

TRANSPORT OF SODIUM, POTASSIUM,
AND CALCIUM ACROSS RABBIT
POLYMORPHONUCLEAR LEUKOCYTE MEMBRANES
Effect of Chemotactic Factor

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ABSTRACT

The transport properties of the rabbit peritoneal polymorphonuclear leukocyte (PMN) plasma membrane to Na^+ , K^+ , and Ca^{2+} have been characterized. The use of a silicone oil centrifugation technique provided a rapid and reliable method for measuring ion fluxes in these cells. Na^+ and K^+ movements across PMN membranes were found to be rapid. The value for the unidirectional steady-state fluxes (in meq/liter cell \times min) were of the order of 3.0 for Na^+ and 7.4 for K^+ . Ouabain inhibited both K^+ influx and Na^+ efflux, the latter being also dependent on the presence of extracellular potassium. The rate constant (in min^{-1}) for ^{45}Ca influx was found to be .05 and that for ^{45}Ca efflux .04. The synthetic chemotactic factor formyl-methionyl-leucyl-phenylalanine (FMLP) was found to affect the fluxes of Na^+ , K^+ , and Ca^{2+} at concentrations as low as 10^{-10} M. FMLP induced a large and rapid increase in the permeability of the PMN plasma membrane to ^{22}Na . Smaller and delayed enhancements of ^{42}K influx and ^{22}Na efflux were also noted. Some evidence that the latter findings are a consequence of the increased ^{22}Na influx is presented. ^{45}Ca influx and efflux were also stimulated by FMLP. In the presence of 0.25 mM extracellular calcium, FMLP induced an increase in the steady-state level of cell-associated ^{45}Ca . In the presence of .01 mM extracellular calcium, however, a transient decrease in the steady-state level of cell-associated ^{45}Ca was induced by FMLP. The curves relating the concentration of FMLP to its effects on cation fluxes are very similar to those found for its enhancement of migration.

The presence of actin- and myosin-like proteins has been demonstrated in almost all mammalian cells that have been investigated (19, 16) including polymorphonuclear leukocytes (PMN) from various species (33, 28). Various investigators have postulated the participation of these contractile elements in the control of cellular shape change, secretion and movement (11, 1). In 1954, Fukushima et al. (13) suggested that the movement of

polymorphonuclear leukocyte cells involved contractile mechanisms similar to those found in muscle cells. Recently, various lines of indirect evidence have been presented in support of this idea. For example, glycerinated rabbit peritoneal PMN have been shown to contract upon addition of ATP in a calcium-dependent manner (15); actin and myosin have been isolated and characterized from leukocytes (33, 28); human neutrophils with

poorly polymerizing actin were found to be defective in their movement and chemotactic responsiveness (7); cytochalasin B, an agent reported to interfere with microfilament function, inhibits the locomotion and chemotactic responsiveness of rabbit and human neutrophils (37), and the chemotactic activity of neutrophils can be significantly altered by changing the extracellular ionic environment and by agents that affect K^+ movement such as ionophores and ouabain (29). In accord with this, it has been suggested (29, 3) that membrane events similar to those found in muscle, such as changes in membrane permeability to Na^+ and Ca^{2+} , might precede and initiate the chemotactic response of PMN.

These studies were undertaken to provide a complete and systematic investigation of the transport properties of the rabbit PMN to Na^+ , K^+ , and Ca^{2+} and also to study the effect of chemotactic factors on the movement of these ions. The object of the latter part of this study is to test directly the hypothesis that membrane permeability of rabbit PMN changes do occur in the presence of a chemotactic factor. The rabbit peritoneal PMN was chosen as the model cell, while the synthetic peptide formyl-methionyl-leucyl-phenylalanine (FMLP) whose chemotactic properties have just been reported (4) was used as the chemotactic factor.

MATERIALS AND METHODS

Preparation of Cells

Polymorphonuclear leukocytes were obtained as previously described (5) from albino rabbits (2-3 kg) which were injected intraperitoneally with 300-500 ml of sterile isotonic saline solution containing glycogen (0.5 g/liter). The peritoneal exudate collected in a heparinized flask was strained through cheesecloth to remove large clumps and debris. For the measurement of ion fluxes, cells were resuspended after washing in an isotonic phosphate-buffered solution whose composition was (mM): NaCl, 150; KCl, 5; $MgCl_2$, 1; $CaCl_2$, 0.25; Na_2HPO_4 , 5.0; NaH_2PO_4 , 1.0; glucose, 10.0; crystalline bovine serum albumin 1 mg/ml; pH, 7.3. All experiments were carried out at 37°C. The cells were maintained in suspension by gentle stirring with micromagnetic stirring bars. All glassware used was siliconized. The majority of experiments were performed with a cell concentration of 5×10^6 cells/ml, although occasionally cell concentration of up to 10^7 cells/ml were used. No cell concentration dependent effects were noted for any of the experiments to be reported. Representative determinations indicated that 90-95% of the cells were viable at the completion of each experiment as shown by Trypan Blue exclusion and

lactic dehydrogenase release (a cytoplasmic enzyme marker). There were no differences in viability between the control cell suspensions and those treated with any of the chemicals used in this study.

Transport Studies

Fluxes were measured by a fast and reproducible technique (12, 20). 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 (Versilube F50, density 1.05 g/ml, Harwick Chemical Corp., Cambridge, Mass.) in 1.5-ml capacity microcentrifuge tubes. The separation of the cell 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 8,000 rpm reached in less than 10 s). It was found that practically no radioactivity could be found in the oil layer and that the trapped extracellular space, as determined by [^{14}C]inulin, was small (less than 0.2% of the counts in 1 ml of the total suspension [27]). Whenever solubilization of the cell pellets was required, the supernate and the oil layer were aspirated by suction, and the bottom of tubes containing the cell pellets was excised and the pellets were transferred to test tubes to which 0.5 ml of 0.1% Triton X-114 in 1N NaOH was added. The samples were then incubated with occasional agitation for 1 h at 50°C. An equal volume of 1N HCl was then added, and aliquots were taken for the measurement of either radioactivity or protein, the latter being measured by the method of Lowry et al. (18). The amount of protein was found to be linearly related to the number of cells layered on top of the oil layer.

For efflux experiments, the cells were preincubated 30-45 min with the desired isotope at 37°C. They were then centrifuged 2 min at 1,000 g in a refrigerated Sorvall RC3 centrifuge (DuPont Instruments, Sorvall Operations, Newtown, Conn.) for two minutes in 15-ml conical tubes. The supernate was aspirated and the pellet was resuspended in 1 ml of the desired buffer containing no radioactivity and transferred to 1.5 ml Eppendorf microcentrifuge tubes with a Pasteur pipet. The suspension was then centrifuged for about 5 s in an Eppendorf microcentrifuge, the supernate was removed, and the pellet was gently resuspended in 1 ml of buffer. This was repeated three times, and it 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 less than 4 min. Occasionally, the integrity of the cells at the end of the washing procedure and also at the completion of efflux experiment was checked. It was generally found that more than 90% of the cells were viable as shown by Trypan Blue exclusion and lactic dehydrogenase release. At a preset time, aliquots were then sampled and centrifuged as described above. The radioactivity in both the supernate and in the pellets was determined. The intra-

cellular concentrations of Na^+ , K^+ , and Ca^{2+} were measured with the atomic absorption spectrophotometer (Perkin-Elmer, Model 107; Perkin-Elmer Corp., Instrument Div., Norwalk, Conn.). Steady-state unidirectional fluxes were calculated by the standard two-compartmental analysis (32, 23, 24, 9). The theory of the kinetics of two-compartment system has been extensively studied. The fundamental equation which governs the movement of a given substance in a closed two-compartment system is equation 1:

$$\ln [1 - S/S_{\infty}] = -Kt \quad (1)$$

in which S and S_{∞} refer to the concentration of radioactivity (counts per unit volume) in the cells at any given time and at equilibrium, respectively. K is a first-order rate constant in reciprocal time and t is time. One of the basic assumptions in this analysis is that the cells as well as the bathing medium are in steady state with respect to the concentration of the ion under study. The linearity between $\ln [1 - S/S_{\infty}]$ and time is the basic characteristic of two-compartment system. Cation fluxes are obtained by multiplying the rate constants by the intracellular concentrations per liter of cell of the respective cation. Identical analysis is followed for efflux studies.

