Desensitization of calcium mobilization and cell function in human neutrophils

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Neutrophils pretreated with the chemoattractant formylmethionyl-leucyl-phenylalanine become unresponsive when re-exposed to the same ligand, a process termed desensitization. We have examined whether desensitization of transduction (Ca^{2+} mobilization) or of other cell functions (superoxide generation, enzyme release, or aggregation) occurs synchronously. Simultaneous studies of Ca^{2+} mobilization and aggregation by using Fura-2-loaded cells indicate that, under conditions where the aggregation response is abolished, most of the Ca^{2+} mobilization is unaltered. Further studies were then carried out to ascertain whether desensitization of Ca^{2+} mobilization could in fact be induced. Desensitization was observed, and was dependent on the number of exposures of the cells to the ligand, the concentration of the ligand, and whether the ligand was left in the medium or was removed. The pattern of resensitization was dependent on the experimental design. Under conditions where ligand was continuously present, no recovery of the Ca^{2+} -mobilization response was seen with subsequent challenges. In contrast, on removal of ligand, this response showed partial recovery. Whereas complete desensitization of aggregation was noted, enzyme release showed a markedly lesser degree of desensitization and required more frequent exposures to the ligand before it was observed. Little or no desensitization of superoxide generation was observed regardless of the conditions utilized. Studies using phorbol myristate acetate as the ligand showed that Ca^{2+} mobilization and aggregation could be simultaneously inhibited. Our results suggest that discrete mechanisms of desensitization are possible in human neutrophils, and that desensitization of one particular function (aggregation) does not imply concomitant desensitization of other functions.

INTRODUCTION

Exposure of cells to a hormone leads to a subsequent loss of activation of the cell by that hormone. This process is referred to as desensitization. The best studied system thus far is the β -adrenergic-receptor-stimulated adenylate cyclase system, in which pathways of desensitization have been defined in some detail [1].

The phenomenon of desensitization has been shown to occur in human neutrophils [2–12]. Pretreatment of neutrophils with fMet-Leu-Phe leads to a loss of aggregation and a diminished enzyme release response. In contrast with the cyclic AMP-adenylate cyclase system, very little is known about the desensitization of receptors linked to Ca^{2+} -mobilizing systems, including the fMet-Leu-Phe receptor. Thus studies of fMet-Leu-Phe-mediated desensitization seem particularly worthwhile, since they impact not only on the understanding of how responses may be regulated in neutrophils (and hence for inflammation in general), but also on the understanding of the regulation of several Ca^{2+} -mobilizing receptors.

A central assumption that is involved in the desensitization studies carried out thus far [2–9] is that receptor desensitization is the central unique contributor to cellular desensitization. According to this view, loss of the ability of the hormone to activate the cell is caused by an uncoupling of the receptor from a target enzyme, possibly owing to the modification of the receptor or its associated

regulatory G-proteins. Although there is evidence, at least for the β -adrenergic receptor, that this is the case, there is little or no evidence concerning this hypothesis for the fMet-Leu-Phe receptor. If receptor desensitization was the unique event determining cellular desensitization, then it would follow that all receptor-mediated events, i.e. loss of Ca^{2+} mobilization, InsP_3 generation, and cell function, would occur synchronously.

To test this hypothesis, we have evaluated the loss of transduction and various cell functions in desensitized neutrophils. Our results indicate that desensitization of aggregation by exposure to fMet-Leu-Phe does not imply a global desensitization of all cell functions, and that distinct conditions are required for the desensitization of Ca^{2+} mobilization and cell aggregation. These results imply that multiple mechanisms contribute to cellular desensitization.

MATERIALS AND METHODS

Materials

Bovine serum albumin, fMet-Leu-Phe, ferricytochrome *c*, superoxide dismutase, cytochalasin B, *Micrococcus lysodeikticus* lysozyme, and phorbol myristate acetate (PMA) were from Sigma. Dextran T-500 and Ficoll were from Pharmacia. Sodium diatrizoate (Hypaque) was from Sterling Drug Co. (New York, NY, U.S.A.), and Hanks' balanced salt solution (HBSS) was

Abbreviations used: fMet-Leu-Phe, formylmethionyl-leucyl-phenylalanine; PMA, phorbol myristate acetate; HBSS, Hanks' balanced salts solution.

