

Regulation of the Affinity State of the *N*-Formylated Peptide Receptor of Neutrophils: Role of Guanine Nucleotide-binding Proteins and the Cytoskeleton

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Abstract. Previous studies have indicated that the receptor for *N*-formylated peptides present on human neutrophils can exist in several ligand-dissociation states at least one of which is sensitive to guanine nucleotides. Human neutrophil membranes rich in cell surface enzyme markers have been isolated from cells pretreated at 37°C with 5 nM fluoresceinated chemotactic peptide (*N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-fluorescein; FI-peptide) or a buffer control and analyzed for receptor-ligand dissociation states using a previously published fluorescence assay for estimating ligand binding and dissociation rates (Sklar, L. A., et al. 1984. *J. Biol. Chem.* 259:5661-5669). Fractionation of crude microsomes derived from homogenates of unstimulated cells by ultracentrifugation on linear D₂O gradients yielded two plasma membrane-rich fractions termed fast and slow microsomes. Analysis of FI-peptide dissociation rates from receptor present in fast membrane fractions of unstimulated cells yielded data that could be best fit by assuming that the receptor exists in three distinct ligand-dissociation states. The intermediate ligand-dissociation state (state B) accounted for 47% of the total and was converted to the fastest ligand-dissociation state (state A) by incubation of membranes with GTP or GTP- γ -S. The remainder of the receptor (17%) present in unstimulated mem-

branes was in a state from which ligand was virtually nondissociable (state C). This form of the receptor was insensitive to GTP- γ -S. When cells were stimulated with FI-peptide, most of the receptor present in slow and fast membranes was of the state C type. In contrast to unstimulated cells, slow membranes derived from cells exposed to FI-peptide contained the majority of the recoverable receptor indicating that receptor was transferred to a physically isolatable membrane domain after ligand binding to the intact cell. The ligand-induced formation of state C in both fast and slow microsome fractions was inhibited by treatment of cells with dihydrocytochalasin B. However, the drug had no effect on translocation of the receptor to slow membranes. Pertussis toxin treatment of intact cells had no effect on ligand-induced formation of state C in either fraction even though other cellular responses were inhibited. Both slow and fast membranes contained a 41-kD G protein as assayed by immunoblot analysis. The data suggest that ligand induces a segregation of receptor-ligand complexes into a membrane domain in which the receptor is functionally uncoupled from the 41-kD neutrophil G protein. This pathway may serve to remove the receptor from the transductional pathway and hereby terminate the initial cellular response.

THE chemotaxis of human neutrophils toward *N*-formylated oligopeptides (56), such as *N*-formyl-L-methionyl-L-leucyl-L-phenyl-alanine (FMLP),¹ is mediated by a stereospecific surface membrane glycoprotein receptor with an *M_r* of 50-60 kD (1, 4, 20, 24, 38, 40, 45, 46, 50, 57). Within several seconds of binding, peptides of this class initiate a variety of rapid biochemical and cellular responses. These responses include decreases in receptor-ligand dissociation rates (28, 29, 63), receptor downregulation (47, 63,

74, 81), activation of membrane phospholipases (12, 70), activation of protein kinases (15, 25, 58), increases in polymerized actin (11, 15, 23, 53, 77, 78), cell shape changes (13, 41, 65, 79), increases in intracellular free calcium levels (30, 31, 67), and release of various mediators and enzymes including oxidants, arachidonate, and proteases (for reviews, 49, 68, 72). If the concentration of peptide increases over time or space, many of these cellular responses are maintained; if not, the initial response terminates within several minutes by a slower, competing process (49, 59, 66-68, 80) that closely mimics the response behavior of a classic adaptive response exhibited by sensory systems such as vision (18, 36, 39, 61).

In its fastest ligand-dissociation state, the receptor appears

1. *Abbreviations used in this paper:* FI-peptide, fluorescein conjugate of *N*-formyl-L-norleucyl-L-leucyl-L-phenylalanyl-L-norleucyl-L-tyrosyl-L-lysine; FMLP, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; 1 KP, 1,000-g pellet; 1 KS, 1,000-g supernatant; SASD; sulfo-succinimidyl-2-(*p*-azidosalicylamido)ethyl-1,3'-dithio-propionate.

somal pellets were resuspended in 1 ml of resuspension buffer and saved for further analysis. In some experiments the slow microsome concentrate was further fractionated into uncoated and clathrin-coated vesicles by isopycnic ultracentrifugation on a linear gradient of 9% D₂O-2% Ficoll to 90% D₂O-20% Ficoll prepared as described by Pearse (52). After 16 h at 26,000 rpm (80,000 g) in an SW 50.1 rotor, 0.5-ml fractions were collected for further analysis. All assays (see below) were performed the same day.

Receptor-binding Assays

The total receptor concentration of cells and subcellular fractions was measured using an adaptation of the procedures described by Sklar et al. (63) for whole cells. Briefly, a 25–150- μ l aliquot of test fraction was diluted with resuspension buffer containing 10- μ g/ml digitonin (the receptor retains full ligand-binding activity when extracted into this detergent [45]) to a final volume of 1.5 ml in a 1-cm acrylic cuvette. A saturating amount of the fluorescein conjugate of *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (FI-peptide) (5 nM final) was added in 15 μ l and allowed to bind for 10 min at 23°C. The total fluorescein fluorescence was measured in a model 8000 spectrofluorometer (SLM Instruments, Moline, IL) configured to minimize interference due to light scattering as described by Sklar et al. (63). After binding equilibrium had been achieved, high affinity rabbit antibody to fluorescein was added (1 nmol) into the stirred sample. As previously shown using intact cells, these antibodies bind exclusively to free FI-peptide, thereby causing better than 99% quenching of its fluorescence (66). Consequently, residual fluorescence is a direct measure of bound ligand because the intrinsic fluorescence of the peptide conjugate is unaffected by its binding to the receptor (66). In addition, the ligand-dissociation rate can be directly measured by following the loss of fluorescence as a function of time after the addition of anti-fluorescein. To calculate the receptor-binding capacity, the residual fluorescence remaining was estimated by extrapolation to time zero (see below). Nonspecific binding was estimated by incubation of test fractions with 1 μ M nonfluorescein-peptide for 5 min before assay. The nonspecific component (typically <10% of total bound) was subtracted and the total specific ligand-binding capacity calculated directly from a linear standard curve relating FI-peptide concentration to observed fluorescence. All fractions were assayed in duplicate.

Calculation of Ligand-Dissociation Rate Constants

Specific binding data calculated as described above were analyzed using a commercially available MS-DOS computer program called KINETIC available from Elsevier-Biosoft, Ltd. (Cambridge, England). This program employs an iterative process to provide the best statistical fit of the data to a general first-order rate equation using procedures described in detail by Rodbard (55) and by McPherson (42). In essence, data were fit to a general first-order model described by Eq. 1 below:

$$[R_{bd}] = \sum_{i=1}^{i=n} [R_{oi}] \cdot e^{-k_{-ii}t} \quad (1)$$

where $[R_{bd}]$ is the concentration of receptor-bound ligand at time t , $[R_{oi}]$ is the amount bound to each receptor state i at time = 0, and k_{-ii} is the respective dissociation rate constant for each site i out of a possible total of n sites. In general, all of our observed dissociation data (over a 5,400-s period) was best fit by three rate constants except where noted (see Results). The respective dissociation half-times ($t_{1/2}$) were calculated from the observed dissociation rate constants by the relation, $t_{1/2} = 0.693/k_{-ii}$.

