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Ionomycin-Induced Neutrophil NADPH Oxidase Activity Is Selectively Inhibited by the Serine Protease Inhibitor Diisopropyl Fluorophosphate

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ABSTRACT

The calcium-specific ionophore ionomycin triggers neutrophils to activate their NADPH oxidase and generate reactive oxygen species. This activation is restricted to intracellular sites and involves the neutrophil granules. Cells that have experienced an ionomycin-induced rise in intracellular calcium will also mobilize their intracellular granules and are primed to subsequent challenge with the chemoattractant formylmethionyl-leucyl-phenylalanine (fMLF), but have lost their ability to become desensitized to the same agonist. We have investigated the involvement of serine proteases in the calcium-induced effector functions using the inhibitor diisopropyl fluorophosphate (DFP). The ionomycin-induced NADPH oxidase activity was abrogated by the protease inhibitor, whereas the activity induced by fMLF was unaffected. The DFP-dependent inhibition was restricted to the NADPH oxidase activity, as all other ionomycin-induced cellular activities were largely unaffected. We thus suggest that a serine protease is of importance for the calcium ionophore-induced signal(s) to reach and activate the dormant NADPH oxidase in the neutrophil granules. Antioxid. Redox Signal. 4, 17–25.

INTRODUCTION

DURING NEUTROPHIL ACCUMULATION at an inflammatory site, the cells are exposed to a number of different mediators of importance for proper cell function (14). Such mediators usually interact with specific receptors exposed on the cell surface, initiating a signal transduction cascade that will ultimately affect different cellular effector proteins and/or effector systems (16). Binding to neutrophil cell-surface receptors of, for example, a chemoattractant activates a transduction cascade that initiates discrete biological responses in the cell, including mobilization of granules, activation of the superoxide-generating NADPH oxidase, and rearrangement of the cytoskeleton (8). Although the precise signals responsible for regulation of the different neutrophil functions have yet to be defined (3), it is known that different regulatory pathways exist. This is illustrated by the fact that the different subsets of neutrophil granules are mobilized hierarchically (4, 26, 27) and that granule secretion may occur without any activation of

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the NADPH oxidase (23). It is currently believed that alterations of phospholipid metabolism, the state of protein phosphorylation, and fluctuation in intracellular concentrations of different ions are key elements in stimulus–response coupling (3, 16). The involvement of Ca^{2+} has been of special interest, owing to the fact that most physiological stimuli induce a rise in the intracellular level of Ca^{2+} ions from nanomolar to micromolar levels (2, 13). An intracellular Ca^{2+} rise induced by Ca^{2+}-specific ionophores has been shown to induce a multitude of effects in the neutrophil (including shape changes, superoxide production, and secretion), further strengthening the concept of Ca^{2+} as a key regulator of cell activation (9, 15). At the molecular level, Ca^{2+} may exert its effect by direct interaction with an effector molecule or indirectly through binding to Ca^{2+}-regulated proteins (*e.g.*, calmodulin, phospholipase A2, grancalcin, and annexins).

Ca^{2+}-regulated cytosolic proteases are also suggested to be involved in the regulation of cellular functions such as cytoskeletal rearrangements, protein phosphorylation, and apoptosis (21), and there is evidence that neutrophil cytosol contains serine proteases (19) of potential importance in signal transduction events. Proteolysis mediated by serine proteases can be inhibited by the membrane-permeable agent diisopropyl fluorophosphate (DFP) (1). Organic fluorophosphates such as DFP specifically inhibit serine protease activity through formation of a fluorophosphate–enzyme complex, which is indefinitely stable. As DFP very effectively inhibits serine protease activity without affecting neutrophil motile functions such as phagocytosis (1), we chose this inhibitor to investigate the role of serine-proteases in Ca^{2+}-mediated neutrophil activities. We found that DFP inhibits the NADPH oxidase activity generated after stimulation with ionomycin, but the protease inhibitor had no effect on ionomycin-induced priming or granule secretion. We thus suggest that a serine protease is involved in the process leading to activation of the NADPH oxidase localized in the neutrophil granules.