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from GIBCO/Bethesda Research Laboratories, West Chester, PA, U.S.A. Fura-2 AM was from Molecular Probes, Eugene, OR, U.S.A. Modified HBSS for enzyme-release studies is HBSS containing 1 mg of bovine serum albumin/ml, 1 mg of glucose/ml, 10 mM-Hepes, pH 7.4, and 1 unit of heparin/ml.

Preparation of neutrophils [13]

Neutrophils from normal human volunteers were isolated by sedimentation on dextran and Ficoll/Hypaque. The cell preparation was > 98% pure, and functional characteristics of enzyme release, chemotaxis and superoxide generation were normal. Cell viability was checked routinely by using Trypan Blue exclusion, and was > 95%.

Aggregation [14]

Aggregation studies were performed with a Sienco DP-247-E aggregometer (Morrison, CO, U.S.A.). Neutrophils ($160\ \mu\text{l}$ of 1×10^7 cells/ml in HBSS) were added to a silicone-treated cuvette. After a 2 min warming period at 37°C in the presence of cytochalasin B ($10\ \mu\text{g}/\text{ml}$) (except for the experiments which involved pretreatment with PMA, in which case cytochalasin B was omitted), fMet-Leu-Phe was added in the concentration range $1\ \text{pM}$ – $10\ \mu\text{M}$, and aggregation was monitored for 10 min.

Enzyme release [15]

Neutrophils (1×10^7 cells/ml) were preincubated in modified HBSS with cytochalasin B ($10\ \mu\text{g}/\text{ml}$) for 5 min at 37°C . fMet-Leu-Phe ($10\ \text{nM}$ – $1\ \mu\text{M}$) was added to a final assay volume of $250\ \mu\text{l}$, and the cells were incubated for 5 min at 37°C . The cells were centrifuged at $400\ g$ for 15 min, and the supernatant containing the released enzymes was assayed for lysozyme. Total releasable enzyme was determined by incubation in a buffer containing 1% Triton X-100. The percentage of lysozyme released was in the range 35–45% of the total releasable.

Superoxide generation [16]

The reduction of cytochrome *c* was monitored for superoxide generation by using fixed-time-point assays. Neutrophils (1×10^7 cells/ml) were washed and resuspended in HBSS. Cytochrome *c* was added to give a final concentration of $76\ \mu\text{M}$, cytochalasin B was added to a final concentration of $10\ \mu\text{g}/\text{ml}$, superoxide dismutase and PMA were used at $10\ \mu\text{g}/\text{ml}$ final concentrations in an assay volume of 1 ml, and the effects of fMet-Leu-Phe were examined. The cells were incubated for 5 min at 37°C and then placed on ice for 5 min. The tubes were then centrifuged in a Beckman Microfuge at full speed for 30 s, and the supernatant was decanted directly into a 1 ml cuvette and the A_{550} was read. Controls included incubation of cells and substrate without fMet-Leu-Phe, and an incubation with fMet-Leu-Phe in the presence of superoxide dismutase. The amount of superoxide generated was assessed with an agent such as PMA, which causes maximal superoxide generation ($47\ \text{nmol}/5\ \text{min}$ per 2×10^6 cells), by using the published absorption coefficient for ferricytochrome *c* [16]. This corresponds to an A_{550} change of 1.0. As far as possible, superoxide generation and enzyme release were tested simultaneously.