For convenience the receptor state associated with rate constants k_{-ii} , k_{-12} , and k_{-13} will be referred to hereafter as dissociation states A, B, and C, respectively.

Measurement of Nondissociable Fluorescent Peptide (State C)

Receptor from which ligand was nondissociable (state C) was operationally defined as bound fluorescein remaining after incubation with antibody for 1 h. In fact, little further change was noted after 3 h. The measurement was performed on the same samples used to measure total receptor binding (see above). In the case of membranes derived from unstimulated cells, the specific binding was determined by subtracting nonspecifically bound fluorescence from the total fluorescence. In those experiments where cells had been preexposed to FI-peptide before membrane preparation, the bound fluorescence was determined by subtraction of the light scatter contributed by the membranes themselves from the total fluorescence of an equal

amount of such membranes. The validity of this estimate was confirmed in studies that employed FI-peptide labeled with ¹²⁵I at the tyrosine residue. In all cases when state C was observed in FI-peptide-stimulated cells, >90–95% of the total was accounted for by the FI-peptide bound during cell stimulation.

Treatment of Neutrophils with Pertussis Toxin

Pertussis toxin obtained from List Biologicals (Campbell, CA) was activated just before use with 1 mM DTT for 30 min. Cells (5×10^8) suspended in 10 ml of Tyrode's-BSA were treated with 5 μ g of activated toxin for 50 min at 37°C. In these experiments untreated cells were similarly incubated without the addition of toxin. Control experiments using aliquots of toxin-treated cells indicated that FI-peptide-dependent superoxide anion production was inhibited >90%.

Immunoblot Analysis of Membrane Fractions for G Proteins

Membrane proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose paper and immunoblotted as described (51). After incubating the paper with 3% gelatin dissolved in 0.5 M NaCl, 0.05 M Tris-HCl (pH 7.5), specific or control antisera (1:100 dilution) were added and the incubation was continued for 18 h. The paper was washed three times with the above buffer containing 1% gelatin and then incubated with peroxidase-conjugated goat anti-rabbit IgG (1:2,000; Bio-Rad Laboratories) for 1 h. After extensive washing the peroxidase-positive bands were visualized by incubation with the peroxidase substrate.

Rabbit antisera specific for G protein subunits were generously provided by Dr. David Manning of the University of Pennsylvania. Antisera with three differing antigenic specificities were used. Antiserum 6460 was prepared by immunizing rabbits with a synthetic peptide corresponding to sequence common to the α subunit of all known G proteins (44). Antiserum 5296 was prepared against purified bovine brain Go (10, 44), cross-reacts with human Go but did not show a positive reaction with neutrophil membrane preparations. Antiserum 5357 was prepared against purified bovine brain Gi β complex and reacts predominantly with the β chain of human G proteins (10, 44).

Photoaffinity Labeling of the Receptor

N-formyl-Met-Leu-Phe-Lys ¹²⁵I-SASD (5 nM; 550 Ci/mmol) was cross-linked to the receptor by exposing membrane preparations which contained previously bound photoaffinity label to ultraviolet light as described by Allen et al. (2). In some experiments, the membranes were additionally incubated with 5 nM *N*-formyl-Met-Leu-Phe-Lys-[¹²⁵I]-SASD in the presence or absence of 1 μ M unlabeled peptide before photolysis to label receptors not occupied by ligand when the cells were exposed to ligand before membrane isolation. The treated membranes were dissolved in SDS sample buffer containing 8 M urea and analyzed by SDS-PAGE in a modified gel system containing 8 M urea and higher than normal concentrations of SDS as previously described (2, 50, 57) to minimize receptor aggregation. The labeled cross-linked species were visualized by autoradiography of the dried gel as described (2, 50, 57).

HPLC Analysis of Fluorescence Associated with Membrane Fractions

Slow and fast membranes were extracted with 10 vol of acetonitrile-0.1% TFA for 1 h at 4°C. After centrifugation, the supernatant solvent was evaporated to dryness in a Speed Vac apparatus (Savant Instruments, Inc., Hicksville, NY). The residue, which contained >80–90% of the original fluorescein fluorescence, was dissolved in 0.1 ml of dimethylformamide, applied to a DuPont ODS C18 reverse-phase column and eluted with a linear gradient of 0–80% acetonitrile containing 0.1% TFA.

Miscellaneous Methods

SDS-PAGE analysis was performed on a mini gel system as previously described (51). Protein concentrations were estimated by the Bradford assay (8) as described by Jesaitis et al. (26). UDP-galactosyltransferase (5), alkaline phosphatase (37), lactate dehydrogenase (32), and β -glucuronidase (16) activities were assayed according to the respective referenced method as previously described (26).

Results

Preparation and Characterization of Neutrophil Microsomal Fractions

Fig. 1 summarizes the scheme used to purify the various neutrophil membrane fractions used in these studies. The data shown in Table I indicate that the fast microsomal fraction contained most of the recovered receptor activity and was enriched 5.2-fold and 6.47-fold in receptor-binding activity and alkaline phosphatase activity, respectively. Corresponding values of 1.11 and 6.03 were obtained for these two markers in slow membrane fractions. Assay of these fractions for enzyme markers specific for other subcellular organelles indicated that fast and slow membranes were enriched 11.6-fold and 7.4-fold, respectively, relative to the homogenate for UDP-D-galactosyltransferase. Both fractions were depleted of cytosolic (lactate dehydrogenase) enzyme markers relative to the starting homogenates. As expected the granule-rich fraction sedimenting at 10,000 *g* (10,000-*g* pellet [10 KP]) contained most of the β -glucuronidase activity.

The overall recovery of receptor-binding sites in the fast and slow membrane fractions was 10–12% when compared with the 1 KS fraction. However, at least 50% or more of the total cellular receptors are localized in the granule-rich 10 KP fraction. These receptors have been shown to be latent in intact cells and therefore represent an internal pool of non-surface localized molecules (Table I and references 26 and 27). When the overall recovery of surface receptor is calculated, >33% of the total surface receptor was recovered in the fast and slow microsomal fractions assuming 60,000 receptors per cell.

Distribution of Receptor-Ligand Dissociation Rate States in Unstimulated Neutrophil Membrane Fractions

Fig. 2 shows a typical assay of FI-peptide binding and subsequent dissociation from fast microsomes in the presence of 10 μ g/ml digitonin. In this assay binding equilibrium was first established (10 min, 23°C) in the stirred fluorometer cuvette in the presence of 5 nM FI-peptide. At time zero, antfluorescein was added in order to quench unbound FI-peptide fluorescence. Control experiments established that >99% of the initial peptide fluorescein fluorescence was

quenched within 2–3 s after antibody addition. As Fig. 2 A shows, this preparation of fast membranes specifically bound 43.5 pmol FI-peptide/mg protein under the conditions employed. Preincubation of membranes with 1 μ M nonfluorescent parent peptide reduced the observed fluorescent signal to that observed with membranes alone (*horizontal line*) indicating that the binding of FI-peptide is receptor-mediated. In contrast, slow membranes showed little specific ligand-binding activity (Fig. 2 B) although nonsaturable binding was more pronounced in this fraction as compared with the fast fraction.

