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REGULATION OF FORMYL PEPTIDE RECEPTOR BINDING TO RABBIT NEUTROPHIL PLASMA MEMBRANES

Use of Monovalent Cations, Guanine Nucleotides, and Bacterial Toxins to Discriminate among Different States of the Receptor[1]

DOUGLAS E. FELTNER AND WAYNE A. MARASCO[2]

From the Departments of Pathology and Internal Medicine, University of Michigan Medical School, Ann Arbor, Michigan 48109

The regulation by monovalent cations, guanine nucleotides, and bacterial toxins of [^{3}H]FMLP binding to rabbit neutrophil plasma membranes was studied by using dissociation techniques to identify regulatory effects on separate receptor states. Under conditions of low receptor occupancy (1 nM [^{3}H]FMLP) and in both Na^+ and K^+ buffers, dissociation is heterogenous, displaying two distinct, statistically significant off rates. [^{3}H]FMLP binding was enhanced by substituting other monovalent cations for Na^+. In particular, enhanced binding in the presence of K^+ relative to Na^+ was caused by additional binding to both rapidly and slowly dissociating receptors.

Three receptor dissociation rates, two of which appear to correspond to the two affinity states detected in equilibrium binding studies, were defined by specific GTP and pertussis toxin (PT) treatments. Neither GTP, nor PT or cholera toxins (CT) had an effect on the rate of dissociation of [^{3}H]FMLP from the rapidly dissociating form of the receptor. Both 100 μM GTP and PT treatments increased the percentage of rapidly dissociating receptors, correspondingly decreasing the percentage of slowly dissociating receptors. CT treatment resulted in only a small increase in the percentage of rapidly dissociating receptors. The observed changes in the rapidly and slowly dissociating receptors after GTP, PT, and CT treatments were caused by an absolute decrease in the amount of binding to the slowly dissociating receptors. However, complete inhibition of slowly dissociating receptor binding by GTP, PT, or both was never observed. Both GTP and PT treatments, but not CT treatment, increased by twofold the rate of dissociation of 1 nM [^{3}H]FMLP from the slowly dissociating form of the receptor, resulting in a third dissociation rate. Thus, slowly dissociating receptors comprise two different receptor states, a G protein-associated guanine nucleotide and PT-sensitive state and a guanine nucleotide-insensitive state.

The neutrophil formyl peptide receptor, which mediates cellular responses to formyl peptides produced at sites of bacterial infection (1), has been reported to exist in two affinity states on the surface of rat, rabbit, and human cells (2–6). Investigations into the mechanistic significance of the two formyl peptide receptor affinity states have resulted in a variety of models that attempt to explain how these receptors function in neutrophil chemotaxis, lysosomal enzyme secretion, and superoxide anion production (7–10). The observation that chemotaxis typically requires 10- to 50-fold lower concentrations of formyl peptides than either lysosomal enzyme secretion or superoxide anion production (11–13) has prompted speculation about the existence of two structurally distinct classes of receptors, with high affinity receptors mediating chemotaxis and low affinity receptors mediating lysosomal enzyme secretion and superoxide anion production. Alternatively, a single class of receptors may exist in different forms that are subject to regulation by a variety of intracellular and extracellular modulators, including monovalent and divalent cations, nucleotides, and a variety of circulating hormones (7–10). Two distinct types of models have been proposed to explain how a single class of dynamically regulated receptors might function in mediating the cellular responses to formyl peptides. Yuli et al. (7) have suggested that both high and low affinity receptors are active in signal transduction, that each receptor state favors a unique cellular response, and that exogenous agents may regulate these receptors to favor either the high or low affinity state. An alternative model for the two affinity states contends that receptors exist in a transiently active low affinity form that is rapidly converted to an inactive high affinity form (8, 9). Inactive high affinity receptors are associated with the cytoskeleton, become internalized, and are unable to couple to cell activation.

The pathways of both receptor regulation and cell activation by the formyl peptides appear to involve the interaction of the ligand-receptor complexes with guanine nucleotide-binding proteins. Evidence of this interaction comes from studies which show that the binding affinity for the formyl peptides is decreased by guanine nucleotides (4, 14, 15), that ligand binding induces GTP hydrolysis as well as guanine nucleotide exchange (14–

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[2] To whom reprint requests should be sent: Wayne A. Marasco, M.D., Ph.D., Division of Infectious Diseases, Brigham and Women's Hospital, 75 Francis St., Boston, Mass 02115.

17), and that PT (17–21), and to a lesser extent CT (22–24), are potent inhibitors of cell activation via these receptors, presumably because of inhibition of G protein-dependent activation of phospholipase C (25–27). Similarly, other experiments performed with both human (28) and rabbit (29) neutrophils have shown that monovalent cations, in particular Na^+, decrease the affinity of the formyl peptide receptors for agonists. In addition, sodium and other monovalent cations have been shown to inhibit, and potassium to increase, the ability of the neutrophils to exhibit both respiratory and secretory responses to FMLP (30) as well as migration toward lower concentrations of chemotactic peptide. The mechanism is unknown but is clearly not related to membrane potential (28, 29). Although sodium, like guanine nucleotides, decreases the affinity of the receptor for agonists, different mechanisms and sites of action might be involved. For example, sodium may act directly on the receptor itself to effect affinity changes (31). Sodium may also exert an effect upon a guanine nucleotide regulatory protein (32, 33), which might in turn influence the relationships between the regulatory protein and the formyl peptide receptor and between the regulatory protein and phospholipase C.

