Activated neutrophils aggregate, generate superoxide (O$_2^-$), and degranulate. The role of Ca as "second messenger" in neutrophil activation was examined using as agonist the chemotactic peptide fMet-Leu-Phe and its antagonist t-butoxycarbonyl-Phe-Leu-Phe to systematically vary the time of receptor occupancy. Release of enzymes from specific and azurophil granules showed a finite requirement for receptor occupancy; the cells were committed to full degranulation after 10 s of receptor-agonist interaction. In contrast, continuous occupation of the receptor by agonist was required to initiate and maintain O$_2^-$ generation and aggregation. Cytosolic Ca (Quin2 fluorescence) increased immediately in response to fMet-Leu-Phe, requiring less than 2 s of agonist-receptor interaction to initiate an optimal response. Mobilization of membrane-associated Ca (chlorotetracycline fluorescence) also demonstrated a finite time requirement; the cells were fully committed after 10 s of agonist-receptor interaction. Increased Ca permeability (44Ca uptake) was fully launched after 15 s of agonist-receptor interaction. The data indicate that Ca movements (Quin2, chlorotetracycline fluorescence, 44Ca uptake) are both necessary and sufficient to account for degranulation by neutrophils activated by fMet-Leu-Phe. However, neutrophil aggregation and the generation and release of O$_2^-$ in response to the same stimulus require a further unknown factor(s) associated with receptor occupancy to maintain these responses.

Occupation of receptors by specific ligands gives rise to "second messengers" which act as a signal for the initiation of the subsequent physiological response, e.g. secretion, contraction (1, 2). Calcium and cyclic AMP have been proposed as classical "second messengers" in many stimulus response sequences (1, 2, 5–7), and increments in either cytosolic Ca or cyclic AMP have been proposed as sufficient signals for subsequent secretory events (7).

In neutrophils, secretory doses of a ligand, e.g. the chemotactic peptide fMet-Leu-Phe, acting at its specific receptor simultaneously triggers three very different responses—aggregation, superoxide anion (O$_2^-$) generation, and release of azurophil and specific granule contents (3, 4). It is not clear, however, how occupation of this single receptor signals the onset of three discrete processes. Nor is it known whether the signalling requirements for each process are identical. A characteristic lag period has been demonstrated between addition of a particular ligand and the onset of each response, a period representing the time required for activation. Previous work has shown that the lag period of the several responses activated by the same ligand are of different lengths (3, 6), suggesting that there are differences in the signalling requirements for the several responses. These differences could represent a requirement for different levels of a common key signal substance, the shorter the lag period the lower the trigger (threshold) level of the key substance. Alternatively, the different lag periods might indicate that different signal intermediates are rate limiting for each physiological response.

In the neutrophil, a role has been proposed for translocation of calcium ions as a signal in stimulus response coupling. A rise in cytosolic Ca, monitored by changes in Quin2 fluorescence, can be recorded immediately after activation by ligands such as fMet-Leu-Phe, concanavalin A, and aggregated IgG (8–11). The increased cytosolic Ca represents principally the mobilization of cell-associated Ca (9). Release of membrane-associated Ca, as measured by changes in chlorotetracycline fluorescence, has been proposed to act as a "trigger pool" of Ca (12). This pool-monitored chlorotetracycline may represent a local change in Ca levels as opposed to the general elevation in cytosolic Ca monitored with Quin2. Finally, although extracellular Ca is not essential to neutrophil activation, enhanced Ca permeability serves to amplify the physiological responses (13). In contrast to these Ca requirements, the activation sequence of the neutrophil does not utilize cyclic AMP as a "second messenger" (3).

