ACTIVATION OF THE RABBIT POLYMORPHONUCLEAR LEUKOCYTE MEMBRANE "Na+,K+-" ATPase BY CHEMOTACTIC FACTOR

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ABSTRACT

Addition of the synthetic chemotactic factor, formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Phe) to medium containing magnesium, sodium, and potassium results in a doubling of the Na+,K+-ATPase activity of the plasma membrane fraction from polymorphonuclear leukocytes (PMN). This activation is sensitive to ouabain inhibition and is dose dependent, maximal activity occurring at 10^-9 M F-Met-Leu-Phe. Equivalent activation was observed with the nonformylated derivative Met-Leu-Phe at 10^-6 M. The dipeptide, carbobenzoxy-methionyl-phenylalanine, which acts as an antagonist for F-Met-Leu-Phe, prevents the stimulation of the "Na+,K+-"-ATPase by F-Met-Leu-Phe.

KEY WORDS Na+,K+-ATPase, neutrophil, polymorphonuclear leukocyte, membrane, chemotactic factor, peptide

The chemotactic activity of a number of synthetic peptides has been described recently (12). These peptides have been shown to interact in a very specific manner with polymorphonuclear leukocytes (PMN); in addition to being chemotactic, they increase random migration and induce lysosomal enzyme release, the latter in the presence of cytochalasin B (12, 13). In studies dealing with the ionic basis of neutrophil functions, we found that external K+ increases the chemotactic responsiveness of the leukocytes, and that this is prevented by ouabain (11). However, cells are still able to react chemotactically in the absence of K+ or the presence of ouabain. Further work has shown that the highly chemotactic synthetic peptide, formyl-methionyl-leucyl-phenylalanine (F-Met-Leu-Phe), was able to produce (5 × 10^-10 M) a large and rapid increase in the permeability of rabbit PMN membrane to Na+ (5, 6). In addition, significant enhancements of K+ influx and Na+ efflux also occurred (5). The enhanced K+ influx and Na+ efflux were prevented by ouabain, and leaving K+ out of the medium prevented the enhanced Na+ efflux, indicating that the peptide enhanced the "Na+,K+-" pumping activity.

There are at least two possibilities as to how the chemotactic peptide enhances the activity of the Na+,K+ pump. One mechanism would be a direct activation of the membrane Na+,K+-ATPase either through the peptide acting on the enzyme directly or, more likely, by perturbing the membrane, and thus activating the enzyme. The other possibility is that the large increased influx of Na+ into the cell consequent to the interaction of chemotactic factor and neutrophil raises the intracellular Na+ concentration sufficiently to increase the Na+,K+ pumping activity.

These studies were undertaken to attempt to
differentiate between those hypotheses by testing whether the chemotactic factor can act on isolated PMN membranes to activate their Na⁺,K⁺-ATPase. Obviously, a positive result would strongly support the first hypothesis.

MATERIALS AND METHODS

PMN were obtained from white albino rabbits by injection of 400 ml of 0.1% glycogen in sterile saline and collecting the leukocyte-rich exudate 12 h after injection. The suspension was centrifuged and the packed cells were resuspended in 20-50 ml of isotonic buffered NH₄Cl. This procedure was necessary to hemolyze erythrocytes (3, 9, 10). The suspension was centrifuged for 3 min at 580 g. The supernate was aspirated and the pellet was gently resuspended in 11.6% sucrose, 1 mM EDTA, and spun at (1600 g) for 3 min in a Sorvall RC-3 centrifuge. The pellet was then gently resuspended in the 11.6% sucrose and spun again for 3 min (1,600 g) at 2,500 rpm. The pellet was homogenized in a T-Line Laboratory Homogenizer (Talboys Engineering Corp., Emerson, N. J.) for 2 min at ~1,000 rpm until all the cells were broken as determined by microscope examination or lactate dehydrogenase release.

