessary in order to obtain at one time a complete dose-response curve. The changes in the steady-state levels of cellular ^{45}Ca as percent of the control level at each FMLP concentration were calculated. The amount of enzyme release as percent of the total was also measured on the same cell suspension and under the same experimental conditions. The results are summarized in Fig. 2. There is no doubt that a significant dose-dependent increase in the amount of intracellular exchangeable calcium occurs over the same concentration range of FMLP that induces lysosomal enzyme release. Furthermore, a linear correlation between the percent change in cellular calcium and the percent change in lysozyme enzyme release can be deduced from the results shown in Fig. 2. Such a relationship makes it difficult to escape the conclusion that these two variables are tightly coupled, and this is therefore consistent with a causal role of Ca^{+2} in the degranulation process but, of course, does not prove it.

The effects of FMLP alone or in combination with cytochalasin B on ^{45}Ca efflux from PMNs were also studied, and the results of a typical experiment are summarized in Fig. 3. It has been reported previously (22) that FMLP increases significantly Ca^{+2} efflux. The effect appears to be
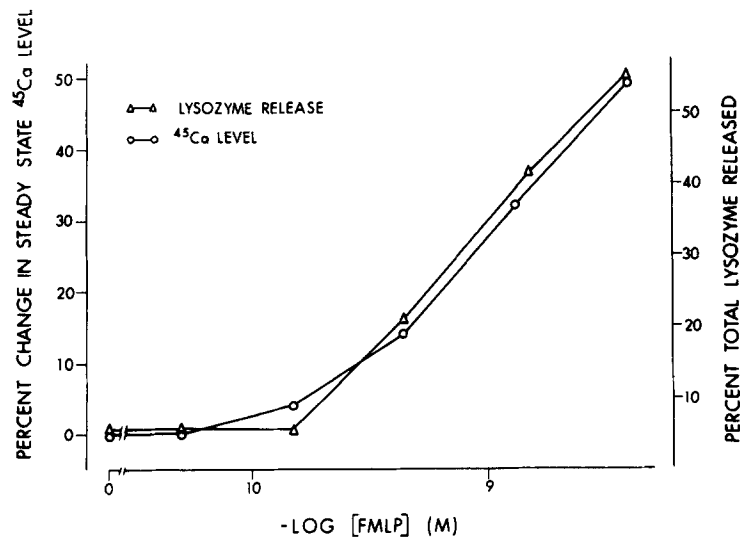

FIGURE 2   Variations of the changes in steady-state intracellular ^{45}Ca level and enzyme release with different concentrations of FMLP. Each point represents the average of duplicate determinations. The maximum variation between duplicate samples was 17%.
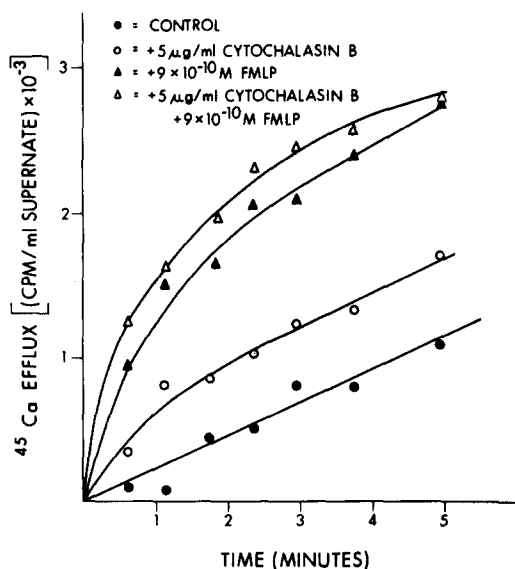
• = CONTROL
o = +5μg/ml CYTOCHALASIN B
▲ = +9×10⁻¹⁰M FMLP
△ = +5μg/ml CYTOCHALASIN B
+9×10⁻¹⁰M FMLP

^{45}Ca EFFLUX $[(CPM/ml\ SUPERNATE)\times10^{-3}]$

TIME (MINUTES)

FIGURE 3 The time-course of ^{45}Ca efflux across rabbit PMN membranes under various experimental conditions. Each point represents the average of duplicate determinations. The maximum variation between duplicate samples was 4%.

on the initial rate. Cytochalasin B alone has only a small but consistent (five experiments) increase on Ca^{+2} efflux. Again, the effect appears to be on the initial rate. This is in contrast to the observation of Gallin and Rosenthal (11) who have found that cytochalasin B inhibits Ca efflux in human PMNs simulated by C5a. The results in Fig. 3 also show that the rate of Ca^{+2} efflux in the presence of both cytochalasin B and FMLP is not statistically different from that observed in the presence of FMLP alone. This is in contrast to the dramatic increase in Ca^{+2} influx when both FMLP and cytochalasin B are present together, compared to the effect of either one of them alone (see Fig. 1).

Effect of Extracellular K^+ on the FMLP and Cytochalasin B-Dependent-Ca^{45} Influx

As already discussed previously, rabbit PMNs incubated in a K^+-free medium are much less sensitive to chemotactic factors in both their chemotactic responsiveness and cytochalasin B-dependent lysosomal enzyme release (26–28). To gain some insight into the mode of action of K^+ in lysosomal enzyme release, we have examined the effect of K^+ on the FMLP- and cytochalasin

B-dependent ^{45}Ca influx; lysosomal enzyme release was also measured simultaneously. The results are summarized in Fig. 4. ^{45}Ca influx was calculated as the difference in the counts between 0 time and 3 min after the addition of the stimuli. As was shown in Fig. 1, the counts taken up by the cells in the presence of cytochalasin B and FMLP at the end of 1 min do not vary significantly with time after 1 min after the addition of the stimuli. The results in Fig. 4 clearly show that FMLP and cytochalsin B-dependent ^{45}Ca influx is sensitive to the presence of extracellular K^+.

