# Kinetic Analysis of Chemotactic Peptide Receptor Modulation

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ABSTRACT    The dynamics of the chemotactic peptide receptor on rabbit peritoneal polymorphonuclear leucocytes were followed using the tritiated peptide N-formylnorleucylleucylphenylalanine (FNLLP). We have used a kinetic analysis to examine the possible interrelationships between receptor loss (down-regulation), receptor-mediated peptide uptake, and receptor recycling. We have previously demonstrated that cells incubated with FNLLP show a dose-dependent reduction in the number of receptors available on the surface. This receptor down-regulation is complete within 20 min and then the number of receptors available for binding remains at a plateau level. Peptide continues to be taken up in a receptor-mediated manner even after down-regulation is complete. If peptide is removed, receptor recovery occurs and does not require protein synthesis. In these studies we have investigated the kinetics of these processes. On the basis of this analysis, we propose that the plateau receptor level is a steady-state in which receptor internalization and return occur continuously. We demonstrate that the rate of receptor-mediated peptide uptake is approximately equal to the rate of receptor recovery measured after peptide removal. In addition, the rate of receptor recovery is proportional to the number of receptors missing from the surface, suggesting receptor recycling may be occurring.

In the process of endocytosis, plasma membrane components are believed to move into an internal membrane pool and then to recycle back to the plasma membrane (12, 13, 19, 21, 23). Recycling has also been suggested for several receptors including those for the low density lipoprotein (LDL) (3, 8, 18), mannose-6-phosphate (9), mannose glycoconjugates (22), and $\alpha_2$-macroglobulin (13). In these systems the receptors are believed to be internalized in the process of receptor-mediated pinocytosis of the ligand that is being transported into the cell (4). And yet receptors remain available on the surface. Since the amount of ligand transported exceeds the available receptor number, the receptors are believed to recycle. In other systems the binding of a polypeptide hormone to its receptor induces a reduction in the number of the receptors available on the surface; the receptor loss has been termed receptor down-regulation (5, 10, 11, 15). The mechanism of receptor loss is also believed to be pinocytosis (6).

With the N-formylated chemotactic peptide receptor in polymorphonuclear leucocytes (PMNs) we see receptor-mediated endocytosis, receptor down-regulation, and possibly receptor recycling (1, 24). We believe that these processes are interrelated.

We have previously shown that when rabbit peritoneal PMNs are first incubated with a given concentration of peptide, they decrease their receptor number, i.e., exhibit down-regulation. However, the receptor number soon stabilizes at a plateau level which is characteristic for a given concentration of peptide (24). This plateau could result from a transient response to addition of peptide, e.g., if receptors are internalized for only a limited period of time after peptide addition. Alternatively, the plateau level may result from a steady-state due to continual receptor internalization along with an equal rate of receptor insertion into the membrane.

When the peptide is removed, the receptor number on the surface increases again. This increase occurs even in the presence of cycloheximide (24), indicating that new synthesis of receptors is not required.

At the time of receptor loss, cells accumulate peptide that can not be removed by a 5-min wash, which normally removes >90% of receptor-bound peptide (24). Peptide continues to accumulate after the receptor number has reached its plateau level. This uptake is believed to be via both receptor-mediated and bulk-fluid-phase pinocytosis (20, 24). The amount of peptide taken up in a receptor-mediated manner can exceed the

number of receptors present on the surface at any time by severalfold. Thus, if the receptor-mediated uptake involves internalization of a receptor-peptide complex, the surface receptors must be replaced. They could be replaced either from a pool of spare receptors or by recycling of the original receptors.

After studying the kinetic rate expressions and parameters governing these processes, we propose that the leucocyte interaction with the chemotactic peptide might involve the features pictured in Fig. 1. The peptide binds to free receptors, $R$, with forward rate constant $k_1$ and dissociates from the complex, $C$, with reverse rate constant $k_{-1}$. Bound receptors may be internalized by the cells with the rate constant $k_i$, which is equal to the observed rate constant for receptor-mediated peptide uptake. Free peptide is taken up into the pinocytic vesicles, with the observed rate constant $k_u$. Once inside the vesicles, peptide is degraded and released, with an observed rate constant $k_d$. Peptide may also be partitioned into a nonreleasable pool, with an observed rate constant $k_p$. The (free) receptors in the vesicles may be recycled back to the cell membrane, with an observed rate constant $k_r$.

Down-regulation, the decrease in the number of surface receptors with increasing extracellular peptide concentrations, can be explained by the mechanisms in Fig. 1. Upon incubation of the cells in extracellular peptide of concentration $A_0$, the receptors go through the cycle of binding, internalization, dissociation, and return to the surface. After a while, a time-independent steady-state is reached.

In this report we will present data to support the general aspects of the model: (a) We have obtained estimates for the rate constants of receptor-mediated peptide uptake, fluid uptake, peptide release and partitioning, and receptor recovery. (b) The rate of receptor removal via receptor-mediated peptide uptake is shown to be approximately equal to the rate of

receptor recovery. This is consistent with the proposal that the plateau receptor number is due to a steady-state. (c) The rate of receptor recovery is shown to be proportional to the number of receptors missing from the surface; this suggests the recovery process acts on a pool of internalized receptors or some related pool. (d) The rate constant of receptor recovery is shown to be a function of the concentration of the extracellular peptide present during receptor loss. Both the rate constant and the final extent of recovery decrease with increasing concentrations of peptide. These results could indicate that only free (unoccupied) receptors recycle back to the surface. High extracellular peptide concentrations result in occupancy of an increased proportion of the internalized receptors, thereby diminishing recycling. (e) Finally, the peptide taken up is shown to be partitioned into at least two pools: one gets released, the other remains in the cell. This partitioning is similar for peptide taken up in a receptor-mediated and a nonsaturable fashion.

## MATERIALS AND METHODS

### Cells

Rabbit peritoneal exudate cells were collected 4 h after injection of 0.1 g of shellfish glycogen (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) in 250 ml of 0.9% saline. Contaminating erythrocytes were lysed by brief (60 s) treatment with hypotonic (0.18%) saline. The cells were washed twice with 0.9% saline and then resuspended in Hanks' balanced salt solution (HBSS) (Grand Island Biological Co., Grand Island, N. Y.) buffered with 2.4 mg/ml of HEPES (Sigma Chemical Co., St. Louis, Mo.) at a concentration of $3.3 \times 10^6$ cells/ml. 3 ml of the cell suspension was placed in $60 \times 15$ mm petri dishes (Falcon Labware, Div. Becton, Dickinson & Co., Oxnard, Calif.) and the cells were allowed to settle for 15 min at 23°C. Dishes were checked with an inverted microscope (Olympus) to ensure that the cells had formed an even monolayer.

### Binding Studies

Each dish containing $10^7$ cells was incubated in 0.5 ml of HBSS with the appropriate concentration of tritiated (12.5 Ci/mM) $N$-formylnorleucylleucylphenylalanine (FNLLP). After incubation, this medium was aspirated and the dish washed quickly (6 s) and vigorously in two baths of 4°C saline. Cell-associated radioactivity was measured by adding 0.5 ml of 0.1 N NaOH to each dish. The cells were scraped into this fluid with a rubber policeman and then each dish was washed with 0.5 ml of distilled water. This 1 ml of cell suspension was counted in 15 ml of scintillation fluid Formula 963 (New England Nuclear, Boston, Mass.) with an efficiency of ~40%. In preliminary experiments, Lowry assays (17) were done to confirm that cells were not lost from any of the dishes during the treatment. In all experiments the dishes were monitored visually for cell loss.

