0026-895X/86/050437-07$02.00/0

MOLECULAR PHARMACOLOGY, 30:437–443



# Characterization of fMet-Leu-Phe Receptor-Mediated $Ca^{2+}$ Influx Across the Plasma Membrane of Human Neutrophils

TOMMY ANDERSSON, CLAES DAHLGREN, TULLIO POZZAN, OLLE STENDAHL and P. DANIEL LEW

*Department of Medical Microbiology, University of Linköping, Sweden (T.A., C.D., O.S.), CNR Unit for Study of Physiology of Mitochondria, c/o Institute of General Pathology, Padova, Italy (T.P.), and Division of Infectious Diseases, Department of Medicine, University Hospital, Geneva, Switzerland (P.D.L.)*

Received November 18, 1985; Accepted July 28, 1986

## SUMMARY

*N*-Formyl-L-methionyl-L-leucyl-L-phenylalanine (fMet-Leu-Phe) stimulation of human neutrophils leads to a rapid increase of the cytosolic free $Ca^{2+}$ concentration, $[Ca^{2+}]_i$, which is significantly reduced by removal of extracellular calcium. In the present study we show that fMet-Leu-Phe-induced $[Ca^{2+}]_i$ increases are, in part, mediated by an increase of the plasma membrane permeability to $Ca^{2+}$. This conclusion is based on the following evidence. (a) In the presence of extracellular calcium, addition of $La^{3+}$ reduced the fMet-Leu-Phe-induced $[Ca^{2+}]_i$ increase to approximately the same level as that observed in the absence of extracellular calcium. (b) A net increase of the plasma membrane permeability for $Mn^{2+}$ could be observed after fMet-Leu-Phe stimulation, as revealed by intracellular quenching of the quin2 signal. The influx of $Mn^{2+}$, like that of $Ca^{2+}$, was inhibited by $La^{3+}$ and was more pronounced in the absence of extracellular $Ca^{2+}$, suggesting competition for the same pathway. (c) Temporal dissociation of intracellular $Ca^{2+}$ release from stores and $Ca^{2+}$ influx from the medium could be demonstrated by readdition of calcium to cells stimulated in the absence of this cation. This second $[Ca^{2+}]_i$ increase could be abolished either by giving the specific chemotactic peptide receptor antagonist, BOC-Met-Leu-Phe, or $Co^{2+}$. We could also show that the fMet-Leu-Phe-dependent $Ca^{2+}$ influx was not due to the activation of voltage-dependent calcium channels since depolarization either by $K^+$ or gramicidin D did not affect the resting $[Ca^{2+}]_i$, nor did it affect a subsequent $[Ca^{2+}]_i$ increase induced by fMet-Leu-Phe. Furthermore, nifedipine and verapamil, at concentrations known to block classical voltage-dependent calcium channels, had no significant effects on the $Ca^{2+}$ influx induced by fMet-Leu-Phe. We suggest that fMet-Leu-Phe promotes influx of $Ca^{2+}$ ions across the plasma membrane of human neutrophils by opening of receptor-dependent calcium channels.

Mature neutrophils play an important role in host defense against microorganisms by virtue of their well known chemotactic, phagocytic, and degranulating properties. Recent studies on the ligand receptor transduction mechanism responsible for initiating these cellular activities have been shown to include polyphosphoinositide breakdown, $[Ca^{2+}]_i$ increases, membrane depolarization, and activation of arachidonic acid metabolism (1–4). The precise time sequence, as well as the interrelationship between these phenomena, are not completely understood. However, evidence is accumulating suggesting that the primary event is the hydrolysis of $PIP_2$ by a GTP-regulated, membrane-bound phospholipase C (for review see Refs. 5 and 6). This scheme is in agreement with the experimental observation that inositol 1,4,5-trisphosphate, the hydrophilic product of $PIP_2$ hydrolysis, is capable of mobilizing $Ca^{2+}$ from a membrane-enclosed intracellular $Ca^{2+}$ store in a variety of cell types (7, 8), including human neutrophils (9). Furthermore, it is well established that a number of agonists linked to $PIP_2$ hydrolysis increase $[Ca^{2+}]_i$ in intact cells (for review see Ref. 6).

In a number of cell types, e.g., human neutrophils, it is generally observed that in the presence of extracellular calcium the amplitude and the duration of the $[Ca^{2+}]_i$ increases are larger and more prolonged (10). These results, together with the observations of an increased $^{45}Ca$ uptake upon fMet-Leu-Phe stimulation, led to the suggestion that receptor triggering also results in an increased plasma membrane permeability to $Ca^{2+}$. However, no definitive experimental proof for such a hypothesis has yet been provided. In the present study evidence will be presented showing that, in human neutrophils, when the chemotactic peptide fMet-Leu-Phe binds to specific recep-

This work was supported by the Swedish Medical Research Council (Grant 5968), the Swiss National Foundation (Grant 3.990.0.84), the Italian Ministry of Public Education (40 and 60%), King Gustav V Foundation, the Swedish Society of Medicine, Ligue Swiss contre le Cancer, and the Nordic Insulin Foundation. T. A. is a recipient of a Post-Doctoral Fellowship from the Swedish Medical Research Council and P. D. L. is the recipient of a Max Cloetta Career Development Award.

**ABBREVIATIONS:** $PIP_2$, phosphatidylinositol bisphosphate; quin2, methoxyquinoline derivate of bis (*o*-aminophenoxyl)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; quin2/AM tetraacetoxymethyl ester of quin2; fura2/AM, an acetoxymethyl ester of fura2; DTPA, diethylenetriaminepentaacetic acid; fMet-Leu-Phe, *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine; BOC-Met-Leu-Phe, *N*-*tert*-butoxycarbonyl-L-methionyl-L-leucyl-L-phenylalanine; TPEN, *N*,*N*,*N*′*N*′-tetrakis (2-pyridylmethyl)ethylenediamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DMSO, dimethyl sulfoxide.

tors on the cell surface, there is an increased influx of $Ca^{2+}$ through the plasma membrane.

The demonstration of an fMet-Leu-Phe stimulated influx of $Ca^{2+}$ naturally raises questions concerning the nature and regulation of this influx. In a number of other cell types the influx of $Ca^{2+}$ across the plasma membrane occurs through voltage-dependent calcium channels (11, 12). This type of calcium channel, which is regulated by the membrane potential, has also been suggested to exist in the plasma membrane of human neutrophils (13, 14). The data indicating the existence of voltage-dependent calcium channels in neutrophils are very weak, although it is well established that the plasma membrane of these cells depolarizes upon stimulation with fMet-Leu-Phe (3). Consequently, alternative influx mechanisms for $Ca^{2+}$ have to be considered. In the present study we will provide evidence indicating that the fMet-Leu-Phe-stimulated influx of $Ca^{2+}$ does not depend on the opening of classical voltage-dependent calcium channels but rather on the activation of "receptor-regulated calcium channels."