Measurement of intracellular Ca^{2+} by using Fura-2 fluorescence [17]

Neutrophils (10^8 cells/ml) are loaded with Fura-2 AM by incubation for 30 min at 37°C with Fura-2 AM ($5\ \mu\text{M}$) in Fura-2 buffer (150 mM-NaCl, 5 mM-KCl, 1.3 mM- CaCl_2 , 1.2 mM- MgCl_2 and 10 mM-Hepes, pH 7.4). The suspension was then diluted 10-fold and incubated for a further 30 min. After loading, the cells were centrifuged at $250\ g$ for 10 min, resuspended in Fura-2 buffer at a concentration of 10^7 cells/ml and kept at room temperature. The assay was as follows: 1 ml of the cell suspension was diluted with 2 ml of the Fura-2 buffer in a 1 cm cuvette and incubated at 37°C . Fluorescence intensities were measured in a Perkin-Elmer LS-5 fluorescence spectrophotometer, by using an excitation wavelength of 335 nm and an emission wavelength of 505 nm. An enhanced sensitivity setting (FS = 10) was utilized for optimal readings. The excitation slit was set to 5 nm and the emission slit to 10 nm to enhance sensitivity. A measure of the total Fura-2 present in the cells was estimated by lysing the cells in the presence of 0.1% Triton X-100. The instrument was auto-zeroed after the reading had stabilized. The respective ligand ($30\ \mu\text{l}$) was added, and the fluorescence was monitored for up to 800 s. Absolute intracellular Ca^{2+} levels were calculated from the changes in fluorescence as described [18,19] by using a value of 224 nm for K_d of Fura 2.

Since the concentration of the dye is an important consideration in assessing the Ca^{2+} -mobilizing responses, we first tested to see if variations of over 50-fold in the concentration of the dye altered the pattern of Ca^{2+} mobilization or desensitization observed. No such dependence on the concentration of Fura-2 was found. A limited number of experiments were carried out with Quin-2-loaded cells, with essentially similar results. Thus buffering effects of the dye, insufficient levels of dye, or the type of dye used do not influence the desensitization patterns observed, and are not considerations relevant to the interpretation of the results.

RESULTS

Neutrophils were pretreated with $0.1\ \mu\text{M}$ -fMet-Leu-Phe and then washed and re-exposed to various concentrations of the same ligand, and the extent of aggregation induced by the second dose was examined. The results shown in Fig. 1(a) (triangles) are qualitatively similar to those of O'Flaherty and colleagues [6,7]. Loss of aggregation response was noted in treated, but not in control, neutrophils. Variations were also tested to see if loss of aggregation is critically dependent on parameters such as extracellular Ca^{2+} and the presence or absence of cytochalasin B (results not shown). Desensitization of aggregation was noted regardless of the pretreatment conditions utilized. The magnitude of the aggregation responses observed in the absence of cytochalasin B-treated cells was lower, but the overall profile of desensitization of aggregation was unaltered. Aggregation was also assessed by using a particle-counting assay [20]. Similar results were obtained, although for reasons which are unclear the dose/response that was obtained by using the transmittance assay was different from that observed with the particle-counting assay.

In addition to the alterations in the aggregation response, other changes also occur when the cell is

Fig. 1. Simultaneous determination of desensitization of aggregation and Ca^{2+} -mobilization responses