### Peptide Uptake and Release

Cells were incubated with $^3$H-FNLLP at 37°C. At the end of the incubation, total peptide uptake was measured as the amount of radioactivity remaining cell associated after a 5-min wash at 4°C. A 5-min wash has been shown to remove >90% of the receptor-bound peptide (24). Nonsaturable uptake was determined from the amount of $^3$H-FNLLP uptake in the presence of $10^{-5}$ M unlabeled FNLLP. To measure release of the cell-associated radioactivity, the cells were incubated at 37°C in HBSS after the five-min wash. After various times at 37°C, the medium was removed, and the tritium in both the medium and the cells was counted.

### Pinocytosis

Fluid uptake was measured by the uptake of tritiated sucrose ($10^{-6}$ M, 11.2 Ci/mM; New England Nuclear) or fluorescein isothiocyanate-labeled dextran, (5 mg/ml; Sigma Chemical Co.) from the medium. Cells were incubated with the probes in the presence or absence of unlabeled FNLLP. At the end of the incubation, the medium was aspirated and the cells were washed for 5 min at 4°C. Cell-associated radioactivity was assayed as described previously; the fluorescence was read in a Aminco-Bownan spectrofluorometer exciting at 480 nm and reading at 520 nm.

### Peptide Digestion

The amount of peptide digestion has been assayed using ethyl acetate extrac-
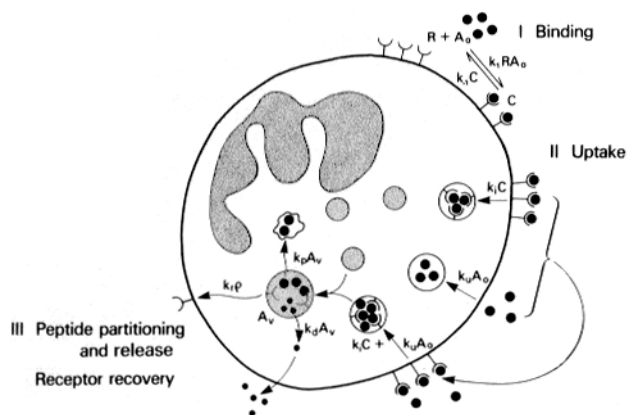


FIGURE 1 Model of hypothesized receptor and peptide movements. The receptor dynamics are separated into three steps: (I) Binding: the peptide at external concentration, $A_0$, binds to the receptor at concentration, $R$, with an on rate $k_1$ and a dissociation rate of $k_{-1}$. (II) Internalization: peptide (and receptors) are internalized by receptor-mediated pinocytosis at a rate equal to the concentration of bound receptors, $C$, times the observed rate constant of receptor-mediated peptide uptake, $k_i$; peptide is also internalized by bulk fluid phase uptake at a rate equal to the concentration of peptide, $A_0$, times the observed rate constant of bulk fluid uptake $k_u$. (III) Partitioning, digestion, and receptor recovery: the internalized peptide in a vesicular compartment $A_v$ is partitioned into a nonreleasable pool with an observed rate constant $k_p$ or released with an observed rate constant $k_d$. The internalized receptors are cycled back to the surface with an observed rate constant $k_r$.

tion and/or thin-layer chromatography (TLC). For ethyl acetate extraction, the medium was brought to pH 2 with 1 N HCl, and an equal volume of ethyl acetate is added. After thorough mixing, the solution was allowed to separate into two phases. The undigested FNLLP partitioned into the ethyl acetate (top) and the phenylalanine (Phe) remained in the aqueous (bottom) phase. The radioactivity (labeled [³H]Phe) in each of the phases was then counted.

To determine the amount of digestion by means of TLC, an aliquot was spotted onto a precoated silica gel 60 F₂₅₄ TLC sheet (EM Reagents, Darmstadt, FRG). The TLC was run in a chloroform:methanol:acetic acid:water (60:30:4:1) solvent. It was then cut into ¼-inch strips which are counted. The ratio to the front of the undigested peptide was ~0.7 and that of the Phe peak was ~0.2.

## Down-regulation

Cells were incubated with unlabeled FNLLP for various lengths of time at 37°C. At the end of the incubation, the medium was aspirated and the cells were washed for 5 min at 4°C with chilled saline (5 × 2 ml washes). The rebinding capacity was measured by adding 0.5 ml of HBSS with tritiated FNLLP for 15 min at 4°C. At the end of this incubation, the cells were washed quickly as outlined above. For receptor recovery experiments, the cells were incubated with unlabeled peptide for 20 min at 37°C and washed for 5 min. Cells were then incubated in HBSS for various times at 37°C before being assayed for rebinding. Control cells were preincubated without peptide but were subjected to the same washing procedure.

## RESULTS

### Receptor-mediated Peptide Uptake

Peptide uptake can be divided into two classes: nonsaturable and receptor-mediated (24). Nonsaturable uptake is directly proportional to the concentration of peptide in the medium. Receptor-mediated uptake is saturable and shows the same concentration dependence as receptor binding. In addition, it is competitively inhibited by unlabeled FNLLP and the competitive antagonist carbobenzoxyphenylalanylmethionine.

To determine the rate constant for the receptor-mediated peptide uptake, the total amount of ³H-FNLLP accumulated in the cells in different peptide concentrations at 37°C was measured between 15 and 60 min. During this period the receptor number is at the plateau level characteristic of that peptide concentration. The nonsaturable uptake was measured by the accumulation of $10^{-8}$ M ³H-FNLLP in the presence of $10^{-5}$ M unlabeled FNLLP over the same time period. The rate of receptor-mediated uptake was then calculated from the difference between the total and the nonsaturable uptake. Rates of receptor-mediated peptide uptake are shown in Table I.

There is a linear relationship between the rate of receptor-mediated uptake and the number of peptide receptors occupied

at a given concentration (Fig. 2). The number of occupied receptors, $C$, is determined from the equation $C = A_0S/(A_0 + K_d)$, when $A_0$ is the concentration of extracellular peptide, $K_d$ is the dissociation constant $(2 \times 10^{-8}$ M), and $S$ is the number of receptors available on the cell surface at plateau in that peptide concentration. $S$ has been previously determined for each external concentration (25). Initially, there are $\sim5 \times 10^4$ receptors available per cell. The percent remaining at plateau is $63 \pm 5$ (mean $\pm$ SEM; $n = 13$) in $1 \times 10^{-8}$ M FNLLP, $90 \pm 10$ $(n = 6)$ in $3 \times 10^{-9}$ M, $96 \pm 10$ $(n = 3)$ in $1 \times 10^{-9}$ M, and $100$ $(n = 2)$ in $3 \times 10^{-10}$ M. The linear relationship between the rate of uptake and receptor occupancy seen in Fig. 2 suggests that if interaction between bound receptors, such as clustering, is required for peptide uptake, it is not the rate-limiting step in this process.

From the slope of the plot in Fig. 2 we estimate the observed rate constant for the receptor-mediated uptake to be $0.13$ min$^{-1}$. However, these uptake data have not been corrected for release of cell-associated peptide that occurs over this time. Cells preloaded with labeled peptide and washed, do release radioactive counts into the medium. Thus an accurate estimate of the forward rate constant of uptake requires correction for release. The time-course of peptide loss from cells and the corresponding accumulation of tritium in the medium is shown in Fig. 3. The amount of peptide taken up during the 30-min incubation period is dependent on the concentration of extracellular peptide. However, when the cells are placed in fresh medium, roughly 40% of the accumulated counts is released over the next 30 min, regardless of the extracellular peptide concentration during preloading (Fig. 4).