Chemicals

The chemotactic factor FMLP was synthesized as previously described (30). 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 (less than .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 Corporation, Boston, Mass. Ouabain and bovine serum albumin were purchased from Sigma Chemical Company, St Louis, Mo., and all other reagents were analytical grade.

RESULTS

Steady-State Na^+ and K^+ Fluxes

We initially studied the kinetics of steady state Na^+ and K^+ movements across rabbit PMN membranes. Figs. 1 and 2 show the time courses of Na^+ and K^+ influx, respectively, in a typical experiment. Both processes are rapid, reaching virtual

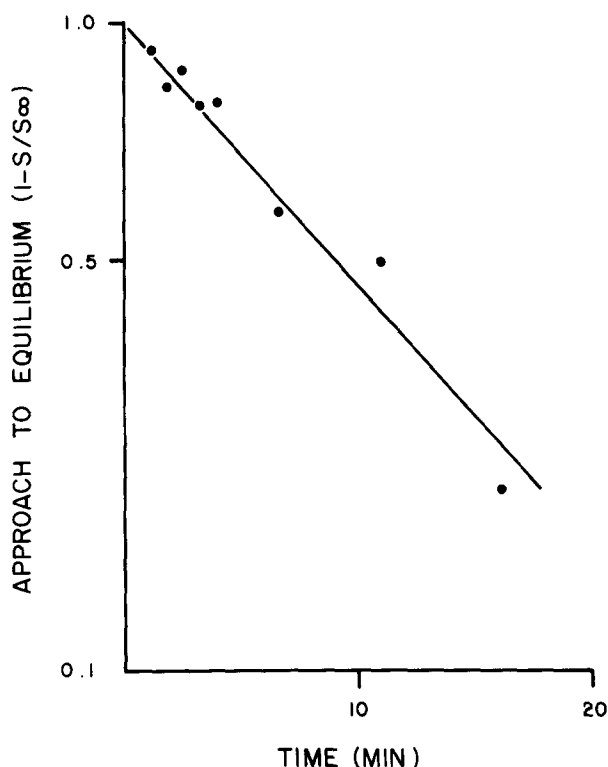


FIGURE 1 The time course of ^{22}Na influx across rabbit PMN membranes. S and S_{∞} refer to the amounts of radioactivity in the cells at any given time and at equilibrium, respectively.

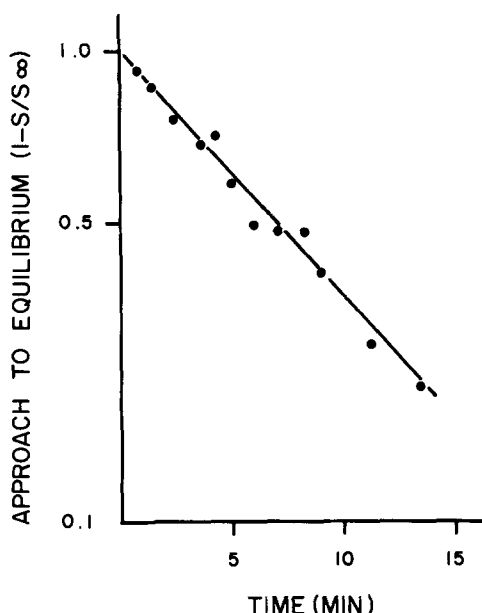


FIGURE 2 The time course of ^{42}K influx across rabbit PMN membranes. S and S_{∞} refer to the amounts of radioactivity in the cells at any given time and at equilibrium, respectively.

completion within less than 30 min. The average value and the standard error of the mean of the rate constant for K^+ influx measured under steady-state conditions is $0.086 \pm 0.01 \text{ min}^{-1}$. This is equivalent to an influx of 7.1 meq/liter of cell \times min. The corresponding value of the rate constant for Na^+ influx is 0.087 ± 0.01 , which is equivalent to an influx of 3.0 meq/liter cells \times min. These fluxes are about two orders of magnitude higher than the corresponding values for mammalian red cells (31, 34). The time courses of Na^+ and K^+ efflux were also studied. The results of a representative experiment for the efflux of ^{42}K are shown in Fig. 3. In the initial experiments, the time course of Na^+ efflux was measured by following both the appearance in the supernate and [or] the disappearance from the cells of ^{22}Na . Although the values of the rate constant calculated by both methods were in good agreement, the value calculated by following the disappearance of the radioactivity from the cell was more reproducible. The results of a representative experiment for the time course of the efflux of ^{22}Na are shown in Fig. 4. The average values for Na^+ and K^+ fluxes measured under steady-state conditions are summarized in Table I.

Effect of Ouabain on Na^+ Efflux and K^+ Influx and the Dependence of Na^+ Efflux on Extracellular K^+ Concentration

These experiments were carried out to test whether or not the observed concentration gradients of Na^+ and K^+ across rabbit leukocyte membranes are maintained by means of a "Na, K pump". The results of two representative experiments on the effect of ouabain on the time course of K^+ influx and Na^+ efflux are depicted in Figs. 5 and 6, respectively. As shown in these figures, the inhibitory effect of ouabain can be readily detected at 10^{-6} mol ouabain. Furthermore, we tested the effect of 10^{-4} M ouabain on both the influx of Na^+ and the efflux of K^+ and, as expected, neither was affected by this concentration of ouabain.

A second important characteristic of the Na, K pump which we have tested is the dependence of Na-efflux on extracellular K^+ concentration. In these experiments, the cells were preincubated for 40 min with ^{22}Na in complete buffer. As described in Methods, they were then washed either in complete buffer or in a buffer from which KCl was omitted and replaced by an isoosmotic amount of NaCl. The results of a typical experiments are shown in Fig. 7. It is quite clear that removal of K^+ from the bathing medium lowers significantly the efflux of Na^+ . Furthermore, the efflux of Na^+ was inhibited to about the same extent by either the addition of ouabain or the removal of K^+ as would be expected should a Na, K pump exist in rabbit PMN membranes.

