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Membrane-Cytoskeleton Interactions and the Regulation of Chemotactic Peptide-Induced Activation of Human Granulocytes: The Effects of Dihydrocytochalasin B

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When N-formyl chemotactic peptides bind to granulocyte receptors at 37°C they rapidly form a high-affinity ligand-receptor complex whose coisolation with cytoskeletal residues of Triton X-100–extracted cells is under cellular control [Jesaitis et al: J Cell Biol 98:1378, 1984]. Experiments were performed to investigate the significance of this coisolation. When the granulocytes were preincubated with dihydrocytochalasin B (dhCB) for 10 min at 37°C and then stimulated with 50 nM N-formyl-Met-Leu-[^{3}H]Phe, the rate of uptake of the radioligand by the cells was inhibited. Colocalization of the retained peptide with the Triton X-100 fraction of these cells was also reduced relative to this fraction of the untreated cells. This inhibition was apparent before the onset of FMLP endocytosis. The inhibition was 50% effective at 0.25 μg dhCB/ml. Maximal inhibition (80–90%) occurred at doses of dhCB > 1 μg/ml. The 90% retention of two plasma membrane markers by the cytoskeleton was marginally affected. These results support the hypothesis that coisolation of the high-affinity receptor-peptide complexes with granulocyte cytoskeletons occurs because of specific association of the complexes with the cytoskeleton at the cell surface. In addition, since these events precede internalization, they suggest that formation of the association between the ligand-receptor complex and cytoskeleton may be necessary for ligand-receptor endocytosis. Experiments were also performed to evaluate other functional consequences of cytoskeletal disruption on chemotactic peptide-stimulated functions. f-Met-Leu-

Abbreviations used: FMLP, N-formyl methionyl leucyl phenylalanine; FML[^{3}H]P, N-formyl methionyl leucyl [^{3}H]phenylalanine; ^{125}I-WGA, ^{125}I-conjugated wheat germ agglutinin; dhCB, dihydrocytochalasin B, DPBS, Dulbecco's phosphate buffered saline.

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Phe stimulation of O_2^- production was potentiated due to a prolongation of and an increase in the rate of O_2^- production. This potentiation had the same dose dependency as the inhibition of receptor modulation. The possible relationship of these various functions is discussed.

Key words: superoxide generation, chemotactic peptide receptor, cytoskeleton, cell activation, polymorphonuclear leukocytes, granulocytes, neutrophils, dihydrocytochalasin B

Cytochalasins are a family of fungal alkaloids that have been observed to have pleiotropic effects on eukaryotic cells [1,2]. They have been used to probe the cytoskeleton and motile apparatus [3], and it is now believed that many of these effects are related to the binding of the cytochalasins to the growing ends of actin filaments and to specific protein complexes in plasma membrane that serve as actin attachment points [4]. In addition, proteins have been isolated from cells that mimic the molecular effects of cytochalasin and are therefore possible structural elements of actin-actin and actin-membrane interaction.

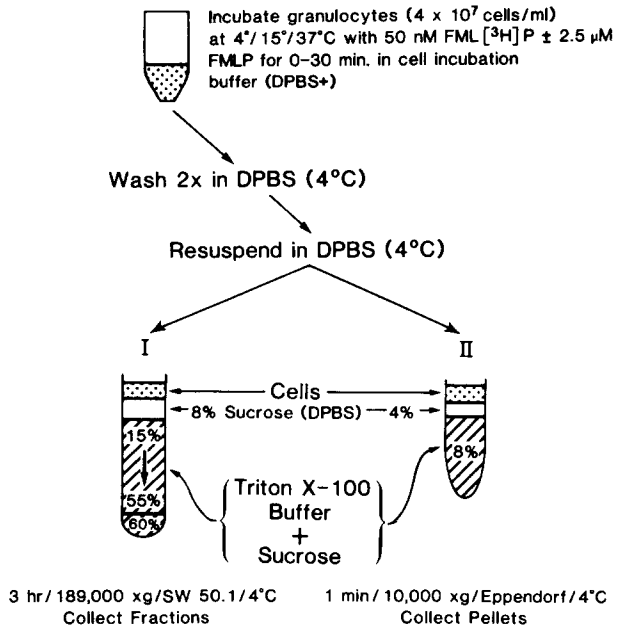
The production of superoxide anion by human neutrophils in response to monovalent chemotactic agents such as N-formyl chemotactic peptide is a classic example of a cellular sensory response [5] and the cytochalasins have been shown to perturb this and other sensory responses of these cells [6–10]. As a result, an unspecified sensory role for the cytoskeleton has been implicated. Recently, we have shown that soon after binding the N-formylated chemotactic peptide f-Met-Leu-Phe forms a ligand-receptor complex that dissociates two orders of magnitude more slowly at 4°C and coisolates with the surface remnants of the cell cytoskeleton [11]. The coisolation of this ligand-receptor complex with the cytoskeleton can be observed very soon after addition of the stimulus (< 5 sec) and continues until it reaches a high steady-state level at 5 min. The steady-state level is reached presumably when receptor expression at the cell surface is balanced by entry of receptor into the cell by endocytosis.