At $1 \times 10^{-5}$ M, 99% of the peptide uptake occurs via the nonsaturable process, whereas at $1 \times 10^{-8}$ M $\sim90\%$ of the peptide uptake occurs via a receptor-mediated process. Since the cells release a similar fraction of the accumulated peptide in both cases, the processing of peptide taken up by the two means must be similar. This is clearly seen in Fig. 5, which shows that the logarithm of the percentage of counts released within 60 min (total released in 60 min minus the number of counts released at a given time divided by the total released) is a linear function of time. Thus the release is a pseudo first-

### TABLE I

#### Peptide Uptake

| Peptide concentration | Total uptake* | Nonsaturable uptake‡ | Saturable uptake§ |
|---|---|---|---|
| M | cpm/2 × 10⁷ cells | cpm/2 × 10⁷ cells | cpm/2 × 10⁷ cells |
| $1 \times 10^{-10}$ | 86 | 18 | 68 |
| $3 \times 10^{-10}$ | 855 | 46 | 809 |
| $1 \times 10^{-9}$ | 4,500 | 138 | 4,362 |
| $3 \times 10^{-9}$ | 10,000 | 413 | 9,587 |
| $1 \times 10^{-8}$ | 138,000 | 1,240 | 136,760 |
| $10^{-5}$ (0.001 sp act) | 1,240 | 1,240‖ | |

* Uptake was measured over the 45 min between 15 and 60 min after peptide addition.
‡ Uptake in the presence of $10^{-5}$ M unlabeled peptide. Actual measurement was done with $1 \times 10^{-8}$ M ³H-labeled peptide.
§ Total uptake minus nonsaturable uptake.
‖ This is uptake of $10^{-8}$ M tritiated peptide in the presence of $10^{-5}$ M unlabeled peptide. The assumption is that essentially all of this uptake is via the bulk fluid phase, i.e., nonsaturable.
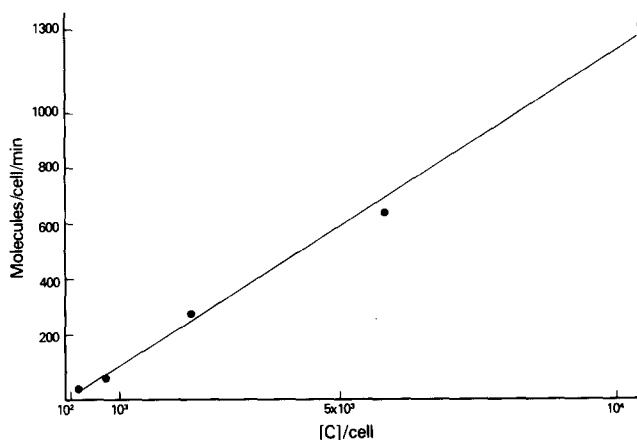
FIGURE 2  Receptor-mediated peptide uptake as a function of receptor occupancy. The rate of peptide uptake was measured after incubating the cells at 37°C in $1 \times 10^{-10}$, $3 \times 10^{-10}$, $1 \times 10^{-9}$, $3 \times 10^{-9}$, and $1 \times 10^{-8}$ M ³H-FNLLP. The number of receptors bound per cell at these concentrations was calculated from the number of receptors remaining on the cell surface at plateau and the $K_d$ of binding, which is $2 \times 10^{-8}$ M.
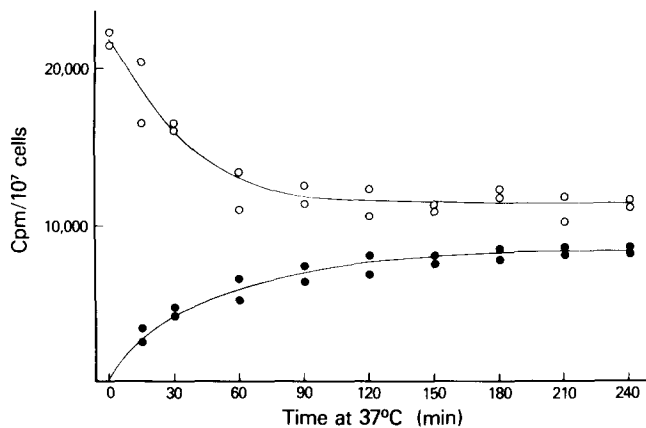
FIGURE 3 Release of cell-associated peptide. The cells were incubated in $2 \times 10^{-8}$ M $^3$H-FNLLP for 30 min at 37°C and washed for 5 min at 4°C to remove the peptide bound to surface receptors. They were then incubated in HBSS for various lengths of times at 37°C. The radioactivity remaining cell associated (O) and that released into the medium (●) were determined.
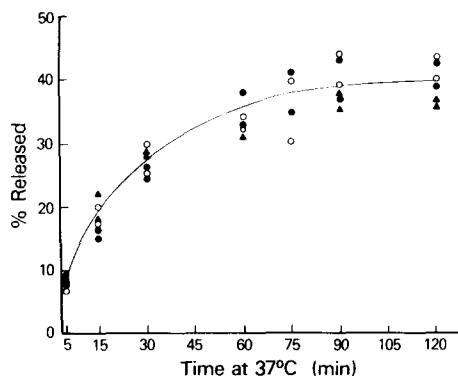


FIGURE 4 Release of cell-associated peptide at different concentrations. Cells were incubated for 30 min at 37°C in $2 \times 10^{-8}$ M $^3$H-FNLLP (O, ●) or with $2 \times 10^{-8}$ M $^3$H-FNLLP and $1 \times 10^{-5}$ M unlabeled FNLLP (▲) and then washed for 5 min at 4°C. They were then incubated in HBSS (O, ▲) or $2 \times 10^{-8}$ M unlabeled FNLLP (●). The percent of cell-associated counts released was plotted as a function of time.

order process and the observed rate constant is independent of the concentration present during either the uptake or the release.

Only a portion of the cell-associated peptide gets released into the medium even after long periods of time, as seen in Fig. 3. The amount of peptide released after uptake in any concentration increases with incubation time up to 60–90 min, at which time it reaches a plateau. However, the cells are also accumulating peptide that they will not release. Thus, as they take up peptide for longer periods of time, the proportion of the accumulated peptide that is subsequently released decreases (Fig. 6). We believe that these data suggest that the peptide being taken up is partitioned into two pools. One pool will be released, with an observed rate constant $k_d$, and the other pool will be placed in a storage pool, with an observed rate constant $k_p$, and stored indefinitely (times up to 240 min were examined). Further analysis shows that most of the released peptide is digested, whereas most of the peptide that remains cell associated is ethanol-extractable intact peptide (manuscript in preparation). The equations describing this process are given in the Appendix.

From this information we can now more accurately describe

the rate of peptide accumulation by cells with the following equation: $dA_t/dt = k_uA_0 + k_iC - k_dA_v$. This describes the rate of peptide accumulation by the cells as a function of the rates of bulk fluid phase uptake, $k_uA_0$, plus the rate of receptor-mediated uptake, $k_iC$, minus the rate of release of the pool of peptide that is available for release, $k_dA_v$, where $A_t$ = total intracellular peptide (moles/cell), $A_0$ = concentration of peptide in the medium (moles/liter), $k_u$ = observed rate constant of nonsaturable uptake (liter/minute/cell), $k_i$ = observed rate constant of receptor-mediated uptake (minute$^{-1}$), $k_d$ = observed rate constant of peptide release from the cells (minute$^{-1}$), $k_p$ = observed rate constant of peptide partitioning into a stored pool (minute$^{-1}$), $A_v$ = cell-associated peptide in a releasable pool (moles/cell). The derivation of this equation is presented in the detailed kinetic analysis in the Appendix.

The pool of cell-associated peptide available for release appears to be governed by the equation: $dA_v/dt = k_uA_0 + k_iC - (k_d + k_p)A_v$, where the rate of change of the pool is a function of the rate of incoming peptide, $k_uA_0 + k_iC$, and the relative rates of release and partitioning of the peptide into a storage pool.