Effect of the Calcium Ionophore A23187 on ^{45}Ca Influx

The calcium ionophore A23187 is known to promote calcium uptake in a number of cell types, and to induce lysosomal enzyme secretion from PMNs (15, 23). This release does not require, although it can be enhanced by, cytochalasin B, but it is critically dependent on extracellular Ca^{+2} (28). Moreover, K^+ enhances the Ca^{+2}-dependent release induced by A23187 in both the absence and the presence of cytochalasin B (28). Accordingly, we investigated the effects of the calcium ionophore A23187 on both ^{45}Ca influx and lysosomal enzyme release in rabbit PMNs in the
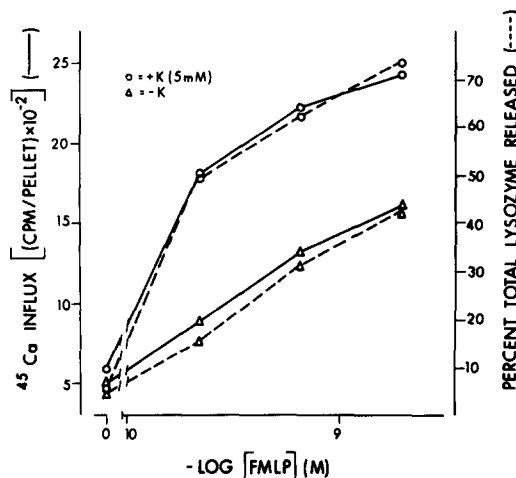

FIGURE 4 Effect of the removal of extracellular K^+ on ^{45}Ca influx and lysosomal enzyme release induced by various concentrations of FMLP. Cytochalasin B (5 μg/ml) is present in each case. Each point represents the average of triplicate determinations. The suspending medium in one case ($^+$K) is HBSS, whereas in the second case ($^-$K) the 4 mM K^+ was replaced by 4 mM Na^+.

presence and absence of extracellular potassium. The results are summarized in Fig. 5 and clearly demonstrate that a good parallelism exists between ^{45}Ca influx and lysosomal enzyme release elicited by A23187. Furthermore, it is quite clear from Fig. 5 that the removal of extracellular potassium shifts the dose-response curve significantly to the right.

As stated earlier, the action of the ionophore A23187 on lysosomal enzyme release does not require the presence of cytochalasin B but can be enhanced by cytochalasin B (28). This is also the case for the induced increase in ^{45}Ca influx as shown in Fig. 6.

Effect of Varying the Extracellular Concentration of Calcium on ^{45}Ca Influx under Different Experimental Conditions

Lysosomal enzyme release by rabbit PMNs in the presence of cytochalasin B induced by FMLP or the ionophore A23187 has been shown to be sensitive to the extracellular calcium concentration (28). To investigate further the relationship between calcium influx and enzyme release, we have

measured the effect of varying the extracellular calcium concentration on ^{45}Ca influx under different experimental conditions. The results are summarized in Table 1 and Fig. 7. In these experiments, enzyme release was measured on the same cell suspensions and under experimental condi-
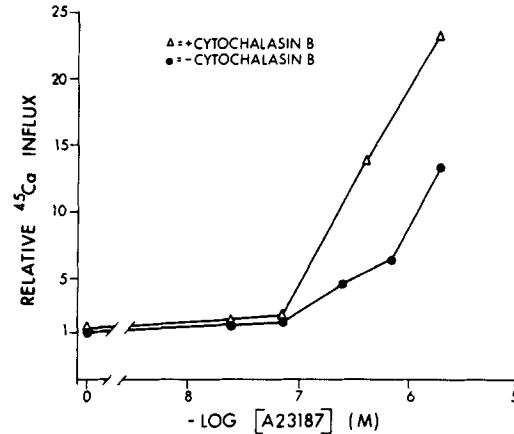

FIGURE 6   Effect of cytochalasin B (5 μg/ml) on ^{45}Ca influx induced by the ionophore A23187. Each point represents the average of triplicate determinations. The suspending medium is HBSS.
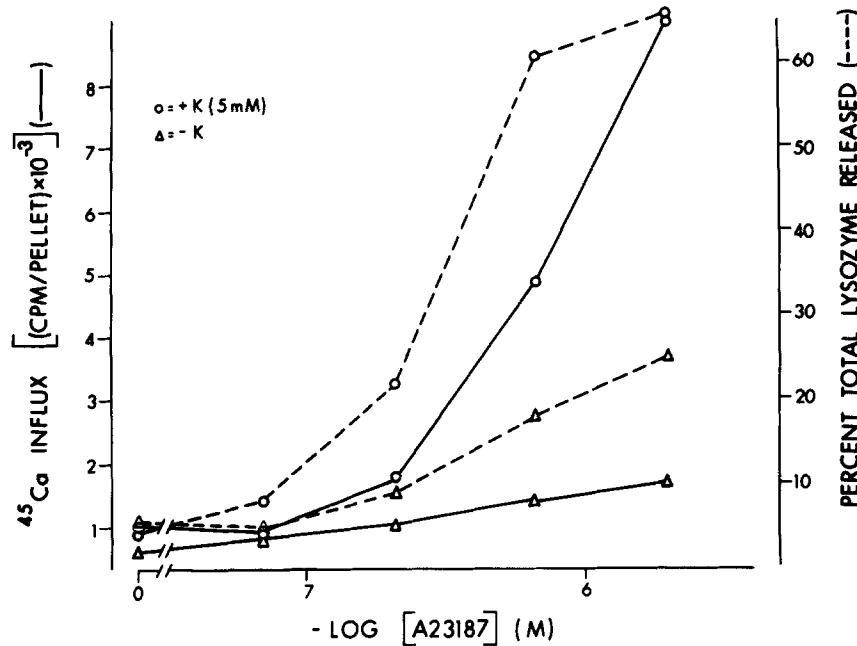