To study the relationship between calcium translocation and the subsequent physiological responses triggered by receptor occupancy, we have systematically varied the amounts of putative signal by varying the duration of occupation of receptor by ligand. The strategy employed was to displace agonist by a specific antagonist at various times while measuring discrete neutrophil responses. By varying the time over which the receptor is occupied, different levels of putative signalling factor(s) should be attained. Using this agonist/antagonist pair, we could determine if continuous occupation of the receptor, as opposed to discrete length of receptor occupancy, was required in order to trigger all three physiological responses and if the accompanying calcium movements

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1 The abbreviations used are: fMet-Leu-Phe, N-formyl methionyl iucyl phenylalanine; Boc, t-butoxycarbonyl; BocPLPLP, N-tert-Butoxycarbonyl-L-alanine-p-nitrophenyl ester; O$_2^-$, superoxide anion; HbO$_2$, 4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid; Nle, norleucine.
Receptor Occupancy and Neutrophil Activation

were necessary and sufficient for their elicitation. A convenient agonist is the surrogate bacterial chemotaxtractant fMet-Leu-Phe. A competitive antagonist of fMet-Leu-Phe is BocPLPLP which has been shown to inhibit fMet-Leu-Phe-activated degranulation and decrements in chlorotetracycline fluorescence (14–16). Using this agonist/antagonist pair to vary the time of receptor occupancy and thus to vary the amount of signalling factor(s), we have shown that the different physiological functions show different requirements for receptor occupancy. O2 generation and aggregation require continuous receptor occupancy to achieve and to maintain an optimal response whereas degranulation does not. Furthermore, under conditions where full calcium mobilization is achieved, the process of O2 generation is not maintained unless the receptor remains occupied, implying that for O2 generation and aggregation a further unknown factor, associated with occupation of the receptor, is an absolute requirement.

**MATERIALS AND METHODS**

Preparation of Neutrophil Suspensions—Neutrophil suspensions containing 98 ± 1% neutrophils were prepared from heparinized venous blood (of heparinized blood) obtained from healthy adult donors. Standard isolation techniques employing Hepsy-Ficol gradients were used (17) followed by dextran sedimentation and hypotonic lysis to remove red cells. The cells were suspended in a Heps buffer, pH 7.45, having the composition of NaCl 150 mM, KCl 5 mM, CaCl2 1.29 mM, MgCl2 0.1M, and Heps 10 mM.

Cell Viability—Cell viability was determined by monitoring release of the cytoplasmic enzyme lactic dehydrogenase according to the method of Wacker et al. (18). In which greater than 4% of total cell lactic dehydrogenase was released was considered unacceptable.

f(3H)[Met-Leu-Phe Binding to Neutrophils—[3H]fMet-Leu-Phe binding was determined by incubating 4 × 106 neutrophils with 5 × 10-6 M [3H]fMet-Leu-Phe (12.5 Ci/mM), in the presence of either 10-6 M fMet-Leu-Phe or buffer for appropriate time intervals. The incubation mixture was then filtered through a Millipore filter (0.5 μm pore size) and the filter washed twice with 3 ml aliquots of ice-cold Hepes buffer. The filter was solubilized in 10 ml of Triton X (National Diagnostics) and counted in a scintillation counter. Addition of 10-4 M fMet-Leu-Phe permitted corrections to be made for nonspecific binding by subtracting the total radioactivity of [3H]fMet-Leu-Phe with 10-4 M fMet-Leu-Phe from the total activity of [3H]fMet-Leu-Phe alone.

Ultrastructural Studies—Cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, and postfixed in 1% OsO4 in 0.1 M sodium cacodylate buffer, pH 7.2. Suspensions were strained on black nitrocellulose and embedded in Spurr's embedding medium. Sections were stained with lead citrate and examined on a Zeiss EM 9 electron microscope. In order to ensure that random cells were examined, the first 10 cells containing centriole plus two nuclear lobes were photographed at a magnification of 5122. Analysis of the photographs was done using the stereological methods described by Weibel (19). Briefly, a coherent multipurpose test lattice was used for surface area estimates. The lattice consists of discrete short lines. 2.3 cm in length and equivalent to 1.48 μm at the print magnification used, whose end points are arranged in a regular triangular lattice. The number of intersections of the test line with the cell surface is used to estimate surface area, and the number of end points of the line that fall within the cell is used to estimate volume.