From the kinetics of peptide loss, $k_d + k_p$ can be determined to be $\sim 0.04$ min$^{-1}$ and $k_d$ is between 0.013 and 0.022 min$^{-1}$ (see Appendix for details on the determination of these rate constants). The $k_u$ can be determined from the peptide uptake
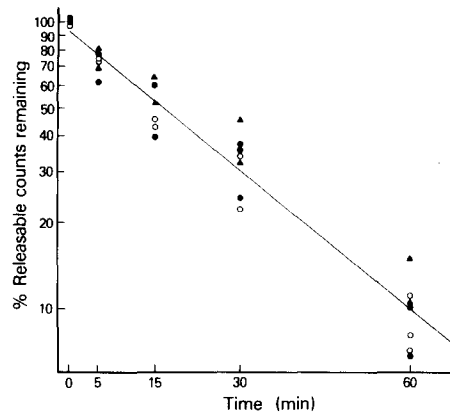


FIGURE 5 Determination of release rate constant. Cells were incubated as described in Fig. 4. The log of the percentage of the total releasable counts (counts released after 90 min) is plotted vs. time.
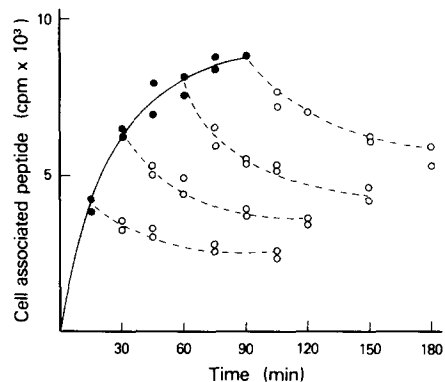


FIGURE 6 Time-course of uptake and release. Cells were incubated at 37°C in $2 \times 10^{-8}$ M $^3$H-FNLLP for various periods of time before they were washed for 5 min at 4°C. Duplicate dishes were then counted for total uptake (●); other dishes were returned to 37°C in HBSS for various times before the supernate was removed and the cells were counted to determine the amount of peptide remaining cell associated (O).

at high concentrations when $k_u A_0 \gg k_i C$ and from calculations described in Appendix. The value of $k_u$ obtained is $\sim 4 \times 10^{-14}$ liter/min/cell. The observed rate constant of receptor-mediated uptake, $k_i$, can now be calculated to be between 0.12 and 0.18 $\text{min}^{-1}$. There does not appear to be a concentration dependence to this rate constant for concentrations between $3 \times 10^{-10}$ and $1 \times 10^{-7}$ M FNLLP.

## Receptor Recovery

Upon removal of peptide, PMNs that have undergone down-regulation rapidly recover their "lost" receptors. This recovery does not require protein synthesis. To better understand the nature of the recovery process, we have investigated the kinetics of recovery and the effects of peptide concentration.

The rate of receptor recovery is a first-order function of the number of receptors missing. The log of the number of missing receptors plotted against the time of recovery gives a straight line (Fig. 7). The number of receptors missing is determined from the number of receptors present at the end of the recovery period (after 80 min when the receptor number is constant) minus the number present at any time during the recovery. The observed rate constant of recovery varies somewhat between experiments. Nevertheless in a series of experiments it could be shown that the observed recovery rate constant (slope of the line) depends on the peptide concentration present during the receptor loss. As the peptide concentration increases the observed rate constant decreases (Fig. 7).

The extent of receptor recovery is also dependent upon the concentration of peptide used to induce the receptor loss (Fig. 8). At concentrations close to that of the dissociation constant, $K_d$, the number of receptors often exceeds that present at the beginning of the experiment, i.e., there is "superrecovery." At concentrations 10–1,000 times the concentration of $K_d$, the amount of recovery decreases.

The superrecovery indicates the presence of a pool of spare receptors. The existence of this pool has been reported by others (7, 16). The spare receptors seem to be inserted into the membrane or become unmasked very shortly after the addition of peptide. Cells incubated with $1 \times 10^{-8}$ M FNLLP for only 1 min, washed, and allowed to recover have more receptors than were present initially. This increase in receptor number can also be observed at early times after peptide addition. Although the major effect of peptide addition is the receptor
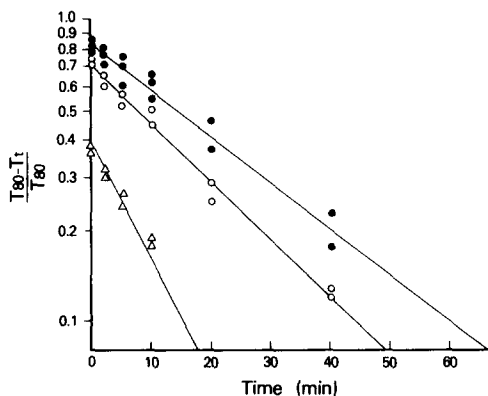
FIGURE 7 Kinetics of receptor recovery. Cells were incubated in unlabeled FNLLP at $2 \times 10^{-7}$ M ($\bullet$), $1 \times 10^{-7}$ M (O), and $1 \times 10^{-8}$ M ($\triangle$). They were washed for 5 min at 4°C and then incubated in HBSS at 37°C for 80 min. The log of fraction of total receptors ($T_{80}$) that is unavailable for binding was plotted as a function of recovery time to give the rate constant of recovery (the slope).
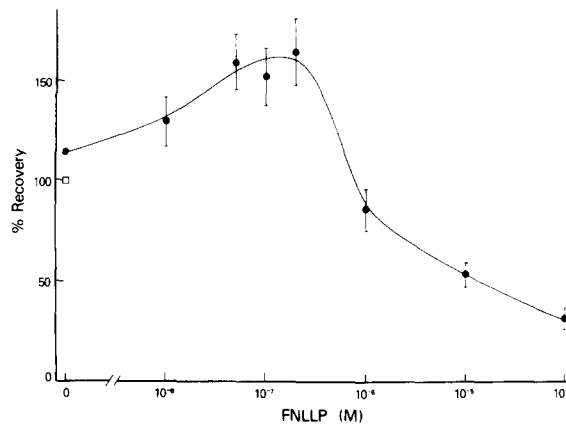
FIGURE 8 Dose response of receptor recovery. Cells were incubated in various concentrations of unlabeled FNLLP for 20 min at 37°C and washed for 5 min at 4°C. They were then incubated in HBSS at 37°C for 80 min. Recovery was plotted as the percent of $T_0$ binding capacity. $\square$, $T_0$ binding before 20 min incubation with no peptide.
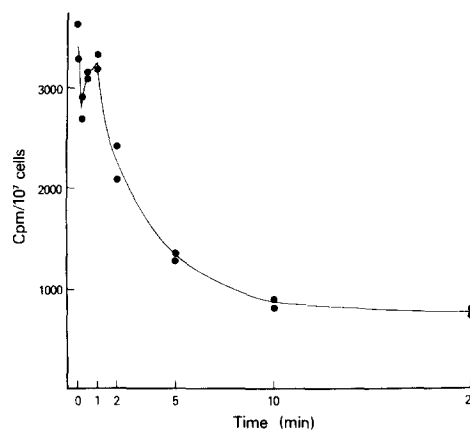
FIGURE 9 Early time-course of receptor loss. Cells were incubated in $10^{-7}$ M unlabeled FNLLP for various times at 37°C. They were then washed for 5 min at 4°C and tested for ability to bind $2 \times 10^{-8}$ M $^3$H-FNLLP at 4°C.

loss, the time-course of receptor loss often shows a pause in the rate of loss or even a transient increase in receptor number. The receptor number then continues to decrease to the plateau level (Fig. 9). The transient increase may be due to the insertion or unmasking of spare receptors. Recent evidence suggests that the total cell surface area is also increasing at this time (S. Hoffstein, personal communication).

