Guanine Nucleotides Modulate the Binding Affinity of the Oligopeptide Chemoattractant Receptor on Human Polymorphonuclear Leukocytes

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ABSTRACT The oligopeptide chemoattractant receptor on human polymorphonuclear leukocyte (PMN) membranes exists in two affinity states. Since guanine nucleotides regulate the binding affinity and transductional activity of several other types of receptors, we examined the effect of nucleotides on the binding of *N*-formyl-methionyl peptides to their receptors on human PMN membranes. The addition of guanylylimidodiphosphate (0.1 mM), a nonhydrolyzable derivative of guanosine triphosphate (GTP), to PMN membrane preparations reduced the fraction of high-affinity receptors detected in equilibrium binding studies from 21.3±0.13 to 11.8±0.05% ($P < 0.03$), without altering the binding affinities. Since the total number of receptors remained unchanged, the effect of guanylylimidodiphosphate was to convert a portion of the receptors from the high-affinity state to the low-affinity state. At the maximal concentration of guanine nucleotide tested, ~50% of the high-affinity sites were converted to low-affinity sites. The findings obtained by equilibrium binding were supported by kinetic studies since the dissociation of the radiolabeled oligopeptide chemoattractant *N*-formyl-methionyl-leucyl-[^{3}H]phenylalanine from PMN membranes was accelerated in the presence of guanine nucleotide. The effect of guanine nucleotides was reversed upon washing, indicating that affinity conversion is bidirectional. The guanine nucleotide effects were greatest with nonhydrolyzable derivatives of GTP followed by GTP then guanosine diphosphate. Neither guanosine monophosphate nor any adenine nucleotide tested had an effect on receptor binding. These data suggest a role for guanine nucleotides in the regulation of stimulus-receptor coupling of chemoattractant receptors on human PMN.

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INTRODUCTION

Chemoattractant receptors initiate a number of cellular responses in leukocytes including shape changes, directed locomotion, lysosomal enzyme secretion, and superoxide anion production (1). The mechanisms of stimulus-response coupling for chemotactic factor receptors on leukocytes are as yet poorly defined. Development of the tritiated form of the potent oligopeptide chemotactic factor *N*-formyl-methionyl-leucyl-phenylalanine (fMet-Leu-Phe) has provided a useful probe for uncovering such mechanisms. We have recently demonstrated that the binding of fMet-Leu-[^{3}H]Phe to human polymorphonuclear leukocytes (PMN) membrane preparations is heterogeneous and consistent with a model postulating the existence of two classes of the receptor with different affinities for the chemotactic peptide (2). Heterogeneous chemoattractant binding has also been reported in guinea pig macrophages and rabbit PMN (3, 4). The two different affinities of the chemotactic factor receptor in human PMN membranes could represent two discrete populations of receptors or interconvertible affinity states of the same receptor.

Guanine nucleotides have been shown to be important regulators of the activities of several hormone receptors, such as glucagon (5), α- and β-adrenergic (6), muscarinic cholinergic (7), and dopaminergic receptors (8). The receptors for a number of neurotransmitters exist in interconvertible high- and low-affinity states with interconversion regulated by guanine nu-

cleotides. To determine whether the high- and low-affinity forms of the chemotactic factor receptor in human PMN are similarly convertible, we examined the effect of guanine nucleotides on the binding of formylated oligopeptide chemoattractants to human PMN membrane preparations.

METHODS

Chemicals. *N*-Formyl-Met-Leu-[^{3}H]Phe (FML[^{3}H]P)[1] with a specific activity of 47.6 Ci/mmol was purchased from New England Nuclear (Biston, MA). FMet-Leu-Phe (FMLP) was obtained from Sigma Chemical Co. (St. Louis, MO). Guanylylimidodiphosphate (p[NH]ppG) and guanosine-5′-*O*-3-thiotriphosphate (GTPγS) were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Guanosine-5′-triphosphate (GTP), guanosine 5′-diphosphate, guanosine 5′-monophosphate, guanosine 3′:5′monophosphate, adenosine 5′-triphosphate, adenosine-5′ diphosphate, adenosine-5′-monophosphate, and adenosine-3′:5′monophosphate were purchased from Sigma Chemical Co. as was 5-adenylylimidodiphosphate (p[NH]ppA).

