# Response of Neutrophils to Stimulus Infusion: Differential Sensitivity of Cytoskeletal Activation and Oxidant Production

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*Abstract.* The responses of human neutrophils to *N*-formyl peptides were studied under conditions where ligand binding was controlled by infusing a cell suspension with the peptide over a time period comparable to the normal half-time for binding. Receptor occupancy was measured in real time with a fluorescently labeled peptide using flow cytometry. This binding was approximated by a simple reversible model using typical on ($7 \times 10^8$ $M^-$ $min^{-1}$) and off (0.35/min) rate constants and the infusion rates (0.02–0.2 nM/min). Under conditions of stimulus infusion intracellular calcium elevation, superoxide generation, and right angle light scatter and F-actin formation were measured. As the infusion rate was decreased into the range of 10 pM/min, lowering the rate of increase of receptor occupancy to ~0.5% per min, the calcium and right angle light scatter responses elongated in time and decreased in magnitude. Superoxide generation decreased below infusion rates of ~100 pM/min (occupancy increasing at a rate in the range of 5% per min). This behavior could contribute to differences between chemotactic responses, which appear to require low rates of receptor occupancy over long periods, and bactericidal or inflammatory responses (free radical generation and degranulation), which require bursts of occupancy of several percent of the receptors.

HUMAN neutrophils have specific receptors for *N*-formyl peptides (1). These peptides are derived from bacterial proteins (20), and reflect the neutrophil's function in seeking out and destroying bacteria at sites of infection. In addition, neutrophils respond to endogenous chemotactic substances such as leukotrienes (34) and the complement component C5a (13) and thus are involved in tissue injury. A neutrophil will migrate up a concentration gradient of stimulus, phagocytose particles, produce superoxide radicals, and release proteolytic enzymes in response to stimuli (22, 23, 26, 33, and references therein). Thus a complex set of functions are set into action by stimulation of the *N*-formyl peptide receptors. How the cell regulates and coordinates this diverse set of functions is not clearly understood. Presumably, the cell must be able to distinguish low concentrations of *N*-formyl peptide which will trigger chemotaxis but not inflammatory responses, since it would be inappropriate for the cell to release injurious substances before it gets to the site of infections. Evidence shows that different neutrophil responses have different receptor occupancy requirements (18, 37).

With recent advances in techniques for analysis of ligand binding and continuously observing cell responses on short time scales, it has become evident that many cell responses are transient. For example, *N*-formyl peptide stimulation of human neutrophils (in the absence of cytochalasin) causes transient changes in right angle light scattering, actin polymerization, cAMP levels, intracellular calcium levels, superoxide generation, membrane depolarization, and aggregation. For several of these responses, the changes reach a maximum well before binding equilibrium have occurred (37).

Moreover, the responses decay while occupied receptors persist on the cell surface (37). The rate of decay of the responses does not appear to be sensitive to the number of receptors which have been occupied. The relative contributions of receptor desensitization, homeostasis, and intracellular adaptation mechanisms to the decay process has not been elucidated.

In a series of experiments observing membrane depolarization it has been shown that a dose of fluorescein-labeled *N*-formyl hexapeptide which was stimulatory when injected as a bolus, was nonstimulatory when slowly infused into the cell suspension over 3 min (38). A similar result has been reported for oxidant production (5). Moreover, it has been reported that under infusion conditions where oxidant production was nearly obliterated, the quin2-detected intracellular $Ca^{++}$ increase reached the same maximum as when the stimulus was injected as a bolus, however, phosphoinositide metabolism and cAMP increases were diminished (6). These results suggest that the number of occupied receptors is not the only factor involved in eliciting and modulating the

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responses, but the temporal history of occupancy must also be considered.

In this manuscript the method of stimulus infusion is used to regulate the time course of receptor occupancy. Real-time assays of high temporal resolution are used to quantify an inflammatory function (superoxide generation), one of its putative signals (intracellular calcium elevation), and chemotaxis-related events (right angle light scattering and actin polymerization). Flow cytometric methods are used to quantify the rate of receptor occupancy for a fluorescently-labeled *N*-formylhexapeptide. We show that the temporal characteristics of receptor occupancy has a profound effect on the responses. The generation of superoxide requires a much faster rate of stimulus infusion than the right angle light scatter response and intracellular calcium elevation. This may contribute to the mechanism by which neutrophils are able to avoid releasing oxidants before they arrive at a site of infection.

