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CHARACTERIZATION OF THE PLASMA MEMBRANE BOUND GTPase FROM RABBIT NEUTROPHILS

I. Evidence for an N_i-like Protein Coupled to the Formyl Peptide, C5a, and Leukotriene B_4 Chemotaxis Receptors[1]

DOUGLAS E. FELTNER, RICHARD H. SMITH, AND WAYNE A. MARASCO[2]

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109

We have characterized the GTPase activity of the N_i-like guanine-nucleotide-binding regulatory protein in rabbit neutrophil plasma membranes. The low K_m ($3.64 \pm 0.87 \times 10^{-7}$ M) GTPase copurified with the formyl peptide receptor in the plasma membrane fraction obtained by discontinuous sucrose density gradient centrifugation. The V_{max} (23.9 ± 2.91 pmol/mg/min) and K_m of the unstimulated enzyme were similar to those reported for N_i in other cell types. The activity of the unstimulated enzyme was both magnesium and sodium dependent and linear over the first 4 min of the assay.

The chemoattractants, formyl-methionyl-leucyl-phenylalanine (fMLP), C5a, and leukotriene B_4 (LTB_4) stimulated the GTPase in purified neutrophil plasma membrane preparations, whereas other secretagogues, such as A23187 and PMA, were without effect. Lineweaver-Burk analysis showed an fMLP-induced increase in V_{max} (31.94 ± 4.80 pmol/mg/min) (33.1 ± 9.5%) but not in K_m. The dose-response curve for fMLP stimulation showed an ED_{50} of $4.1 \pm 1.0 \times 10^{-8}$ M and an overall 22.2 ± 3.1% maximal stimulation. C5a (30 μg/ml) increased the activity of the GTPase 21.3 ± 5.7% and 10^{-7} M LTB_4 produced a 32.2 ± 5.4% increase.

Activated pertussis toxin treatment of neutrophil plasma membranes inhibited by 72.5 ± 14.3% the stimulation of GTPase activity induced by fMLP; however, activated cholera toxin had no effect on the inhibition of fMLP stimulation, suggesting a direct role for an N_i-like protein in the coupling process. In contrast to the lack of inhibition of fMLP stimulation by activated cholera toxin treatment of plasma membranes, both pertussis toxin and to a lesser extent cholera toxin treatment reduced fMLP, C5a, and LTB_4 stimulation of the GTPase in sonicates prepared from pretreated whole cells. Pertussis toxin inhibited fMLP stimulation of the GTPase by 75 ± 7%, C5a stimulation was inhibited by 83 ± 13%, and LTB_4 stimulation was inhibited completely. Sonicates prepared from neutrophils treated similarly with cholera toxin showed a smaller inhibition of GTPase activity (50 ± 4% and 14 ± 9% for fMLP and LTB_4, respectively) with the exception of C5a, where CT inhibition (81 ± 32%) equaled pertussis toxin inhibition. Similarly, pertussis toxin completely inhibited the release of the granule enzyme *N*-acetyl-glucosaminidase by all three chemoattractants, whereas cholera toxin, except with C5a stimulation, had little or no effect. Thus, our results identify and characterize the GTPase activity of an N_i-like guanine-nucleotide-binding protein in the rabbit neutrophil plasma membrane and implicate the N_i-like protein as an important mediator of the neutrophil functional responses induced by the chemoattractants fMLP, C5a, and LTB_4. Our results also suggest an indirect effect of cholera toxin on the GTPase activity in these same cells.

A number of soluble mediators of inflammation, including the chemoattractants formyl-methionyl-leucyl-phenylalanine (fMLP),[3] C5a, and leukotriene B_4 (LTB_4) are known to stimulate multiple neutrophil functional responses through binding to specific cell surface receptors (1–4). In contrast, other neutrophil secretagogues, such as phorbol 12-myristate 13-acetate (PMA) and the calcium ionophore A23187, do not act through specific cell surface receptors, but exert their effect at a point distal to cell surface receptor interaction in the activation pathway (5, 6). Following the recent discovery that guanine nucleotides modulate the affinity of the formyl peptide receptor on the human neutrophil (7) and guinea pig macrophage (8), much effort has been focused to define the biochemical basis of this receptor/GTP-binding protein interaction. As in other receptor systems (9–11), the use of specific bacterial toxins has partially clarified this receptor/GTP-binding protein interaction. For example, cholera toxin treatment of human neutrophils, which is known to cause a persistent elevation in cAMP and to inhibit neutrophil chemotaxis, and lysosomal enzyme

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[1] This work was supported by National Institutes of Health Grant HL-28445.
[2] To whom reprint requests should be sent: Wayne A. Marasco, M.D., Ph.D., University of Michigan Medical School, Department of Pathology, Box 0602, 1500 East Medical Center Drive, Ann Arbor, MI 48109.

[3] Abbreviations used in this paper: fMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; LTB_4, leukotriene B_4; N_i, the inhibitory, guanine-nucleotide-binding, regulatory component of adenylate cyclase; N_s, the stimulatory, guanine-nucleotide-binding, regulatory component of adenylate cyclase; AppNHp, 5′-adenylylimido-diphosphate; fMLP-ME, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine methyl ester; DIFP, diisopropylfluorophosphate; DTT, dithiothreitol; EGTA, ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid; TLC, thin-layer chromatography; PMA, phorbol 12-myristate 13-acetate.

release (12, 13), has been shown to catalyze the ADP-ribosylation of a 42,000 m.w. membrane protein, which is believed to be the α-subunit of an "N_s[3]-like" protein (14). In addition, treatment of human and guinea pig neutrophils with pertussis toxin, which in other receptor systems ADP-ribosylates the α-subunit of N_i, a 41,000 m.w. membrane protein involved in the inhibition of adenylate cyclase (15), catalyzes the ADP-ribosylation of a 41,000 m.w. "N_i-like" protein (16–18). The N_i-like protein appears to be a distinct entity, separate from the 42,000 m.w. "N_s-like" cholera toxin substrate involved in the activation of membrane adenylate cyclase. Pertussis toxin treatment also results in inhibition of a multitude of biologic responses and biochemical reactions (16–22). Thus, although both N_s- and N_i-like regulatory proteins are found in the neutrophil, it remains controversial as to whether only N_i or perhaps both of the N-regulatory proteins are involved either directly or indirectly in the activation process.

To elucidate directly the role of the N-regulatory proteins (N_s and N_i) in the activation process, we examined the plasma membrane bound GTPase in rabbit neutrophils. Both N_s and N_i have been purified and characterized in other cell systems (23–28), and each has been shown to have an intrinsic GTPase activity (28–30). By studying both the cation requirements for stimulation and the effects of both cholera and pertussis toxin on the membrane bound GTPase activity, we have been able to document several important experimental findings that suggest that an N_i-like protein is directly coupled to multiple chemotaxis receptors, whereas an N_s-like protein is not directly coupled to these receptors, but may still influence receptor activation of N_i indirectly, perhaps through the rise in the intracellular level of cAMP.