Fig. 2 C represents a first-order plot of the specific binding data shown in Fig. 2 A. As is evident, the measured ligand-dissociation rate was best explained by a rate equation comprising two dissociation constants, K_{-11} and K_{-12} . The faster of the two dissociation rate components (rate A) is on the order of 5.9 min^{-1} , which corresponds to a $t_{1/2}$ of 14 s. The second component (type B) corresponds to a rate of 0.25 min^{-1} or a $t_{1/2}$ of 167 s. In Fig. 2 A there is a small component of ligand-binding activity (type C), which does not measurably dissociate for up to 1 h. The rate constant for this component is greater than $6 \times 10^{-5} \text{ min}^{-1}$. The relative proportions of A, B, and C dissociation rates in this preparation of membranes were 27.3%, 42.6%, and 30.1%, respectively.

Table II summarizes the means and standard deviations of such data obtained for fast and slow microsomes obtained from unstimulated neutrophils in five separate preparations. The data show that the FMLP receptor exhibits at least three distinguishable dissociation rate constants. In most fast microsomes preparations examined $\sim 47.1\%$ (SD = 8.7%; $n = 5$) of the receptor exhibited dissociation rate characteristic of type B dissociability. With the exception of a small percentage (17.2%; SD = 15.2%; $n = 5$) of nondissociable binding (type C), the remainder was of the A type (35.6%, SD = 4.4%, $n = 5$). In contrast, slow microsomes, which are enriched in plasma membrane and Golgi markers, did not exhibit consistent receptor-binding activity of any type. The variability of the binding activity (three of five preparations had no measurable activity) suggested that the small amounts of ligand-binding activity seen were due to contamination by fast membranes.

The extrapolation of these results to the intact cell depends

Table I. Distribution of FI-peptide-binding Activity and Marker Enzymes in Subcellular Fractions of Unstimulated Neutrophils

Fraction	Protein mg	Specific enrichment of marker in fraction relative to 1,000- <i>g</i> supernatant*				
		FI-peptide binding	Alkaline phosphatase	UDP-galactosyltransferase	β -Glucuronidase	Lactate dehydrogenase
1 KS	55.0	1	1	1	1	1
1 KP	10.9	0.39	0.98	0.86	1.96	1.17
10 KS	34.2	0.89	0.57	1.18	0.33	3.54
10 KP	18.0	1.48	1.80	0.86	1.71	0.27
123 KS	36.6	0.17	0.35	0.12	0.17	1.94
123 KP	2.39	4.15	7.42	4.53	2.74	1.2
Fast MS	1.08	5.17	6.47	11.60	3.70	0.2
Slow MS	0.48	1.11	6.03	7.44	6.33	0.6

* Specific enrichment of each activity is equal to the activity per milligram of protein of the specified fraction divided by the activity per milligram of protein of the 1,000-*g* supernatant (1 KS). See Fig. 1 for the definition of the fractions.

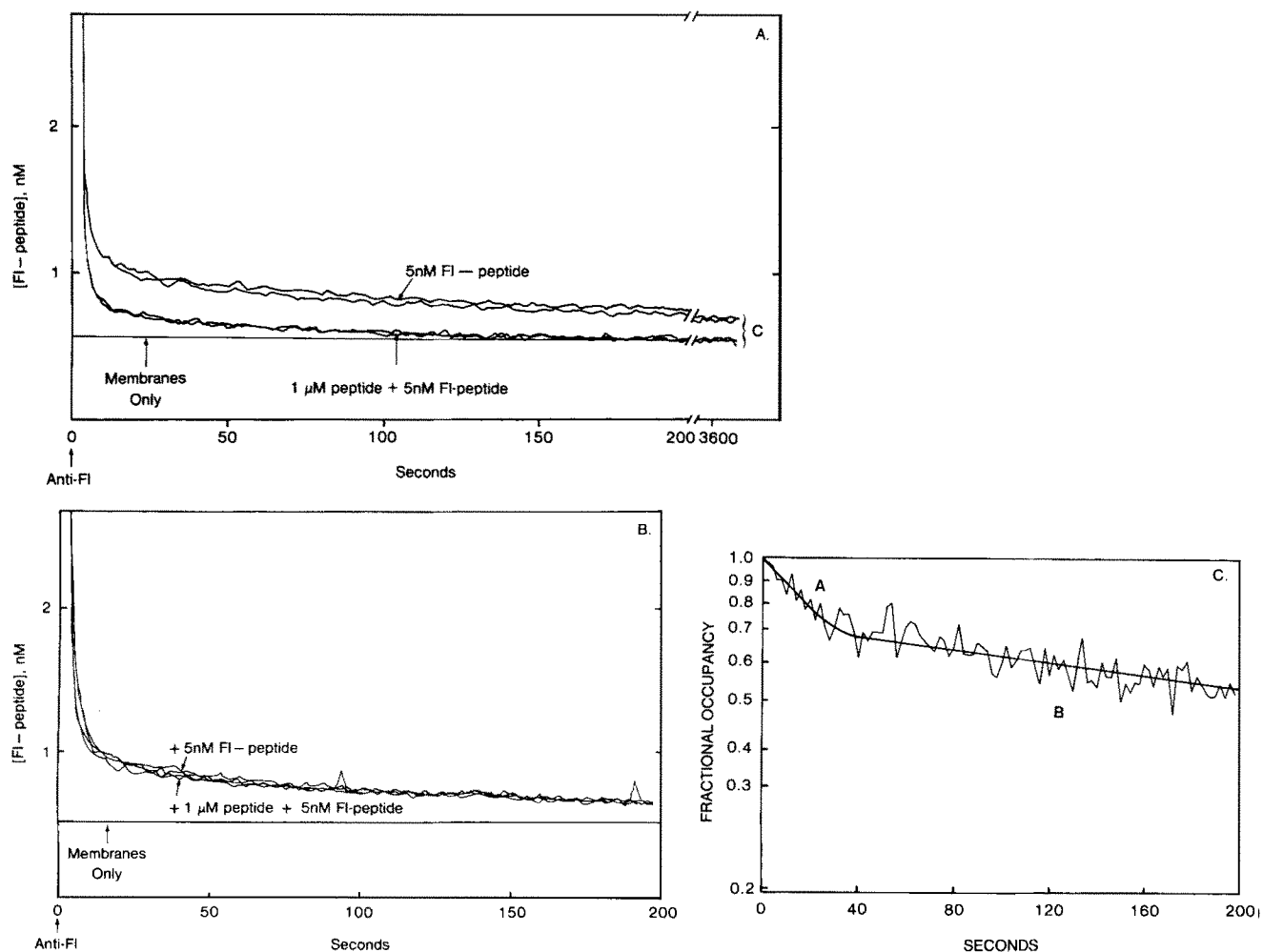


Figure 2. Dissociation of FI-peptide from fast (*A* and *C*) and from slow (*B*) microsomes derived from unstimulated human neutrophils. Fast (16.8 μg) and slow (9.2 μg) membrane fractions (see Fig. 1) were exposed to 5 nM FI-peptide either in the presence or absence of 1 μM unlabeled peptide as indicated for 10 min in incubation buffer at 23°C. At time = 0, 1 nmol of rabbit anti-fluorescein was added with continuous stirring. After data had been collected for 200 s, the cuvettes were further incubated for 1 h and the remaining fluorescent signal was measured to determine the amount of specifically bound ligand that did not dissociate in that time period (labeled *C* in panel *A*). (*C*) A semilog first-order plot of the data shown in panel *A*. Note the presence of two distinct dissociation rates labeled *A* and *B*. In this experiment slow membranes (panel *B*) had no detectable specific ligand-binding activity.

