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Competitive Binding Kinetics in Ligand-Receptor-Competitor Systems

Rate Parameters for Unlabeled Ligands for the Formyl Peptide Receptor

L. A. SKLAR, J. SAYRE, V. M. MCNEIL, AND D. A. FINNEY

Department of Immunology, Scripps Clinic and Research Foundation, La Jolla, California 92037

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SUMMARY

We describe the real-time kinetics of the competition of different ligands for the same receptor and use the computer routine SAAM to simulate this competition. Based on the simulation, we have developed two experimental approaches whereby the parameters of the interaction of nonlabeled ligands with their receptor can be detected; briefly, the analysis of the nonlabeled ligands depends on the perturbation of the kinetics of interaction of labeled ligands with the receptor with which they are in competition. The approach relies primarily upon an analysis of the kinetics of the competition between fluorescent and nonfluorescent ligands using a real-time, homogeneous binding assay in the fluorescence flow cytometer. A secondary approach depends upon an examination of the kinetic impact of antagonists on the responses of cells stimulated by agonists at the same receptor. Experimental verification of these approaches has been obtained using the *N*-formyl peptides (and their antagonists) which bind to receptors on human neutrophils and produce rapid cell stimulation. We find that agonistic *N*-formyl peptides have residence times of minutes while nonstimulatory antagonists have residence periods of, at most, a few seconds at these receptors. The limitations and general range of applicability of these procedures are discussed. The main advantage of these approaches is that they permit the evaluation of kinetic parameters of unlabeled ligands, even those which bind weakly or which have brief residence times—properties which make analyses by conventional methods difficult.

INTRODUCTION

Competitive equilibria for the binding sites on cellular receptors have been of interest for several decades (1, 2) and the kinetics of competition has recently been analyzed by both exact and numerical methods (3, 4). In our preliminary experiments (4) we observed that the binding kinetics of a labeled ligand were influenced by the presence of an unlabeled ligand with which the labeled ligand was in competition. We proposed that we could estimate the binding rate parameters of the unlabeled ligand by analyzing the perturbation of binding kinetics of the labeled ligand as a function of the order of addition of these ligands.

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In the present study we have used the computer routine "SAAM" (5–8) to simulate, analyze, and model the kinetics of the competition between a fluorescent formyl peptide and several agonists and antagonists for the formyl peptide receptor on human neutrophils. These analyses take advantage of new, real-time measurements of ligand-receptor interaction using the fluorescence-activated cell sorter (9, 10) and provide high resolution analysis of ligand binding and competition compared to standard radioligand assays which involve a separation step. These approaches are particularly useful in examining competition when the unlabeled ligand is of low affinity, has high nonspecific binding, or has a short residence period at the receptor (compared to the wash step), conditions which would obviate even direct kinetics measurements with those radioligands.

MATERIALS AND METHODS

Cells. Neutrophils, prepared by the method of Henson and Oades (11), were handled as described previously (12). Free radical production by neutrophils was measured by the fluorescent assay described elsewhere (13).

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Reagents. The fluoresceinated hexapeptide, FNLPNTL-FL,[1] and high affinity antibody to fluorescein were prepared and characterized as described (10, 14). Other *N*-formyl peptides and inhibitors were obtained as follows and used without further purification: tBoc-Phe-Leu-Phe-Leu-Phe and *N*-formyl-Nle-Leu-Phe (Vega Biochemicals, Tucson, AZ); *N*-formyl-Met-Phe, and Cbz-Phe-Met (Sigma, St. Louis, MO); *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys (Bachem Fine Chemicals, Torrance, CA).

Cytometry experiments. Both kinetic and equilibrium binding interactions were analyzed by flow cytometry as described previously (9, 10) using the Becton-Dickinson FACS IV.

The essential elements of cytometric analyses of the binding of fluorescent ligands to cell surface receptors are provided here. Neutrophils are selected in flow cytometry on the basis of forward angle and right angle scattering. For analyses of ligand binding, fluorescence histograms of 2500–5000 cells are recorded either as a function of time (15-, 30-, or 60-sec intervals) or as a function of ligand concentration at steady state. In kinetic analyses, the histogram is acquired at a rate of 1000–2000 cells/sec or over an interval of 2–3 sec.

The increase in the mean fluorescence channel number of the histogram, ΔM, in the absence of ligand internalization at 4°, is related to the fractional receptor occupancy LR/R_{TOT} as:

$$LR/R_{TOT} = \Delta M/\Delta M_{SAT} \quad (1)$$

ΔM is the mean fluorescence channel number (as a function of time or ligand concentration) of the specifically bound ligand minus the mean fluorescence channel number of cells exposed to fluorescent ligand under identical conditions but in the presence of excess nonfluorescent ligand (at a concentration of at least 1000 × K_d). The nonspecific or background increase in the mean channel number is typically less than 10% of the specific binding under all of our experimental conditions. ΔM_{SAT} refers to the specific increase in mean channel number at receptor saturation.

The number of receptors per cell was calibrated using Fluorotrol GF (Ortho Diagnostics, Westwood, MA) as described previously (10). Briefly, Fluorotrol GF consists of thymocyte nuclei stained with 0, 20,000, or 72,000 fluorescein equivalents/particle. In our hands, these reflect roughly 0, 35,000, and 140,000 equivalents of fluorescein isothiocyanate (as complexed to the fluoresceinated peptide, Ref. 10). The FACS IV instrumental sensitivity is adjusted so that the brightest Fluorotrol peaks are roughly full scale (i.e., ΔM ~200 channels or 700 fluorescein isothiocyanate equivalents/channel). This calibration is used in the calculation of receptor number for every cell preparation. We have found that cells from the same donor yield values for receptor number which are reproducible to within about 10%. Normal donors were found to have averages of ~50,000 to 200,000 receptors/cell. For cell concentrations of 10^6/ml, the receptor concentration ranges up to 0.4 nM.