MATERIALS AND METHODS

Materials. Oyster glycogen, Type II, diisopropylfluorophosphate (DIFP), fMLP, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), GTP, ATP, 5'-adenylylimido-diphosphate (AppNHp), dithiothreitol (DTT), creatine phosphokinase, phosphocreatine, calcium ionophore A23187, PMA, charcoal (Norit A), and fMLP methyl ester (fMLP-ME) were from Sigma Chemical Co., St. Louis, MO. Guanosine 5'-[γ-^{32}P] triphosphate, 10 to 50 Ci/mmol, was purchased from either Amersham, Arlington Heights, IL or ICN Biomedicals, Irvine, CA. Formyl-L-methionyl-L-leucyl-L-[^{3}H]phenylalanine, 40 to 60 Ci/mmol, was obtained from Dupont NEN Research Products, Boston, MA. Guanosine 5'-triphosphate [8-^{3}H] was from ICN. Polyethyleniminetreated cellulose thin-layer chromatography (TLC) plates were obtained from MC/B Manufacturing Chemists, Gibbstown, NJ. Human C5a was a generous gift from Dr. Steven L. Kunkel (University of Michigan); LTB_4 was kindly supplied by Dr. J. Rokach, Merck-Frosst Canada. Drs. C. N. Shih and Larry Winberry of the Michigan Department of Public Health were generous in their gift of purified pertussis toxin.

Preparation of crude sonicate and purified neutrophil plasma membranes. Rabbit neutrophils were harvested 4 to 16 hr after peritoneal injection of 0.1% oyster glycogen. The cells were washed twice in Hanks' buffer, 10 mM HEPES, and 0.3 mM EDTA (pH 7.2) and were resuspended at 1×10^8 cells/ml in 12.1% sucrose (w/v) with Hanks' buffer, including 10 mM HEPES and 1 mM DTT (pH 7.2). They were then treated with DIFP (1.5×10^{-3} M) for 30 min at 4°C, divided into 20-ml aliquots, and sonicated on ice in four 15-sec bursts with a W-375 sonicator (setting 4) (Heat Systems-Ultrasonics, Inc., Farmingdale, NY).

Purified plasma membranes were prepared from a low-speed supernatant (10 min at 200 × G) of the crude sonicate by fractionation over discontinuous sucrose density gradients (12 ml 75.6% (w/v); 8 ml 54.1% (w/v); 8 ml 21.6% (w/v); sucrose) by using an SW28 rotor in a Beckman L5 ultracentrifuge (27,000 rpm for 120 min). These fractions have been extensively characterized (31). Plasma membranes are found primarily at the 22/54% interface. Each of the bands were collected and washed in 12.5 mM Tris-HCl, 150 μm EGTA,[3] pH 7.4, assayed for protein by using a Bio-Rad protein assay kit (32), and either used immediately or frozen at −70°C.

Measurement of f Met-Leu-[^{3}H]Phe binding to neutrophil cell fractions. All binding studies were performed at 24°C in phosphate-buffered saline (PBS), pH 7.2, after thermal equilibration at 24°C for 15 min. Thirty micrograms of crude sonicate or cell fractions from discontinuous sucrose density gradient centrifugation were incubated with 1.0 to 80 nM ^{3}H-fMLP in 12 x 75-mm siliconized glass test tubes. After 30 min, 4 ml of ice-cold PBS were added to the tube, the mixture was vortexed, and the cells were harvested by a glass-fiber vacuum filtration method (33). The filters were washed with an additional 6 ml of ice-cold PBS, dried, and quantified for membrane-bound radioactivity by liquid scintillation counting. Nonspecific binding was defined as the amount of ^{3}H-fMLP bound in the presence of a 1000-fold or greater molar excess of nonradiolabeled fMLP and was always less than 10% of total binding. Throughout this article, "specific binding" refers to total binding minus nonspecific binding. All data points were determined in duplicate or triplicate, and the standard error of the mean was consistently less than ±5%. All binding data were analyzed by using the NONLIN computer program as described (34).

GTPase assay. We used two separate assays to define GTPase assay activity. The standard assay was a modification of the GTPase originally described by Cassel and Selinger (35). Briefly, plasma membranes or crude sonicate were washed once at 4°C and then resuspended to 0.25 to 2.5 mg/ml in 12.5 mM Tris-HCl, 5 mM $MgCl_2$, 150 μM EGTA, 1 mM DTT, 1 mM ouabain, 150 mM sodium phosphate, pH 7.4 (buffer A). The assay was initiated by adding 20 μl of membranes in buffer A to 80 μl of 0.625 mM ATP, 1.25 mM AppNHp, 1 mM creatine phosphate, 0.04 to 1.0 μM GTP[γ-^{32}P], and 5 U creatine phosphokinase in buffer A and placing the incubation mixture at 37°C. Except for the Lineweaver-Burk experiments, the final concentration of GTP[γ-^{32}P] was always 0.1 μM. These assay conditions minimize the possibility that contaminating nucleotide triphosphatases (NTPases) contribute to the measured GTPase activity. The gross excess of ATP and AppNHp over GTP inhibit nonspecific NTPases and adenylate cyclase (35). The presence of EGTA and the absence of Ca^{2+} inhibit the Ca^{2+}-dependent ATPase in the neutrophil membrane. Furthermore, higher concentrations of these reagents produced no further reductions in nonspecific GTP hydrolysis. All reactants were kept at 4°C before initiating the assay to minimize hydrolysis. The reactions were quenched by adding a 5% (w/v) charcoal:1 M formate mixture, and the preparations were placed on ice until centrifugation for 10 min at 1900 × G. One hundred or 200 μl of the resulting supernatant were sampled and quantified in a Beckman LS7500 counter by using Safety Solve liquid scintillation cocktail. Under these assay conditions, no protein phosphorylation was detectable by autoradiography of assay samples separated by SDS-PAGE.

Assay products were also analyzed by polyethylenimine (PEI)-cellulose TLC (36, 37) to positively determine that the observed activity was due to a GTPase and not guanylate cyclase (38) or pyrophosphate cleavage. Analysis of unstimulated GTPase activity by TLC indicated that >98% of the product was GDP. Furthermore, TLC additionally identified the product produced by fMLP, C5a, and LTB_4 stimulation as GDP. Reactions were initiated and run as described above (using GTP-[8-^{3}H] in place of GTP-[γ-^{32}P]), but were stopped by adding 11.1 μl of ice-cold 50% trichloracetic acid and then placing the tubes on ice. The precipitated proteins were pelleted at 1900 × G for 10 min, and 20 μl of the supernatant were added to the TLC plates in 5-μl units with intermediate drying. The plates were then soaked for 5 min in a Tris-methanol solution (600 mg/500 ml) to neutralize the acids, dried, soaked 10 min more in methanol to solubilize the $MgCl_2$, dried, and chromatographed in a 1 M formate:0.25 M LiCl solution. Then, 15 nmol each of GMP, cGMP, GDP, and GTP were run in each lane to allow detection under a 253-nm ultraviolet light. The nucleotides were eluted with 20 mM Tris-HCl containing 0.7 M $MgCl_2$ (pH 7.4) and were quantified by liquid scintillation counting.

In both assays, control hydrolysis in the absence of membranes, always less than 20% of total hydrolysis, was subtracted from the total cpm of inorganic phosphate liberated. Data points were determined in triplicate or quadruplicate. Standard deviations typically varied between 1 and 10%.