FIGURE 5   Variations of ^{45}Ca influx and enzyme release with varying concentrations of the divalent cation ionophore A23187, and the effect of the removal of extracellular K^+ on these two dose-response curves. Each point represents the average of triplicate determinations. The suspending media were identical to those in Fig. 4.

TABLE 1

*Effect of Varying the Extracellular Calcium on ⁴⁵Ca Influx across Rabbit PMNs under Different Experimental Conditions**

Experimental condition	⁴⁵Ca Influx			
	$[Ca]_o = 0.01$	$[Ca]_o = 0.1$	$[Ca]_o = 0.5$	$[Ca]_o = 1.0$
	nmol/ml packed cells × 3 min			
Control	5.6	20.7	57.0	41.7
+5 μg/ml Cytochalasin B	5.3	20.5	37.0	44.0
+5 × 10⁻¹⁰ M FMLP	6.2	20.2	51.2	35.7
+5 μg/ml Cytochalasin B and 5 × 10⁻¹⁰ M FMLP	6.8	45.2	199.0	390.0

Fluxes were calculated using the following relation: Flux = $[CPM]_i/[Ca]_o$ where $(CPM)_i$ represents the counts in 1 ml of packed cells in 3 min, and $[Ca]_o$ represents the specific activity (counts/mole) of calcium ion in the suspending medium.

* $[Ca]_o$ refers to the extracellular calcium concentration in millimolar. The values for fluxes are the average of at least triplicate determinations.
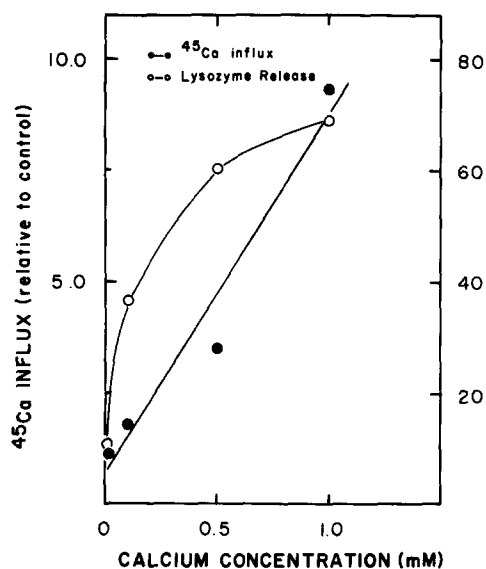

FIGURE 7 Effect of varying the extracellular Ca^{+2} concentration on ⁴⁵Ca influx and lysosomal enzyme release induced by 2 × 10⁻⁹ M FMLP. Each point represents the average of duplicate determinations. The suspending medium was HBSS in which the concentration of Ca^{+2} was varied from 0 to 1 mM.

tions identical to those used for flux measurements. The most important conclusion to be drawn from these studies is that the ⁴⁵Ca influx almost doubles whereas only a small increase in enzyme release is found when the extracellular concentration of calcium increases from 0.4 to 1 mM. This is important since it indicates that additional increases in ⁴⁵Ca influx can occur in the absence of appreciable corresponding amounts

of release suggesting that the observed change in ⁴⁵Ca influx is not due to a nonspecific generalized consequence of the release process.

Effect of FMLP and A23187 on ²²Na Influx in PMNs

Fig. 8 summarizes the results of a typical experiment dealing with the effect of the chemotactic factor FMLP in the presence and absence of cytochalasin B on ²²Na influx. As previously reported, FMLP by itself enhanced the rate of ²²Na influx in PMNs (21, 22). The presence of cytochalasin B significantly potentiated, however, both the onset and the magnitude of the FMLP-induced enhancement of ²²Na influx. The same concentration of cytochalasin B alone produced only a small enhancement of ²²Na influx. It is quite evident that the addition of FMLP to cells already incubated for 1 min with cytochalasin B elicits a large and a rapid enhancement of ²²Na influx which is significantly larger than the sum of the two increases obtained by the two agents separately. In addition, the time-course of FMLP-induced ²²Na influx can be seen to closely parallel that of ⁴⁵Ca influx (Fig. 1).

