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MINI-REVIEW

Activation of the Neutrophil Respiratory Burst by Chemoattractants: Regulation of the N-Formyl Peptide Receptor in the Plasma Membrane

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Abstract

The N-formyl peptide receptor mediates a number of host defensive responses of human neutrophils that result in chemotaxis, secretion of hydrolytic enzymes, and superoxide generation. Inappropriate activation or defective regulation of these responses can result in pathogenic states responsible for inflammatory disease. The receptor is a 50 to 70-kD, integral plasma membrane glycoprotein with intracellular and surface localization. Its abundance in the membrane is regulated by membrane flow and recycling processes. Cytoskeletal interactions are believed to control its organization in the plane of the membrane and interaction with other proteins. The receptor's most important interaction is with guanyl nucleotide binding proteins that serve as signal transduction partners ultimately leading to activation of effector responses. Because the interaction of the receptor with G proteins is necessary for transduction, control of this interaction may be at the root of understanding the molecular control of responses in these cells. This review briefly summarizes some of the molecular properties, dynamics, and interactions of this receptor system in human neutrophils and discusses how these characteristics may pertain to the activation and control of superoxide generation.

Key Words: Chemoattractants; N-formyl peptide receptor; neutrophil respiratory burst; plasma membrane.

Introduction

There exists significant evidence directly implicating leukocyte-generated oxygen metabolites in inflammatory tissue damage (Weiss and LoBuglio,

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1984; Freeman and Crapo, 1984). Chemoattractants, including the N-formyl peptides and complement fragment C5a, have been implicated as mediators in such injury (Desai *et al.*, 1979; Kreutzer *et al.*, 1979: 109), yet the same generation of oxygen metabolites, when activated appropriately, at a site of injury or infection is essential for health (Curnutte, 1988). Since human neutrophils stimulated with chemoattractants are capable of producing superoxide at a rate > 100 nmol/min/10^7 cells (Karnovsky and Badwey, 1983), then a relatively simple estimate of the upper limit of the concentration of toxic oxygen products (primarily H_2O_2) in the vicinity of the plasma membrane can be made. Assuming every 2 mol of O_2^- is converted by dismutation into 1 mol of H_2O_2, then solution of the diffusion equation for a continuous point source (Crank, 1975) producing O_2^- at a rate of 0.17 fmol/sec and diffusion constant of 10^{-5} cm^2/sec translates into a rapidly equilibrating steady-state concentration of H_2O_2 of 2 μM in the immediate vicinity of the cell. Considering that this concentration falls inversely with the radial distance from the cell center and that such production may be prolonged with many cells accumulating at such sites of activation, then this upper limit would be greatly magnified to levels toxic to adjoining cells or microbes.

What, then, allows neutrophils to be activated in the blood or bone marrow and to migrate to the site of infection or injury without causing inflammatory damage to the tissues and fluid on route? There are, no doubt, a number of processes that may interact to prevent the unregulated generation of superoxide. Part of the answer may be found in the ability of the cell to demonstrate classical adaptation to chemoattractant concentrations over $4\frac{1}{2}$ orders of magnitude (Keller *et al.*, 1976; Seligmann *et al.*, 1982; Sklar *et al.*, 1984a), necessarily implying that, if the rate of stimulus presentation is slow enough, then the cell will not be activated (Sklar *et al.*, 1981). Such adaptive processes, nearly universal in sensory cells, have been shown in other cell types to result from direct receptor modification such as by phosphorylation (Sibley and Lefkowitz, 1985) or methylation (Koshland *et al.*, 1982). Thus, by analogy, regulation of receptor activity is probably one mechanism of control used by the neutrophil to keep the superoxide generation under control without compromising the sensitivity of the cell to chemoattractants.

Another answer to the question of regulation of chemoattractant-induced superoxide production may also lie in the observation that, in suspension, the neutrophil's oxidative response to chemotactic factors is transient, lasting only a few minutes. Thus again if the system's control is such that the turn-off process proceeds much more rapidly than the activation process, the activation would be held to a minimum.

The transient response can be perturbed by agents or conditions affecting the surfaces of these cells such as the binding of lectins (Kitagawa *et al.*,

1980), the attachment neutrophils to surfaces (Dahinden *et al.*, 1983), by drugs that disrupt the integrity of the attachment of the membrane to the cytoskeleton such as the cytochalasins (Jesaitis *et al.*, 1985; English *et al.*, 1981; Goldstein *et al.*, 1973; Lehmeyer *et al.*, 1979) and by "priming" substances such as other chemoattractants (Bender *et al.*, 1983), phorbol esters (McPhail and Snyderman, 1984; McPhail and Sugarman, 1983), lymphokines (Klebanoff *et al.*, 1986; Weisbart *et al.*, 1985, 1986), and bacterial lipopolysaccharide (Guthrie *et al.*, 1984), which also can affect the surface composition and organization of the neutrophil.

All of these perturbations have pleiotropic effects on the cell. Thus it would not be incorrect to ascribe their effects to perturbation of second messenger levels or intermediate steps of signal transduction. However, another common feature of their action is to perturb the organization or composition of the plasma membrane. Since the initial and terminal molecular entities responsible for the activation (receptors, G proteins) and production of superoxide anion (NADPH-oxidase, cytochrome *b*) are, in part, localized to the plasma membrane (Jesaitis *et al.*, 1982a; Bokoch *et al.*, 1988; Segal and Jones, 1979; Babior *et al.*, 1981), then an examination of the events occurring at this level may uncover distinct mechanisms of regulation that might contribute to the characteristic kinetics of chemoattractant-activated superoxide production.

This review summarizes some of the features of the N-formyl chemotactic peptide receptor system of human neutrophils and relates these features to potential mechanisms of regulation in the plasma membrane. It examines the structure, organization, and dynamics of the N-formyl peptide receptor system and its relationship to the activation of superoxide generation. A unifying theme in this review is a discussion of the compositional and organizational constraints imposed by the plasma membrane on receptor–G–protein interactions. It is also shown that this theme can be extended to include the superoxide-generating system. In so doing, it is hoped that discussion can be generated leading to the elucidation of regulatory mechanisms whose appropriate function leads to the killing of microbes and minimal damage to host tissue and whose inappropriate function results in unregulated production of superoxide.

Sensory Transduction Cascade of Neutrophils

The initial event in the activation of superoxide generation in human neutrophils by N-formyl peptide chemoattractants is the occupancy of specific receptors for those peptides termed N-formylated chemotactic peptide receptors (Allen *et al.*, 1988; Sklar *et al.*, 1984a). They interact with a class

of guanyl nucleotide binding proteins called G proteins (Gilman, 1987). This class of proteins is highly conserved and acts as a transducer that activates a variety of functions in different cell types, mediated by, for example, the β-adrenergic receptor system and the rhodoposin photoreceptor. In these latter sensory systems, the G proteins are comprised of a heterotrimeric complex of polypeptides of approximate molecular weights 41,000, 35,000, and 10,000. The N-formyl peptide receptor forms a stable complex with at least one component (α subunit) of this heterotrimeric transducer protein variously called Gn (Bokoch *et al.*, 1988), Giα (Rotrosen *et al.*, 1988), or Gc (Polakis *et al.*, 1988) that serves as a pertussis toxin substrate. This