Measurement of granule enzyme release from rabbit neutrophils. Rabbit neutrophils (1×10^7/ml) were first preincubated at 37°C for 10 min. Then, 250 μl of cells in Hanks' buffer without cytochalasin B were pipetted into tubes containing 250 μl of the appropriate stimulus and 10 μg/ml cytochalasin B at 37°C. After incubation for 5 min at 37°C, the tubes were placed on ice and then centrifuged to pellet the cells. The supernatants were removed and assayed for either lysozyme or β-glucosaminidase activity. Total enzyme release was determined by cell lysis with 0.1% Triton deter-

gent. Results for lysozyme paralleled those for β-glucosaminidase and are not shown. Next, 200 μl of each supernatant were assayed for glucosaminidase activity by adding 300 μl of 1.4 mg/ml substrate (*p*-nitrophenyl *N*-acetyl-D-glucosaminide) in 0.05 M sodium citrate buffer, pH 4.5, and incubating at 37°C for 30 to 60 min. The reactions were quenched with 500 μl 0.4 M ice-cold glycine, pH 10.5, and read at 410 nm.

Treatment of neutrophil membranes with activated pertussis and cholera toxins. Pertussis and cholera toxins were first activated by incubation with 20 mM DTT for 30 min at 30°C in buffer A. Neutrophil plasma membranes (1 mg/ml) were then incubated at 24°C for 30 min in 12.5 mM Tris-HCl, 5 mM DTT, 1 mM ATP, 10 mM NAD, 10 mM thymidine, 5 U creatine phosphokinase, 1 mM phosphocreatine, 5 mM $MgCl_2$, 150 mM $NaHPO_4$, and 1 mM EGTA (pH 7.5), with or without activated pertussis or cholera toxin (50 μg/ml). Toxin treatment was quenched by the addition of 1 ml of ice-cold buffer A. The membranes were pelleted for 15 min at 4°C in an Eppendorf microcentrifuge and then resuspended to 1.5 mg/ml in buffer A.

Pertussis and cholera toxin treatment of viable rabbit neutrophils. Rabbit neutrophils, obtained as described, were suspended at 5×10^6 cells/ml in Hanks' buffer, including 0.1% dextrose and 1.7 mM Ca^{++}. The cells were treated with pertussis or cholera toxin (1 μg/ml) for 90 min at 37°C. Two different pertussis toxin preparations were used in these studies, and the most purified preparation was up to eightfold more active per nanogram in its ability to inhibit neutrophil functional responses. Under these conditions, pertussis toxin inhibits neutrophil granule enzyme release stimulated by fMLP, C5a, and LTB_4. The cells were washed once, then sonicated, and a low-speed supernatant was obtained. Control cells were subjected to similar conditions in the absence of toxin. Cell viability, determined by trypan blue exclusion, was ≥95%.

In two control experiments, we found no changes in the total number of ^{3}H-fMLP binding sites in sonicates of rabbit neutrophils that had been treated with pertussis toxin compared with untreated controls (data not shown), indicating that no significant destruction or blocking of the binding site had occurred and that the inhibitory effects of pertussis toxin were not due to direct action on the fMLP binding site. Moreover, pertussis toxin does not appear to interact directly with the LTB_4 binding site (22).

RESULTS

Copurification of the GTPase with the plasma membrane fraction. The GTPase enzyme measured in the different sucrose density fractions obtained from sonicated rabbit neutrophils displayed an increase in specific activity similar to the increase in ^{3}H-fMLP binding capacity in the same fractions. In the experiment shown in Figure 1, GTPase activity in the plasma membrane fraction increased approximately 10-fold compared with the low-speed supernatant (from 1.58 to 15.85 pmol/mg/min). ^{3}H-fMLP binding capacity was increased 5.4-fold, from 2.3 ± 0.21 to 12.4 ± 0.99 pmol/mg. In several experiments, unstimulated GTPase activity in the plasma membrane fraction increased 8.1-fold compared with the low-speed supernatant (from 1.35 ± 0.13 to 10.99 ± 2.40 pmol/mg/min, n = 5). Similarly, ^{3}H-fMLP binding capacity in the same fractions increased 6.9-fold (from 1.17 ± 0.40 to 8.04 ± 2.2 pmol/mg, n = 4). The fMLP-stimulated GTPase activity showed a similar degree of purification. GTPase and ^{3}H-fMLP binding activity showed similar, low levels of activity in the granule fraction (band III, 54:75% sucrose interface) as well.

Ionic requirements of the plasma membrane-bound GTPase. The ionic requirements of the unstimulated GTPase were initially determined. The GTPase was Mg^{++} dependent, increasing nearly threefold, from 1.77 ± 0.89 pmol/mg/min to 4.88 ± 0.65 pmol/mg/min (n = 2), with the addition of 5 mM $MgCl_2$ (Fig. 2). Similarly, the unstimulated GTPase was also Na^+ dependent (Fig. 3). Fifty to 150 mM Na^+ increased unstimulated GTPase activity 40 to 60% above sodium-free controls. Very low doses of Na^+ (1 mM) had little or no effect.

GTPase stimulation by fMLP (as described below), like unstimulated GTPase activity, was dependent on Mg^{++} and Na^+. Stimulation of GTPase activity by fMLP was evident only at 100 mM or greater concentrations of Na^+ and was not dependent on whether Cl^- or PO_4^- was the corresponding anion (Fig. 3). No fMLP stimulation was evident in the presence of equivalent concentrations of either KCl or ChCl (choline chloride) (data not shown). Stimulation of the N_i-GTPase associated with opiate receptors has been shown to require similar concentrations of sodium (10, 39, 40), whereas stimulation of the N_s-GTPase coupled to dopamine (D_1) (10) and β-adrenergic (35) receptors has no requirement for sodium. Stimulation by fMLP (above control) was evident only in the presence of a 1 mM or higher concentration of $MgCl_2$ (Fig. 2). Both N_i and N_s are known to require Mg^{++} for activation (23–25, 41, 42). In general, lesser concentrations of Mg^{++} are required to promote activation of N_i than N_s (41, 42).