Effects of the Chemotactic Factor FMLP on Na^+ and K^+ Fluxes

The synthetic peptide, FMLP, is highly chemotactic. Its activity can be detected at concentrations as low as 10^{-11} M, reaching optimum activity at 5×10^{-10} M, and has an ED_{50} of about 8×10^{-11} M as determined in modified Boyden chambers (30). The effects of FMLP on the various fluxes described above were studied over a concentration range that covered all phases of the dose-response curve for chemotaxis. For influx studies the chemotactic factor was added at the same time as the isotope, whereas for the efflux studies it was added when the cells which had been preloaded with the isotope under study were

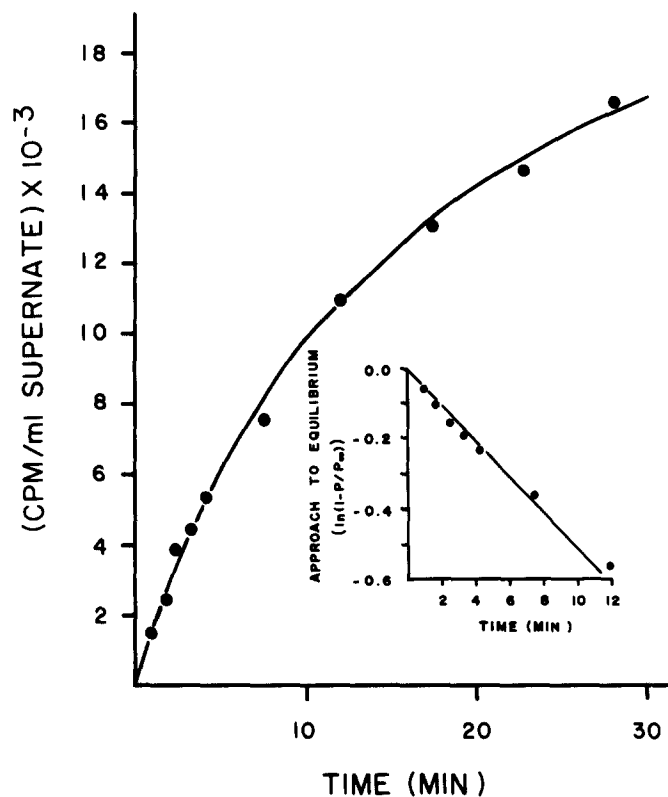


FIGURE 3 The time course of ^{42}K efflux across rabbit PMN membranes. *Inset* represents the same data in a semilogarithmic plot. P and P_{∞} , respectively, refer to the counts per minute per milliliter of supernate at any given time and at equilibrium.

washed and transferred to suspending medium containing no radioactivity. In the initial experiments, a complete time course for Na^+ influx or efflux was followed at two different concentrations of the chemotactic factors. It was found that a slight enhancement of the rate of Na^+ -influx was seen at 3×10^{-11} M, whereas, at 3×10^{-9} M FMLP, ^{22}Na was completely equilibrated in 4 min as opposed to 30 min for the control curve. Since it is not feasible experimentally to follow a complete time course with all the concentrations of FMLP needed for a dose response curve, we chose to measure the radioactivity in the cell at two preset points in time (5 and 30 min). From the slopes of the lines joining the origin and the first point, estimates of the "initial rates" can be obtained. These are most probably underestimates in those cases where FMLP exerted a large effect. Attempts at sampling at shorter times after the addition of FMLP were not possible, owing to the number of samples being taken. These initial rates were then normalized to that of the control curve.

This was done by dividing the value of the initial rate obtained in the presence of a given concentration of the chemotactic factor by the corresponding value of the initial rate obtained in the absence of any chemotactic factor. Fig. 8 shows a dose-response curve so obtained for both Na influx and efflux. The increase in the rate of Na influx is linear from 1×10^{-10} M on, reaching a value at 3×10^{-9} M FMLP that is at least four times larger than that of the control. A dose-dependent increase in the rate of efflux of ^{22}Na is also apparent over the same concentration range. These increases are, however, of a much smaller magnitude than those observed with the influx rates. Furthermore, removing extracellular K^+ was found, in preliminary experiments, to abolish the enhancement of ^{22}Na efflux caused by 5×10^{-10} M FMLP. In five determinations, we found the ratio of ^{22}Na efflux in the presence of 5×10^{-10} M FMLP to that of control to be equal to 1.25 ± 0.1 in the presence of 5 mM extracellular K^+ and 1.05 ± 0.1 in the absence of K^+ .

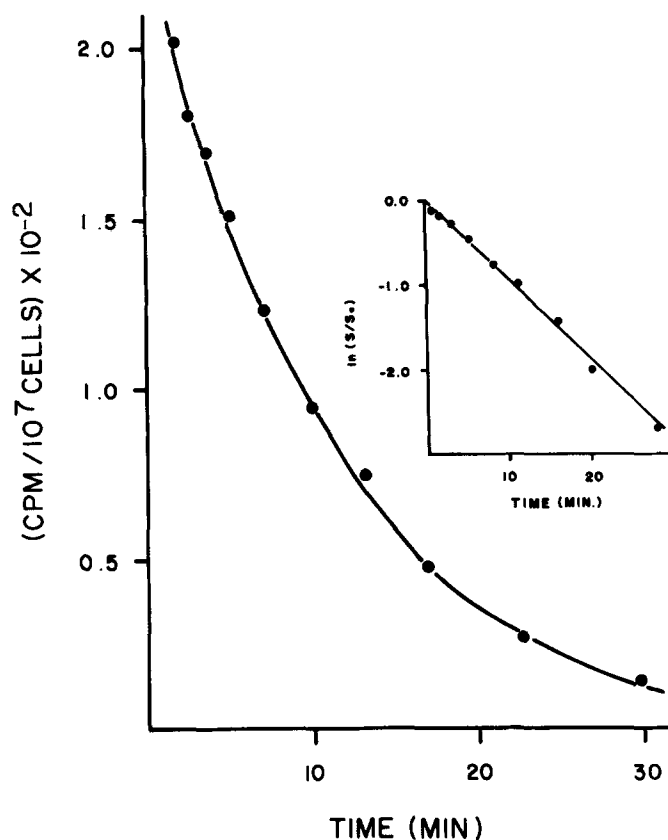


FIGURE 4 The time course of the ^{22}Na efflux across rabbit PMN membranes. *Inset* displays the same data in a semilogarithmic plot. S and S_0 , respectively, refer to the radioactivity in the cells at any given time and at zero time.

TABLE I
Steady-State Na^+ and K^+ Fluxes across Rabbit Neutrophil Membranes

K-Fluxes		Na-Fluxes	
Influx	Efflux	Influx	Efflux
<i>meq/L cell \times min</i>		<i>meq/L cell \times min</i>	
7.1 ± 0.1 (12)*	7.4 ± 0.2 (3)	3.0 (2)	2.9 ± 0.2 (4)

* The numbers in parentheses refer to the number of experiments. Errors are the standard error of the mean.

In the case of K^+ movements, a small (<20%) but consistent effect on the rate but not on the final level reached was observed. This means that no detectable accumulation or net loss of intracellular K^+ occurs in the presence of the chemotactic factor. As shown in Table II, this was also confirmed by measuring directly the intracellular K^+ concentration in the presence and absence of the chemotactic factor. On the other hand, the rate of

K^+ efflux was not affected by the chemotactic factor. The results of these studies obtained in the same manner as discussed above are summarized in Fig. 9. The slight increase in ^{42}K influx induced by FMLP is ouabain sensitive, and can be completely inhibited by 10^{-5} M ouabain as seen in Fig. 10. This figure shows how the uptake of K^+ by PMN is affected by 5×10^{-10} M FMLP in the presence and absence of 10^{-5} M ouabain. It is

