Cell Surface Expression of fMet-Leu-Phe Receptors on Human Neutrophils

Correlation to Changes in the Cytosolic Free Ca^{2+} Level and Action of Phorbol Myristate Acetate

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Abstract

We have studied how cytosolic free Ca^{2+} ($[Ca^{2+}]_i$) changes and phorbol myristate acetate (PMA) exposure affects ligand-independent cell surface expression of fMet-Leu-Phe receptors on human neutrophils. Mere incubation primed neutrophils to double their binding of fMet-Leu-Phe. This spontaneous increase of peptide binding was unaffected by changes in the extracellular calcium concentration. However, depression of the $[Ca^{2+}]_i$ totally abolished the increased binding of fMet-Leu-Phe. Scatchard-Plot analysis revealed that the observed increase of peptide binding was due to an increased number of receptors. Normalization of the $[Ca^{2+}]_i$ in cells where it was initially depressed resulted in a slow but progressive increase in fMet-Leu-Phe binding. The rate of receptor recruitment could be enhanced by rapidly increasing the $[Ca^{2+}]_i$ by addition of ionomycin.

Addition of PMA to cells with near maximal receptor expression led to a marked reduction of fMet-Leu-Phe binding without affecting $[Ca^{2+}]_i$. These observations suggest the existence of a dual regulatory mechanism for up- and down-regulation of fMet-Leu-Phe receptors on the cell surface of human neutrophils.

Introduction

Human neutrophils are equipped with a number of specific cellular functions, and all are of crucial importance for host defense against invading microorganisms. Initiation of these various neutrophilic activities usually occurs as a result of interaction between a ligand and its specific plasma membrane receptor, as is the case for the chemotactic peptide fMet-Leu-Phe (for review see references 1 and 2). Receptor activation initiates a number of intracellular signals, and special attention has been given to the increase in the $[Ca^{2+}]_i$ and the increased activity of protein kinase C (3–6). Using quin2, a high affinity fluorescent probe for Ca^{2+}, it has been shown that interaction between the chemotactic peptide fMet-Leu-Phe and its membrane receptor on human neutrophils results in a biphasic rise of the $[Ca^{2+}]_i$ (7, 8). Several pieces of evidence indicate that after the initial interaction between fMet-Leu-Phe and its specific receptors, which are already present on the cell surface at the time of exposure, the ligand–receptor complexes disappear from the cell surface via endocytosis (9, 10). At the same time, up-regulation of unoccupied receptors are thought to occur from an intracellular reservoir (for review see reference 1). Possible involvement of the $[Ca^{2+}]_i$ in receptor recruitment and internalization has been discussed in a number of recent papers. It was shown that a high concentration of the calcium ionophore A23187 increases the recruitment of four different cell surface receptors in mouse tumor macrophages and CR1 receptors in human neutrophils, while it resulted in inhibition of epidermal growth factor–receptor internalization in three different cell lines (11–13). In addition to changes in the $[Ca^{2+}]_i$, another intracellular signal has been intimately connected with both receptor internalization and recruitment. This is the activity of the phospholipid and Ca^{2+}-dependent enzyme protein kinase C. Direct activation of this enzyme with phorbol esters has been shown to stimulate the internalization of transferrin receptors both in K562 cells (14) and in HL-60 cells (15, 16). However, in human neutrophils it has been shown that phorbol myristate acetate (PMA) exposure results in an increased expression of fMet-Leu-Phe receptors (17). Furthermore, two recent reports claim that, depending upon the concentration used, PMA can increase both externalization and internalization of CR1 receptors in human neutrophils (18, 19).

Due to the obvious controversy about how receptor expression is regulated, the aims of the present investigation were to correlate changes in the $[Ca^{2+}]_i$ and PMA exposure to alterations of fMet-Leu-Phe cell-surface receptor expression on human neutrophils. The results presented suggest the existence of a dual regulatory control mechanism for fMet-Leu-Phe receptor expression, i.e., $[Ca^{2+}]_i$ increases up-regulation and PMA stimulates down regulation of fMet-Leu-Phe receptors on the cell surface of human neutrophils.

Methods

Chemicals. All reagents were of an analytical grade. Dextran and Ficoll-Paque were from Pharmacia Fine Chemicals Inc. (Uppsala, Sweden). Ionomycin, quin2, and quin2/AM were from Calbiochem-Behring Corp. (La Jolla, CA) and were stored as stock solutions in dimethylsulfoxide (DMSO) at −20°C. fMet-Leu-Phe, PMA, luminol, cytochrome *c*, superoxide dismutase, and glucose oxidase were all obtained from Sigma Chemical Co. (St. Louis, MO). New England Nuclear (Boston, MA) provided ^{3}H-fMet-Leu-Phe. ^{125}I was obtained from Amersham International (Buckinghamshire, U. K.).

Isolation of human neutrophils. Blood was obtained from healthy adult volunteers. The whole blood was allowed to settle on dextran after which the neutrophil granulocytes were isolated according to the method described by Böyum (20). The rest of the contaminating erythrocytes were eliminated by hypotonic lysis after which the polymorphonuclear leukocyte (PMN) cells were washed twice before resuspension in the calcium containing medium that has been described.