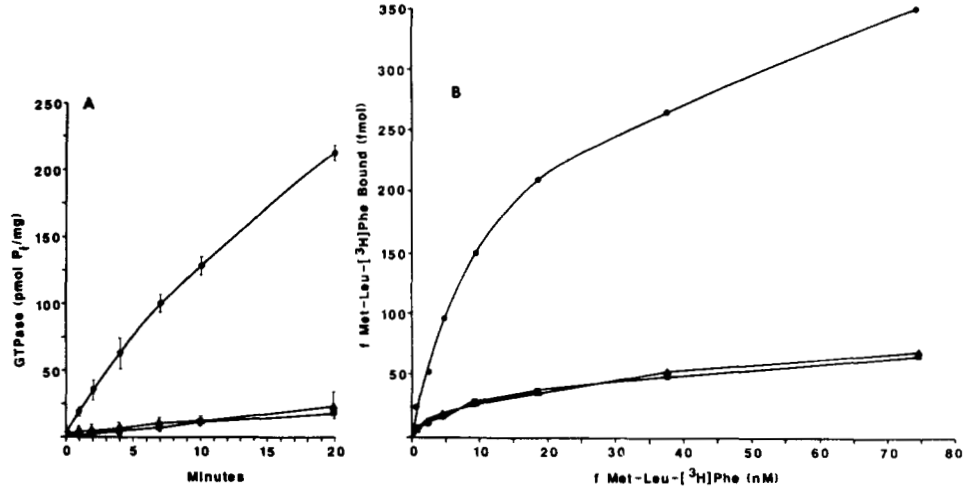

Figure 1. Copurification of GTPase with the plasma membrane fraction. *A*, GTPase activity in 30 μg protein was determined from 0 to 20 min in the low-speed supernatant (▲), the granule (54:75% sucrose interface) (■), and plasma membrane (22:54% sucrose interface) (●) fractions. GTPase activity was determined by linear regression of the initial 4 min of each reaction. *B*, ^{3}H-fMLP binding to 30 μg of neutrophil particulate fractions was performed as described in *Materials and Methods*. The data were analyzed by using the NONLIN computer program as described (34). Binding capacities (B.C.) and equilibrium dissociation constant (K_D) values in this experiment were: (●) plasma membranes K_D = 10.42 ± 1.88 nM. B.C. = 12.40 ± 0.99 pmol/mg; (■) granule fraction K_D = 15.04 ± 2.91 nM, B.C. = 2.5 ± 0.22 pmol/mg; (▲) low-speed supernatant K_D = 12.50 ± 2.58 nM, B.C. = 2.3 ± 0.21 pmol/mg. Although the Scatchard plot for ^{3}H-fMLP binding to the plasma membrane fraction appeared curvilinear with upward concavity, suggesting the presence of two classes of binding sites (as previously reported (60, 69)), that conclusion was not statistically justifiable in this experiment.

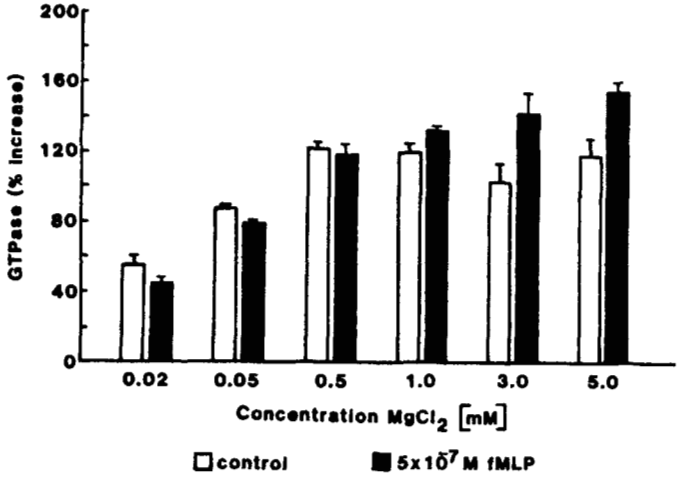

Figure 2. Magnesium dependence of fMLP-stimulated and unstimulated GTPase activity. GTPase activity was measured in 30 μg of plasma membranes after 4 min in the presence (■) or absence (□) of 5×10^{-7} M fMLP. The reaction conditions were as described in *Materials and Methods* except that the concentration of $MgCl_2$ was varied from 0 to 5 mM. Data are expressed as percent increase in GTPase activity compared with control GTPase activity in the absence of Mg^{2+} (2.66 ± 0.07 pmol/mg/min). In this experiment, specific fMLP stimulation was 1.0 pmol/mg/min (+18.3%) at 5 mM $MgCl_2$ (standard assay conditions). Student's *t*-test showed all data points were statistically different from the no Mg^{2+} control ($p < 0.01$). Error bars equal mean ± SD (n = 4).

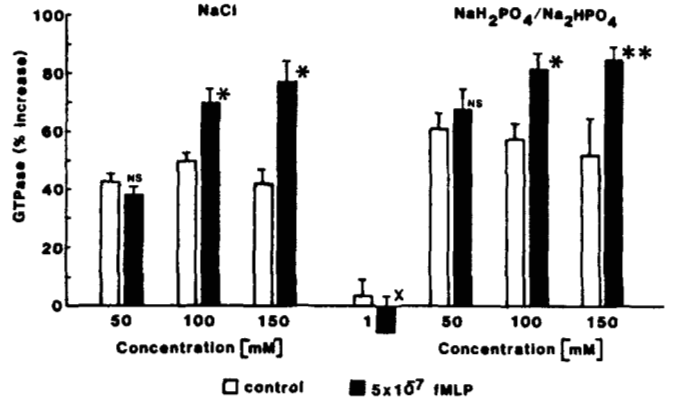

Figure 3. Sodium dependence of fMLP-stimulated and unstimulated GTPase activity. GTPase activity in 30 μg of plasma membranes was measured in the presence (■) or absence (□) of 5×10^{-7} M fMLP. The reaction conditions were as described in *Materials and Methods* except that the Na^+ concentration was varied from 0 to 150 mM by using either NaCl or NaH_2PO_4/Na_2HPO_4. Data are expressed as percent increase in GTPase activity compared with control activity in the absence of Na^+ (7.10 ± 0.52 and 7.39 ± 0.44 pmol/mg/min for NaCl and NaH_2PO_4/Na_2HPO_4, respectively). Statistical significance was calculated by using Student's *t*-test on cpm data comparing GTPase activity in the presence of 5×10^{-7} M fMLP to GTPase activity in the absence of fMLP at the same Na^+ concentration ($p < 0.005$ [*], $p < 0.01$ [**], not significant [NS]). In this experiment, specific fMLP stimulation was 2.49 and 2.47 pmol/mg/min in 150 mM NaCl and 150 mM NaH_2PO_4/Na_2HPO_4 (standard conditions), respectively. Error bars equal mean ± SD (n = 3).

Our finding that GTPase activity plateaued at approximately 1 mM $MgCl_2$ is consistent with the requirement for micromolar concentrations of free Mg^{++} to activate N_i. Finally, ouabain had no effect on either unstimulated or fMLP-stimulated GTPase, indicating that an Na^+/K^+ ATPase was not responsible for the observed activity.

Characteristics of the unstimulated and chemotactic factor-stimulated GTPase in purified neutrophil plasma membranes. Unstimulated GTPase activity was linear for at least the first 4 min of the assay at all concentrations of substrate tested (0.03 to 1.0 μM) (Fig. 4, *closed circles*). In addition, unstimulated GTPase activity varied linearly over a wide range of protein concentrations (5 to 50 μg/100 μl). To determine the extent of coupling of this enzyme system to several known receptor-mediated (formyl peptides, C5a, LTB_4) and nonreceptor-mediated (PMA, A23187) secretagogues, we next investigated their ability to stimulate the membrane bound GTPase. Figure 4 demonstrates that stimulation of GTPase activity by fMLP (*open circles*) was evident after 2 min and continued for at least the first 10 min of the assay. The concentrations of fMLP that stimulated the GTPase range from 10^{-9} M to 5×10^{-6} M (Fig. 5). Stimulation was half-maximal at $4.1 \pm 1.0 \times 10^{-8}$ M fMLP (n = 3) and plateaued at approximately 5×10^{-7} M. Maximal stimulation, measured at 5×10^{-7} M fMLP, was 22.2 ± 3.1% (n = 10). Similar results were obtained with fMLP-ME (half-maximal stimulation = 2.5×10^{-8} M, 14.5% maximal increase in activity after 4 min) (data not shown). The chemoattractants LTB_4 and C5a also stimulated the GTPase in purified neutrophil plasma membranes (Table I). The GTPase was stimulated 32.2 ± 5.4% by 10^{-7} M LTB_4, compared with a paired 0.5% methanol control; 30 μg/ml C5a stimulated the GTPase 21.3 ± 5.7%. Large doses of PMA (100 ng/ml) and A23187 (10^{-6} M) had little or no effect.

