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Characterization of the Membrane-Associated GTPase Activity: Effects of Chemotactic Factors and Toxins

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Membranes prepared from rabbit neutrophils exhibit GTPase activity which can be stimulated by the chemotactic factor fMet-Leu-Phe. The maximum contribution of the ATPase activities to the basal and the fMet-Leu-Phe-stimulated GTPase activities are less than 20% and 9%, respectively. The basal GTPase activity has a V_{max} = 34.2 ± 1.3 (pmol/mg protein, min) and a K_m = 0.39 ± 0.03 μM; and the fMet-Leu-Phe-stimulated has a V_{max} = 52.3 ± 2.5 (pmol/mg protein, min), and a K_m = 0.29 ± 0.02 μM. The GTPase activity can be stimulated by fMet-Leu-Phe and leukotriene B_4. Unlike these two chemotactic factors, concanavalin A does not stimulate this GTPase activity. In addition, the rise in intracellular concentration of free calcium produced by concanavalin A is not inhibited by pertussis toxin treatment. Both the basal and stimulated GTPase activities are affected by pertussis toxin, cholera toxin and N-ethylmaleimide.

Key words: neutrophil, GTPase, fMet-Leu-Phe, leukotriene

Much of the current interest in the field of signal transduction in calcium-mobilizing systems is focused on the roles of guanine-nucleotide binding regulatory proteins in the mediation of the receptor dependent hydrolysis of the inositol lipids [1–17]. In neutrophils, it is generally hypothesized that a guanine-nucleotide binding protein, which has been given the symbol G_c [18–20] and is presumably composed of three subunits ($\alpha\beta\gamma$), mediates the effects of most chemotactic factors. α_c has a molecular weight of 40–41 kDa, pI of 5.7 [19–24]; it can be ADP-ribosylated by pertussis toxin [3,4] and is immunologically distinct from α_i, transducin, and α_o [23]. The amino acid sequence of α_c deduced from cDNA clones exhibits 90% and 77% homology to α_i and α_o, respectively [25].

Abbreviations used: [App(NH)p], 5′-adenylylimidodiphosphate; Con A, concanavalin A; [$Gpp(NH)_p$], 5′-guanylylimidodiphosphate; NEM, N-ethylmaleimide.

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Analogous to other G-proteins, it is assumed that activation of G_c by fMet-Leu-Phe leads to the dissociation of the complex (GDP-$\alpha\beta\gamma$) into its three subunits and the exchange of GDP on the α_c subunit for GTP [22,24]. As is the case for other guanine-nucleotide binding proteins, the termination of the action of GTP-α_c is accomplished by a GTPase system.

The presence of what appeared to be a GTPase activity in neutrophils has been demonstrated [22,24,26–28]. The possibility of an ATPase system which may be contributing to this activity has not been investigated. The GTPase activity is slightly stimulated (15–25%) by the chemotactic factor fMet-Leu-Phe [22,26–28]. Because of this weak stimulation, it has been difficult to characterize properly the stimulated activity of this system.

The present work was undertaken to develop a method to increase the net amount of stimulation by chemotactic factors, to estimate the maximum possible contribution of the various ATPase systems, to characterize completely the kinetic properties of this enzyme, and to examine the effects of various toxins on the GTPase activity.

MATERIALS AND METHODS

Cell Preparation

Rabbit neutrophils were collected and handled as previously described [22]. Briefly, the neutrophils were obtained from peritoneal exudates 16 h after intraperitoneal injection of 500 ml of 0.1% glycogen-saline solution. Erythrocytes were lysed by hypotonic shock, and neutrophils were washed and resuspended in Hanks' balanced salt solution (10 mM HEPES, 123 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 5 mM dextrose, pH 7.35). When the toxins were used, cell suspensions (10^7 cells/ml) were treated with either pertussis toxin (0.5 μg/ml) or cholera toxin (2.0 μg/ml) for 1 h at 37°C or the equivalent amount of dimethylsulfoxide (0.05%) at 37°C. The cells were then washed twice in the Hanks' buffer previously described and resuspended at 10^8 cells/ml in buffered sucrose solution (0.25 M sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.5) containing 1 mM GDP, 1 mM diisopropyl fluorophoshate (DFP), and 50 μg/ml leupeptin.