The hypothesis that superrecovery is a result of an early insertion or unmasking of extra receptors is also supported by the recovery kinetics. As mentioned above, the rate of recovery is proportional to the number of receptors yet to be recovered. Even at early times in the recovery, the total receptor number is better represented by the number present after 80 min (the superrecovered number) than the number of receptors present at time zero (Fig. 7).

At concentrations above that of the dissociation constant, the extent of recovery decreases until at very high concentration ($10^{-4}$ M) there is no recovery at all. Again, the effect of the addition of peptide is rapid. Incubation with $10^{-4}$ M FNLLP for 1 min is sufficient to prevent receptor recovery. This inhibition of recovery may indicate that only free receptors are able to return to the surface. At increasing peptide concentrations, a greater fraction of intracellular receptors should be bound, since a large amount of peptide is taken up through the

bulk fluid uptake as well as bound to the receptors (see Appendix for details).

At low or moderate concentrations of peptide, receptor loss and recovery can occur repeatedly. However, as shown in Fig. 10, the extent of recovery decreases somewhat with each subsequent down-regulation. The extent of recovery is also a function of time the cells were maintained at 37°C in peptide. These results suggest that even at moderate peptide concentrations some receptors are lost during the cycling.

## Plateau Receptor Number

If the plateau receptor number is due to a steady-state, the rate of receptor internalization should equal the rate of receptor return at any given concentration of peptide. We can test this hypothesis, given the following assumptions: (a) The rate of receptor-mediated peptide uptake represents the rate of receptor internalization. (b) The rate constant of receptor recovery after removing peptide is the same as the rate constant of receptor recovery in the continued presence of that peptide concentration (we can measure receptor recovery only after removing the peptide).

In Table II the mean observed rate constants of receptor recovery and receptor-mediated peptide uptake are shown along with the mean receptor occupancies and plateau receptor numbers for each concentration. As can be seen, the rates of receptor internalization and initial rates of receptor recovery are very close. The estimates of net receptor recovery are about twice the rates of net receptor loss. This difference could be due to the inaccuracy of the experimental measurements or a
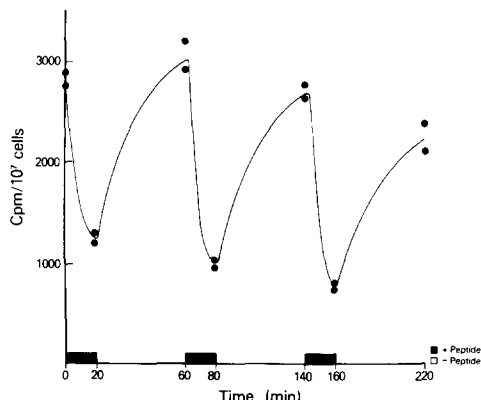


FIGURE 10 Sequential receptor loss and recovery. Cells were repeatedly incubated in $10^{-7}$ M unlabeled FNLLP at 37°C for 20 min, followed by a 5-min wash at 4°C and incubation in HBSS for 60 min at 37°C. The cells were tested for their ability to bind $2 \times 10^{-8}$ M $^3$H-FNLLP at 4°C at each of the times indicated.

modulation of receptor recovery rates by peptide presence. Thus the results are consistent with the hypothesis that the plateau receptor number is really a steady-state.

## DISCUSSION

Kinetic analyses of chemotactic peptide uptake and release and receptor loss and recovery using rabbit peritoneal PMNs have been carried out to examine the possible interrelationships between these processes. The results obtained are consistent with the model shown in Fig. 1.

### Receptor Loss

The model shows the receptors being lost from the surface through a pinocytic mechanism. This is supported by the observations that receptor loss is associated with peptide uptake (24) and that fluorescence-labeled peptide accumulates in intracellular vesicles (20). In receptor-mediated pinocytosis, the receptor is believed to be internalized in the vesicle with the ligand. Indeed, the LDL receptor has recently been shown to be present in the coated vesicles transporting LDL into cells (18). The fact that the peptide accumulated in both receptor-mediated and nonsaturable uptake is processed similarly suggests the peptide is taken up by a similar mechanism in both, i.e., pinocytosis. (Thus the vesicular accumulation seen with the fluorescent peptide should include receptor-mediated and nonsaturable uptake). However, our kinetic analysis is not dependent on the mechanism of receptor loss.

Fig. 1 also suggests that peptide binding induces receptor internalization. This seems reasonable since peptide addition induces receptor down-regulation and stimulates the rate of pinocytosis. However, a down-regulated steady-state could be as much a result of a process in which peptide acts to slow the receptor return to the membrane as of one in which peptide induces receptor internalization. As will be discussed below, both processes may play a role.

### Receptor Recovery

The course of receptor recovery is an exponential function of time. The rate of recovery depends on the pool of internalized receptors or some equivalent such as "receptor vacancies" in the membrane. The dependence on missing receptors is consistent with the idea of receptor recycling. The decrease in the recovery rate constants after incubation in increasing concentrations of peptide could result from detrimental effects of high peptide concentrations. Alternatively, the rate-limiting step in the receptor recovery could be the dissociation of the receptor from the peptide. In increasing concentrations of peptide, the pinosome contains increased peptide concentra-

TABLE II

Plateau Receptor Number as a Steady-State

| Peptide concentration | $\rho^*$ | $k_r$ | $k_r\rho$ | C‡ | $k_i$§ | $k_iC$ |
|---|---|---|---|---|---|---|
| M | molecule/cell | min$^{-1}$ | molecule/cell/min | molecule/cell | min$^{-1}$ | molecule/cell/min |
| $1 \times 10^{-8}$ | $3.1 \times 10^4$ | 0.11 | $3.4 \times 10^3$ | $1.1 \times 10^4$ | 0.15 | $1.6 \times 10^3$ |
| $3 \times 10^{-8}$ | $4.8 \times 10^4$ | 0.086 | $4.1 \times 10^3$ | $1.3 \times 10^4$ | 0.15 | $2.0 \times 10^3$ |
| $2 \times 10^{-7}$ | $6.5 \times 10^4$ | 0.038 | $2.5 \times 10^3$ | $0.9 \times 10^4$ | 0.15 | $1.4 \times 10^3$ |

* $\rho$, The number of molecules of receptor in an internal pool is equal to the number of surface receptors lost during down-regulation (37% at $1 \times 10^{-8}$ M, 56% at $3 \times 10^{-8}$ M, and 80% at $2 \times 10^{-7}$ M plus the additional receptors seen in recovery [superrecovery] [an additional 25% at $1 \times 10^{-8}$ M, 40% at $3 \times 10^{-8}$ M, and 50% at $2 \times 10^{-7}$ M]).

‡ C, the number of occupied receptors on the surface is calculated from the number of receptors remaining on the surface after down-regulation in the different concentrations times the calculated percent occupancy, using $2 \times 10^{-8}$ M as $K_d$.

§ $k_i$ has a range of 0.12–0.18.

tions. Any receptors in the pinosome or pinolysosome will therefore be occupied a higher proportion of the time. Sly and co-workers (9) have suggested that the mannose-6-phosphate receptor must dissociate from its ligand to recycle. Thus, the decrease in receptor recovery seen after down-regulation with high concentrations of peptide might result from destruction of bound receptors that remain trapped in a lysosome. Even at moderate concentrations of peptide there is less recovery after each round of down-regulation. This could indicate incomplete recovery of the cycled receptors under these conditions. This progressive decrease is difficult to explain on the basis of an internal pool of new receptors. If this pool was not sufficient to result in complete recovery after the second round of down-regulation, it could not account for the recovery seen after a third round of down-regulation.