The dependency of the enhancement of the ²²Na influx on the concentration of FMLP was investigated next. In addition, in order to determine whether the ²²Na influx is a secondary effect of the degranulation process, or possibly a causative event, conditions were sought under which enzyme release but not ²²Na influx would be inhibited. Such conditions were met with those cell preparations that were highly dependent on
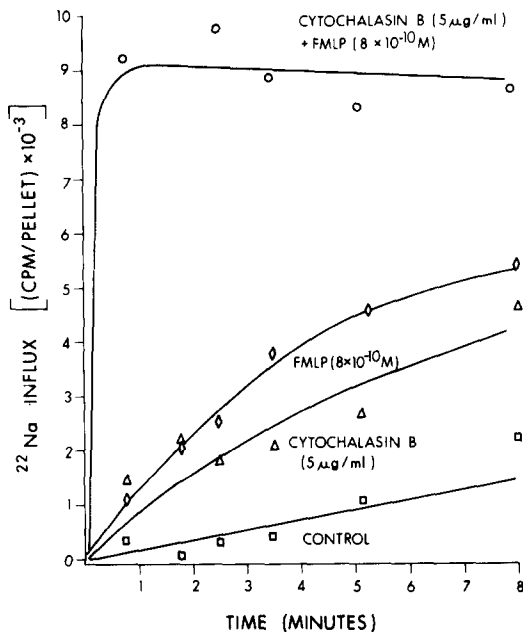
FIGURE 8 The time-course of ^{22}Na influx across rabbit PMN membranes under various experimental conditions. Cytochalasin B (5 μg/ml) was added 1 min before the addition of FMLP and the isotope. Each point represents the average of duplicate determinations. The maximum variation between duplicate samples in this experiment was 6%.

extracellular calcium. The results of such an experiment are shown in Fig. 9. ^{22}Na influx was calculated as the difference in the counts between 0 time and 3 min after the addition of the stimuli. FMLP clearly enhances ^{22}Na influx over the same concentration range in which it induces lysosomal enzyme release. In addition, it can be seen that the FMLP-induced enhancement of sodium influx still occurs to the same extent under conditions of reduced enzyme release, i.e., in the absence of added calcium.

Slightly different results are obtained when the above experiment is performed with A23187 instead of FMLP as a stimulus. A23187 can be seen in Fig. 10 to induce a calcium-dependent enhancement of ^{22}Na influx. The removal of extracellular calcium abolishes both the enzyme release and the enhancement of ^{22}Na influx.

Effect of FMLP and Cytochalasin B on ^{42}K Fluxes

FMLP, by itself, has been shown previously to induce a small, ouabain-sensitive enhancement of

^{42}K influx across membranes of rabbit PMNs (22). In the present studies, we examined the effect of FMLP, of cytochalasin B alone, and of both together, on ^{42}K influx. As shown in Fig. 11, FMLP induces an increase in ^{42}K influx that is abolished in the presence of cytochalasin B. No significant differences, however, can be observed between the control points and those obtained in the presence of cytochalasin B alone or together with FMLP. If, as have been suggested in previous studies (22), the enhanced ^{42}K influx in the presence of FMLP is a reflection of an increase in the "Na$^+$, K$^+$" pump activity, the latter could result from an increase in intracellular sodium concentration.

We have previously shown that FMLP by itself has no effect on ^{42}K efflux (22). We have repeated these experiments and found that whereas FMLP by itself has no effect, in the presence of cytochalasin B it induces a rapid and dramatic enhancement of ^{42}K efflux from PMNs. In fact, as shown in Fig. 12, both FMLP and A23187, in the presence of cytochalasin B, produce similarly large increases in ^{42}K efflux. The time-course of each closely resembles their respective time-courses for enzyme release. ^{42}K efflux was affected only slightly by cytochalasin B alone.

The effects of FMLP and cytochalasin B on ^{42}K efflux in the presence and absence of extracellular calcium were next studied, in an attempt to dissociate this enhancement from the secretory response. As can be seen in Fig. 13, however, the magnitude of the increase in ^{42}K efflux was found to be closely related to the extent of the degranulation.