The present investigations use monovalent cations, PT and CT, and guanine nucleotides to examine alterations in receptor dissociation rates and were designed to delineate the mechanisms by which formyl peptide receptors are regulated. In this report we show that 1) monovalent cations alter [^{3}H]FMLP binding by a mechanism distinct from the G protein-mediated action of GTP and PT;[3] 2) rapidly dissociating receptors correspond to low affinity guanine nucleotide-insensitive receptors; and 3) slowly dissociating receptors are heterogenous and represent two states of the receptor: a state sensitive to GTP, PT and, to a lesser extent CT, corresponding to GTP-sensitive high affinity receptors, and a guanine nucleotide-insensitive state, associated with the new dissociation rate that follows GTP and PT treatments.

MATERIALS AND METHODS

Oyster glycogen (type II), diisopropylfluorophosphate, FMLP, HEPES, GTP, GDP, GMP, cGMP, ATP, AppNHp, CT, and DTT were from Sigma Chemical Co., St. Louis, MO. [^{3}H]FMLP (40 to 60 Ci/mmol) was obtained from Du Pont NEN Research Products, Boston, Mass. PT was from either List Biochemicals, Irvine, California or Drs. C. N. Shih and Larry Winberry of the Michigan Department of Public Health.

Preparation of crude sonicate and purified neutrophil plasma membranes. Rabbit neutrophils were harvested 4 to 16 h after peritoneal injection of 0.1% oyster glycogen. The cells were washed twice in HBSS, 10 mM HEPES, and 0.3 mM EDTA (pH 7.2), and resuspended at 1×10^8 cells/ml in 12.1% sucrose (w/v) with HBSS, including 10 mM HEPES and 1 mM DTT (pH 7.2). They were then treated with diisopropylfluorophosphate (1.5×10^{-3} M) for 30 min at 4°C, divided into 20-ml aliquots, and sonicated on ice in 4- × 15-s 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) with the use of an SW28 rotor in a Beckman L5 ultracentrifuge (27,000 rpm × 120 min). These fractions have been extensively characterized (17, 34). Plasma membranes are found primarily at the 22/54% interface. Each of the bands was collected and washed in 12.5 mM Tris-HCl, 150 μM EGTA, pH 7.4, assayed for protein by using a Bio-Rad protein assay kit (35) and either used immediately or frozen at −70°C. Membranes were stable at −70° for at least 1 mo. The amount of specific [^{3}H]FMLP binding varied at most fivefold between separate membrane preparations.

Detection of endogenous nucleotide triphosphates in neutrophil plasma membranes. To determine if endogenous nucleotide triphosphates could be detected in the membrane preparations, a coupled bioluminescence assay was used (36). Nucleotides were extracted from 1 mg each of three separate neutrophil plasma membrane preparations in a final volume of 250 μl, and GTP was separated from other nucleotides that might have been present by thin layer chromatography as described by Goodrich (36). The subsequent eluate was diluted to 900 μl for transfer of γ-phosphates to ADP by using nucleotide diphosphate kinase. The final 900-μl volume contained: 5 mM Tris-HCl, pH 7.8; 0.1 mM EDTA; 10 mM magnesium acetate; 0.125 μM ADP; and 0.01 mg/ml nucleotide diphosphate kinase, in addition to the nucleotide extract. After incubation for 30 min at room temperature, 100 μl of a luciferin-luciferase solution containing 50 mM Tris-HCl, pH 7.8; 5 mM magnesium acetate; 0.17 mM EDTA; 5 mM DTT; 0.1% BSA; 0.04 mg/ml D-luciferin; and 0.14 mg/ml luciferase were added. Fluorescence was measured after 15 s in a Chrono-log Whole Blood Aggregomete (Chrono-Log Corp. Havertown, PA). The results were then compared to an ATP standard curve, after correcting for background fluorescence by using buffer controls. The total amount of endogenous GTP was 2.6×10^{-10} mol/mg, which corresponds to 5.0×10^{-8} M in each reaction vessel in the [^{3}H]FMLP-binding experiments. This concentration of GTP has little or no effect on [^{3}H]FMLP binding (Figs. 1 and 2), and thus it is unlikely that it produced any appreciable changes in the dissociation kinetics described here.

PT and CT treatment of viable rabbit neutrophils. Rabbit neutrophils, obtained as described, were suspended at 5×10^6 cells/ml in HBSS, including 0.1% dextrose and 1.7 mM Ca^{2+}. The cells were treated with PT (0.3 to 1 μg/ml) or CT (1 μg/ml) for 90 min at 37°C. PT concentrations were varied depending upon the purity of the individual preparations. Similar results were obtained with all preparations of PT. Under these conditions, PT completely inhibits neutrophil granule enzyme release stimulated by FMLP (17). The cells were washed once and then used to prepare plasma membranes. Control cells were subjected to similar conditions in the absence of toxin. Cell viability, determined by trypan blue exclusion, was ≧95%.

Measurement of [^{3}H]FMLP binding to neutrophil plasma membranes. All binding studies were performed at 24°C in 50 mM Tris, 5 mM $MgCl_2$, and 150 mM of either NaCl, KCl, LiCl, or ChCl at pH 7.3, after thermal equilibration for 15 min. From 5 to 15 μg of