MATERIALS AND METHODS

Isolation of phagocytic cells

Neutrophil polymorphonuclear leukocytes were isolated from buffy coats obtained from apparently healthy adults. After dextran sedimentation at 1 *g*, hypotonic lysis of the remaining erythrocytes, and centrifugation in a Ficoll–Pacque gradient (6), the neutrophils were washed twice and resuspended in physiological saline. Neutrophil samples were then subjected to DFP treatment (1). The protease inhibitor was added to give a final concentration of 5 mM, and the cells were incubated on ice for 10 min. The DFP-treated cells were sedimented by centrifugation (300 *g* for 10 min), washed twice in saline, and resuspended in Krebs–Ringer phosphate buffer containing glucose (10 m*M*), Ca^{2+} (1 m*M*), and Mg^{2+} (1.5 m*M*) (KRG; pH 7.3). The cells were kept on a melting ice bath and used within 120 min of preparation. The curves shown are from representative experiments repeated at least five times with essentially the same results.

Chemiluminescence measurements of neutrophil superoxide anion production

Chemiluminescence was measured in a six-channel Biolumat LB 9505 (Berthold Co., Wildbad, Germany), using disposable 4-ml polypropylene tubes with a 0.9-ml reaction mixture. The tubes were placed in the Biolumat and allowed to equilibrate for 5–10 min at 37°C. To activate the system, 0.1 ml of a stimulus was added and the light emission was recorded continuously. Tubes used to measure intracellular activity contained cells (10^6 neutrophils), superoxide dismutase (SOD; 200 U), catalase (2,000 U), and luminol (5.6×10^{-5} *M*). Tubes used to measure extracellular activity contained cells, horseradish peroxidase (HRP; 4 U), and isoluminol (5.6×10^{-5} *M*) (11).

Neutrophil production of hydrogen peroxide

Production of hydrogen peroxide was assayed continuously at 37°C with *p*-hydroxyphenylacetate (11), using a Perkin–Elmer fluo-

rometer (LS50B) with a thermostated cuvette holder. Each cuvette contained 1.8 ml of a cell suspension (2×10^6 cells) with HRP (8 U), azide (1 m*M*), and *p*-hydroxyphenylacetate (PHPA; 1 mg). The cells were allowed to equilibrate for 5 min at 37°C before the stimulus (0.2 ml) was added. The increase in emission was measured at 400 nm with the excitation wavelength 317 nm.

Granule mobilization

Mobilization of neutrophil granules was determined by measuring the up-regulation to the cell surface of the granule membrane localized complement receptor 3 (CR3). After preincubation of the cells for 5 min at 37°C, ionomycin (5×10^{-7} *M*) was added and the incubation was continued for 5 min. The cells were fixed for 30 min in ice-cold paraformaldehyde [4% (wt/vol) in PBS]. To determine the exposure of CR3 (CD11b/CD18), 10 µl of phycoerythrin-conjugated mouse anti-human CD11b antibody (Becton–Dickinson, San Jose, CA, U.S.A.) was added to a cell pellet (~100 µl) of 10^6 cells. The cells were incubated on ice for 30 min, washed twice, and analyzed for the amount of cell-bound probe (correlating to the amount of CR3 exposed on the cell surface) by flow cytometry (FACScan, Becton–Dickinson, Mountain View, CA, U.S.A.).

Priming and desensitization

Neutrophils were metabolically primed by treatment of the cells with ionomycin. After preincubation of the cells for 5 min at 37°C, ionomycin (5×10^{-7} *M*) was added and the incubation was continued for 5 min. The cells were washed twice, resuspended in KRG, and stored on melting ice until used for stimulation with the chemoattractant formylmethionyl-leucyl-phenylalanine (fMLF).