Degranulation—The extracellular release from cytochalasin B-treated neutrophils of the granule-associated enzymes, lysozyme, and β-glucuronidase was measured as described previously (20). β-Glucuronidase, an azurophil granule marker, was determined by incubation with phenylthialine glucuronidate as substrate (21). Lysozyme was determined as the rate of lysis of Micrococcus lysodeikticus, measured by the decrease of absorbance at 450 nm (22). Total enzyme activity was determined simultaneously in duplicate reaction mixtures containing detergent Triton X-100 (0.2%).

Elastase Assay—Activity of elastase (an azurophil granule marker) was monitored continuously by determining the release of p-nitrophenol from the substrate N-t-Boc-L-alanine-p-nitrophenyl ester (23). The reaction was followed in a twin beam spectrophotometer at 400 nm. Neutrophils (1.25 × 107/ml) were preincubated at 37 °C for 5 min with 5 μg/ml of cytochalasin B. Cells plus 150 μM N-t-Boc-L-alanine-p-nitrophenyl ester were added to both reference and sample cuvettes, and the reaction was started by the addition of 5 × 10-6 M fMet-Leu-Phe to the sample cuvette.

Superoxide Anion (O2) Generation—The generation of O2 by cytochalasin B-treated neutrophils was measured as superoxide dismutase-inhibitable cytochrome c reduction by a continuous recording method (24).

Aggregometry—Neutrophil aggregation in response to fMet-Leu-Phe was monitored by determining changes in light transmission in a dual channel aggregometer (Payton Associates) according to the method described by Craddock et al. (25). Cells (1 × 106) were placed in a siliconized microcuvette containing a siliconized stirring bar rotating at 900 rpm and preincubated in the presence of cytochalasin B (5 μg/ml) at 37 °C for 5 min. The stimulus was then added and the changes in light transmission monitored continuously. The aggregation wave was quantitated by planimetry and aggregation expressed as area under the curve in the first minute per mg of protein.

Quin2 Fluorescence Spectroscopy—Neutrophils were preloaded with Quin2, and changes in fluorescence of an unstirred suspension of preloaded cells upon stimulation were monitored as previously described (26).

Chlorotetracycline Fluorescence Spectroscopy—Chlorotetracycline fluorescence was measured by a modification of the method of Naccache et al. (26, 27). Neutrophils were preincubated at 37 °C for 10 min in the presence of 100 μM chlorotetracycline, washed, and the fluorescence changes monitored at 37 °C at emission wavelength 590 nm and excitation wavelength 380 nm (20).

"Ca Uptake Measured by a Rapid Millipore Filtration Technique—Neutrophils (3–4 × 107) were preincubated in the presence of cytochalasin B for 10 min at 37 °C and the uptake initiated by the addition of 50 μM CaCl2 in the presence or absence of stimulus. The filtration procedure was carried out as previously described (31).

Protein—Protein was determined by the method of Lowry et al. (28) using lysozyme as the standard.

Reagents—N-fMet-Leu-Phe employed at 5 × 10-8 M and t-Boc-L-Phe-d-Leu-L-Phe-d-Leu-L-Phe were obtained from Vega Biochemicals, stored as concentrated stock solutions in dimethyl sulfoxide and diluted into buffer before use. N-t-Boc-L-alanine-p-nitrophenyl ester (Vega Biochemicals) was made fresh as an ethanol stock solution and diluted into Hepes buffer. Cytochalasin B (Aldrich) was prepared as a stock solution in dimethyl sulfoxide. Chlorotetracycline was purchased from Sigma. "Ca and [3H]fMet-Leu-Phe from New England Nuclear, and Quin2 from Lancaster Synthesis.