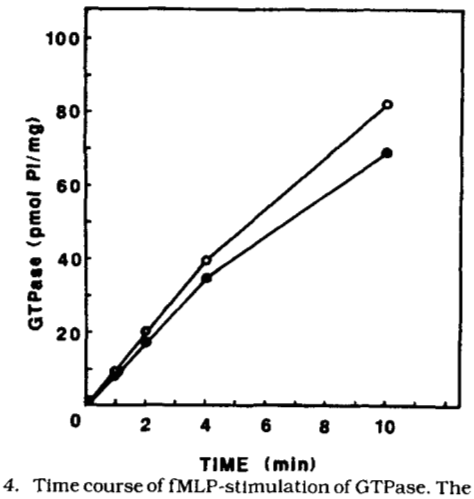

Figure 4. Time course of fMLP-stimulation of GTPase. The time course of GTPase activity was measured in 30 μg of plasma membranes that were either unstimulated (●) or stimulated with 5×10^{-7} M fMLP (○). In this experiment, unstimulated GTPase activity was 69.1 ± 1.3 pmol/mg at the 10-min time point; fMLP-stimulated GTPase activity was 82.5 ± 5.5 pmol/mg at 10 min ($p < 0.02$ by Student's *t*-test).

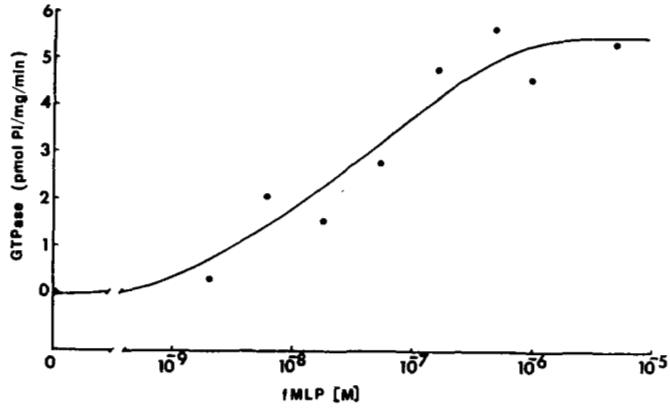

Figure 5. Dose-response curve for fMLP stimulation of GTPase. GTPase activity was measured in 30 μg of plasma membranes after 4 min of stimulation by 0 to 5×10^{-6} M fMLP. In this experiment, data points were determined in quadruplicate and are expressed as mean stimulation above buffer control. Stimulation was half maximal at 3.6×10^{-8} M fMLP.

TABLE I

Effect of various neutrophil secretagogues on GTPase activity in the plasma membrane fraction[a]

Stimulus	% GTPase Activity Relative to Control
5×10^{-7} M fMLP	122.2 ± 3.1 (n = 10)
10^{-7} M LTB_4	132.2 ± 5.4 (n = 4)
0.5% Methanol	91.1 ± 5.7 (n = 4)
30 μg/ml C5a	121.3 ± 5.7 (n = 4)
PMA 100 ng/ml	94.0 ± 5.7 (n = 3)
A23187 10^{-6} M	106.7 ± 6.3 (n = 3)
Control	100.0

[a] Rabbit neutrophil plasma membranes were incubated with the indicated neutrophil activators and 0.1 μM GTP[γ-^{32}P] under standard assay conditions; 0.5% methanol is included as a control for LTB_4, which was stored as a 2×10^{-5} M stock in methanol. Data points were determined in quadruplicate and are expressed as the mean ± SEM.

Lineweaver-Burk analysis of the unstimulated GTPase revealed an enzyme with a single, low K_m ($3.64 \pm 0.87 \times 10^{-7}$ M) (n = 4). In a separate experiment, performed by using unlabeled GTP with trace amounts of GTP^{32} to attain much higher substrate concentrations ($\geq 10^{-4}$ M), a high K_m GTPase, similar to those previously reported (43), was also detected ($K_m \geq 5 \times 10^{-5}$ M). The high K_m GTPase did not make a significant contribution to GTPase activity measured in standard assay conditions. Lineweaver-Burk analysis of the unstimulated GTPase and GTPase stimulated by 5×10^{-7} M fMLP showed a statistically significant rise in V_{max} in three of four experiments (23.97 ± 2.91 to 31.94 ± 4.80 pmol/mg/min) (33.1 ± 9.5%) (n = 4) and a consistent, although not statistically significant (except in one experiment), increase in K_m (3.64 ± 0.87 to $4.06 \pm 0.96 \times 10^{-7}$ M) (11.5 ± 4.8%). These results are shown in Table II and Figure 6. The fMLP-induced increase in V_{max} detected by Lineweaver-Burk analysis is consistent with a ligand-induced increase in the availability of the GTPase catalytic site of N_i in its active state, or more specifically, the ligand-induced dissociation of the α-subunit of N_i from its β-subunit, as has been suggested by other studies of N_i (24, 41, 44). The potential meaning, if any, of the observed increase in K_m is unknown, but it clearly accounts for only a fraction of the observed increase in V_{max}. An increase in V_{max} due to opiate stimulation of the GTPase in NG108-15 hybrid cells has been reported to be due to an increase in K_m (39).

Pertussis toxin and cholera toxin inhibition of glucosaminidase release from rabbit neutrophils induced by the chemoattractants fMLP, C5a, and LTB_4. Pertussis toxin inhibited completely the ability of the chemoattractants fMLP, C5a, and LTB_4 to stimulate the release of glucosaminidase from rabbit neutrophil lysosomal granules (Fig. 7). Inhibition by pertussis toxin of glucosaminidase release induced by all three chemoattractants was dependent on the concentration of toxin and was not reversible even with extensive washing. The ID_{50} values for pertussis toxin induced inhibition were 6.8 ng/ml for fMLP-induced glucosaminidase release, 3.5 ng/ml for C5a-induced release, and 2.8 ng/ml for LTB_4-induced release. In addition, fMLP-induced granule enzyme release was inhibited by 1 μg/ml of pertussis toxin throughout the dose-response range for fMLP (data not shown). Pertussis toxin had no effect on glucosaminidase release induced by 10^{-7} M A23187 or by 100 ng/ml PMA (data not shown).

In contrast to pertussis toxin, cholera toxin had only a minimal effect on glucosaminidase release induced by fMLP and LTB_4 at concentrations of toxin (1 μg/ml) used in the GTPase experiments (Fig. 7). Higher concentrations of toxin (5 μg/ml) produced a small amount of inhibition. Cholera toxin inhibited C5a-induced glucosaminidase release substantially (approximately 40%), but not in a dose-response manner over the range of toxin concentration tested (0.15 to 5 μg/ml). The selective inhibition of C5a-induced glucosaminidase release by cholera toxin paralleled the greater inhibition of C5a-stimulated (compared with fMLP or LTB_4) GTPase activity by cholera toxin (see below and Table IV) and may imply that a portion of the C5a-stimulated GTPase activity is due to an N_s-like protein. Inhibition by pertussis toxin indicates that an N_i-like guanine nucleotide binding protein may couple ligand-receptor binding of these chemoattractants to neutrophil functional responses.