Instrumental drift is a factor in our experiments. Over the 4 hr period typically required for real-time cytometric analyses in competitive binding experiment, the instrumental sensitivity has been observed to decay up to 10 to 30%. The drift is monitored by examining both the peak channel values for Fluorotrol and the mean channel values, ΔM_{SAT}, for cells exposed to 3 nM FNLPNTL-FL, maintained at 4°, and re-examined periodically. Drift was found to be generally linear with time. In order to minimize the impact of drift two precautions have been taken. First, the drift is calibrated by periodic examination of the same sample. Second, the several competed and noncompeted binding protocols (see below) are examined in the order I, II, III, and then re-examined in duplicate in reverse order, III, II, I. In general, experiments reflect at least quadruplicate determinations, corrected for instrumental drift, corrected for both kinetic and steady state components of "nonspecific" binding, and calibrated for receptor number. Throughout the course of these experiments, standard deviations of replicate determinations were on the order of "fractional occupancy ± 0.02 to 0.05." The size of the symbols used in the kinetic experiments approximates the typical standard deviations.

[1] The abbreviations used are: FNLPNTL-FL, fluoresceinated *N*-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine; FNLPNTL or F-Nle-Leu-Phe-Nle-Tyr-Lys, *N*-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine; FNLP or F-Nle-Leu-Phe, *N*-formyl-norleucyl-leucyl-phenylalanine; FMP or F-Met-Phe, *N*-formyl-methionyl-phenylalanine; Cbz-Phe-Met, carbobenzyoxy-phenylalanyl-methionine; tBoc-Phe-Leu-Phe-Leu-Phe, tert-butyloxyphenylalanyl-leucyl-phenylalanyl-leucyl-phenylalanine.

SIMULATION OF COMPETITIVE BINDING

Modeling Competitive Binding Experiments

The reversible equilibria between ligand (L) and receptor (R) and competitor (C) and receptor are:

$$(R) + (C) \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} (CR);\ K_{dC} = k_{-1}/k_1 \quad (2)$$

$$(R) + (L) \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (LR);\ K_{dL} = k_{-2}/k_2 \quad (3)$$

Given nonequilibrium, initial concentrations of the reactants, the equilibrium is achieved according to the simultaneous equations

$$d(R)/dt = k_{-1}(CR) - k_1(R)(C) + k_{-2}(LR) - k_2(R)(L) \quad (4a)$$

$$d(CR)/dt = k_1(C)(R) - k_{-1}(CR) = -d(C)/dt \quad (4b)$$

$$d(LR)/dt = k_2(L)(R) - 1_{-2}(LR) = -d(L)/dt \quad (4c)$$

In preliminary calculations (4), we used numerical

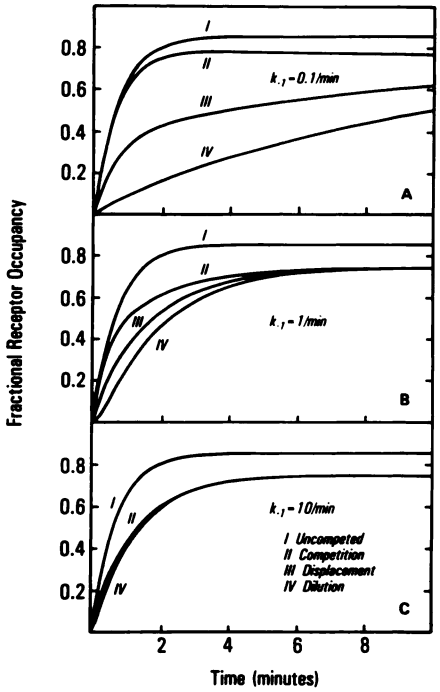

FIG. 1. *Model calculations for competition of 3 nM fluoresceinated ligand and a competitor with K_d = 100 nM.*

The calculations used: L, 3 nM; C, 100 nM; k_2, 4 × 10^8 M^{-1} min^{-1}; k_{-2}, 0.2; K_d, 100 nM. The calculations indicated by I, II, III, and IV correspond to binding, competition, displacement, and dilution. In *A*, k_{-1} = 0.1/min; in *B*, k_{-1} = 1/min; in *C*, k_{-1} = 10/min. When dissociation and association are rapid as in *C*, the competed binding of the fluoresceinated ligand in protocols II, III, and IV are nearly coincident.

integration to simulate the kinetics of competition. These calculations were verified and extended using the routine for compartmentation analysis, SAAM (5–8).

These simulations show that the binding of the fluorescent ligand to the receptor is sensitive to the rate parameters of the unlabeled ligand as well as the order of addition and timing of the fluorescent and nonfluorescent ligands (Fig. 1). The experimental protocols are described below.

Protocol I (*binding*). The kinetics of binding of the fluorescent ligand (L) in the absence of competitor (C) provides information about the on and off rate constants of the ligand (k_2, k_{-2}).

Protocol II (*direct competition*). The kinetics of binding of the fluorescent ligand (L) when the competitor (C) and fluorescent ligand are introduced simultaneously is sensitive to the competitor on rate constant (k_1).