## Materials and Methods

**Chemicals.** All reagents used were of analytical grade. quin2/AM and quin2 free acid were purchased from Calbiochem, La Jolla, CA. Ionomycin was kindly provided by Dr. C. M. Liu of Hoffman, La Roche, and bis-oxonol was provided by Dr. Roger Tsien, University of California, Berkeley, CA. fura2, fura2/AM, and TPEN were obtained from Molecular Probes, Junction City, OR. fMet-Leu-Phe, BOC-Met-Leu-Phe, DTPA, verapamil, and gramicidin D were obtained from Sigma Chemical Co., St. Louis, MO. Dextran and Ficoll-Paque were from Pharmacia Fine Chemicals, Uppsala, Sweden. Nifedipine was kindly provided by Bayer, Germany.

**Preparation of Human Neutrophils.** Blood was obtained from healthy adult volunteers, using citrate-containing tubes. Whole blood was allowed to sediment on dextran after which the neutrophils were isolated according to the method described previously (15). The rest of the contaminating erythrocytes were eliminated by a 20-sec hypotonic lysis in distilled water after which the polymorphonuclear leukocytes were washed twice before resuspension in the described calcium-containing medium. Cell counting of polymorphonuclear leukocytes was performed either in a Burker chamber or with a Coulter Counter ZF with a 100-channel pulse height analyzer from Coulter-Electronics, Ltd., England.

**Determination of Cytosolic Free $Ca^{2+}$.** The usual medium contained: 138 mM NaCl, 6 mM KCl, 1 mM $MgSO_4$, 1.1 mM $CaCl_2$, 0.1 mM EGTA, 1 mM $NaHPO_4$, 5 mM $NaHCO_3$, 5.5 mM glucose, and 20 mM Hepes (pH 7.4). This medium is defined as "calcium-containing medium" throughout this paper. When using $La^{3+}$ (see Fig. 1C), $PO_4^-$ was excluded from the medium. In some experiments $Ca^{2+}$ was omitted and this medium is defined as "calcium-free medium." Upon addition of 1 mM EGTA to this latter medium the extracellular calcium concentration decreased to less than $10^{-9}$ M. The technique of loading the cells with quin2 has been described previously (10). In short, the cells were suspended at a concentration of $5 \times 10^7$ cells/ml and were allowed to equilibrate at 37° for 5 min, after which quin2/AM was added to a final concentration of 30 μM, from a stock solution suspended in DMSO. The maximal final DMSO concentration during this part of the incubation never exceeded 0.3% (v/v). Ten min after the addition of the probe, the cell suspension was diluted to $1 \times 10^7$ cells/ml in the calicum-containing medium supplemented with 0.5% (w/v) albumin. This procedure gave an intracellular quin2 concentration of 0.34 ± 0.07 nmol/$10^6$ cells ($n$ = 19). For autofluorescence, control cells were incubated with a similar concentration of DMSO, but without quin2/AM. Loading the cells with fura2 was performed in the same way over a wide range of concentrations, i.e., 2–50 μM. After the loading procedure the cells were kept at room temperature until used. Just before use, a sample of the cell suspension was centrifuged and resuspended in medium, either calcium containing or calcium free. All of our experiments were performed within 3 hr of quin2 or fura2 loading, during which no significant elevation of cytosolic free calcium was observed. Fluorescence measurements were performed with a Perkin-Elmer fluorimeter (LS3). The cuvette holder was thermostated at 37° and equipped with a continuous stirring device. The cell number in each cuvette was 1.5–2.0 × $10^6$ cells/ml. Excitation and emission wavelengths for quin2 measurements were 339 nm and 492 nm, respectively, and for fura2 measurements, 340 nm and 510 nm, respectively. To minimize light scattering artifacts, we used two cut-off filters, UV D25 and UV 35, for excitation and emission respectively. quin2 and fura2 fluorescence as a function of the $[Ca^{2+}]_i$ concentration was determined as previously described (10, 16). The intracellular concentrations of quin2 or fura2 free acid were measured by comparing the $Ca^{2+}$-dependent fluorescence of unloaded cells, treated with 0.1% (v/v) Triton with the fluorescence of a standard solution of quin2 or fura2 free acid in the presence of unloaded cells, also treated with 0.1% (v/v) Triton in a calcium-containing medium (10, 16). All graphs shown are representative experiments of at least five very similar experiments.

**Determination of Membrane Potential.** For measurements of membrane potential, the lipophilic anion, bis-oxonol, was used as previously described (17). In short, $10^6$ neutrophils were suspended in 2 ml of buffer and incubated with 100 μM bis-oxonol for 5 min to allow a homogeneous distribution of the dye. Membrane potential changes upon various stimulations were monitored at 37° in a fluorimeter equipped with a continuous stirring device. Excitation and emission wavelengths were 540 and 580 nm, respectively. As mentioned in Ref. 17, bis-oxonol can only be used to measure qualitative and not quantitative changes of the plasma membrane potential.

## Results

We have previously reported that the duration and amplitude of the fMet-Leu-Phe-stimulated $[Ca^{2+}]_i$ increase in neutrophils are dramatically different if the stimulus is applied in the presence or absence of extracellular calcium (10). The traces from a typical experiment performed under these conditions are shown in Fig. 1. The maximal $[Ca^{2+}]_i$ in the presence of extracellular calcium in a number of experiments of this type (Table 1) is reached in approximately 90 sec ($n$ = 19) and its amplitude is nearly 3-fold higher than that obtained in the absence of extracellular calcium, where the maximal cytosolic free $Ca^{2+}$ concentration is reached in about 30 sec. Similar results were obtained with cells loaded with the new fluorescent $Ca^{2+}$ indicator, fura2 (Fig. 1, *inset*). These differences have been generally taken as evidence for an increased influx of $Ca^{2+}$ ions across the plasma membrane (2, 10). However, other explanations could be offered for the longer duration and the larger amplitude of the $[Ca^{2+}]_i$ transients observed in the presence of extracellular calcium: (a) inhibition of $Ca^{2+}$ efflux; (b) $Ca^{2+}$-induced $Ca^{2+}$ release, as suggested by Fabiato (18) for muscle sarcoplasmic reticulum (18); or (c) slow release of $Ca^{2+}$ from an intracellular pool which cannot be compensated for in the absence of a basal $Ca^{2+}$ influx.