To desensitize the neutrophils, the cells were first incubated for 5 min at 15°C, after which fMLF (10^{-7} *M*) was added. The incubation was continued for another 10 min at 15°C, the cells were then added directly to prewarmed (37°C) chemiluminescence vials, and the production of reactive oxygen species was monitored as described above (20).

Superoxide production in subcellular fractions

Disintegrated neutrophils were fractionated on Percoll gradients as described earlier (5, 12). The fractions containing the specific granules (β-fraction) and plasma membranes/secretory vesicles (γ-fraction) were used in a cell-free superoxide generation assay composed of cytochrome C, a membrane fraction, an excess of the cytosolic NADPH oxidase components, and sodium dodecyl sulfate. The absorbance change accompanying reduction of cytochrome C was converted to nanomoles of O_2^- formed using a millimolar extinction coefficient for cytochrome C of 21.0. The content of the NADPH oxidase membrane component, the b cytochrome, was determined in the membrane fractions from absorbance spectra (as described in 24), and these values are given together with the maximal rate of superoxide production (nanomoles O_2^- produced per minute) in each fraction.

Reagents

Dextran and Ficoll–Paque were purchased from Pharmacia (Uppsala, Sweden). fMLF, isoluminol, luminol, and DFP were obtained from Sigma (St. Louis, MO, U.S.A.). Ionomycin and lactacystin were bought from Calbiochem (La Jolla, CA, U.S.A.).

RESULTS

DFP inhibits the ionomycin-induced production of reactive oxygen metabolites

The metabolic response induced in neutrophils upon interaction with the Ca^{2+} ionophore ionomycin was followed with a luminol/isoluminol chemiluminescence technique. We have previously shown that the measured activity is peroxidase-dependent, and that isoluminol and luminol differ with respect to the ability to pass neutrophil membranes (11). This knowledge can be exploited to determine the relationship between intracellularly and extracellularly produced reactive oxygen species. Most of the activity induced by ionomycin was of an intracellular origin (Fig. 1). This contrasts the stimulation by the

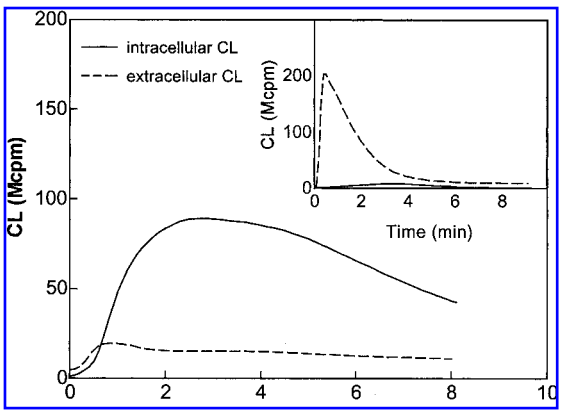


FIG. 1. Time course of the neutrophil chemiluminescence (CL) response induced by ionomycin (5×10^{-7} *M*) and fMLF (inset; 1×10^{-7} *M*). The curves are from a representative experiment, and describe superoxide release following stimulation of the neutrophils as measured in the presence of HRP and isoluminol (dashed lines), and intracellular production of reactive oxygen species as measured in a system containing SOD, catalase, and luminol (solid lines), respectively.

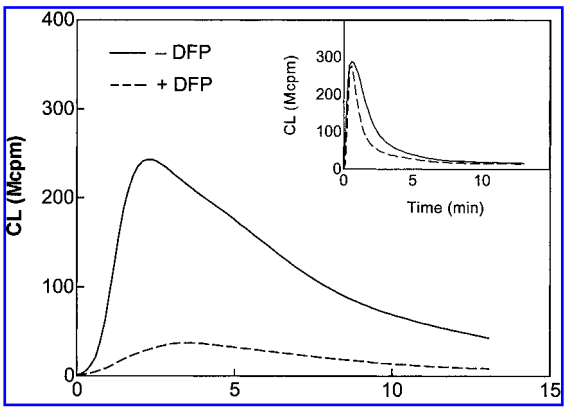


FIG. 2. Effect of DFP on the intracellular production of reactive oxygen species in neutrophil activated by ionomycin (5×10^{-7} *M*). The curves are from a representative experiment and show the results obtained with DFP-treated cells (dashed lines) and untreated control cells (solid lines). The **inset** shows the effect of DFP on superoxide release from neutrophils activated by fMLF (5×10^{-7} *M*). CL, chemiluminescence.

chemoattractant fMLF (shown for comparison in the inset in Fig. 1), which results in release of the generated reactive oxygen species.