Membrane Isolation

Membranes were prepared by nitrogen cavitation and ultracentrifugation. Cells were disrupted in the buffered sucrose solution at 250 psi for 20 min at 4°C. The homogenates were centrifuged at 8,000*g* for 30 min. The supernatants were then centriguted at 100,000*g* for 60 min at 4°C, and the pellets were resuspended in the buffered sucrose solution containing 1 mM 5′-adenylylimidodiphosphate [App(NH)p] and 1 mM 5′-guanylylimidodiphosphate [Gpp(NH)p] and incubated at room temperature for 30 min. Membrane suspensions were then washed twice by centrifugation at 100,000*g* for 60 min at 4°C and the membrane pellets were resuspended in the buffered sucrose solution and stored at −70°C.

GTPase Activity

GTPase activity was calculated by measuring the liberation of ^{32}P from [γ-^{32}P]-GTP. The final assay mixture (100 μl) contained 10 μg protein, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA (pH 7.5), 1.14 mM ATP, 0.5

mM [App(NH)p], 0.25 mM ouabain, 0.45 μM GTP, and 0.05 μM ^{32}P-GTP, as well as the appropriate stimulus. The reaction was carried out for 10 min at room temperature and terminated by the addition of 0.5 ml of a 5% charcoal mixture containing 0.1% dextran (MW 200,000) and 0.5% bovine serum albumin in 20 mM phosphate buffer (pH 7.5). The tubes were vortexed and centrifuged to sediment the charcoal and 0.31 ml of the supernatant was counted for ^{32}P content.

Quin 2 Measurements

Intracellular concentration of free calcium was measured using quin 2 fluorescence [29]. Fluorescence was measured using the SLM 8000 [29]. Fluorescence excitation and emission wavelengths were 339 and 492 nm, respectively.

Chemicals

Formyl-methionyl-leucyl-phenylalanine was obtained from Peninsula Labs (San Carlos, CA). GTP, concanavalin A, [App(NH)$_p$], [Gpp(NH)$_p$], leupeptin, DFP, and cholera toxin were obtained from Sigma (St. Louis, MO). ^{32}P-NAD and ^{32}P-GTP were obtained from New England Nuclear (Boston, MA). Pertussis toxin was a generous gift from Dr. J.J. Munoz (NIH, Rocky Mountain Laboratory, Hamilton, MT).

RESULTS

Membrane Associated GTPase Activity

The basal and stimulated GTPase activities of the membrane preparation isolated as described in the Materials and Methods section have been measured, and the results are summarized in Table I. Note that fMet-Leu-Phe stimulates the GTPase activity by 79%. This is in contrast to previously published data where the stimulation by fMet-Leu-Phe was less than 30%. Unlike fMet-Leu-Phe or leukotriene B_4, concanavalin A (Con A) does not increase the GTPase activity. This would indicate that, in these cells, the rise in the intracellular concentration of free calcium produced by concanavalin A would not be inhibited by pertussis toxin. The results shown in Figure 1 clearly demonstrate this point.

In order to establish that this activity is indeed due to the liberation of ^{32}P by a GTPase rather than an ATPase system, we have carried out the same experiment

TABLE I. Basal and Stimulated GTPase Activities in Rabbit Neutrophil Membrane Preparation

Stimulus[a]	GTPase activity (pmol/mg protein, min)[b]	Percent stimulation
Control[c]	28.5 ± 2.5 (80)	—
+ fMet-Leu-Phe (5×10^{-8} M)	51.1 ± 4.7 (80)	79
+ Leukotriene B_4 (5×10^{-7} M)	44.7 ± 2.0 (3)	57
+ Concanavalin A (100 μg/ml)	30.0 ± 3.0 (3)	5

[a]The stimulus was present throughout the reaction period.