Membrane preparations. PMN membrane suspensions were prepared as previously described (2). Briefly, human PMN were isolated from peripheral blood by dextran sedimentation followed by Ficoll-Hypaque gradient centrifugation. The contaminating erythrocytes were removed by three hypotonic lyses and the PMN were disrupted in Tris-HCl buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.7), with a tissue homogenizer (Tekmar Co., Cincinnati, OH). The suspension was centrifuged at 200 *g* for 10 min at 4°C. The supernatant was decanted and centrifuged at 41,000 *g* for 10 min at 4°C. The pellet was washed once with incubation buffer (140 mM NaCl, 1.0 mM KH_2PO_4, 5 mM Na_2HPO_4, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, pH 7.4).

Binding assays. All experiments were performed at 25°C and each determination was performed in duplicate. The mean of the duplicates were used to calculate all data. The standard error of the mean of the replicates did not extend beyond the symbols used to represent the data points on the accompanying figures. The equilibrium binding assay conditions were as previously described (2). The membrane preparations were kept on ice until assayed, at which time the preparations were incubated for 15 min with buffer or p[NH]ppG in order that the temperature of the preparations be equilibrated at 25°C. The membranes were then incubated with the radioligand for 30 min at 25°C. Nonspecific binding was defined as the amount of radioligand bound in the presence of at least 1,000-fold excess of unlabeled ligand. The highest concentration of the radioligand used was 50 nM and thus 50 μM FMLP was the concentration used to establish nonspecific binding. Incubation was stopped by rapid filtration through Whatman GF/C filters (Whatman Laboratory Products, Inc., Clifton, NJ) followed by four 5-ml washes with ice-cold incubation buffer. The filters were then counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, CA).

To see if any FML[^{3}H]P degradation products could either contribute to or interfere with the binding of the native radioligand, several experiments were performed. Supernatants from membrane preparations containing FML[^{3}H]P, incubated under binding conditions, were fractionated by high-pressure liquid chromatography (HLPC) using a μBondapack C_{18} column (Waters Associates, Milford, MA) and methanol/acetic acid (0.05 M) as the solvent. The stock FML[^{3}H]P preparation contained a radioactive peak comprising 85% of the applied counts at a position corresponding to native FMLP. A smaller peak contained ~15% of the counts eluted earlier. Incubation of the FML[^{3}H]P in buffer alone under binding conditions reduced the native peak to 73%. The native FML[^{3}H]P peak contained ~59% of the counts following incubation with membranes and was accompanied by a corresponding increase in the earlier radioactive peak. Supernatants of membranes made from PMN pretreated with 1.0 M diisopropylfluorophosphate and containing the protease inhibitors phenylmethylsulfonyl fluoride (1 mM), soybean trypsin inhibitor (0.25 mg/ml), and α_1-antitrypsin (0.25 mg/ml) (9) contained 61% native ligand. Since some ligand degradation did occur during the binding assay in the presence or absence of protease inhibitors, the degradation products formed were tested for their ability to bind to the membranes and thus compete with the native ligand. Degradation products formed upon incubation of a 10-fold concentration of membranes with fMet-Leu-(^{35}S)Phe (sp act ~ 700 Ci/mmol) were isolated by HPLC and tested for binding activity to PMN membranes. The degradation products, standardized to the same number of counts as the chromatographed native ligand, contained negligible binding activity.

Dissociation of FML[^{3}H]P from human PMN membranes was assayed by first equilibrating the membranes with FML[^{3}H]P. Membranes were incubated at 25°C with FML[^{3}H]P for 30 min, at which time unlabeled FMLP was added to give a final concentration of 10^{-4} M. 150-μl aliquots were removed in duplicates at indicated time intervals thereafter and assayed for bound radioligand.