Although the coisolation of the ligand-receptor complex with the cell cytoskeleton was shown to be under cellular control and, therefore, a physiologically relevant phenomenon, additional supportive evidence was required to exclude the possibility that nonspecific interactions might be trapping the ligand-receptor complex with other insoluble components at the periphery of the cytoskeleton. Moreover, since little was known about the functional relevance of the high-affinity ligand-receptor complex, functional correlations needed to be established.

In the study reported here, we have used the dihydro derivative of cytochalasin B [12] to perturb the interaction between the neutrophil plasma membrane and the cell cytoskeleton. We have shown that this drug inhibits both the formation of the high-affinity complex and receptor-mediated endocytosis in these cells. In addition, we have correlated these effects with the effects of the drug on the FMLP-stimulated production of O_2^-. The possible relationships of these various processes is discussed.

MATERIALS AND METHODS

Isolation of neutrophils and surface labelling of cells have been previously described [11]. A summary of the procedures used is shown in Figure 1. Alkaline phosphatase activity was measured as described by Bretz and Baggiolini [13].



BUFFERS

Cell Incubation Buffer (DPBS+)	Triton X-100 Buffer
Dulbecco's PBS pH 7.4 } DPBS	20 mM Tris-HCl pH 7.4
0.1% BSA	3 mM $MgCl_2$
10 μg/ml Superoxide Dismutase	0.5% Triton X-100
7 μg/ml Catalase	

Fig. 1. Summary of the preparation of granulocyte cytoskeletons and washed cells.

Determination of f-Met-Leu-Phe Uptake

In this study the nondissociable component of f-Met-Leu-Phe absorption by cells was measured. We define this component as *uptake*. The determination of the specific and nonspecific contributions to the cellular uptake was accomplished by measuring the tritium content in cells that had been incubated with 50 nM f-Met-Leu-[^{3}H]Phe ± 2.5 μM f-Met-Leu-Phe under various conditions, rapidly cooled by dilution in ice-cold buffer, and then thoroughly washed. The radioactivity coisolating with the Triton X-100–insoluble (cytoskeletal) fraction of the cell was also determined.

Isolation of cytoskeleton-enriched cell fractions, based on a modification of the method of Koch [14], was carried out in two ways. Each used the lysis buffer of Vale and Shooter [15] with 0.5% Triton X-100 in buffer containing 3 mM $MgCl_2$, 20 mM Tris-HCl, pH 7.4, and varying amounts of sucrose. All steps were performed at 0–4°C. Twice-washed cells were layered and centrifuged in gradients containing sucrose and Triton X-100. In some experiments 2.5 μM FMLP was included in the washing buffer to expedite removal of loosely bound ligand. In order to measure total cell-associated radioactivity Triton X-100 was omitted from the appropriate buffers.

In one method 0.5 ml of DPBS containing 1.5×10^8 washed cells was layered onto sucrose gradients with the following composition: 1 ml of 60%, 10 ml of 55–20%, and 1 ml of 15% w/w sucrose in 10 mM Hepes pH 7.4. The two densest layers contained 0.5% Triton. The gradients were centrifuged at 4°C at 31,000 rpm (100,000g at Rav). All steps subsequent to the incubation of the cells was performed at 4°C. Smaller sucrose gradients were also run in an SW50.1 rotor as has been described previously in Jesaitis et al [11]. Measurement of kinetics was performed by replacing the linear gradients with step gradients and quantitating the tritium and protein content of the pellet fractions. In this method 0.2 ml of DPBS containing 1×10^7 washed cells was layered onto 0.2 ml of DPBS containing 4% sucrose overlaid onto 1 ml of lysis buffer containing 8% sucrose with 0.5% Triton X-100 in 20 mM Tris-HCl, 3 mM $MgCl_2$, pH 7.4 (for cytoskeletons) or without Triton (for intact cells). Centrifugation was for 1 min at 8000g in a Beckman or Eppendorf microfuge.

Pellets were resuspended either in 1 N NaOH (10^7 cell equivalents per 0.2 ml) or in the same volume of 20 mM Tris-HCl, 3 mM $MgCl_2$ pH 7.4. For kinetic studies the content of f-Met-Leu-Phe per microgram protein was determined. The number of molecules per cell equivalent was calculated assuming 50 pg protein per intact cell and 30 pg per cytoskeletal residue.