In Fig. 1C, $La^{3+}$, a trivalent cation known to inhibit $Ca^{2+}$ binding and transport in a number of cellular systems (19–21), was added to the calcium-containing medium prior to fMet-Leu-Phe stimulation of quin2-loaded cells. In the presence of $La^{3+}$, even if $Ca^{2+}$ was present in the extracellular medium, the duration and amplitude of the $[Ca^{2+}]_i$ increase were very similar but not identical to that observed in $Ca^{2+}$-free medium (Fig. 1B), and was not statistically different from this latter condition (Table 1). Similar results were also obtained using fura2-loaded human neutrophils (Fig. 1, *inset*). Interestingly, $La^{3+}$

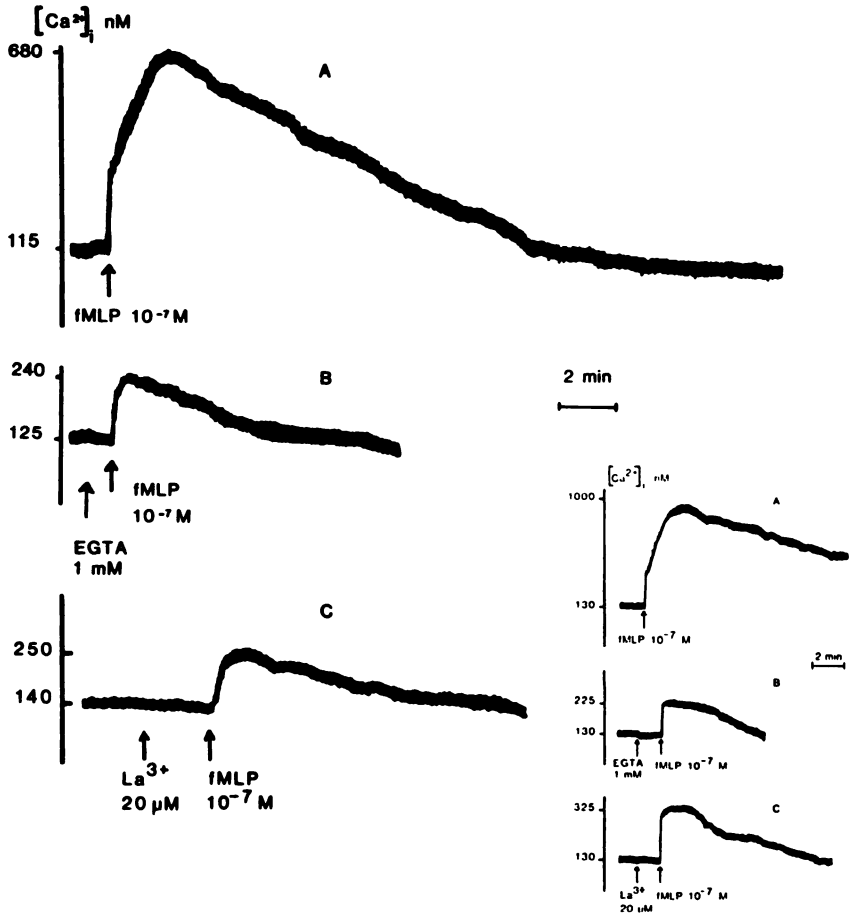


**Fig. 1.** Effects of extracellular $Ca^{2+}$ and $La^{3+}$ on fMet-Leu-Phe-induced $[Ca^{2+}]_i$ transients. Human neutrophils, loaded with quin2, were stimulated with fMet-Leu-Phe (*fMLP*, $10^{-7}$ M) in the presence (A and C) or absence (B) of extracellular calcium as described in Materials and Methods. Where indicated (C), 20 μM $La^{3+}$ was added to the cells. The *inset* shows the same experiments performed with human neutrophils loaded with fura2.

TABLE 1

**Cytosolic free $Ca^{2+}$ under the various experimental conditions**

This table summarizes the resting $[Ca^{2+}]_i$, $[Ca^{2+}]_i$ after the first 10 sec, peak $[Ca^{2+}]_i$, and times to reach peak $[Ca^{2+}]_i$ under the various experimental conditions reported in the figures. Unless indicated, the experiments were performed in a medium containing $Ca^{2+}$ as described in Materials and Methods. Mean ± standard error are given for the number of experiments indicated in parentheses. Using Student's *t* test, no statistically significant differences were found for any of the parameters either between fMet-Leu-Phe stimulation in calcium-free medium and fMet-Leu-Phe stimulation in the presence of $La^{3+}$, or fMet-Leu-Phe stimulation in the absence or presence of $K^+$ or nifedipine. The indicated differences are given in comparison to control, i.e., fMet-Leu-Phe stimulation in a medium containing $Ca^{2+}$.

| Experimental conditions | $[Ca^{2+}]_i$ | | | Time to reach peak levels |
|---|---|---|---|---|
| | Resting levels | Level after 10 sec | Peak levels | |
| | *nM* | | | *sec* |
| $Ca^{2+}$ medium | 118 ± 13 (19) | | | |
| $Ca^{2+}$-free medium | 110 ± 21 (12) | | | |
| fMet-Leu-Phe ($10^{-7}$ M) in $Ca^{2+}$-free medium | | 178 ± 5 (12) | 265 ± 22 (12)[a] | 32 ± 2 (12)[a] |
| $La^{3+}$ (20 μM) + fMet-Leu-Phe ($10^{-7}$ M) | | 185 ± 9 (8) | 290 ± 42 (8)[a] | 37 ± 2 (8)[a] |
| fMet-Leu-Phe ($10^{-7}$ M), control | | 190 ± 8 (19) | 783 ± 52 (19) | 87 ± 5 (19) |
| $K^+$ (40 mM) + fMet-Leu-Phe ($10^{-7}$ M) | | 175 ± 20 (5) | 739 ± 69 (5) | 88 ± 7 (5) |
| Nifedipine ($10^{-6}$ M) + fMet-Leu-Phe ($10^{-7}$ M) | | 185 ± 16 (5) | 710 ± 74 (5) | 85 ± 8 (5) |

[a] $p < 0.001$.

did not alter the basal $[Ca^{2+}]_i$. Similar results were obtained when $Co^{2+}$ or $Ni^{2+}$, instead of $La^{3+}$, were added before the fMet-Leu-Phe stimulation (not shown). Whereas $La^{3+}$, as well as $Co^{2+}$ and $Ni^{2+}$, has been used as a nonpermeable inhibitor of calcium channels (20–22), other divalent cations such as $Mn^{2+}$ and $Ba^{2+}$ have been shown to be permeable through calcium channels (20–21). Contrary to $Ca^{2+}$, however, $Mn^{2+}$ binding to quin2 results in quin2 fluorescence quenching rather than in fluorescence increase (22). It was thus predicted that if fMet-Leu-Phe induces $Ca^{2+}$ as well as $Mn^{2+}$ influx across the plasma membrane, then the influx of the latter cation should cause a decrease rather than an increase of the quin2 signal. Fig. 2B shows that in the presence of both $Mn^{2+}$ and $Ca^{2+}$ addition of fMet-Leu-Phe resulted in an initial fluorescence increase, similar to that observed in the absence of extracellular $Ca^{2+}$, followed by a decrease of the quin2 signal well below basal levels. Fig. 2D shows that this fluorescence decrease required the addition of fMet-Leu-Phe, since the $Mn^{2+}$-dependent decay of the quin2 signal in unstimulated cells was much less pronounced over the same period of time. Furthermore, the fluorescence quenching induced by fMet-Leu-Phe was larger when $Mn^{2+}$ was added in the absence of extracellular $Ca^{2+}$ (not shown), suggesting a competition between the influx of $Mn^{2+}$ and $Ca^{2+}$. It can be argued that the quenching of quin2 fluorescence is not due to $Mn^{2+}$ influx into the cytoplasm but rather to quin2 release into the medium containing $Mn^{2+}$. However,