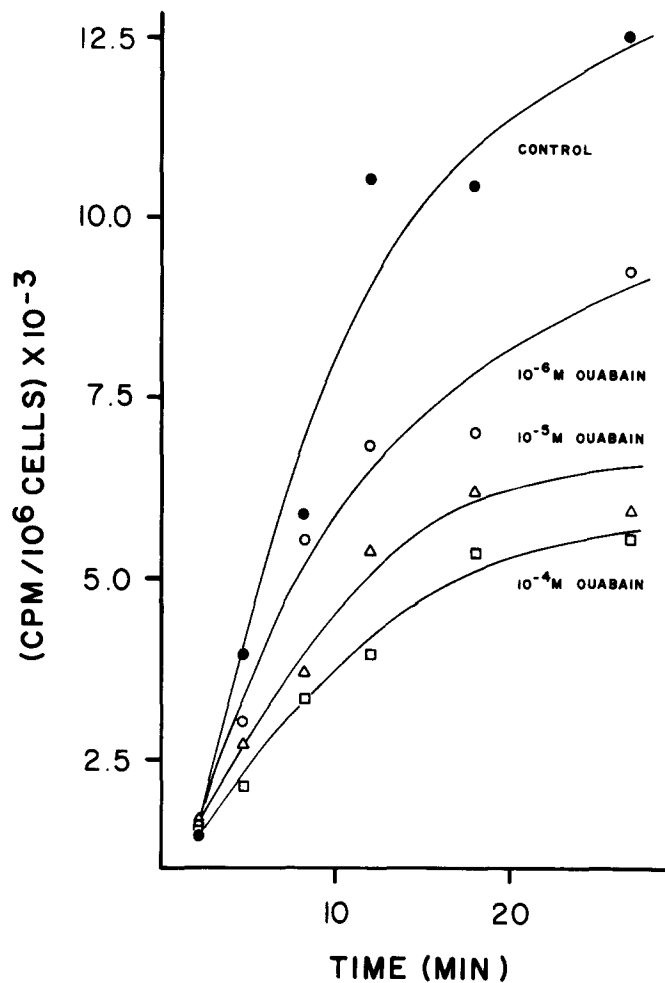


FIGURE 5 Effect of ouabain on the time course of ^{42}K influx across rabbit PMN membranes. Ouabain at the desired concentration was added at the start of the experiment. Each point represents the mean of duplicate determinations.

quite clear that the two curves (with or without FMLP) in the presence of 10^{-5} M ouabain are indistinguishable.

^{45}Ca Movements

The method described above proved suitable for following the kinetics of ^{45}Ca -movement into and out of the cells. The results shown in Fig. 11 represent a typical experiment of the time course of ^{45}Ca influx in these cells. As evident from the figure, ^{45}Ca exchange is rapid, reaching steady-state at about 40 min. This is in contrast to calcium movement in mammalian red cells which is quite slow (25, 21). The rate of efflux of ^{45}Ca from PMN as seen both through the appearance of

radioactivity in the supernate and through the loss of radioactivity from the cells is illustrated in Fig. 12. It can be seen that both methods gave identical results. The rate of efflux of ^{45}Ca was not affected by 10^{-5} M ouabain. The values of the average rate constants and their standard errors of the means in reciprocal minutes for ^{45}Ca inward and outward movements in the presence of 0.25 mM Ca^{2+} are 0.05 ± 0.003 and 0.04 ± 0.002 , respectively. Actual fluxes cannot be calculated since the intracellular concentration of exchangeable calcium is not known.

These studies also provided a way to approximately calculate the magnitude of the intracellular exchangeable Ca^{2+} concentration, using the following relationship:

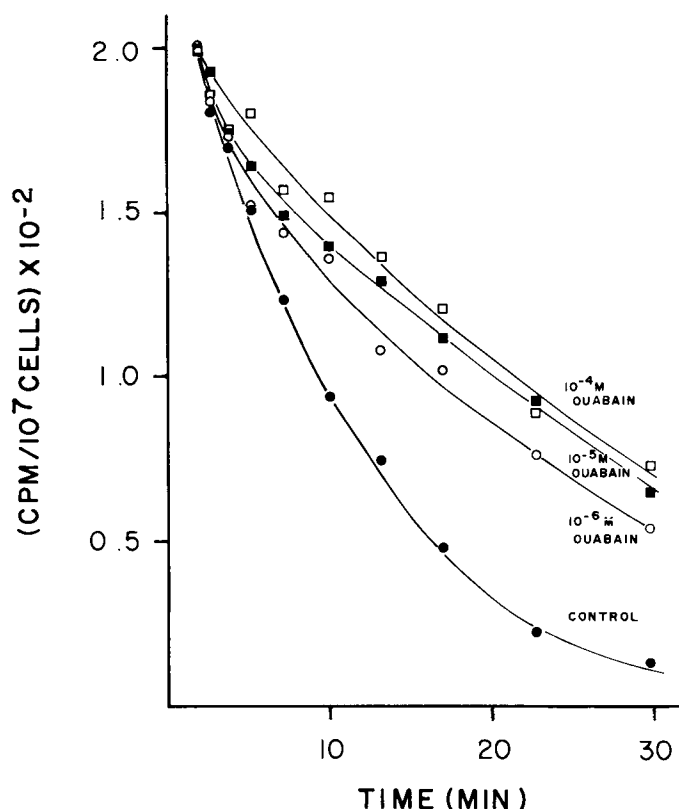


FIGURE 6 Effect of ouabain on the time course of ^{22}Na efflux across rabbit PMN membranes. Ouabain was added at zero time. Each point represents the mean of duplicate determinations.

$$\frac{(CPM)_0}{[Ca^{2+}]_0} = \frac{(CPM)_{i,\infty}}{[Ca^{2+}]_{i,E}}$$

where $(CPM)_0$ = the counts in the extracellular medium (CPM/ml); $(CPM)_{i,\infty}$ = the counts in the cell at steady-state (CPM/mg protein); $[Ca^{2+}]_0$ = the concentration of calcium in the medium (mol/ml); $[Ca^{2+}]_{i,E}$ = the concentration of calcium in the cell (mol/mg protein).

This relationship is nothing more than a formulation of the statement that when steady-state is reached, the specific activity of ^{45}Ca in the bathing medium must equal the ^{45}Ca -specific activity in the cell. Using this equation and the value of $1.2 \mu\text{g}/10^8$ cells for total intracellular concentration of Ca^{2+} which we have measured, we calculate that at steady-state (after 60 min of incubation with ^{45}Ca) <20% of the intracellular Ca^{2+} is exchangeable.

Effect of the Chemotactic Factor

FMLP on ^{45}Ca -Movement

The effect of the various concentrations of

FMLP on ^{45}Ca uptake was studied. In these experiments, the chemotactic factor was added immediately after the addition of the radioactive calcium and aliquots were taken at two different preset times (6 and 45 min). The initial rate of ^{45}Ca -influx was calculated from the difference in the counts between the 0 and 6-min time periods. In the case of the efflux studies, the cells were preincubated with ^{45}Ca for 40 min. The cells were then washed free of the radioactivity, as described, except that the buffered solution used for washing contained 1 mM EGTA and no Ca^{2+} . The cell suspension was divided into various flasks, each containing the desired concentration of FMLP. The initial rates were calculated in the same manner as for influx studies. The results of two representative experiments are shown in Fig. 13. It is quite evident that the chemotactic factor FMLP dramatically increases the ^{45}Ca -efflux and causes a less pronounced but still significant increase in the cells uptake of ^{45}Ca . The increase in ^{45}Ca -efflux is similar to that observed by Gallin and Rosenthal

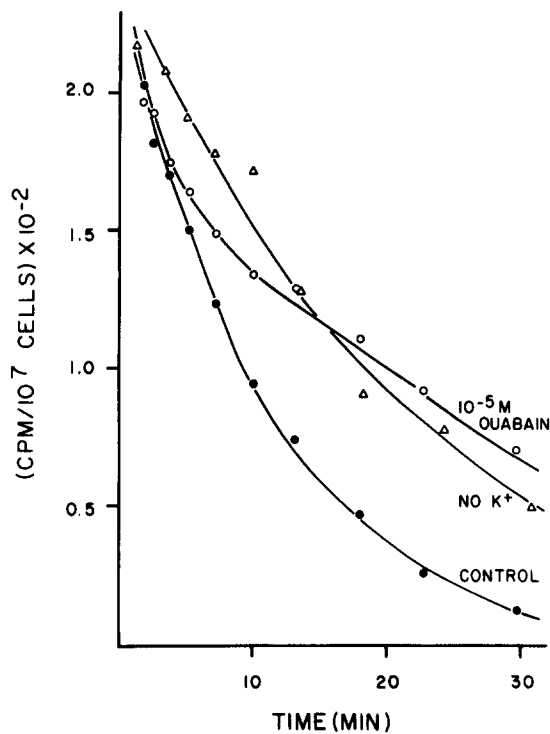


FIGURE 7 Effect of ouabain and lack of extracellular potassium on the time course of ^{22}Na efflux across rabbit PMN membranes. Ouabain was added at zero time. Each point represents the mean of duplicate determinations.