Protocol III (*displacement*). The kinetics of binding of the fluorescent ligand when a prior equilibrium between the competitor and the receptor has been established is sensitive to the competitor off rate constant (k_{-1}). A fourth protocol (dilution) is theoretically very sensitive to k_{-1}. This protocol involves pre-equilibrating a (10-fold) concentrated cell suspension in the presence of (10-fold) concentrated (50–100 K_d) competitor. The binding of FNLPNTL-FL is initiated by a (1:10) dilution of the cell suspension and competitor into the FNLPNTL-FL. This protocol enables receptors to be nearly saturated by competitor when the fluorescent ligand binding is initiated. Binding can occur only as competitor dissociates from the receptor.

Fig. 1 illustrates a representative calculation for a competitor with a fixed K_d. When the competitor has a small off rate (<1/min, panel A) compared to the half-time for ligand binding, the displacement protocol (III) diverges from the competition protocol (II). This occurs because the ligand can bind only after the prebound competitor dissociates. As the off rate constant increases (Fig. 1, B and C), the ligand binding rate in protocol III is less limited by the extent of competitor dissociation.

In contrast, when the on rate constant for the competitor is small, competitor has little effect on the initial rate of ligand binding in competed (II) compared to uncompeted (I) binding protocols (Fig. 1A). As the on rate constant for the competitor increases (Fig. 1, B and C), the competitor reduces the initial binding rate of the ligand by rapidly decreasing the number of available sites.

Values for Dissociation Constants in Equilibrium Competitive Binding Calculations

The dissociation constant of the fluorescent ligand, K_{dL} (0.4×10^{-9} M), was determined directly from equilibrium binding measurements of the fluorescent ligand in the absence of competition (9, 10). The equilibrium values for the binding of the competitors are determined by examination of the fractional receptor occupancy by the fluorescent ligand in equilibrium with various competitor concentrations as shown in Fig. 2 assuming (for simplicity) single classes of binding sites. The fractional occupancy (Y) of receptor by ligand (L) in the presence of competitor (C) is given as

$$Y_{L(C)} = [1 + (1 + (C)/K_{dC}) \cdot K_{dL}/(L)]^{-1} \text{ (Ref. 1)} \quad (5)$$

From the dependence of $Y_{L(C)}$ on (C) a value of K_{dC} is derived. For example, the competitor, tBoc-Phe-Leu-Phe-Leu-Phe, was determined to have a K_{dC} ~500 nM. The stimulatory ligand *N*-formyl-Nle-Leu-Phe had a K_{dC} ~300 nM.

Fitting of Kinetic Data

The kinetics of the uncompeted binding of the fluorescent ligand (protocol I) were simulated simultaneously with the competed binding protocols (II and III, competition and displacement, respectively) using SAAM. All four rate parameters were optimized simultaneously by SAAM without constraint and standard deviations were calculated (Table 1 and figure legends). The values for the fluorescent peptide in Table 1 are averaged from all the determinations (see figure legends).

RESULTS

Equilibrium inhibition. The inhibition of the binding of the fluorescent peptide (3 nM) to neutrophil receptors by several stimulatory and nonstimulatory ligands was evaluated at 4° at equilibrium by fluorescence flow cytometry (Fig. 2). The values of the dissociation constants for several ligands are summarized in Table 1. The stimulatory ligands (Fig. 2, *open symbols*) vary in dissociation constant from <1 nM to 1 μM and overlap the inhibitory ligands which have values >100 nM.

Kinetics of association and dissociation of competitors. We compared the association of fluorescent ligand with neutrophils in the presence of competitors on nine separate occasions. The stimulatory ligands tested were F-Nle-Leu-Phe-Nle-Tyr-Lys (three times), F-Nle-Leu-Phe (once), and F-Met-Phe (twice); the inhibitory ligands were CBZ-Phe-Met (twice) and tBoc-Phe-Leu-Phe-Leu-Phe (once). In each case, the fluorescent ligand concentration was 3 nM (i.e., 8 K_{dL}) and the competitor concen-

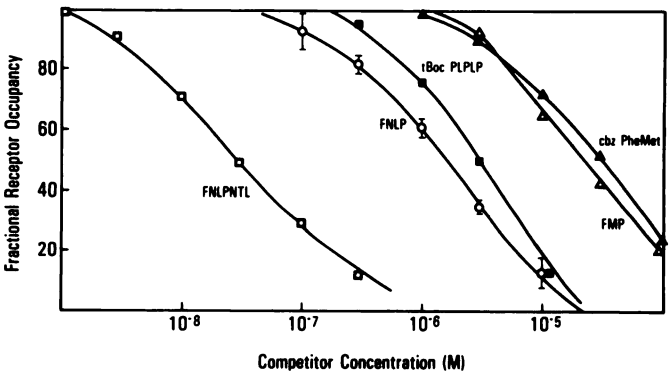

FIG. 2. *Equilibrium inhibition of binding of the fluoresceinated ligand by various concentrations of competitory ligands.*

The data are plotted as the reduction in binding (100% = unblocked) of the fluorescent ligand by the nonfluorescent competitors. The data were obtained from a cytometric analysis. Cells (2×10^6/ml) were exposed to the indicated concentrations of competitor at 4° (15 min) then exposed to 3 nM fluoresceinated ligand (1 hr).