[b]All the reactions were carried out for 10 min. The values were calculated as the differences between zero and 10 min. Each value represents the mean ± SEM, and the no. in parentheses refers to the no. of experiments, each carried out in duplicate. The reaction mixture contained 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 0.25 mM ouabain, 0.5 mM [App(NH)$_p$], 1.14 mM ATP, 0.45 μM GTP, 0.05 μM ^{32}P-GTP, 150 mM NaCl, and 10 μg protein.

[c]The solvents used (DMSO, methanol) had no effect on the activity.

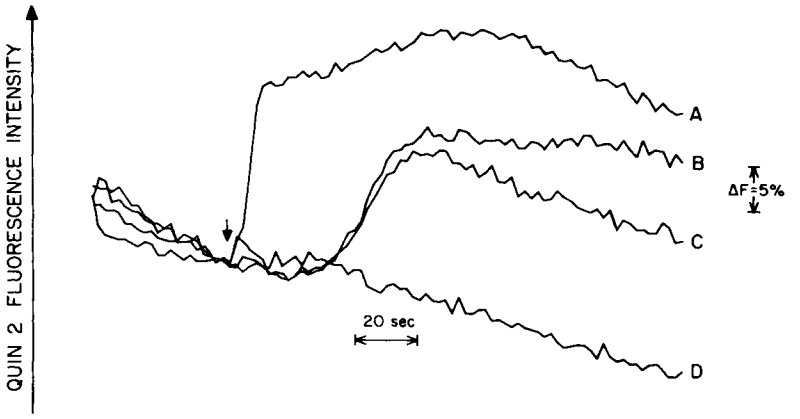


Fig. 1. Effect of concanavalin A on the fluorescence of cell-associated quin 2 in control and pertussis toxin treated cells. The cells were incubated with pertussis toxin (500 ng/ml) for 1 h at 37°C. **A** and **B** are control cells stimulated with 1.0 nM fMet-Leu-Phe and 100 μg/ml concanavalin A, respectively. **C** and **D** are pertussis-toxin-treated cells stimulated with 100 μg/ml Con A and 1.0 nM fMet-Leu-Phe, respectively.

TABLE II. Basal and fMet-Leu-Phe-Stimulated ^{32}P Release by Rabbit Neutrophil Membranes Using Either ^{32}P-Labelled ATP or ^{32}P-Labelled GTP as Substrate

	Basal amount of ^{32}P released in each sample (% of total)[b]			Stimulated amount of ^{32}P released in each sample (% of total)[b]		
Condition[a]	Using ^{32}P-ATP	Using ^{32}P-GTP	Ratio	Using ^{32}P-ATP	Using ^{32}P-GTP	Ratio
No addition	47.1 ± 0.8	32.0 ± 2.4	1.47	48.4 ± 0.5	32.7 ± 3.1	1.48
[App(NH)p] (0.5 mM)	5.2 ± 0.3	8.4 ± 0.9	0.62	5.0 ± 0.2	12.6 ± 1.5	0.40
[App(NH)p] (0.5 mM) ATP (1.1 mM)	0.9 ± 0.1	4.5 ± 0.9	0.20	0.9 ± 0.1	7.7 ± 1.1	0.09

[a]The reaction mixture also contained 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.1 mM EGTA, 0.25 mM ouabain, and 40 μg protein. Each value represents the mean ± SEM of at least three separate experiments, and each experiment was done in duplicate.
[b]The same count of either ^{32}P-ATP or ^{32}P-GTP was added to each reaction, which also contained either 0.45 μM ATP or GTP.

using [γ-^{32}P]ATP. The results are summarized in Table II. These data clearly show that, under the conditions employed in the present studies [App(NH)p], 0.5 mM, and ATP, 1.14 mM, the maximal possible amount of ^{32}P released by the various ATPase activities is no more than 20% of the total amount of ^{32}P released. In the case of fMet-Leu-Phe, this value is only 9%.