(14) following stimulation of human PMN by the complement-derived chemotactic factor C5a. The increased ^{45}Ca efflux was observed whether or not the cells were washed in the absence of calcium and magnesium and in the presence of 1 mM EGTA. At the end of the washing procedure and before the addition of FMLP, the level of radioactivity retained in the samples washed with EGTA was only 70% of that of samples washed in the absence of EGTA in normal buffer. The enhancement of ^{45}Ca -efflux develops linearly over roughly a range of concentration 1×10^{-10} – 7×10^{-10} M FMLP, as does that of ^{22}Na influx. The slopes of these two processes are, furthermore, very similar.

TABLE II

Effect of the Chemotactic Factor FMLP on the Steady-State Intracellular K^+ Concentration in Rabbit Neutrophil

Experimental Condition	K^+ Concentration*
Control	110 ± 5 (3)‡
+ 5×10^{-10} M FMLP§	101 ± 7 (3)‡

* Concentration is expressed as meq/L cells.

‡ The number in parentheses refers to number of experiments; in every experiment, each point was done in triplicate, and errors are the standard error of the mean.

§ The cells were incubated in the presence of FMLP for 10 min before intracellular K^+ concentration was measured.

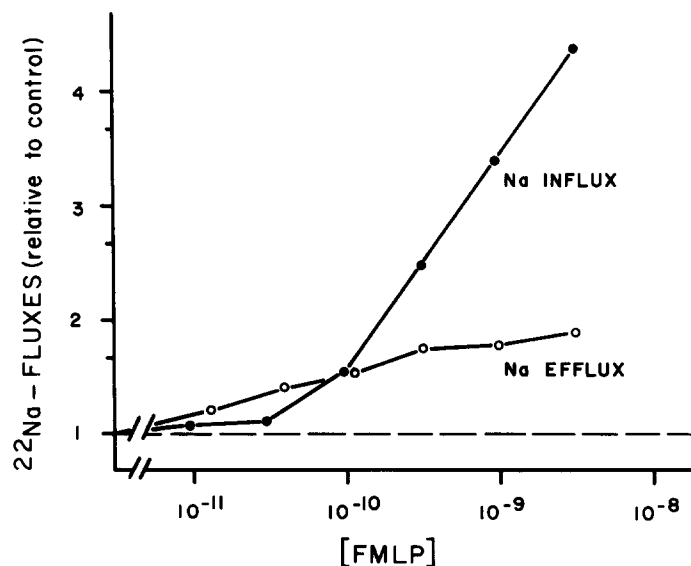


FIGURE 8 Variations of ^{22}Na fluxes with different concentrations of FMLP. Each point represents the mean of quadruplicate determinations.

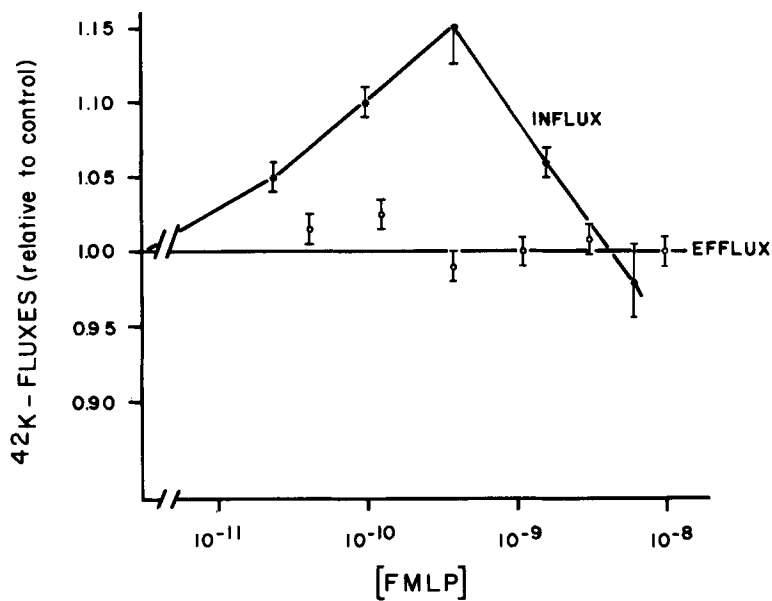


FIGURE 9 Effect of varying concentrations of FMLP on ⁴²K fluxes. Each point represents the mean of quadruplicate determinations and the vertical bars represent the standard error of the mean.

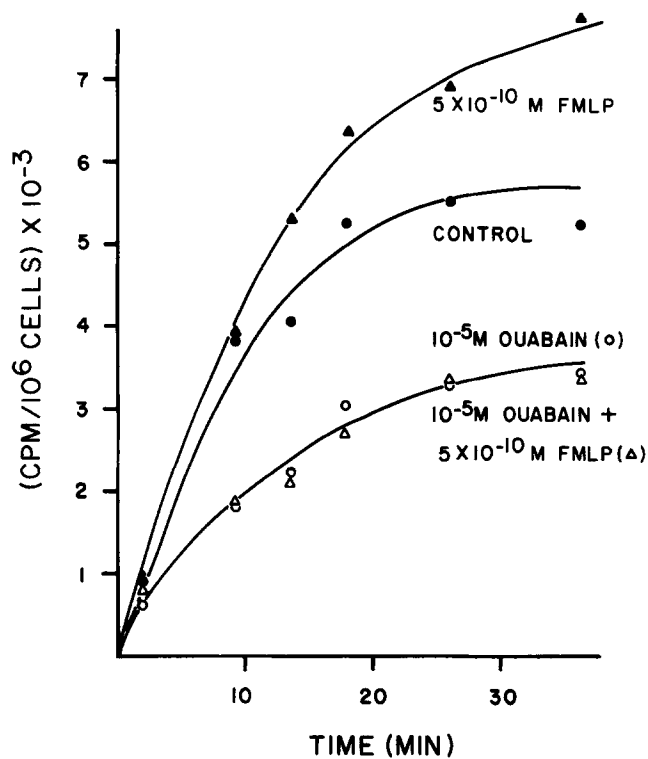


FIGURE 10 Effect of FMLP and ouabain on the time course of ⁴²K influx across rabbit PMN membranes. Ouabain and/or FMLP were added at zero time. Each point represents the mean of duplicate determinations.

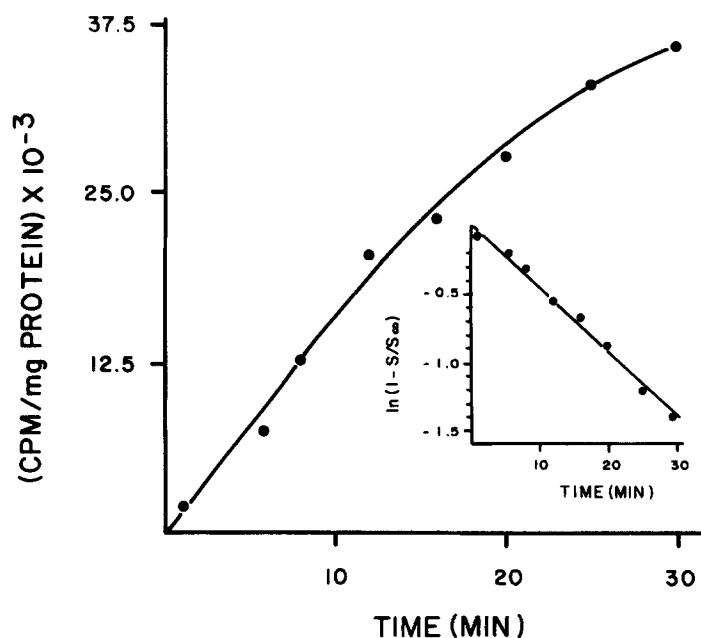


FIGURE 11 Time course of the ^{45}Ca influx across rabbit PMN membranes. *Inset* displays the same data in a semilogarithmic plot. S and S_{∞} , respectively, refer to the amount of radioactivity in the cells at any given time and at equilibrium.