**RESULTS**

Reversibility of fMet-Leu-Phe Binding—The concentrations of agonist/antagonist were chosen by determining the concentration of BocPLPLP that would inhibit aggregation activated by a given concentration of fMet-Leu-Phe. When 5 × 10-8 M fMet-Leu-Phe was the stimulus, it was necessary to add 10-4 M BocPLPLP in order to prevent aggregation. Due to solubility restrictions of the BocPLPLP, it was not possible to work at 10-7 M fMet-Leu-Phe, since this would have required too high a concentration of dimethyl sulfoxide, the solvent used to solubilize BocPLPLP. The time course of [3H]fMet-Leu-Phe binding during the initial 5 min is shown in Fig. 1A. Binding of the ligand was continuous for over 60 min (results not shown). The capacity was studied of the antagonist BocPLPLP to displace previously bound fMet-Leu-Phe. When 10-4 M BocPLPLP was present in the incubation mixture, it completely prevented the specific binding of 5 × 10-8 M [3H]fMet-Leu-Phe (Fig. 1A). Next [3H]fMet-Leu-Phe was allowed to bind to neutrophils for 1 min, then BocPLPLP added, and the time course of [3H]fMet-Leu-Phe displacement monitored (Fig. 1B). The [3H]fMet-Leu-Phe prebound to cells for 1 min was displaced by subsequent addition of BocPLPLP, the half-life of displacement under these conditions being approximately 15 s.

Degranulation and Receptor Occupancy—Release of granule
enzymes provoked by fMet-Leu-Phe can be monitored extracellularly by treating neutrophils with cytochalasin B. Release was monitored of the azurophil granule enzyme β-glucuronidase and the specific azurophil granule marker lysozyme in response to fMet-Leu-Phe (5 × 10⁻⁸ M) 5 min after addition of stimulus. Release of both β-glucuronidase and lysozyme was profoundly inhibited when BocPLPLP (10⁻⁴ M) was added simultaneously with the stimulus (Table I). Addition of BocPLPLP at progressively longer time periods after the addition of Met-Leu-Phe (2, 5, and 7 s), yielded progressively less inhibition (Table I). When antagonist was added 10 s after addition of fMet-Leu-Phe, no inhibition of β-glucuronidase or of lysozyme release was observed. Thus by 10 s, the cell was fully committed to complete degranulation.

In order to monitor continuously the release of azurophil granule contents, elastase release was determined by measuring the rate of cleavage of p-nitrophenol from the substrate N-t-Boc-L-alanine-p-nitrophenyl ester (Fig. 2). The stimulus, 5 × 10⁻⁸ M fMet-Leu-Phe, was added at zero time. When BocPLPLP was added at zero time almost complete inhibition of elastase release (Fig. 2) was observed. As shown for β-glucuronidase and lysozyme release, addition of antagonist at 2 and 5 s caused progressively smaller inhibition of elastase release. By 10 s, the cell was fully "committed" to release of elastase.

**Ultrastructural Changes and Receptor Occupancy—**We next determined the increase in surface to volume ratio (S/V) of the neutrophil subsequent to stimulation, since these increases have been correlated with addition of membrane to the plasmalemma during degranulation (29). Suspensions of cytochalasin B-treated neutrophils were activated by 5 × 10⁻⁸ M fMet-Leu-Phe plus substrate N-t-Boc-L-alanine-p-nitrophenyl ester for 5 min at 37 °C before addition of 5 × 10⁻⁸ M fMet-Leu-Phe (zero time). The cleavage of p-nitrophenol from N-t-Boc-L-alanine-p-nitrophenyl ester was monitored at 400 nm. Boc-Phe-Leu-Phe-Leu-Phe (10⁻⁴ M) was added at the indicated times (0, 2, 5, 10 s).