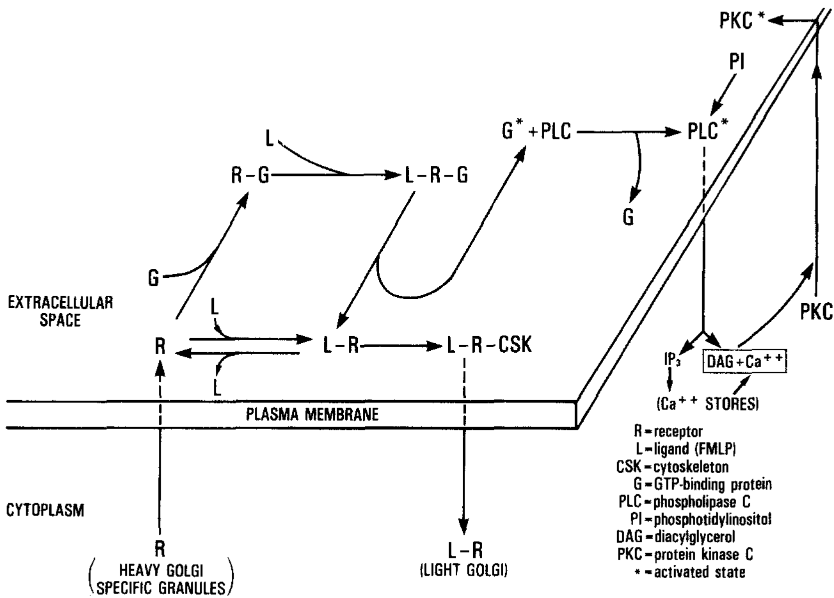

Fig. 1. A speculative model of the chemoattractant activation pathway of human neutrophils. The activation of human neutrophils by chemoattractants results in an orchestration of spatial and temporal events leading to superoxide production. Occupancy of the chemoattractant receptor R with ligand L results in formation of ligand–receptor complex. The complex interacts with guanyl nucleotide binding proteins (G) resulting in the formation of activated G protein, G*, which then activates phospholipase C (PLC) in the plasma membrane. PLC activation results in phosphatidylinositol (PI) hydrolysis to generate 1,4-5-inositoltrisphosphate (IP_3) and diacylglycerol (DAG). IP_3 induces the release of Ca^{++} from intracellular stores, which in concert with DAG, activate and cause the translocation of intracellular protein kinase C (PKC) to the plasma membrane. These events result in phosphorylation of membrane proteins, calcium-dependent secretory events, remodeling of the plasma membrane, and changes in cytoskeletal organization. Such changes feed back to the receptor level, providing more R from intracellular stores (heavy Golgi/specfic granules) or recycling loci. Additionally, they dampen the response by a lateral sequestration of L-R complex from G proteins by cytoskeletal–membrane interactions and ultimate internalization to a light Golgi or endosomal fraction. From Allen *et al.*, (1988), with permission.

multimeric structure is sensitive to guanyl nucleotides (Koo *et al.*, 1983; Lad *et al.*, 1985), in particular GTP, the presence of which causes the dissociation of the receptor from the G protein (Sklar *et al.*, 1988b; Polakis *et al.*, 1988). The dissociation occurs in concert with exchange of bound GDP for GTP (Lad *et al.*, 1985) and, in other systems, the dissociation of the 40-kD α subunit from the G-protein complex (Gilman, 1987). The α subunit with bound GTP then is thought to initiate the next step in the transduction cascade, which is the activation of phosphoinositide turnover by phospholipase C (Smith *et al.*, 1986; Okajima *et al.*, 1985). Hydrolysis of GTP by the α subunit GTPase (Hyslop *et al.*, 1984; Lad *et al.*, 1985) results in a inactive GDP-bound subunit which, it is assumed, reassociates with the β and γ subunits and receptor (Bokoch, 1987).

Activation of phospholipase C results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate and production of diacylglycerol (Brandt *et al.*, 1985; Honeycutt and Niedel, 1986), inositol 1,4,5-trisphosphate (Brandt *et al.*, 1985; Brandford and Rubin, 1986), and inositol 1,3,4,5-tetrakisphosphate (Traynor-Kaplan *et al.*, 1988a). The kinetics of this hydrolysis are compatible with activation of superoxide production (Traynor-Kaplan *et al.*, 1988b). Inositoltriphosphate (IP_3) has been shown to have specific receptors on membranes of internal stores of calcium that, when occupied, release the ion elevating the intracellular free calcium concentration (Bradford and Rubin, 1986; Prentki *et al.*, 1984; Spat *et al.*, 1986). Diacylglycerol in combination with calcium results in the activation of protein kinase C and its translocation to the plasma membrane (Robinson *et al.*, 1984; DiVirgilio *et al.*, 1984; McPhail *et al.*, 1984). The relationship between these events and the stimulation of superoxide production in the plasma membrane is not fully resolved and is covered in another section of this volume. The transduction pathway is summarized in Fig. 1.

The Structure of the N-Formyl Peptide Receptor

The N-formyl peptide receptor of human neutrophils is an integral membrane protein that migrates as a diffuse band on SDS–polyacrylamide gel electrophoresis with an apparent M_r or 50–70,000 (Niedel *et al.*, 1980; Schmitt *et al.*, 1983; Allen *et al.*, 1986b). The receptor has been resolved into two isoforms of M_r 50,000 with an isoelectric point (pI) of 6.0 and M_r 60,000 with a pI of 6.5 (Schmitt *et al.*, 1983a). The receptor is a glycoprotein (Painter *et al.*, 1982) containing two N-linked carbohydrate chains that can be removed sequentially with endo-β-*N*-acetylglucosaminidase F (endo F) generating initially a 40–50,000 fragment and subsequently a 33,000 fragment that is capable of ligand binding (Malech *et al.*, 1985). The pI differences of

the receptor isoforms remained after deglycosylation (Malech *et al.*, 1985), suggesting that the isoforms arise due to differences in the peptide structure of the receptor and not due to differential glycosilation.

Allen *et al.*, (1986a) have reported some of the physicochemical properties of the receptor. The solubilized, photoaffinity-labelled receptor has a Stokes radius of 40 Å, a molecular weight of 63,000 Daltons, determined by sedimentation equilibrium analysis, and an anomalously high partial specific volume, indicating that the receptor may be associated with tightly bound endogenous lipid or that it is a very hydrophobic membrane protein. A frictional ratio of 1.1 : 1.3 was determined for the receptor, which is consistent with an elongated detergent–protein complex having an axial ratio of $\sim 3:1$. This value together with the Stokes radius suggests that the receptor is capable of spanning the 35–30 Å nonpolar center of the lipid bilayer (Levine and Wilkins, 1971; Caspar and Kirschner, 1971). Further evidence of the transmembrane nature of the receptor comes from work by Dolmatch and Niedel (1983) in which papain and pronase produced different cleavage fragments from cells and plasma membranes, indicating the presence of protease-sensitive receptor sites on the cytoplasmic face of the membrane. The receptor fragment of M_r 29–31,000, produced by papain treatment of whole cells, was no longer susceptible to endo F (Malech *et al.*, 1985), indicating that the oligosaccharide chains are located distally on the extracellular domain of the receptor. Further structural characterization must await pure receptor preparations, which thus far have been elusive, although partial purifications have been reported (Allen *et al.*, 1985; Huang, 1987).

Organization of N-Formyl Peptide Receptors

In the "unstimulated" human neutrophil isolated for laboratory use, the reported number of cell surface N-formyl peptide binding sites varies widely, ranging from 3000 (Williams *et al.*, 1977) to 75,000 per cell (Sklar *et al.*, 1984b). This variation may be related to the perturbation of the cell by exogeneous agents used in the purification of the cells, their handling, and storage. Dahinden (personal communication) has found that, if neutrophils are very carefully isolated and maintained under conditions that perturb the cell minimally, they express no sensitivity to formyl peptides in terms of superoxide production and probably have very few cell surface receptors. Exposure of the cell to certain exogenous agents (see below) appears to increase the receptor number at the cell surface.