TABLE 1

Summary of binding parameters for ligands for the formyl peptide receptors

Ligand	K_d			k_{on}[a] at 4°	k_{off}[a] at 4°
	Equilibrium[b]	Kinetics[a]	Function		
	nM			$M^{-1}\ min^{-1}$	min^{-1}
Agonists					
FNLPNTL-FL	0.4	0.57 ± 0.1	0.3[c]	$6.1 \pm 1.8 \times 10^8$	0.35 ± 0.12
FNLPNTL	3.0	4.7	4[c]	$5.9 \pm 0.8 \times 10^7$	0.28 ± 0.03
FNLP	300	150	400[c]	$8.6 \pm 1.8 \times 10^{6}$[d]	1.3 ± 0.3
		120		$5.8 \pm 0.9 \times 10^{6}$[e]	0.72 ± 0.11
FMP	3,000	2,200	20,000[c]	$2.3 \pm 0.8 \times 10^6$	5.1 ± 0.6
Antagonists					
CbzPM	4,000	2,400	2,000[f]	$2.8 \pm 1.4 \times 10^6$	6.6 ± 3.8
tBocPLPLP	400	540	100–200[g]	$1.0 \pm 0.2 \times 10^7$	5.4 ± 1.2
			250[f]	$\geq 8 \times 10^{7}$[g]	≥ 10[g]

[a] Based on the simulations shown in Figs. 3, 4, or 6 at 4°.
[b] From Eq. 5 and Fig. 2.
[c] ED_{50} for superoxide production (Fig. 7); 37°.
[d] For 10 min.
[e] For 60 min.
[f] Based on inhibition of cell function at 37°; Ref. 18.
[g] Based on Figs. 8 and 9 at 37°.

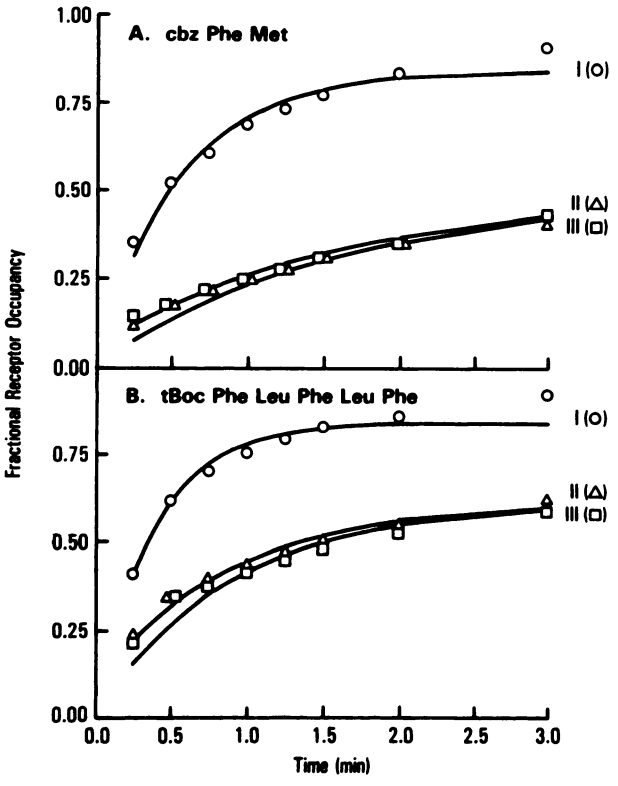

FIG. 3. *The kinetics of the competed binding of the fluoresceinated peptide in the presence of the antagonists Cbz-Phe-Met (A) or tBoc-Phe-Leu-Phe-Leu-Phe (B).*

The data are plotted as the fractional receptor occupancy versus time for binding protocols I (uncompeted), II (competed), and III (displacement, 5- or 60-min preincubation) at 4°. The *solid lines* are the fit to the data. In A, the experimental parameters are L = 3 nM, C = 10 μM, and R = 0.4 nM. The fit values for L are: $k_2 = 5.0 \pm 0.25 \times 10^8\ M^{-1}\ min^{-1}$, $k_{-2} = 0.33 \pm 0.04\ min^{-1}$. In B, the experimental parameters are C = 1 μM and R = 0.25 nM. The fit values for L are $k_2 = 7.2 \pm 0.4 \times 10^8\ M^{-1}\ min^{-1}$, $k_{-2} = 0.49 \pm 0.05\ min^{-1}$. When competitor was allowed to pre-equilibrate for at least 1 hr, protocol III was unchanged.

tration was 5–10 times greater than its own K_{dC}. The data are summarized in Table 1.

The results for the inhibitors are shown in Fig. 3. The similarity of binding in protocols II (competition) and III (displacement) is indicative of systems where the off rate constants are large, >5/min. Regardless of the length of time that inhibitor is allowed to associate with the receptor (up to at least 1 hr), protocol III (displacement) remains unaffected. Thus, there is no detectable change in the inhibitor dissociability as a function of time.

The behavior of the stimulatory ligands F-Nle-Leu-Phe and F-Nle-Leu-Phe-Nle-Tyr-Lys differs markedly from the inhibitory ligands in two respects (Fig. 4, A and B). First, the competition protocols II and III diverge strongly. The binding data are consistent with off rate constants in the range of <1/min. Second, the longer the ligands associated with the receptor, the slower the ligand binding in protocol III (displacement) (Fig. 4C). This loss of dissociability occurs with a half-time of approximately 60 min and the off rate is reduced considerably.

This loss of dissociability at 4° can be observed directly with fluorescent ligand as shown in Fig. 5. This occurs while the ligand-receptor complex is trapped on the surface because internalization is inhibited at 4° (10). The change in off rate appears to be considerably more rapid at physiological temperatures and may reflect an interaction of the ligand-receptor complex with the biochemical apparatus responsible for cell function (10).

The peptide F-Met-Phe, a low affinity stimulus, is anomalous, as judged in Fig. 6. The convergence of protocols II and III, under several experimental conditions, is consistent with a competitor off rate constant >5/min.