Determination and manipulation of cytosolic free Ca^{2+}. The medium used 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). As stated in the legends, Ca^{2+} was

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omitted in some experiments, with and without the addition of 1 mM EGTA. This resulted in extracellular calcium concentrations in the range of micromolars and nanomolars, respectively. The technique of loading the cells with quin2 has previously been described (7). The cells were suspended at a concentration of 5×10^7 cells/ml and after 5 min at 37°C, quin2/AM was added so that there was a final concentration of 100 μM. 10 min after the addition of the probe, the cell suspension was diluted to 1×10^7 cells/ml with the same type of medium supplemented with 0.5% (wt/vol) albumin. The loading procedure was completed after incubation for 50 min at this cell concentration. This loading procedure yielded a cellular quin2 concentration of 1.48±0.21 nmol/10^6 cells (n = 12). Due to the relatively high concentration of quin2/AM which was added, it was possible to manipulate the $[Ca^{2+}]_i$ by changing the extracellular calcium concentration in the loading medium. Control experiments showed that the viability of cells loaded with 100 μM did not significantly differ from controls when assessed with trypan blue exclusion tests.

Measurements of fluorescence were performed with a fluorimeter (model MPF-3L; Perkin-Elmer Corp., Pomona, CA). The cuvette holder was maintained at 37°C by means of a thermostat and was equipped with a continuous stirring device. The excitation and emission wavelengths were 339 and 492 nm, respectively. To minimize light-scattering phenomena, we used two cut-off filters, UV D25 and UV 35, for excitation and emission, respectively. Quin2 fluorescence as a function of the $[Ca^{2+}]_i$ was determined as previously described (7). The intracellular concentrations of quin2 were measured by comparing the Ca^{2+}-dependent fluorescence of quin2-loaded cells, treated with 0.1% (vol/vol) Triton, with the fluorescence of a standard solution of quin2 in the presence of unloaded cells, also treated with 0.1% (vol/vol) Triton, in a calcium-containing medium.

Determination of ^{3}H-fMet-Leu-Phe binding. The different cell suspensions, in triplicates for each experiment, were placed in an ice-bath and 20 nM ^{3}H-fMet-Leu-Phe was added (this gave ~ 30% saturation of fMet-Leu-Phe binding). To evaluate the contribution of nonspecific binding, samples containing the above components plus 10^3 times more nonlabeled fMet-Leu-Phe, were run in parallel. Scatchard-plot analyses were performed using concentrations of ^{3}H-fMet-Leu-Phe between 2 and 200 nM. The cell suspensions (final volume 200 μl) were incubated in the ice-bath with the radiolabeled peptide for 60 min. This was followed by rapid filtration (15 s) of the cell suspensions using a cell harvester (Skatron AS, Lier, Norway) and including a washing with an ice-cold medium (total volume, 4 ml). The filters were then placed in liquid scintillation vials for determination of the cell-bound radioactivity. As a control for the filtration technique, we performed identical ^{3}H-fMet-Leu-Phe–binding studies but separated cells from unbound peptide by centrifugation through an oil layer (in a microfuge; Beckman Instruments Inc., Fullerton, CA). This technique allowed cells to be separated from unbound ligands within a few seconds. The results obtained with the centrifugation method did not significantly differ from those of the previously described filtration technique.

Measurements of chemiluminescence and O_2^- production. The generation of oxidative metabolites was determined by measuring the production of chemiluminescence in a six-channel Biolumat LB 9505 (Berthold, Wildbad, W. Germany). Human neutrophils were suspended in a calcium-containing medium supplemented with luminol (final concentration 2×10^{-5} M). These cell suspensions were placed in the Biolumat (maintained at 37°C by means of a thermostat) and after addition of ionomycin and different concentrations of PMA, the light emission for each tube was recorded continuously for 60 min. Superoxide production was measured continuously, essentially as described by Cohen and Chovaniec (21), using a DU-6 spectrophotometer (Beckman Instruments Inc.) with a temperature regulator set at 37°C. In the standard assay, both sample and reference cuvettes contained 2×10^6 cells and 1.5 mg cytochrome c in 1 ml of the calcium-containing medium. Furthermore, the reference cuvette contained 200 U of superoxide dismutase. After the addition of ionomycin and different concentrations of PMA, the absorbance change accompanying cytochrome c reduction was monitored continuously for 60 min at 550 nm.