Cytochalasin B Treatment

Dihydrocytochalasin B (Sigma Chemical Co., St. Louis, MO) was dissolved in 95% ethanol or dimethylsulfoxide at 5 mg/ml and diluted to the appropriate levels in Dulbecco's phosphate-buffered saline. The maximum concentration of dihydrocytochalasin B used was 5 μg/ml. The concentration of ethanol and dimethylsulfoxide was adjusted to 0.04% in all incubations; at this concentration of solvent there was no observable effect on responses. Cells were resuspended in the appropriate cytochalasin concentration and warmed to 37°C for 10 min. Uptake studies were performed by the addition of f-Met-Leu-[^{3}H]Phe to a concentration of 50 nM in the presence and absence of nonradioactive f-Met-Leu-Phe as described above. The details of each experiment are given in the figure legends.

Measurement of O_2^- Generation

Production of O_2^- was determined spectroscopically (Cary Spectrophotometer Model 219; Varian, Inc., San Jose, CA) by reduction of cytochrome C as measured by change in absorbance at 550 nm; $1.85 \times 10^4 M^{-1} cm^{-1}$ was used as the reduced minus oxidized extinction coefficient at 550 nm to calculate absolute levels of reduction. Cells (5×10^5 to 2×10^6) from a stock suspension (1×10^8/ml) were diluted into 2 ml of DPBS with 0.62 mg/ml cytochrome C (horse heart type VI; Sigma Chemical Co.) and the appropriate concentration of cytochalasin B. Cells were allowed to incubate in a stirred plastic cuvette in buffer for 10 min at 37°C. Activation was initiated by the addition of 2 μl of 0.5 mM FMLP in 10% DMSO in water. Parallel controls were run in the presence of 100 μg/ml superoxide dismutase (human erythrocyte; Sigma Chemical Co.).

RESULTS

If human granulocytes are incubated with N-formyl peptide at 37°C then a significant percentage of the occupied receptors is converted to a slowly dissociating

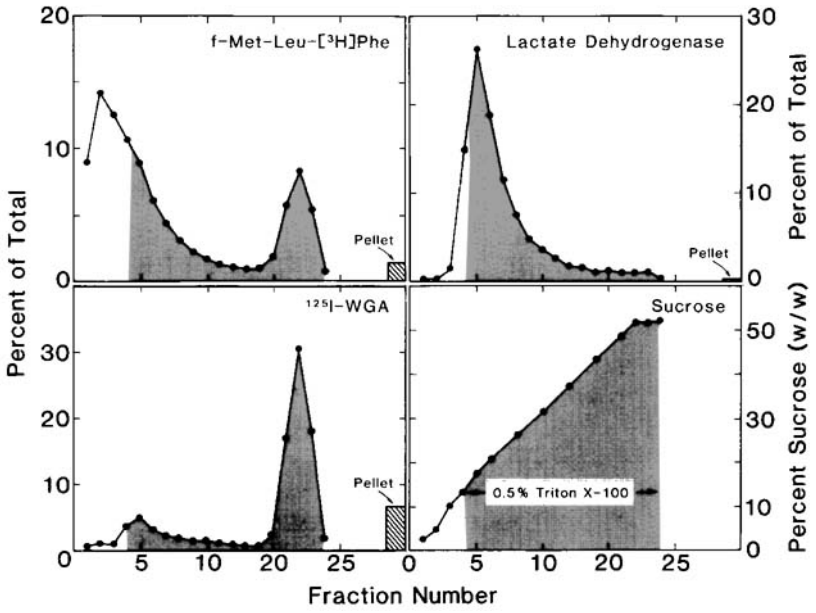


Fig. 2. Detergent fractionation of human granulocytes on linear sucrose gradients: 3×10^7 cells were trace labeled with ^{125}I-WGA, loaded with 50 nM FML[^{3}H]P for 4 min at 37°C, washed, layered onto a sucrose gradient containing Triton X-100, and centrifuged in an SW50.1 rotor as described in Materials and Methods. The total activity of each fraction is plotted as percent of the total measured in the gradient for FML[^{3}H]P, ^{125}I-WGA, and lactate dehydrogenase. Sucrose densities are also shown. Shaded areas indicate presence of 0.5% Triton X-100. Hatched bars indicate percentages recovered in pellets. Recoveries of activity ranged from 85% to 95%.