**Fig. 2. Effect of Boc-Phe-Leu-Phe-Leu-Phe addition at varying time intervals on elastase release from human neutrophils.** Cells were preincubated with 5 μg/ml of cytochalasin B and fixed with glutaraldehyde at the appropriate time. Stereological analysis of changes in S/V ratio (Fig. 3A) demonstrated a continuous increase in S/V ratio subsequent to activation, ranging from a value of 1.13 ± 0.18 in resting cells, to 2.96 ± 0.22 at 30 s. Neutrophils were then activated with 5 × 10⁻⁸ M fMet-Leu-Phe, BocPLPLP was added at varying times (Fig. 3B), and the suspension allowed to incubate for 30 s. At this time the cells were fixed with glutaraldehyde and stereological analysis of changes in S/V ratio carried out. The addition of BocPLPLP at 2 s caused a profound inhibition of the S/V change decreasing the response to 40% of control at 30 s (p < 0.01). However, when BocPLPLP was added 5 s after fMet-Leu-Phe, no inhibition of S/V change was observed. Therefore, as found for enzyme release, changes in S/V ratios (the morphological

<table>
<thead>
<tr>
<th>Table I</th>
<th>Effect of BocPLPLP addition at varying times on β-glucuronidase and lysozyme release from fMet-Leu-Phe-activated neutrophils</th>
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</thead>
<tbody>
<tr>
<td>Time of BocPLPLP addition</td>
<td>β-Glucuronidase release</td>
</tr>
<tr>
<td>Before fMet-Leu-Phe*</td>
<td>6.1 ± 1.5%</td>
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<tr>
<td>2 s after fMet-Leu-Phe</td>
<td>33.1 ± 5.7</td>
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<tr>
<td>5 s after fMet-Leu-Phe</td>
<td>64.5 ± 7.1</td>
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<tr>
<td>7 s after fMet-Leu-Phe</td>
<td>85.8 ± 4.7</td>
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<tr>
<td>10 s after fMet-Leu-Phe</td>
<td>98.8 ± 2.1</td>
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* Cells were preincubated at 37 °C for 5 min with 5 μg/ml of cytochalasin B before the addition of 5 × 10⁻⁸ M fMet-Leu-Phe (zero time). Release of β-glucuronidase and lysozyme into the medium was monitored after a further incubation for 5 min. Background release (without fMet-Leu-Phe) was 4.2 ± 1.1% of control for β-glucuronidase and 15.3 ± 0.4% of control for lysozyme.

* Per cent of control expressed as mean ± S.E. (n = 4).
Fig. 3. A, time course of changes in S/V ratio in fMet-Leu-Phe (5 × 10^{-8} M) activated cytochalasin B-treated human neutrophils. Stirred suspensions of cells were fixed at appropriate times by addition of 2% glutaraldehyde and prepared for ultrastructural studies (see under "Materials and Methods"). Data is expressed as the percent of increase in S/V ratio ± S.E. (n = 3) over the S/V ratio of resting cells. B, effect of addition of Boc-Phe-Leu-Phe-Leu-Phe at varying times after fMet-Leu-Phe (5 × 10^{-8} M) addition on changes in S/V ratio. Cells were incubated for a total of 30 s after addition of fMet-Leu-Phe fixed by addition of glutaraldehyde. Data is expressed as the percent of increase in S/V ratio ± S.E. (n = 3) over the S/V ratio of resting cells.

correlate of degranulation) show a finite requirement for receptor occupancy.

Superoxide Anion Generation and Receptor Occupancy—The generation of O_2^{-} can be readily monitored continuously as superoxide dismutase-inhibitable cytochrome c reduction. As found for degranulation, when BocPLPLP was added with the stimulus, i.e. zero time, there was a profound inhibition of O_2^{-} generation (Fig. 4). However, unlike degranulation, which was no longer inhibitable after 10 s, it was possible to inhibit the O_2^{-} generation process at any time during its course. Thus BocPLPLP added at 2, 10, and 30 s (Fig. 4), as well as 60 s (not shown), after the addition of fMet-Leu-Phe inhibited the ongoing generation of O_2^{-}. Therefore, unlike degranulation of specific and of azurophil granules, O_2^{-} generation requires continuous occupation of the receptor in order to maintain an activated state of the generating system.