To understand the implication of this anomalous behavior, the ability of all four agonistic peptides to stimulate free radical production at 37° (a response which involves nearly all the cell surface receptors) (15) was

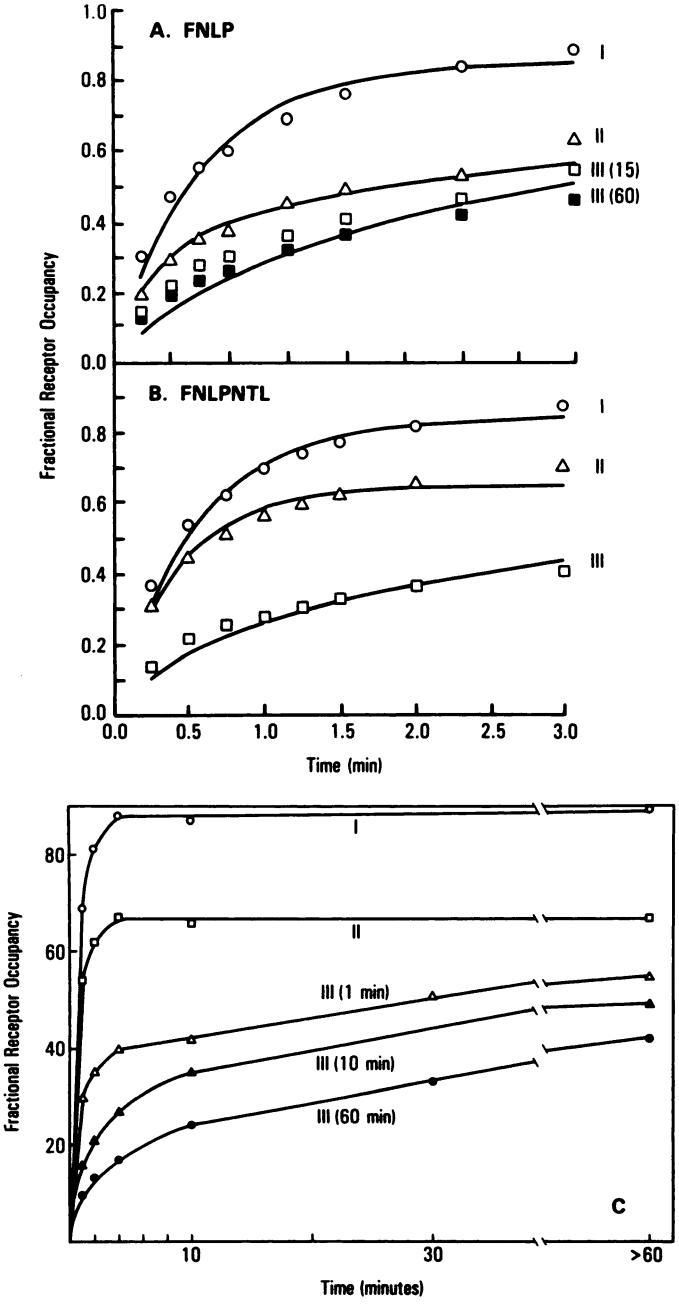

FIG. 4. *The kinetics of the competed binding of the FNLPNTL-FL in the presence of the agonists F-Nle-Leu-Phe (A) and F-Nle-Leu-Phe-Nle-Tyr-Lys (B and C).*

The data are plotted as fractional receptor occupancy versus time at 4° for the binding protocols I, II, and III. The *solid lines* are the fit to the data in A and B. In A, the experimental parameters are C = 300 nM, R = 0.28 nM, and L = 3. The fit parameters are $k_2 = 4.0 \pm 0.2 \times 10^8$ M^{-1} and $k_{-2} = 0.19 \pm 0.03$ min^{-1}. Displacement protocols (III) used 15 or 60 min of preincubation. In B, the experimental parameters are L = 3 nM, C = 10 nM, and R = 0.42 nM. The fit parameters are $k_2 = 5.5 \pm 0.2 \times 10^8$ M^{-1} min^{-1} and $k_{-2} = 0.25 \pm 0.04$ min^{-1}. Displacement protocol (III) used 5 min of preincubation of competitor. In C the competition with FNLPNTL, performed on cells on a separate occasion, is shown for periods of pre-equilibration of 1, 10, and 60 min. The early time points in C, not shown, are similar to B.

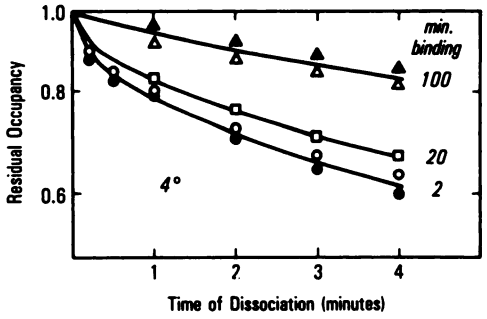

FIG. 5. *The dissociability of FNLPNTL-FL (3 nM) shown as a function of the time of pre-equilibration (2, 20, and 100 min) at 4°.*

The data are plotted as the residual receptor occupancy (using a value of 1.0 at the time dissociation was initiated) as a function of time following the addition of 1 μM FNLPNTL (*open symbols*) or 25 nM antibody to fluorescein (*closed symbols*).