Computer modeling. The data from saturation binding assays, that is, the amount of radioligand bound vs. amount of radioligand added, were subjected to nonlinear least squares curve fitting using a computer method developed by DeLean et al. (10, 11). This method is based on the law of mass action and allows the analysis of the binding of a radioligand to multiple classes of binding sites. The data was fitted to one- and two-site models successively and a two-site model was accepted only when the fit of the data was significantly improved ($P < 0.01$). The computer analysis yields affinity constants and concentrations for each class of receptors. The dissociation constants reported are the geometric means±SE (10).

RESULTS

Effect of guanine nucleotide on equilibrium binding characteristics. Membranes were preincubated with or without the guanine nucleotide (10^{-4} M) for 15 min at 25°C and then assayed for radioligand binding under equilibrium conditions. Fig. 1 shows that the guanine nucleotide p[NH]ppG reduced the binding of FML[^{3}H]P to PMN membrane preparations. Computer analysis of experiments such as that shown in Fig. 1 revealed that the altered binding isotherm was

[1] *Abbreviations used in this paper:* FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; GTP, guanosine-5′-triphosphate; GTPγS, guanosine-5′-*O*-3-thiotriphosphate; HPLC, high-pressure liquid chromatography; p[NH]ppA, 5-adenylylimidodiphosphate; p[NH]ppG, guanylylimidodiphosphate.

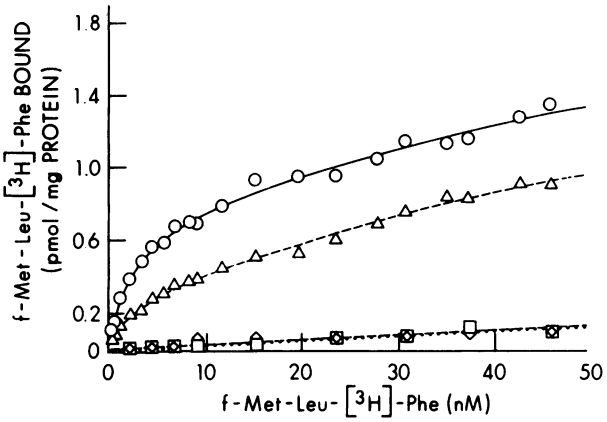

FIGURE 1 FML[^{3}H]P binding isotherms to human PMN membrane preparations. PMN membranes, treated with buffer (○) or 10^{-4} M p[NH]ppG (△) were incubated with varying concentrations of FML[^{3}H]P at 25°C for 30 min. Both isotherms represent two-site fits to the data that were significantly better than one-site fits ($P < 0.01$). (□) and (◇) represent nonspecific binding in the presence of buffer or 10^{-4} M p[NH]ppG, respectively.

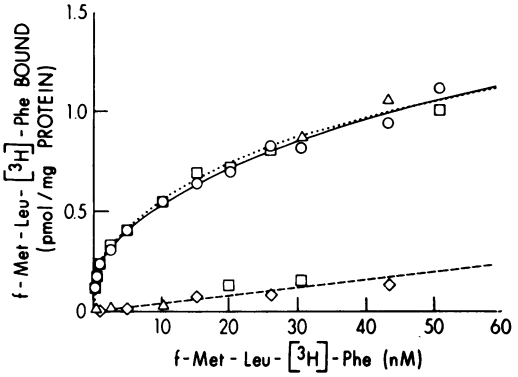

FIGURE 2 FML[^{3}H]P binding isotherms to PMN membrane preparations following removal of guanine nucleotides. PMN membranes were incubated with buffer or p[NH]ppG at 25°C. The membranes were then washed and FML[^{3}H]P binding was assayed. Before removal of p[NH]ppG, binding isotherms similar to Fig. 1 were obtained. Removal of guanine nucleotides restored FML[^{3}H]P binding to control levels. (○) buffer; (△) p[NH]ppG; (□) and (◇) nonspecific binding for buffer and p[NH]ppG-treated membranes, respectively.