Aggregation and Receptor Occupancy—Aggregation of neutrophils is also elicited by fMet-Leu-Phe (Fig. 5). In the presence of BocPLPLP added at zero time, cell aggregation was profoundly inhibited (Fig. 5). Addition of the antagonist BocPLPLP at any time during the aggregation response caused a rapid inhibition of any further response. Thus aggregation resembled O_2^{-} generation, but differed from degranulation, in requiring prolonged receptor occupancy in order to activate and to maintain the response. It should be noted, however, that although the addition of BocPLPLP halted aggregation, aggregation of the cells was not reversed, even when the cells were monitored for prolonged times.

Quin2 Fluorescence Changes and Receptor Occupancy—One of the earliest measurable events in neutrophil activation is an elevation of cytosolic Ca as measured by changes in Quin2 fluorescence. When neutrophils were preloaded with Quin2 and then exposed to 5 × 10^{-8} M fMet-Leu-Phe, there was an immediate rise in fluorescence (lag, <1 s), reflecting a rapid rise in cytosolic Ca (Fig. 6). However, when 10^{-7} M BocPLPLP was added before the addition of fMet-Leu-Phe, only minimal inhibition of the Quin2 fluorescence change was observed. However, when the BocPLPLP concentration was raised 5-fold (results not shown), a much greater inhibition of the Quin2 fluorescence changes was observed. When BocPLPLP was added 2 s after the addition of fMet-Leu-Phe, no inhibition of the rise in cytosolic Ca was noted. Thus under conditions where O_2^{-} generation, degranulation, and aggregation were profoundly inhibited, the rise in cytosolic Ca was only modestly affected, reflecting different requirements for receptor occupancy for the several responses.

Chlorotetracycline Fluorescence Changes and Receptor Occupancy—Interactions of ligands with neutrophils triggers a rapid release of membrane-associated calcium as monitored by changes in chlorotetracycline fluorescence. When fMet-Leu-Phe was added to chlorotetracycline-loaded neutrophils
Receptor Occupancy and Neutrophil Activation

FIG. 6. Effect of Boc-Phe-Leu-Phe-Leu-Phe (B) addition at varying time intervals on the changes in cytosolic calcium of human neutrophils activated with 5 x 10^{-6} M fMet-Leu-Phe (F). Cells were preincubated with 5 μg/ml of cytochalasin B for 5 min at 37 °C before addition of 5 x 10^{-6} M fMet-Leu-Phe (zero time). Cytosolic calcium was monitored as the fluorescence of Quin2 preloaded neutrophils (see under "Materials and Methods"). Boc-Phe-Leu-Phe-Leu-Phe (10^{-4} M) was added at the indicated times: A, 0 s; B, 2 s.

FIG. 7. Effect of Boc-Phe-Leu-Phe-Leu-Phe (B) addition at varying time intervals on the changes in fluorescence of chlorotetracycline preloaded human neutrophils activated with 5 x 10^{-6} M fMet-Leu-Phe (F). Cells were preincubated with 5 μg/ml of cytochalasin B for 5 min at 37 °C before addition of 5 x 10^{-6} M fMet-Leu-Phe (zero time). Chlorotetracycline fluorescence was monitored as described under "Materials and Methods." Boc-Phe-Leu-Phe-Leu-Phe (10^{-4} M) was added at the indicated times: 0, 2, 5, and 10 s.

(Fig. 7), fluorescence was rapidly lost, an event probably due to loss of membrane-associated calcium. When BocPLPLP was added at zero time, it profoundly inhibited the chlorotetracycline fluorescence response to fMet-Leu-Phe (Fig. 7). However, when BocPLPLP was added to cells already depleted of calcium, i.e., after the loss of fluorescence subsequent to activation with fMet-Leu-Phe, BocPLPLP did not permit reaccumulation of calcium. When BocPLPLP was added 2 and 5 s after fMet-Leu-Phe, progressively less inhibition of the chlorotetracycline response was observed. Finally, addition of BocPLPLP 10 s after fMet-Leu-Phe no longer inhibited the chlorotetracycline response (Fig. 7). This time course of susceptibility to BocPLPLP addition is strikingly similar to the findings for degranulation.