## Materials and Methods

### Preparation of Neutrophils

Neutrophils were obtained from fresh human blood by the gel sedimentation method of Henson and Oades (11) or by a modification (43) of the elutriation method described by Berkow et al. (2). Cells were kept on ice in calcium-free buffer until assayed, usually within 3 h of purification. The buffer for the experiments contained 5 mM KCl, 1.9 mM $KH_2PO_4$, 1.1 mM $Na_2HPO_4$, 5.5 mM glucose, 1.5 mM $CaCl_2$, 0.3 mM $MgSO_4$, 1 mM $MgCl_2$, and 147 mM NaCl, at pH 7.2. When ammonium chloride was added to the buffer, the concentration of NaCl was decreased to maintain a constant ionic strength. Thus $NH_4$-buffer contained 29.4 mM $NH_4Cl$ and 117.6 mM NaCl.

### Reagents

*N*-formyl-norleucyl-leucyl-phenylalanine (FNLP)[1] (Vega Biochemicals, Tucson, AZ), *N*-formyl-methionyl-leucyl-phenylalanine (FMLP), and *N*-formyl-methionyl-phenylalanine (FMP) (Sigma Chemical Co., St. Louis, MO) were used without further purification. *N*-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine was obtained from Bachem Fine Chemicals (Torrance, CA). The FITC derivative of this hexapeptide (FLPEP) was prepared as previously described (36). quin2 acetoxytetramethyl ester was obtained from Lancashire Synthesis, Ltd. (Lancashire, UK). Rhodamine-phalloidin was obtained from Molecular Probes, Inc. (Junction City, OR). Phosphoramidon was obtained from Sigma Chemical Co.

### Binding of FLPEP to Neutrophils

The kinetics of FLPEP binding to neutrophils was measured on a Becton-Dickinson FACS IV cell sorter as described by Sklar et al. (36). Because FLPEP internalization becomes significant within 3 min of binding and because fluorescein fluorescence is quenched at low pH, it was necessary to neutralize acidic intracellular compartments so all cell-associated FLPEP could be measured. Thus 29.4 mM $NH_4^+$, a lysozomotropic agent (30), was added to the buffer as described by Sklar et al. (37). This concentration of $NH_4^+$ did not alter the dose-response curves of the assays used in this study (data not shown). For studies of binding under the infusion protocol, a specially designed sample chamber was used which allowed linear infusion of the FLPEP into the cell suspension while on line in the flow cytometer (24). The amount of FLPEP bound to the cells was calibrated using fluorotrol GF as a reference (36). On and off rate constants at 37°C were determined by measuring the kinetics of binding after injecting various concentrations of FLPEP. This data was then fit using an iterative calculation scheme (36, 39) assuming only a simple reversible-binding model adequate to account qualitatively for the data: FLPEP + receptor ⇌ FLPEP-receptor complex. The program for calculating binding under conditions of linear infusion of the stimulus was the same except that the free ligand concentration was updated at each time interval to include the additional ligand which had been infused during that interval (iterations were done at 0.01-min time intervals).

### Functional Assays

Cell functions were assayed kinetically at 37°C with 2 × $10^6$ cells/ml (unless otherwise stated) using an SLM 8000 or 4800 spectrofluorometer. Cells were incubated in calcium-containing buffer at 37°C for 5 min before stimulation. The sample was continuously stirred by a magnetic stir bar in the cuvette. A typical assay for cell function used 2 ml of cells which were stimulated by injecting 20 μl of a concentrated solution of *N*-formylpeptide. The stimulus was injected using a Hamilton microliter syringe attached to tubing which was inserted through the spectrofluorometer's cover and into the sample. Under the infusion protocol, this 20 μl was injected slowly by driving the syringe with a Harvard infusion pump. Hence the *N*-formylpeptide concentration was linearly increased, and the total time required to reach a given total concentration could be varied. The linearity of the concentration gradient was verified using FLPEP by observing fluorescein fluorescence (excitation 490 nm and emission 520 nm) during the course of an infusion (data not shown).

Free radical production was determined using the chromophore para-hydroxyphenylacetic acid as described by Hyslop and Sklar (14). Intracellular calcium levels were monitored and calibrated with the fluorescent dye quin2 (29, 45). Actin polymerization was determined by the method of Howard and Meyer (12) using rhodamine-phalloidin as an indicator of F-actin formation. Right angle light scatter (49) was measured in the spectrofluorometer as described by Sklar et al. (40) using a wavelength of 340 nm. Because ambient light contains comparatively little light at 340 nm, it was possible to leave the cover off of the sample chamber of the spectrofluorometer and observe right angle light scatter while removing aliquots of cells for fixing and staining for the F-actin assay (41). Thus it was possible to observe right angle light scatter and actin polymerization on the same sample.

It should be noted that all experiments were done in the absence of cytochalasin B.