due to a decreased number of receptors in the high-affinity state with a concomitant increase in the number of receptors in the low-affinity state. Averaging the results of seven experiments, it was found that in the absence of p[NH]ppG, 21.3±0.13% of the receptors on PMN membranes were in the high-affinity state, but in the presence of 10^{-4} M p[NH]ppG, only 11.8±0.05% of the receptors were in the high-affinity states ($P < 0.03$) while the total number of receptors remained unchanged (0.435±0.07 nM for control membranes vs. 0.425±0.06 nM for p[NH]ppG-treated membranes). All binding isotherms were significantly better fitted to two-site models than one-site models. The equilibrium dissociation constant (K_D) for each of the two affinity states from control and p[NH]ppG-treated membranes were found to be not significantly different by paired t test as well as by the paired Wilcoxon test (0.66 and 34.7 nM for control; 0.62 and 38.4 nM for p[NH]ppG treated). Thus, the effect of p[NH]ppG is to convert receptors originally in the high-affinity state to those in a low-affinity state. p[NH]ppG exerted its effect only when present during the receptor binding assay. Membranes incubated with p[NH]ppG and then washed to remove the nucleotide regained initial binding characteristics. The two binding isotherms were then superimposable (Fig. 2).

When PMN membranes were equilibrated with FML[^{3}H]P in the presence of 10^{-4} M p[NH]ppG, the dissociation of the radioligand was accelerated. In Fig. 3, the filled circles (●) represent the dissociation of FML[^{3}H]P in the absence of p[NH]ppG from membranes equilibrated with the radioligand in the absence of the guanine nucleotide. The open circles (○) represent radioligand dissociation in the presence of p[NH]ppG from membranes equilibrated with the radioligand FMLP, in the presence of the guanine nucleotide. The rate of dissociation of radioligand in the second case was accelerated in comparison with the dissociation rate observed when no guanine nucleotide was added.

A direct demonstration of the effect of p[NH]ppG on FML[^{3}H]P binding is shown in Fig. 4. PMN mem-

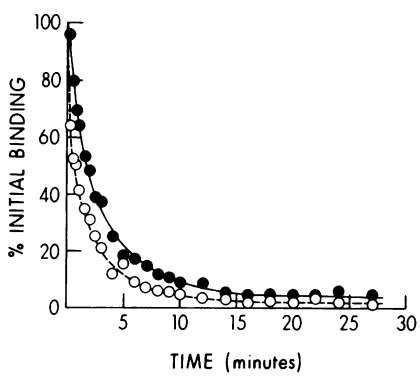

FIGURE 3 Dissociation of FML[^{3}H]P from PMN membrane preparations. FML[^{3}H]P was bound to PMN membrane preparations at 25°C for 30 min in the presence of buffer (●) or 10^{-4} M p[NH]ppG (○). When the mixtures have reached equilibrium (30 min at 25°C), 1,000-fold excess unlabeled FMLP was added to the preparations and the amount of radiolabel remaining bound to the membranes was determined at the indicated times thereafter.

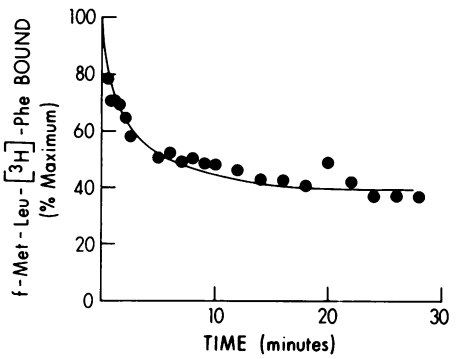

FIGURE 4 Effect of p[NH]ppG on FML[^{3}H]P binding. FML[^{3}H]P (1 nM) was incubated with PMN membranes for 30 min at 25°C and 10^{-4} M p[NH]ppG was added to the equilibrated mixture. Binding was assayed at the indicated times after the addition of the guanine nucleotide as indicated by the arrow.

branes were first equilibrated with 1 nM FML[^{3}H]P at 25°C (30 min), and then p[NH]ppG was added to give a final concentration of 10^{-4} M. Addition of the nucleotide alone without excess unlabeled FMLP resulted in an immediate dissociation of a portion of the bound FML[^{3}H]P to a new equilibrium level.