Membranes were prepared from the homogenate by a slight modification of the method of Woodin and Wieneke (9, 15). In this method, the homogenate was diluted to 21 ml with a solution of 11.6% sucrose, 1 mM EDTA, and 3.4 ml was layered on a 30% (3.6 ml), 40% (2.75 ml), and 50% (3.6 ml) (by weight) sucrose gradient. All of the sucrose solutions contained 1 mM EDTA. The tubes were placed in a SW40 rotor (Beckman Instruments, Inc., Spinco Div. Palo Alto, Calif.) for 1 h at 4°C. Band I was formed between the suspending medium and 30% sucrose, band II between the 30 and 40% sucrose, and band III between the 40 and 50% sucrose. The material designated as band II is referred to as plasma membrane and that designated as band III is the cytoplasmic membrane fraction (9, 15). The bands were transferred to clean centrifuge tubes and diluted with cold 16.4 mM Tris-HCl (pH 7.6), and centrifuged at 70,000 g for 40 min. The pellets were then resuspended in 16.4 mM Tris solution. Protein concentration was determined by the method of Lowry et al. (4) using bovine serum albumin as standard. We have shown previously that only band II contains ATPase activity (9) which meets all the requirements: (a) Mg²⁺; (b) Mg²⁺,Na⁺; (c) Mg²⁺,K⁺; (d) Mg²⁺,Na⁺,K⁺; (e) Mg²⁺,Na⁺,K⁺,ouabain. Final ionic concentrations were: Mg²⁺, 0.26 mM; Na⁺, 100 mM; K⁺, 10 mM; ouabain 0.1 mM. All samples contained 10 mM Tris-HCl, pH 8.6, and 1 mM EDTA. The final ATP concentration was 0.4 mM and the final volume was 0.5 ml. All samples, before and after incubation, were kept on ice.

The reaction was started by the addition of the membranes, with final protein concentrations ranging from 0.02 to 0.06 mg protein per ml. Incubation was carried out at 37°C for 10 min. The reaction was stopped by the addition of 0.15 ml of 30% TCA (wt/vol). The phosphate concentration of the supernate was determined by the method of Ames, except that the color development was done at 4°C or 15 h to minimize ATP hydrolysis (1, 9).

The synthetic factors, F-Met-Leu-Phe, methionyl-leucyl-phenylalanine (Met-Leu-Phe), and carbobenzoxo-phenylalanine-methionine (CBZ-Phe-Met) were synthesized as previously described (12). Stock solutions of these factors were made in dimethyl sulfoxide (DMSO) and used throughout this study. Subsequent dilutions were made before use. No effect of DMSO on ATPase activity could be observed at any of the dilutions used.

RESULTS

The various components of the ATPase activities found in the band II fraction and the effect of 10⁻⁹ M F-Met-Leu-Phe on these activities are summarized in Table 1. In confirmation of previous results (9), Table I shows that band II contains Na⁺,K⁺-ATPase activity. Moreover, it is also quite clear from the table that the chemotactic synthetic peptide, F-Met-Leu-Phe, produces a significant enhancement of the Na⁺,K⁺-sensitive ATPase activity. This increase in activity is inhibitable by 10⁻⁴ M ouabain.

Fig. 1 represents a dose-response curve of the percent activation of the Na⁺,K⁺-sensitive ATPase activity over a concentration range that covers all phases of the dose-response curves of chemotaxis and lysosomal enzyme release. Significant enhancement in the activity of this mem-
TABLE I  
Effect of the Chemotactic Factor F-Met-Leu-Phe on the ATPase Activity in Fraction II of Rabbit PMN *