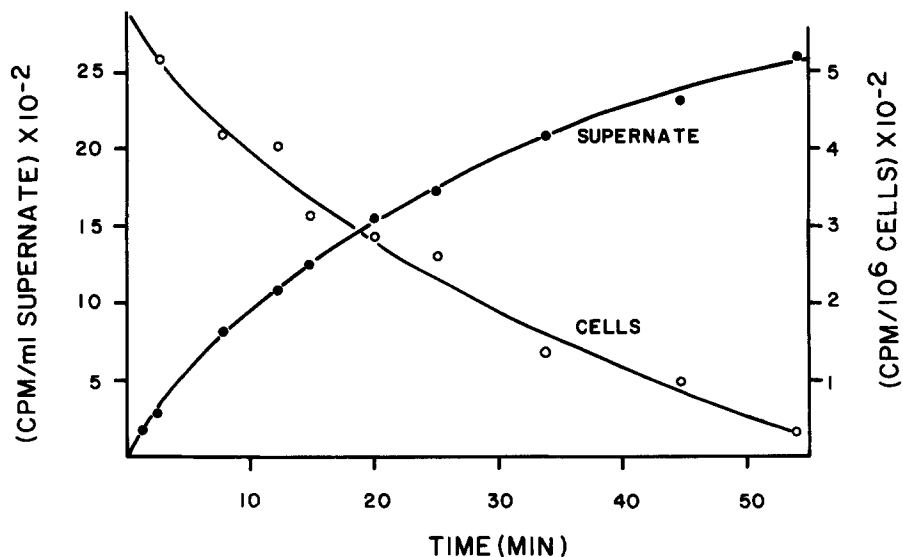


FIGURE 12 The time course of ^{45}Ca efflux across rabbit PMN membranes as followed both in the cells and in the supernate. Each point represents the mean of duplicate determinations.

The enhancement of ^{45}Ca -efflux by FMLP was found not to be sensitive to 10^{-5} M ouabain.

Since the system under these conditions is certainly not in a steady-state, no actual fluxes can be calculated. Furthermore, it is not possible to de-

cide from these experiments whether the chemotactic factor causes a net loss or a net gain of calcium from the cell. In addition, it is not possible to decide from these experiments whether the chemotactic factor causes an increase in the

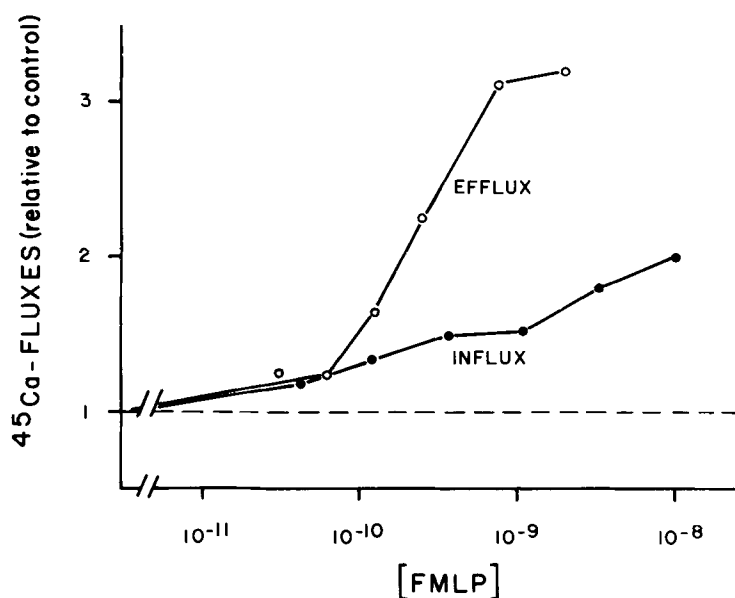


FIGURE 13 Variations of ^{45}Ca fluxes induced by varying concentrations of FMLP. Each point represents the mean of quadruplicate determinations.

permeability of the leukocyte membranes to calcium and/or an increase in the amount of intracellular exchangeable calcium. The observed increased ratios of influx or efflux of ^{45}Ca seen in Fig. 13 could be a reflection of an increase in the concentration of the intracellular exchangeable pool of Ca^{2+} resulting from a net uptake of calcium from the extracellular compartment and/or a shift in the intracellular calcium from a bound to exchangeable pool. To obtain a better understanding of this, we carried out the following three sets of experiments. In the first, the effect of various concentrations of FMLP on ^{45}Ca -influx was measured under conditions where the extracellular calcium concentration was very low (10^{-5} mol). This was intended to minimize the contribution to the ^{45}Ca -exchange by net calcium uptake. The results of these studies are shown in Fig. 14. It is clear from the data in Fig. 14 that the enhancing effect of FMLP on ^{45}Ca -influx diminishes considerably when the extracellular concentration of Ca^{2+} is low. This suggests that at least part of the observed increased ratio of ^{45}Ca -uptake by the chemotactic factor seen in the presence of extracellular Ca^{2+} is due to a net gain of Ca^{2+} . Another possibility is that there is no net gain in Ca^{2+} but that the effect of FMLP is Ca^{2+} dependent.

In the second set of experiments, a known volume of cells at a final concentration of 5×10^6

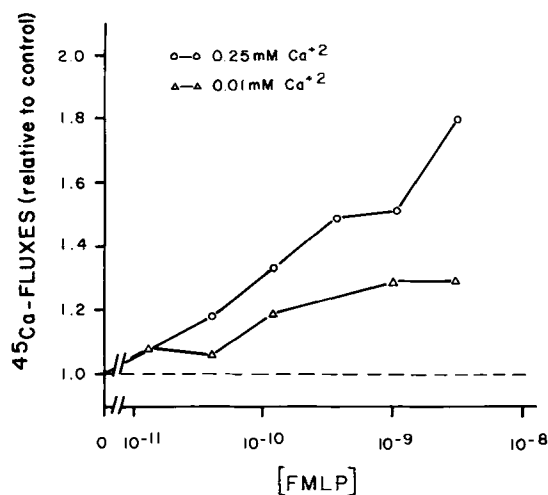


FIGURE 14 Variations of ^{45}Ca influx induced by varying concentrations of FMLP at two different concentrations of extracellular calcium. Each point represents the mean of quadruplicate determinations.

cells/ml was added to an isotonic buffered solution containing 0.25 mM calcium. The suspension was allowed to equilibrate at 37°C for 15 min. A known volume of the ^{45}Ca -isotope stock solution was added to the suspension, and at preset times aliquots in quadruplets from the suspension were removed for the analysis of radioactivity. After 40

min, the suspension was equally divided into two flasks. (This time was sufficient for the radioactivity to reach steady-state, i.e., $dCMP/dr = 0$; where CMP is the counts in the cell/mg protein and $t = \text{time}$.) A known amount of FMLP to give a final concentration of 10^{-9} M was added to one flask and an equivalent amount of DMSO and/or buffer was added to the second. Sampling was continued for another 30 min. The results are summarized in Fig. 15.