Dose dependence and specificity of guanine nucleotide effects on FML[^{3}H]P binding. PMN membranes were incubated with varying concentrations of p[NH]ppG and then FML[^{3}H]P binding was assayed using 1 nM FML[^{3}H]P. Fig. 5 shows that the inhibition of radioligand binding is dose dependent. The concentration at which p[NH]ppG produced a half-maximal effect was 10^{-6} M. Similar experiments were performed using GTP and the concentration producing half-maximal inhibition was 5×10^{-6} M.

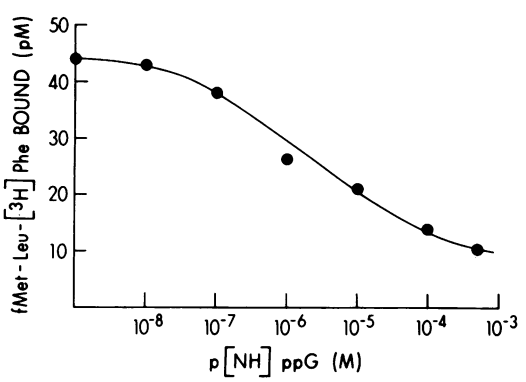

FIGURE 5 FML[^{3}H]P binding to human PMN membrane preparations in the presence of varying concentrations of p[NH]ppG. The membranes were incubated with the indicated concentrations of the nucleotide, then with 1 nM FML[^{3}H]P for 30 min at 25°C, and specific binding was determined.

TABLE I
Effects of Nucleotides on High-Affinity FML[^{3}H]P Binding to Human PMN Membranes

10^{-4} nucleotide*	Inhibition*
	%
GTP	68
p[NH]ppG	81
GTPγS	71
GDP	69
GMP	8
cGMP	0
ATP	0
p[NH]ppA	0
ADP	0
AMP	0
cAMP	0

* PMN membranes were incubated with 10^{-4} nucleotide at 25°C for 15 min and then assayed for FML[^{3}H]P binding in the presence of the nucleotides. Results are expressed as percent inhibition of binding of FML[^{3}H]P to membranes preincubated with buffer alone.

To determine the specificity of the p[NH]ppG effect on FML[^{3}H]P binding, several other nucleotides were tested. Table I shows the percent inhibition of FML[^{3}H]P binding produced by the indicated nucleotides. The adenosine nucleotides: ATP, ADP, AMP, cyclic (c) AMP as well as the nonhydrolyzable analogue p[NH]ppA did not produce any significant inhibition of FML[^{3}H]P binding. The guanine nucleotides GMP and cGMP also had no effect, while GDP, GTP, and its nonhydrolyzable analogues, p[NH]ppG and GTPγS, produced significant inhibition.

DISCUSSION

The binding of FML[^{3}H]P to human PMN membranes is heterogeneous and is compatible with the notion that there are two classes of receptor sites with distinct affinities (2). The two classes of sites could represent two distinct and independent populations of receptors or two interconvertible states of a single population of receptors. The data presented in this report indicate that the two classes of binding sites are at least in part composed of interconvertible states of one population of receptors. The evidence for this conclusion is based on the effects of guanine nucleotides on the binding of FML[^{3}H]P to PMN membranes. In the presence of GTP, GDP, or nonhydrolyzable derivatives of GTP, FML[^{3}H]P binding to PMN membranes was decreased. Analysis of equilibrium studies suggested that the decreased binding could not be accounted for by a decrease in the number of receptors per se. Rather, the