Calcium Permeability Changes and Receptor Occupancy—Enhanced permeability of the plasmalemma to calcium is also associated with receptor-ligand interaction. The changes in permeability lead to enhanced calcium uptake by the cell which serves to enhance the physiological responses (13). Uptake of 46Ca stimulated by fMet-Leu-Phe was a rapid event and was continuous over 5 min (Fig. 8A). When BocPLPLP
was added at the same time as the fMet-Leu-Phe. 4Ca uptake was profoundly inhibited (Fig. 8B). The degree of inhibition of the 4Ca uptake response was progressively diminished as the BocPLLP was added at 2, 5, 10, and 15 s after the fMet-Leu-Phe. This was true whether the 4Ca uptake was measured at 1, 2, or 5 min after the addition of fMet-Leu-Phe. Finally by 20 s after the addition of fMet-Leu-Phe, addition of BocPLLP was no longer capable of inhibiting calcium uptake, and the cell was fully committed to 4Ca uptake. Thus calcium uptake resembled degranulation and the chlorotetracycline response with respect to a finite time requirement for receptor occupancy in triggering a full response. The time required for occupancy was, however, 15–20 s as opposed to the 10 s required for degranulation. Calcium uptake, therefore, could also be distinguished from O2 generation and aggregation which displayed no finite time requirement and which required continuous receptor occupancy for the maintenance of an ongoing response.

**DISCUSSION**

These studies demonstrate different time requirements for receptor occupancy by fMet-Leu-Phe for three discrete physiological responses: degranulation, O2 generation, and aggregation. The length of the receptor occupancy requirement for the secretion of both azurophil and specific granule contents, under the conditions of these experiments, was 10 s, i.e. there was a finite requirement for occupation of the receptor by fMet-Leu-Phe before the cell was committed to full degranulation. No difference was observed between secretion of enzymes from the two granule types. A similar finding of a finite requirement for receptor occupancy for elastase release from neutrophils has been determined by Sklar et al. (30) using a different agonist/antagonist pair.

Changes in S/V ratio have been correlated with the addition of membrane to the plasma membrane which accompanies degranulation (29), implying that the source of this added plasma membrane may be the membranes of azurophil and/or the specific granules. Both degranulation and S/V ratio changes show finite requirements for receptor occupancy, 5 s for S/V changes and 10 s for degranulation. Although technical factors might account for this difference in time of occupancy required, the difference in time requirements may also indicate that there are a number of different granule populations that contribute to the additional plasmalemma, the contents of which have not been measured in this study. Alternatively, the additional surface membrane may not originate from granules.

In contrast to degranulation, O2 generation required continuous occupation of the receptor by fMet-Leu-Phe to initiate and maintain this response. This requirement for continuous receptor occupancy in initiating and maintaining O2 generation is not unique to fMet-Leu-Phe-activated cells; similar results have been obtained by other workers using ConA/α-methylmannoside and CHO-Nle-Leu-Phe-Nle-Tyr-Lys antibody (31, 32). Therefore, a factor that is not required for degranulation is evidently needed for the maintenance of O2 generation. Aggregation shows a similar requirement for continuous occupation of the receptor for the ongoing process to be maintained. Since Ca has been proposed as a "second messenger" in the activation sequence of the neutrophil, the temporal relationship between Ca movements and receptor occupancy was also studied. Indeed, it has been proposed that a rise in cytosolic Ca is the trigger for secretion (6). The rise in Quin2 fluorescence suggests that a rapid increment in cytosolic Ca is in fact associated with cell activation. This response was, however, only partially inhibited when BocPLLP was added at the same time as fMet-Leu-Phe, a finding in accord with the dose-response curve already reported (9). An optimal Quin2 fluorescence response was obtained at fMet-Leu-Phe concentrations as low as 5 × 10−8 M, whereas optimal secretion was not attained until the fMet-Leu-Phe concentration was raised to 10−7 M. At the antagonist/agonist ratio used in this study, the equilibrium favors occupation of the receptor by antagonist; however, a small number of receptors was occupied by agonist. This small number of agonist-occupied receptors is apparently sufficient to trigger a Quin2 fluorescence change. For responses such as degranulation, O2 generation, and aggregation, a finding that reinforces the dose-response data and leads to the conclusion that although rises in cytosolic Ca might be necessary for neutrophil activation, such an elevation in cytosolic Ca is not sufficient to trigger secretion. Indeed the low dose of fMet-Leu-Phe required to trigger changes in cytosolic Ca and the short time requirement for occupation of the receptor in order to trigger a maximal change in cytosolic Ca may indicate that changes in cytosolic Ca might more appropriately be designated a chemotactic rather than a secretory signal. However, this evidence does not rule out a requirement for locally elevated levels of Ca, perhaps released from the plasmalemma.