Inhibition of fMLP-stimulated GTPase activity in plasma membranes by activated pertussis toxin. Treatment of rabbit neutrophil plasma membranes with activated pertussis toxin completely inhibited stimulation of the GTPase by 5×10^{-7} M fMLP (Fig. 8). As can be seen, pertussis toxin treatment caused a concentration-dependent inhibition of fMLP stimulation, with maximal inhibition occurring at 50 μg/ml. In three experiments, activated pertussis toxin treatment (50 μg/ml) caused 72.5 ± 14.3% inhibition of fMLP-stimulated GTPase. In contrast, treatment of membranes with activated cholera toxin (50 μg/ml) resulted in no inhibition of the fMLP-stimulated GTPase (data not shown). Membranes treated with activated cholera toxin were stimulated 106.6 ± 16.4% (n = 2) compared with stimulation in untreated membranes.

Inhibition of fMLP-, C5a-, and LTB_4-stimulated GTPase activity by toxin treatment of rabbit neutrophils. In contrast to the lack of inhibition of fMLP stimulation by activated cholera toxin treatment of plasma membranes, both pertussis toxin and to a lesser extent

TABLE II

Lineweaver-Burk analysis of fMLP-stimulated and unstimulated GTPase activity for rabbit neutrophil plasma membranes[a]

Experiment	V_{max} Unstim.	V_{max} Stim.	% Increase	P Value[b]	K_m Unstim.	K_m Stim.	% Increase	P Value[b]
1	28.06	34.66	23.5	NS	6.18	6.73	8.9	NS
2	29.49	44.34	50.4	$p < 0.01$	3.31	4.16	25.7	$p < 0.01$
3	21.23	23.54	10.9	$p < 0.01$	2.44	2.59	6.1	NS
4	17.10	25.22	47.5	$p < 0.01$	2.63	2.77	5.3	NS
Mean	23.97 ± 2.91	31.94 ± 4.80	33.1 ± 9.5		3.64 ± 0.87	4.06 ± 0.96	11.5 ± 4.8	

[a] GTPase activity in 30 μg of plasma membranes was determined at 0, 2, and 4 min. Initial velocities at each concentration of GTP[γ-^{32}P] were determined by averaging the velocities (pmol/mg/min) calculated at 2 and 4 min. The concentration of GTP[γ-^{32}P] ranged from 1.0 to 0.03 μM; 5×10^{-7} M fMLP was used to stimulate GTPase activity. Data points were determined in triplicate or quadruplicate.

[b] P values comparing unstimulated and fMLP stimulated V_{max} as well as unstimulated and fMLP stimulated K_m were calculated by analysis of covariance techniques. NS = nonsignificant.

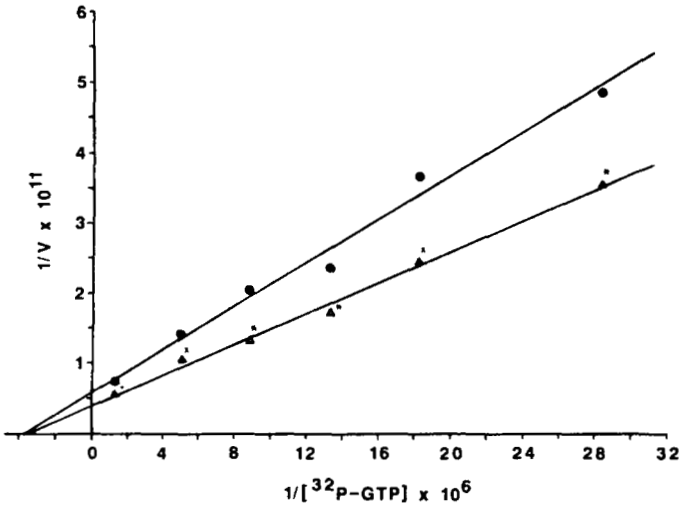

Figure 6. Lineweaver-Burk plot of fMLP-stimulated vs unstimulated GTPase activity. Unstimulated GTPase activity (●) and GTPase activity stimulated by 5×10^{-7} M fMLP (Δ) was determined at 0, 2, and 4 min in 30 μg of plasma membrane protein. Initial velocities at each concentration of GTP[γ-^{32}P] were determined by averaging the velocities calculated at 2 and 4 min. In this experiment (Expt. 4 in Table III), the concentration of GTP[γ-^{32}P] ranged from 0.75 to 0.035 μM. Data points were determined in quadruplicate. Statistical significance, comparing fMLP stimulated vs unstimulated activity at a given substrate concentration, was calculated by Student's *t*-test, using cpm data from the 4-min time points ($p < 0.005$ [*], $p < 0.01$ [x], $p < 0.05$ [+]).

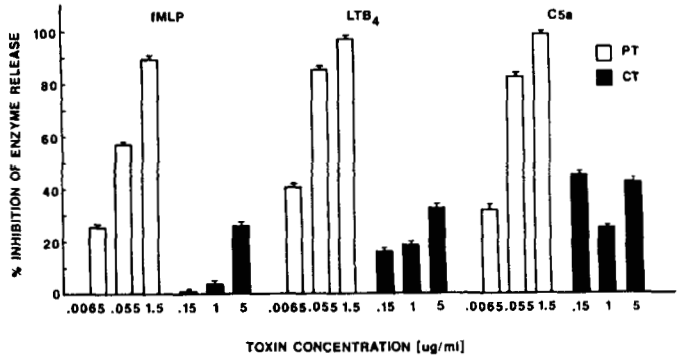

Figure 7. Pertussis and cholera toxin inhibition of glucosaminidase release from rabbit neutrophils induced by the chemoattractants fMLP, C5a, and LTB_4. Rabbit neutrophils (5×10^6 cells/ml) were treated with pertussis toxin, cholera toxin, or buffer for 90 min at 37°C as described in *Materials and Methods*. Glucosaminidase release was then measured after stimulation of the toxin or buffer-treated neutrophils with various concentrations of fMLP, C5a, or LTB_4. The concentrations of fMLP (5×10^{-8} M), C5a (1.25 μg/ml), and LTB_4 (5×10^{-7} M) used in the inhibition experiment each gave maximal glucosaminidase release for the given ligand, as determined by a dose-response experiment on untreated cells (data not shown). Toxin concentrations are shown underneath each bar. Data points were determined in duplicate or triplicate.

cholera toxin treatment (1 μg/ml) of rabbit neutrophils significantly impaired the ability of fMLP to stimulate GTPase activity in sonicates prepared from the pretreated cells. The results of four individual experiments are shown in Table III. As can be seen, pertussis toxin inhibited the GTPase activity (range of inhibition = 47 to 100%) stimulated by 5×10^{-7} M fMLP in all four experiments. Cholera toxin treatment of rabbit neutrophils also inhibited fMLP stimulation of the GTPase in sonicates prepared from those cells, but in all cases to a lesser extent than did pertussis toxin treatment. The inhibition by pertussis and cholera toxin of C5a and LTB_4 stimulation of GTPase activity is shown in Table IV. As can be seen, pertussis toxin completely inhibited stimulation of GTPase activity by 10^{-7} M LTB_4, whereas cholera toxin was without effect. In two of the LTB_4 experiments,