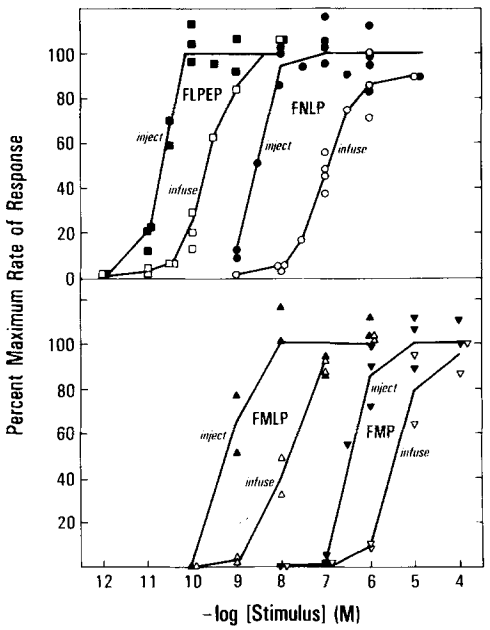


*Figure 1.* Dose-response curves for *N*-formylpeptide-stimulated right angle light scatter under conditions of stimulus injection or infusion over 75 s. The response was quantified as the rate of the initial decrease in right angle light scatter and plotted as the percent of the maximal rate observed at high doses. The same maximal rate was observed for all stimuli.

1. *Abbreviations used in this paper*: FLPEP, *N*-formyl-norleucyl-leucyl-phenylalanyl-norleucyl-tyrosyl-lysine-fluorescein; FMLP, *N*-formyl-methionyl-leucyl-phenylalanine; FMP, *N*-formyl-methionyl-phenylalanine; FNLP, *N*-formyl-norleucyl-leucyl-phenylalanine; quin2, (2-([2-bis(carboxylmethyl)-amino-5-methylphenoxy]-methyl)-6-aminoquinoline.

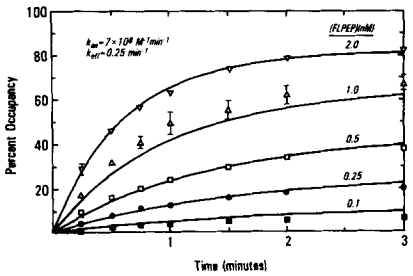


*Figure 2.* Ligand-binding rates for FLPEP-receptor interaction at 37°C in buffer with 29 mM $NH_4^+$. At time zero FLPEP was injected into the stirred cell sample, and the fluorescence histograms were collected at the indicated time points. 2,000 cells were counted in 2 s. The mean channel number was calculated form these fluorescence histograms. Symbols denote actual measurements, solid curves are theoretical-binding data assuming $k_{on} = 7 \times 10^8$ $M^{-1}$ $min^{-1}$ and $k_{off} = 0.25$ $min^{-1}$ using the model described under Materials and Methods. The data are the averages of duplicate determinations from one donor, and representative of three different experiments. The range of the duplicates, when larger than the symbols, is given by error bars.

## Results

### Generality of the Infusion Response for Various N-Formylpeptide Agonists

Four different *N*-formylpeptides (FLPEP, FNLP, FMLP, and FMP) were used to stimulate the actin-associated right angle light scatter response. Fig. 1 shows the right angle light scatter dose-response characteristics for injection or infusion over 75 s of these *N*-formylpeptides which range in estimated $K_d$ from $4 \times 10^{-10}$ M to $3 \times 10^{-6}$ M ($K_d$ at 4°C, reference 34). The responses in Fig. 1 were quantified as the rate of the initial decrease in right angle light scatter (see Fig. 4). Under conditions where the stimuli are infused over 75 s, the dose-response curves for FNLP, FMLP, FMP, and FLPEP (when measured as the initial rate of the polymerization phase) are shifted to higher concentrations by ~10-fold when compared with injections. In other words, for each stimulus there was a concentration of peptide that caused a near-maximal response when injected, but almost no response when infused over 75 s (e.g., $10^{-10}$ M FLPEP, $10^{-9}$ M FMLP, $10^{-8}$ M FNLP, and $10^{-6}$ M FMP). The similarity in the shapes of these curves demonstrated that this loss of responsiveness under infusion conditions was a general phenomenon of the *N*-formylpeptide receptor.

To understand the basis of this infusion response we turned to the fluorescent hexapeptide FLPEP because we could also characterize the binding of this peptide using real-time flow cytometric methods. Fig. 2 shows the kinetics of FLPEP binding at 37°C in $NH_4$-buffer. For this experiment, the data were fit with an on rate of $7 \times 10^8$ $M^{-1}$ $min^{-1}$ and an off rate of 0.25 $min^{-1}$. For seven separate determinations $k_{on}$ varied from 5–7 $\times 10^8$ $M^{-1}$ $min^{-1}$ and $k_{off}$ varied from 0.25–0.4 $min^{-1}$. These values were similar to those determined in the buffer without ammonium ($1 \times 10^9$ $M^{-1}$ $min^{-1}$ and 0.25 $min^{-1}$, reference 31). For the theoretical calculations on Figs. 3–5, average values of 0.35 $min^{-1}$ and $7 \times 10^8$ $M^{-1}$ $min^{-1}$ were used for $k_{off}$ and $k_{on}$, respectively.