(a) Desensitization by fMet-Leu-Phe pretreatment. The aggregation response in fMet-Leu-Phe-pretreated cells ($\blacktriangle$) is significantly diminished compared with that in buffer-treated cells (Δ). The Ca^{2+} -mobilization response, however, is almost identical in the fMet-Leu-Phe-pretreated ($\bullet$) and buffer-treated ($\circ$) samples. The cells were pre-loaded with Fura 2 in the buffer used for the aggregation measurements. Neutrophils (1×10^7 cells/ml) were buffer-treated (Δ , $\circ$) or exposed to $0.1 \mu\text{M}$ -fMet-Leu-Phe ($\blacktriangle$, $\bullet$) for 5 min at 37°C in HBSS. The cells were then washed by centrifugation for 15 min at $400g$, resuspended in HBSS and evaluated for aggregation responses resulting from different doses of fMet-Leu-Phe as described in the Results section. Aggregation is measured as a fMet-Leu-Phe-specific increase in transmittance, and Ca^{2+} mobilization was measured on the same sample of neutrophils by measuring the increase in fluorescence at 550 nm as described in the Materials and methods section. The aggregation and fluorescence changes induced by varying the test doses of fMet-Leu-Phe are presented as percentages of the maximum respective responses that occur in buffer-treated cells. The corresponding calculated intracellular Ca^{2+} levels are also shown on the right. (b) Desensitization by PMA pretreatment. Both the aggregation ($\blacktriangle$) and calcium-Fura 2-fluorescence ($\bullet$) responses in PMA-pretreated cells are significantly diminished compared with those in buffer-treated cells (Δ , $\circ$, respectively). Pretreatment for the aggregation studies consisted of addition of PMA (final concn. $1 \mu\text{g/ml}$) and incubation at 37°C for 5 min before the aggregation studies. For the Ca^{2+} -mobilization studies, similar conditions were used, except that the treatment was for 10 min and the PMA was washed out and the cells were resuspended before the Ca^{2+} -mobilization measurement. Results are presented as percentages of the maximum respective responses occurring in buffer-treated cells. Corresponding intracellular Ca^{2+} levels are also shown on the right.

desensitized. Desensitization of receptors is the prominent hypothesis thought to explain the diminished responsiveness of neutrophils. Moreover, binding studies have already shown diminution in receptor number [2,4], although the significance of the binding profiles is unclear. We therefore focused our attention on the transduction response of the fMet-Leu-Phe receptor, i.e. the Ca^{2+} -

mobilizing activity, since this is thought to be the first response after mobilization of the PtdInsP_2 pathway, which occurs after stimulation of the receptor.

Desensitization of both the aggregation and the Ca^{2+} -mobilization responses were compared. Ca^{2+} mobilization was carried out with Fura-2-loaded cells, whereas the aggregation studies were carried out either with the

Fig. 2. Evaluation of fMet-Leu-Phe-induced desensitization of Ca^{2+} mobilization

Neutrophils were repeatedly exposed to $0.1 \mu\text{M}$ -fMet-Leu-Phe (a) at the times indicated by the arrows, and the Ca^{2+} mobilization was monitored. The cells were then centrifuged, resuspended in loading buffer and the Ca^{2+} response was further examined (b). The cycle of exposures followed by washing was again repeated (c). Ca^{2+} mobilization after the fMet-Leu-Phe doses was monitored as described in the legend to Fig. 1. The ordinate denotes fluorescence intensity in arbitrary units. The corresponding calculated Ca^{2+} levels are also shown on the right.

same cells not loaded with Fura-2 (results not shown) or with the same Fura-2-loaded cells (Fig. 1a). The variations were present to ensure that the same phenomenon is observed in both the native and Fura-2-loaded cells. The results shown in Fig. 1(a) suggest that, under conditions where aggregation is substantially decreased, most of the Ca^{2+} -mobilizing activity is not lost. It was consistently found that the Ca^{2+} -mobilizing activity observed at low levels of fMet-Leu-Phe (approx. 0.1 – 10 pM) was lower in the desensitized cells, although this effect was small and subject to the greatest difficulty in measurement. A small shift in the apparent K_{act} , or a selective desensitization of a limited group of receptors seems probable, in view of the results reported by others [2,10].

One of the postulates for the mechanism of fMet-Leu-Phe activation is that it stimulates kinase C activity. PMA is another agent believed to act via this mechanism. The effect of PMA pretreatment on aggregation of neutrophils and on release of internal Ca^{2+} was therefore determined to see if fMet-Leu-Phe and PMA may be acting through similar pathways. The results for the PMA pretreatments shown in Fig. 1(b) indicate that, unlike fMet-Leu-Phe, PMA pretreatment essentially abolishes both aggregation and release of Ca^{2+} . These experiments were done without cytochalasin B, as PMA is known to cause degranulation of neutrophils in its presence. As has been consistently observed, this abolishes all of the neutrophil's responses at low (approx. 1 pM – 10 nM) concentrations of fMet-Leu-Phe, resulting in a higher apparent K_{act} .