on the assumption that the recovered receptor is representative of the total surface receptor with respect to ligand-dissociation properties. To address this issue, we measured the proportion of states *A*, *B*, and *C* present in the initial cell homogenates. These studies indicated that the 1 KS superna-

tant contained 12%, 61%, and 27% of states *A*, *B*, and *C* as compared with 28%, 51%, and 21%, respectively for the combined fast and slow membrane fractions. In the case of states *B* and *C*, which are inherently easier to measure than state *A*, these values are in reasonable agreement with one

Table II. Summary of FI-Peptide-Dissociation Properties of *N*-Formyl Peptide Receptor of Fast and Slow Microsomes Derived from Unstimulated Neutrophils

Microsome fraction	Amount of receptor associated with:			Total
	State A	State B	State C	
Fast microsomes (<i>pmol/mg</i> \pm <i>SD</i>)	12.3 \pm 1.7	16.3 \pm 3.0	5.9 \pm 5.2	34.5 \pm 7
(% \pm <i>SD</i>)	35.6 \pm 4.4	47.1 \pm 8.7	17.2 \pm 15.2	100
Slow microsomes (<i>pmol/mg</i> \pm <i>SD</i>)	6.6 \pm 7.6	5.3 \pm 4.9	<0.5	11.9 \pm 12
(% \pm <i>SD</i>)	60 \pm 55	39 \pm 45	<0.5	100
<i>k</i> ₋₁ (<i>min</i> ⁻¹ \pm <i>SD</i>)	4.1 \pm 1.8	0.18 \pm 0.08	<6 \times 10 ⁻⁵	—

Mean of five separate membrane preparations from five individual donors. See text for description of the methods used for calculation of each individual *k*₋₁.

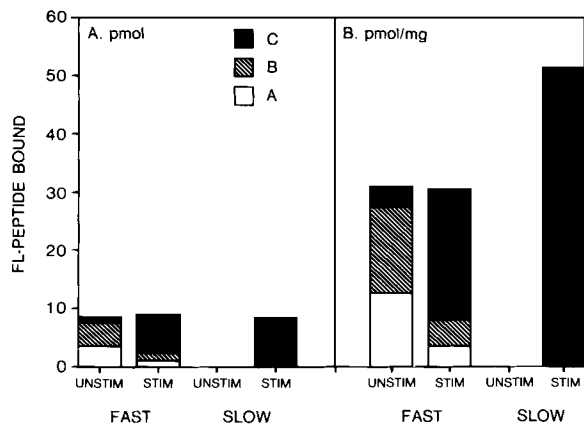


Figure 3. Treatment of cells with FI-peptide at 37°C for 2 min induces an increase in the percentage of receptor that is present in state C. Neutrophils (5×10^8 in each case) were exposed at 37°C for 2 min to 5 nM FI-peptide or buffer. After washing with ice-cold buffer, fast and slow membranes were isolated as described in the text and 14.9–27.1 μg was assayed for the percentage of affinity states A, B, and C that were present. (A) The total picomoles of receptor recovered in the respective fractions. (B) The corresponding receptor-specific activities in picomoles per milligram of protein. Note that in the case of FI-peptide-treated cells, most of the receptor recovered with the fast and slow membranes was of the state C type.

another and with values published for intact cells by Sklar et al. (63).

Distribution of Ligand-Dissociation Rate States A, B, and C in Fast and Slow Microsomes Derived from Cells Stimulated with FI-Peptide at 37°C

As Fig. 3 documents, exposure of cells to 5 nM FI-peptide for 2 min at 37°C before washing and homogenization yield-

ed fast and slow membrane preparations that exhibited a markedly different pattern of receptor-ligand dissociation rate states as defined above.

Slow membranes that initially contained little, if any, saturable ligand-binding activity (Tables I and II) contained more than half of the total bound ligand recovered in the fast and slow membrane fractions. In addition, nearly all of the recovered receptor exhibited type C receptor with little or no detectable state A or B receptor. Unlike slow microsomes, most of the remaining receptor in this fraction was type B. Nearly all (90–95%) of the measured state C component in both fractions was due to FI-peptide bound to the intact cells before membrane preparation and thus did not dissociate during membrane preparation. In contrast to slow membranes, over half of the receptor activity present in fast membranes was of the state C variety. Extraction and analysis of the bound fluorescence present in both fractions by HPLC indicated that the fluorescence was associated with intact peptide. We also noted that the total receptor recovered in membranes obtained from FI-peptide stimulated cells was 20–30% higher than that recovered from identical amounts of fractions (on a protein basis) obtained from unstimulated cells. This effect appeared to be associated with increasing times of exposure of cells to FI-peptide at 37°C (data not shown) suggesting that upregulation of the known pool of intracellular FMLP receptors into the surface membrane (17, 26) may be responsible. Nevertheless, the quantitative increase observed in state C binding at the expense of state A and B binding could not be quantitatively accounted for by the observed increases in total receptor recovery. This result indicates that increase in type C binding was due in large part to the conversion of state A and B receptors to state C receptors.

The overall distribution and recoveries of various subcellular markers did not change after stimulation of cells with FI-peptide (Table III; also see Jesaitis et al. [26]).

Table III. Distribution of Plasma Membrane and Golgi Markers in Neutrophils Exposed to Dihydrocytochalasin B and FI-Peptide

Fraction	Control	FI-peptide	diHCB	diHCB + FI-peptide
<i>pmol · min⁻¹ · mg⁻¹</i>				
Alkaline phosphatase				
10KS	4.3	6.2	7.6	5.0
10KP	36.5	18.9	24.8	20.0
123KS	3.7	5.6	4.6	3.9
123KP	92.7	75.0	54.7	48.7
Fast	182.2	171.3	124.6	189.3
Slow	295.1	268.8	207.5	103.8
UDP-galactosyltransferase				
10KS	19.9	14.6	15.0	17.1
10KP	6.6	8.7	12.1	11.9
123KS	0.7	0.3	0.2	0.3
123KP	83.9	66.1	66.7	76.0
Fast	70.2	58.9	64.9	91.1
Slow	264.0	244.0	228.4	157.8

Cells were treated with or without dihydrocytochalasin B (diHCB) or buffer (control) as indicated for 5 min. FI-peptide was added to a final concentration of 5 nM and incubated at 37°C for an additional 2 min. The cells were then diluted into ice-cold buffer, homogenized, and fractionated according to the scheme depicted in Fig. 1. Protein yields were comparable for all four treatment conditions for each listed fraction. Data given are from one of two such experiments.