Fig. 3 shows data from flow cytometric measurements of FLPEP binding to neutrophils using the infusion protocol at 37°C. FLPEP was injected or infused into the cell suspension in $NH_4$-buffer to give a final concentration of 0.2 nM. The FLPEP was infused over different time intervals to vary the rate of binding. Even for infusions as long as 3.5 min, bind-

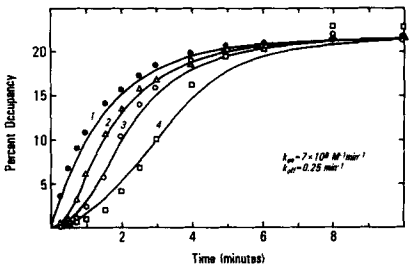


*Figure 3.* Binding of FLPEP to PMNs in buffer with 29 mM $NH_4^+$ using the stimulus infusion protocol. Binding kinetics were observed as described in Fig. 1. 0.2 nM FLPEP was either injected into the cells (●) or linearly infused over time intervals of 0.92 (△), 1.83 (○), or 3.57 (□) min. The solid curves are calculated using $k_{on} = 7 \times 10^8$ $M^{-1}$ $min^{-1}$ and $k_{off} = 0.25$ $min^{-1}$ and the model described in Materials and Methods. Data is the average of duplicate determinations from the donor and representative of three donors.

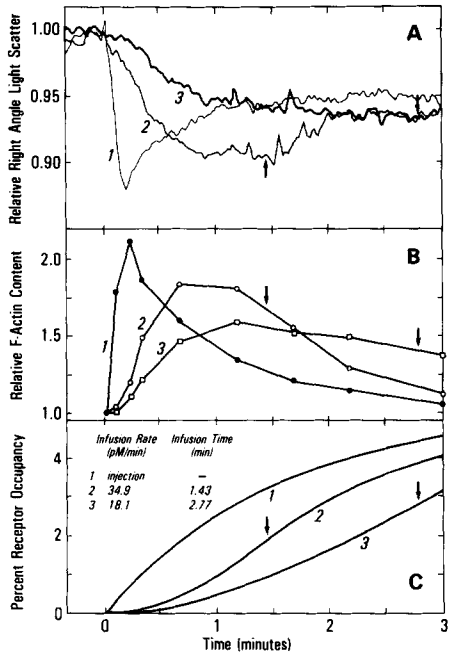


*Figure 4.* Right angle light scatter (*A*) and F-actin polymerization (*B*) under conditions of stimulus infusion. $4 \times 10^6$ neutrophils/ml buffer without $NH_4^+$ were stimulated with 0.05 nM FLPEP added as a bolus (*1*) or infused over 1.43 min (*2*) or 2.77 min (*3*). The arrow for each curve indicates the time at which the infusion stopped. *C* shows the analogous theoretical-binding curves, calculated for $k_{off} = 0.35$ $min^{-1}$, $k_{on} = 7 \times 10^8$ $M^{-1}$ $min^{-1}$, and the number of receptors/cell = 60,000. Data shown are the averages of duplicate determinations for one donor. Right angle light scatter and F-actin polymerization were determined simultaneously as described in Materials and Methods. The right angle light scatter experiment was repeated eight times with actin being monitored simultaneously in two experiments.

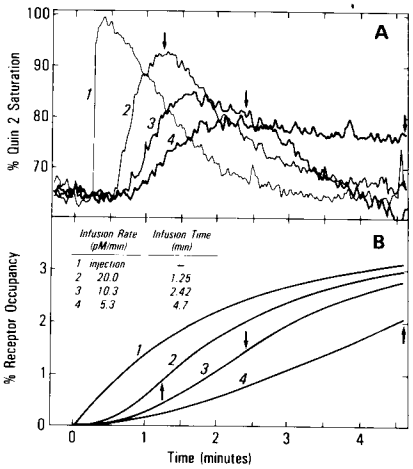


*Figure 5.* Intracellular free calcium levels under conditions of stimulus infusion. Cells were loaded with quin2 and resuspended at $2 \times 10^6$/ml $NH_4^+$-free buffer. 0.025 nM FLPEP was added as a bolus (*1*) or over 1.25 (*2*), 2.4 (*3*), or 4.7 min (*4*). The arrow for each curve indicates the time at which the infusion stopped. *B* shows the analogous theoretical binding curves calculated for $k_{off} = 0.35$ min$^{-1}$, $k_{on} = 7 \times 10^8$ M$^{-1}$ min$^{-1}$ and the number of receptors/cell = 60,000. Data are averages of duplicate determinations from one donor and are representative of six donors.