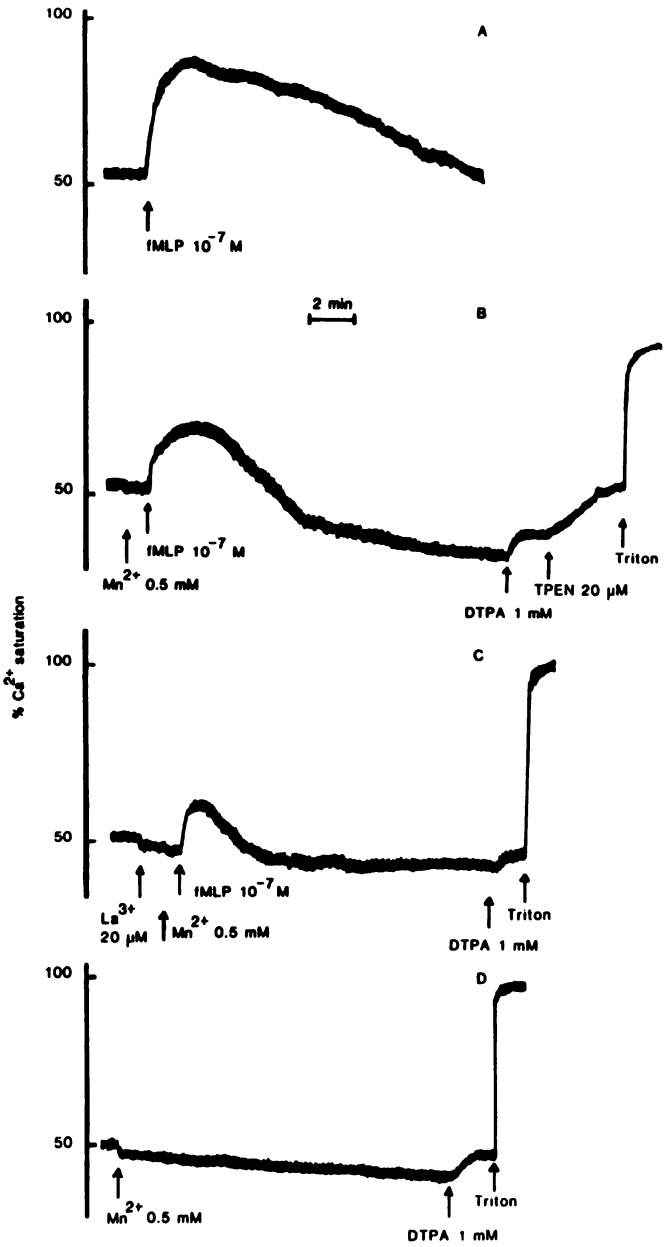


**Fig. 2.** Effects of $Mn^{2+}$ and $La^{3+}$ on fMet-Leu-Phe-induced $[Ca^{2+}]_i$ transients. Human neutrophils, loaded with quin2, were stimulated with fMet-Leu-Phe (*fMLP*, $10^{-7}$ M) in a medium containing a calcium concentration as described in Materials and Methods. Where indicated, $Mn^{2+}$ (0.5 mM), DTPA (1 mM), TPEN (20 μM), Triton X-100 (0.1%), and $La^{3+}$ (20 μM) were added to the cells. The *ordinate* is labeled as % $Ca^{2+}$ saturation since, in the presence of $Mn^{2+}$, the decrease of fluorescence is due to quenching and does not necessarily reflect a decrease of $[Ca^{2+}]_i$.

three lines of evidence strongly argue against this possibility. 1) Fig. 2B shows that addition of DTPA, a nonpermeant high affinity heavy metal chelator (23), resulted in a negligible fluorescence increase. However, after DTPA addition, lysis of the cells with Triton X-100 increases the fluorescence up to full $Ca^{2+}$ saturation (Fig. 2D). This indicates that DTPA would have been effective if the $Mn^{2+}$-quin2 complex had been in the extracellular medium. 2) Fig. 2B shows that TPEN, a membrane-permeant heavy metal chelator devoid of any $Ca^{2+}$ binding and ionophoric capacity (23), when added after DTPA, increases quin2 fluorescence well above resting level. 3) Fig. 2C shows that $La^{3+}$ largely prevents the fMet-Leu-Phe-induced quenching of quin2 fluorescence in the presence of $Mn^{2+}$.

Temporal dissociation of $Ca^{2+}$ release from stores and $Ca^{2+}$ influx has been demonstrated in other cell types (24, 25). Fig. 3A shows that, in the absence of extracellular $Ca^{2+}$, addition of fMet-Leu-Phe caused the usual small and transient increase of $[Ca^{2+}]_i$.

Addition of $Ca^{2+}$ back to the medium, 2 min after stimulation with fMet-Leu-Phe, resulted in a second phase of $[Ca^{2+}]_i$ increase, up to a level similar to that observed in the continuous presence of extracellular $Ca^{2+}$. This second phase depended on the presence of fMet-Leu-Phe (Fig. 3B) and could be antagonized by either treatment with BOC-Met-Leu-Phe (Fig. 3C) or $Co^{2+}$ (Fig. 3D). Taken together, these results (Figs. 1–3) strongly support the hypothesis that the interaction of fMet-Leu-Phe with its receptor results in increased plasma membrane permeability to $Ca^{2+}$.