Consistent with this modulation is the existence of an intracellular pool or pools of receptor (Fletcher and Gallin, 1983; Jesaitis *et al.*, 1982a; Gardner *et al.*, 1986). Although no morphological evidence has yet been obtained,

biochemical evidence based on subcellular fractionation analysis suggests that receptor binding activity exists in fractions enriched in specific granules or trans-Golgi. Independent comparisons from two laboratories (Fletcher and Gallin, 1983; Jesaitis *et al.*, 1982a) of the plasma membrane activity with that isolated in specific granule/Golgi fractions indicates that the intracellular pool contains from one to two times the number of high-affinity formyl peptide binding sites found in the surface pool. Of course, the actual ratio determined by any individual laboratory may depend on the treatment of cells during isolation for the reasons cited above.

The large number of lower-affinity binding sites ($K_d > 10^{-7}$ M) for f-Met-Leu-Phe detected in the intracellular pool by Gallin and coworkers (Fletcher and Gallin, 1980) may have resulted from binding to a highly abundant granule content protein of molecular weight 24,000 that has recently been studied in our laboratory using a photoaffinity derivative of f-Met-Leu-Phe-Lys (Allen and Jesaitis, 1988). In these studies, another species was detected with an M_r of 33,000 and localization to the light Golgi or endosomal internalization locus $\varrho = 1.11$ g/cc) (Jesaitis *et al.*, 1982b, 1983). We speculate that this species may be a deglycosylated form of the 50 to 70-kD receptor. Recently, several laboratories have reported the existence of alternative secretory granules having different sedimentation properties than specific granules. These organelles may or may not be related to one another and variously contain gelatinase (Dewald *et al.*, 1982), latent alkaline phosphatase activity (Borregaard *et al.*, 1987), ubiquinone (Crawford and Schneider, 1983), and tetranectin (Borregaard, personal communication). Whether any of these contain formyl peptide receptors is yet to be determined.

The Dynamics of the N-Formyl Peptide Receptor

The N-formyl peptide receptors or the functions that they activate appear to be modulated by interaction with cellular mediators derived from invasive organisms, other host cells, or neutrophils themselves. Such factors include colony-stimulating factor (Weisbart *et al.*, 1985, 1986), interferon-gamma (Shalaby *et al.*, 1985; Cassatella *et al.*, 1988), tumor necrosis factor (Shalaby *et al.*, 1985; Klebanoff *et al.*, 1986), Raji cell supernatant factor (Cross *et al.*, 1985), and lipopolysaccharide (Goldman *et al.*, 1986; Haslett *et al.*, 1985). Exposure of neutrophils to colony-stimulating factor (GM-CSF) modulates the number and affinity of receptors in a manner highly dependent on the length of exposure (Weisbart *et al.*, 1986). Lipopolysaccharide has a major effect on neutrophils, enhancing the surface expression of receptors as much as two- to eightfold (Goldman *et al.*, 1986; Zimmerli *et al.*, 1986). Most

of these factors tend to activate the neutrophils by increasing the effect of f-Met-Leu-Phe on superoxide production. Because the priming requires times that are long, relative to neutrophil responses, and for some agents appears to be inhibited by inhibitors of protein synthesis (Casatella *et al.*, 1988), the increase in receptor expression or function resulting from exposure of neutrophils to these types of priming agents may result from an increased biosynthesis stimulated by these factors.

Another class of stimuli, which act very rapidly, also modulate the number of receptors at the cell surface. These include the f-Met-Leu-Phe itself, which activates receptor-mediated endocytosis (Niedel *et al.*, 1979b; Jesaitis *et al.*, 1982b, 1983; Zigmond *et al.*, 1982; Sklar *et al.*, 1984b; Anderson and Niedel, 1984) and recycling (Zigmond *et al.*, 1982) of receptors from intracellular storage pools (Fletcher and Gallin, 1983; Jesaitis *et al.*, 1982a; Gardner *et al.*, 1986) or internalized pools of receptor (Jesaitis *et al.*, 1982b, 1983; Anderson and Niedel, 1984). Fletcher and Gallin (1980) found that degranulating stimuli causing limited granule release increased receptor expression on the surface of neutrophils twofold. Zigmond *et al.*, (1982) also found a rapid transient increase in receptor binding activity within minutes after stimulation of rabbit neutrophils with formyl peptides. Thus, the source of these receptors is probably not increased biosynthesis, but the intracellular pools described above.

Following occupancy, the ligand–receptor complex is internalized after a short delay of 30 sec to 1 min (Jesaitis *et al.*, 1982b; Sklar *et al.*, 1984b). The internalization in the neutrophil and differentiated HL-60 cells proceeds via a Golgi-like or endosomal compartment probably containing galactosyl transferase activity (Jesaitis *et al.*, 1983; Anderson and Niedel, 1984). Subcellular fractionation analysis of neutrophil cavitates has shown that this compartment is distinct from, and has a lower density ($\varrho = 1.11$ g/cc) than, the compartment enriched in glactosyl transferase activity that cosediments with specific granule markers (Jesaitis *et al.*, 1982a, 1983). In a kinetic analysis of receptor modulation, Zigmond and coworkers (Zigmond *et al.*, 1982; Sullivan and Zigmond, 1980) also demonstrated that the N-formyl peptide receptor of rabbit peritoneal neutrophils was internalized with similar kinetics as for human peripheral blood neutrophils. Uptake of radioligand, however, was not blocked by dihydrocytochalasin B as it was in the human system (Zigmond and Tranquillo, 1986). The resultant quantitative analysis of these kinetics suggested that receptors were reexpressed at the cell surface at a rate proportional to the number of down-regulated receptor binding sites (Zigmond *et al.*, 1982).

The source of these receptors remains unclear. Studies performed by Dolmatch and Niedel (1983) employing papain cleavage of photoaffinity-labeled surface receptor suggested that the resultant active 35-kD fragment

was not recycled. In differentiated HL-60 cells, Anderson and Niedel (1984) indicated that internalization is similar to that observed in neutrophils, but that no recovery from receptor down regulation occurred. The HL-60 cells do not contain an intracellular specific granule pool that might contain receptors. In this cell, the internalized, nonphysiologic, (covalently coupled) ligand–receptor complexes are degraded with a $t_{1/2}$ of 7 h. Thus, it could be inferred that receptors are not recycled in neutrophils, but only replenished from intracellular stores. This speculation, however, is only weakly supported.

Prior to internalization, the occupied N-formyl peptide receptor undergoes complex transformations of affinity and organization that result from interaction with G proteins and possibly the cytoskeleton. Within seconds after receptor occupancy, formation of a high-affinity, slowly dissociating complex of ligand and receptor has been observed (Jesaitis *et al.*, 1984; Sklar *et al.*, 1984b). This high-affinity form of receptor coisolates with the Triton X-100-insoluble fraction of the cell and has been interpreted to be indicative of a cytoskeletal association of the ligand–receptor complex (Jesaitis *et al.*, 1983, 1984). This form of receptor is transient, since pulse-chase experiments indicated that radiolabel taken up by the cell remained at constant levels in intact cells while the amount coisolating with the cytoskeleton declined with a $t_{1/2}$ ranging between 0.5 to 2 min (Jesaitis *et al.*, 1984). At 4°C, the conversion still occurs, but much more slowly (Zigmond and Tranquillo, 1986; Sklar *et al.*, 1984b). At 15°C, a temperature not permissive to internalization, secretion, or superoxide production, the high-affinity form accumulates at the cell surface, down regulating receptor binding activity *without internalizing the ligand–receptor complex* (Jesaitis *et al.*, 1986). These conversions were confirmed by Sklar *et al.* (1987) in a kinetic analysis using fluorescent derivatives of N-formyl peptides.