ing reached a plateau after 8 min, and the final levels of FLPEP bound were the same as for an injection. The data were fit using a $k_{on}$ of $7 \times 10^8$ M$^{-1}$ min$^{-1}$ and a $k_{off}$ of 0.25 min$^{-1}$. Slight deviations between the calculations and the observed binding are detected in Figs. 2 and 3. This probably resulted from uncertainties in the measurement of the binding and the simplistic-binding model assumed. However, overall binding under the stimulus infusion protocol approximates the simple-binding model.

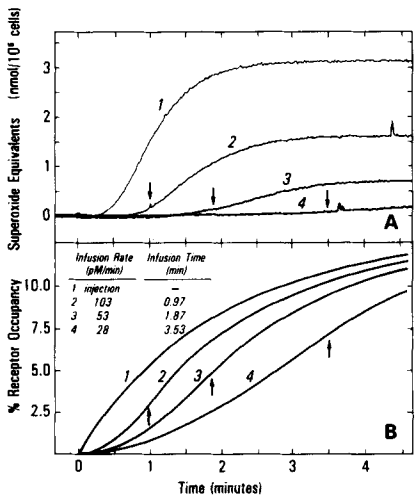


*Figure 6.* Superoxide production under conditions of stimulus infusion. $2 \times 10^6$ cells/ml buffer were stimulated with 0.1 nM FLPEP added as a bolus (*1*) or over 0.97 min (*2*), 1.87 min (*3*), or 3.53 min (*4*). The arrow for each curve indicates the time at which the infusion stopped. Superoxide equivalents were determined using the parahydroxyphenyl acetic acid assay described in Materials and Methods. *B* shows binding curves calculated for $k_{off} = 0.35$ min$^{-1}$, $k_{on} = 7 \times 10^8$ M$^{-1}$ min$^{-1}$, and the number of receptors/cell = 60,000. Data are averages of duplicate determinations from one donor and are representative of six donors.

## *Responses Under Conditions of Stimulus Infusion*

Figs. 4–6 show typical results for the responses under conditions of stimulus infusion. The results were the same in buffer with or without $NH_4^+$ (data not shown). For these experiments, the rates of the infusion and the infusion times were varied such that the final total concentration of FLPEP was the same at the end of the experiment. Figs. 4 and 5 show that the F-actin-associated right angle light scatter response and calcium response were rapid, transient responses. The analogous theoretical binding curves show that the responses were initiated and maximal changes observed long before the binding reached its plateau. When the infusion rate was below ~50 pM/min such that the rate of receptor occupancy

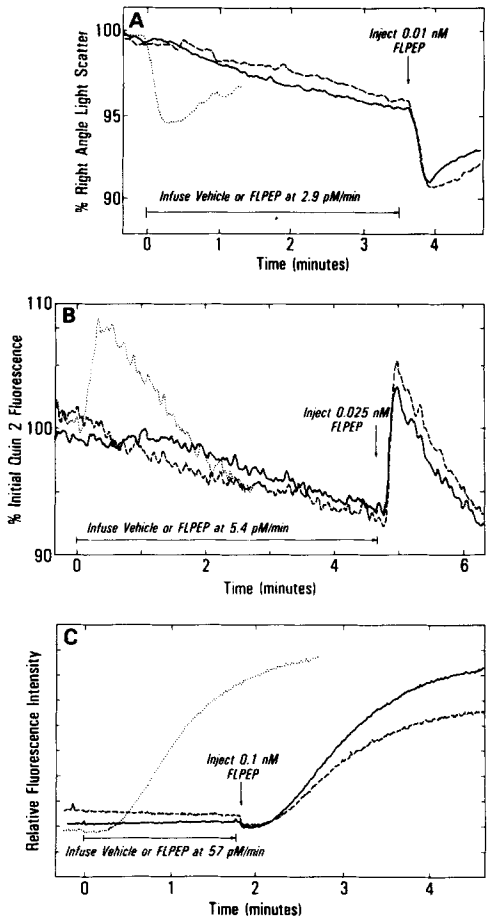


*Figure 7.* Stimulation of neutrophil responses after infusion of FLPEP at a substimulatory rate. $2 \times 10^6$ cells/ml buffer were assayed as described in Materials and Methods. FLPEP was injected at time zero (*dotted line*) or FLPEP was infused at a substimulatory rate, then an equivalent amount injected at the end of the infusion (*solid line*). As a control, cells were infused with an equal volume of vehicle, then FLPEP was injected (*dashed line*). (*A*) Right angle light scatter response. 0.01 nM FLPEP was injected or infused at a rate of 2.9 pM/min. (*B*) Intracellular calcium response. 0.025 nM FLPEP was injected or infused at a rate of 5.4 pM/min. (*C*) Superoxide production. 0.1 nM FLPEP was injected or infused at a rate of 57 pM/min. Data are averages of duplicate determinations from one donor and representative of at least two donors.