To determine a role of serine proteases in the activation process, we used DFP. DFP specifically inhibits serine protease activity through the formation of a DFP–enzyme complex that is indefinitely stable. Neutrophils were first pretreated with the serine protease inhibitor and washed to remove nonbound DFP. After challenge with ionomycin, the response in DFP-treated cells was reduced by at least 90% (Fig. 2). No difference was observed between DFP-treated and nontreated cells when stimulated with fMLF and the release of oxygen metabolites was monitored (Fig. 2, inset).

No inhibitory effect was obtained on the ionomycin-induced NADPH oxidase activity with the proteasome inhibitor lactacystin (at concentrations up to 10 μ*M*, and preincubation times up to 30 min; not shown by figure).

The luminol-amplified chemiluminescence system containing SOD and catalase allows us to measure intracellular activation of the NADPH oxidase. However, the chemiluminescence reaction is dependent not only on NADPH-oxidase activity, but also on the availability of a functional myeloperoxidase (MPO). DFP was without effect on the activity of MPO (data not shown), but we know nothing about the availability. Hence, the precise mechanism involved in the observed inhibition cannot be investigated using the chemiluminescence system. Instead we used hydrogen peroxide-induced reduction of PHPA for this purpose. Hydrogen peroxide can pass biological membranes, and intracellularly generated hydrogen peroxide can thus be measured on the outside of the cells, under the condition that the intracellular consumption of hydrogen peroxide by MPO and catalase is reduced (11, 18). Both MPO and catalase can be inhibited by azide, which is a small molecule that gains access to intracellular sites.

When both nontreated and DFP-treated neutrophils were stimulated with ionomycin, the level of hydrogen peroxide release (*i.e.*, measured in the absence of azide) was very low (Fig. 3). In the presence of azide, the nontreated control cells responded with a massive hydrogen peroxide production, whereas the level of hydrogen peroxide leakage from DFP-treated cells was low (Fig. 3). Taken together, these data imply that DFP treatment results in an impaired intracellular production of hydrogen peroxide.

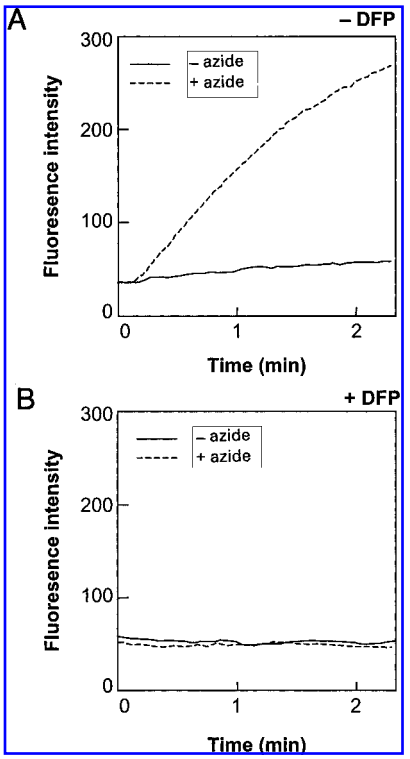


FIG. 3. Effect of DFP on hydrogen peroxide production and release from neutrophils activated by ionomycin. The production of hydrogen peroxide was measured with the HRP-PHPA technique in the presence (dashed lines) or absence (solid lines) of azide (inhibitor of the hydrogen-consuming enzymes MPO and catalase) to allow intracellularly generated peroxide to be determined. The curves are from a representative experiment with untreated (**A**) and DFP-treated (**B**) neutrophils.