or "high-affinity" form that coisolates with Triton-insoluble cytoskeletons. In order to analyze the fate of other plasma membrane proteins during isolation of cytoskeletons, the distribution of two plasma membrane markers was measured in the detergent-containing sucrose gradients used to fractionate the cells. Figure 2 shows the distribution of radioactivity on Triton X-100–containing sucrose gradients from the sedimentation of cells prelabeled with trace amounts of ^{125}I-WGA, stimulated with 50 nM N-formyl-Met-Leu-[^{3}H]Phe for 4 min at 37°C (FML[^{3}H]P) and then sedimented through the detergent gradient to separate soluble and insoluble cell components. A significant fraction of the cell-associated tritium remains sedimentible in the detergent. At early times before the onset of endocytosis, most of the ligand (0.8 ± 0.2; $n = 9$ at 1 min) is sedimentible; at later times, as ligand is accumulated in intracellular compartments, a smaller proportion remains sedimentible. At 4 min, as in Figure 2, the fraction of the slowly dissociating ligand remaining sedimentible is 0.6 ± 0.1 ($n = 7$). Ninety percent of the surface-labeled ^{125}I-WGA remains sedimentible for up to 5 min of incubation at 37°C (see also Fig. 4). In addition, the distribution of the granulocyte plasma membrane marker alkaline phosphatase [16] is nearly identical to the surface label, with 90% remaining sedimentable (see also Fig. 4). Cell lysis is evidenced by the distribution of lactate dehydrogenase, which remains at the Triton X-100 interface, indicating that the cytosol is extracted when the cells impact the interface.

The association of N-formyl chemotactic peptide with these actin-containing Triton-insoluble cytoskeletal structures appears to be specific and under cellular control [11]. Specific linkage of the peptide to the cytoskeleton, however, has only been inferred. In order to provide evidence supporting involvement of the cytoskeleton in the control of this receptor modulation the effect of dihydrocytochalasin B (dhCB) on peptide cytoskeletal association was investigated. Purified neutrophils were incubated in the presence and absence of 2 μg/ml dhCB at 37°C for 10 min, followed by stimulation of the cells with 50 nM FML[^{3}H]P. From each incubation mixture aliquots of cells were taken and washed cells and cytoskeletons were prepared according to the step gradient protocol described in Materials and Methods. The uptake of the radiolabeled peptide by cells and its incorporation into the cytoskeletal fraction are shown in Figure 3, which is the average of three experiments from three donors. Standard deviations of the points were approximately 30%, reflecting variability in overall rates of uptake of cells. In the upper panel it is evident that dhCB causes an inhibition in uptake that is significant even after 1 min of incubation with the peptide (66 ± 7%). At this time most of the ligand would normally be at the cell surface [5, 11]. At later times when an increasingly greater proportion of the ligand would have been internalized the inhibition is even greater, between 70% and 90%. Even greater inhibition is observed in the amount of peptide coisolating with the

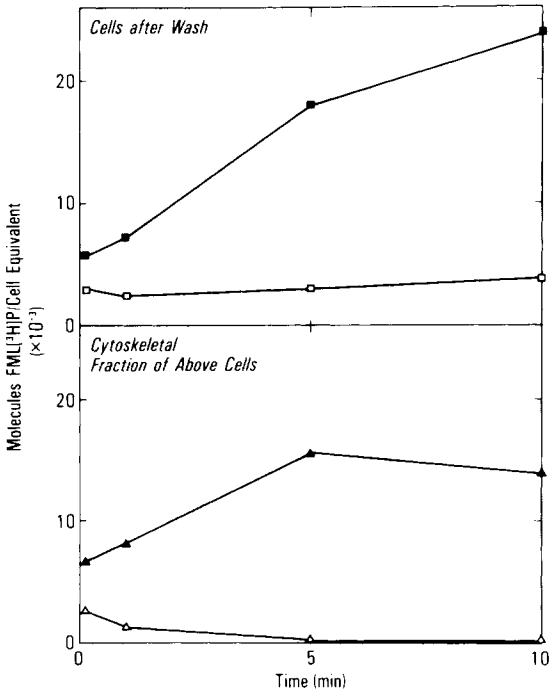


Fig. 3. Effect of dihydrocytochalasin B on the uptake of FMLP and its coisolation with cytoskeleton. Human granulocytes were incubated with 50 nM FML[^{3}H]P ± 2.5 μM FMLP for the times indicated in the presence (open symbols) and absence (closed symbols) of 2 μg/ml dhCB. Aliquots containing 4 × 10^7 cells were removed and the incubation was terminated by dilution in five volumes of ice-cold buffer. The cells were washed in the presence of 2.5 μM FMLP before centrifugation through an 8% sucrose cushion free of or containing 0.5% Triton X-100. The cell (no Triton X-100)- and cytoskeleton (with Triton)- associated radioactivity is plotted as a function of time. Average of three experiments. Standard deviations were approximately 30%, reflecting donor variability.