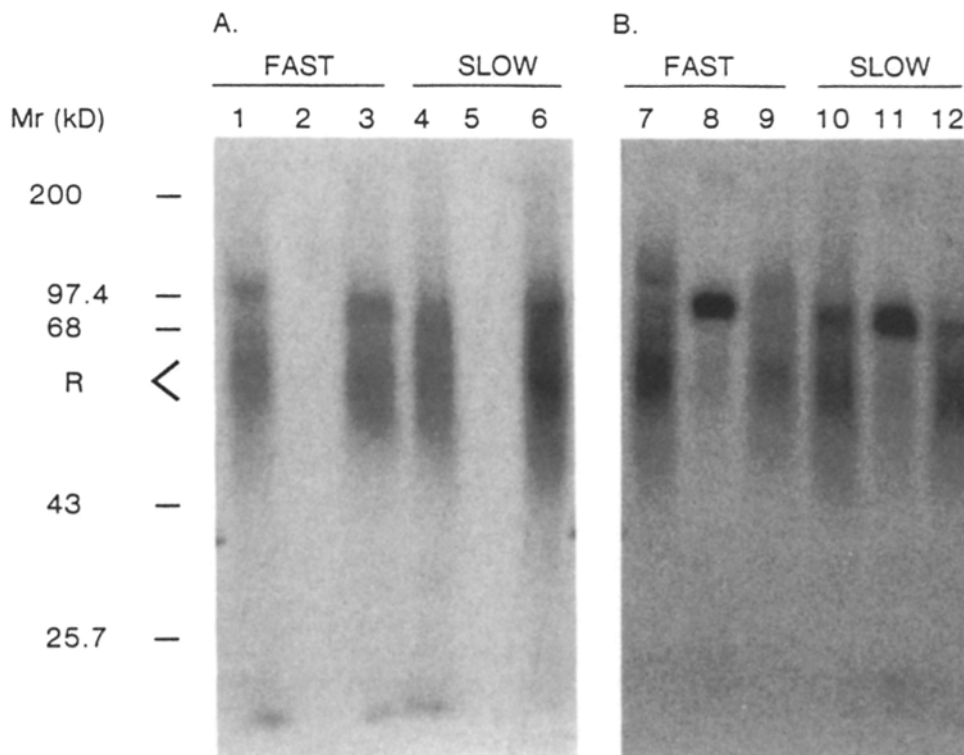


Figure 4. Photoaffinity labeling of fast and slow membranes with *N*-formyl-Met-Leu-Phe-Lys-¹²⁵I-SASD. (A) Irreversibly bound ligand. Fast (lanes 1–3) and slow microsomes (lanes 4–6) were prepared from cells stimulated (in the dark) for 2 min at 37°C with 5 nM *N*-formyl-Met-Leu-Phe-Lys-¹²⁵I-SASD. Fast and slow membranes containing the residual-bound photoligand were photolyzed with UV light before and after incubation for 1 h with 1 μM unlabeled parent peptide. In the above autoradiogram, note the incorporation of ~80% of the specifically bound label into the typically broad receptor band (*R*) centered between 50 and 65 kD (lanes 1 and 4). The remainder is associated with a 100–110-kD protein which may represent a dimer. Incubation of the cells with 1 μM nonradioactive parent peptide before addition of photo-

label abolished its incorporation into the receptor (lanes 2 and 5). In contrast, incubation with unlabeled peptide for 1 h before photolysis did not reduce labeling (lanes 3 and 6) indicating that the bound ligand is nondissociable (i.e., state C). (B) Dissociably bound ligand. Fast microsomes (lanes 7–9) and slow microsomes (lanes 10–12) were prepared as described for A. The membrane preparations were then treated with 5 nM *N*-formyl-Met-Leu-Phe-Lys-¹²⁵I-SASD for 15 min before photolysis (lanes 7 and 10). Note the increase in covalent incorporation of photolabel into fast membranes as compared with the corresponding lane in A. Slow membranes show less increase in labeling over that seen in A. Nonradioactive peptide added before the photolabel blocks this incremental increase in labeling (lane 2). A chase with 1 μM parent peptide for 1 h after photolabel has been allowed to bind but before photolysis, partially reduces incorporation of radioactivity into fast membranes (lane 3) but had no effect on label incorporated into slow membranes (lane 6).

Dissociable and Nondissociable Ligand Is Associated with the 50–65-kD Receptor

The above analysis assumed that nondissociable ligand was, in fact, associated with a receptor. To determine whether this was indeed the case, cells were incubated for 2 min at 37°C with 5 nM *N*-formyl-Met-Leu-Phe-Lys-¹²⁵I-SASD and fast and slow membranes isolated as described above except that DTT was omitted from all buffers. Membrane fractions were cross-linked by irradiation with UV light just before solubilization with SDS sample buffer.

Fig. 4 A shows that nondissociable photolabel that isolated with the membranes was cross-linked into a 50–65-kD polypeptide that has been previously identified as the receptor in this system (2, 46, 50, 57). A higher molecular mass species of 100–110 kD was also seen which may represent an aggregate of the 50–65-kD protein. Quantitation of the radioactivity contained in each band revealed that the total percentage of label bound to the 100–110-kD protein accounted for not >15–30% of the total label in both membrane fractions. Addition of 1 μM unlabeled photolabel to the cells completely blocked the incorporation of radioactivity into these two proteins. In addition, if 1 μM unlabeled photolabel was incubated with these membranes for 1 h before photolysis no appreciable decrease in receptor labeling was observed for both slow and fast membranes indicating the presence of state C receptors. In conclusion, at least 70% of nondissocia-

bly bound ligand is associated with the 50–65-kD FMLP receptor even if one assumes that the 100–110-kD labeled species is not a dimer of the receptor.

In order to photolabel unoccupied receptors (i.e., state A and B type receptors), the above membrane fractions were incubated (23°C) with 5 nM *N*-formyl-Met-Leu-Phe-Lys-¹²⁵I-SASD in the presence or absence of 1 μM unlabeled photolabel (Fig. 4 B) and then photolyzed. As judged by the increase in labeling intensity of the 50–65-kD protein, fast membranes contained additional photolabelable receptors. Addition of 1 μM unlabeled ligand blocked labeling of the receptor but appeared to increase labeling of a 90-kD protein indicating that this component binds photolabel with high capacity and very low affinity (i.e., nonspecific binding). Labeling of this nonreceptor protein was seen previously in membrane preparations but not in intact cells (see Fig. 4 of reference 2) and very likely represents a protein accessible only in broken cell preparations. Subsequent incubation with 1 μM nonradioactive peptide for 1 h before photolysis, reduced the observed radiolabeled receptor present, indicating that the bound ligand is exchangeable.