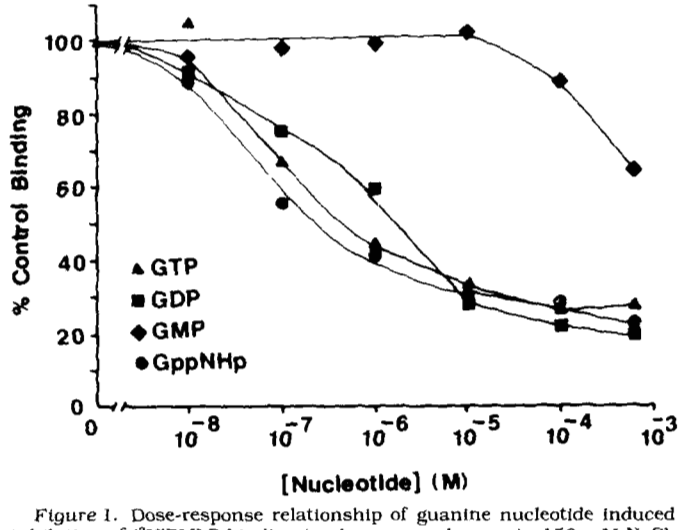

Figure 1. Dose-response relationship of guanine nucleotide induced inhibition of [^{3}H]FMLP binding to plasma membranes in 150 mM NaCl. Guanine nucleotide inhibition of specific [^{3}H]FMLP binding to rabbit neutrophil plasma membranes. Membranes were incubated with GTP, GDP, GMP or GppNHp (10^{-8} M to 8×10^{-4} M) for 30 min as described, before binding 1 nM [^{3}H]FMLP for 30 min. Nonspecific binding was determined by using 1 μM FMLP and was subtracted from total binding to obtain specific binding. Results are expressed as percent of specific [^{3}H]FMLP binding to buffer-treated control membranes. Data points were determined in triplicate.

[3] Abbreviations used in this paper: PT, pertussis toxin; Ns, the stimulatory, guanine-nucleotide binding, regulatory component of adenylate cyclase; Ni, the inhibitory, guanine-nucleotide-binding, regulatory component of adenylate cyclase; GppNHp, guanosine 5′-(β,γ-imino) triphosphate; GTPγS, guanosine 5′-(3-0-thio) triphosphate; DTT, dithiothreitol; K_D, equilibrium dissociation constant; CT, cholera toxin; AppNHp, 5′-adenylylimido-diphosphate.

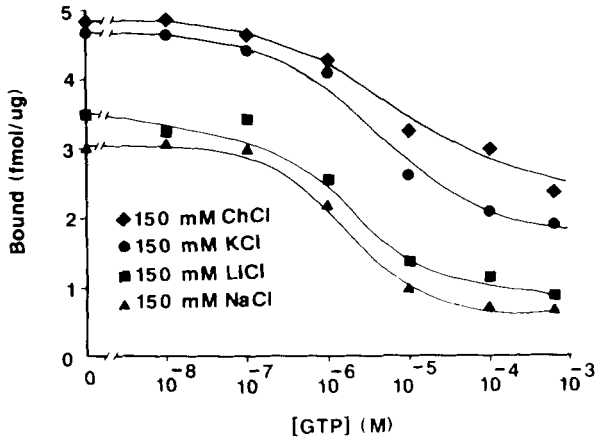

Figure 2. Dose-response relationship of GTP-induced inhibition of [^{3}H]FMLP binding to plasma membranes in the presence of various monovalent cations. Neutrophil membranes were incubated with GTP (10^{-8} M to 8×10^{-4} M) for 30 min in 150 mM Na^+, Li^+, K^+, or Ch^+ buffer as described, before binding 1 nM [^{3}H]FMLP for 30 min. Nonspecific binding was determined by using 1 μM FMLP and was subtracted from total binding. Results are expressed as specific [^{3}H]FMLP binding (fmol/μg membrane protein). Data points were determined in triplicate. The amount of maximal inhibition was similar irregardless of what cation was present and was 2.36, 2.77, 2.62, and 2.45 fmol/μg for 150 mM NaCl, KCl, LiCl and ChCl, respectively.

neutrophil plasma membranes were incubated with 1.0 nM fMet-Leu-[^{3}H]Phe in 12- × 75-mm siliconized glass test tubes for 30 min after a 30-min preincubation with either guanine or adenine nucleotides or buffer. Binding was terminated by the addition of 4 ml of ice-cold buffer to the tube, vortexing, and rapid filtration through Whatman GF/C glass-fiber filters (12). The filters were washed with an additional 6 ml of ice-cold buffer, dried and quantified for membrane-bound radioactivity by liquid scintillation counting. Measurement of [^{3}H]FMLP dissociation from its receptor was as follows: Binding was allowed to reach equilibrium, and 5 ml of 24°C binding buffer with either 150 mM KCl or 150 mM NaCl, with or without 100 μM GTP, or with 10^{-5} M FMLP was then added. These conditions approximate infinite dilution, so that significant re-binding does not occur (2). Association effects are thus not a component of the dissociation data presented here. Dissociation was allowed to proceed for 0 to 15 min and was terminated by rapid filtration followed by three 2-ml washes of ice-cold binding buffer containing either 150 mM KCl or 150 mM NaCl. 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 paper, "specific binding" refers to total binding minus nonspecific binding. All data points were determined in duplicate or triplicate, and the SEM was consistently less than ± 5%. Binding data used to determine equilibrium dissociation rate constants and the percentage of slowly and rapidly dissociating receptors were analyzed by using the NONLIN computer program as previously described (2).

RESULTS

Inhibition of [^{3}H]FMLP Binding to Neutrophil Plasma Membranes by Guanine Nucleotides

Analysis of receptor binding at a concentration of 1 nM [^{3}H]FMLP will reflect, with most sensitivity, changes in binding to the high affinity receptor state that we (2, 3) and others (4–6) have previously reported. Preincubation of neutrophil plasma membranes with guanine nucleotides diminished equilibrium binding of 1 nM [^{3}H]FMLP. The decrement in binding depended upon the dose of guanine nucleotide added. These showed a rank order of potency of GTP ≈ GppNHp ≈ GDP ≫ GMP (Fig. 1). This rank order of binding was attributed directly to an effect of their occupancy of the guanine nucleotide-binding site on the "Ni-like" protein, since this paralleled directly the rank order of inhibition of [^{35}S]GTPγ binding to the G-protein (data not shown) (37). In addition, inhibition of [^{3}H]FMLP binding was specific to guanine nucleotides; a wide range of adenine nucleotides were without effect (Table I).