decreased into the range of 2–0.5%/min the responses decreased in amplitude and elongated in time. Hence the stimulus infusion rate was an important factor in modulating the time courses of these responses. Fig. 6 shows superoxide production under conditions of stimulus infusion. When the infusion rate was below several hundred picomolar per minute such that the occupancy rate decreased into the range of 10–3% the rate of superoxide production decreased and the final amount of superoxide produced decreased. At the end of the assay, occupancy of receptors under the condition of the slowest infusion rate was within 83% of the occupancy observed for a bolus injection. However, total superoxide produced under the condition of the slowest infusion rate was less than 10% of that produced for a bolus injection.

Fig. 7 shows that the decrease in response at slower infusion rates was not due to a global inactivation of the cells by low levels of FLPEP, because cells which had been infused with a substimulatory rate of FLPEP could still respond to an injection of FLPEP. Under the conditions used for these experiments, the number of receptors occupied by the infused peptide was less than 10%. Thus a second addition of FLPEP caused a burst of receptor occupancy comparable to an injection which was not preceded by an infusion. In addition, the response to an injection made after infusion of vehicle was comparable to the response to an injection made early in the time course of the experiment, thus cell death during the time course of the experiment was not occurring.

The decrease in response at longer infusion times could not be due to stimulus decomposition. Because the FLPEP does not contain methionine, degradation due to methionine oxidation (3, 32) can be ruled out. A peptidase activity in neutrophils has been reported which can cleave *N*-formylpeptides (4, 50). If this peptidase activity were prominent and FLPEP was being degraded to a significant extent during the infusion, the specific binding at the plateaus in Fig. 3 could not be the same for the infusion and injections. Moreover, 10 μM phosphoramidon, which has been shown to inhibit FMLP cleavage by neutral endopeptidase (4), did not alter the response (as assayed by the right angle light scatter response) when FLPEP was infused into the cell suspension and did not alter the amount of FLPEP bound over similar time periods (data not shown). Further, there was great similarity in the shapes of the dose-response curves for injections and infusion for the four *N*-formyl peptides in Fig. 1 even though they vary greatly in their effective concentrations. Since peptide decomposition is likely to vary depending on the peptide structure and concentration, again it seems unlikely that stimulus decomposition was an important factor. Taken together, these considerations lead to the conclusion that peptide decomposition was not a significant mechanism by which cell responsiveness was lost under infusion conditions.

## Discussion

### *Physiological Implications of the Temporal Characteristics of Receptor Occupancy in the Neutrophil*

Different neutrophil responses have specific dependencies on the rate of stimulus infusion. For example, Fig. 7 *C* shows that superoxide production does not occur when FLPEP is infused at 57 pM/min. However, right angle light scatter, actin, and calcium responses are observed at infusion rates much below this level (Figs. 4 and 5). The superoxide response is nearly obliterated at infusion rates during which receptor occupancy increases at rates below ~5%/min. However, calcium elevation and right angle light scatter are dramatically reduced only when the rate of increase of receptor occupancy drops into the range of 0.5%/min. Thus the sensitivity to the temporal aspects of stimulus presentation may be an important factor in the neutrophil's ability to chemotax without exhibiting inflammatory functions until it has arrived at the site of infection.

It has been shown that neutrophils chemotax in response to a spatial gradient of chemoattractant (52, 53). Using the data presented by Zigmond (52) and typical on and off rates for *N*-formyl peptides, one can approximate the rate of increase of receptor occupancy for the conditions used in her experiments. Such calculations indicate that chemotaxis occurs when new receptors are occupied at rates in the range of 1%/min. The right angle light scatter and F-actin responses observed in this study are thought to be related to morphological and biochemical changes in the neutrophil which are a prelude to and/or contributory to chemotaxis (40, 41, 49). Thus it is possible that, although the direction of movement is determined by a spatial gradient, the underlying morphological and biochemical events which are necessary for movement are nonetheless sensitive to the temporal aspects of binding.