The time course of the GTPase activity, as measured by the amount of ^{32}P released from radiolabelled GTP by 10 μg membrane protein, was investigated. The results are summarized in Figure 2. It is clear from the data shown in this figure that the amount of released radioactivity varies linearly with time in the first 15 min.

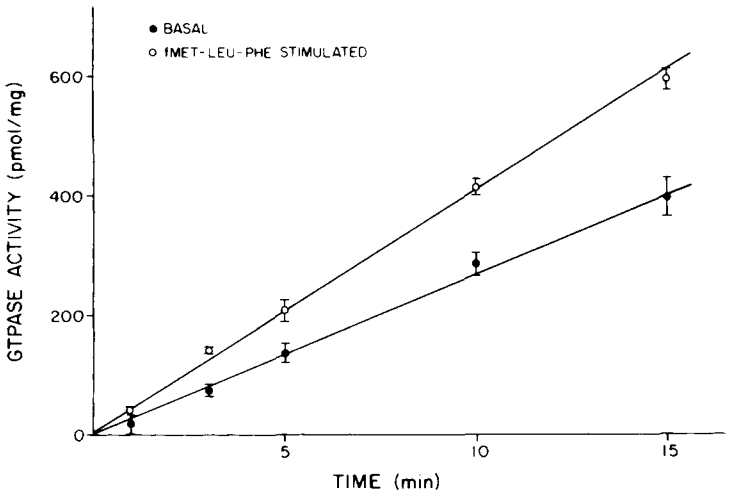


Fig. 2. Time course of fMet-Leu-Phe stimulation of rabbit neutrophil membrane GTPase activity. The time course of GTPase activity in 10 μg of membrane protein was measured in unstimulated and 10^{-8} M fMet-Leu-Phe-stimulated membranes. Data points were taken from two separate experiments done in triplicate. Values represent the mean ± SEM and were calculated as the differences between zero and the time point indicated. The reaction mixture has the same composition as in Table I.

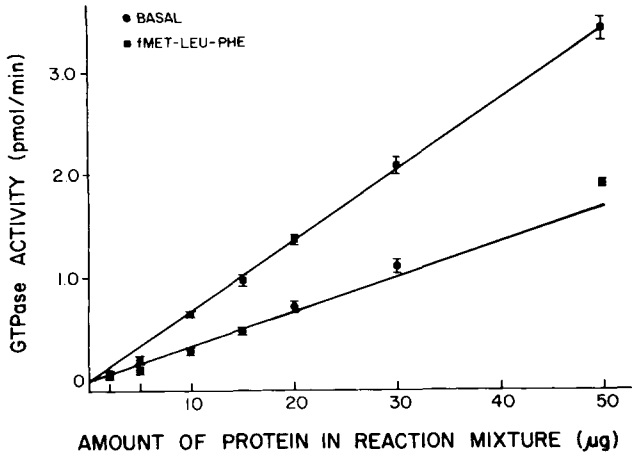
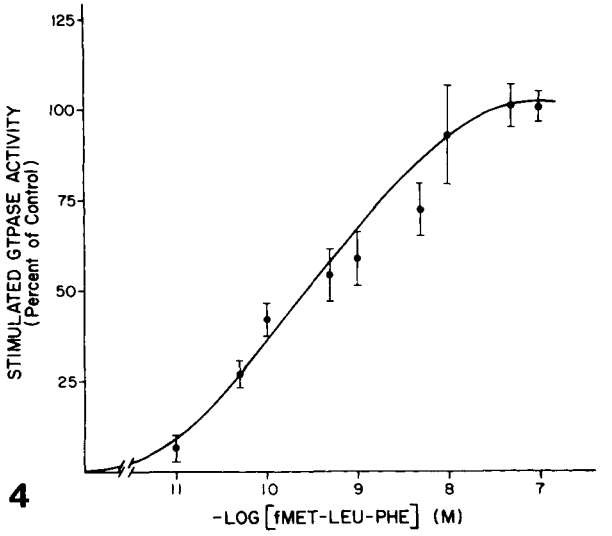


Fig. 3. Effect of membrane protein concentration on basal and fMet-Leu-Phe-stimulated GTPase activity. Unstimulated and fMet-Leu-Phe-stimulated GTPase activity was measured under assay conditions of zero to 50 μg of rabbit neutrophil membrane protein. fMet-Leu-Phe concentration was 5×10^{-8} M. Values represent the mean ± SEM of at least three separate experiments done in duplicate. The reaction mixture has the same composition as in Table I.