Analysis of Slow and Fast Microsomes by Isopycnic Ultracentrifugation on Ficoll-D₂O Gradients

Slow membranes derived from cells exposed to 2 nM Fl-peptide (Fig. 5 B) or to buffer (Fig. 5 A) for 2 min at 37°C

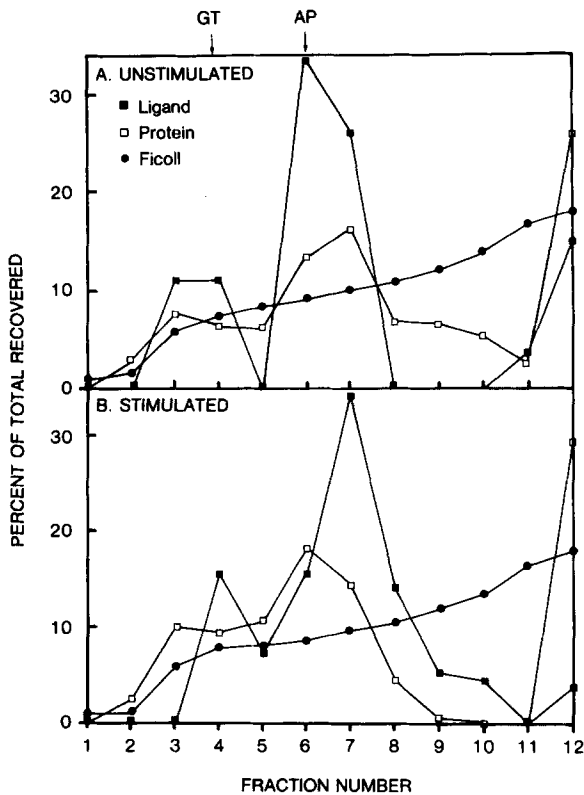


Figure 5. Isopycnic ultracentrifugation of slow membranes from (A) buffer or (B) FI-peptide-treated cells on linear gradients of 2–20% Ficoll, 9–90% D₂O. Slow membranes were prepared from cells treated with (A) buffer or (B) FI-peptide as described in the legend to Fig. 3. Membranes (93 μ g of protein in 1.0 ml of resuspension buffer) were applied to Ficoll–D₂O gradients and ultracentrifuged to their isopycnic points as described in Materials and Methods. Fractions (0.5 ml each) were assayed for protein (\square), Ficoll concentration (\bullet), and total receptor concentration (\blacksquare). The top of the gradient is on the left; fraction 12 is the pellet fraction. Total receptor binding activity recovered was 2.7 and 13.7 pmol for control and FI-peptide-pretreated cells, respectively. In the case of pretreated cells, this includes the contribution of the peptide bound during stimulation which amounted to >90% of the recovered activity in this case. Note that receptor-bearing vesicles banded at Ficoll concentrations of 9–10% and cosedimented with alkaline phosphatase (AP). Less than 15% of the total receptor cosedimented with UDP-galactosyltransferase activity (GT).

were ultracentrifuged to their isopycnic densities on linear gradients of Ficoll–D₂O. Fig. 5 shows that the majority of the recovered ligand-binding activity sedimented with alkaline phosphatase-rich fractions (AP) rather than with the less dense UDP-galactosyltransferase-rich fractions (GT) in both cases. The data were expressed as a percentage of the total activity applied to the gradients for ease of comparison. Similar analyses of fast membranes derived from stimulated and unstimulated cells showed that the ligand-binding activity was likewise associated with vesicles of identical isopycnic banding characteristics as shown in Fig. 5 for slow membranes (data not shown). These results further show that the slower sedimentation rate observed in the velocity gradient step used to separate slow and fast membranes is due to differences in vesicle size rather than vesicle density.

Effect of Dihydrocytochalasin B and GTP- γ -S on Receptor–Ligand Dissociation Rates

The question arose as to the relationship of ligand-dissociation rate states B and C to the GTP-sensitive state described by Snyderman and co-workers (29, 35, 72, 73) and the dihydrocytochalasin B-sensitive state described by Jesaitis et al. (27, 28). To address this question, we examined the effect of dihydrocytochalasin B and GTP on the distribution of receptor–ligand dissociation rate states. As Fig. 6 shows, dihydrocytochalasin B pretreatment (5 min, 37°C) of intact cells prior to addition of FI-peptide (10 nM) markedly suppressed the ligand-induced formation of state C contained in both slow and fast membrane fraction derived from such cells. The total recovered ligand-binding activity was similar in both cases, indicating that the drug affected the formation of state C without affecting the ligand-induced translocation of receptors to the slow membrane compartment. Addition of similar concentrations of dihydrocytochalasin B to membrane preparations rich in state C receptor did not cause dissociation of FI-peptide (data not shown).

The above alterations in the distribution of receptors could be explained by changes related to secretion induced by the combined action of cytochalasin and peptide. However, as Table III shows, the overall distribution of enzymes associated with the plasma membrane and the Golgi complex was not dramatically different when cytochalasin-treated cells are compared with cells treated with both cytochalasin B and FI-peptide. In contrast to the dramatic increases observed in FI-peptide binding activity, slow membranes showed a 35–50% decrease in alkaline phosphatase and UDP-galactosyltransferase activities; however, these same markers were not significantly altered in fast membranes fractions indicating that the increases in receptor activity seen in Fig. 6 can not be explained by secretion-related alterations in plasma membrane isolation characteristics. As expected, β -glucuronidase

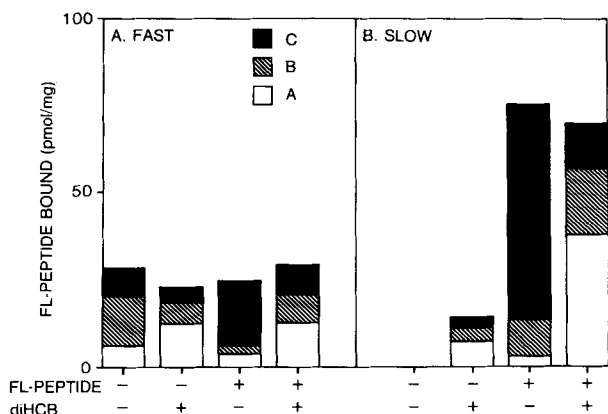


Figure 6. Dihydrocytochalasin B (diHCB) inhibits FI-peptide-induced formation of state C affinity. After incubation at 37°C for 5 min in the presence (+) or absence (–) of diHCB (5 μ g/ml) cells (1×10^8 in each case) were exposed to 10 nM FI-peptide (+) or buffer (–) for 2 min at 37°C as indicated below the abscissa. Fast and slow membranes were prepared and assayed for protein and for the proportion of receptor in states A, B, and C. Total recovery of protein (fast and slow membranes combined) was as follows: unstimulated cells, no diHCB, 269 μ g; unstimulated cells + diHCB, 231 μ g; stimulated cells, no diHCB, 220 μ g; and stimulated cells + diHCB, 244 μ g. One of three representative experiments.

dase was decreased by >60% in these cell homogenates due presumably to secretion of this marker into the medium during the initial incubation (not shown).