These observations raised the question of whether conditions could be devised such that Ca^{2+} mobilization was abolished in neutrophils after treatment with fMet-Leu-Phe, and, if so, whether abolitions of cell function and of Ca^{2+} mobilization occur synchronously.

Neutrophils were exposed to sequential doses of fMet-Leu-Phe without removal of the ligand. This resulted in diminishing responses of Ca^{2+} mobilization. The cells were then washed to remove free ligand and then again evaluated for their Ca^{2+} -mobilization response. Ca^{2+} -mobilizing activity was again present, although the maximal activity which was observed was noticeably less than was observed in the first cycle. After another wash, a third such cycle of sequential exposures resulted in cells in which Ca^{2+} mobilization was essentially abolished. The pattern of Ca^{2+} mobilization through three cycles is

shown in Fig. 2. The viability of the cells was $> 95\%$, as judged by ethidium bromide/Acridine Orange staining.

Two conclusions derived from such experiments were: (a) the sequential additions of ligand within each cycle yield progressively lower levels of Ca^{2+} mobilization; (b) multiple cycles of such additions are required to yield cells in which the Ca^{2+} mobilization is significantly diminished.

The simplest explanation for such a phenomenon would be that it is related to the progressive saturation of the fMet-Leu-Phe receptor sites available on the cell surface. This was tested as follows. The dose/response curve was determined with fresh cells for each determination; then a dose/response curve was determined in which ligands were added sequentially to a single sample of cells (Fig. 3). When the two dose/response curves are compared, it is clear that, with individual doses, the response increases with increasing dosage, whereas with

Fig. 3. Desensitization of Ca^{2+} mobilization: effect of using the same cell population to determine the dose/response curve

One sample of cells was tested for the Ca^{2+} -mobilization response by using doses of 1 nM -, 10 nM -, $0.1 \mu\text{M}$ -, $1 \mu\text{M}$ - and $10 \mu\text{M}$ -fMet-Leu-Phe sequentially at 200 s intervals (●). In parallel, the Ca^{2+} -mobilization responses produced by the same dosages, tested on fresh cells for each dosage, were also determined (○). At high concentrations of fMet-Leu-Phe, the cells that had received previous doses of fMet-Leu-Phe consistently showed less Ca^{2+} mobilization than did untreated cells. Results are presented as the change in fluorescence (left) and the corresponding Ca^{2+} level (right).

Fig. 4. Desensitization of Ca^{2+} mobilization: influence of concentrations of fMet-Leu-Phe and the number of 'hits' on desensitization in the absence of washing

Neutrophils were treated repeatedly with fixed doses of fMet-Leu-Phe for multiple exposures, and the Ca^{2+} -mobilization responses were measured. The ordinates show second and subsequent responses as percentages of the first response. At $0.1 \mu\text{M}$ -fMet-Leu-Phe (a), the primary response varied from 70 to 157 arbitrary units, at 10 nM (b) it was 36 units, and at 1 nM (c) 15 units. The corresponding Ca^{2+} levels are shown on the right.

sequential doses (i.e. with cells previously exposed to fMet-Leu-Phe), the response falls off with subsequent higher doses after an initial responsive state.

Some parameters of the sequential addition were then explored further. The number of 'hits' required to cause desensitization and the influence of the ligand concentration on the number of hits required is shown in Fig. 4. Desensitization occurs even at doses as low as 1 nM and appears to occur more rapidly if the dosages used are higher.