The data presented in this figure clearly show that the addition of the chemotactic factor causes an increase in the steady-state level of intracellular ^{45}Ca . This increase must be due to an increase in the intracellular exchangeable pool of Ca^{2+} resulting from either a net uptake of Ca^{2+} from the extracellular compartment or a shift in the intracellular Ca^{2+} from a bound to exchangeable pool, or both. A further possibility which may give rise to an increase in the intracellular exchangeable pool is a swelling of the cell resulting from a nonselective net inward flow of isoosmotic fluid from the extracellular compartment. This last possibility is ruled out because if this were indeed the

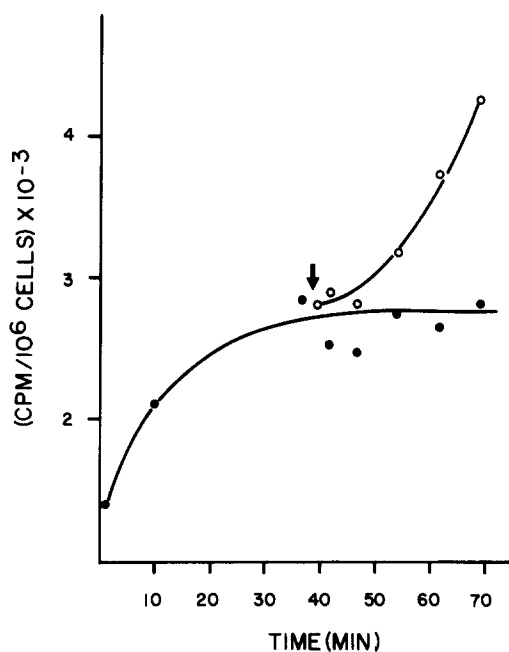


FIGURE 15 Effect of FMLP on the time course of ^{45}Ca influx across rabbit PMN membranes. Arrow indicates time of addition of FMLP (10^{-9} M). Extracellular calcium concentration: 0.25 mM. Each point represents the mean of duplicate determinations before and of triplicate determinations after the addition of FMLP.

explanation, the cell would have to double in size to account for the observed increase in the $^{45}\text{Ca}^{2+}$ level. This is extremely unlikely, since the maximum increase in cell volume observed after the addition of FMLP is 15% (15).

In the third set of experiments, a known volume of cells to give a final concentration of 5×10^6 cell/ml was added to an isotonic buffered solution with no Ca^{2+} . The suspension was allowed to equilibrate at 37°C for 15 minutes. A known volume of the ^{45}Ca -isotope stock solution was added to the suspension so that the concentration of extracellular Ca^{2+} became 0.01 mM. At preset times, quadruplicate aliquots from the suspension were removed for the analysis of radioactivity. After 40 min, the suspension was equally divided into two flasks. A known amount of FMLP to give a final concentration of 10^{-9} M was added to one flask and an equivalent amount of buffer and/or DMSO was added to the second. Sampling was continued for another 30 min. The results, which are summarized in Fig. 16, clearly demonstrate that when the external Ca^{2+} concentration is low, the addition of FMLP causes a transient decrease in the steady-state level of ^{45}Ca -influx. On the other hand, the addition of FMLP causes an increase in the intracellular Ca^{45} when the extracellular Ca^{2+} is relatively high. This transient decrease in ^{45}Ca is most interesting since it indicates that part of the chemotactic factor effect is to cause a displacement of Ca^{2+} from the cell, most likely from the membrane.

To facilitate comparison of the effect of FMLP on ^{45}Ca -influx under the two conditions (0.25 mM Ca^{2+} , 0.01 mM Ca^{2+}), we have taken the results shown in Figs. 15 and 16 and have calculated the changes (each relative to its control) in the steady-state level of ^{45}Ca -influx after the addition of FMLP. The results are summarized in Fig. 17. Two important conclusions can be drawn from the results shown in this figure. First, it is clear that the addition of the chemotactic factor causes an increase in the amount of the intracellular exchangeable calcium. This increase is quite significant, and most of it is probably due to a net gain of calcium from the extracellular medium. This is supported by the finding that no significant increase in the exchangeable calcium can be demonstrated when the concentration of the extracellular calcium is low. Second, the initial effect of the chemotactic factor is to cause a transient displacement of calcium (most likely from the membrane). This is clearly evident when the extracellular cal-

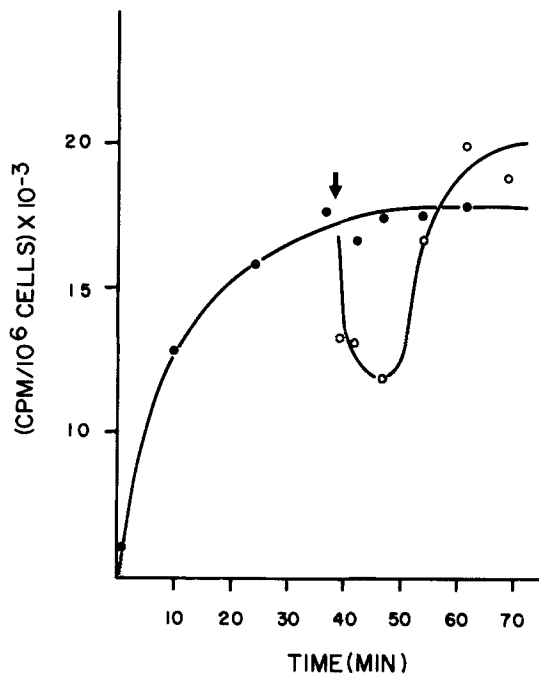


FIGURE 16 Effect of FMLP on the time course of ^{45}Ca influx across rabbit PMN membranes. Arrow indicates time of addition of FMLP (10^{-9} M). Extracellular calcium concentration: 0.01 mM. Each point represents the mean of duplicate determinations before and of triplicate determinations after the addition of FMLP.

cium concentration is low so that this effect is not masked by a net gain in Ca^{2+} by the cells.

We have also studied the effect of varying the concentration of FMLP on the maximum amount of ^{45}Ca displaced. In these experiments, a suspension of cells was prepared exactly in the same manner as described above. After steady state was achieved, the suspension was divided equally into various containers, each containing the desired concentration of FMLP. One minute later, duplicate samples from each container, starting with the container which had the highest concentration of the chemotactic factor, were removed for radioactivity analysis. The sampling process took about 0.5 min. The number of counts in each sample was divided by the number of counts in the control sample. The results are given in Fig. 18. It is clearly evident from the figure that the amount of ^{45}Ca displaced increases with increasing concentration of the chemotactic factor.

DISCUSSION

The rates at which Na^+ and K^+ equilibrate across the plasma membrane of rabbit polymorphonu-

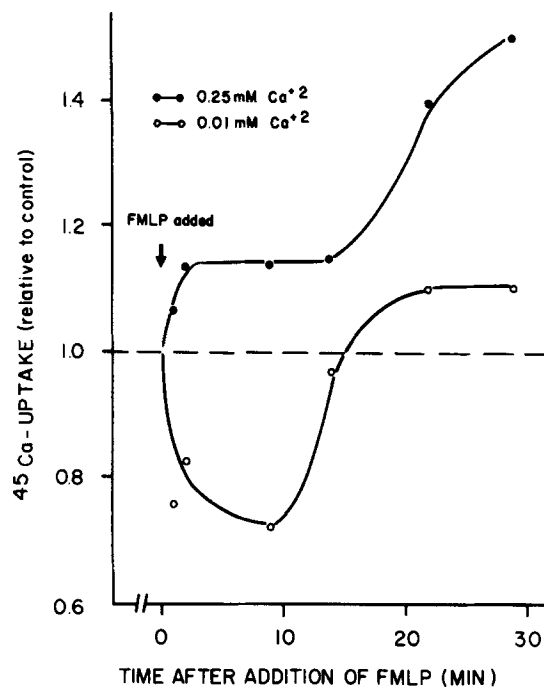


FIGURE 17 Time course of the relative changes in the steady-state levels of ^{45}Ca after the addition of FMLP at two concentrations of extracellular calcium. Arrow indicates time of addition of FMLP.