Next we examined the distribution of ligand-dissociation rate states when membrane preparations obtained from unstimulated cells were assayed with 10 μ M GTP- γ -S included in the binding assay buffer (Table IV). Under the conditions of our assay, GTP- γ -S appeared to abolish the state B dissociation rate. In three such experiments the total recovery of receptor was somewhat diminished (20–30% of control levels) in the presence of GTP- γ -S. However, it was apparent that the majority of state B receptor was converted to state A-like receptor; the fraction that was lost after GTP- γ -S treatment was very likely not detected in our assay system due to its lowered affinity. AMP-imidodiphosphate (10 μ M) and ATP- γ -S (10 μ M) were without effect (data not shown) indicating that the effect of GTP- γ -S was nucleotide specific.

In sharp contrast, treatment of fast membranes rich in state C receptor (i.e., derived from Fl-peptide-stimulated cells) with similar concentration of GTP- γ -S did not cause appreciable change in the amount of receptor present in that state (Table IV).

In summary, the data indicate that state B is GTP-sensitive and is converted at least in part to state A by GTP and its analogues. State C in contrast is GTP-insensitive and is not formed in cytochalasin B-treated cells in response to chemotactic peptide. Once formed it is insensitive to cytochalasin B in vitro.

Distribution of α and β Subunits of the Neutrophil G Protein in Slow and Fast Microsomes

The lack of GTP-dependence of state C dissociability and lack of state B receptor in slow membranes suggested that neutrophil G protein might be absent in this membrane fraction. Alternatively, the receptor could be functionally and physically uncoupled from G proteins present in slow membrane preparations. To test this possibility, slow and fast microsomes from unstimulated cells were assayed for α and β subunits of the neutrophil G protein by immunoblotting the SDS-PAGE separated proteins with antisera specific for the

Table IV. Effect of GTP- γ -S on Receptor Affinity States of Fast Membranes Derived from Unstimulated and Fl-Peptide-treated Cells*

Fl-peptide	GTP- γ -S	Receptor affinity state		
		State A	State B	State C
<i>nM</i>	μ M	<i>pmol/mg protein</i>		
0	0	39.6	39.8	21.0
	10	73.5	0 \ddagger	26.5
5	0	23.4	18.8	40.5
	10	10.9	0 \ddagger	44.3

* Fast membranes (19 μ g) were derived from cells treated with or without 5 nM Fl-peptide as indicated above in Tyrode's-BSA for 2 min at 37°C. Membranes were treated with or without GTP- γ -S for 10 min at 23°C before the fluorometric assay for ligand-dissociation rate states as described in the legend to Fig. 3.

\ddagger A two-state model that assumed the presence of states A and C was a better fit of the data than the conventional three-state model ($P < 0.001$). The dissociation rate constants were not held constant during the iterative calculations; however, the calculated constants were within the ranges reported for states A, B, and C in Table II.

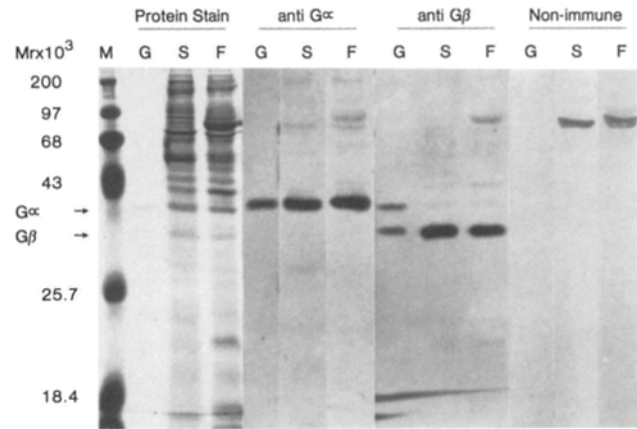


Figure 7. Immunoblot analysis of G protein composition of fast and slow membranes. Fast (F) and slow (S) microsomes prepared from unstimulated cells were applied in equal protein loads (10.5 μ g) to 8% SDS polyacrylamide cells. A mixture of 50 ng each of purified brain Gi and Go (lane G) was applied as a control. After electrophoretic transfer of the separated proteins to nitrocellulose paper, the samples were immunostained with rabbit anti-G protein α -chains (*anti G α*), anti-G beta-gamma complexes (*anti G β*), and nonimmune rabbit serum, all diluted 1:100 prior to use. The Coomassie Blue protein staining pattern is shown in the panel at the far left. Note the staining of a 41- and a 35-kD protein (arrows) with the two respective antisera. Both comigrated with the G protein standards. Molecular mass standards in kilodaltons are shown in lane M.

α and β chains of Gi protein after electrophoretic transfer to nitrocellulose paper.

As Fig. 7 shows, fast and slow membranes contain, on an equal protein basis, similar amounts of α and β subunits of G protein. The staining of the bovine brain α -chain of the Gi and Go standards is presumably due to contamination of the antisera with antibodies that react with bovine α -chain but not with human α -chain. The major immunoreactive species which stained with the α -chain-specific antibody had an M_r of 41 kD. Since these membranes failed to react with antibodies specific for Go (not shown), these data indicate that the 41-kD species is more closely related immunologically to Gi than Go. In experiments not shown, this species comigrated with the 41-kD peptide which was ADP-ribosylated by pertussis toxin. Membranes derived from stimulated cells yielded similar results to that shown in Fig. 7 (data not shown). These results indicate that fast and slow microsomes contain comparable amounts of G protein. Furthermore, the activation state of the cells either in the presence or absence of cytochalasin does not affect the amount of G protein associated with either membrane fraction.

Pertussis Toxin Fails to Block Fl-Peptide-induced Formation of State C

Neutrophils were incubated with or without DTT-preactivated pertussis toxin (0.5 μ g/ml) for 50 min at 37°C. Parallel assays confirmed that this concentration and incubation time was sufficient to inhibit Fl-peptide-induced superoxide anion production by >90% when compared with control cells incubated similarly in the absence of toxin. After 50 min of exposure to toxin, Fl-peptide (5 nM) was added and after

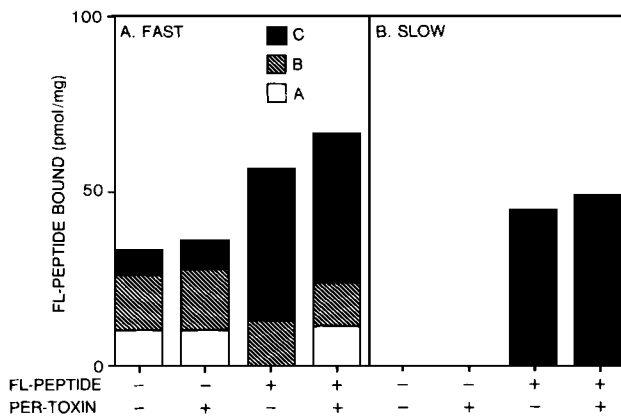


Figure 8. Pretreatment of neutrophils with pertussis toxin fails to inhibit Fl-peptide-induced formation of state C affinity. After incubation at 37°C for 50 min in the presence (+) or absence (-) of pertussis toxin (PT) (5 µg), cells were exposed to 5 nM Fl-peptide (+) or buffer (-) for 2 min at 37°C as indicated below the abscissa. Fast and slow membranes were prepared and assayed for protein and for the proportion of receptor in states A, B, and C. Total recovery of protein (fast and slow membranes combined) was as follows; unstimulated cells, no PT, 86 µg; unstimulated cells + PT, 71.4 µg; stimulated cells, no PT, 83 µg; and stimulated cells + PT, 74 µg. One of two representative experiments.