Iodination. Enzyme-mediated iodination of intact human neutrophils was performed essentially as previously described (22–24). A modification of the medium described above, lacking Mg^{2+} and Ca^{2+} and with an EGTA concentration of 2 mM, was used during the labeling procedure. Human neutrophils (2×10^7/ml) were incubated in this EGTA-containing medium at room temperature for 30 min together with 10 μg/ml lactoperoxidase, 5.5 mM glucose, 1.4 U glucose oxidase, and 50 μCi ^{125}I. Immediately after labeling, the cells were extensively washed five times in the EGTA-containing medium. The cells were then incubated at 37°C for 50 min in the same medium used for all other experiments done in this study, either with or without PMA (10^{-9} or 10^{-7} M), as described in the legend to Table II. After this incubation for 50 min, the cells were centrifuged (500 g for 10 min) and washed twice. The pellets were lysed in 0.5 ml of a 10% Triton solution and stored together with the medium at −20°C. Triplicate 100-μl aliquots of cells and medium from each group were precipitated with 25% TCA and placed on Millipore HA filters (pore size, 0.45 μm), and then washed with 4 ml of 25% TCA and dried. The respective radioactivity levels were determined using 1282 CompuGamma universal gamma counter (LKB Instruments, Inc., Gaithersburg, MD).

Results

Fig. 1 shows how incubation of human neutrophils before determination of ^{3}H-fMet-Leu-Phe binding almost doubled the binding capacity for the peptide, indicating a spontaneous increase of receptor expression. This observation corresponds well to previous functional results (25). A similar protocol has also recently been shown to increase both the number of CR1 and

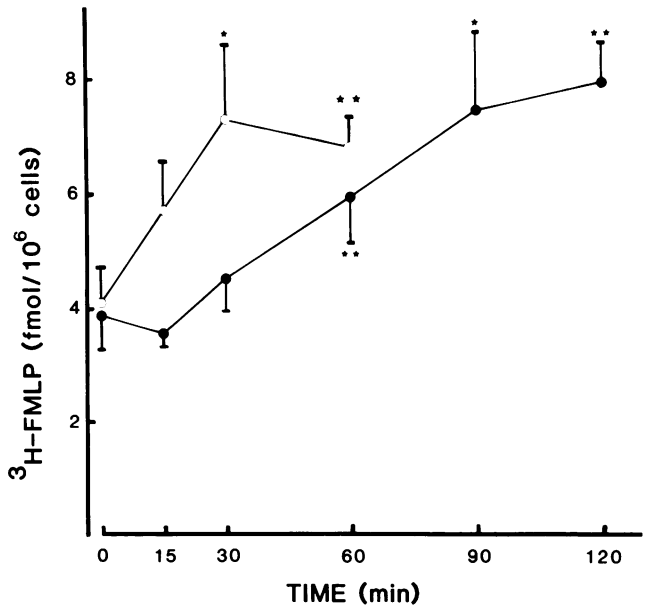

Figure 1. Binding of ^{3}H-fMet-Leu-Phe to human neutrophils after incubation in a medium containing calcium as described in Methods. Purified human neutrophils were incubated at 22°C (*solid circles*) or 37°C (*open circles*) for various periods of time. After 0 (control), 15, 30, 60, 90, and 120 min, samples were taken for determination of cellular ^{3}H-fMet-Leu-Phe binding. To measure peptide binding, the cells were incubated with ^{3}H-fMet-Leu-Phe in an ice-bath for 60 min, followed by a rapid filtration of the cell suspension with a cell harvester. The filters were washed with ice-cold medium and then placed in liquid scintillation vials for determination of the specific cell-bound radioactivity. Mean values±SEM are given for 6–10 separate experiments. Statistical analyses were performed using the t test. * $P < 0.05$. ** $P < 0.01$.

CR3 receptors on human neutrophils (26). Under the conditions used in this investigation, there was an increase from ~ 3.9 to 7.9 fmol bound ^{3}H-fMet-Leu-Phe/10^6 cells. This effect of incubation before peptide binding was shown to be temperature dependent, because increasing the temperature from 22° to 37°C during incubation resulted in a more rapid rise to maximal peptide binding. To avoid unnecessarily long incubations of the cells and to allow for optimal conditions for the quin2 loading, all the subsequent experiments were performed at 37°C.

Fig. 2 shows how changes in the extracellular and the intracellular calcium concentrations during 60 min of incubation at 37°C affected the increased receptor expression for the chemotactic peptide. Reduction in the extracellular calcium concentration from the millimolar to the micromolar range had no effect on receptor expression (Fig. 2 *A*). A further decrease in the extracellular calcium concentration to the nanomolar range, by supplementation with 1.0 mM EGTA, was also without effect on receptor expression (not shown). However, modulation of the $[Ca^{2+}]_i$, with the use of high quin2 loading, yielded quite different results. Loading human neutrophils with 100 μM quin2/AM in the presence of 1.0 mM Ca^{2+}, resulting in a normal resting level of the $[Ca^{2+}]_i$ (legend to Fig. 2), had no effect on receptor expression (Fig. 2 *B*). This observation indicates that the probe itself had no nonspecific effects on the expression of fMet-Leu-Phe receptors. However, when the quin2 loading was performed in the absence of extracellular calcium, resulting in a significant reduction in the $[Ca^{2+}]_i$ to half of normal resting levels (see legend to Fig. 2), the previously observed increase in receptor expression was totally abolished (Fig. 2 *B*). Further reduction in the $[Ca^{2+}]_i$ concentration to 9.1 nM (n = 5) after the addition of 1 mM EGTA to a calcium-free medium during the quin2 loading had no additional effect on the receptor expression (not shown). To determine whether these changes of receptor expression resulted from a change in receptor affinity or a change in receptor number, we performed a Scatchard-plot analysis of cells from the two groups marked *A* and *B* in Fig. 2 *B*. Fig. 3 shows a Scatchard-plot analysis of one representative experiment, treating the data as representing binding to one population of receptors (2). The K_D values were; 6.9×10^{-8} M (*A*) and 5.9×10^{-8} M (*B*). The number of receptors were 2.8×10^4/cell (*A*) and 1.3×10^4/cell (*B*), respectively. The affinity and the number of fMet-Leu-Phe receptors in this study are in the same range as those previously reported (17, 27). This analysis revealed that the depressed receptor expression after reduction in the $[Ca^{2+}]_i$ is most readily explained by a decreased number of fMet-Leu-Phe receptors on the cell surface.