### *Potential Mechanisms for Differential Sensitivities of Actin Polymerization, Calcium Elevation, and Oxidant Production to the Rate of Stimulus Infusion*

***Mechanisms at the Receptor Level: Different Receptor Classes.*** Studies of *N*-formylpeptide–receptor binding in neutrophil membrane preparations (16) suggested there are two classes of binding sites for FMLP, a small fraction of high-affinity sites (20–30%, $K_d$ 1 nM) and a large fraction of low-affinity sites ($K_d$ 20–60 nM). Because the concentration of FMLP required to stimulate chemotaxis is much lower than that required to stimulate oxidant production and degranulation, it was suggested that the high affinity receptors transduce chemotaxis and low affinity receptors transduce oxidant production and degranulation (16). However, more recent studies indicate that the different *N*-formylpeptide receptor forms are not due to intrinsically different receptor forms but rather are due to receptor G-protein interactions and the ability of G-protein to modulate receptor affinity for its ligand. Koo et al. (17) and Lane and Snyderman (19) have reported that the relative proportion of the two classes of sites on neutrophil membranes was sensitive to GTP, with the fraction of low affinity sites increasing upon addition of GTP or analogs. Moreover, real-time fluorescence spectroscopic studies of fluorescent *N*-formylhexapeptide (FLPEP) dissociation from permeabilized cells indicates that the addition of nonhydrolyzable GTP analogs causes the conversion of receptors from a slowly dissociating form (high affinity) to a rapidly dissociating form (low affinity), and essentially all the receptors were capable of being in either form (35). In our hands both $O_2^-$ production and cytoskeletal activation are absent when G proteins are exhaustively ribosylated (Omann, G. M., and L. A. Sklar, unpublished results). Hence

the data strongly indicates that there is a single receptor whose binding affinity is modulated by GTP via guanine-nucleotide-binding proteins. Thus we view differential sensitivity of responses to infusion of stimulus based on binding of ligand to different receptor classes which transduce different cell responses as unlikely.

*"Rate Receptor" vs. "Occupancy Receptor."* Classical receptor models use an "occupation theory" of receptor function in which the receptor is active for the duration of ligand occupancy. In contrast, in the early 1960s, Paton proposed a "rate theory" of receptor function in which the receptor binding event produces one "quantum" of excitation and the ligand-receptor complex then becomes inactive (27, 28). In simple cases where the receptor is directly linked to the measured response, the rate theory predicts that the response will be greatest at the time of stimulus addition when the rate of ligand-receptor binding events is the greatest, and the occupation theory predicts that the response will be the greatest after equilibrium has been reached when the most receptors are occupied (46). The rate theory has been implicated in several systems (10, 27, 28) including cyclic AMP stimulation of the chemotactic receptor in *Dictyostelium discoideum* (47, 48).

Because many neutrophil responses to *N*-formylpeptides are transient with the maximum being reached well before receptor binding plateaus, it was appropriate to consider neutrophil function in terms of a rate theory. Under stimulus infusion conditions, the rate theory predicts that the response would elongate in time and reach a maximum that is less than the maximum response under injection conditions. Qualitatively, this is consistent with the data. However, the loss of magnitude of responses observed under infusion conditions does not correlate well with the total rate of receptor occupancy ($[L][R]k_{on}$) predicted by theoretical calculations. For example, under the conditions of Fig. 6, where nearly 100% of the superoxide response is lost at the slowest infusion rate, the maximum value of $[L][R]k_{on}$ occurs near the end of the infusion and is reduced by only 10% when compared with the injection. Thus the differential sensitivity data cannot be explained by a simple rate theory of receptor occupancy.

Van Haastert has pointed out that the rate characteristics of responses may be determined by the biochemical details of the signal transduction pathway (46). For example, in a system such as the neutrophil, where receptors are coupled to G-proteins, the simple rate prediction would apply specifically to GTPase activity if each receptor activates a single G-protein. The occupation theory would apply to GTPase activity if each receptor activates G-proteins continuously while it is occupied by agonist. The situation in the neutrophil is yet more complicated than the models derived by Van Haastert (46) since cytoskeletal activation and oxidant production proceed through branching pathways in which receptors are transiently activated. In these pathways, the branches may be differentially amplified, the signals may be controlled by homeostatic mechanisms which function on a time scale comparable to ligand binding, and adaptive mechanisms may play a role.