DISCUSSION

The experiments reported here were designed to examine, under conditions which induce lysosomal enzyme release in PMNs, the effects of FMLP on cation (Ca^{+2}, Na$^+$, and K$^+$) fluxes in these cells. In addition, the effects on cation fluxes of one other secretagogue, the calcium ionophore A23187, were also examined. It is quite clear from the results presented, that the two secretory stimuli tested here enhanced ^{45}Ca influx by PMNs and increased the amount of intracellular free Ca^{+2}. As expected, A23187 causes a large enhancement of the rate of ^{45}Ca influx in PMNs. The similarity of the changes in calcium metabolism induced by two secretagogues as diverse as FMLP and A23187 provides evidence for the involvement of calcium at some later common
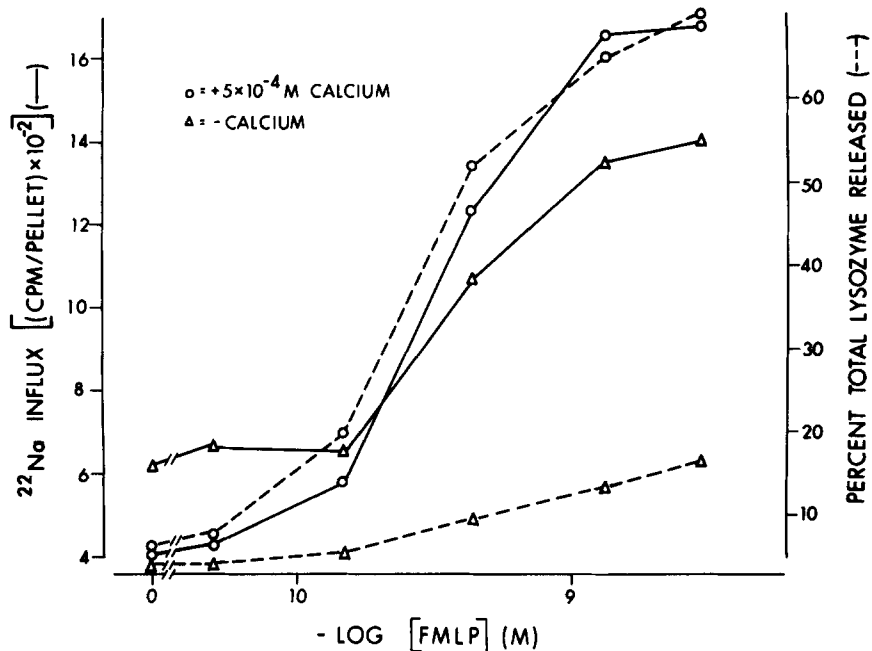
FIGURE 9   Variations of ^{22}Na influx and lysosomal enzyme release induced by FMLP in the presence and absence of 5×10^{-4} M extracellular Ca^{+2}. Each point represents the average of duplicate determinations. Cytochalasin B (5 μg/ml) was present 1 min before the addition of FMLP and the isotope in all cases. The suspending medium is HBSS.
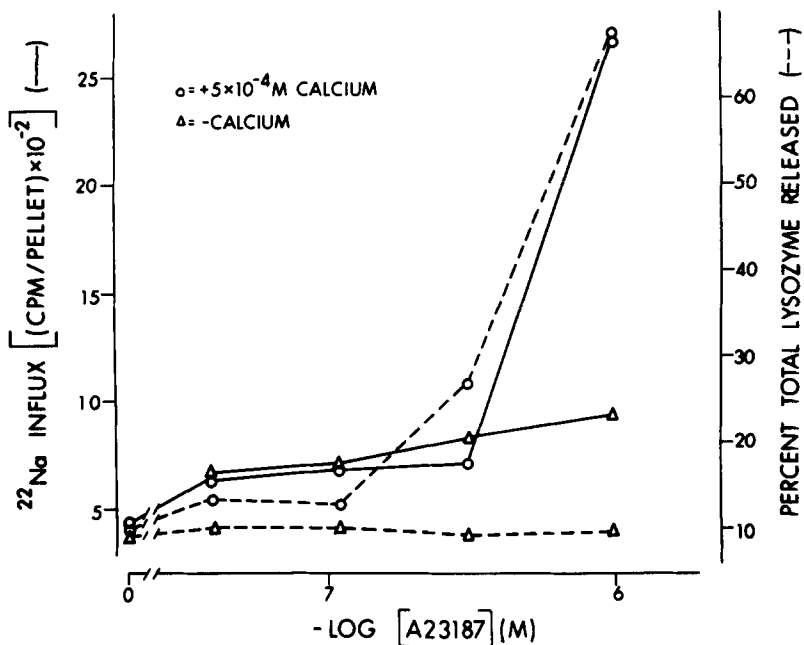

FIGURE 10   Variations of ^{22}Na influx and lysosomal enzyme release induced by the divalent cation ionophore A23187 in the presence and absence of 5×10^{-4} M extracellular Ca^{+2}. Each point represents the average of duplicate determinations. The maximum difference between duplicate samples was 5%. Cytochalasin B (5 μg/ml) was present 1 min before the addition of the ionophore and the isotope in all cases. The suspending medium is HBSS.
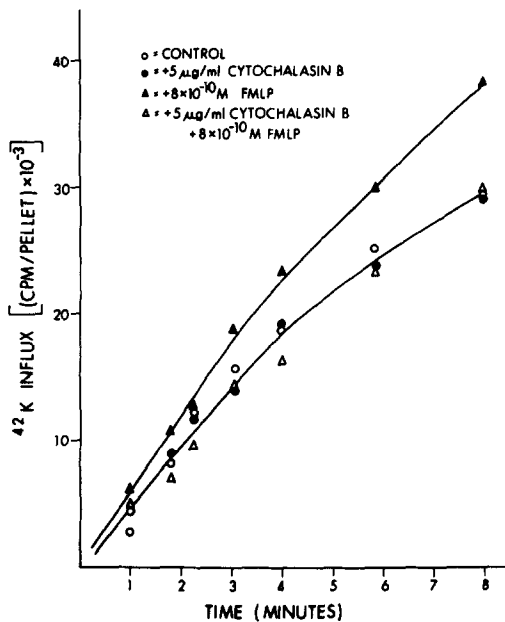
FIGURE 11 The time-course of ^{42}K influx across rabbit PMN membranes under various experimental conditions. Conditions are identical to those in Fig. 1. The results are from a single experiment, and each point represents the average of duplicate determinations. The maximum variation between duplicate samples was 5%. The suspending medium is HBSS.

step of the release process. The nature of the calcium-sensitive biochemical steps (microfilament activation, membrane fusion, etc.), however, is still conjectural.