To further investigate the role of $[Ca^{2+}]_i$ in receptor up-regulation, we reconstituted a normal resting $[Ca^{2+}]_i$ after initially having reduced it to ~ 50 nM (Fig. 4 *B*). This was done by restoration of the extracellular calcium concentration to 1.0 mM (indicated by an arrow in Fig. 4 *B*). The normalization of the $[Ca^{2+}]_i$ occurred within 5 min, and the number of ^{3}H-fMet-Leu-Phe–binding sites started to increase simultaneously. Control experiments with the same batch of cells showed no increase in peptide binding sites when the $[Ca^{2+}]_i$ was maintained at ~ 50

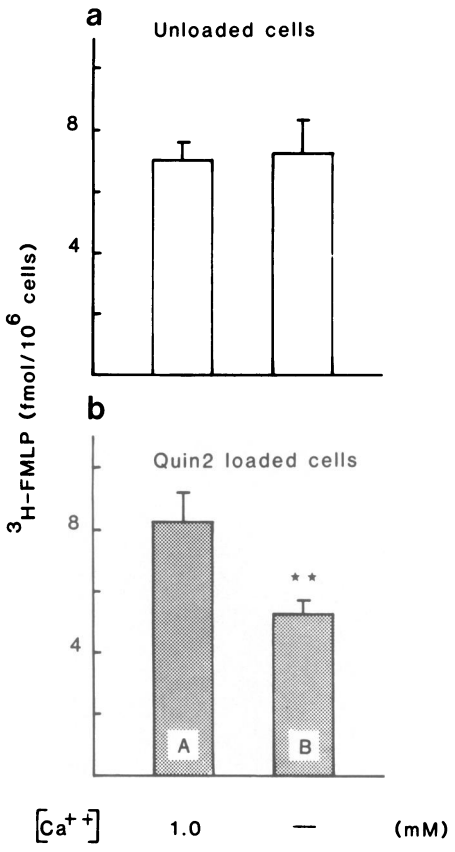

Figure 2. Effects of changes in extracellular and cytosolic-free Ca^{2+} concentrations on cellular binding of ^{3}H-fMet-Leu-Phe. Purified human neutrophils were incubated with either 100 μM quin2/AM, dissolved in DMSO (*B*), or in equal volumes of DMSO alone (*A*). The medium used during these incubations differed only with regard to the concentration of calcium, as indicated. Immediately after the loading procedure, samples were taken for determination of both ^{3}H-fMet-Leu-Phe binding and $[Ca^{2+}]_i$, as previously described. The mean values±SEM for the $[Ca^{2+}]_i$ of the groups marked with *A* and *B* were 129±11 nM and 52±9 nM, respectively. The ^{3}H-fMet-Leu-Phe binding data are given as mean values±SEM for six to nine separate experiments. The two bars in *b* marked with *A* and *B*, respectively, were chosen for further Scatchard-plot analysis (see Fig. 3). The effect of lowering the $[Ca^{2+}]_i$ was evaluated with the *t* test. * $P < 0.05$; ** $P < 0.01$.

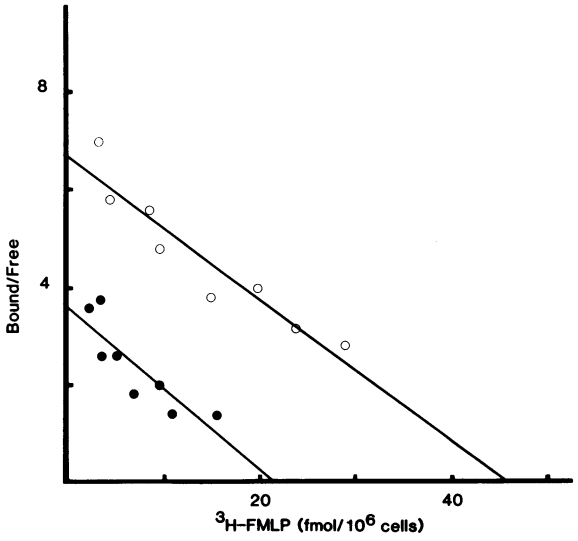

Figure 3. Scatchard-plot analysis of ^{3}H-fMet-Leu-Phe binding to cells with normal or depressed $[Ca^{2+}]_i$, marked A and B in Fig. 2. The Scatchard-plot analysis in this figure is based on data from one representative experiment out of six.