Ca mobilized from cellular membranes, as monitored by changes in chlorotetracycline fluorescence, has been proposed as a "trigger" pool that is essential in the activation sequence (12). This pool of Ca, however, cannot appreciably contribute to cytosolic Ca as monitored by Quin2; the two probes Quin2 and chlorotetracycline appear to monitor separate and distinct pools of Ca that are functionally distinct (10). The requirements for receptor occupancy of the chlorotetracycline response were the same as those determined for degranulation, i.e. 10 s. In addition, the dose-response for fMet-Leu-Phe for both processes was similar. Nevertheless, although the full chlorotetracycline response occurred after 10 s of interaction of the agonist with its receptor, this was not sufficient to fully trigger and maintain O2 generation and aggregation.

Ca uptake is needed for optimal physiological responses (13). The receptor occupancy requirement for Ca uptake was 15–20 s, different again from the chlorotetracycline response. Thus at 15–20 s there appears to be full Ca mobilization, i.e. rise in cytosolic Ca, chlorotetracycline fluorescence change, and Ca uptake, but this is not sufficient for full generation of O2 and aggregation, suggesting that while mobilization of Ca may be necessary and sufficient for degranulation, this is not so for O2 generation and aggregation; some other factor, possibly non-Ca associated and having a short half-life, is necessary to maintain the generation of O2 and aggregation.

The signalling requirements for the three physiological responses of degranulation, aggregation, and O2 generation are clearly different. Calcium movements may be a sufficient trigger for degranulation and for the shape changes as reported by increases in S/V. Indeed the inhibitory action of the Ca antagonist TMB8 on all three physiological responses (13) demonstrates a common requirement for Ca mobilization. However, for O2 generation and aggregation a further "factor" or "second messenger" in addition to Ca is essential in order
to maintain an ongoing response. Silinsky (33) has suggested that signalling for the release of preformed substances differs from that of secretion of a substance that must be generated. The suggestion was made that an influx of extracellular Ca is important in the secretion of a preformed product, while mobilization of intracellular Ca is important in synthesis and aggregation or if each response has its own unique activating factor serving to turn on the O₂-generating system or to maintain an ongoing response. Silinsky (33) invoked a possibility that an influx of extracellular Ca is linked secretion. We do not find such a difference in calcium requirements for the neutrophil, i.e. for degranulation versus O₂ generation. However, our data support the general concept that signalling differs for these two classes of secretion. The nature of the factor required for O₂ generation and aggregation is unknown. The factor(s) may represent either a released soluble factor(s) by analogy with the other "second messengers" calcium and cyclic AMP. Alternatively, it may represent an altered membrane state, perhaps due to phospholipid remodelling, which is dependent on the receptor being occupied by the agonist. This factor/condition may represent an activating factor serving to turn on the O₂-generating system or may represent the depression of a factor that serves to inhibit the O₂-generating system. A precedent for such an inhibitory factor is found for the adenylyl cyclase of the adipocyte (34). Present evidence does not allow us to determine if an identical "signalling factor" is involved in maintaining both O₂ generation and aggregation or if each response has its own unique factor, the activating levels of which are associated with an agonist-occupied receptor.

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