An obvious question to be answered is whether the desensitization of either cell function or Ca^{2+} mobilization is reversible. The experiments in Fig. 5 were designed to test this. If resensitization occurs, then some degree of restoration of the lost response should be discernible. Recovery of Ca^{2+} -mobilization response in the continued presence or absence of ligand was therefore determined. When ligand is left in the medium with cells that are no longer capable of another response (after one cycle of hits or after three cycles), then recovery is not observed even when the incubation was carried out for times up to 2 h (results not shown). Cells which are exposed to a single dose of fMet-Leu-Phe (where desensitization is not complete) and from which ligand is

Fig. 5. Recovery of the Ca^{2+} -mobilization response to fMet-Leu-Phe in desensitized cells

Neutrophils were treated with one hit of $0.1 \mu\text{M}$ -fMet-Leu-Phe, washed, resuspended in fresh buffer, and the time course of recovery of the Ca^{2+} -mobilizing response to a subsequent dose of $0.1 \mu\text{M}$ -fMet-Leu-Phe was monitored (Δ). A second sample was treated with three similar doses of fMet-Leu-Phe at approx. 300 s intervals, then washed and resuspended, and the time course of recovery of the Ca^{2+} mobilization was monitored ($\bullet$). A third sample was taken through three such cycles of exposures to fMet-Leu-Phe followed by washings. After the final wash, the time course of recovery of the Ca^{2+} -mobilization response was monitored ($\circ$). Fluorescence responses produced by the test dose are presented as percentages of the response elicited from a single dose in the naive cell. Beginning and end values for the corresponding calculated intracellular Ca^{2+} levels for the single-hit sample (Δ) are 793 and 517 nM respectively, for the sample hit multiple times and then washed ($\bullet$) 293 and 365 nM, and for the sample taken through three cycles of multiple hits and washing ($\circ$) 240 and 328 nM.

not washed away are still capable of showing subsequent responses (see the peak at 400 s in Fig. 2, and also see Fig. 3), but the magnitude of this response increases rapidly from 30 % of the initial response at 150 s after the desensitizing dose to 60 % at 700 s (results not shown). In contrast, when the ligand is removed by washing, a rapid (within 60 s) but partial return of the lost Ca^{2+} mobilization is observed (Fig. 5). The resensitization experiments also suggest two components, one which is rapid and occurs upon removal of the ligand, and the other which is a slower, time-dependent, continuing recovery.

The last set of experiments presented here were directed to see whether different cell functions initiated by fMet-Leu-Phe-receptor stimulation were desensitized synchronously. Our results in Figs. 6(a)–6(d) describe the reactions of enzyme release and superoxide generation. Under conditions where the aggregation response is almost completely lost, enzyme release is only marginally inhibited. After multiple cycles of hits where Ca^{2+} mobilization is substantially decreased, enzyme release is also found to be decreased, but superoxide generation is unaffected.

DISCUSSION

The prevailing view suggests that desensitization is mediated predominantly at the receptor level. A simple

Fig. 6. Effect of fMet-Leu-Phe pretreatment(s) on enzyme release and superoxide generation

(a) Lack of desensitization of fMet-Leu-Phe-induced enzyme release after prior exposure of cells to fMet-Leu-Phe. Cells were buffer-treated (○) or pre-treated with $0.1 \mu\text{M}$ -fMet-Leu-Phe (●) and then washed as described in the legend for Fig. 1. Enzyme release is measured as the amount of lysozyme assayable in the medium 5 min after subsequent fMet-Leu-Phe stimulation with various dosages, and is presented as a percentage of lysozyme releasable by treatment with Triton X-100. Averages of duplicate determinations are shown. (b) Desensitization of fMet-Leu-Phe-induced enzyme release after multiple cycles of exposures of cells to fMet-Leu-Phe. Cells were buffer-treated (○) or pre-treated with $0.1 \mu\text{M}$ -fMet-Leu-Phe (●) for three cycles of hits as described in the legend to Fig. 2, and then evaluated for enzyme release. Enzyme release is measured as described in (a). (c) Lack of desensitization of fMet-Leu-Phe-induced superoxide generation after prior exposure of cells to fMet-Leu-Phe. Cells were buffer-treated (○) or pre-treated with $0.1 \mu\text{M}$ -fMet-Leu-Phe (●) and then washed as described in the legend for Fig. 1. Superoxide generation is measured as the change in A_{550} caused by reduction of cytochrome *c* during a 5 min incubation of a cytochrome *c* solution with treated cells. Addition of superoxide dismutase to block cytochrome *c* reduction resulted in a reading of 0.253 for buffer-treated and 0.226 for fMet-Leu-Phe-pretreated cells. Data are averages of triplicate determinations $\pm$ S.E.M. (error bars do not extend beyond the symbol diameter in most cases). (d) Lack of desensitization of fMet-Leu-Phe-induced superoxide generation after multiple cycles of exposures of cells to fMet-Leu-Phe. Cells were buffer-treated (○) or pre-treated with $0.1 \mu\text{M}$ -fMet-Leu-Phe (●) for three cycles of hits as described in the legend to Fig. 2, and then evaluated for superoxide generation as described for (c). Addition of superoxide dismutase resulted in a reading of 0.006 for buffer-treated and -0.001 for fMet-Leu-Phe-pretreated cells.