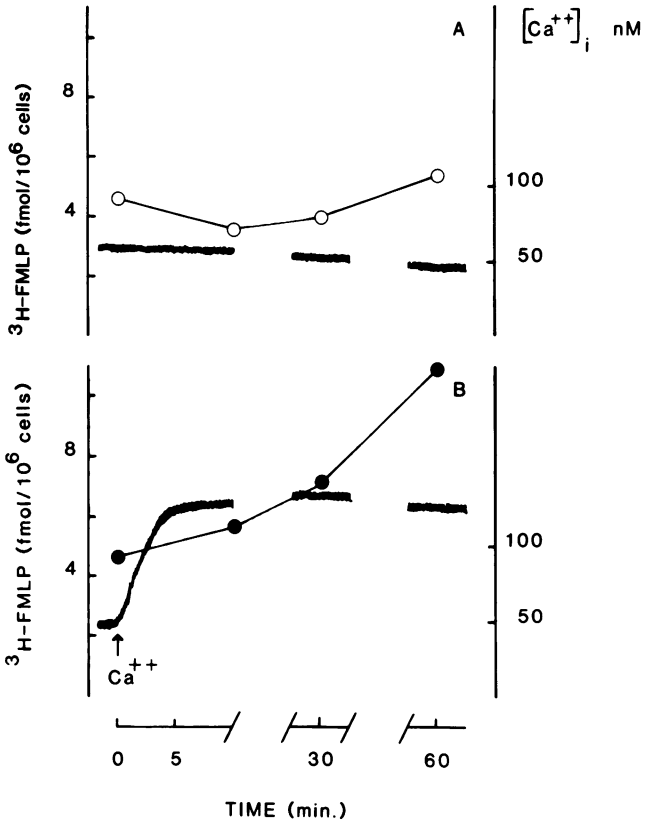

Figure 4. Correlation between $[Ca^{2+}]_i$ and cellular binding of ^{3}H-fMet-Leu-Phe. Human neutrophils were loaded with quin2/AM under conditions similar to those for the group marked B in Fig. 2. (*A*) Cells were kept in a calcium-free medium. (*B*) Extracellular calcium concentration was reconstituted to 1 mM (*arrow*). At different time points (0, 10, 30, and 60 min) samples were taken from both groups for determination of their cellular ^{3}H-fMet-Leu-Phe-binding capacity. Furthermore, cells were taken from each group to follow changes in the $[Ca^{2+}]_i$. To avoid photobleaching of the quin2 signal, the $[Ca^{2+}]_i$ were not followed continuously, as indicted by the broken lines in this figure. Data are from the same batch of cells. Table I presents the compiled results of the ^{3}H-fMet-Leu-Phe-binding data from this series of experiments.

nM (Fig. 4 *A*). Furthermore, when 1 mM Ca^{2+} was introduced together with ionomycin (250 nM) to quin2-loaded cells, a more rapid increase in the $[Ca^{2+}]_i$ to micromolar levels was obtained (Fig. 5, *A* and *B*). This rapid increase in the $[Ca^{2+}]_i$ to well above the resting value resulted in an even more rapid (already significant after 10 min) increase of fMet-Leu-Phe receptor expression on the cell surface (Table I and Fig. 5, *A* and *B*). A problem worth mentioning in this context, although not addressed in the present study, is whether the Ca^{2+}-induced effects observed are direct or indirect. It could well be that the effect of a rise in the $[Ca^{2+}]_i$ could be due to the consequent production of other intracellular messengers. For example, a rise in the $[Ca^{2+}]_i$ has been implicated in activating phospholipase A2 (28) and also in increasing the breakdown of polyphosphoinositides (29).