We correlated the conversion of receptors to the high-affinity cytoskeleton-associated form at 15°C with the ability of these cells to produce superoxide anion in response to f-Met-Leu-Phe at 37°C (Jesaitis *et al.*, 1986). It was found that the log of the maximum rate of superoxide production was directly proportional to the number of receptors *not* converted to the high-affinity form coisolating with the cytoskeleton. This relationship is shown in Fig. 2. Cells that were desensitized in this way retained full responsiveness to phorbol myristate acetate (PMA) and partial responsiveness to C5a. The desensitization and conversion of receptors was also slowed in the presence of dihydrocytochalasin B (dhCB) as is shown in Fig. 3. This relationship suggested that the process was receptor specific and involved cytoskeleton. Since the same conversions were apparent and more rapid at 37°C, we suggested that the process might also be involved in termination of the response of the cell to formyl peptides and that the cell need control only a

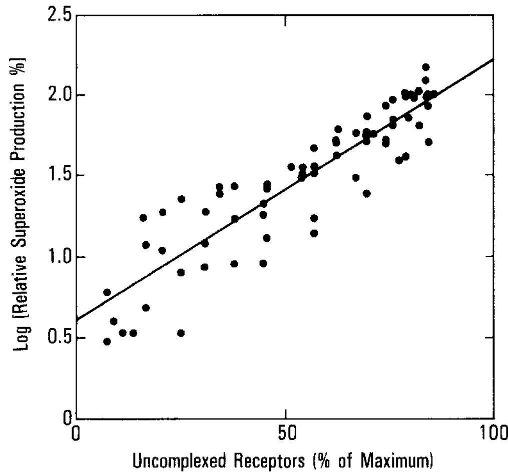

Fig. 2. Relationship between the ability of human granulocytes to respond to the chemoattractant f-Met-Leu-Phe and the calculated number of receptors not complexed with ligand and cytoskeleton in cells desensitized by prior incubation with f-Met-Leu-Phe at 15°C for various intervals of time. Their ability to respond at 37°C was measured by dilution in superoxide assay buffer at 37°C, containing the same amount of f-Met-Leu-Phe. In parallel or simultaneous experiments (Hedo *et al.*, 1987) n = the amount of radioligand coisolating with the Triton X-100-insoluble fraction of the cell, was used to calculate the complementary number of receptors not coisolating with this fraction. Values were calculated from figures 7 and 8 of Jesaitis *et al.*, (1986), which represent data from seven donors of blood. From Jesaitis *et al.*, (1988c), with permission.

fraction of total cell receptor population in order to control the superoxide response fully (Jesaitis *et al.*, 1986, 1987a).

In addition to the affinity conversions resulting in these surface ligand–receptor complexes that do not participate in transduction, significant progress has been made in the analysis of the affinity conversions resulting from interaction of the N-formyl peptide receptors with G proteins. First observed in membranes (Koo *et al.*, 1983), the study of these interactions was extended to digitonin-permeabilized cells (Sklar *et al.*, 1987). It became clear that receptors associated with G proteins interact with their ligand in a high-affinity state that results in dissociation of receptor–G-protein complex and a lowering of the receptor's affinity for the ligand. These interactions are sensitive to ionic conditions and are blocked by pertussis toxin. They have been formalized by Sklar and coworkers (1988a) and Painter *et al.* (1987) into a model proposing three states of occupied receptor similar to the one proposed by us and shown in Fig. 1. The model suggests that the L-R-G complex is the one that triggers transduction and the L-R-CSK complex represents the inactive surface ligand–receptor complex associated with the cytoskeleton. In our model, a pathway exists between L-R and L-R-CSK, which was postulated to account for cytoskeleton-mediated desensitization

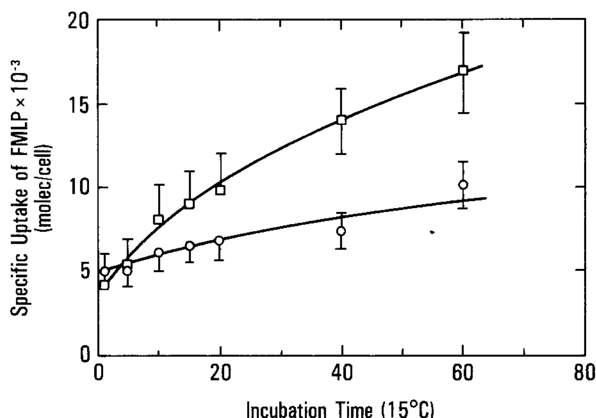

Fig. 3. Effect of dihydrocytochalasin B on uptake of f-Met-Leu-Phe by human neutrophils at 15°C. Human neutrophils (10^7 cells/ml in Dulbecco's phosphate-buffered saline) were incubated with 50 nM f-Met-Leu-Phe plus 50 mM f-Met-Leu-[^{3}H]-Phe ($\pm 5\,\mu$M f-Met-Leu-Phe) for 1 h at 15°C in the presence (○) or absence (□) of 2 μg/ml dihydrocytochalasin B. Aliquots were removed at the indicated times, diluted fivefold in ice-cold buffer, washed in the presence of 5 μM f-Met-Leu-Phe, and pelleted. The pellets were solubilized over night in 0.1 ml 1 M NaOH and counted in a liquid scintillation counter. The specifically retained radioactivity (total–nonspecific) is plotted as a function of incubation time with ligand at 15°C. Methods and materials for this experiment are given in Jesaitis *et al.* (1986).

and control of activation. The model also predicts the existence of pre-associated unoccupied receptor–G-protein complexes.

To study the molecular basis for this control in a system depleted of nuclear and irrelevant cytoplasmic cytoskeletal proteins, a comparison of purified plasma membranes from responsive and desensitized cells that had been prelabeled with the photoaffinity derivative of f-Met-Leu-Phe-Lys-SASD-[^{125}I] at 4°C and 15°C, respectively, was carried out (Jesaitis *et al.*, 1988a). Receptors from fully responsive cells colocalized with standard plasma membrane markers and guanyl nucleotide binding activity. Surprisingly, receptors from desensitized cells were shifted in density and colocalized with membrane-associated cytoskeletal proteins actin and fodrin, well resolved from the distribution of G proteins. When these receptors were solubilized in octyl glucoside and subjected to ultracentrifugation in detergent-containing sucrose gradients, they behaved as would a 4S particle (Jesaitis *et al.*, 1988b). Receptors from responsive cells, and the lighter plasma membrane fractions, sedimented like 6S particles. The 6S receptor form could be converted to the 4S form if the extraction was performed in the presence of 10 μM GTPγ S, suggesting that receptors in the heavy fraction of desensitized cells were unassociated with, and therefore uncoupled from, G proteins.

The N-formyl peptide receptor system must be capable of conversion to a lower-affinity state upon prolonged incubation at 37°C with formyl peptide

to account for the dynamic range in sensitivity of human neutrophils (Seligmann *et al.*, 1982). To date, virtually no evidence is available about such a conversion. Weak evidence obtained from our laboratory suggests that the tenfold decrease in sensitivity to formyl peptides observed in cytochalasin-treated cells (Elferink and Riersma, 1981; A. J. Jesaitis, unpublished) is correlated to a decrease in binding affinity and expression of the 50-kD isoform of the receptor from intracellular, cytoskeletal-associated stores (Allen and Jesaitis, 1987). In the rhodopsin visual system, and the dictyostelium chemotactic system, multiple phosphorylation leading to a slight shift in M_r results in such an adaptive modulation.

As stated in the section on the receptor structure, the N-formyl peptide receptors are comprised of at least two isoforms determined by isoelectric focusing techniques. When resting neutrophils are radiolabeled with a photoaffinity derivative, the characteristic diffuse band of 50–70 K is seen. This band sometimes contains two or three discrete bands. A 50-kD isoform is observed when cytoskeletons from unlabeled cells are radiolabeled. Using tritiated f-Met-Leu-Phe to measure the affinity of this population of receptors, we found that their affinity was tenfold higher ($K_d \sim 1$ mM vs. 10 mM) than the surface receptors of intact cells (Allen and Jesaitis, 1987).