The effect of varying the membrane protein concentration in the reaction mixture on the amount of radioactivity released at the end of 10 min was also studied. The results are summarized in Figure 3. The kinetics of the release of the radioactivity indicate that the reaction is linear in the range of interest.

Effects of varying the concentrations of fMet-Leu-Phe and leukotriene B_4 on GTPase activity. The effects of various concentrations of fMet-Leu-Phe and leukotriene B_4 on the membrane-associated GTPase activity have been investigated.

In these experiments, the reaction mixture contains 10 μg protein, and the reaction is carried out for 10 min. The dose-response curve of fMet-Leu-Phe stimulation is shown in Figure 4, and that for leukotriene B_4 is shown in Figure 5. In both cases, the degree of GTPase stimulation increases with the increasing concentration of the stimulus, and then reaches a plateau at higher concentrations. As expected, leukotriene B_4 is less effective than fMet-Leu-Phe. The concentrations that give 50% stimulation (EC_{50}) are 0.5 nM for fMet-Leu-Phe and 91 nM for leukotriene B_4.



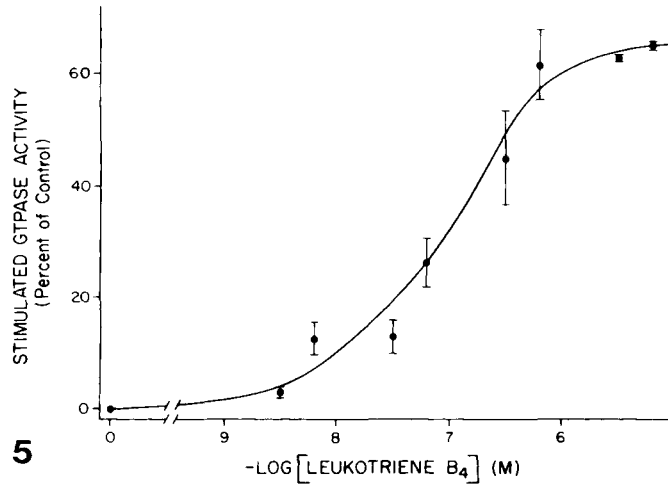


Fig. 4. Dose-response curve for fMet-Leu-Phe stimulation of the rabbit neutrophil membrane GTPase. The reaction mixture has the same composition as Table I. Values represent the mean ± SEM and were calculated as the differences between zero and 10 min. Data points were taken from at least three separate experiments done in duplicate. Stimulation was half-maximal at 5×10^{-10} M fMet-Leu-Phe.

Fig. 5. Dose-response curve for stimulation of the rabbit neutrophil membrane GTPase by leukotriene B_4. The reaction mixture has the same composition as Table I. Values represent the mean ± SEM were calculated as the differences between zero and 10 min. Data points were taken from three separate experiments done in duplicate. Half-maximal stimulation was reached at 9.1×10^{-8} M leukotriene B_4.

Kinetics of the basal and fMet-Leu-Phe-stimulated GTPase activity. The effect of varying the concentration of GTP (0.1–2 μM) in the reaction mixture on the GTPase activity was studied. In these experiments, the reaction mixture contained 10 μg protein, and the reaction was carried out for 4 min. The shorter time was necessary to avoid the hydrolysis of a large fraction of the substrate. At the lowest GTP concentration used (0.1 μM), the maximal amounts of the substrate that were hydrolyzed were less than 15% and 23% for the basal and stimulated conditions, respectively. Lineweaver-Burke analysis is shown in Figure 6. The values for K_m and V_{max} (means ± SEM of four separate experiments) for basal GTPase activity are $K_m = 3.9 \pm 0.3 \times 10^{-7}$ M and $V_{max} = 34.2 \pm 1.3$ pmol/mg protein, min. The corresponding values for fMet-Leu-Phe (50 nM)-stimulated GTPase activity are $K_m = 2.9 \pm 0.2 \times 10^{-7}$ and $V_{max} = 52.3 \pm 2.5$ pmol/mg protein, min.