PMA, considered and often used as a direct activator of protein kinase C (30, 31), has previously been shown to increase the expression of fMet-Leu-Phe receptors in human neutrophils (15), while it has been reported to stimulate receptor internalization in other cell types (14–16). To study whether PMA exposure could decrease receptor expression also in human neutrophils, we chose a condition in which the number of cell surface receptors had reached a near maximal level, i.e., the process of receptor up-regulation was negligible. Furthermore, because a rise in the cytosolic free Ca^{2+} level has been suggested to increase the translocation of protein kinase C from the cytosol to the plasma membrane and thereby potentiate the effect of PMA (32), our protocol also ought to provide optimal conditions for protein kinase C–mediated effects. Fig. 5 *B* shows the effects on fMet-Leu-Phe receptor expression of two different concentrations of PMA (10^{-7} and 10^{-9} M) introduced 10 min after the addition of Ca^{2+} (1 mM) and ionomycin (250 nM). Both concentrations resulted in a prompt reduction of peptide binding to the cells. The lower concentration of PMA reduced the binding to approximately the same level as observed before the addition of Ca^{2+} and ionomycin, whereas the higher concentration of PMA reduced peptide binding to a much lower level (Table I). The effect of PMA on peptide binding could also be observed at normal resting $[Ca^{2+}]_i$ (i.e., 120 nM), if this compound was added after the cell surface expression of fMet-Leu-Phe receptors had reached near maximal values (i.e., 1 h at 37°C). Because pretreatment with ionophore also substantially potentiates PMA-induced superoxide production (5), we tested the effect of these two PMA concentrations on the generation of O_2^-. Exposure to ionomycin (250 nM) + PMA (10^{-7} M) resulted in a marked production of superoxide anions (Fig. 5 *C*), so that it is possible that part of the decreased peptide binding at this concentration could be due to an autooxidative process. However, the addition of scavengers, i.e., catalase and superoxide dismutase, had no effect on the reduced receptor expression (not shown). Furthermore, ionomycin (250 nM) + PMA (10^{-9} M) had a minimal effect, if any, on the generation of O_2^- (Fig. 5 *C*), but nevertheless reduced the fMet-Leu-Phe binding by ~ 50% (Table I). Identical results were obtained when the PMA-induced metabolic activity was assessed with luminol-dependent chemiluminescence. After incubation and before determination of ^{3}H-fMet-Leu-Phe binding, the number of cells was always calculated for each group with the aid of a Coulter counter. No loss of cell numbers could be detected in any of the samples and could consequently not explain the PMA-induced decrease of fMet-Leu-Phe–receptor expression. Furthermore, to exclude the possibility that the PMA-induced decrease of fMet-Leu-Phe–receptor expression was due to a loss of cell surface membrane by shedding to the media, whole cells were labeled with ^{125}I by means of a lactoperoxidase-catalyzed reaction, essentially as described previously (22–24). Iodinated cells were exposed for 50 min to either of the two PMA concentrations which were used, after which cells were separated from the media by means of centrifugation. Table II shows that PMA exposure did not significantly decrease the cellular content of radioactivity nor did it increase the radioactivity in the media.

Discussion

The cellular mechanisms involved in receptor recruitment and internalization are of fundamental importance for various aspects of cell biology. In human neutrophils these processes are essential for the understanding of chemotaxis, phagocytosis, and secretion (1, 2). During locomotion of neutrophils, a continuous receptor cycling and asymmetric distribution on the cell surface are thought to be critical for sensing the chemotactic gradient and maintaining a directed movement (33, 34). Although our knowledge about the systems that control membrane flow and