A large portion of the high affinity receptor state was sensitive to guanine nucleotide additions since we observed greater than 70% inhibition of 1 nM [^{3}H]FMLP binding at high concentrations of guanine nucleotides (Fig. 1; Table I). However, in none of our experiments did we observe complete inhibition of 1 nM [^{3}H]FMLP binding by guanine nucleotides. This guanine nucleotide-insensitive high affinity binding was not caused by entrapment and/or resealing of membranes because greater than 95% of the [^{3}H]FMLP binding was reversed by 10 μM unlabeled FMLP when in the same experiment 100 μM GTP reversed binding by only 77% (data not shown).

Regulation of [^{3}H]FMLP Binding by Monovalent Cations and Their Lack of Effect on Inhibition of Binding by Guanine Nucleotides.

Substitution of 150 mM NaCl with 150 mM KCl, 150 mM LiCl or 150 mM ChCl resulted in increased binding of 1 nM [^{3}H]FMLP to neutrophil plasma membranes (Fig. 2). Under conditions of both low receptor occupancy (high affinity binding) and where ionic concentration was maintained constant, substitution of Na^+ with Ch^+, K^+ or Li^+ resulted in increased binding with $Ch^+ > K^+ > Li^+$. To determine whether the increased binding seen when other monovalent cations were substituted for NaCl was caused by changes in receptor coupling to a guanine nucleotide-binding protein, we measured the guanine nucleotide inhibition of [^{3}H]FMLP binding in the presence of various monovalent cations. Inhibition by guanine nucleotides was evident in the presence of all four monovalent cations tested (Fig. 2; Table I). The maximal inhibition by GTP (100 μM) in the presence of 150 mM NaCl, KCl, LiCl, or ChCl was not statistically different, and in a representative experiment equaled in NaCl (2.10 ± 0.07 fmol/μg); KCl, (1.97 ± 0.34 fmol/μg); LiCl, (2.04 ± 0.18 fmol/μg); and ChCl, (1.61 ± 0.23 fmol/μg). These results indicate that substitution of NaCl with KCl, LiCl, or ChCl does not impair or substantially alter the ability of guanine nucleotide to inhibit [^{3}H]FMLP binding and imply that receptor interactions with guanine nucleotide-binding proteins are unchanged by substituting various monovalent cations for Na^+. These results also demonstrate that the effects on [^{3}H]FMLP binding by monovalent cations are independent of the effects on binding by guanine nucleotides.

Dissociation of 1 nM [^{3}H]FMLP from Neutrophil Plasma Membranes

Effect of sodium and potassium buffers. To further characterize the nature of formyl peptide receptor regulation by monovalent cations, we examined the dissociation of [^{3}H]FMLP from its receptor on neutrophil plasma membranes in the presence of both 150 mM NaCl and 150 mM KCl. [^{3}H]FMLP (1 nM) dissociates from its receptor on neutrophil plasma membranes heterogeneously, with two statistically significant off rates, in both 150 mM NaCl and 150 mM KCl (Table II, Fig. 3*A* and *B*). Substitution of NaCl with KCl increased the proportion of rapidly dissociating receptors from 18.2 ± 2.7 to 31.2 ± 3.0% ($p < 0.02$) (Table II). Although the dissociation

TABLE I

Guanine nucleotide inhibition of specific [^{3}H]FMLP binding to neutrophil plasma membranes in the presence of various monovalent cations[a]

Nucleotide	NaCl %I ± SEM	KCl %I ± SEM	LiCl %I ± SEM	ChCl %I ± SEM
GppNHp	69.8 ± 1.9 (4)[b]	36.0 ± 4.9 (4)	44.8 ± 6.5 (2)	33.2 ± 9.8 (3)
GTP	72.7 ± 1.7 (4)	41.1 ± 7.5 (4)	58.3 ± 5.3 (3)	33.6 ± 4.6 (4)
GDP	68.9 ± 4.4 (4)	34.7 ± 4.4 (4)	43.2 ± 6.9 (2)	20.6 ± 3.9 (3)
GMP	8.6 ± 1.1 (3)	3.0 ± 5.1 (3)	+2.0 ± 8.5 (2)	4.8 ± 2.9 (3)
cGMP	+7.8 ± 5.1 (2)	+1.4 ± 4.0 (2)		
AppNHp	5.6 ± 1.3 (2)	6.0 ± 1.8 (2)		
ATP	+2.4 ± 5.0 (2)	4.5 ± 0.9 (2)		
ADP	4.7 ± 5.3 (2)	9.4 ± 1.2 (2)		
AMP	+6.3 ± 2.3 (2)	8.3 ± 0.3 (2)		
cAMP	+7.4 ± 1.5 (2)	10.3 ± 3.4 (2)		
dBcAMP	+11.2 ± 3.6 (2)	10.2 ± 2.9 (2)		

[a] Rabbit neutrophil membranes were preincubated with 10^{-4} M nucleotide at 25°C for 30 min. 1 nM [^{3}H]FMLP was then allowed to bind for 30 min with the nucleotides still present. Nonspecific binding was determined by using 1 μM FMLP and was subtracted from total binding. Results are expressed as percent inhibition of specific 1 nM [^{3}H]FMLP binding to membranes preincubated with buffer containing 150 mM of the indicated monovalent cations. Numbers in parentheses show the number of experiments. The amount of control binding in a representative experiment was: NaCl (2.88 fmol/μg); KCl (4.82 fmol/μg); LiCl (3.49 fmol/μg); ChCl (4.82 fmol/μg); dBcAMP, Dibutyryl cyclic AMP.