When cells are stimulated with PMA or with f-Met-Leu-Phe in the presence of dhCB, a large increase in the 50-kD and 60-kD isoforms is observed on the cell surface. Neither or these forms coisolated with the Triton X-100-insoluble cytoskeletal fraction of cytochalasin-treated cells in marked contrast to cells stimulated with f-Met-Leu-Phe in the absence of dhCB, where both forms are Triton X-100 insoluble. The detection of these various forms of receptors may be of interest in the study of the activation of superoxide production in neutrophils treated with cytochalasin B, as the ED_{50} for f-Met-Leu-Phe is shifted in order of magnitude to *higher* concentrations. Measurement of the affinity of f-Met-Leu-Phe receptors on cytochalasin-B-treated cells stimulated with the peptide indicates that receptors in these cells, indeed, have a lowered affinity (Jesaitis *et al.*, 1983). It would be of interest to demonstrate that the affinity of the receptor for its ligand is modified in a major way when a 50-kD form was removed from the cytoskeleton by stimulation with f-Met-Leu-Phe in the presence of cytochalasin B.

Regulation of Sensory Transduction in the Plasma Membrane

As is evident in Fig. 1, the signal transduction cascade initiated by occupancy of N-formyl peptide receptors is a complex spatial and temporal orchestration of biochemical events mediated by specific molecules. Thus, regulation could be effected at each step in the process by perturbation of the

relevant enzyme or molecules. This review, however, considers those events occurring at the receptor level that could influence activation of the process. It also peripherally discusses the possibility that some of the mechanisms that might be operative at receptor level might also be relevant to control of the interaction of the components of superoxide-generating system.

Regulation at the receptor level controls the fruitful interaction of occupied receptors and their guanyl nucleotide-binding transduction partners, the G proteins. This control can be achieved by modulating the *activity* of the receptor, its *concentration* in the membrane, or its *accessibility* to G proteins. As described above, modulation of the intrinsic activity of receptors has been demonstrated in other receptor systems, but never fully demonstrated in the formyl peptide receptor system. Aside from the classical adaptation observed for formyl peptides, little information exists suggesting that the receptor is structurally modified during activation of the neutrophil. Currently, several pieces of evidence exist which suggest that receptor isoforms exist. These include work that has demonstrated that there are two isoforms of the receptor of differing pI (Schmitt *et al.*, 1983a; Malech *et al.*, 1985), those that are differentially extracted from neutrophils by nonionic detergent (see above), and receptors in the cell that may be differentially acylated (Lobo *et al.*, 1988).

Modulation of Receptor Abundance

In a biophysical analysis of mitochondrial electron transport, Hackenbrock and coworkers (Gupte *et al.*, 1984; Hackenbrock *et al.*, 1986) have suggested that the transfer of electrons proceeds via collisions between individual electron carriers freely mobile in the mitochondrial membrane. Assuming that this general principle applied not only to electron transfer, but to transduction in the neutrophil plasma membrane, then direct control of superoxide production could be determined by the surface concentration, and accessibility (distribution and lateral mobility) of receptors and, by analogy, components of superoxide production. Such a scheme would, therefore, provide for elegant control of a surface response system in the plane of the plasma membrane.

We have made a simple calculation to examine the plausibility of a hypothesis of such lateral control in neutrophil membranes using the equation for calculating the frequency of interaction of two diffusing species in a two-dimensional membrane of a cell (see Fig. 4 and Table I). From published data, we estimate that, in the unstimulated state, the plasma membrane of human neutrophils contains $\sim 50{,}000$ formyl peptide receptors and $\sim 600{,}000$ guanyl nucleotide binding proteins (Bokoch *et al.*, 1988). If we

Table 1. Calculation of Theoretical Collision Frequency of Chemotactic Receptors and G Proteins in the Plane of the Neutrophil Plasma Membrane: $F = 2\, N_r N_g \{D_r/d_g + D_g/d_r\}/A^2$

Symbol	Quantity	Units	Value used	References
F	Collision frequency	collision/sec-cm^2	See Fig. 4	Hackenbrock *et al.* (1986)
N_r	Surface receptor number	molecule/cell	5×10^4	Sklar *et al.* (1984a)
N_g	Surface G-protein number	molecule/cell	$0–1.2 \times 10^6$	Bokoch *et al.* (1988)
D_r	Receptor lateral diffusion coefficient	cm^2/sec	$(10, 3, 1) \times 10^{-10}$	(assumed)
D_g	G-protein lateral diffusion coefficient	cm^2/sec	1×10^{-9}	(assumed)
d_r	$\ln[(N_r + a_g)^2/A)^{-1/2}]$	—	—	Hackenbrock *et al.* (1986)
d_g	$\ln[(N_g(a_r + a_g)^2/A)^{-1/2}]$	—	—	Hackenbrock *et al.* (1986)
a_r	Receptor collision radius[a]	nm	3.1	Allen *et al.* (1986a), Kyte (1972)
a_g	G-protein collision radius[a]	nm	3.5	Bokoch *et al.* (1983), Kyte (1972)
A	Surface area of neutrophil	cm^2	2.9×10^{-6}	Schmid-Schonbein *et al.* (1980)

[a] Calculated by assuming that the detergent-free protein is a hard sphere of partial specific volume 0.74 as previously calculated for other integral membrane proteins (Kyte, 1972).

assume that they are all freely diffusing in the membrane with two-dimensional diffusion constants typical of other integral membrane proteins [D = 1–10 × 10^{-10} cm^2/sec (Peters, 1981)], then we can calculate the collisional frequencies to determine what kind of amplification factor or efficiencies of interaction might be required to permit activation of superoxide production. If one is immobilized, the frequencies are approximately halved.

Figure 4 describes the collision frequency as a function of receptor number assuming 100% receptor occupancy, given a fixed number of transducer proteins (600,000 per cell) and diffusion limited reaction rates. Such a relationship would suggest that the collisional frequency between occupied receptors and G proteins should be very sensitive to the relative concentration of receptor and G proteins at the cell surface and, therefore, by extrapolation to the rate of superoxide production. Clearly the collision frequency would be altered if new receptors were added to the membrane from intracellular stores, thus possibly increasing their concentration as may occur during degranulation (Fletcher and Gallin, 1983; Jesaitis *et al.*, 1986) or prior exposure to lymophokines or endotoxin (see above). On the other hand, the collision frequency would be decreased if receptors were removed from the plasma membrane by endocytosis (Jesaitis *et al.*, 1982b, 1983; Zigmond *et al.*, 1982; Sullivan and Zigmond, 1980; Sklar *et al.*, 1984b; Niedel

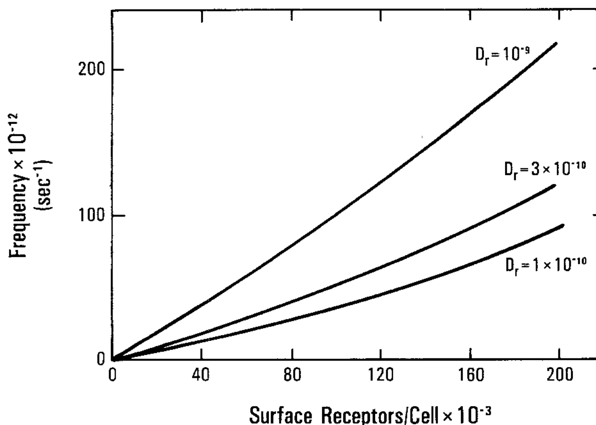

Fig. 4. Relationship between the collisional frequency (F) between freely mobile receptors and G proteins in the two-dimensional plane of the plasma membrane as a function of G-protein content of the plasma membrane N_g. Three different values of the lateral diffusion content for the receptor were used in the calculation. G protein was assumed to be mobile with a D_r = 1 × 10^{-9} cm^2/sec. We have applied the equations used by Hackenbrock and coworkers (Gupta *et al.*, 1984) for calculation of the collisional frequency of electron transport components of the respiratory chain of mitochondrial inner membrane that is defined for our system in Table I. If G protein is assumed to be immobilized, then the dependency is linear and the maximal rates are ~140, 40, and 10 × 10^{12} sec^{-1} for the three cases, respectively.