Effects of pertussis and cholera toxins on GTPase activity. The effects of both pertussis and cholera toxins were examined. In these studies, two sets of experiments were carried out. In the first set, the intact cells were incubated with the toxin for a specific length of time and then the cells were used for membrane preparation. The results of these studies are summarized in Table III. In the second set, the isolated membrane preparation was first treated with the toxin under ribosylating conditions, and then was used to measure GTPase activity. The results are shown in Table IV. It is quite clear that both toxins inhibit the basal and fMet-Leu-Phe stimulated GTPase activity.

DISCUSSION

Membrane preparations isolated from rabbit neutrophils are able to cleave phosphate from a [γ-^{32}P]GTP. This hydrolysis is due mainly to membrane associated

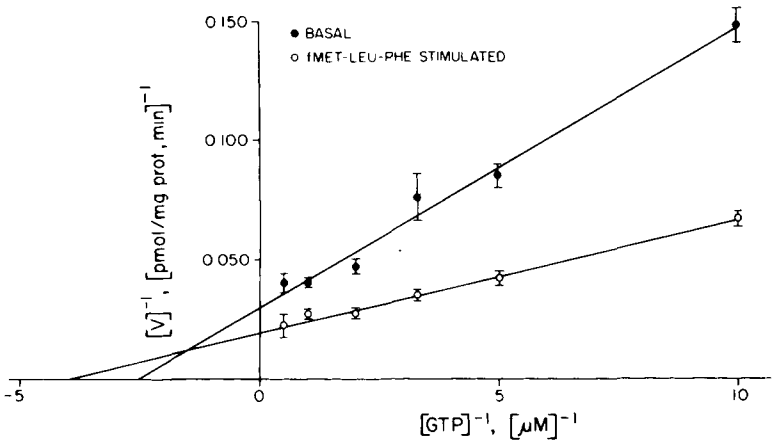


Fig. 6. Lineweaver-Burk plot of basal and fMet-Leu-Phe-stimulated GTPase activity of rabbit neutrophil membranes. The fMet-Leu-Phe concentration was 5×10^{-8} M. GTPase activity was measured at 10 min with 10 μg of membrane protein. The concentration of GTP ranged from 0.1 to 2.0 μM. Aside from GTP concentration, the reaction mixture has the same composition as Table I. Values represent the mean ± SEM of four separate experiments done in duplicate. Calculations show a basal V_{max} of 34.2 ± 1.3 pmol/mg, min with a K_m of $3.9 \pm 0.3 \times 10^{-7}$ M, whereas the stimulated V_{max} was 52.3 ± 2.5 pmol/mg, min with a K_m of $2.9 \pm 0.2 \times 10^{-7}$ M.

TABLE III. Basal and fMet-Leu-Phe-Stimulated GTPase Activities in Membranes Prepared From Control, Pertussis-Toxin-Treated or Cholera-Toxin-Treated Intact Rabbit Neutrophils

	GTPase activity (pmol/mg, min)[b]	
Conditions[a]	Basal	+ fMet-Leu-Phe[c]
Control cells	30.2 ± 2.5	61.4 ± 3.2
PT-treated cells	17.2 ± 1.2	24.8 ± 4.0
CT-treated cells	18.1 ± 1.1	31.2 ± 3.0

[a]The cells were incubated at 37°C for 1 h with 0.5 μg/ml pertussis toxin (PT) or 2.0 μg/ml cholera toxin (CT) or an equivalent amount of toxin solvent in the control. The reaction mixture has the same composition as in Table I.
[b]Each value represents the mean ± SEM of at least four separate experiments. All experiments were done in duplicate. Experiments were carried out for 10 min. Values were calculated as the differences between zero and 10 min.
[c]fMet-Leu-Phe concentration was 50 nM.