TABLE II

Dissociation rate constants for 1 nM [^{3}H]FMLP dissociation from neutrophil plasma membranes[a]

Sample	Rapid Dissociation k_1 (min-1)	Slow Dissociation k_2 (10^{-2} min-1)	k_1 (%)[××]
150 mM KCl			
Untreated	2.68 ± 0.29[++]	3.68 ± 0.36[++]	31.2 ± 3.0[••] (6)[•]
Plus 100 uM GTP	3.60 ± 0.70[+]	7.08 ± 1.50[•]	59.7 ± 4.4[×] (3)
Plus PT treated	2.89 ± 0.27[+]	6.24 ± 0.96[•]	67.7 ± 9.5[×] (3)
Plus CT treated	2.05 ± 0.55[+]	3.50 ± 1.35[+]	43.6 ± 2.0[o] (3)
PT treated plus 100 uM GTP	2.72 ± 0.22[+]	6.42 ± 0.66[×]	85.4 ± 5.8[×] (2)
150 mM NaCl			
Untreated	5.36 ± 2.13	4.88 ± 0.50	18.2 ± 2.7 (4)
Plus 100 uM GTP	6.72 ± 2.04[+]	8.70 ± 0.36[×]	48.3 ± 6.9[×] (2)
Plus PT treated	6.60 ± 2.46[+]	5.17 ± 1.12[+]	46.8 ± 3.7[×] (3)
PT treated plus 100 mM GTP	7.14 ± 3.24[+]	10.0 ± 1.80[•]	64.7 ± 5.4[×] (2)

[a] Rabbit neutrophil plasma membranes, prepared from toxin- or buffer-treated cells, were preincubated with 100 μM GTP or buffer, bound for 30 min with 1 nM [^{3}H]FMLP and then allowed to dissociate for 0 to 15 min as described. Dissociation plots were analyzed by using the NONLIN program. In all experiments, two dissociation rate models were statistically justifiable using the F-ratio test ($p < 0.05$). Dissociation rate constants and % rapidly dissociating receptors of GTP- and toxin-treated groups were compared to untreated control groups for the appropriate cation by using Student's t-test ($x = p < 0.01$; $• = p < 0.02$; $o = p < 0.05$; + = not significant). Data points were determined in triplicate and were corrected for nonspecific binding. All numbers are mean ± SEM. KCl control values were also compared to NaCl control values by using Student's t-test to ascertain the effect of replacement of NaCl with KCl ($•• = p < 0.02$; $++$ = not significant).

[•] Numbers in parentheses indicate the number of separate experiments.

[××] K_1 (%) is the percent of binding sites that are rapidly dissociating (amount bound to rapidly dissociating sites divided by the total amount bound).

TABLE III

Amount of rapidly and slowly dissociating forms of the formyl-peptide receptor[a]

Cation	Amount of [^{3}H]FMLP Bound		
	Total (fmol/μg)	Rapid dissociation (fmol/μg)	Slow dissociation (fmol/μg)
150 mM NaCl	0.57 ± 0.08	0.10 ± 0.02	0.47 ± 0.06
150 mM KCl	0.86 ± 0.06[o]	0.27 ± 0.03[×] (59)[•]	0.59 ± 0.04[+] (41)

[a] Rabbit neutrophil plasma membranes were incubated with 1 nM [^{3}H]FMLP as described and then allowed to dissociate for 0 to 15 min. Dissociation plots were analyzed by using the NONLIN program. Binding in NaCl and KCl was compared by using Student's t-test ($x = p < 0.01$; $o = p < 0.05$; + = not significant). The amount of rapidly and slowly dissociating receptors was determined by multiplying the total amount of 1 nM [^{3}H]FMLP specifically bound by the fraction of rapidly dissociating receptors (x) and by $1 - x$, respectively, for each experiment. Data are mean fmol/μg ± SEM for both NaCl (four experiments) and KCl (six experiments), and are from two separate membrane preparations that showed similar binding capacities.

[•] The numbers in parentheses are the percentage of the total increase in binding (0.29 fmol/μg) accounted for by the rapidly and slowly dissociating receptors.

rate constants for the rapidly and slowly dissociating forms of the receptor decreased by 50 and 25%, respectively, in the 150 mM KCl buffer, this decrease was not statistically significant (Table II). Examination of the amount of rapidly and slowly dissociating forms of the receptor in 150 mM NaCl and 150 mM KCl revealed that the increased binding seen in the presence of KCl relative to NaCl was caused primarily by increased binding to the rapidly dissociating form of the receptor (Table III). The statistically significant increase in binding to the rapidly dissociating receptors accounted for 59% of the total increase in binding under conditions (low receptor occupancy) that favored detection of changes in the number of slowly dissociating (high affinity) receptors relative to changes in rapidly dissociating (low affinity) receptors. The increase in binding to the slowly dissociating receptors was not statistically significant.

Effect of GTP and bacterial toxins. To further examine the nature of formyl peptide receptor interaction with the guanine nucleotide-binding proteins, we examined the effect of GTP and PT or CT on the dissociation of [^{3}H]FMLP in the presence of both 150 mM NaCl and 150 mM KCl buffers (Figs. 3*A* and 3*B*, respectively). Both 100 μM GTP pretreatment of neutrophil membranes and preparation of membranes from cells treated with PT promoted a statistically significant increase in the proportion of receptors in the rapidly dissociating state (Table II). Preincubation with 100 μM GTP of membranes prepared from PT-treated cells resulted in a further increase in the proportion of rapidly dissociating receptors, and in the KCl buffer, ≈85% of the receptors were rapidly dissociating (Table II). The increase in the percentage of rapidly dissociating receptors was caused by an absolute decrease in the amount of binding to the slowly dissociating receptors, whereas binding to the rapidly dissociating receptors showed no consistent change under these assay conditions (Table IV). These effects were similar both in the presence of 150 mM NaCl and 150 mM KCl (Tables II to IV). Membranes prepared from CT-treated cells showed a small though statistically significant increase in the