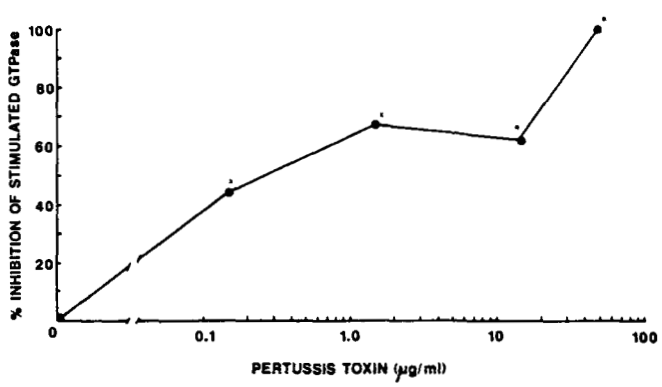

Figure 8. Inhibition of GTPase activity in plasma membranes treated with activated pertussis toxin. Purified neutrophil plasma membranes (30 μg/ml) were pretreated with varying concentrations of activated pertussis toxin (0.15 to 50 μg/ml) for 30 min as described in *Materials and Methods*. After treatment, the membranes were washed and resuspended, and GTPase activity was measured at 0.1 μM GTP [γ-^{32}P]. Protein was assayed in each sample after resuspensions. The amount of specific stimulation by 5×10^{-7} M fMLP in control samples was 2.43 pmol/mg/min. Data points were determined in quadruplicate. Statistical significance was calculated by using Student's *t*-test. ($p < 0.005$ [*], $p < 0.02$ [x]).

GTPase activity in the presence of stimulus was less than GTPase activity in the absence of stimulus after pertussis toxin treatment. This resulted in the >100% inhibition associated with LTB_4. Pertussis toxin also inhibited C5a-stimulated GTPase activity by 83%. However, sonicates prepared from neutrophils treated similarly with cholera toxin showed a substantially greater inhibition of C5a-stimulated GTPase activity than of fMLP- and LTB_4-stimulated activity. In these experiments, cholera toxin inhibition of the C5a-stimulated GTPase (81 ± 32.8%) equaled pertussis toxin inhibition.

DISCUSSION

We have measured a multiple chemotactic factor-stimulatable GTPase that copurifies with the rabbit neutrophil plasma membrane fraction and with the formyl peptide receptor (Fig. 1). This enzyme shares several characteristics with the GTPases known to transduce ligand-receptor signals in other cell systems. It is a membrane-associated enzyme with a K_m (0.36 μM) that matches closely the reported K_m values for N_i in C6 glioma (0.21 μM) (11), NG108-15 (0.14 μM) (39), and platelet (0.3 to 0.4 μM) (45) membranes. A similar K_m (0.24 μM) has recently been reported for the formyl-peptide-stimulatable GTPase in crude guinea pig neutrophil homogenates (43). The lack of effect of PMA and A23187 on the neutrophil GTPase is consistent with reports that pertussis toxin has no effect on PMA- and A23187-induced neutrophil responses (19, 22). These results support the notion that the actions of PMA and A23187 are not mediated by a guanine-nucleotide-binding protein. PMA is known to bind directly to protein kinase C (5), and the role of A23187 as a calcium ionophore has been well established (6).

One question that arises is whether N_s contributes to the unstimulated and stimulated GTPase we have measured. An N_s-like guanine nucleotide-binding protein has been shown to exist in the human neutrophils (14). Moreover, in the neutrophil, cholera toxin treatment has been shown to produce persistent increases in cAMP (12). However, several pieces of evidence both in the literature

TABLE III

GTPase activity in sonicates prepared from pertussis and cholera toxin-treated and control rabbit neutrophils[a]

Expt.	Stimulus	Control GTPase (pmol/mg/min)	Pertussis Toxin Treated		Cholera Toxin Treated	
			GTPase (pmol/mg/min)	% Inhibition of Control Stimulation	GTPase (pmol/mg/min)	% Inhibition of Control Stimulation
1	−	5.12	2.94		4.93	
	+	6.44 (1.32)**	3.17 (0.23)$^+$	83	5.74 (0.81)*	39
2	−	0.84	0.58		0.72	
	+	1.12 (0.28)*	0.73 (0.15)*	47	0.90 (0.18)*	36
3	−	6.32	2.61		3.71	
	+	7.22 (0.90)$^+$	2.76 (0.15)$^+$	83	4.19 (0.48)**	47
4	−	1.12	1.43		1.17	
	+	1.37 (0.25)*	1.12 (−0.31)*	100	1.27 (0.10)$^+$	60

Protein (30 to 50 μg) from the low-speed supernatant of sonicated, toxin- or buffer-treated neutrophils was measured for GTPase activity in the presence (+) or absence (−) of 5×10^{-7} M fMLP under standard assay conditions. Data points were determined in quadruplicate. The numbers in parentheses are the amount of stimulation (pmol/mg/min) induced by fMLP. Pertussis toxin inhibited formyl-peptide stimulation of the GTPase by 78.2 ± 11.2%. Cholera toxin inhibited 45.5 ± 5.4% of the fMLP stimulated activity. Protein assays were performed after each experiment to correct for protein loss during centrifugation and resuspension. In individual experiments, stimulated and control means within each treatment group were compared to determine if the amount of stimulation induced by fMLP was significant. Significance was determined by Student's *t*-test. (*), $p < 0.01$; (**), $p < 0.02$; (*), $p < 0.05$; ($^+$), not significant $p > 0.05$.

TABLE IV

Inhibition of fMLP-, C5a-, and LTB_4-stimulated GTPase activity by toxin treatment of whole cells[a]

Treatment	% Inhibition of Stimulation of Untreated Cells
PT + fMLP	75.1 ± 6.6 (n = 8)
CT + fMLP	50.0 ± 3.7 (n = 7)
PT + LTB_4	118.6 ± 29.4 (n = 4)
CT + LTB_4	14.1 ± 9.3 (n = 4)
PT + C5a	83.2 ± 13.4 (n = 3)
CT + C5a	81.0 ± 32.8 (n = 3)

[a] Rabbit neutrophils were treated with either 1 μg/ml pertussis toxin (PT), 1 μg/ml cholera toxin (CT), or buffer as described in *Materials and Methods*. The crude sonicate of these cells was then assayed for GTPase activity by using 10^{-7} M LTB_4, 5×10^{-7} M fMLP, or 30 μg/ml C5a as the stimulus. Data are presented as percent inhibition by toxin of the amount of stimulation in buffer-treated cells. For each ligand, an appropriate control was included to measure base-line activity: buffer for fMLP, 0.5% methanol for LTB_4, and 0.02% BSA for C5a. The data represent mean ± SE; n = number of experiments.