Up to now, the results have been discussed on the assumption that the increase in Ca^{+2} influx is a cause of the release. One alternative is that the influx is a consequence of the release. We have been unable, under all circumstances we have tested, to prevent release and not inhibit Ca^{+2} influx. Thus, we have no direct evidence that eliminates the latter hypothesis. There is, however, indirect evidence that makes it improbable. There are at least three not necessarily independent ways by which the release process may give rise to the observed rapid increase in ^{45}Ca influx. First, new Ca^{+2}-binding sites may appear on the surface of the membrane. Second, the membrane may become transiently very leaky as a result of the release. Third, a combination of both the first and second ways could be operating. The following arguments suggest that the observed increase in ^{45}Ca influx cannot be entirely due to these nonspecific changes:

(a) If the first possibility is correct, the amount of new binding sites exposed as a result of the release must be tremendous. As the results summarized in Table 1 clearly demonstrate, ^{45}Ca influx, when the outside concentration of calcium is 1 mM, in cells treated with 5 μg/ml cytochalasin B and 5 × 10^{-10} M FMLP, is about one order of magnitude higher than control. Simple quantitative calculation indicates that even if all the membrane surface area becomes available for binding, it could not account for this increase in influx. Furthermore, the results in the same table clearly show that the effect of 5 × 10^{-10} M FMLP on ^{45}Ca influx in cells treated with cytochalasin B is comparable with that of a large concentration of the divalent cation ionophore A23187 on cells also treated with cytochalasin B. It is well established by now that this ionophore causes a significant increase in net calcium movement in a variety of cells (23). In addition, the ^{45}Ca influx almost doubles whereas only small increase in enzyme release is found when the extracellular concentration of calcium increases from 0.5 to 1 mM (Fig. 7).
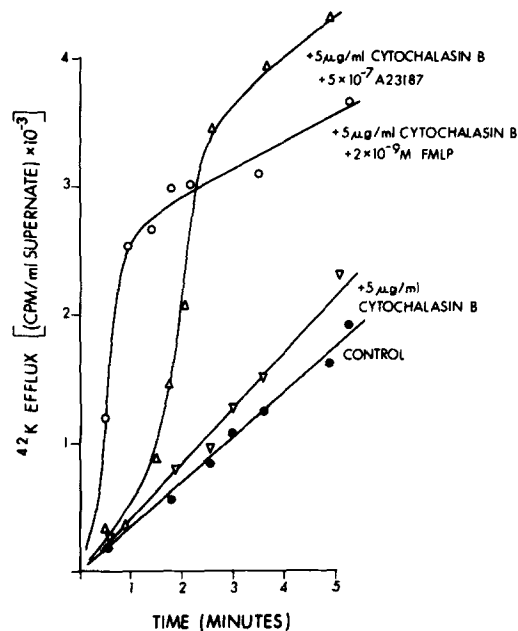

FIGURE 12 The time-course of ^{42}K efflux across rabbit PMN membranes under various experimental conditions. The agents (cytochalasin B, 5 μg/ml; FMLP, 2 × 10^{-9} M; and A23187, 5 × 10^{-7} M) were added after the cells had been washed free of extracellular radioactive potassium. The suspending medium is HBSS.
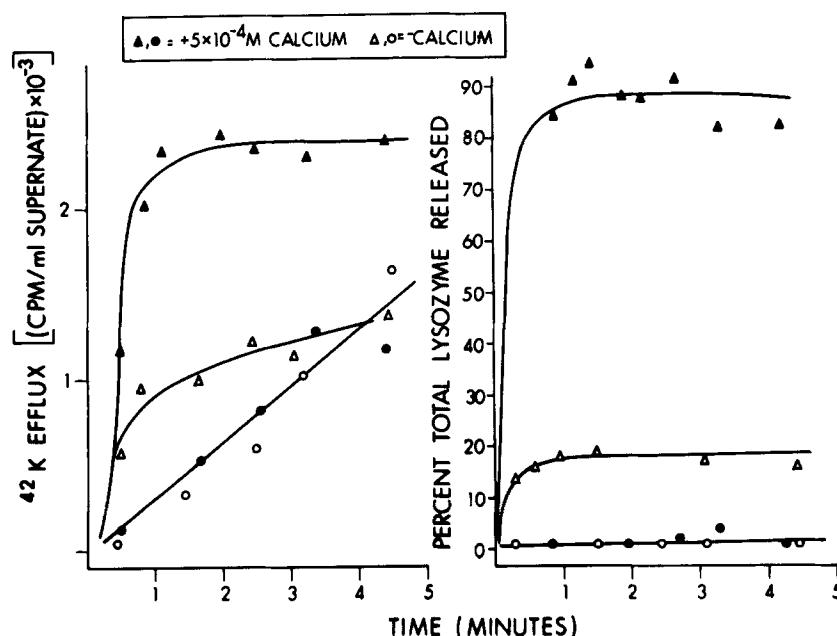
FIGURE 13   Effect of the removal of extracellular Ca^{+2} on the time-course of ^{42}K influx and lysosomal enzyme release induced by 2×10^{-9} M FMLP. Each point represents the average of at least two determinations. The variation between duplicate samples is <5%. $\triangle$, Control in complete HBSS. $\blacktriangle$, In the presence of FMLP and cytochalasin B in complete HBSS. $\bigcirc$, Control in HBSS from which calcium has been deleted. $\triangle$, In the presence of FMLP and cytochalasin B in HBSS from which calcium has been deleted.