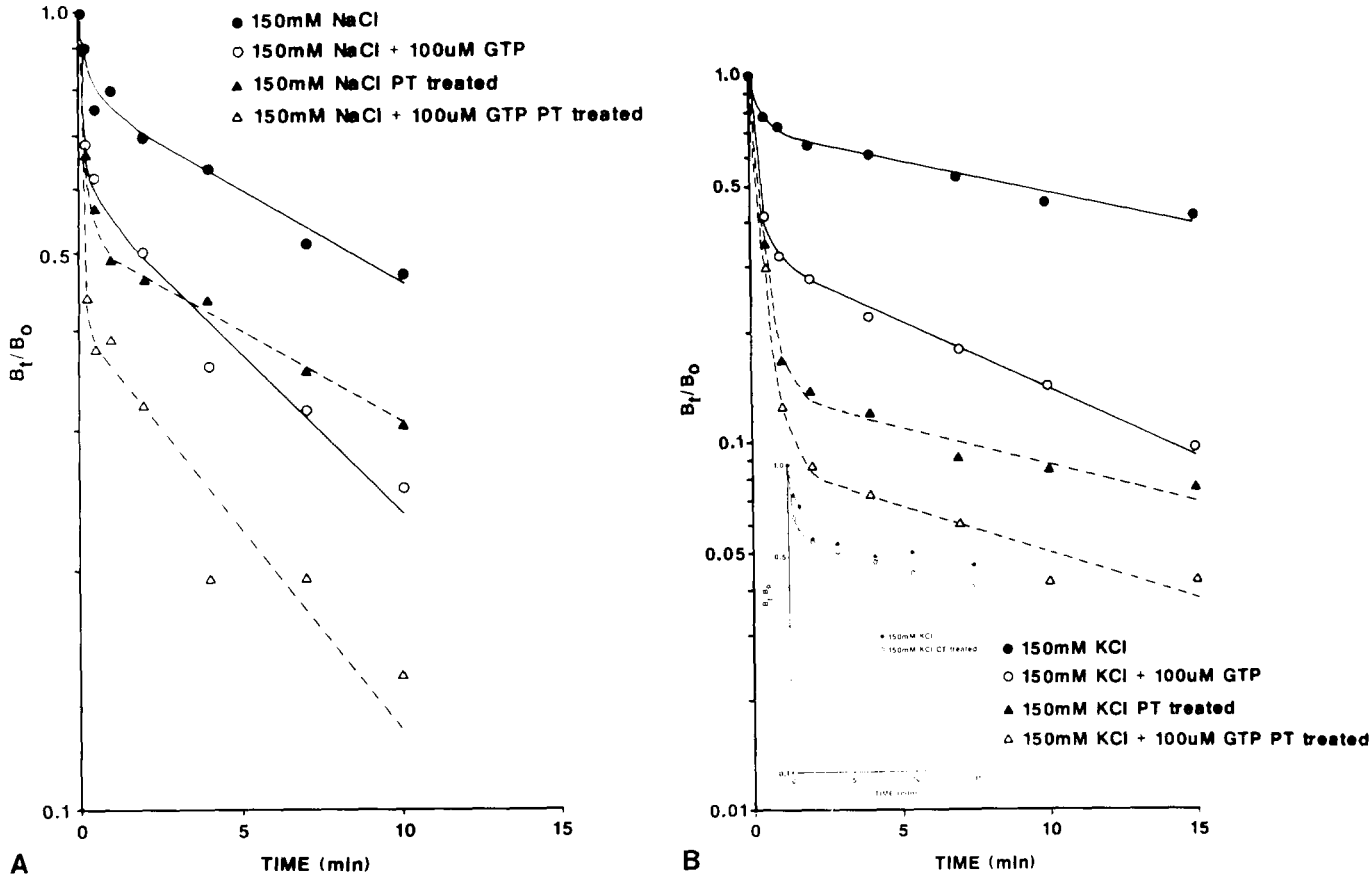

Figure 3. Effect of GTP, PT, and CT on dissociation of 1 nM [^{3}H]FMLP from neutrophil plasma membranes. Neutrophil plasma membranes that had been prepared from cells incubated with buffer, PT or CT, were preincubated with 100 μM GTP or buffer and then equilibrated with 1 nM [^{3}H] FMLP for 30 min at 25°C with either 150 mM NaCl or 150 mM KCl. Dissociation was from 0 to 15 min. Single, representative experiments are shown. Data points were determined in triplicate and have been corrected for nonspecific binding (determined at T = 0 with 1 μM unlabeled fMLP). *A*, effect of 100 μM GTP and PT pretreatment on dissociation of 1 nM [^{3}H]FMLP from neutrophil membranes in 150 mM NaCl. PT-treated (*triangles*) or buffer-treated control (*circles*) membranes were preincubated in either 150 mM NaCl (*solid symbols*) or 100 μM GTP + 150 mM NaCl (*open symbols*) before 1 nM [^{3}H]FMLP binding and dissociation (as described). *B*, effect of 100 μM GTP and PT pretreatment on dissociation of 1 nM [^{3}H]FMLP from neutrophil membranes in 150 mM KCl. PT-treated (*triangles*) or buffer-treated control (*circles*) membranes were preincubated with 150 mM KCl (*solid symbols*) or 100 μM GTP + 150 mM KCl (*open symbols*) before 1 nM [^{3}H]FMLP binding and dissociation (as described). The inset shows the relative lack of effect of CT pretreatment (*open circles*) on dissociation of 1 nM [^{3}H]FMLP in 150 mM KCl compared to 150 mM KCl buffer control (*closed circles*).

proportion of rapidly dissociating receptors (Table II and Fig. 3*B*, *Insert*). A corresponding, small decrease in the amount of binding to the slowly dissociating receptors was observed in membranes prepared from CT-treated cells (Table IV).