| Condition                        | Mg$^{2+}$ | Mg$^{2+}$ + Na$^+$ | Mg$^{2+}$ + K$^+$ | Mg$^{2+}$ + Na$^+$ + K$^+$ | Na$^+$ + K$^+$-ATPase |
|----------------------------------|-----------|-------------------|-----------------|-----------------------------|----------------------|
| Control                          | 4.0 ± 0.3 (14) | 3.9 ± 0.4 (10) | 3.6 ± 0.4 (10) | 5.3 ± 0.3 (20) | 4.2 ± 0.6 (11) | 1.5 ± 0.5 |
| +10$^{-4}$ M FMLP                | 3.9 ± 0.3 (9) | 4.1 ± 0.4 (8) | 3.8 ± 0.4 (8) | 6.6 ± 0.4 (20) | 4.7 ± 0.6 (11) | 2.7 ± 0.6 |
| °                                | NS        | NS                | NS              | <0.05                      |                      |

FMLP, F-Met-Leu-Phe.
* The activity is expressed as µmol P$_i$ per mg protein per hour. Concentrations are as follows: Mg$^{2+}$, 0.26 mM; Na$^+$, 100 mM; K$^+$, 10 mM; ouabain 10$^{-4}$ M. Means are given with the standard error of the mean. The number in parentheses refers to the number of experiments, and in every experiment each sample was measured in duplicate. The variation between duplicates was <3%. Na$^+$, K$^+$-ATPase activity refers to the difference in activities when Mg$^{2+}$ + Na$^+$ + K$^+$ are present and that when Mg$^{2+}$ is present.

Using paired sample test.

![Figure 1](image-url)

**Figure 1**: A dose-response curve for the percent activation of the Na$^+$, K$^+$-ATPase activity by 10$^{-4}$ M of the chemotactic factor F-Met-Leu-Phe (FMLP). The data on the dose-response curve dealing with K$^+$ influx were taken from Naccache et al. (5).

brane-bound enzyme can be observed at a concentration as low as 10$^{-12}$ M. This enhancement increases with increasing F-Met-Leu-Phe concentration, reaching a maximum at 10$^{-8}$ M, and then drops off rapidly at concentrations greater than 10$^{-9}$ M, with the baseline being reached at 10$^{-7}$ M. As indicated in the figure, these results parallel those of Naccache et al. on K$^+$-influx measurements (5), although it is clear that stimulation of ATPase activity is a more sensitive response to the peptide than is K$^+$ influx.

Previously, it has been found that the nonformylated synthetic peptide Met-Leu-Phe is three orders of magnitude less active in producing chemotaxis or lysosomal enzyme release in rabbit polymorphonuclear leukocytes than the formylated peptide F-Met-Leu-Phe (5, 12). In addition, it was also found that the dipeptide, CBZ-Phe-Met, acts as a competitive antagonist to the synthetic chemotactic peptides, specifically binding to the receptor but producing neither chemotaxis nor lysosomal enzyme release (8). On the basis of these observations, we have tested the effect of these two compounds on both the Na$^+$, K$^+$-ATPase activity and the stimulation of this activity by F-Met-Leu-Phe. The results are summarized in Table II. It is quite evident that the effects of F-Met-Leu-Phe and Met-Leu-Phe in stimulating the Na$^+$, K$^+$-ATPase and of CBZ-Phe-Met in inhibiting the ATPase activity in

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1 O’Flaherty, J. T., H. J. Showell, D. L. Kreutzer, P. A. Ward, and E. L. Becker. Inhibition of in vivo and in vitro neutrophil responses to chemotactic factor by a competitive antagonist. Manuscript submitted for publication.
TABLE II

Na⁺,K⁺-ATPase Activity in Fraction II of Rabbit PMN Membranes under Different Experimental Conditions

| Addition       | Na⁺,K⁺-ATPase activity (μmol Pi, mg protein⁻¹·h⁻¹) |
|----------------|--------------------------------------------------|
| None           | Normal + 10⁻⁹ M FMLP                               |
|                | 1.3 ± 0.21 (5)                                    |
| + 10⁻⁹ M MLP   | 2.6 ± 0.30 (6)                                    |
| + 10⁻⁶ M MLP   | 2.5 ± 0.22 (5)                                    |
| + 10⁻⁴ M CBZ   | 2.4 ± 0.26 (5)                                    |
| + 10⁻⁴ M CBZ   | 1.3 ± 0.17 (3)                                    |
| + 10⁻⁴ M CBZ   | 1.7 ± 0.15 (3)                                    |