Triton-insoluble material. This progressive increase in inhibition suggests that dhCB is preventing formation of the high-affinity ligand-receptor conversion and interfering with isolation of the ligand-receptor complex on the cytoskeleton.

Such an inhibition might be the result of a general decrease in the association of membrane proteins with the cytoskeleton. To explore this possibility the detergent fractionation of dhCB-treated cells was compared to control cells. In this fractionation, the subcellular partition into the Triton X-100–soluble and–insoluble fractions was determined for two plasma membrane markers and the cell-associated FML[^{3}H]P. To restrict the analysis to the effect of dhCB on the modulation of the peptide-receptor interaction at the cell surface [11], the incubation of the cells with radioligand was performed for only 1 min at 37°C. Figure 4 shows the distribution of alkaline

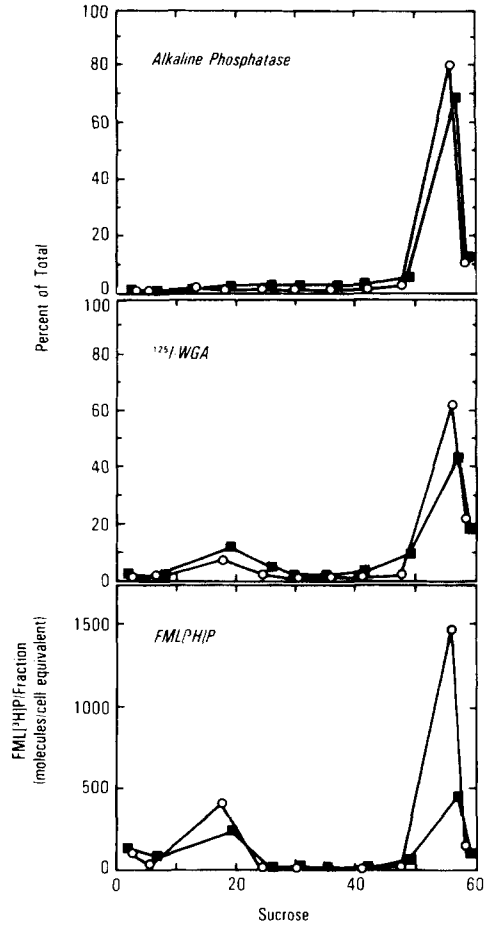


Fig. 4. The effect of dihydrocytochalasin B in the coisolation of cytoskeleton and plasma membrane receptors and enzymes: 1×10^8 cells were preincubated for 10 min at 37°C in 3 ml of DPBS containing 2 μg/ml dhCB, 50 nM FML[^{3}H]P was then added, and the incubation continued for 1 min. Parallel runs were performed on control cells preincubated without dhCB (○—○). The incubation was quenched with five volumes of ice-cold DPBS and the cells were washed twice. The cells were resuspended in 1.5 ml and kept at 0–4°C before and during centrifugation on SW41 Triton X-100–containing sucrose gradients. The percent activity in each fraction relative to the total measured in the gradient is plotted for ^{125}I-WGA and alkaline phosphatase. The absolute content of FML[^{3}H]P in each fraction is plotted in the lowest panel. Inhibition of cellular uptake and cytoskeletal retention of FML[^{3}H]P by dhCB was 65% and 70%, respectively.

phosphatase, ^{125}I-WGA surface label, and specific cellular FML[^{3}H]P on linear sucrose gradients containing 0.5% Triton X-100. The *distribution* of the activities was only marginally affected by the presence of dhCB during the incubation, since the percent remaining sedimentible was reduced only 3%, 10%, and 16%, respectively. The *amount* of the peptide remaining sedimentible in detergent, however, was reduced to 46% of the control level. This result suggests that the dhCB did not cause a major irreversible breakdown in cytoskeletal association of membrane proteins and confirms the inhibition of formation of the high-affinity ligand-receptor complex at the cell surface.

In order to quantitate the effective dosage range for the inhibition of receptor modulation by dhCB, the uptake was measured after 10 min of incubation with FMLP[^{3}H]P as a function of drug concentration. Figure 5 shows the dose dependency of inhibition of FML[^{3}H]P uptake on dhCB concentration. Fifty percent of the maximum inhibition was achieved at approximately 0.25 μg/ml. These concentrations probably correspond to the high-affinity binding sites (K_d = 0.3 μM) for cytochalasins measured by Howard et al [17] and suggest participation of the cytoskeleton in the formation of the "higher-affinity" state of the chemotactic receptor-ligand complex.