(b) The finding that ^{42}K influx, under the same conditions used in ^{45}Ca-influx measurement, is not strongly affected argues against the possibility that the observed increase in the latter influx is due to a generalized leakiness of the cell membrane. In addition, the observation that the increase in ^{45}Ca efflux is small and not rapid supports this conclusion. Also, the fact that no significant amount of LDH release can be detected under these conditions is consistent with this view.

(c) We have shown previously that FMLP can produce significant increases in both Na^+ and Ca^{+2} influxes under conditions in which enzyme release does not occur, i.e., cytochalasin B is not present (22).

The results shown in Fig. 8 show that, in the presence of cytochalasin B, FMLP induces a rapid concentration-dependent enhancement of ^{22}Na influx in PMNs. As in the case of calcium influx, both the time-course and the concentration dependence of the enhancement of ^{22}Na influx by FMLP in the presence of cytochalasin B are consistent with a direct role of sodium in the degranulation process. In addition, as can be seen

in Fig. 9, the FMLP-induced enhancement of the rate of ^{22}Na influx persists, albeit slightly reduced, under conditions which greatly inhibit lysosomal enzyme release. The observed increase in ^{22}Na influx is most likely due to a direct increase in the inward membrane permeability to Na^+. A second possibility, which may account for at least part of the observed increase, is that Na^+ influx is coupled to a Ca^{+2} efflux pump. This is a good possibility since a Na^+, Ca^{+2} exchange mechanism has been shown to be present in many membrane systems (5). However, until a similar mechanism is shown to be present in neutrophils, this possibility must remain conjectural.

The enhancement of ^{22}Na influx induced by A23187 is, on the other hand, even more perplexing. A23187 enhances ^{22}Na influx only in the presence of extracellular calcium. This strictly Ca^{+2} dependent increase in ^{22}Na influx strongly supports the hypothesis that a "Ca^{+2} efflux, Na^+ influx" exchange mechanism is present in rabbit PMN membrane. According to this interpretation, only part of the FMLP-induced enhancement of Na^+ influx would be directly caused by the chem-

otactic factor; the other part would be a secondary effect due to the increase in the intracellular concentration of free calcium. This idea is similar to that proposed by Gallin et al. (12). The slight reduction in the magnitude of the enhancement of the rate of ^{22}Na influx in the absence of extracellular calcium observed in Fig. 12 is consistent with this interpretation. The existence of such a Na$^+$, Ca^{+2} exchange mechanism would, furthermore, help explain the enhanced rate of spontaneous release observed in the absence of extracellular sodium (28). Basal secretory rates from a variety of tissues, including catecholamine release at adrenergic synapses (19) and from the adrenal medulla (2) and insulin release from β cells (17), are likewise stimulated by the removal of extracellular sodium. Some evidence is given to this suggestion by the finding that the presence of Ca^{+2} increases the spontaneous release in the absence of Na$^+$ (28).

The role of potassium and/or potassium fluxes in lysosomal enzyme release in PMNs is still unclear at the present time. FMLP has previously been shown to cause a ouabain-sensitive, concentration-dependent increase in the rate of K$^+$ influx in PMNs (22), and this was confirmed here. Furthermore, while the addition of FMLP alone does not affect ^{42}K efflux from PMNs (22), in the presence of cytochalasin B it induces a large and rapid loss of ^{42}K from the cells. In fact, FMLP and A23187 both induced similar enhancements of ^{42}K efflux from PMNs, the time-course of which closely resembled that of enzyme release. In addition, this enhancement could be observed only when enzyme release occurred. Two possible mechanisms may account for this rapid ^{42}K efflux. First, this increase is merely due to the exocytosis induced by the various secretory stimuli. A second and more likely possibility is that these stimuli lead to a significant increase in the amount of intracellular free calcium. This increase in free calcium could lead, as generally found in other systems, to a rapid and specific net K$^+$ efflux (13), giving rise to membrane hyperpolarization. Gallin et al. advanced a similar idea to account for the substantial hyperpolarization that they have recorded in macrophages upon stimulation (11). Although this phenomenon has been observed and studied extensively, it is still not clearly understood (6, 10, 13, 20). In any case, in view of the results previously obtained (22) and those described above, it does not appear likely that potassium fluxes are directly involved in either

the chemotactic or the secretory response of rabbit peritoneal PMNs. However, a modulating influence of extracellular potassium, possibly via membrane potential changes, on the response of PMNs to FMLP and A23187 cannot be ruled out.

A flow diagram of the events that are thought to accompany the stimulation of PMNs by chemotactic or degranulating stimuli is presented in Fig. 14. This hypothesis was designed to accommodate the data presented in this report and the previously described results concerning the effects of chemotactic factors on ionic fluxes in PMNs and should be taken as a tentative working hypothesis. On the basis of this hypothesis, the sequence of events starts with the binding of a given stimulus with a specific membrane receptor, the presence of which on the cell membrane of PMNs has started to be documented (1, 30). As a result of this initial interaction, the inward permeabilities of the plasma membrane to sodium and calcium are transiently increased, leading to a membrane depolarization. The magnitude of these changes may be much greater in the presence of cytochalasin B. Direct evidence for an increase in the permeability of PMNs to ^{22}Na and ^{45}Ca after the addition of chemotactic and degranulating stimuli has been presented here and elsewhere (7, 11, 22). In addition, Gallin et al. have obtained intracellular recordings from macrophages showing small transient depolarizations upon the exposure to partially purified C5a (12). As a result of these events, intracellular calcium is released from previously bound stores. That the plasma membrane may act as at least one such store is consistent with the effects of chemotactic factors on ^{45}Ca efflux in the absence of extracellular calcium (7, 11) and with the recent report by Gallin et al. of increased submembranous calcium deposits at the leading edge of cells oriented in a chemotactic gradient (12).