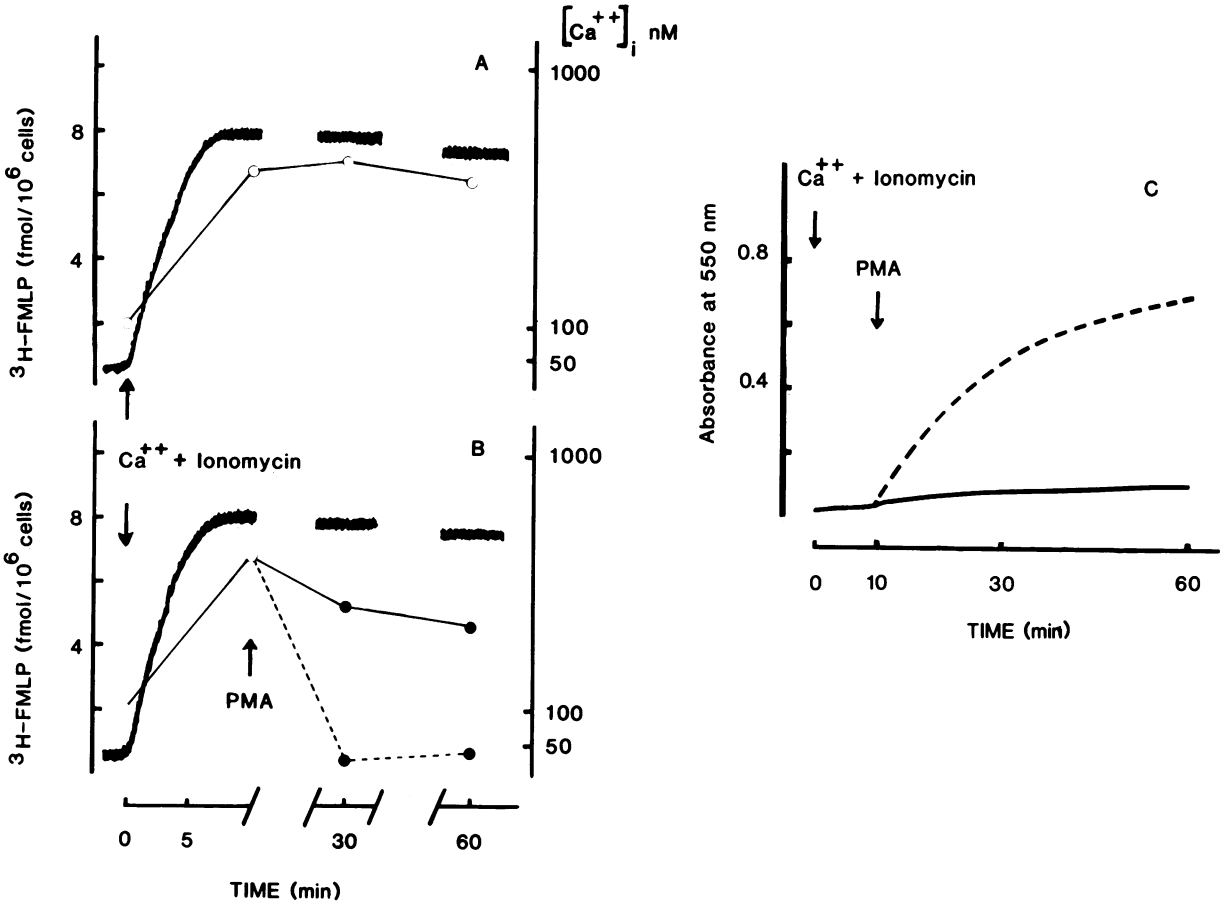

Figure 5. Correlation between rapid changes in the $[Ca^{2+}]_i$ and exposure to PMA vs. cellular binding of ^{3}H-fMet-Leu-Phe and superoxide production. Human neutrophils were loaded with quin2/AM under conditions similar to those for the group marked B in Fig. 2. *A*, cells were initially kept in a calcium-free medium, but at the point indicated with the arrow the extracellular calcium concentration was reconstituted to 1 mM and 250 nM ionomycin was added. *B*, cells were treated similarly for up to 10 min, and then the cell suspension was split into two and PMA, 10^{-9} (*solid line*) was added to one group and 10^{-7} M (*broken line*) was added to the other group, as indicated in the figure. At different time points (0, 10, 30, and 60 min) samples were taken from each group for determination of their cellular ^{3}H-fMet-Leu-Phe–binding capacity. Furthermore, cells were taken from each group to follow changes in the $[Ca^{2+}]_i$. To avoid photobleaching of the quin2 signal, the $[Ca^{2+}]_i$ were not followed continuously, as indicated by the broken lines in this figure. The compiled results of the ^{3}H-fMet-Leu-Phe–binding data from this series of experiments are presented in Table I. *C*, O_2^- production in response to ionomycin and PMA. *Solid line*, response to 10^{-9} M; *broken line*, response to 10^{-7} M of PMA. *C*, one representative experiment out of five.

receptor cycling is limited, much information has been gained about signals that are triggered after ligand binding to the chemotactic receptor (6, 7, 35, 36). The catalytic unit of this transduction mechanism appears to be a membrane bound phospholipase C linked to an *N* regulatory protein. Increased phospholipase C activity has been shown in a number of cells to result in an increased production of inositol trisphosphate, which mobilizes Ca^{2+} from intracellular stores, and diacylglycerol, which activates protein kinase C (for review see references 3, 37, and 38). In this study we have bypassed the plasma membrane receptors and have directly manipulated the $[Ca^{2+}]_i$ and exposed the cells to PMA, a phorbol ester known to activate protein kinase C (30, 31), and have studied their effects on cell surface expression of fMet-Leu-Phe receptors on human neutrophils. Our results address not only the question of whether the signals triggered by chemotactic receptor activation modulate subsequent receptor availability, i.e., as a positive or negative feed-back mechanism, but also the more general question as to how receptor distribution is controlled independent of ligand binding.

The development of quin2, a high affinity indicator of free Ca^{2+}, has also made it possible to both monitor and manipulate with the $[Ca^{2+}]_i$ in small cells (6, 7, 39). Using the latter property of quin2, it has been possible to show that IgG-mediated phagocytosis, but not C3bi-mediated phagocytosis, is a calcium-dependent process in human neutrophils (40). To make use of the chelating properties of quin2, high intracellular concentrations of this probe must be used. In a previous report we have shown that under the conditions of our studies the effects of quin2 can be attributed mainly, if not totally, to buffering of the $[Ca^{2+}]_i$ and not to any nonspecific side effects (7).

Direct evidence that the increased fMet-Leu-Phe receptor expression is modulated by the $[Ca^{2+}]_i$ was obtained from the three following observations. First, receptor up-regulation was entirely abolished when the basal $[Ca^{2+}]_i$ was decreased to ~ 50 nM. Second, this process was entirely reversible by reconstituting

Table I. Effects of Cytosolic Free Ca^{2+} Changes and PMA Exposure on Chemotactic Peptide Receptor Expression

Additions			^{3}H-fMet-Leu-Phe			
Ca^{2+}	Ionomycin	PMA	0 min	10 min	30 min	60 min
mM	*nM*	*nM*	*fmol/10^6 cells*	*fmol/10^6 cells*	*fmol/10^6 cells*	*fmol/10^6 cells*
—	—	—	4.6±0.2	3.7±0.3	3.4±0.2	4.6±0.5
1.0	—	—	4.6±0.2	4.5±0.3	6.8±1.0*	7.3±0.7§
1.0	250	—	4.6±0.2	6.1±0.7‡	8.4±0.8§	10.8±1.1§
1.0	250	1	—	—	4.8±0.4§	4.6±0.5‡
1.0	250	100	—	—	0.6±0.3§	0.8±0.1§