altered binding isotherm was the result of a decrease in the fraction of receptors in the high-affinity state. This decrease in the fraction of high-affinity receptors coupled with no decrease in the total number of receptors suggests that in the presence of guanine nucleotides the reduction of high-affinity receptors is due to the conversion of receptors from the high-affinity state to those in a low-affinity state. Guanine nucleotides also enhanced the rate of dissociation of FML[^{3}H]P from PMN membranes. The effects of the nucleotides were reversible by washing, indicating interconvertibility of the receptor from low to high affinity as well.

Guanine nucleotide regulation of interconvertible receptor affinity states has been demonstrated in a number of instances where receptors are coupled to the enzyme adenylate cyclase (5–8). At the β-adrenergic receptor, guanine nucleotides effect conversion of receptors from high to low affinity and have been shown to regulate the agonist-induced formation of a receptor-guanine nucleotide regulatory protein complex, which is involved in the signal transduction process (6). Guanine nucleotides, at a sufficiently high concentration cause conversion of all the β-adrenergic receptors to a single low-affinity population of receptors. This was not observed for the chemoattractant receptors, where only half of the high-affinity sites could be converted to low-affinity. However, the muscarinic cholinergic receptor, which appears to be regulated by guanine nucleotides, has also been reported not to be completely converted to a homogeneous low-affinity receptor population by high concentrations of p[NH]ppG (7). The possibility exists that for the muscarinic cholinergic and chemotactic receptors, even the high concentration of guanine nucleotides used was not sufficient for the conversion to be completed. Alternatively, a subpopulation of high-affinity receptors may exist, which are independent of guanine nucleotide control. This contention is supported by the finding that FMLP preincubation increases the fraction of guanine nucleotide-insensitive high-affinity binding sites in human PMN membranes (12). It has also been shown that human PMN are not a homogeneous population of cells (13) and this heterogeneity could be reflected in heterogeneous regulation of chemoattractant receptors on the membranes of these cells.

Nonetheless, the widespread occurrence of guanine nucleotide modulation of agonist binding has suggested that this property reflects early activation steps that subsequently result in the observed biological responses. In the β-adrenergic system, the formation of the receptor-guanine nucleotide regulatory protein complex facilitates the activation of the enzyme adenylate cyclase by the nucleotides, while the muscarinic cholinergic receptor has been demonstrated to inhibit the activation of adenylate cyclase (14, 15). Although chemoattractants have been shown to produce an accumulation of intracellular cAMP (16, 17), attempts to demonstrate a direct activation or inhibition of adenylate cyclase by chemoattractants have been unsuccessful (18).

Guanine nucleotide regulatory proteins are not solely involved with modulation of adenylate cyclase activity. Recently, light activation of rhodopsin has been shown to result in a guanine nucleotide-regulated activation of a cGMP phosphodiesterase (19). Thus, guanine nucleotide regulation appears to be involved in linking a variety of receptors to the activation or inhibition of effector enzymes required for the function of these receptors. The data presented here suggest that guanine nucleotide regulatory protein may be required for certain aspects of stimulus-receptor coupling of the chemoattractant receptor. The effector units to which these receptors may be coupled by nucleotide regulatory proteins remain to be determined.