prediction which stems from this view is that all cell functions dependent on fMet-Leu-Phe-receptor stimulation would be simultaneously and equivalently desensitized by virtue of receptor down-regulation. Our results suggest that the prevailing view is incorrect, and that more than one mechanism is involved in the desensitization of human neutrophils.

Fig. 7 summarizes the different mechanisms which are compatible with the results presented. One conclusion that can be drawn is that loss of various cell functions are not coupled in an obligatory way to loss of Ca^{2+} mobilization. For desensitization of aggregation, the proposed mechanism, based on our results, is that a small component of the Ca^{2+} mobilization may be required for aggregation, and desensitization of this small component would lead to desensitization of aggregation. The diminution in Ca^{2+} -mobilizing activity that is observed at low (0.1 – 10 pM) fMet-Leu-Phe concentrations and the upward shift in the apparent K_{act} for fMet-Leu-Phe-mediated Ca^{2+} mobilization in the cells desensitized to aggregation (Fig. 1) suggests that only a very limited pool of the total Ca^{2+} mobilization might be relevant for cell aggregation. The results with the re-

sensitization studies (Fig. 5) also point in a similar direction, in that (a) a small portion of the Ca^{2+} signal is rapidly and irreversibly lost, as is the aggregation response, and (b) distinct rapid and slow components of recovery are observed.

An alternative interpretation of the results would be that no Ca^{2+} mobilization is required for aggregation. The studies by Sklar [11] and Omann *et al.* [12] suggest that actin polymerization and aggregation occur in an earlier time frame which is distinguishable from the time course of release of internal Ca^{2+} . External Ca^{2+} , which is important to some cellular processes, is not required for aggregation. Results from other laboratories suggest that PtdInsP_2 can act as a second messenger in the absence of increased Ca^{2+} and that the predominant target of PtdInsP_2 is the cytoskeleton [21,22]. In short, our results, which suggest that desensitization to aggregation occurs without loss, or with minimal loss, of Ca^{2+} mobilization, are entirely compatible with the above observations.

Desensitization of enzyme release appears to occur concomitantly with desensitization of Ca^{2+} mobilization. This would occur if enzyme release was a direct proportional consequence of the released Ca^{2+} or if both

Fig. 7. Mechanisms for desensitization of Ca^{2+} mobilization and cellular functions in human neutrophils

The proposed mechanism for Ca^{2+} mobilization is that fMet-Leu-Phe binding to the fMet-Leu-Phe receptor initiates signal transduction via G-proteins to activate PtdInsP_2 lipase C. This enzyme generates InsP_3 [and diacylglycerol (DAG)], which activates Ca^{2+} release. The released Ca^{2+} and DAG together are proposed to activate a kinase C which may subsequently act on the G-protein, thereby desensitizing this pathway. The aggregation response is proposed to be initiated either via a separate pathway directly from the G-protein or from an earlier metabolite in the Ca^{2+} -activation pathway, such as PtdInsP_2 . Desensitization of aggregation could occur in the absence of desensitization of the Ca^{2+} -mobilization pathway. Degranulation and enzyme release are proposed to be regulated by the mobilization of intracellular Ca^{2+} . Hence desensitizing the cells for Ca^{2+} mobilization would also desensitize the cells for enzyme release. Superoxide generation is proposed to occur as a result of two processes, the first of which is an irreversible translocation of the NADPH oxidase-cytochrome system to the cell surface, which occurs when intracellular Ca^{2+} is mobilized. Since this step is irreversible, it is not desensitized when Ca^{2+} mobilization is desensitized. The second requirement is a direct induction of the NADPH oxidase activity by a step which is not sensitive to intracellular Ca^{2+} and which may be directly stimulated by the activated G-protein. This would thus occur in the presence of a desensitized Ca^{2+} -mobilization pathway.