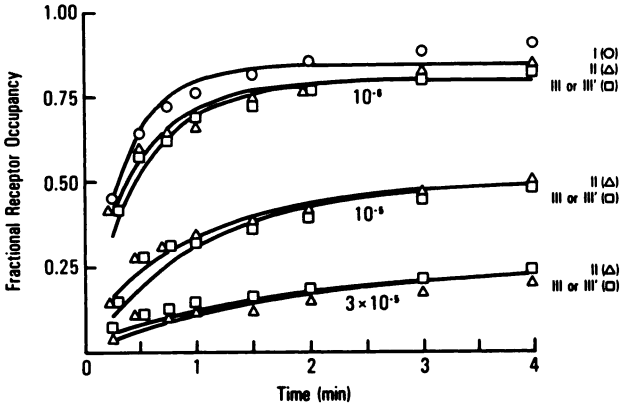

FIG. 6. *The competed binding of the FNLPNTL-FL in the presence of F-Met-Phe.*

The data represent two pooled experiments on cells from the same donor in which there were six determinations of protocol I; for C = 1 or 10 μM, there were four determinations of II, four determinations of III (5 min), two determinations of III (1 hr-preincubation); for C = 30 μM there were duplicate determinations of protocols II and III each. The *solid lines* are the fit to the data. The experimental parameters are L = 3 nM and R = 0.44 nM. The fit parameters are $k_2 = 8.8 \pm 0.6 \times 10^8$ M^{-1} min^{-1} and $k_{-2} = 0.45 \pm 0.07$ min^{-1}. If the data of protocols II and III differ by ≤0.01 the *symbols* are shown side by side.

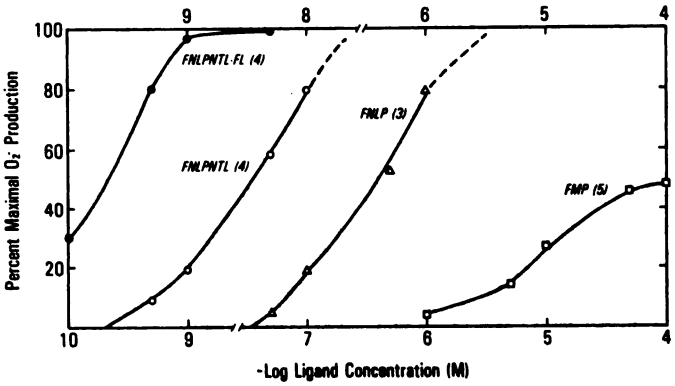

FIG. 7. *The dose-response curves for the production of superoxide anion by agonists at 37°.*

The production is compared to a saturating dose (3 nM) of FNLPNTL-FL. The numbers in parentheses represent the number of donors examined, with at least duplicate determinations for each dose.

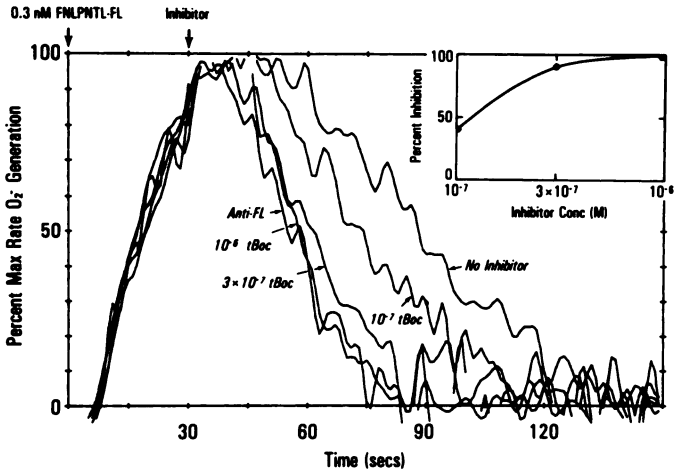

FIG. 8. *The kinetics of inhibition of superoxide production at 37° by antibody to fluorescein or tBoc-Phe-Leu-Phe-Leu-Phe (tBoc) when the stimulus is the fluoresceinated peptide.*

The data are plotted as the rate of production (the first derivative) versus time. In each case, the inhibition was provided at $t = 30$ sec, a time sufficient to permit an optimal rate of production for 0.3 nM FNLPNTL-FL.

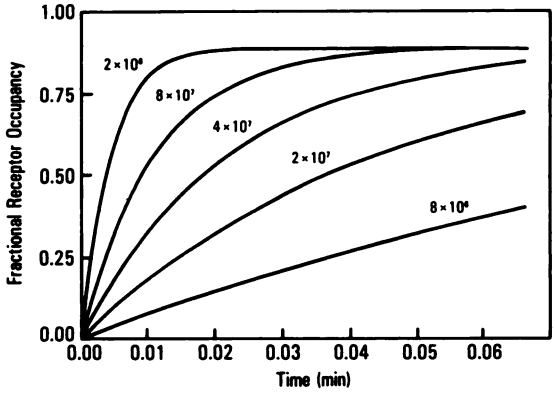

FIG. 9. *The calculated dependence of the kinetics of association of tBoc-Phe-Leu-Phe-Leu-Phe on the kinetic rate constants at 37°.*

The fractional receptor occupancy as a function of time for 1 μM tBoc-Phe-Leu-Phe-Leu-Phe (K_d = 125 nM) is calculated for several off rate constants (25, 10, 5, 2.5, 1/min).

examined (Fig. 7). The fluorescent peptide, F-Nle-Leu-Phe-Nle-Tyr-Lys, and F-Nle-Leu-Phe all elicited half-maximal responses at concentrations similar to their K_d. In contrast, F-Met-Phe elicits only half-optimal responses even at >10 K_d. F-Met-Phe at 37° by this criterion is a partial agonist. At 4°, however, no evidence of induced receptor conformational change is detected.

Independent estimates of the on rate constant for inhibitory ligands. In order to verify the kinetic parameters for the antagonists, we have developed an independent analysis based on the kinetics of inhibition of cell function normally stimulated by the fluoresceinated ligand. Neutrophils generate superoxide anion within seconds of the binding of *N*-formyl peptides (16, 17). We can deliver a "pulse" of stimulation in which cells are exposed to the fluoresceinated ligand and stimulation is interrupted by a high affinity antibody to fluorescein (14, 15, 18). The antibody works by complexing the ligand in solution with a half-time of 1 sec and inhibiting further binding to the receptor. The superoxide generation stops prior to agonist dissociation, while agonist remains receptor-bound but with the receptor in an inactive state (15).