TABLE IV. Effect of Treatment of Rabbit Neutrophil Membranes With Pertussis Toxin, Cholera Toxin, or N-Ethylmaleimide on Basal and fMet-Leu-Phe-Stimulated GTPase Activity

	GTPase activity (pmol/mg, min)[b]	
Conditions[a]	Basal	+ fMet-Leu-Phe[c]
Control membranes	27.5 ± 2.5	54.2 ± 5.0
Control membranes + ribosylation solution	16.7 ± 1.6	29.6 ± 1.3
CT-treated membranes	9.7 ± 0.7	19.6 ± 1.1
PT-treated membranes	7.6 ± 0.6	11.3 ± 0.9
NEM-treated membranes	3.7 ± 1.3	9.1 ± 1.4

[a]The membranes were incubated for 20 minutes at 30°C under ribosylation conditions with either pertussis toxin (17 μg/ml), cholera toxin (30 μg/ml), ribosylation solution without toxin, or N-ethylmaleimide (NEM, 100 μM). Control membranes represent GTPase activity prior to treatment with ribosylation solution.
[b]Each value refers to the means ± SEM of at least four different experiments. All experiments were done in duplicate.
[c]fMet-Leu-Phe concentration was 50 nM.

GTPase and not ATPase activity. This conclusion is supported by several lines of evidence. First, the reaction was carried out in the presence of 0.25 mM ouabain and the absence of K^+. These conditions inhibit any Na^+/K^+ ATPase. Second, the chemotactic factor fMet-Leu-Phe does not stimulate other ATPases. Third, both the basal and stimulated activities are inhibited by pertussis toxin. Fourth, stimuli such as Con A, which does not mediate the effect through the pertussis toxin G-protein, do not stimulate the activity. Fifth, the activity was measured in a mixture containing 0.5 mM unlabelled [App(NH)p] and 1.14 mM unlabelled ATP.

The values for K_m and V_{max} for the basal GTPase activity are $K_m = 3.9 \pm 0.3 \times 10^{-7}$ M and $V_{max} = 34.2 \pm 1.3$ pmol/mg protein, min. The GTPase activity can be greatly stimulated by chemotactic factors that mediate their effects through the pertussis-toxin-sensitive guanine-nucleotide binding protein G_c, such as fMet-Leu-Phe and leukotriene B_4, but not by stimuli that are not coupled to G_c, such as concanavalin A. In the case of fMet-Leu-Phe (10^{-11} to 10^{-7}), the stimulation begins

at 10^{-11} M, increases with increasing concentration and plateaus at 7×10^{-8} M. For leukotriene B_4 (5×10^{-8} to 10^{-5}), the effect is observed at 5×10^{-8} M, and it reaches maximum at 10^{-6} M. The chemotactic factor fMet-Leu-Phe (50 nM) increases the V_{max} by 51% and decreases the K_m by 22%. While the increase in V_{max} is highly significant ($P < 0.001$), the decrease in K_m is not.

Both the basal and fMet-Leu-Phe-stimulated activities of membrane preparations isolated from pertussis or cholera toxin treated intact neutrophils are significantly ($P < 0.001$) decreased. In addition, pretreatment of the membrane preparations with either pertussis or cholera toxin, under ribosylating conditions, inhibits both the basal and fMet-Leu-Phe activities. As expected, the inhibition is greater when the toxins are added to the membranes. This is due to the higher concentrations of the toxins that can be used. Furthermore, the addition of N-ethylmaleimide, a compound which reacts with sulfhydryl groups, as expected, abolishes the GTPase activity. The decreased GTPase activity in the ribosylation solution is most likely due to the ability of some reagents in this solution to react with sulfhydryl groups. Membranes isolated from human neutrophils and from myeloid differentiated HL60 cells contain a 40-KDa protein which is a substrate for ADP ribosylation by both pertussis toxin and cholera toxin [30,31].

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