depended on an earlier intracellular messenger. The mechanism by which Ca^{2+} -mobilizing activity is lost can be inferred from the results of other investigators, who have shown that activation of protein kinase C causes (a) an activation of InsP_3 monoesterase [23] and (b) modification of G-proteins [24,25]. InsP_3 monoesterase activation will lead to lower effective levels of InsP_3 when production of this messenger is stimulated. Since the release of Ca^{2+} is proportional to the activation of InsP_3 production, a decrease in the levels of calcium mobilization will be observed. Alteration of G-proteins will lead to the inhibition of the PtdInsP_2 lipase C activation

by fMet-Leu-Phe or guanine nucleotide stimulation, resulting in a loss of production of InsP_3 and loss of Ca^{2+} mobilization. The decrease in degranulation responses (enzyme release) suggests that at least a component of the degranulation process is Ca^{2+} -dependent. It has already been shown that threshold effects of Ca^{2+} are important in the release of primary and secondary granule constituents [11,12], and our results are certainly compatible with these findings. Thus concomitant desensitization of degranulation (enzyme release) and Ca^{2+} mobilization is proposed to occur as a consequence of kinase C activation.

Superoxide generation is stimulated by fMet-Leu-Phe treatment, but is entirely unaffected under conditions in which Ca^{2+} mobilization is lost. A special consideration involved here is that the superoxide generation measured in the presence of cytochalasin B involves the translocation of the NADPH oxidase system to the plasma membrane, possibly along with the translocation of kinase C. Phosphorylation of at least one component of the electron-transfer system which makes up the oxidase has previously been noted as being functionally important, since this particular phosphoprotein is absent from chronic-granulomatous-disease neutrophils which lack this constituent [26]. The mechanism whereby superoxide generation is maintained rather than being desensitized is therefore related to the fact that irreversible activation by translocation of the oxidase has occurred after initial fMet-Leu-Phe stimulation and Ca^{2+} mobilization. Subsequent loss of Ca^{2+} mobilization by the mechanisms discussed above (InsP_3 monoesterase activation and/or G-protein phosphorylation) will occur without loss of superoxide-generating activity if superoxide generation occurs as a result of both translocation of granules to the cell surface (Ca^{2+} -dependent but irreversible step) and triggering of the superoxide generation by a Ca^{2+} -independent step [26]. Our results are compatible with those of Sha'afi and colleagues, who have defined 'cytochalasin B-dependent and -independent' forms of desensitization [8].

The importance of our results is that they suggest that the prevailing view, that cellular desensitization and receptor desensitization are synonymous and that global desensitization of all cell functions might occur, is obviously incorrect. Indeed, several discrete mechanisms involving separate elements are very likely to be involved. The study of transduction and of cell function in other cell types may also produce similar patterns. Preliminary studies of the monocyte system show similarities to the results obtained in granulocytes. Receptor- and function-specific desensitization processes may prove to be more widely applicable to other cellular systems in the future.

We are grateful to Jimmie Jefferson for secretarial assistance. This work was supported by NIH grants AM30878 and AM34550, and by a Biomedical Research Support grant RR-05521-19 from the Kaiser Foundation Research Institute to P.M.L.

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Received 7 November 1988/12 April 1989; accepted 26 April 1989