and in our studies suggest that the contributions of N_s are both minimal and indirect. First, although fMLP and C5a have been shown to induce a transient rise in cAMP in human (46) and guinea pig neutrophils (16), it has recently been demonstrated that the fMLP-induced transient rises in cAMP in human neutrophils are mediated by a Ca^{2+}-dependent pathway independent of N_s, possibly involving transient inhibition of phosphodiesterases (47). Our finding that activated cholera toxin had no effect on the fMLP-stimulated GTPase in treated plasma membranes is consistent with the notion that the formyl peptide receptor is not coupled to N_s. Second, double reciprocal plots of fMLP-stimulated and unstimulated GTPase activity were consistently linear, indicating that only a single enzyme was measured. Third, we have also found that fMLP stimulation of the rabbit neutrophil GTPase depends on the concentration of sodium ion present in the reaction mileau. This is consistent with the sodium dependence of [D-Ala^2-Met^5] enkaphalinamide stimulation of GTPase activity associated with N_i coupled to opiate receptors in NG108-15 hybrid cells (39, 40) and rat striatal membranes (10) and with the requirement for sodium in the inhibition of adenylate cyclase by ligands acting through N_i coupled to opiate (39, 40), α-adrenergic (48), and muscarinic cholinergic (49, 50) receptors. Furthermore, these similarities suggest that sodium acts on N_i or N_i-receptor coupling rather than on N_i-adenylate cyclase coupling, since the N_i-like protein associated with the formyl-peptide receptor appears to interact with phospholipase C and not adenylate cyclase (21, 51). Additionally, the influx of sodium that occurs with fMLP activation of the neutrophil (52) may serve to regulate fMLP activation of N_i. These observations do not negate our findings that cholera toxin treatment of whole neutrophils inhibited fMLP and C5a stimulation of the GTPase in sonicates prepared from those cells. However, because treatment of neutrophil membranes with activated cholera toxin had no effect on fMLP stimulation of the GTPase, these results suggest that the inhibition of fMLP-stimulatable GTPase in sonicates prepared from cells treated with cholera toxin is mediated by an intracellular process, perhaps the increase in intracellular concentration of cAMP known to be induced by cholera toxin treatment (46, 53). High concentrations of cAMP are known to inhibit neutrophil functional responses (54), and cAMP regulation of N_i could potentially account for the inhibition. De Togni et al. (55) have suggested that cAMP regulates phosphatidylinositol turnover in the neutrophil, and phosphatidylinositol turnover is now thought to be regulated by N_i (21, 51, 56). The greater inhibition by cholera toxin of both C5a stimulation of the GTPase (81%, Table IV) and release of the granule enzyme *N*-acetyl-glucosaminidase (40%, Fig. 6) suggests either that the control of the C5a receptor/guanine-binding protein complex by the presumed indirect actions of increased cAMP is more pronounced or that the C5a receptor is directly coupled to N_s in addition to N_i.

The majority of our data support the contention that the chemotaxis receptors are coupled directly to an N_i-like guanine-binding protein that is responsible for the stimulatable GTPase activity. This has been ascertained by pertussis toxin treatment of both whole cells and plasma membranes. Stimulation by fMLP, C5a, and LTB_4 of the GTPase in sonicates prepared from treated cells were inhibited 75%, 83%, and 100%, respectively (Table IV) by pertussis toxin, indicating that an N_i-like protein was responsible for the stimulated activity. Toxin inhibition of *N*-acetyl-glucosaminidase release stimulated by fMLP, C5a, and LTB_4 paralleled the results seen for toxin

inhibition of GTPase. Pertussis toxin inhibited enzyme secretion by LTB_4, C5a, and fMLP nearly completely at the concentration of toxin (1 μg/ml) used to inhibit the GTPase activity. C5a-induced enzyme release, like C5a-stimulated GTPase activity, was more sensitive to inhibition by cholera toxin than were fMLP- or LTB_4-induced activities. PMA and A23187 had little or no effect on the GTPase, and their induction of enzyme release was not affected by either pertussis or cholera toxins. Finally, activated pertussis toxin inhibited the fMLP-stimulated GTPase in neutrophil plasma membranes nearly completely (72.5 ± 14.3%), and did so in a dose-response manner (Fig. 8). In support of this conclusion, the effects of pertussis toxin have been correlated with the NAD-dependent ADP-ribosylation of a 41,000 m.w. protein in human (18, 22) and guinea pig (16, 17) neutrophil membranes. The neutrophil protein comigrates with pure N_i from rabbit liver when examined by SDS-PAGE (16).

Several other important observations have been documented in our studies. First, our results show that a 10^{-7} M dose of LTB_4 gave as large an increase or larger in GTPase activity than did 5×10^{-7} M dose of fMLP (Table I). This is significant because a 10^{-7} M dose of LTB_4 is approximately fivefold less than the concentration of LTB_4 required for maximal granule enzyme release, whereas a 5×10^{-7} M dose of fMLP is approximately 100-fold greater than the concentration of fMLP required for maximal granule enzyme release (19, 57) (data not shown). Thus, LTB_4 is either relatively more potent or substantially more efficacious than fMLP in eliciting GTPase activity. One possible explanation is that LTB_4 may act on N_i not only through its cell surface receptors, but also either directly or indirectly through its role as an intracellular effector in the neutrophil. LTB_4 is known to be the primary product of arachidonic acid metabolism in the neutrophil (58) and is a potent molecule in eliciting neutrophil functional responses (59). Second, the dose-response curve (10^{-9} to 10^{-6} M) for fMLP stimulation of GTPase activity occurs over a range of concentrations nearly identical to that required to saturate its receptor on the rabbit neutrophil (60). These concentrations are approximately 500-fold higher than the ligand concentrations required to stimulate chemotaxis (61). There are two immediate implications. First, amplification of the ligand-induced signal has not yet occurred at the N-protein level of stimulus-response coupling. Second, neutrophils, capable of maximal functional responses at low levels of receptor occupancy (1 to 10%), and thus containing "spare receptors" (62), also seemingly contain an excess supply of N_i-like proteins. The exact purpose or function of the "spare N-proteins" remains obscure, although they might be utilized in repetitive stimulation by the same, or even different, ligands if, in the latter case, a common pool of N-proteins were available.

It is not clear from our experiments whether a single pool of N_i-like binding proteins can interact with all three chemotaxis receptors as has been demonstrated (63) for prostaglandin E_1, nicotinic acid, and α-adrenergic receptors in hamster adipocyte membranes, or whether each type of chemotaxis receptor interacts with a separate group or pool of N_i-like proteins. Although the latter possibility would provide an explanation for the observed desensitization of the neutrophil to stimulation by a given ligand after pretreatment with the same (i.e., fMLP, C5a, or LTB_4) but not a different ligand (64, 65), further experiments are needed to clarify this possibility. In addition, other mechanisms have been suggested for desensitization phenomena, including ligand-receptor internalization (66) and modulation of chemoattractant receptor affinity independent of, or in concert with, other mechanisms (67).

Stimulation of the neutrophil GTPase by these three chemoattractants provides direct evidence for coupling of these receptors to a guanine-nucleotide-binding protein that is "N_i-like" in character. The coupling of formyl-peptide, C5a, and LTB_4 chemotaxis receptors to the "N_i-like" guanine-nucleotide-binding proteins demonstrates that neutrophils respond to multiple types of chemotactic signals by a common or similar mechanism at very early stages of signal transduction. The pertussis toxin inhibition of neutrophil responses like polyphosphoinositol turnover, Ca^{++} influx, and Na^+/H^+ exchange induced by fMLP, C5a, and LTB_4 indicates that the site of action of pertussis toxin, the N_i-like protein, is involved in the very early stages of signal-response coupling for all three of these chemoattractants (16–22, 43, 51, 52, 56, 68). The multiple chemotaxis receptor-mediated stimulation of GTPase activity thus serves as a measure of an important early step in neutrophil activation.

The identification and characterization of an N_i-like GTPase that copurified with the formyl peptide receptor in the plasma membrane fraction provides a foundation for further studies designed to understand the interactions of the N_i-like protein and the different chemotaxis receptors in their purified forms (69) and should help to elucidate the initial steps involved in the activation of neutrophils and their recruitment to sites of infection and inflammation.

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