In the experiments presented in Figs. 4–6 we investigated whether this influx pathway could be identified as a classical

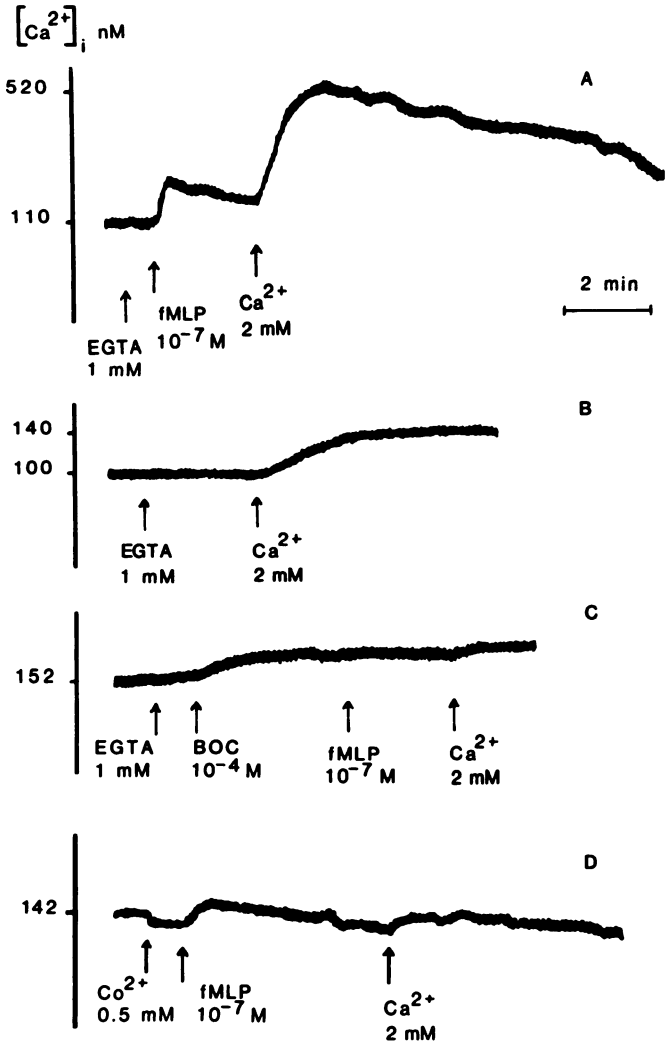


**Fig. 3.** Temporal dissociation of $Ca^{2+}$ mobilization from intracellular stores and influx across the plasma membrane. Human neutrophils, loaded with quin2, were stimulated with fMet-Leu-Phe (*fMLP*, $10^{-7}$ M) in a calcium-free medium as described in Materials and Methods. Where indicated, EGTA (1 mM), $Ca^{2+}$ (2 mM, giving a $Ca^{2+}$ concentration in the medium of approximately 1 mM after saturation of EGTA), BOC-Met-Leu-Phe (*BOC*, $10^{-4}$ M), and $Co^{2+}$ (0.5 mM) were added to the cells.

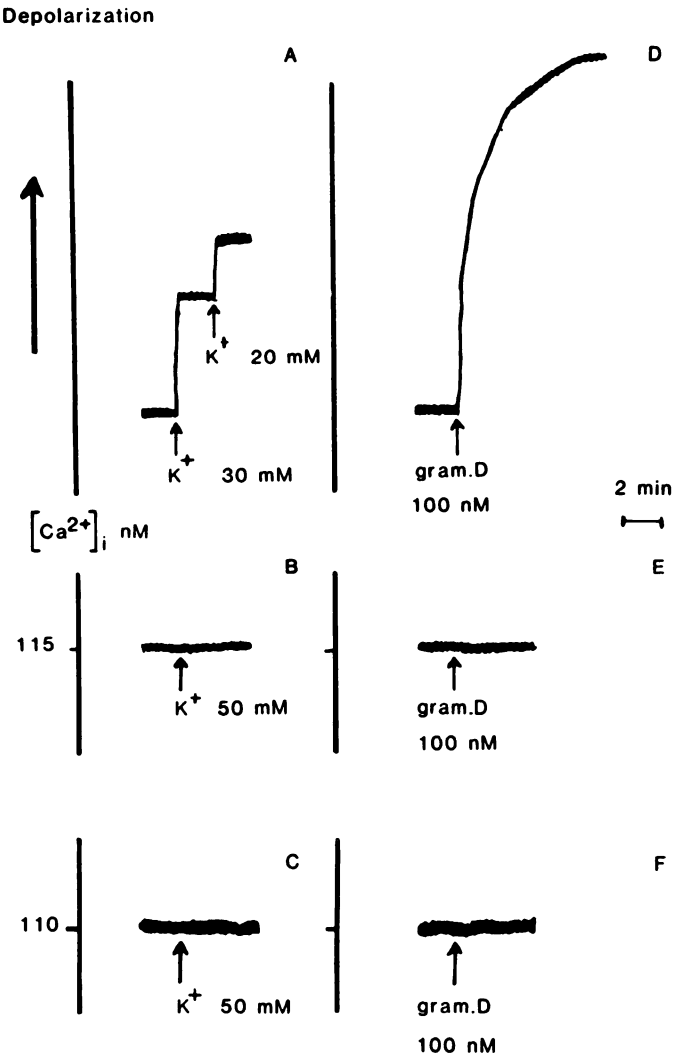


**Fig. 4.** Effects of $K^+$ and gramicidin D on the membrane potential and resting $[Ca^{2+}]_i$. Human neutrophils, loaded with bis-oxonol (A and D), quin2 (B and E) or fura2 (C and F) were suspended in a medium containing a calcium concentration as described in Materials and Methods. Where indicated, $K^+$ (20 mM, 30 mM, or 50 mM) and gramicidin D (*gram.D*, 100 nM) were added to the cells. The *arrows* in A and D indicate bis-oxonol fluorescence increase which qualitatively corresponds to plasma membrane depolarization (arbitrary units).

voltage-dependent calcium channel, similar to that known to exist in a number of neurosecretory cell types (11, 12). Fig. 4, A–C shows that depolarizing concentrations of KCl had no effect on $[Ca^{2+}]_i$, nor did it change the subsequent response to fMet-Leu-Phe (Fig. 5B and *inset*). Similar results were obtained when a larger plasma membrane depolarization was induced with the pore-forming ionophore, gramicidin D (Fig. 4, D–F). It should be mentioned that gramicidin D, by increasing dramatically the permeability of the plasma membrane to $Na^+$, should collapse the membrane potential to approximately 0 mV. Furthermore, when concentrations of nifedipine (1 $\mu$M) or verapamil (20 $\mu$M), considered optimal for specifically blocking voltage-dependent calcium channels in other cell types, were used, the responses to fMet-Leu-Phe were unaltered (Fig. 5C and *inset*, and Fig. 6). Normal fMet-Leu-Phe-induced $[Ca^{2+}]_i$ transients could also be elicited when higher concentrations of both nifedipine (10 $\mu$M, not shown) and verapamil (40 $\mu$M, Fig. 6) were used. However, above 50–100 $\mu$M verapamil, some inhibition of fMet-Leu-Phe-dependent $[Ca^{2+}]_i$ increase could be observed (Fig. 6).

The traces presented in the figures are typical examples of experiments performed with qualitatively identical results on at least five other occasions. In Table 1 the means (± standard error) of resting levels, the levels of $[Ca^{2+}]_i$ after the first 10 sec, peak $[Ca^{2+}]_i$, and times to reach peak $[Ca^{2+}]_i$ are given for all of the experiments described in the figures.