In order to estimate the competitor on rate constant (k_1) on the basis of function, we examined how rapidly cellular responses were inhibited by tBoc-Phe-Leu-Phe-Leu-Phe. Since at 37° the ID_{50} for superoxide production is 100–200 nM (*inset*, Fig. 8), we used concentrations of the antagonist (up to 10 K_{dC}) at which it is effective in inhibiting cell response and where it is expected to nearly saturate (in this case, reversibly occupy) the receptors (19). We find that the inhibition of function by tBoc-Phe-Leu-Phe-Leu-Phe at 10^{-6} M is as rapid as antibody to fluorescein (Fig. 8). Since the antibody has its effect by complexing the fluorescent peptide in solution in a period of 2 sec or less (14), we calculated the association rate constant for tBoc-Phe-Leu-Phe-Leu-Phe which was required for it to occupy the receptors over a few seconds (Fig. 9). Greater than 90% of the available receptors will be occupied in 2 sec if $k_1 > 8 \times 10^7$ M^{-1} min^{-1} (i.e., $k_{-1} > 10$ min^{-1}). Thus, the speed of the functional impact of the inhibitor is consistent with the large dissociation rates determined directly from the competitive binding experiments.

DISCUSSION

Rate parameters for unlabeled ligands. Our goal was to define experimental conditions for the analysis of the rate parameters of unlabeled competitors. Using as a standard a fluoresceinated hexapeptide, several experimental protocols were found to provide information about the rate constants of a competitor (Fig. 1). Protocols in which competitor is prebound to the receptor (displacement, III; and dilution, IV) are influenced by the competitor off rates because the ligand can bind only after the competitor dissociates. Since our ligand binds with a half-time of ~1 min, its binding kinetics are sensitive to the presence of competitors which dissociate over a similar time frame ($k_{-1} < 10$/min). In contrast, if ligand and competitor are added to the receptors simultaneously (II, competition), then the initial rate of ligand association [(L)(R) × k_2] will be influenced by the rate at which the receptors are depleted by the competitor [(C)(R) × k_1]. If ligand association is considerably more rapid, the initial binding in protocol II resembles the uncompeted protocol (I). As both on (k_1) and off (k_{-1}) rate constants increase for a given competitor affinity, protocol II diverges from protocol I; when k_{-1} exceeds 10/min, protocols II, III, and IV (dilution) are nearly indistinguishable.

Comparison of agonists and antagonists (*see Table I*). For stimulatory ligands the K_d values obtained from equilibrium binding (Fig. 2) are comparable to the ED_{50} values for superoxide production (Fig. 7) or the K_d values obtained from the kinetic analyses (Fig. 4) (with the exception of the partial agonist F-Met-Phe). For the inhibitors, the K_d values obtained from equilibrium binding (Fig. 2) or kinetics analyses (Fig. 3) and the ID_{50} values for inhibition of cell function (Figs. 8 and 9) are comparable. Binding experiments were performed at 4° to avoid the complication of agonist internalization.

Full agonists have three characteristics in common. First, agonists are long lived at the receptor at 4° (Fig. 4). Second, a loss of dissociability is observed at 4° when the ligand is allowed to interact with the receptor for periods longer than a few minutes. These effects are observed directly in the uncompeted binding and dissociation of F-Nle-Leu-Phe-Nle-Tyr-Lys-FL (Fig. 5). They are also observed in competed binding on the basis of the divergence between displacement (III) and competition (II) protocols and by the loss of dissociability as a function of time (Fig. 4).[2] Third, the differences in K_d are accounted for largely by different on rate constants (Table 1). These observations, taken together with parallel shifts in ED_{50} for free radical production (a response which involves occupancy of nearly all the receptors for the agonist F-Nle-Leu-Phe-Nle-Tyr-Lys-FL; Ref. 15), imply that the efficacies of these ligands are comparable.[3]

The behavior of the antagonists is markedly different. There is no loss of dissociability with time and there is no discernible difference between displacement and competition protocols (Fig. 3). This latter phenomenon is indicative of short residence periods (seconds). The kinetic parameters have been confirmed by analysis of the rate at which one antagonist, tBoc-Phe-Leu-Phe-Leu-Phe, inhibits cell function at 37° (Figs. 8 and 9). At concentrations approaching 1 μM, the action of inhibitor is as rapid as antibody to fluorescein. The antibody, which inhibits ligand binding to the receptor, requires no more than 1–2 sec to fully complex free ligand. Effective kinetic competition by the antagonists can occur only when its on and off rate constants are large enough so that free receptors are saturated over a similar time period. In this case, we calculate that the off rate constant (k_{-1}) at 37° must be >10 min^{-1}.

The behavior of F-Met-Phe is anomalous. At 4° it has a short residence period (like the antagonists, Fig. 6), but at 37° it has partial agonist character (Fig. 7). It may be that at 37° it partially fulfills the structural requirements for an agonist which leads to cell stimulation, loss of ligand dissociability, receptor internationalization, and receptor down-regulation. These questions have yet to be investigated.