### *Mechanisms Within the Signal Transduction Pathway*

To consider signal mechanisms which contribute to the differential temporal sensitivity it is necessary to describe the signal pathway explicitly (see details reviewed in references 23 and 32). Responses to formylpeptides appear to be transduced via a pertussis toxin sensitive G-protein. G-proteins activate phospholipase C which acts on phosphatidylinositol 4,5-bisphosphate to yield diacylglycerol and inositol trisphosphate. Inositol trisphosphate causes the release of calcium from intracellular stores into the cytosol (31). This cytosolic calcium rise appears to be necessary but not sufficient for generation of oxidants (8, 29). Diacylglycerol stimulation of protein kinase C (8, 15) and other phosphoinositides (44) have been implicated as possible additional signals for oxidant production. Calcium and diacylglycerol may act synergistically to activate protein kinase C and stimulate oxidant production (8, 9). However, the signal transduction sequence for actin polymerization is not known in detail. It is known that this response requires G-protein, however, it does not require an increase in cytosolic calcium (41). Whether it is mediated by phospholipase C products or some unknown G-protein-activated enzyme is not known. However, it is evident that the signal pathways for oxidant production and cytoskeletal activation diverge at a level before the calcium elevation step.

***Signal Synergy.*** We show here that superoxide is not generated at infusion rates which do stimulate the actin polymerization and calcium responses. We might expect that responses which require more than one transient signal will depend upon the interplay between all of those signals or will be limited by the signal which is most sensitive to the infusion. While maximal calcium elevation may require relatively few inositol trisphosphate molecules, the sustained stimulation of protein kinase C may require the generation of a relatively large pool of diacylglycerol. Thus under the infusion protocol, it is possible that a decrease in the level of both or either signal for oxidant production will result in a loss of synergistic coordination of these two signals. A loss of synergy in converging pathways due to the more transient activation of signals has been suggested to explain why leukotriene $B_4$ stimulation of oxidant production is deficient when compared with *N*-formylpeptide stimulation (25).

***Homeostasis.*** Homeostatic mechanisms appear to exist which function to return signal levels to resting values. For example, it has been shown that the elevated cytosolic calcium concentration (as well as other responses) rapidly returns to resting levels if new ligand-receptor binding is blocked (18, 37). The rate of decay of calcium to baseline is relatively insensitive to the ligand concentration and to the duration of exposure to the stimulus (at least within the first few minutes after stimulation, 37). Thus homeostatic mechanisms are likely to play a role in regulating responses under infusion conditions by lowering signal levels in the intervening pathways. Differential sensitivity of responses to stimulus infusion may result from different rates of recovery for different signals.

***Adaptive Pathways.*** Adaptation may occur by the stimulation of inhibitory pathways which could block activation anywhere within the activation sequence. Agents which elevate cAMP are potent inhibitors of oxidant produciton but have relatively little effect on calcium or actin polymerization responses (42). Since cAMP rises in response to *N*-formylpeptide, cAMP could play a role in the differential sensitivity of responses to stimulus infusion. De Togni et al. (6) have shown that cAMP levels rise under infusion conditions, but

are diminished compared with an injection. However, it is possible that this inhibitory pathway is saturated by submaximal cAMP levels. It is not clear if the balance between the rate of activation of the oxidant pathway and cAMP mediated inhibition of the response is altered under stimulus infusion conditions.

Pretreatment of neutrophils with phorbol esters, which stimulate protein kinase C, inhibit subsequent stimulation by *N*-formylpeptides (21). Since *N*-formylpeptide itself stimulates protein phosphorylation, it is possible that these phosphorylation events are adaptive. Phosphorylation of receptor or phospholipase C have been postulated as adaptive/inhibitory events in other cell systems (7, 51). Under conditions of stimulus infusion it has been shown that the phosphatidyl inositol turnover is dimished (6). Thus it appears that phospholipase C activation may be reduced under conditions of stimulus infusion. This suggests that adaptation may occur at least in part at the level of the receptor-G-protein-phospholipase C interactions.

Uncoupling of G-protein from the receptor may be regulated by alteration of the ratio of GTP to GDP, or other unidentified mechanisms. Mechanisms which function at the level of the receptor or uncouple G-protein from the receptor may have a differential effect on cell responses based on the difference in the number of receptors required to generate a response.

***Differential Amplification of Activation Pathways.*** Oxidant production is nearly proportional to the number of receptors occupied until all receptors are occupied. However, the actin polymerization response is saturated when less than 1% of the receptors are occupied (37). This would appear to be due to saturation of the biochemical pathway resulting in actin polymerization and differences in the extent of signal amplification which occurs in the pathways. Since it requires more occupied receptors to generate the oxidant response, we might expect oxidant production to be more sensitive to the rate of stimulus infusion.

In summary, the rate of stimulus infusion can have a significant impact on the magnitude of the resulting responses with different responses having differential sensitivity to the rate of receptor occupancy. This differential sensitivity is likely to result from the interplay of the divergent and reconverging pathways that lead to activation of the responses, the inhibitory mechanisms which result in adaptation, and homeostatic mechanisms which return signals to resting levels. This differential sensitivity may play a significant role in a cell's ability to coordinate responses such as in the neutrophil where the cell must migrate to a site of infection before it releases toxic compounds.

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