## Discussion

Whereas the mechanism of fMet-Leu-Phe-induced $Ca^{2+}$ release from intracellular stores in human neutrophils is reasonably well understood (1, 9, 26, 27), the mechanism and existence of an increased $Ca^{2+}$ influx from the extracellular medium upon receptor stimulation remains a matter of controversy. The previous evidence in favor of an increased influx of $Ca^{2+}$ originates from studies with $^{45}Ca$. Flux studies using radioactive isotopes have, in the past, often given contradictory results and, if not supported by direct measurements of $[Ca^{2+}]_i$, might lead to erroneous interpretations. The larger amplitude and longer duration of the $[Ca^{2+}]_i$ rise observed in the presence of extracellular $Ca^{2+}$ are often taken as evidence for an increased $Ca^{2+}$ influx from the extracellular milieu as a consequence of receptor stimulation. However, it could be argued that either an inhibition of $Ca^{2+}$ extrusion or a $Ca^{2+}$-induced release of intracellular $Ca^{2+}$ might similarly explain such experimental findings.

In this report several pieces of evidence strongly support the hypothesis that in human neutrophils the chemotactic peptide fMet-Leu-Phe increases the permeability of the plasma membrane to calcium ions. 1) Addition of $La^{3+}$, as well as $Co^{2+}$ and $Ni^{2+}$, reduced the fMet-Leu-Phe-induced $[Ca^{2+}]_i$ increase to an amplitude similar to that observed in calcium-free medium. 2) The extent of $Mn^{2+}$ influx, measured as quenching of the intracellular quin2 signal, is very low in unstimulated cells, but increases severalfold upon fMet-Leu-Phe addition. 3) $La^{3+}$, as well as $Co^{2+}$ and $Ni^{2+}$, largely prevents the fMet-Leu-Phe-induced influx of $Mn^{2+}$. 4) $Ca^{2+}$ release from intracellular stores and increased influx of $Ca^{2+}$ can be temporally dissociated by applying the stimulus in a calcium-free medium and adding $Ca^{2+}$ back when $[Ca^{2+}]_i$ has returned to resting levels. The $Ca^{2+}$ influx is dependent upon receptor occupancy since it is not observed in the absence of fMet-Leu-Phe or in the presence of BOC-Met-Leu-Phe, a specific antagonist of fMet-Leu-Phe receptor binding (28).

The observation that $Co^{2+}$, an effective inhibitor of neural voltage-dependent calcium channels (20, 21), is an effective inhibitor of $Ca^{2+}$ influx raises the question whether in neutrophils the $Ca^{2+}$ influx actually occurs through this types of channel. This hypothesis has previously been suggested on the basis of two experimental observations. First, receptor stimulation with fMet-Leu-Phe strongly depolarizes the plasma membrane of neutrophils (3). Second, verapamil, a classical inhibitor of voltage-gated calcium channels, has been shown to inhibit $^{45}Ca$ influx and neutrophil functions (13, 14). Our present data argue, however, against the possibility that voltage-dependent calcium channels, if present, participate in the fMet-Leu-Phe-induced increase of $[Ca^{2+}]_i$ in human neutrophils and suggest, rather, that the fMet-Leu-Phe-induced $Ca^{2+}$ influx is due to opening of receptor-operated calcium channels. This

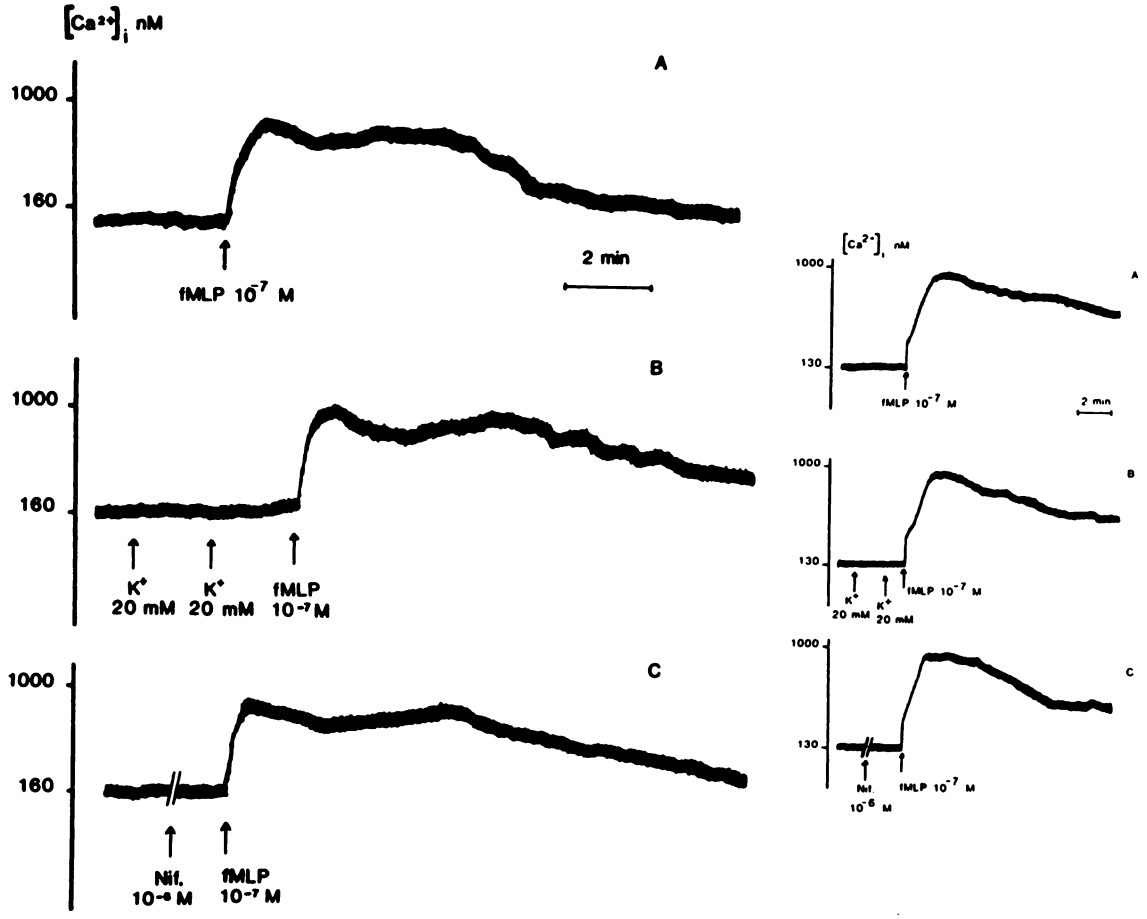


**Fig. 5.** Effects of $K^+$ and nifedipine on the resting level and fMet-Leu-Phe-induced increase of the $[Ca^{2+}]_i$. Human neutrophils, loaded with quin2, were stimulated with fMet-Leu-Phe (*fMLP*, $10^{-7}$ M) in a medium containing a calcium concentration as described in Materials and Methods. Where indicated, $K^+$ (20 mM) and nifedipine ($10^{-6}$ M) were added to the cells. The *broken trace* in B indicates correction for a drop in autofluorescence. The *inset* shows the same experiments performed with human neutrophils loaded with fura2.