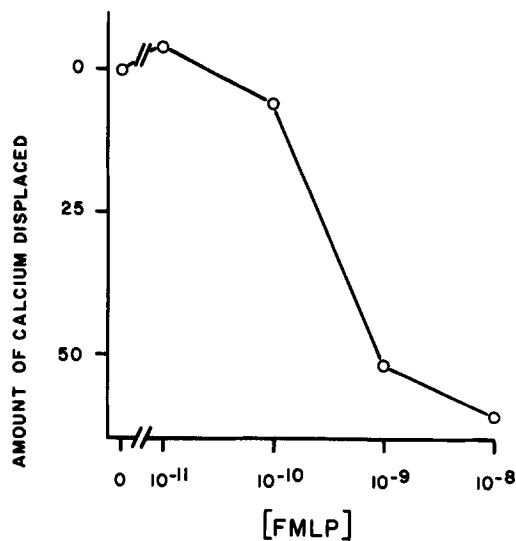


FIGURE 18 Concentration dependence of the effect of FMLP on the steady-state level of ^{45}Ca in the presence of low extracellular calcium. Extracellular calcium concentration: 0.01 mM. Each point represents the mean of duplicate determinations. Amount of calcium displaced expressed as percent of control final uptake.

clear leukocytes are very high compared to those found for most mammalian red cell membranes. For example, the half-times of equilibration of these ions across rabbit PMN membranes are of the order of hours. This conclusion was first reached by Wilson and Manery (35). They found that the equilibration time for Na^+ across rabbit PMN membranes was less than 1 h. However, the experimental techniques then available precluded any actual measurements of the kinetics of ^{22}Na influx by rabbit PMN. More recently, using human PMN, Cividalli and Nathan (9) and Dunham et al. (10) arrived at the same conclusion. The present values for Na^+ and K^+ steady-state fluxes across rabbit PMN are about 50% higher than those reported by Cividalli and Nathan (9) for human PMN. This result probably reflects species differences.

It has been suggested (36) that the "Na, K pump" commonly found in mammalian membranes is not involved in the regulation of the intracellular concentrations of Na^+ and K^+ in rabbit PMN. The results presented here strongly suggest that this is not the case. This conclusion is based on the following observations: (a) both K^+ influx and Na^+ efflux are inhibited by ouabain; (b) Na^+ efflux is significantly reduced when extracellular K^+ is removed; and (c) in a separate study (26), we have been able to demonstrate the presence of Na^+ , K^+ -activated, ouabain-inhibited ATPase activity in rabbit polymorphonuclear leukocyte membranes. Since these three observations show the classical characteristics of the Na, K^+ pump commonly found in various biological membranes, we conclude that the observed concentration gradients across rabbit leukocyte membrane are maintained partially by means of the Na^+ , K^+ pump which is driven by metabolic energy derived from the hydrolysis of ATP by membrane-associated Na^+ , K^+ -activated, ouabain-inhibited ATPase. We use the word "partially" since it appears that part of K^+ influx may not be linked to Na^+ efflux since the steady-state value of K^+ influx is higher than the corresponding value of Na^+ efflux.

The chemotactic activity of a number of synthetic peptides has been recently described (30, 22, 2). These peptides have been shown to interact in a very specific manner with the PMN; in addition to being chemotactic, they increase random migration and induce lysosomal enzyme release, the latter in the presence of cytochalasin B (30). The concentrations of these peptides that caused 50% of maximum induced movement var-

ied from 7×10^{-11} M for FMLP (the most active peptide studied) to 2.7×10^{-3} M for Met-Leu-Glu (the least active), a difference in activity of about 26×10^6 -fold. The studies reported here show for the first time that the chemotactic factor FMLP induces a rapid and dramatic increase in the rate of influx of ^{22}Na across the PMN plasma membrane but only slightly enhances ^{22}Na efflux from preloaded cells. This selective increased permeability to Na^+ could lead to a slow membrane depolarization. The effect of FMLP on ^{22}Na efflux may be just a reflection of the activation of the (Na, K) pump caused by the increased Na^+ influx. That the enhancement of ^{22}Na efflux is sensitive to the presence of extracellular potassium is consistent with this interpretation. Potassium influx is also slightly enhanced by FMLP, and this effect can be abolished by ouabain. The slight enhancement of K^+ influx again could be a reflection of the activation of the Na, K pump by the increased Na influx. The findings that this enhancement is abolished by ouabain and that K^+ efflux is not affected by the chemotactic factor are consistent with this interpretation. On the other hand, a direct effect of the chemotactic factor in increasing the activity of the Na, K pump cannot be ruled out from these studies.

With respect to calcium transport across leukocyte membranes, the only study of which we are aware is that of Gallin and Rosenthal (14). Since those authors did not measure steady-state fluxes of calcium, we cannot compare our results directly with theirs. On the other hand, the present value for the time of equilibration of ^{45}Ca across the rabbit PMN membrane is very similar to that reported by those authors (14) for human PMN. Calcium efflux has been found to be not sensitive to ouabain (data not shown) precluding its linkage to the Na, K pump. This, however, does not rule out the existence of a "calcium pump" whose role would be to keep the free intracellular calcium at a low enough level for metabolism to proceed as usual under resting conditions.

The two major effects of the chemotactic factor FMLP on Ca movement across rabbit PMN membrane seem to be an enhanced membrane permeability to calcium and, later, a net increase in the intracellular exchangeable calcium pool. Boucek and Snyderman (6) have recently shown that calcium influx in human neutrophils is markedly stimulated by chemotactically active serum. The increased pool of exchangeable calcium seen in the presence of extracellular calcium may be due

partially to a net uptake of calcium as a result of increased membrane permeability to calcium. It is not clear from these studies whether the net influx of Ca^{2+} is necessary for the chemotactic activity of PMN.

An attractive working hypothesis is the postulation that the primary effect of the chemotactic factor is to cause a selective increase in membrane permeability to Na^+ . The increased Na influx may in turn release bound calcium from intracellular compartments by a mechanism possibly similar to the Na -induced calcium release from heart mitochondria (8) or the veratridine (a Na ionophore)-induced insulin release from pancreatic islets of Langerhans (17). However, in view of the relative scarcity of mitochondria in PMN, further speculations about the identity of the intracellular calcium stores seem unwarranted at the present.

The present work does not purport to deal with the question of whether the changes in cation transport and the increased neutrophil movement induced by chemotactic factor are related. Yet, there are indications that they may be, and these bear mentioning.

The removal of Na^+ from the extracellular medium greatly decreases or even abolishes the ability of chemotactic factor to stimulate neutrophil movement (29); this is compatible with the hypothesis that the increased movement is related to the ability of the chemotactic factor to enhance Na^+ influx.

The curves relating the concentration of FMLP to K^+ , Na^+ , and Ca^{2+} influxes (Figs. 8, 9, 14) are very similar, if not identical, to the curve relating the concentration of the same peptide to the enhancement of migration (Fig. 1 of reference 30). Showell and Becker (24) demonstrated that K^+ in the extracellular medium causes a two-fold increase in the sensitivity of the PMN to the migration-stimulating effect of all chemotactic factors studied, including FMLP. Moreover, this increased sensitivity is abolished by ouabain. Thus, to the extent of present evidence, it is likely that the changes in cation transport and the increased neutrophil movement induced by chemotactic factors are related either directly or indirectly.

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