In 150 mM KCl buffer, GTP and PT, but not CT, also increased the rate of dissociation of [^{3}H]FMLP from the slowly dissociating form of the receptor (Table II), suggesting the existence of a dissociation state previously unrecognized. In 150 mM NaCl, GTP had a similar effect, although PT, which showed a large amount of variation in this parameter, did not. In both 150 mM KCl and 150 mM NaCl, GTP pretreatment of membranes prepared from PT-treated cells increased the rate of dissociation from the slowly dissociating form of the receptor. The new rate of dissociation of [^{3}H]FMLP from the slowly dissociating receptors detected after GTP pretreatment of membranes prepared from PT-treated cells was similar to the new rate seen after GTP or PT treatment alone.

DISCUSSION

Further evidence of the Ni-like character of the G Protein coupled to the formyl peptide receptor. G proteins resembling both Ns (24) and Ni (14, 17–21) are found in the neutrophil. The Ni-like G protein has been labeled Ni, Gn (38), Nc (39) and has been shown to be immunochemically distinct from Ni (40). Both PT and, to a lesser extent, CT treatment of cells results in inhibition of several FMLP-mediated biologic activities. It has been controversial as to whether only the Ni-like or perhaps both of the G regulatory proteins are involved either directly or indirectly in the activation process. Here we show that the PT-sensitive G protein is primarily involved in receptor coupling. However, we also show that CT pretreatment diminishes slightly [^{3}H]FMLP binding to slowly dissociating receptors. The change in the rate of dissociation from the slowly dissociating receptors, as is seen after PT or GTP treatment, is not observed (Table II).

The mechanism of this CT effect is not established, however, several possibilities exist. First, CT may ADP ribosylate an "Ns-like" substrate which is coupled to a small fraction of formyl peptide receptors (41). This would explain our finding that GTP treatment of membranes prepared from PT-treated cells produced an additional decrease in binding (Table II) to the slowly dissociating receptors. This would occur if not all of the receptor associated G proteins serve as PT substrates. Second, CT may ADP ribosylate the Ni-like protein itself (39), producing a different effect than does PT, perhaps

TABLE IV

Effect of GTP and bacterial toxins on the amount of rapidly and slowly dissociating forms of the formyl-peptide receptor[a]

	Amount of [^{3}H]FMLP Bound		
	Total (fmol/μg)	Rapid dissociation (fmol/μg)	Slow dissociation (fmol/μg)
Experiment 1			
150 mM NaCl			
Untreated	0.393 ± 0.012	0.082 ± 0.015	0.311 ± 0.057
Plus 100 μM GTP	0.148 ± 0.013$^{\times\times}$	0.061 ± 0.006^{+}	0.087 ± 0.008$^{\times}$
Plus PT treated	0.167 ± 0.011$^{\times\times}$	0.080 ± 0.001^{+}	0.087 ± 0.002$^{\times}$
PT treated			
Plus 100 μM GTP	0.098 ± 0.010$^{\times\times}$	0.058 ± 0.003^{+}	0.040 ± 0.002$^{\times}$
Experiment 2			
150 mM KCl			
Untreated	0.770 ± 0.034$^{\times\times}$	0.228 ± 0.037$^{\times}$	0.542 ± 0.087†
Plus 100 μM GTP	0.346 ± 0.069$^{\times}$	0.205 ± 0.011^{+}	0.141 ± 0.007$^{\times}$
PT treated	0.438 ± 0.020$^{\times\times}$	0.241 ± 0.014^{+}	0.197 ± 0.011$^{\times}$
CT treated	0.60 ± 0.075°	0.24 ± 0.030^{+}	0.36 ± 0.045°

[a] Rabbit neutrophil plasma membranes were incubated with 1 nM [^{3}H]FMLP as described and allowed to dissociate for 0 to 15 min. Dissociation plots were analyzed using the NONLIN program. Because of variability in the amount of binding between different membrane preparations, single representative experiments are shown. Data are mean fmol/μg ± SD for triplicate determinations. SD for rapidly dissociating receptors are given by the NONLIN program. Standard deviations for total and slowly dissociating receptors are calculated from standard deviations of rapidly dissociating receptors. Data for GTP- and toxin-treated groups were compared to untreated control groups for the appropriate cation using Student's *t*-test. KCl control values were also compared to NaCl control values using Student's *t*-test. ($+$ = not significant, $\circ = p < 0.05$; $\dagger = p < 0.02$; $\times = p < 0.01$; $\times\times = p < 0.001$).

because of a different ribosylation site. Third, CT may modify the Ni-like protein indirectly through a rise in intracellular cAMP (17, 41).

Insight into the role of Na^+ and GTP in signal transduction through the formyl peptide receptor. Replacement of Na^+ with K^+, Ch^+, or Li^+ resulted in increased binding of 1 nM [^{3}H]FMLP to its receptors (Table I; Fig. 2). The dissociation experiments demonstrate that 59% of the additional binding in the presence of K^+ relative to Na^+ is to the rapidly dissociating form of the receptor (Table III). Cation substitution thus alters [^{3}H]FMLP binding in a manner distinct from GTP and PT, which diminish the amount of binding only to the slowly dissociating receptors. These data suggest that monovalent cations change receptor binding characteristics by a direct effect on the receptor itself, rather than indirectly by a nonspecific membrane effect or through changes in a receptor coupling to a guanine nucleotide-binding protein. Sodium has been shown to decrease the affinity of both the high and low affinity states of the platelet α-adrenergic receptor raising the possibility that sodium may act directly on the α-adrenergic receptor as well (32).