2 min of further incubation, the cells were washed with ice-cold buffer and slow and fast microsomes prepared as before.

Even though the levels of toxin used have been shown to block a variety of *N*-formylated peptide-stimulated responses (3, 6, 7, 9, 12, 21, 33, 34, 43, 48, 70) slight enhancement of state C receptor was found when either slow or fast microsomes were assayed for ligand-binding activity (Fig. 8). Thus, ligand-induced formation of state C is not inhibited by pertussis toxin in the face of profound inhibition of other neutrophil physiological responses to chemotactic peptide.

Discussion

A large body of literature has clearly implicated guanine nucleotide-binding proteins in the regulation of the response of neutrophils to chemotactic factors (3, 6, 7, 9, 12, 21, 29, 33, 34, 43, 48, 70, 71). This class of receptor regulatory proteins also appears to render the ligand-dissociation rate of the receptor sensitive to GTP and its nonhydrolyzable analogues (29, 35, 62, 72, 73). The data presented here confirm and extend these earlier observations in a number of significant ways. First, the data clearly document the existence of at least three distinct ligand-dissociation rate states for this receptor. The faster two of the three states, termed A and B here, account for 85% of the total receptor found on the surface membrane of unstimulated neutrophils. State B appears to be converted to state A in the presence of GTP. State C, a receptor state from which ligand is virtually nondissociable, is induced by ligand binding to cells and, based on its sensitivity to cytochalasin B, may involve interactions with elements of the cytoskeleton. Secondly, our data indicate that formation of state C is associated with the segregation of receptor-ligand complexes into a membrane domain which can be isolated in the form of small membrane vesicles (slow

microsomes) that are rich in plasma membrane markers but not Golgi markers (Fig. 5). Interestingly, the translocation of receptor from the fast to the slow membrane compartment is not blocked by this drug, indicating that this process does not depend upon state C formation. Thirdly, pertussis toxin, which inhibits most neutrophil responses to formyl-peptides, does not affect the formation of state C (Fig. 8) when cells are exposed to Fl-peptide, a finding that suggests that its formation is independent of G protein regulation and of any other pertussis-sensitive cellular response to Fl-peptide.

Evidence for the existence of at least two ligand-dissociation rate states for the receptor in intact neutrophils and neutrophil membrane preparations has been presented by a number of laboratories (27-29, 35, 62, 63, 72, 73). Using crude microsome preparations derived from unstimulated neutrophils, Snyderman and his colleagues showed that equilibrium binding data could be best fit by at least two affinities (29, 35, 72, 73). The higher of the two affinities was converted to the lower state by GTP and its nonhydrolyzable analogs (29, 35, 72, 73). Evidence has been presented by Sklar et al. (62) showing that at least two similar ligand dissociation rate states could be demonstrated in a permeabilized neutrophil model (12, 71), the slower of which is also GTP sensitive. The GTP-sensitive state described by these workers represented ~70% of the total measured receptor. Based on the reported K_D , its GTP sensitivity, and the relative proportions reported by these groups, we conclude that state B described herein represents the same GTP-sensitive receptor state with state A representing the lower of the two receptor affinity states. Because of its relatively low affinity and correspondingly fast dissociation rate (14 s), state A is intrinsically difficult to quantitate by the methods used here. Thus, it is difficult to assess if state B is quantitatively converted to state A. However, Koo et al. (29) have clearly shown using equilibrium binding methods that GTP lowered receptor affinity in this system with no detectable change in number of ligand-binding sites.

It has remained unclear whether the ligand-induced high affinity state first reported by Jesaitis et al. (27) was identical to that reported by Snyderman et al. (72) and Sklar et al. (63). Jesaitis et al. (27) showed that ligand induced a ligand-receptor interaction that was virtually nondissociable ($t_{1/2} > 3$ h). Furthermore, this complex, which was of a noncovalent nature, formed only when cells were incubated with ligand at temperatures of 15°C or greater and was inhibited by dihydrocytochalasin B (27, 28). Our own data indicate that the affinity state described by Jesaitis et al. is identical to state C based on its dihydrocytochalasin B sensitivity, its nondissociability, and its relative paucity in membranes obtained from unstimulated neutrophils.

This form of the receptor appears to be resistant to guanine nucleotide analogues (Table IV). Furthermore, its formation in the presence of concentrations of pertussis toxin (Fig. 8), which inhibit secretion and other neutrophil responses to formyl peptides (3, 6, 7, 9, 12, 21, 33, 34, 43, 48, 70) proves that the expression of state C is not due to an upregulation of a granule-localized receptor population with unusual binding characteristics. By similar logic, the formation of state C must be independent of any other cellular process that is inhibited by pertussis toxin. Finally, this observation, together with its insensitivity to GTP- γ -S (Table IV), indicates that the formation of state C is not mediated by a G protein. Since

the fast and slow fractions contain equivalent amounts of G protein, it must be functionally uncoupled from the receptor.

In addition, state C appears to be ligand-induced and resides on a physically separable subpopulation of membrane vesicles that are of smaller size (as judged by their rate of sedimentation in D₂O gradients) but of similar density when compared with that exhibited by the bulk plasma membrane. The vesicles bearing state C receptors are rich in plasma membrane markers but deficient in Golgi markers (Fig. 5); Under conditions that inhibit receptor downregulation but not formation of state C (e.g., 15°C), this fraction of receptor-bearing membranes still forms suggesting that they represent plasma membrane-derived vesicles rather than endosomes or Golgi (Painter, R. G., unpublished observations). Thus, this form of the receptor appears to have been sequestered into a distinct subdomain of the plasma membrane. Indeed, the β -adrenergic receptor has been shown to exhibit ligand-dependent increases in affinity which are associated with functional desensitization of cells to agonist and sequestration of receptors into an isolatable subpopulation of small vesicles that are remarkably similar to those reported here (76).

The failure of pertussis toxin to inhibit the ligand-induced formation of state C indicates that receptor-ligand complexes are processed by two parallel and competing pathways. One of these pathways is characterized by the functional coupling of the receptor to a neutrophil G protein which is pertussis-inhibitable; the other pathway leads to the formation of state C receptor and is cytochalasin B-sensitive (but not pertussis-sensitive), suggesting the possible involvement of the cytoskeleton.

The inhibitory effect of cytochalasins upon the formation of state C is consistent with its involvement in the desensitization (i.e., adaptation) process. Previously published findings that show these drugs inhibit the normal termination of the cellular response are compatible with this hypothesis and suggest that cytoskeletal interactions may be involved (27, 28). It is also possible, in analogy with other hormone receptor systems controlled by G proteins, that phosphorylation of the receptor by specific protein kinases may be involved in the desensitization pathway (for reviews, 14, 19, 36, 39, 69). The ultimate biochemical mechanisms involved in the adaptation response (49, 59, 67, 68, 80) of neutrophils to chemotactic peptides should be facilitated by the ability to isolate the inactivated receptor in biochemically useful amounts employing the procedures described herein.

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