* Means are given with the standard error of the mean. The number in parentheses refers to the number of experiments and in every experiment each sample was measured in duplicate. The variation in duplicates was <4%. The Na⁺,K⁺-ATPase activities were calculated as indicated in Table I.
† F-Met-Leu-Phe.
‡ Met-Leu-Phe.
§ CBZ-Phe-Met. This compound has no effect by itself on ATPase activity.

produced by F-Met-Leu-Phe parallel their actions on chemotaxis and lysosomal enzyme release.

DISCUSSION

These studies allow the conclusion that the reason why K⁺ enhances the chemotactic responsiveness of the PMN is that the chemotactic factor directly stimulates the Na⁺,K⁺-ATPase activity of the membrane, and, for this to be manifest, external K⁺ must be present. The evidence for this conclusion is the following: K⁺ enhances the chemotactic responsiveness of rabbit PMN; this enhancement is abolished by ouabain (11). The chemotactic peptide F-Met-Leu-Phe stimulates the influx of K⁺ and the efflux of Na⁺, and these are abolished by ouabain, (5, 6, 12). Moreover, the concentration dependence of the enhancement of K⁺ influx is precisely the same as the concentration dependence of the stimulation of locomotion over the ascending portion of both curves (12). As shown in this work, F-Met-Leu-Phe induces a significant dose-dependent increase in the Na⁺-ATPase activity of isolated rabbit PMN membranes. The stimulation of ATPase activity by F-Met-Leu-Phe is specific to the Na⁺,K⁺-ATPase, being abolished by ouabain and not occurring in the presence of Mg²⁺ alone, or Na⁺ or K⁺ alone, but only when Na⁺ and K⁺ are present together. The stimulation of Na⁺,K⁺-ATPase activity is due to interaction of the peptide with the same receptor responsible for stimulation of locomotion as evidenced by the following: the dose dependency of the enhancement of the Na⁺,K⁺-ATPase activity and the stimulation of K⁺ influx parallel each other, both having an optimum at 10⁻⁹ M F-Met-Leu-Phe (Fig. 1). The specific binding activity, and thus, at least part of the chemotactic receptor, are found in the membrane fraction. To the extent studied, the relation between the structure of the peptide and its activity in stimulating locomotion or the Na⁺,K⁺-ATPase is the same (Table II). A specific, competitive antagonist of receptor function, CBZ-Phe-Met, inhibits the ability of F-Met-Leu-Phe to stimulate Na⁺,K⁺-ATPase activity at the same concentrations at which it inhibits the ability of F-Met-Leu-Phe to induce locomotion (8). It must be pointed out, however, that we are not suggesting that the Na⁺,K⁺-ATPase is the chemotactic factor receptor.

As mentioned, there is a 10–100% increase in sensitivity of the Na⁺,K⁺-ATPase response of the isolated membrane to chemotactic peptide compared to either the simulation of K⁺ influx (Fig. 1) or locomotion (5, 12). This increased sensitivity, although not too surprising in view of the fact that an enzyme reaction of the isolated membrane is being compared to the responses of the whole cell, is, nevertheless, noteworthy.

The finding that the Na⁺,K⁺-ATPase activity of isolated membranes can be stimulated by interaction of the peptide with its receptor provides a tool for the detailed study of the mechanisms by which receptor interaction with chemotactic peptides induces various membrane functions. For example, in such a study it is hoped that an explanation will arise for the very interesting, rapid decrease in the ability of F-Met-Leu-Phe to stimulate Na⁺,K⁺-ATPase activity at concentrations above 10⁻⁹ M.

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