Dihydrocytochalasin B also has profound effects on the regulation of the generation of O_2^- in response to N-formyl Met-Leu-Phe and may provide a way of analyzing the relationship between receptor state, regulation of O_2^- production, and the cell cytoskeleton. To begin analysis of these relationships, the effect of dhCB on the generation of O_2^- by granulocytes was also investigated. Figure 6 shows the effect of dhCB on the kinetics of production of O_2^- in response to a saturating dose of

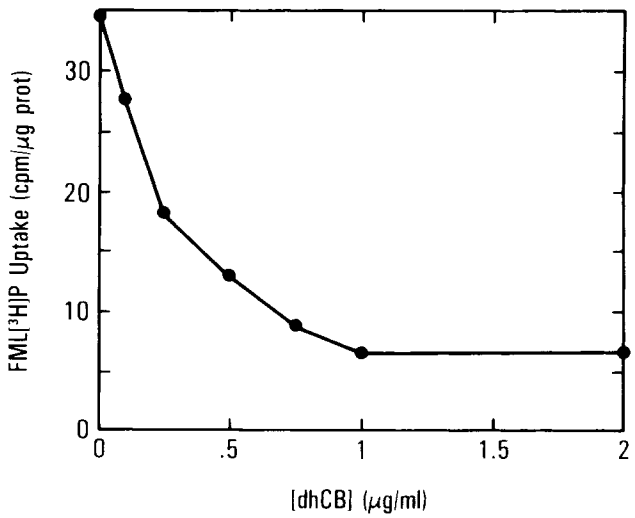


Fig. 5. Effect of dihydrocytochalasin B on receptor-mediated uptake of FMLP. Cells were preincubated with buffer or dihydrocytochalasin B for 10 min at 37°C at a cell density of 2 × 10^7 cells/ml. At time 0, 50 nM FML[^{3}H]P ± 2.5 μM FMLP was added to each in an equal volume dose in the corresponding buffer. The incubation was terminated at 2 min by dilution with five volumes of ice-cold buffer. Cells were washed by pelleting twice and spun through an 8% sucrose cushion to remove loosely bound FML[^{3}H]P. Specifically associated radioactivity is plotted as a function of concentration of dhCB.

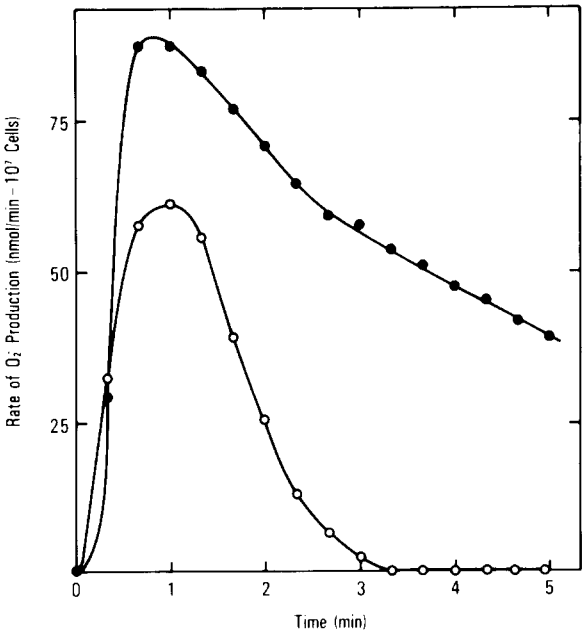


Fig. 6. Effect of dihydrocytochalasin B on the kinetics of O_2^- production by granulocytes stimulated by FMLP. At time 0, 500 nM FMLP was added to a stirred suspension of granulocytes incubated in the presence (●—●) or absence (○—○) of 2 μg/ml of dihydrocytochalasin B, at 37°C in cytochrome C buffer, as described in Materials and Methods. The rate of O_2^- production was calculated from the rate of superoxide dismutase-inhibitable reduction of cytochrome C, assuming an extinction coefficient of $1.85 \times 10^4 m^{-1} cm^{-1}$ for the reduced-oxidized form at 550 nM.

FMLP. The normal response in the lower curve shows that the rate of production reaches a maximum by 0.75–1 min after the addition of FMLP and then relaxes to no production by 3–5 min. If the cells are first incubated with dhCB and then stimulated, there is normally a *potentiation* in the response resulting from an increase in the rate and duration of O_2^- production. The degree of potentiation is variable from preparation to preparation. On the average, the ratio of the maximum rates of O_2^- production with and without dhCB is 1.7 ± 0.5 (n =7, four donors) and the time until 50% of the response has relaxed is increased from t = 2.0 ± 0.5 to t = 3.9 ± 1.2 min (n = 7, four donors). These effects of dhCB are entirely reversible if cells are washed once before stimulation, indicating that irreversible degeneration processes are not involved in the potentiation.