Table 2. Estimated Collision Frequencies of Selected Pairs of Human Neutrophil Plasma Membrane Proteins Important in Chemoattractant-Stimulated Superoxide Production[a]

Collision pair	*Hypothetical collision frequency* $D = 1 \times 10^{-10} cm^2/sec$	$D = 1 \times 10^{-9} cm^2/sec$
Receptor–G protein	10	100
Flavoprotein–cytochrome *b*	130	1,300

[a]Calculations based on the mathematical relationship derived by Hardt (1979) and applied by Gupte *et al.* (1984) for mitochondrial electron transport. This equation was recalculated to give collision frequencies in molar units/per 10^7 cells/min assuming a cell radius of 4.5 μm. To obtain frequencies in molecular collision/sec/cm^2, multiply values by 4×10^{11}. The quantities used for the calculation of the different reactive pairs are:

Plasma membrane receptor density = 50,000/cell (Sklar *et al.*, 1984a)
Plasma membrane G-protein density = 600,000/cell (Bokoch *et al.*, 1988)
Plasma membrane NADPH flavoprotein = 300,000/cell (Parkos *et al.*, 1985)
Plasma membrane cytochrome *b* = 1×10^6/cell (Parkos *et al.*, 1985)

Lateral diffusion rates were assumed to be for unrestricted motion i.e. $\sim 1 \times 10^{-9} cm^2/g$. Collision frequencies are approximately halved if one partner is completely immobilized, and reduced by a factor of $\sim$ 10 if both partners show a restricted lateral mobility of $1 \times 10^{-10} cm^2$/sec. This latter rate is observed for many membrane proteins while the former is observed for certain receptor systems (Peters, 1981).

et al., 1979a) or separated from their G-protein partners in the plane of the membrane by lateral sequestration (Jesaitis *et al.*, 1987c, 1988a). Table II shows that such interaction would require amplification of approximately one- to tenfold.

This model also can be extended to the case of the putative components of electron transfer involved in superoxide production. If we make the same calculation assuming 1,000,000 molecules of cytochrome b_{559} and 300,000 flavin bearing NADPH-oxidase molecules in the plasma membrane (Parkos *et al.*, 1985) participate in electron transfer as freely mobile partners, then as Table II shows the electron transfer from flavoprotein oxidase to cytochrome *b* would need only to be $\sim$ 10% efficient. Such efficiencies for protein interaction are close to those observed in the mitochondrial systems described by Hackenbrock and colleagues.

Modulation of Receptor Accessibility to G-Protein Transduction Partners

It must be remembered, of course, that the plasma membrane is not just a fluid bilayer in which integral membrane proteins freely diffuse. It is, instead, a two-dimensional mosaic of membrane proteins and lipids. Some of these components are anchored to a subsurface membrane skeleton composed of a complex network of cytoskeletal proteins such as actin, fodrin, vinculin, and talin to name just a few (Marchesi, 1985; Rindler *et al.*, 1985).

Thus, the diffusion of proteins in the membrane is probably constrained by direct or indirect attachment of proteins to the membrane skeleton, restriction of certain proteins to lateral regions or domains of the membranes, or by interactions with other fixed or diffusing proteins in the membrane (Almers and Stirling, 1984; Axelrod, 1983). Consequently, regulation of receptor accessibility to G proteins in the plasma membrane reduces to control of the compositional and organizational parameters of the membrane that determine formation of their interactive pairs. Such control could of course also extend to other interactive protein pairs such as G protein–phospholipase C and the components of superoxide production.

Modulation of Membrane Composition

There are numerous ways that the composition of the plasma membrane can be altered. Since the time scale of neutrophil activation is on the order of minutes, synthesis of new plasma membrane components is probably not directly involved in response regulation. Membrane flow processes such as degranulation, endocytosis, and recycling, however, appear to alter significantly the levels of major and minor constituents in the membrane. Willinger *et al.* (1979) identified 13 surface iodinated proteins on rabbit neutrophils by SDS–PAGE and showed that three (M_r ~ 200,000, 105,000, 90,000) become depleted after phagocytosis. Thrall *et al.* (1980) demonstrated that chemoattractant induced the appearance of at least one surface protein (M_r ~ 54,000) and degranulation stimuli induced the disappearance of another (M_r ~ 44,000) along with intensification of other bands. In addition, Brown *et al.* (1983) identified two major proteins of specific granules (M_r ~ 145,000 and 96,000), one of azurophil granules (M_r ~ 48,000) that migrated with surface iodinatable protein from intact cells. Degranulation and cell activation increased the relative abundance of these proteins again suggesting that there is significant modulation of the composition of the surface proteins by membrane flow processes.

An additional compositional change in the plasma membranes that occurs upon activation is the translocation of cytosolic protein kinase C to the plasma membrane (Wolf *et al.*, 1985; Wolfson *et al.*, 1985). The resulting protein phosphorylation, if directed to cytoskeletal proteins (Naccache *et al.*, 1979; Anderson, 1986), could then have a major impact on the organization of the membrane and hence interaction of receptors and G proteins.

The lipid composition of the plasma membrane also alters as a result of activation of phospholipase C (Smith *et al.*, 1986) in the chemoattractant-activated neutrophil (Traynor-Kaplan *et al.*, 1988a, 1988b). Membranes from stimulated cells (f-Met-Leu-Phe, phorbol myristate acetate, Echo 9 virus)

have been found to have increased lipid order possibly due to changes in membrane lipid composition or organization (Burkhard *et al.*, 1984; Stocker *et al.*, 1982). Because modulation of the lipid composition of the neutrophil plasma membrane can affect the cytoskeletal control of membrane protein organization (see below), this would appear to be another interesting candidate for control of receptors and G proteins.

Finally, compositional control of plasma membrane may also occur after exposure of neutrophils to various natural lymphokines and growth factors mentioned above that appear to potentiate the responses of neutrophils to chemoattractants. Such agents may affect the biosynthesis of plasma membrane proteins as well as their expression from intracellular stores. It remains to be shown, however, whether the observed potentiation results from expression of new proteins or lipids or other parameters of sensory transduction.

Control of the Mobility of Membrane Proteins by Cytoskeletal Interaction

Another factor that could play a role in control of the accessibility of reactive membrane pairs is alteration of the mobility of the components. As can be seen in the equation given in Table I, the diffusion coefficient influenced the collision frequency in a major way. This factor would be important in determining the rate of interaction of two components of the activation pathway and could be perturbed by altering the interaction of those components with a membrane lattice or anchoring structures of cytoskeleton that might impede lateral diffusion (Axelrod, 1983). Clearly immobilization of one collisional partner in some domain or region of the membrane where is could no longer undergo collision with its partner (because it too is immobilized elsewhere in the membrane) would control the effective concentration of a component as observed in Fig. 1.

Numerous examples of control of mobility are available. Hafeman *et al.* (1982), measuring the translation of diffusion of antibody-labeled C3b receptors on neutrophils, have shown that the mobility of these receptors is under sensitive control of the activation state of the cell. They found that, in cells adherent to quartz, glass, or alkylated glass surfaces, C3b receptors are patched and do not show random distribution whereas, on nonspreading cells on lipid-coated glasses, the receptors are uniformly distributed and freely diffusing.

After occupancy of the N-formyl peptide receptor with a rhodamine peptide derivative (Niedel *et al.*, 1979a), the transformation of the surface peptide distribution from uniform to patchy suggests that the receptors may be under similar control and thus become anchored to the cytoskeleton, possibly in coated pits after the occupancy-initiated activation. In support of

this view is our finding that, after occupancy at 37°C, more and more surface-localized receptor–ligand complex is found to coisolate with the Triton X-100-insoluble fractions of the cell until a steady state is reached. At this point during ligand uptake, endocytosis rates presumably match the rates of formation of the complex (Jesaitis *et al.*, 1984).