Since cells can recover more receptors than were present initially, there must be at least a limited pool of extra receptors. The insertion or unmasking of these receptors appears to be rapidly induced by peptide addition and by addition of alcohols (7, 16). Control cells incubated in buffer at 37°C for 60 min also increase their receptor number by ~20%. Thus the in vitro incubation conditions may induce receptor increases or the cells may be recovering from in vivo down-regulation.

## Digestion and Release

One of the unexpected observations of this study was that only a portion of the cell-associated peptide was digested and released. The studies on the peptide release are consistent with the hypothesis that the incoming peptide is divided into releasable and nonreleasable pools, possibly the two granules of the PMN (2). At the beginning of incubation in peptide, there is no intracellular peptide, so the pool of releasable peptide is empty. After a period of time, between 60 and 90 min, the amount of peptide in the cell that can be released becomes constant. The model presented in the Appendix describes this pool as approaching a steady-state in which the amount of peptide being released equals the rate of peptide uptake into this pool. As pinocytosis has only recently been recognized in PMNs, the fate of the pinocytic vesicles and the site of the stored peptide pool are unknown.

## Plateau Receptor Number

The model suggests that the relative rates of receptor internalization and recovery determine the receptor number available on the cell surface at any time. Our kinetic examination of this hypothesis assumes (a) that receptor-mediated peptide uptake can be used as a measure of receptor internalization and (b) that receptor recovery occurs equally in the presence of peptide as in its absence. If these assumptions are valid, the rate of receptor internalization (receptor-mediated peptide uptake) should equal the initial rate of receptor recovery at plateau in any peptide concentration. In the data presented the rates are very similar (Table II), with the rate of receptor recovery being about twice the rate of loss. These differences are not significant, considering the errors in the various measurements involved. Nevertheless, the fact that the recovery always seems greater than loss may suggest that some factor was systematically overlooked.

A corollary of the steady-state hypothesis is that receptor loss occurring during down-regulation and receptor-mediated peptide uptake is part of the same process. If this is true, the rate constants for these processes should be the same. Since the rate of receptor loss upon addition of peptide is complicated

by the simultaneous addition of receptors, we were not able to get a measurement of the initial rate. However, the half-time of receptor loss in several concentration of peptide is ~2 min. The rate constant estimated from this half-time is 0.34 min$^{-1}$. This is higher than the 0.15 min$^{-1}$ obtained for the peptide uptake. Using this faster rate constant, the rates of receptor loss would nearly equal rates of recovery. The discrepancy between the two rate constants could indicate that (a) an additional process for receptor internalization occurs initially, (b) there is positive cooperativity in the rate of receptor internalization, (c) receptor-mediated peptide uptake is systematically underestimated for some reason, or (d) the two processes are mechanistically different. The possibility of an additional process affecting the internalization at early times is consistent with the large number of transient responses that are known to occur shortly after peptide addition. To what extent these processes are transient because of receptor loss and to what extent they modulate the rate of receptor loss is not clear. Certainly, changes such as the raised levels of cAMP seen within 30 s of peptide addition could alter the rate of receptor movements. Although we can not rule out positive cooperativity of the receptor loss, our finding that the half-life of receptor loss does not show a significant concentration dependence argues against cooperativity. The linear relationship between receptor occupancy and the rate of peptide uptake suggests that there is no cooperativity in the rate-limiting step in peptide uptake. Thus, it is likely that receptor loss and peptide uptake are two aspects of the same process.

A consequence of receptor recycling as proposed here is that the binding of peptide to receptor might not be in equilibrium. If only bound receptors are internalized and only unbound receptors return, then at plateau the steady-state equations predict that the ratio of free to bound receptors, will be $(R/C)_{plateau} = (k_{-1} + k_i)/k_1A_0$, while at equilibrium binding with no receptor cycling it will be $(R/C)_{equilibrium} = k_{-1}/k_1A_0$. Therefore, $(R/C)_{plateau} = (R/C)_{equilibrium} 1 + (k_i/k_{-1})$. The deviation from equilibrium binding is a function of peptide concentration and the fractional deviation of the number of occupied receptors from equilibrium is equal to $1 - (1 + K_d/A_0)/(1 + [1 + k_i/k_{-1}][K_d/A_0])$. As $A_0$ becomes very large, the binding approaches equilibrium, while as $A_0$ gets very small, the fractional deviation from equilibrium binding approaches $1 - 1/(1 + k_i/k_{-1})$.

The deviation from equilibrium binding between $10^{-10}$ and $10^{-8}$ M FNLLP is between 27 and 20%. Thus the slope of Fig. 2 becomes 0.16 min$^{-1}$ rather than 0.13 min$^{-1}$. This will result in about a 20% increase in the estimate of $k_i$ and a rate of receptor internalization somewhat closer to the rate of recovery shown in Table II.

## APPENDIX

### Kinetic Analysis

We propose that the leucocyte response to the chemotactic peptide might involve mechanisms pictured in Fig. 1. The peptide binds to free receptors, $R$, with forward rate constant $k_1$ and dissociates from the complex, $C$, with reverse rate constant $k_{-1}$. Bound receptors may be internalized by the cells with the rate constant $k_i$, which is equal to the rate constant for receptor-mediated peptide uptake. Free peptide is taken up into the pinocytic vesicles with rate constant $k_u$. Once inside the vesicles, peptide is released with a rate constant $k_d$. Peptide may also be partitioned into a nonreleasable pool with a rate constant $k_p$. The free receptors in the vesicles may be recycled

back to the cell membrane with rate constant $k_r$. Given these facts and assumptions, we can write kinetic equations for the rates of change of important forms of receptor and peptide. For free receptors on the membrane surface, $R$:

$$\frac{dR}{dt} = -k_1 R A_0 + k_{-1} C + k_r \rho. \qquad \text{(A-1)}$$

We assume that there is no net internalization of free receptors. We acknowledge the possibility that free receptors are also being internalized. However, further data will be required to determine whether this is the case. At the present time the available data are consistent with our model.

For receptor-peptide complex on the cell surface, $C$:

$$\frac{dC}{dt} = k_1 R A_0 - k_{-1} C - k_i C. \qquad \text{(A-2)}$$

We assume that no complexes recycle back to the cell surface and that internalization is proportional to the number of complexes on the surface.

For free receptors inside the cell, $\rho$:

$$\frac{d\rho}{dt} = -k_1 \rho A_f + k_{-1} \chi - k_r \rho. \qquad \text{(A-3)}$$

This assumes that the receptor has the same affinity inside the vesicle as on the cell surface; we have evidence that the binding is insensitive to pH between pH 4 and 8.

For receptor-peptide complex inside the vesicles, $\chi$:

$$\frac{d\chi}{dt} = k_1 \rho A_f - k_{-1} \chi + k_i C. \qquad \text{(A-4)}$$

For free peptide in a releasable pool, $A_f$:

$$\frac{d(N_A V A_f)}{dt} = -k_1 \rho A_f + k_{-1} \chi$$
$$- N_A V (k_d + k_p) A_f + N_A k_u A_0. \qquad \text{(A-5)}$$

For total peptide in the releasable pool, $A_v$:

$$\frac{d(N_A A_v)}{dt} = \frac{d}{dt} (N_A V A_f + \chi)$$
$$= k_i C + N_A k_u A_0 - N_A V (k_d + k_p) A_f. \qquad \text{(A-6)}$$

For peptide in the "partitioned" pool, $A_p$:

$$\frac{dA_p}{dt} = k_p A_f. \qquad \text{(A-7)}$$

We emphasize that these equations are intended to model cell behavior only during the time period of the experiments.