TABLE 1. SUPEROXIDE PRODUCTION INDUCED IN PLASMA MEMBRANE AND SPECIFIC GRANULE FRACTIONS ISOLATED FROM UNTREATED AND DFP-TREATED NEUTROPHILS AND MEASURED IN A CELL-FREE ASSAY SYSTEM

	Plasma membrane	*Specific granules*
Control cells		
Superoxide generation	1.8 nmol/min*	6.6 nmol/min
b cytochrome†	12.3 pmol	29.1 pmol
DFP-treated cells		
Superoxide generation	1.5 nmol/min	9.6 nmol/min
b cytochrome	10.5 pmol	30.9 pmol

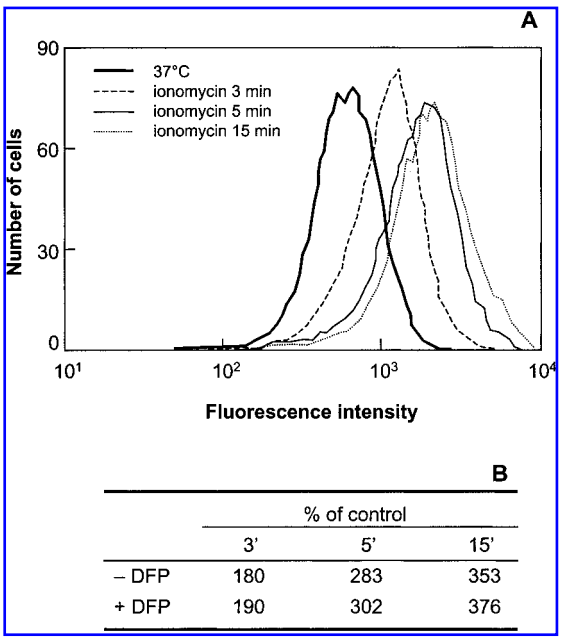
*Values are from a representative experiment.
†Amount of b cytochrome in the sample.

DFP has no direct effect on the NADPH oxidase

Subcellular fractionation of disintegrated neutrophils combined with a cell-free NADPH oxidase activity assay revealed that DFP had no effect on the assembly of the oxidase, neither when specific granules were used as the source for the membrane component of the NADPH oxidase (the b type cytochrome), nor when the plasma membrane was used as membrane source (Table 1).

Mobilization of CR3 is unaffected by DFP

The exposure of CR3 on neutrophils was determined by FACS analysis after binding of a CR3-specific antibody to the cells. Ionomycin treatment induced mobilization of CR3 from intracellular stores to the same degree in nontreated and DFP-treated cells (Fig. 4). Thus, Ca^{2+}-induced triggering of degranulation is independent of serine protease activity.



B

	% of control		
	3'	5'	15'
– DFP	180	283	353
+ DFP	190	302	376

FIG. 4. DFP has no effect on surface exposure of CR3 in neutrophils activated by ionomycin. DFP-treated neutrophils were activated with ionomycin (5×10^{-7} *M*) for 3, 5, and 15 min, paraformaldehyde-fixed, incubated with phycoerythrin-conjugated antibodies directed against CR3, and analyzed by flow cytometry. The histograms (**A**) were used for calculation of percent increase of CR3 exposure in stimulated cells as compared with control cells (**B**). Values are given for both nontreated and DFP-treated neutrophils.

DFP has no effect on priming or desensitization of the NADPH oxidase response

Neutrophils pretreated with ionomycin were primed with respect to the NADPH oxidase activity induced by the chemoattractant fMLF (Fig. 5), and this priming effect was intact in DFP-treated cells (Fig. 5).