Detection of this ternary complex of ligand, receptor, and cytoskeleton resulted from the conversion of the receptor–ligand complex to a virtually nondissociating form that was stable in detergent extract at 4°C. Formation of this complex was inhibited by cytochalasins, yet, in each case where this inhibition was observed, there appeared to be a potentiation in both the duration and magnitude of the chemoattractant-stimulated superoxide production (Jesaitis *et al.*, 1985). It was therefore hypothesized that formation of the ligand–receptor–cytoskeleton complex was somehow involved in termination of the response.

The effects of cytochalasins were shown to be more complex than inhibition of simple receptor immobilization since cytochalasins also promoted a major increase in receptor numbers of f-Met-Leu-Phe-stimulated neutrophils (Jesaitis *et al.*, 1986), large increases in diacylglycerol levels (Honeycutt and Niedel, 1986; Rider and Niedel, 1987), and prolonged increases in intracellular calcium concentration (Naemura *et al.*, 1986; Omann *et al.*, 1987). These mediator increases, however, may also result from increased receptor expression and transduction.

Our most recent results suggest that the cytochalasin may not inhibit conversion of the receptor–ligand complex to the high-affinity nondissociating form, but only the coisolation of receptor–ligand complex with the bulk cytoskeleton of the cell (Omann *et al.*, 1987). Further clouding the issue is our recent observation (A. J. Jesaitis, unpublished) that activation of neutrophils with formyl peptides in the presence of cytochalasin B results in a major degranulation event that not only releases intracellular granule content, but also receptor–bearing plasma membrane blebs. It is not known whether these blebs consist of receptor-bearing "cytoskeleton" fragments or whether their superoxide-generating activity is comparable to that of the membrane remaining attached to the degranulated cell.

In spite of the ambiguities resulting from analysis of the potentiating effects of cytochalasin B, additional evidence was obtained suggesting that the high-affinity, cytoskeletal-associated form of the receptor was not participating in transduction. Our analysis of the desensitization of neutrophils showed that this desensitization accompanied conversion of most of the surface receptor to the occupied, high-affinity from coisolating with the cytoskeleton (Jesaitis *et al.*, 1986). Correlation studies suggested that the log of superoxide production rates was directly proportional to the fraction of receptors not converted to the high-affinity, nondissociating form.

Independently, Sklar and coworkers (1987) showed that the converted high-affinity form of the receptor–ligand complex was insensitive to guanyl nucleotides in permeabilized cells. In collaboration with Sklar, we confirmed this finding for the isolated ligand–receptor–cytoskeleton complexes (A. J. Jesaitis and L. A. Sklar, unpublished). Our most recent evidence suggests that it is premature to call this latter form of receptor inactive since it may only be physically separated from G proteins, but may be capable of interacting with them (Jesaitis *et al.*, 1988a) (see below).

Modulation of Membrane Organization

The last aspect of the regulation of chemoattractant activation of neutrophils to be considered in this review is the role of plasma membrane organization. Because the organization of the plasma membrane depends on cytoskeletal association, it is difficult to separate its discussion from the previous section. The main difference, however, will be a greater emphasis on the substructure of the plasma membrane rather than the mobility of the individual components. Diverse evidence from many systems suggests this membrane organelle is not simply a two-dimensional fluid, random mixture of lipids, integral and peripheral membrane proteins. Instead, it is a highly organized structure exhibiting specific and dynamic interactions between cytoskeletal proteins and membrane and lipids that determine the mobility and distribution of these constituents (Fowler, 1986; Lux and Shohet, 1985; Marchesi, 1985).

Analysis of the erythrocyte membrane structure has revealed that different integral membrane proteins such as glycophorin and the anion transport protein (band III) are anchored by independent interaction with complexes of band 4.1, spectrin, and actin or ankyrin and spectrin, respectively. Since such cytoskeletal association could be influenced by modulation of membrane lipids or activation of protein kinases, control of a collisional interaction of such integral membrane proteins could be envisioned. Thus, one might speculate that such control could be possible in the neutrophil system for certain reactive pairs in the plasma membrane such as receptor–G proteins, G proteins–phospholipase C, or flavoproteins–cytochrome for which there is some evidence to suggest cytoskeletal association (B. Babior, personal communication; Jesaitis *et al.*, 1987b).

Evidence for the lateral differentiation of the plasma membrane in leukocytes is abundant. The formyl peptide receptor system demonstrates distributional heterogeneities such as patching (Niedel *et al.*, 1979a) and the development of polarity of its surface density (Walter and Marasco, 1987; Sullivan *et al.*, 1984; Zigmond *et al.*, 1981; Gerisch and Keller, 1981). Actin

(Stewart and Crawford, 1985), ubiquinone (Crawford and Schneider, 1983), 5′-nucleotidase (Steward and Crawford, 1985), and other proteins (Crawford, 1985) all demonstrate differential purification into phagosomal membranes relative to surface membrane, suggesting segregation on the surface. Interaction of monocytes with antigen–antibody complexes induced a rapid lateral redistribution of Fc receptors to the plasma membrane domain in contact with the immune complexes (Michl *et al.*, 1983). Lectins are capped in classical fashion toward the uropod or "tail end" of the neutrophil. Of particular interest is the subcellular localization, by sedimentation methods, of the superoxide generation system. Tsunawaki *et al.* (1983) observed that superoxide generation activity isolated in membrane fraction from guinea pig polymorphonuclear leukocytes had lower sedimentation rates than bulk plasma membrane markers.

Our most recent results suggest that vesicle formation due to N_2 cavitation produces population of vesicles of surface origin, but of slightly different buoyant density (Jesaitis *et al.*, 1988a, 1988c). The light fractions contain a population of vesicles, enriched in most of the plasma membrane markers including alkaline phosphatase, surface iodinatable proteins, surface lectin binding sites, and surface cytochrome *b*. The heavy fraction, however, is enriched in the cytoskeletal proteins actin, fodrin, flavins, and in PMA-activated cells, superoxide-generating activity. When cells are unstimulated by formyl peptides or exposed to the chemoattractants at 4°C, the receptors for these peptides are found in the light fraction. However, if the cells are densensitized by prior incubation with ligand at 15°C, most of the receptors are found in the heavy fraction. Since this fraction is depleted in G proteins, this shift in receptor localization represents a major change of environment for the receptor and a decreased probability of interaction with G proteins. Indeed, our most recent results suggests that the receptors found in the heavy plasma membrane fraction are not associated with G proteins and uncoupled (see above), whereas receptors from the light fraction are associated with G proteins. This highly organized distribution of receptors in the membrane may therefore be very relevant when considered in the light of potential for regulation of receptors in the membrane. A working model that we have proposed is shown in Fig. 5.

Each of these observations is a strong statement for the existence of plasma membrane "domains" of differing composition and possibly function. Evidence continues to accumulate suggesting that perturbation of this lateral order may be a key mechanism of regulation of the activation of the superoxide generation system. Digitonin (Cohen and Chovaneic, 1978), free fatty acids (Badwey *et al.*, 1984), retinoids (Robinson *et al.*, 1987), and exogeneously added phospholipase C (Grzeskowiak *et al.*, 1985) induce superoxide production when presented to neutrophils at concentrations that

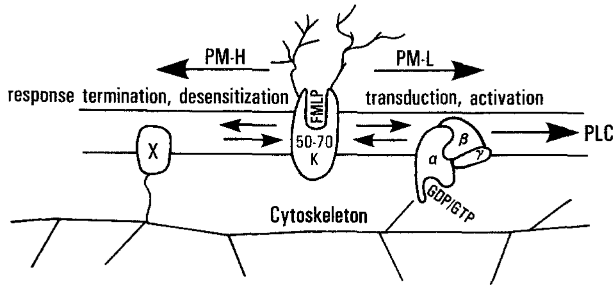

Fig. 5. A possible mechanism of regulation of chemoattractant-induced activation of human granulocytes involving cytoskeleton-mediated lateral segregation of receptors from their transducing partners in the plasma membrane. After occupancy, the formyl peptide receptor may interact with either its guanyl nucleotide transduction partner (α, β, γ) or unknown proteins of the cytoskeleton (χ). In the former case, transduction of occupancy ensues resulting in activation of the cell presumably via activation of phospholipase C (PLC). In the latter case, receptors become segregated away from the G proteins terminating transduction and halting activation. At 15°C or more slowly at 4°C, the process would also desensitize the cell to further stimulation by formyl peptides or other ligands whose receptors might be associated with the formyl peptide receptors or trapped in the same "domain." From Jesaitis *et al.* (1988c), with permission.

would perturb the membrane lipid composition or order. In fact, arachidonic acid (Curnutte, 1985; McPhail *et al.*, 1985) and sodium dodecyl sulfate (Bromberg and Pick, 1985) will promote activation of superoxide generation when added to isolated membranes in the presence of cytosolic cofactors.