The accumulated results of peptide binding from experiments also presented in Figs. 4 and 5. Mean values±SEM are given for five to seven different experiments. The effects of calcium addition alone (1 mM) or calcium (1 mM) plus ionomycin (250 mM) were statistically analyzed in comparison with the binding before these additions, i.e., time zero values. The PMA-induced effects (20 and 50 min after PMA addition) were statistically analyzed in comparison with the binding 10 min after addition of calcium (1 mM) and ionomycin (250 nM). The *t* test was used for statistical analysis. * $P < 0.05$; ‡ $P < 0.01$; § $P < 0.001$.

the $[Ca^{2+}]_i$ back to normal, i.e., ~ 120 nM. Third, receptor expression was markedly accelerated and also enhanced by rapidly raising the $[Ca^{2+}]_i$ to micromolar levels by the addition of the calcium ionophore ionomycin. A similar regulatory mechanism for receptor expression has previously been proposed for other receptors and/or other cells (11, 12, 41). Further support for our findings is provided by the recent demonstration that the $[Ca^{2+}]_i$ appears to be elevated primarily in the anterior part of moving neutrophils (42), and in accordance with this observation, an increased number of chemotactic peptide receptors have also been reported to exist on the front half of moving neutrophils (33). The present observation of increased fMet-Leu-Phe receptor expression most likely reflects a true externalization of receptors, i.e., a membrane flow from the interior of the cell to the plasma membrane. The reasoning behind this assumption comes primarily from the work by Gallin and coworkers (for review see reference 43) and is based on the following observations: (*a*) 30 times more fMet-Leu-Phe receptors can be demonstrated in a subcellular fraction that is enriched in specific granules than in the plasma membrane; (*b*) degranulation has been shown to be an important source of membrane addition; (*c*) degranulation is associated with an increased expression of fMet-Leu-Phe receptors and is independent of protein synthesis; and (*d*) cells from patients who lack specific granules, differentiated HL60 cells also lacking specific granules, and cells that have been artificially devoided of their intracellular organelles, i.e. cytoplasts, all lack the ability to increase their expression of fMet-Leu-Phe receptors. Furthermore, the rate and extent of secretory granule exocytosis is closely associated with the rate and extent of the $[Ca^{2+}]_i$ elevation (7), although additional signal(s) provided by receptor activation has recently been shown to markedly lower the Ca^{2+} requirements of the exocytotic process (44).

Table II. Distribution of Radioactivity after Incubation in the Absence or Presence of PMA

	Acid-insoluble radioactivity	
Cellular conditions	Cells	Medium
	%	%
Control	98.4±0.5	1.6±0.5
PMA (10^{-9} M)	98.5±0.2	1.6±0.2
PMA (10^{-7} M)	98.3±0.3	1.7±0.3

Isolated human neutrophils were iodinated and then transferred to the previously described calcium containing medium and incubated at 37°C for 50 min. This incubation occurred in the absence (control) or presence of PMA. After the incubation the cells were separated from the medium by centrifugation and the radioactivity levels were determined. The cell and medium-associated radioactivity are expressed as percent of total and given as mean±SEM for four different experiments. Statistical analysis of possible PMA-induced effects was performed using the *t* test.

The origin of the increased production of 1,4,5-IP_3, which mobilizes Ca^{2+} from intracellular stores, is a receptor-mediated increase in phosphoinositol breakdown which also results in an increased production of diacylglycerol, the natural activator of protein kinase C (3). A vast amount of knowledge has recently been gained concerning the effects of protein kinase C activity on receptor functions. PMA exposure has been shown to stimulate receptor internalization in a variety of cell types (14–16), presumably because of its ability to activate protein kinase C. However, in neutrophils PMA has been shown to promote secondary granule exocytosis and fMet-Leu-Phe receptor recruitment (17). To circumvent this initial exocytotic event, we selected an experimental condition in which receptor recruitment would not blunt a possible PMA-induced decrease of receptor expression, i.e., we first increased the $[Ca^{2+}]_i$, which resulted in a rapid increase of cell surface receptors, and then exposed the cells to PMA. Using this protocol, PMA exposure led to a marked decrease of fMet-Leu-Phe receptor expression, although the $[Ca^{2+}]_i$ was maintained at a high level. The cause of the observed decrease of receptor expression is unclear although it was apparently not due to cell loss, membrane shedding, or autooxidation. Because it has been shown in previous studies, using photoaffinity-labeled ligands, that the chemotactic receptor can be internalized in both human neutrophils and differentiated HL60 cells (9, 10), it is tempting to imagine that PMA has a dual role in the regulation of fMet-Leu-Phe receptor expression, by both affecting the up-regulation (17) and down-regulation.

The opposite effects of the $[Ca^{2+}]_i$ elevation and PMA exposure on cell surface receptor expression do not necessarily constitute a contradictory finding. It has been suggested that in various cell systems the $[Ca^{2+}]_i$ increases are transient and are followed by a more persistent protein kinase C activity (for review see references 45 and 46). It is thus possible that the dual control of receptor expression on the cell surface of human neutrophils might in part be the cause of the transient nature of cellular responses elicited by fMet-Leu-Phe.

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