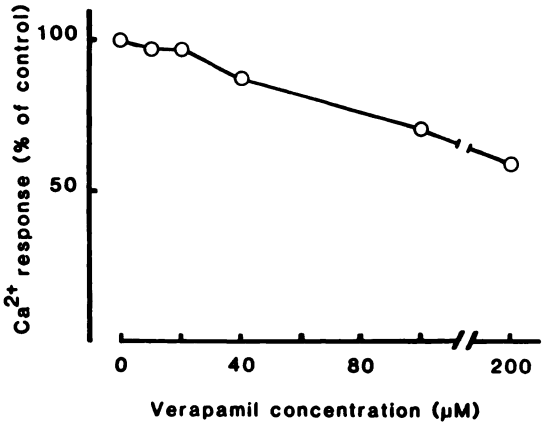


**Fig. 6.** Effects of verapamil on the fMet-Leu-Phe-induced $[Ca^{2+}]_i$ transient. Human neutrophils, loaded with quin2, were stimulated with fMet-Leu-Phe ($10^{-7}$ M) in a medium containing a calcium concentration as described in Materials and Methods. Three min before the fMet-Leu-Phe stimulation various concentrations of verapamil (20–200 μM) were added to the cell suspensions. The response in the absence of verapamil was set to 100% and all other responses were calculated as per cent of this control. All values were from the same batch of cells.

conclusion is based on the following observations. 1) The concentrations of verapamil necessary for effectively inhibiting $Ca^{2+}$ influx in neutrophils are at least 1 order or magnitude higher than those necessary to block classical voltage-dependent calcium channels in nerve, muscle, and endocrine cells (20, 21), In a recent study by Della Bianca *et al.* (29), it was concluded that the inhibition of neutrophil functions induced by verapamil is complex and included effects on cAMP levels, inhibition of protein kinase C, and binding of $Ca^{2+}$ to cellular components. 2) Another specific blocker of voltage-gated calcium channels, nifedipine, is ineffective in neutrophils at concentrations of 10 μM or more, whereas it completely blocks $Ca^{2+}$ influx induced by plasma membrane depolarization in several neuroendocrine cells at nM levels (11, 12). 3) Plasma membrane depolarization either with high $K^+$ concentrations or with the pore-forming ionophore, gramicidin D, which exchanges intracellular $K^+$ with extracellular $Na^+$, are without effect on both the resting $[Ca^{2+}]_i$ and on the $[Ca^{2+}]_i$ increases induced by maximal stimulatory doses or fMet-Leu-Phe. Incidentally, the lack of effect of gramicidin D argues against a major role of $Na^+$ in regulating $Ca^{2+}$ homeostasis in human neutrophils. Two other experimental observations from our and other laboratories also argue against the participation of classical voltage-dependent calcium channels in the fMet-Leu-Phe-induced increase of $[Ca^{2+}]_i$ in human neutrophils. First,

phorbol myristate acetate has no effect on resting $[Ca^{2+}]_i$ levels despite being very powerful in depolarizing the plasma membrane of neutrophils (30, 31). Second, normal $[Ca^{2+}]_i$ increases in response to fMet-Leu-Phe are observed in neutrophils from patients with chronic granulomatous disease, even though their cells depolarize only slightly upon stimulation (32). Using an experimental approach similar to that employed in this study, Hallam (33) and Pozzan *et al.* (25) concluded that in platelets and PC12 cells, receptor-operated calcium channels are opened upon stimulation of receptors linked to polyphosphoinositide breakdown. The present data, however, do not offer any clues as to the mechanism of regulation of these receptor-operated calcium channels in human neutrophils. A receptor-operated channel is in fact a practical definition of an experimental observation and no evidence regarding the molecular nature of this channel is presented. Neither do we know whether the increase of $Ca^{2+}$ influx is brought about by a channel or depends on the generation of an endogenous $Ca^{2+}$ ionophore. The existence of receptor-operated calcium channels has been postulated for a number of agonists in a variety of cell types. Direct (electrophysiological) evidence for their existence, however, has not yet been provided. There are a number of particularly important questions regarding receptor-operated $Ca^{2+}$ influx. a) Is this channel part of the receptor itself or does it comprise a unit separate from the receptor? b) Does it open as a consequence of ligand binding or does it require the generation of intracellular messengers? Relevant to this second question is the observation that, in neutrophils, pertussis toxin entirely inhibits fMet-Leu-Phe-induced $Ca^{2+}$ influx and mobilization from intracellular stores, as well as the increased production of inositol phosphates (26). This makes it tempting to speculate that products of polyphosphoinositide breakdown, apart from mobilizing $Ca^{2+}$ from intracellular stores, might also be involved in the regulation of $Ca^{2+}$ influx.

**Acknowledgments**

We are indebted to Ms. Johanna Gustavsson and Antoinette Monod for excellent technical assistance.