These types of perturbation of the plasma membrane may, of course, be the result of direct effects on a critical enzyme activity of the transduction cascade or oxidase system. Such direct interaction has been reported with other membrane proteins such as the Na^+, K^+-ATPase (Sinensky *et al.*, 1979; Charnock and Bashford, 1975). Alternatively the fractional millimolar levels required with arachidonic acid, and the ensuing gross perturbation of the plasma membrane, implicate a major reorganization of the membrane lipids or membrane skeleton (Burn and Burger, 1987; Burn *et al.*, 1985). Moreover, the effects of retinoids and other lipid products (Burn *et al.*, 1985; Lassing and Lundberg, 1985; Burn and Burger, 1987) suggest that these agents may even have an effect on the state of the membrane skeleton (Rao, 1985; Robinson *et al.*, 1987) magnifying the organizational perturbation of the membrane without, in the latter case, promoting compositional change due to secretion and fusion of endomembrane vesicles. The actual mechanism of this perturbation is unknown and it remains to be determined whether it acts by removing inhibitory organizational constraints or by promoting activation-linked association of individual enzymes.

The effects of cytochalasins on f-Met-Leu-Phe-stimulated superoxide production provide additional support for the role of surface receptor organization in control of activation. As described above, cytochalasin

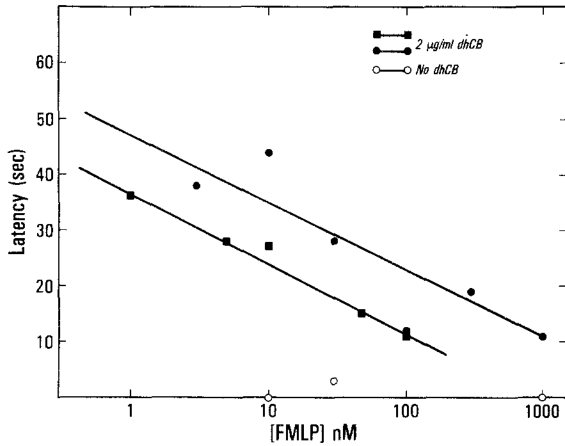

Fig. 6. Effect of dihydrocytochalasin B on the latency of f-Met-Leu-Phe-stimulated superoxide production. Human neutrophils (10^7 cells/ml in Dulbecco's phosphate-buffered saline) were incubated at 4°C with the indicated concentration of f-Met-Leu-Phe for 5 min in the presence (●) or absence (○) of 2 μg/ml dihydrocytochalasin B. The cells were then diluted directly into cytochrome-*c*-containing superoxide assay buffer (Jesaitis *et al.*, 1987a) at 37°C containing the same concentrations of f-Met-Leu-Phe and cytochalasin. Thus, prior to warming, receptor occupancy is at equilibrium and the fractional receptor occupancy can be calculated. Latency was determined graphically from the absorbance change at 550 nm (cytochrome *c* reduction).

treatment of neutrophils results in a significant potentiation of f-Met-Leu-Phe-superoxide production in both rate and magnitude (Lehmeyer *et al.*, 1979b; Jesaitis *et al.*, 1985; English *et al.*, 1981; Goldstein *et al.*, 1975). The potentiation is accompanied by large changes in membrane composition (A. J. Jesaitis, unpublished). In addition, however, anchorage of receptor–ligand complexes to the cytoskeleton is abolished, suggesting that the complexes remain unanchored longer, perhaps allowing them to interact with their partners in the transduction cascade for increased intervals. The net result of such a putative consequence of inhibited anchorage would be a prolonged period of activation.

Recent experiments in our laboratory have examined the effects of cytochalasins on the latency of the f-Met-Leu-Phe-induced oxidative burst and support this hypothesis. In Fig. 6, the latency of the f-Met-Leu-Phe response is plotted as a function of receptor occupancy. If cells are pretreated with dihydrocytochalasin B, a major dependence is observed. In contrast, in the absence of dhCB, virtually no dependence is observed. Although normally a small 3- to 5-sec latency is observed when neutrophils are stimulated at 37°C with a saturating dose of f-Met-Leu-Phe, this latency can be abolished if receptors are occupied prior to stimulation at 4°C as was performed in this experiment. Again these results are consistent with an

enhanced diffusional coupling in cytochalasin-treated cells. The results also might suggest that receptors are tightly coupled to the transducing system prior to occupancy.

These experiments, as all experiments employing so-called "specific" perturbing agents such as the cytochalasins, must of course be interpreted with caution (see above in section on mobility). Cytochalasins have pleiotropic effects on cells that could confirm their interpretation. In neutrophils, these effects include elevation of intracellular Ca^{++} (Naccache *et al.*, 1979; White *et al.*, 1983) and other second messengers (Bennett *et al.*, 1980) such as diacylglycerides (Honeycutt and Niedel, 1986). As stated above, these enhancements contribute to increased degranulation and consequent higher receptor expression, and separating the effects of enhancement of receptor or oxidase populations on the surface membrane from the enhanced second messenger levels becomes additionally complicated by possible feedback effects.

In summary, the unifying mechanism of control in our hypothesis combines three features. Membrane traffic of components to and from the plasma membrane by fusion and pinocytotic processes permits changes of plasma membrane composition. This process may involve the cytoskeleton as the mechanochemical translocator of membrane vesicles. The cytoskeleton and phospholipases could also be the effectors of lateral organization in the plasma membrane. Together these processes could directly change the plasma membrane architecture during the activation of neutrophils by chemoattractants. This architecture would in turn control the initiation, magnitude, and termination of the response by selectively controlling the amount of interaction between the participating receptors, transducers, and catalytic components in the mobile or immobile domains of the plasma membrane. An attractive feature of the lateral control hypothesis is that it can easily incorporate second messengers into the mechanism be they Ca^{++}-dependent phosphorylation of specific membrane proteins, polyphosphatidyl inositol regulation of cytoskeletal membrane interactions, alterations in membrane lipid composition, or activation of membrane "G" proteins or "transducin"-like proteins. In each case, the second messengers could either exhibit control of the membrane organization, and thus the interaction of participating proteins, or control of the "activation state" of the participating proteins. Another feature of this hypothesis is that the events take place at the surface and therefore have the potential to be directly affected by external "abnormal" conditions that might result in inappropriate activation or unregulated superoxide generation with pathological consequences.

It is hoped that research along these lines will provide insight into the biochemical and biophysical control mechanisms operative in the neutrophil respiratory burst, describe the location and orientation of the essential

protein components that activate the superoxide-generating system as well as the electron carriers themselves, and ultimatey lay the foundation for reconstituting the system in artificial membranes. The forthcoming information might then permit identification of surface determinants and abnormalities important in the regulation of superoxide generation that have special relevance to oxidative injury and inflamation.

Acknowledgments

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