The (localized) increase in the concentration of calcium will then, in a basically unknown manner, induce locomotion. The microfilament and/or microtubular system of the PMNs are most probably involved at this step. The increase in the amount of intracellular free Ca^{+2} could lead to an increase in the activity of the Na$^+$, Ca^{+2} exchange pump, resulting in an additional elevation in the concentration of intracellular Na$^+$. The increase in intracellular Na$^+$ concentration will further activate the Na$^+$, K$^+$ pump of the PMNs, resulting in a delayed, ouabain-sensitive enhancement of ^{22}Na efflux and K^{42} influx (22). Cytochalasin B en-
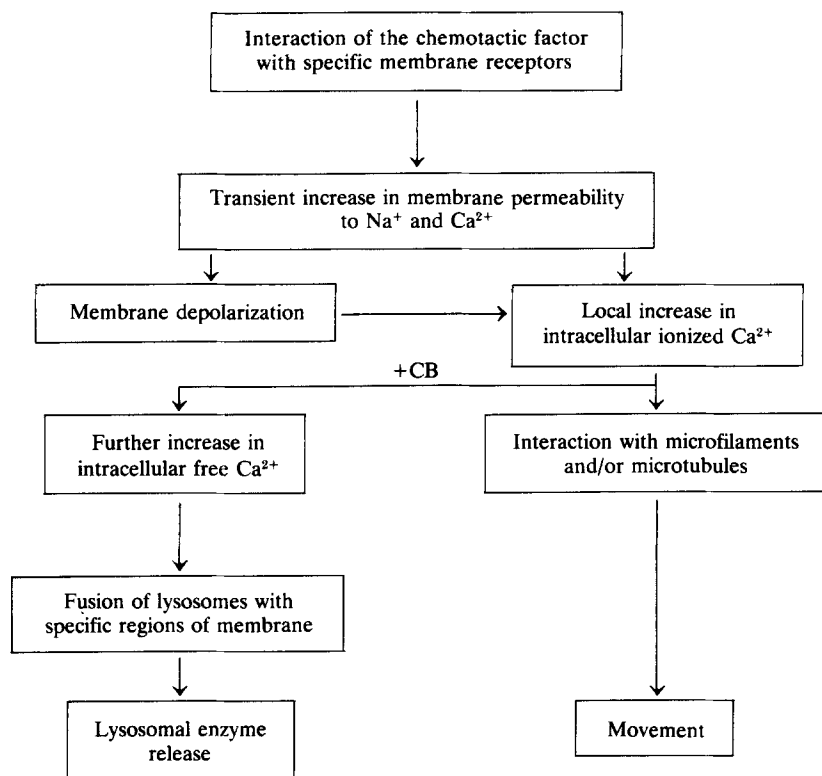
FIGURE 14 A flow diagram of the events concerning ion movements that are thought to accompany chemotaxis and lysosomal enzyme release in rabbit neutrophils. *CB* refers to cytochalasin B.

hances the ^{45}Ca influx response of the PMNs. It is conceivable, within the above-described framework, to think of cytochalasin B as affecting the state of a membrane constituent which either would normally lower the permeability of the plasma membrane to calcium and Na^+ or, alternatively, would buffer the incoming calcium ions, rendering them unavailable to the cytoplasm. The nature of this constituent and its possible relationships to the effect of cytochalasin B on microfilaments (18, 29) are, of course, completely conjectural. Nevertheless, in the presence of cytochalasin B, more free calcium becomes locally available in the cytoplasm, leading to a fusion of the lysosome with a specific region of the plasma membrane and resulting in a lysosomal release. Cytochalasin B may produce its effect either by potentiating the ability of FMLP to release calcium and/or by inhibiting those processes (calcium pump, the calcium-binding capacity of certain intracellular proteins) which are responsible for maintaining a very low intracellular concentration of ionized calcium. The fact that lysosomal en-

zyme release can be induced by the calcium ionophore A23187 in the absence of cytochalasin B argues strongly against a central role for cytochalasin B in the sequence of events leading to release.

Finally, it is interesting to speculate that in vivo, at the site of injury where "chemotactic agent" is at a very high concentration and the process of phagocytosis very prominent, these conditions may give rise to a significant increase in the amount of intracellular free calcium. Similar ideas have been advanced by Gallin et al. (12). The process of phagocytosis results in the formation of a phagocytic vacuole, the membrane of which is probably different structurally from that of its precursor, the plasma membrane. The phagocytic vacuole membrane could contain specific membrane regions with which the granules can fuse and could lack a significant amount of the enzyme normally responsible for the removal of Ca^{+2} from the cytoplasm, the "calcium pump." The absence of the calcium pump from the phagocytic vacuole membrane may allow the calcium

ions, which are brought from the outside medium into the vacuole, to leak into the cytoplasm, giving rise to a local increase in the cytoplasmic free calcium and thus enhancing the fusion of the granules with the phagocytic vacuole membranes.

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