The additional [^{3}H]FMLP binding observed in the K^+ buffer may reflect changes in formyl peptide receptor affinity, number, or both. Previous studies (28, 29) have produced somewhat conflicting results. De Togni et al. (28) have shown that human neutrophils suspended in buffers lacking Na^+ and containing physiologic K^+ exhibit an approximately 50% increase in the number and a threefold increase in the affinity of their formyl peptide receptors, whereas Zigmond et al. (29) have shown with both intact and broken rabbit neutrophils that receptor affinity, but not number, is increased 3- to 10-fold when sodium ions are removed. In our studies (Table II), the dissociation rate constant for the rapidly and slowly dissociating forms of the receptor increased by 50 and 25%, respectively, in the 150 mM NaCl buffer, although this increase was not statistically significant. Based on our findings (Table III), the observation that lower concentrations of FMLP are required to produce a given functional response in a K^+ vs a Na^+ buffer (28–30) may result from binding to a greater number of rapidly dissociating, low affinity receptors since agonist occupancy of unoccupied receptors appears to promote formyl peptide receptor/G-protein coupling (D. E. Feltner, R. Craig, and W. A. Marasco, unpublished results) as has been suggested for adrenergic receptors (42, 43).

Evidence for multiple states of the formyl peptide receptor. Our finding that neither the high concentration of PT (1 μg/ml), a concentration that maximally inhibits FMLP-induced neutrophil enzyme release and chemotaxis (17, 20), nor a maximally inhibitory concentration of GTP (100 μM) (Fig. 3*A* and *B*; Table II), nor these two agents combined, convert all of the slowly dissociating receptors to the rapidly dissociating form suggests that a second class of slowly dissociating receptors, structurally distinct from those coupled to the Ni-like protein and insensitive to guanine nucleotides and PT may exist. In support of these findings, Koo et al. (4) have reported that GTP converts, incompletely, high affinity formyl peptide receptors to the low affinity form in neutrophil membranes. Similarly, Painter et al. (44), using fast microsomes prepared from membrane fractions of both unstimulated and formyl peptide-stimulated human neutrophils, showed that in the presence of 10 μg/ml digitonin 10 μM GTPγS was unable to completely convert all of the slowly dissociating receptors to rapidly dissociating receptors. In contrast, Sklar et al. (10) reported complete conversion of slow to rapidly dissociating formyl peptide receptors in digitonin-permeabilized human neutrophils after GTPγS treatment. The mechanism responsible for the apparent discrepancy between the membrane (4, 44) and cell (10) data is unknown, however, it is interesting to note that the addition of GTPγS to rabbit neutrophil plasma membranes has been reported to solubilize several membrane proteins, including actin and myosin (45). This effect is not mediated by a PT-sensitive guanine nucleotide-binding protein (45). Whether in the permeabilized cell experiments (10) the effects of GTPγS and/or digitonin on cytoskeletal components are responsible for altering the otherwise GTP- and PT-insensitive third receptor state seen in membranes (4, 44) must be considered. A number of previous reports have provided evidence that formyl peptide receptor does interact with the neutrophil cytoskeleton (8, 9, 44, 46).

The enhanced rate of dissociation from the slowly dissociating form of the receptor after GTP and PT treatment provides further evidence for the existence of a third state of the formyl peptide receptor. It seems unlikely that GTP and PT treatments, two independent manipulations of the G protein, would produce, in both K^+ and Na^+ buffers, similar artifactual changes in the slow dissociation rate. Sklar et al. (10) have shown that complex dissociation intermediate in rate between the slowly and rapidly dissociating receptor states occurs in the presence of nonsaturating neutrophil concentrations or incomplete ribosylation. Our studies do not favor this explanation because both saturating concentrations of GTP and conditions favoring complete ribosylations were used (D. E. Feltner, R. Craig, and W. A. Marasco, unpublished results). Our data indicate further that the third receptor state exists even after treatment with both GTP and PT

(Table II).

Possible molecular explanations for the new dissociation rate include 1) an altered form of the slowly dissociating state; 2) an intermediate, transition state in the GTP- and PT-induced conversion from slowly to rapidly dissociating states. This implies that complete receptor conversion requires at least two discrete, discernible events. These events might include GTP- and PT-altered tertiary structure of Ni-like protein, or enzymatic modification of either the Ni-like protein, receptor, or both. Recently, multisite phosphorylation of the α-subunit of transducin by the insulin receptor kinase and protein kinase C has also been demonstrated (47); 3) occupied receptors, which are presumably in equilibrium with the Ni-like protein in untreated membranes, are similarly in an altered equilibrium with the GTP- and PT-modified Ni-like molecule.

Conclusions

Monovalent cations, in particular Na^+, alter receptor affinity by a mechanism distinct from the G protein-mediated action of GTP and PT. The effect of Na^+ appears to be on the receptor itself (because both receptor states exhibit diminished binding) and not caused by alterations in receptor/G protein interaction. Examination of [^{3}H] FMLP dissociation from plasma membranes identifies three receptor states, two of which appear to correspond to those detected in equilibrium-binding studies. Rapidly dissociating receptors are guanine nucleotide insensitive, and represent receptors that are uncoupled from G proteins and/or receptors through which signal transduction has already occurred. Slowly dissociating, high affinity receptors represent two different receptor states that can be differentiated by their dissociation rates and by their sensitivity to guanine nucleotides and bacterial toxins. One slowly dissociating state represents receptors coupled to the Ni-like G protein in the absence of guanine nucleotide and is presumably primed to initiate signal transduction after ligand binding. A second slowly dissociating state, insensitive to guanine nucleotides, becomes apparent after treatment with GTP or PT, and it may represent receptors that have become associated with the cytoskeleton. These receptors, although no longer able to mediate enzyme secretion or superoxide production (9, 10), may still be "active" in mediating chemotaxis, or other neutrophil functions.

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