Reliability of the rate parameters. The simplest possible binding model has been used to fit the data. Since internalization is limited at 4° we have used reversible (single component) equilibria for both agonist- and antagonist-receptor interactions. This model does not explicitly take into account the time-dependent loss of agonist dissociability. One consequence of this model is that the observed uncompeted binding of the fluorescent ligand continues to rise compared to the calculated fit since ligand is "trapped" by receptor in a more slowly dissociating state. This second phase of nonexponential uncompeted binding (in protocol I, Figs. 3, 4, and 6) contributes up to an additional 10% of the receptors.

Since the loss of dissociability is relatively slow compared to the half-time for binding of the fluorescent ligand or the half-time of dissociation for the antagonists, the reversible model approximates antagonist behavior. However, the values of antagonist off rates (k_{-1}) generated by SAAM ($\geq$5/min) must be viewed as minimal values. When k_{-1} is 10/min (compare Figs. 1C and 3B), protocols II and III are nearly coincident. The fits for protocols II and III generated by SAAM in Figs. 3 or 6 do not superimpose eventhough the experimental data do. Increasing k_{-1} to be $\geq$10/min in Fig. 3 causes protocol II to converge toward protocol III without a marked impact in the overall fit to the data (not shown). We have not attempted to influence the weighting of data by SAAM to place relatively more emphasis on the convergence of data in protocols II and III than on the overall point-by-point fit.

We are now attempting to generate a more complete compartmental model of agonist binding which resolves the possible interconvertibility of (both occupied and unoccupied) receptors between states of differing dissociability (20). The data of Fig. 4, A and C (compare protocol III for 15 and 60 min which are barely separated in A and for 10 and 60 min in C which are widely separated), raise the possibility that the rate of interconversion is ligand-dependent. For data generated by the simple model the calculated off rate parameters for the agonists reflect weighted averages of the dissociation rates for interconverting states. Nonetheless, it is clear that very slow dissociation rates ($\ll$1/min) are detectable after prolonged ligand-receptor contact.

Generality of the procedures to analyze the binding of unlabeled competitors to receptors. The experimental protocols derived here are based on the application of a high affinity fluorescent ligand competing with unlabeled ligands of varying affinities. Several elements of the analysis are particularly appropriate for the neutrophil *N*-formyl peptide system. First and foremost, the analysis of binding of the fluoresceinated ligand is based on a continuous, homogeneous binding assay made possible by the fluorescence-activated flow cytometer. As pointed out previously, the cytometer discriminates between dilute ligand in solution (<10 nM) and receptor-bound ligand (10, 11). Such analyses are appropriate for ligands with $K_d < 10$ nM and cells with 50,000 or more receptors. With lower affinity ligands, the high fluorophore concentrations which are required cause the cell suspension to become so highly fluorescent that the cytometer fails to discriminate effectively between free and bound ligand.

The second element of these studies relates to the particular properties of the ligand used. *N*-formyl-Nle-Leu-Phe-Nle-Tyr-Lys-FL binds to its cellular receptors at 4° with a half-time of 1 min or less over the concentration range of interest. This time scale makes it possible to approach equilibrium within a few minutes. Binding curves acquired with a resolution of a few seconds provide data sufficient for a detailed analysis of

[2] The calculated off rate constants for the agonists at 4° ($\leq$1/min) reflect a weighted average dissociation from slowly interconverting states. At 37° the conversion is considerably more rapid (10, 20) and the bulk dissociation is governed by the slowly dissociating state. It is fortuitous that the bulk dissociation rates at 4° (governed at early times by the first state) and at 37° (governed by the second state) are similar.

[3] If the on rate constants of the agonists have similar temperature dependencies, it would imply that similar numbers of receptors are required for each of the full agonists to elicit superoxide anion production.

binding under the competed and noncompeted protocols. While such an approach has general applicability, the rate constants for the particular ligand will largely determine the time frame of the competitive binding. If the time frame is long, then it will be possible to use standard radiolabel binding assays.

One feature of these present analyses which may not be entirely generalizable to other systems is the utility of cellular activation as a probe of antagonist interaction with the receptor. The neutrophil *N*-formyl peptide receptor is uniquely suited to these experiments in at least two ways: 1) the cell responses to ligand binding are very rapid; 2) the cell responses to ligand binding may be interrupted, in time, by interrupting ligand binding. In the neutrophil, the binding of the fluoresceinated *N*-formyl peptide to its receptor may be interrupted in two ways. The peptide can be complexed in solution by antibody to fluorescein with a $t_{1/2}$ <1 sec (10, 14, 20). Alternatively, its binding to the receptor can be interrupted by saturating the receptor with antagonist. By using antagonist concentrations which inhibit responses as fast as the antibody, an estimate of the rate of antagonist binding can be made. These measurements require parallel analyses of cell function with the same time resolution (14, 17).

CONCLUSIONS

Methods have been developed to analyze in real-time the kinetic aspects of the binding of unlabeled ligands. These methods have been used in conjunction with static methods. Based on the K_d values (which vary between 1 nM and 1 μM) and on the association rate constants (which vary between 10^6 and 5×10^8 M^{-1} min^{-1}), there is no clear distinction between stimulatory and inhibitory ligands for the formyl peptide receptor. However, the off-rate constants seem to provide a measure of those properties. Full agonists reside at this receptor for minutes, even at 4°, and show a time-dependent loss of dissociability. At 37°, these ligands undergo a more rapid loss of dissociability, internalization, and induce a modulation of receptor number (21–23). The prolonged residence may reflect an induced conformational change in the receptor characteristic of the process of cell activation. Antagonistic ligands reside only transiently at the receptor both at 4° and 37°. One low affinity partial agonist with respect to cell stimulation at 37°, F-Met-Phe, exhibits antagonistic binding behavior at 4°.

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Send reprint requests to: Dr. L. A. Sklar, Department of Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Rd., La Jolla, CA 92037.