For total receptors on the cell surface, $S$:

$$\frac{dS}{dt} = \frac{d}{dt} (R + C) = k_r \rho - k_i C. \qquad \text{(A-8)}$$

The symbols are defined as follows: $S$ = number of total receptors, free and occupied, on the cell surface = $R$ + $C$; receptors/cell. $R$ = number of free receptors on the cell surface; receptors/cell. $C$ = number of receptor-peptide complexes on the cell surface; complexes/cell. $A_0$ = concentration of extracellular peptide (assumed to remain constant); moles/liter. $\rho$ = number of free receptors inside the cell; receptors/cell. $A_f$ = concentration of free peptide in a vesicle; moles/liter. $\chi$ = number of receptor-peptide complexes in the vesicles; complexes/cell. $A_v$ = amount of incoming peptide; presumably

in vesicles, equal to the sum of free and complexed peptide in the vesicle; moles/cell. $A_p$ = amount of peptide sequestered in a partitioned pool; moles/cell. $V$ = vesicular volume; volume/cell. (We have no estimate for this at the moment but fortunately this quantity is not important in most of the following analysis.) $N_A$ = Avogadro's number; number/mole.

The decrease in the number of surface receptors with increasing extracellular peptide concentration can be explained by our model. Upon incubation of the cells in the peptide concentration $A_0$, the receptors go through the cycle of binding, internalization, dissociation, and returning to the surface. After a sufficiently long time, a time-independent situation, or steady-state, is reached. We can discover the properties of this steady-state by finding the solutions of Eqs. A-1 through A-7 with all time derivatives set equal to zero.

The quantity of interest is the total number of surface receptors, $S = R + C$, which at the steady-state or "plateau" is given by $S_p$:

$$S_p = C \left\{ 1 + [1 + \beta] \left( \frac{K_d}{A_0} \right) \right\}, \qquad \text{(A-9)}$$

where

$$C = \frac{R_0}{2\phi} \left\{ - \left[ 1 + \beta \left( 1 + \frac{1}{\alpha} \right) + (1 + \beta) \left( \frac{K_d}{A_0} \right) + \frac{\beta\delta}{\alpha\gamma} \left( \frac{A_0}{K_d} \right) \right] \right.$$

$$\left. + \sqrt{1 + \beta \left( 1 + \frac{1}{\alpha} \right) + (1 + \beta) \left( \frac{K_d}{A_0} \right) + \frac{\beta\delta}{\alpha\gamma} \left( \frac{A_0}{K_d} \right)} \right\}$$

and $\phi = (r\beta^2)/(\alpha\gamma)$, $\beta = k_i/k_{-1}$, $\alpha = k_r/k_{-1}$, $r = R_0/(N_A V K_d)$, $\gamma = (k_d + k_p)/k_{-1}$, and $\delta = k_u/(V k_{-1})$. $R_0$ is the total number of surface receptors immediately present per cell after incubation in peptide.

The value of $S_p$ represents the plateau receptor number found in the down-regulation experiments. Fig. 11 gives a plot
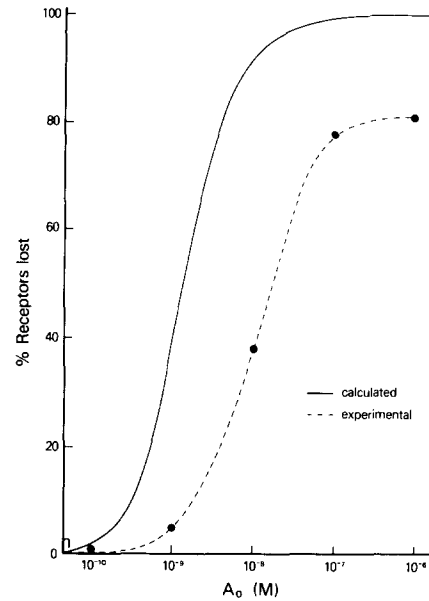


FIGURE 11   Surface receptors at plateau. The surface receptors at plateau, $S_p$, are plotted vs. extracellular peptide concentration, using Eq. A-9. The parameter values used in this plot were estimated in the body of the paper: $k_d = 0.025$ min$^{-1}$, $k_p = 0.015$ min$^{-1}$, $k_u = 4 \times 10^{-14}$ liter/min/cell, $k_i = 0.15$ min$^{-1}$, $K_d = 2 \times 10^{-8}$ M, $k_1 = 2 \times 10^7$ M$^{-1}$ min$^{-1}$, $k_{-1} = 0.4$ min$^{-1}$, $k_r = 0.3$ min$^{-1}$, $R_0 = 5 \times 10^4$ receptors/cell, $V = 10^{-14}$ liter/cell. The calculated values are compared with experimental values (25).

of receptor loss, i.e., $1 - S_p/R_0$, vs. $A_0/K_d$ for a set of parameter values estimated from our kinetic data. It is clear that as $A_0/K_d$ increases, the plateau receptor number decreases. The calculated values of receptor loss are consistently greater than the experimentally determined values. The most probable explanation is that our calculations neglect the increase in surface receptors immediately after incubation in peptide.

The total amount of intracellular peptide, $A_t$, is equal to the incoming peptide $A_v$ plus the sequestered peptide $A_p$, so that $A_t = A_v + A_p$. Its rate equation is found by adding Eqs. A-6 and A-7:

$$\frac{dA_t}{dt} = \frac{1}{N_A} k_i C + k_u A_0 - V k_d A_f. \qquad \text{(A-10)}$$

We now assume that all the incoming peptide $A_v$ is susceptible to release and partitioning whether free or bound to receptors. This could have the effect of yielding underestimates for $k_d$ and $k_p$ if bound peptide is not susceptible. Also, we assume that the time for approach to receptor plateau steady-state is short relative to the time of peptide uptake and release experiments. Then $C$ will be approximately constant during the course of these experiments. Under such conditions Eqs. A-6 and A-10 can be rewritten as:

$$\frac{dA_v}{dt} = (k_u A_0 + k_i C) - (k_d + k_p) A_v \qquad \text{(A-11)}$$

and

$$\frac{dA_t}{dt} = (k_u A_0 + k_i C) - k_d A_v, \qquad \text{(A-12)}$$

where $C$ now has units of *moles* of complexes/cell.

At the beginning of incubation in extracellular peptide, $t = 0$, there is no intracellular peptide, so that $A_v(0) = A_t(0) = 0$. We can now determine the time-course of intracellular peptide from the transient solutions of Eqs. A-11 and A-12:

$$A_v(t) = \frac{k_u A_0 + k_i C}{k_d + k_p} [1 - e^{-(k_d+k_p)t}] \qquad \text{(A-13)}$$

and

$$A_t(t) = \frac{k_d(k_u A_0 + k_i C)}{(k_d + k_p)^2} [1 - e^{-(k_d+k_p)t}]$$
$$+ (k_u A_0 + k_i C)\left[1 - \left(\frac{k_d}{k_d + k_p}\right)t\right]. \qquad \text{(A-14)}$$

Fig. 12 gives a plot of $A_v(t)$ and $A_t(t)$ during the period of incubation in peptide. Notice that after a period of time the amount of peptide being taken up into the releasable compartment, $A_v$, becomes constant, reflecting the steady-state. The total amount of intracellular peptide then increases linearly with time with a slope equal to $(k_u A_0 + k_i C) (1 - [k_d/(k_d + k_p)])$.