To compare the effects of dhCB on the O_2^- response and receptor modulation, its dose dependency was measured in tandem with measurements on receptor-mediated FML[^{3}H]P uptake. Potentiation of the O_2^- response by dhCB is defined as the elevated level of O_2^- production at times when production has relaxed to the basal level in control cells stimulated in the absence of dhCB. Figure 7 shows the percent potentiation of O_2^- production as a function of drug concentration. On the same scale, the inhibition of uptake, as shown in Figure 5, is replotted as percent of maximum inhibition. These two curves were obtained from the same preparation of cells and

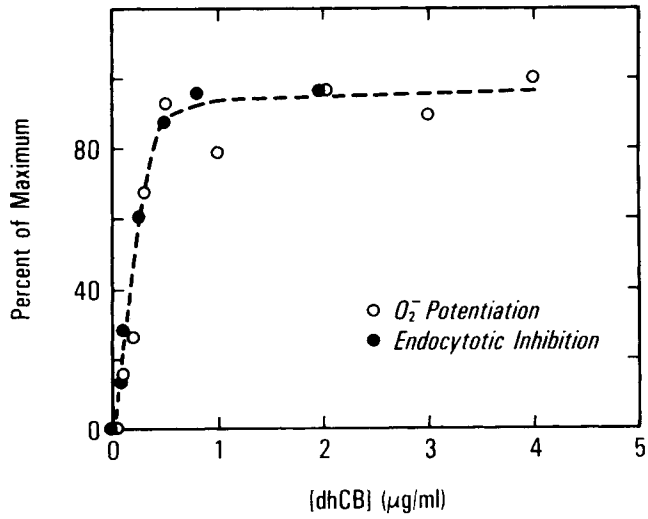


Fig. 7. Comparison of the dose dependency of dihydrocytochalasin B inhibition of receptor-mediated FMLP uptake and potentiation of FMLP-stimulated O_2^- production. The rate of O_2^- production was measured at its maximum (~30 sec) and at 4 min after the stimulation with 500 nM FMLP at different concentrations of dhCB. The ratio of the rates at the respective times was calculated. The percents of the maximum ratio observed were plotted as a function of dhCB concentration (○—○). On the same scale is plotted the percent of the maximum inhibition of FMLP uptake (●—●) as performed in Figure 5.

averaged for two blood donors. The ED_{50} of the inhibitor for both responses was not significantly different.

DISCUSSION

The purpose of this study was to explore the role of the cytoskeleton of human granulocytes in regulating two functions of the cell that are stimulated by N-formyl chemotactic peptides. The focus of the investigation was restricted to an analysis of the effects of the cytoskeleton-perturbing agent dihydrocytochalasin B on the N-formyl peptide receptor modulation and N-formyl peptide-stimulated superoxide production. Dihydrocytochalasin B was used because it shows greater selectivity than cytochalasin B in its effects on the cytoskeleton. It does not bind to or inhibit the hexose transport system [12, 18]. Our results suggest that the inhibitor has profound effects on both O_2^- production and FMLP receptor modulation with a similar dose dependency and imply an involvement of the cytoskeleton.

We have shown that dhCB inhibits uptake of FMLP by granulocytes. The uptake is inhibited both before and after the onset of endocytosis (1 min). Consequently the inhibition probably affects steps preceding internalization but necessary for the ligand-receptor endocytosis. Our results also suggest that dhCB inhibits the conversion of the occupied receptor to its slowly dissociating or "high-affinity" form.

Vale and Shooter [15], using an interpretation of the mobile receptor hypothesis [19,20], have suggested that such conversions might directly be the result of receptors

binding to cytoskeleton. In such a scheme, the cytoskeleton would have a binding site in the receptor that, when occupied, would cause the receptor's ligand-binding site to increase in affinity. Conversely, the occupancy of the ligand-binding site would increase the affinity of the receptor for the cytoskeleton. Further studies are required, however, perhaps utilizing covalent photoaffinity-labelled receptor [21, 22] to show that the occupied receptors are prevented from associating with the cytoskeleton by dhCB.

Cytochalasin B prevents FMLP–dependent rapid association of cellular G actin with the cytoskeleton obtained from rabbit and human neutrophils and even causes a decrease in the basal level of actin associated with cytoskeleton [23, 24]. In addition, it inhibits the rapid and transient polymerization of subcortical actin filaments in response to FMLP in human cells [24, 25]. However, the latter effects of cytochalasin B may be more relevant to morphological and chemolocomoting events because they occur in a dose range that is an order of magnitude higher than the effects observed here. Investigation of the dose range for dhCB action on the NBD-phallacidin detection of subcortical actin assembly [24–26] should reveal whether it is related to the association of actin to the cytoskeleton as measured by White et al [23] or to the functional effects observed here.