Neutrophils that are allowed to interact with fMLF at 15°C become desensitized (20), *i.e.*, when these cells are transferred to 37°C, they do not respond to fMLF. This desensitization phenomenon is abrogated if the cells have been pretreated with ionomycin (20). DFP treatment did not affect the ability of the cells to become desensitized when incubated with fMLF at 15°C (Fig. 6A). Furthermore, the ionomycin-induced abrogation of the desensitization was intact in DFP-treated cells (Fig. 6B).

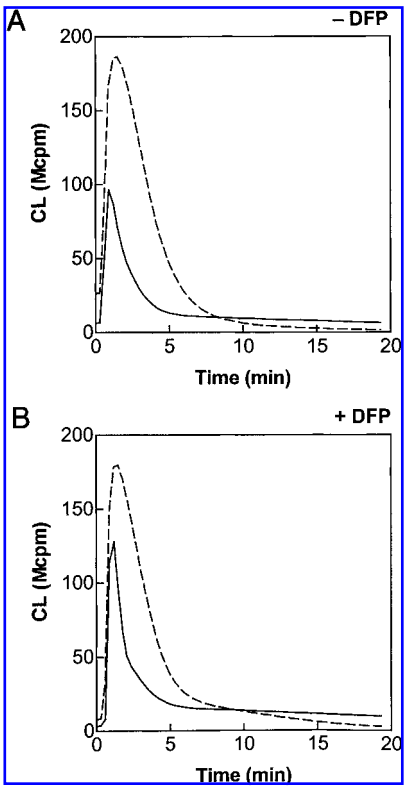


FIG. 5. DFP has no effect on ionomcyin-induced neutrophil priming. Control neutrophils (**A**) and DFP-treated cells (**B**) were incubated with ionomycin ($5 \times 10^{-7} M$) for 3 min and then challenged with fMLF ($1 \times 10^{-7} M$; dashed lines). The release of superoxide was determined and compared with that released by nontreated cells activated by fMLF (solid lines). The curves are from a representative experiment. CL, chemiluminecence.

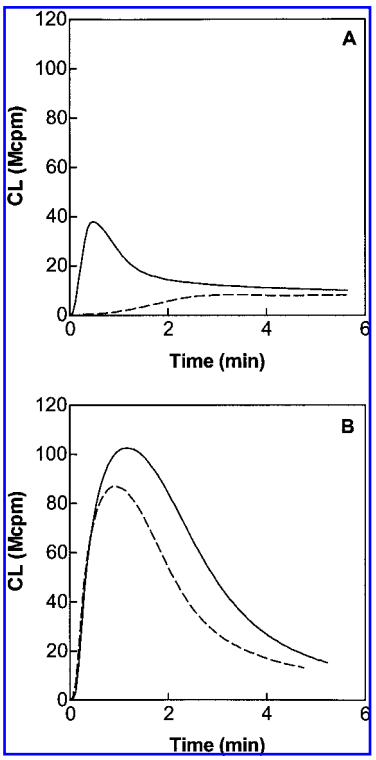


FIG. 6. Desensitization of the formyl peptide receptor is abolished by ionomycin also in DFP-treated cells. DFP-treated neutrophils were incubated at 15°C with (dashed lines) or without (solid lines) fMLF ($1 \times 10^{-7} M$) and were then transferred to measuring vials kept at 37°C (time = 0) containing the same concentration of fMLF. As seen from the lack of responsiveness in cells preincubated with fMLF, these cells were desensitized (**A**). The desensitization is abolished in Ca^{2+} ionophore-primed neutrophils (**B**). The curves are from a representative experiment.

DISCUSSION

The presented data show that the cell-permeable serine protease inhibitor DFP greatly reduces the intracellular NADPH oxidase activity induced by the Ca^{2+}-specific ionophore ionomycin, whereas it lacks effect on the release of oxidants following activation by the chemoattractant fMLF. The intracellular Ca^{2+} increase induced by ionomycin has multiple effects on neutrophil function. However, DFP affected no other ionomycin-induced cellular response tested than the NADPH oxidase activity.