**References**

1. Di Virgilio, F., L. M. Vicentini, S. Treves, G. Riz, and T. Pozzan. Inositol phosphate formation in fMet-Leu-Phe-stimulated human neutrophils does not require an increase in the cytosolic free $Ca^{2+}$ concentration. *Biochem. J.* **229**:361–367 (1985).
2. Pozzan, T., D. P. Lew, C. Wollheim, and R. Y. Tsien. Is cytosolic ionized calcium regulating neutrophil activation? *Science (Wash. D. C.)* **221**:1413–1415 (1983).
3. Seligmann, B. E., E. K. Gallin, D. L. Martin, W. Sjain, and J. I. Gallin. Interaction of chemotactic factors with human polymorphonuclear leukocytes: studies using a membrane potential-sensitive cyanide dye. *J. Membr. Biol.* **52**:257–272 (1980).
4. Samuelsson, B. Leukotrienes: mediators of immediate hypersensitivity reactions and inflammation. *Science (Wash. D. C.)* **220**;568–575 (1983).
5. Berridge, M. H. Inositol trisphosphate and diacylglycerol as second messengers. *Biochem. J.* **220**:345–360 (1984).
6. Williamson J. R., R. H. Cooper, S. K. Joseph and A. P. Thomas. Inositol trisphosphate and diacylglycerol as intracellular second messengers in liver. *Am. J. Physiol.* **248**:C203–C216 (1985).
7. Prentki, M., T. J. Biden, D. Janjic, R. F. Irvine, M. J. Berridge, and C. B. Wollheim. Rapid mobilization of $Ca^{2+}$ from rat insulinoma microsomes by inositol-1,4,5-trisphosphate. *Nature (Lond.)* **309**:562–564 (1984).
8. Burgess, G. M., P. P. Godfrey, J. S. McKinney, M. J. Berridge, R. F. Irvine, and J. W. Putney, Jr. The second messenger linking receptor activation to internal $Ca^{2+}$ release in liver. *Nature (Lond.)* **309**:63–66 (1984).
9. Prentki, M., C. B. Wollheim, and P. D. Lew. $Ca^{2+}$ homeostasis in permeabilized human neutrophils: characterization of $Ca^{2+}$-sequestering pools and the action of inositol 1,4,5-trisphosphate. *J. Biol. Chem.* **259**:13777–13782 (1984).
10. Lew D. P., C. B. Wollheim, F. A. Waldvogel, and T. Pozzan. Modulation of cytosolic free calcium transients by changes in intracellular calcium buffering capacity: correlation with exocytosis and $O_2^-$ production in human neutrophils. *J. Cell Biol.* **99**:1212–1220 (1984).
11. Capponi, A. M., P. D. Lew, L. Jornot, and M. B. Vallotton. Correlation between cytosolic free $Ca^{2+}$ and aldosteron production in bovine adrenal glomerulosa cells. Evidence for a difference in the model of action of angiotensin II and vasopressin. *J. Biol. Chem.* **259**:8863–8869 (1984).
12. Wollheim, C. B., and T. Pozzan. Correlation between cytosolic free $Ca^{2+}$ and insulin release in an insulin secreting cell line. *J. Biol. Chem.* **259**:2262–2267 (1984).
13. Steiner, R. D., A. Pratt, and W. W. Buse. Cytochalasin B facilitates the inhibition of human polymorphonuclear leukocyte generation of superoxide by verapamil. *J. Lab. Clin. Med.* **103**:949–958 (1984).
14. Simchowitz, L., and I. Spilberg. Generation of superoxide radicals by human peripheral neutrophils activated by chemotactic factor. Evidence for the role of calcium. *J. Lab. Clin. Med.* **93**:583–593 (1979).
15. Böyum, A. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* **97**:77–89 (1968).
16. Tsien, R. Y., T. Pozzan, and T. J. Rink. Calcium homeostasis in intact lymphocytes: cytoplasmic free calcium monitored with a new, intracellularly trapped fluorescent indicator. *J. Cell Biol.* **94**:325–334 (1982).
17. Rink, T. J., C. Montecucco, T. R. Hesketh, and R. Y. Tsien. Lymphocyte membrane potential assessed with fluorescent probes. *Biochim. Biophys. Acta* **595**:15–30 (1985).
18. Fabiato, A. Calcium-induced release of calcium from cardiac sarcoplasmic reticulum. *Am. J. Physiol.* **245**:C1–C14 (1983).
19. Boucek, M. M., and R. Snyderman. Calcium influx requirements for human neutrophil chemotaxis: inhibition by lanthanum chloride. *Science (Wash. D. C.)* **193**:905–907 (1976).
20. Borle, A. B. Control, modulation, and regulation of cell calcium. *Rev. Physiol. Biochem. Pharmacol.* **90**:14–153 (1981).
21. Rosenberger, L., and D. J. Triggle. Calcium, calcium translocation, and specific calcium antagonists, in *Calcium and Drug Action* (G. B. Weiss, ed.). Plenum Press, New York, 1–32 (1978).
22. Beaven, M. A., J. Rogers, J. P. Moore, T. R. Hesketh, G. A. Smith, and J. C. Metcalfe. The mechanism of the calcium signal and correlation with histamine release in 2H3 cells. *J. Biol. Chem.* **259**:7129–7136 (1984).
23. Arslan, P., F. Di Virgilio, M. Beltrame, R. Y. Tsien, and T. Pozzan. Cytosolic $Ca^{2+}$ homeostasis in Erlich and Yoshida carcinomas: a new, membrane-permeant chelator of heavy metals reveals that these ascites tumor cell lines have normal cytosolic free $Ca^{2+}$. *J. Biol. Chem.* **260**:2719–2727 (1985).
24. Capponi, A. M., P. D. Lew, and M. B. Vallotton. Cytosolic free calcium levels in monolayers of cultured rat aortic smooth muscle cells: effects of angiotensin II and vasopressin. *J. Biol. Chem.* **260**:7836–7842 (1985).
25. Pozzan, T., F. Di Virgilio, L. M. Vicentini, and J. Meldolesi. Activation of muscarinic receptors in PC12 cells. Stimulation of $Ca^{2+}$ influx and redistribution. *Biochem. J.* **234**:547–553, (1986).
26. Krause, K.-H., W. Schlegel, C. B. Wollheim, T. Andersson, F. A. Waldvogel, and D. P. Lew. Chemotactic peptide activation of human neutrophils and HL60 cells: pertussis toxin reveals correlation between inositol triphosphate generation, $Ca^{2+}$ transients, and cellular activation. *J. Clin. Invest.* **76**:1348–1354 (1985).
27. Dougherty, R. W., P. P. Godfrey, P. C. Hoyle, J. W. Putney, Jr., and R. J. Freer. Secretagogue induced phosphoinositide metabolism in human leukocytes. *Biochem. J.* **222**:307–314 (1984).
28. Korchak, H. M., C. Wilkenfeld, A. M. Rich, A. R. Radin, K. Vienne, and L. E. Rutherford. Stimulus response coupling in the human neutrophil: differential requirements for receptor occupancy in neutrophil responses to a chemoattractant. *J. Biol. Chem.* **259**:7439–7445 (1984).
29. Della Bianca, V., G. Grzeskowiak, P. De Togni, M. Cassatella, and F. Rossi. Inhibition by verapamil of neutrophil response to formylmethionylleucylalanine and phorbol myristate acetate. Mechanisms involving $Ca^{2+}$ changes, cyclic AMP and protein kinase C. *Biochim. Biophys. Acta* **845**:223–236 (1985).
30. Lagast, H., T. Pozzan, F. A. Waldvogel, and P. D. Lew. Phorbol myristate acetate stimulates the ATP-dependent calcium transport by the plasma membrane of neutrophils. *J. Clin. Invest.* **73**:878–883 (1984).
31. Sha'afi, R. I., J. R. White, T. F. P. Molski, J. Schefcyk, M. Volpi and P. H. Naccache. Phorbol 12-myristate 13-acetate activates rabbit neutrophils without an apparent rise in the level of intracellular free calcium. *Biophys. Biochem. Res. Commun.* **114**:638–645 (1983).
32. Lew, P. D., C. B. Wollheim, R. A. Seger, and T. Pozzan. Cytosolic free calcium changes induced by chemotactic peptide in neutrophils from patients with chronic granulomatous disease. *Blood* **63**:231–233 (1984).
33. Hallam, T. J. Agonists increase the permeability of the platelet plasma membrane to divalent cations: evidence for receptor-operated channels. *Eur. J. Clin. Invest.* **15**:A64 (1985).

**Send reprint requests to:** Dr. Tommy Andersson, Department of Medical Microbiology, University of Linköping, S-581 85 Linköping, Sweden.