When the extracellular peptide is removed the kinetic equations for the intracellular peptide become:

$$\frac{dA_v}{dt} = -(k_d + k_p) A_v \qquad \text{(A-15)}$$

and

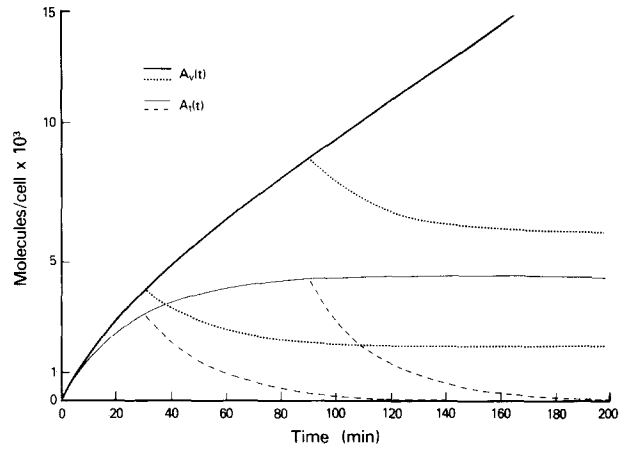$$\frac{dA_t}{dt} = -k_d A_v. \qquad \text{(A-16)}$$



FIGURE 12   Time-course of intracellular peptide. Total intracellular peptide, $A_t$, and vesicular peptide, $A_v$, are plotted vs. time, using Eqs. A-13 and A-14 for $t < t^*$ and Eqs. A-17 and A-18 for $t > t^*$. $t^*$ is the time at which extracellular peptide is removed. Parameter values used are the same as for Fig. 11. $A_t$ is comparable to the experimental data for the cell-associated peptide in Fig. 6.

If the peptide is removed after incubation time $t^*$, then we know the values of $A_v(t^*)$ and $A_t(t^*)$ from Eqs. A-13 and A-14. The transient solutions to Eqs. A-15 and A-16 are then:

$$A_v(t) = A_v(t^*)e^{-(k_d+k_p)(t-t^*)} \qquad \text{(A-17)}$$

and

$$A_t(t) = A_t(t^*) - \frac{k_d A_v(t^*)}{k_d + k_p} [1 - e^{-(k_d+k_p)(t-t^*)}]. \qquad \text{(A-18)}$$

Fig. 12 also shows $A_v(t)$ and $A_t(t)$ after the removal of extracellular peptide. From Eq. A-18, we see that as $t \to \infty$, $A_t(t) \to A_{t\infty} = A_t(t^*) - k_d A_v(t^*)/(k_d + k_p)$. Therefore, the total releasable peptide ($TRP$) is

$$TRP = A_t(t^*) - A_{t\infty} = \frac{k_d A_v(t^*)}{k_d + k_p}. \qquad \text{(A-19)}$$

$TRP$ is a measurable quantity. Now, using Eqs. A-17 and A-19, we can rewrite Eq. A-16 as

$$\frac{dA_t}{dt} = -(k_d + k_p)[TRP]e^{-(k_d+k_p)(t-t^*)} \qquad \text{(A-20)}$$

and its solution becomes

$$A_t(t^*) - A_t(t) = [TRP][1 - e^{-(k_d+k_p)(t-t^*)}]. \qquad \text{(A-21)}$$

Thus, a plot of $\ln\{[TRP] - [A_t(t^*) - A_t(t)]\}$ vs. $(t - t^*)$ should yield a straight line with slope $-(k_d + k_p)$, so this quantity is now also known. We now could compute the initial downward slope of the $A_t(t)$ vs. $t$ curve immediately after removal of peptide (from Eq. A-20 with $t = t^*$):

$$\text{"down-slope"} = -(k_d + k_p)[TRP]. \qquad \text{(A-22)}$$

At that same time $t^*$, the upward slope of the $A_t(t)$ vs. $t$ curve without removing peptide can be estimated experimentally (from Fig. 6) and is given by combining eqs. A-12, A-13, and A-19:

$$\text{"up-slope"} = (k_u A_0 + k_i C) - (k_d + k_p)[TRP]. \qquad \text{(A-23)}$$

Therefore, we have an expression for the total peptide uptake rate, $U$, which is the apparent uptake rate plus the release rate:

$$U = k_u A_0 + k_i C = \text{``up-slope''} + (k_d + k_p)[TRP]. \quad \text{(A-24)}$$

We can now compute the release rate constant, $k_d$, from a combination of Eqs. A-19 and A-13:

$$k_d = \frac{(k_d + k_p)^2 [TRP]}{U[1 - e^{-(k_d + k_p)t^*}]}. \quad \text{(A-25)}$$

Also, $k_i$ can be estimated from Eq. A-24.

Finally, recovery of surface receptors after removal of extracellular peptide can be analyzed mathematically. In this case the analysis is simplified by assuming, temporarily, that the peptide-receptor binding is at equilibrium. Under this assumption, $C = A_0 S/(A_0 + K_d)$, and the surface receptor number is governed by an equation simpler than Eqs. A-1 through A-8:

$$\frac{dS}{dt} = -k_i \left[ \frac{A_0 S}{A_0 + K_d} \right] + k_r'(R_0 - S), \quad \text{(A-26)}$$

where $R_0$ is the total available cell receptors and $k_r'$ is the apparent receptor recovery rate constant. For the receptor recovery experiments, extracellular peptide is removed at time $t = t^*$, when the number of surface receptors is at its plateau level. With this simplified model, the plateau receptor number is given by solving Eq. A-26 at steady-state, $dS/dt = 0$:

$$S_p = \left\{ \frac{1}{R_0} \left[ 1 + \left( \frac{k_i}{k_r'} \right) \left( \frac{A_0}{A_0 + K_d} \right) \right] \right\}^{-1}. \quad \text{(A-27)}$$

Then, after peptide removal, $A_0 = 0$ and the solution to Eq. A-26 is:

$$\frac{(R_0 - S[t])}{(R_0 - S_p)} = e^{-k_r'(t - t^*)}. \quad \text{(A-28)}$$

Thus a plot of the log of the fractional recovery of receptors vs. the time after removal of peptide should yield a straight line with slope $-k_r'$, as in Fig. 8. This is the recovery rate constant measured by the experiments mentioned in the body of the paper. There it is shown to be dependent upon the extracellular peptide concentration, and, in fact, $k_r'$ decreases as $A_0$ increases. This can be explained by reference to the more detailed model, in which the number of surface receptors is governed by the equation:

$$\frac{dS}{dt} = k_r \rho - k_i C \quad \text{(A-8)}$$

Since $\rho = R_0 - S - \chi$, we can write

$$\frac{dS}{dt} = -k_i C + k_r(R_0 - \chi - S). \quad \text{(A-29)}$$

Comparing this with Eq. A-26 and assuming the receptor-peptide binding to be close to equilibrium, we see that $k_r'$ depends upon the true recovery rate constant $k_r$ as well as upon $\chi$, the amount of intracellular receptor-peptide complex. Clearly, $\chi$ is a function of $A_0$, so $k_r'$ will also be a function of $A_0$. More precisely, we can equate the recycle terms of Eqs. A-8 and A-29 to get

$$k_r'(R_0 - S) = k_r(R_0 - \chi - S), \quad \text{(A-30)}$$

which implies that

$$\frac{k_r'}{k_r} = 1 - \left( 1 + \frac{\rho}{\chi} \right)^{-1} \quad \text{(A-31)}$$

Now, as $A_0$ increases, the ratio $\rho/\chi$ will decrease because a greater fraction of receptors will be bound by peptide. Therefore, as $A_0$ increases, the ratio $k_r'/k_r$ must decrease. At very high extracellular peptide concentrations, $\rho/\chi$ becomes extremely small and the receptor recovery rate extremely low. Notice that since the value of $\rho/\chi$ will change during the course of the recovery experiments, the value of $k_r'$ is not expected to be constant.

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