We have previously shown that the tertiary complex between the cytoskeleton/receptor/FMLP is formed at the cell surface, precedes endocytosis, and has a transient lifetime compatible with a role in the endocytotic process [11]. Dihydrocytochalasin B also inhibits the endocytosis or uptake of the peptide by the cells. If the rapid, transient polymerization of subcortical cellular actin is related to these events then they may signal the initial step in modulation and endocytosis of the receptor. Establishment of such a correlation would, therefore, not only support the hypothesis that the tertiary complex between ligand, receptor, and cytoskeleton is a vehicle for receptor-mediated endocytosis of N-formyl chemotactic peptides but also would suggest a functional role for receptor-cytoskeletal interaction in the activation process.

Do such fundamental changes in receptor modulation induced by dhCB have functional consequences in responses stimulated by N-formyl peptides? Cytochalasin B has been used routinely to "enhance" the generation of O_2^- in response to the formyl peptides [7, 27]. We have confirmed these results by demonstrating that dhCB causes enhancement of production of O_2^- in response to FMLP by prolonging the duration of the response and by increasing its magnitude. Of interest is the fact that the dose dependency of the effects matches very closely the dependency observed for the inhibition of receptor-mediated endocytosis and formation of the "high-affinity" tertiary complex between ligand, receptor, and cytoskeleton. The dose dependency, however, does not match those observed for the inhibitors' effects on phagocytosis, morphology [17], or association of actin with the bulk cytoskeleton [23].

The dihydro derivative of cytochalasin B has been shown not to affect hexose transport in other cells [12] and to have specific high-affinity sites probably associated with contractile proteins on the cytoskeleton or plasma membrane of the cell [4, 17, 18]. We therefore infer that dhCB binding to a single class of high-affinity sites affects two seemingly independent functions, namely receptor modulation and superoxide anion production. We also infer from these results that these sites correspond to the higher-affinity cytochalasin binding sites observed by Howard et al [17] in ^{3}H-cytochalasin B binding studies on human granulocytes. The subcellular localization of these sites may provide clues as to their nature.

Currently the interpretation of the effects of cytochalasins on granulocytes is difficult because of their pleiotropic effects [6–10]. These include elevation of Ca^{2+} levels; disruption of exocytotic regulation; alteration of morphology, surface charge, and adherence properties; and the inhibition of locomotion. Consequently extreme caution must be exercised in evaluating the results reported here. However, three possible relationships between cytoskeletal function and the effects of dhCB observed need consideration. The first is that inhibition of actin polymerization or its attachment to the cell membrane affects a metabolic regulating process such as generation of second messenger, which could, in one case, interfere with the activation of the processes involved in receptor modulation and endocytosis and, in another case, result in activation of the superoxide anion response. A possible candidate for such a role would be intracellular Ca^{2+}, which has been shown to become elevated by exposure of neutrophils to cytochalasin B [8].

A second possibility is that independent mechanochemical interactions are involved in both receptor modulation and regulation of O_2^- production. Thus a receptor cytoskeletal interaction might be required to lock ligand into receptor so that it can be pulled into the cell by a cytoskeletal contraction event. Similarly, a cytoskeletal interaction might be required to restrict interaction of surface components of, for example, the plasma membrane electron transport system believed to be involved in the production of O_2^- [28–31].

A third possibility is that receptor modulation is directly involved in regulation of the O_2^- response. Thus dhCB inhibits the formation of the high-affinity complex or its endocytosis and thus prolongs the interaction of the active receptor-ligand complex with either catalytic or regulatory elements in the plasma membrane, thus prolonging the O_2^- response. In addition, since the cytochalasin also results in a major FMLP-induced secretory event fusing membranes containing internal stores of receptors [32, 33] and O_2^- generating system components [34, 35] with the plasma membrane, their concentration in the plasma membrane would be increased. Consequently, the prolonged and enhanced production of O_2^- may be the result of increased interaction in both duration and number of components of the stimulus pathway in the environment of the plasma membrane.

We are currently investigating ways to distinguish between these possibilities and to analyze the functional role of cytoskeleton in cell activation. We have already shown that putative components of the O_2^- generating system are not subject to FMLP-induced endocytosis and exocytotic modulation comparable to that observed with formyl peptide receptor [16, 36]. Our preliminary work suggests that the tertiary complex between the cytoskeleton, receptor, and N-formyl peptide plays a major role in regulating the plasma membrane O_2^- generating system.

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