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REFERENCES

1. Snyderman, R., and E. J. Goetzl. 1981. Molecular and cellular mechanisms of leukocyte chemotaxis. *Science (Wash. DC).* 213:830–837.
2. Koo, C., R. J. Lefkowitz, and R. Snyderman. 1982. The oligopeptide chemotactic factor receptor on human polymorphonuclear leukocyte membranes exists in two affinity states. *Biochem. Biophys. Res. Commun.* 106:442–449.
3. Snyderman, R., S. Edge, and M. C. Pike. 1982. Macrophage chemotactic factor receptor exists in two interconvertible affinities modulated by guanine nucleotides. *Clin. Res.* 30:521*a*. (Abstr.)
4. Mackin, W. M., and E. L. Becker. 1982. The formylpeptide chemotactic receptor on rabbit peritoneal neutrophils. *J. Immunol.* 129:1608–1611.
5. Lad, P. M., A. F. Welton, and M. Rodbell. 1977. Evidence for distinct guanine nucleotide sites in the regulation of the glucagon receptor and of adenylate cyclase activity. *J. Biol. Chem.* 252:5942–5946.
6. Stadel, J. M., A. DeLean, and R. J. Lefkowitz. 1982. Molecular mechanisms of coupling in hormone receptor-adenylate cyclase systems. *Adv. Enzymol.* 53:1–43.
7. Burgisser, E., A. DeLean, and R. J. Lefkowitz. 1982. Reciprocal modulation of agonist and antagonist binding to muscarinic cholinergic receptor by guanine nucleotides. *Proc. Natl. Acad. Sci. USA.* 79:1732–1736.
8. DeLean, A., B. F. Kilpatrick, and M. G. Caron. 1982. Dopamine receptor of the porcine anterior pituitary gland. *Mol. Pharmacol.* 22:290–297.
9. Amrein, P. C., and T. P. Stossel. 1980. Prevention of degradation of human polymorphonuclear leukocyte proteins by diisopropylfluorophosphate. *Blood.* 106:442–447.

10. DeLean, A., A. A. Hancock, and R. J. Lefkowitz. 1982. Validation and statistical analysis of computer modeling method for quantitative analysis of radioligand binding data for mixtures of pharmacological receptor subtypes. *Mol. Pharmacol.* 21:5–16.
11. Hancock, A. A., A. DeLean, and R. J. Lefkowitz. 1979. Quantitative resolution of beta adrenergic receptor subtypes by selective ligand binding. Application of a computerized model fitting technique. *Mol. Pharmacol.* 16:1–9.
12. Koo, C., and R. Snyderman. 1983. The oligopeptide chemoattractant receptor on human neutrophils converts to an irreversible high-affinity state subsequent to agonist exposure. *Clin. Res.* 31:491*a*. (Abstr.)
13. Seligmann, B., T. M. Chused, and J. I. Gallin. 1981. Human neutrophil heterogeneity identified using flow microfluorometry to monitor membrane potential. *J. Clin. Invest.* 68:1125–1131.
14. Watanabe, A. M., M. M. McConnaughey, R. A. Strawbridge, J. W. Fleming, L. R. Jones, and H. R. Besch. 1978. Muscarinic cholinergic receptor modulation of β-adrenergic receptor affinity for catecholamines. *J. Biol. Chem.* 253:4833–4836.
15. Birdsall, N. J. M., C. P. Berrie, A. S. V. Burgen, and E. C. Hulme. 1980. Modulation of binding properties of muscarinic receptors: evidence for receptor-effector coupling. *In* Receptors for Neurotransmitters and Peptide Hormones. G. Pepeu, M. S. Kuhar, and S. J. Enna, editors. Raven Press, New York. 107–116.
16. Simchowitz, L., L. C. Fischbein, I. Spilberg, and J. P. Atkinson. 1980. Induction of a transient elevation in intracellular levels of adenosine-3′-5′ cyclic monophosphate by chemotactic factors: an early event in human neutrophil activation. *J. Immunol.* 124:1482–1491.
17. Marx, R. S., C. E. McCall, and D. A. Bass. 1980. Chemotaxin-induced changes in cyclic adenosine monophosphate levels in human neutrophils. *Infect. Immun.* 29:284–288.
18. Verghese, M. W., and R. Snyderman. 1983. Hormonal regulation of adenylate cyclase in macrophage membranes is regulated by guanine nucleotides. *J. Immunol.* 130:869–873.
19. Bennett, N. 1982. Light-induced interactions between rhodopsin and the GTP-binding protein. Relation with phosphodiesterase activation. *Eur. J. Biochem.* 123:133–139.