DFP exhibits good selectivity for many serine-dependent proteases, including those of the chymotrypsin family, prolyl oligopeptidases, dipeptidyl-peptidase IV, and serine-

type carboxypeptidases. Neutrophils contain a great number of different serine proteases localized mainly in the storage granules, but possibly also in the cytosol (4, 19). One way to characterize further the protease(s) involved in the activation of the NADPH oxidase would be to use different types of inhibitors. However, very few available serine protease inhibitors are membrane-permeable, which is why this approach is not possible for determining the inhibitory profiles in intact cells. There is one membrane-permeable inhibitor besides DFP, lactacystin, which inhibits the protease activities associated with the proteasome. This inhibitor was found to be without effect on neutrophil oxidase activity.

The neutrophil production of reactive oxygen species induced by ionomycin is largely intracellular, whereas the oxidants produced upon fMLF activation are released from the cells. We know from earlier studies (11, 18) that to obtain an intracellular chemiluminescence reaction, the neutrophil peroxidase (MPO) and the oxygen radical-generating system have to be present in the same subcellular compartment (which is not yet identified). Theoretically, the reduced chemiluminescence reaction observed in DFP-treated cells could be due to an impaired formation of such a compartment. However, we favor the idea that the reduced chemiluminescence activity is related to an effect of DFP on the intracellular production of reactive oxygen species. The fact that DFP-treated neutrophils produced very low levels of hydrogen peroxide intracellularly gives support to this suggestion.

The membrane component of the NADPH oxidase, the b cytochrome, is present mainly in the specific granules (~75%, the rest being localized in the secretory vesicles and the plasma membrane) (5). In spite of this, the current, widespread belief is that the NADPH oxidase in intact cells is assembled at the plasma membrane. The activated NADPH oxidase would then either reside in the plasma membrane and deliver the oxygen metabolites to the extracellular space or be translocated to an intracellular membrane vesicle during phagocytosis, resulting in delivery of the oxygen metabolites into the phagosome. Several lines of evidence suggest, however, that cytoplasmic granules may play an important role as host for an assembled and active NADPH oxidase. We have earlier suggested that intraphagosomal production of reactive oxygen metabolites (17), as well as that induced by Ca^{2+} ionophores (10, 12), involves primarily the granule-associated NADPH oxidase. In contrast, NADPH oxidase activation induced by soluble stimuli that act through receptors of the formyl peptide receptor family is associated with a rapid and substantial release of reactive oxygen species (7, 8), primarily mediated by the plasma membrane-associated oxidase.

As DFP specifically inhibits the intracellular production of oxygen metabolites (induced by ionomycin) and not the extracellular release (induced by fMLF), a possible explanation for our results could be that the NADPH oxidase at the two sites in the cell (the plasma membrane and the granule membranes, respectively) are differently affected by the protease inhibitor. However, using a cell-free NADPH oxidase assay system, we could show that there was no difference in NADPH oxidase activity between DFP-treated and nontreated cells, irrespective of whether the membrane component of the NADPH oxidase was derived from the plasma membrane/secretory vesicle or the specific granule-enriched fraction. In addition, these results indicate that the protease inhibitor does not exert its effect directly on the assembly of the NADPH oxidase, but rather on the signal(s) responsible for activation of the NADPH oxidase.

The precise signal(s) mediating an assembly of the NADPH oxidase in the plasma membrane and in the specific granule membrane, respectively, are not known. We have earlier presented data indicating that the signal(s) responsible for activation of the NADPH oxidase differ depending on the subcellular localization of the membrane NADPH oxidase component (22). A rise in intracellular Ca^{2+} is sufficient to activate the oxidase in the granule fraction. The assembly of the NADPH oxidase is not induced by Ca^{2+} *per se* (25), but rather by additional signaling molecules generated in response to an elevation of intracellular Ca^{2+}. These molecule(s) have yet to be identified,

but the data presented here imply that a serine protease is involved in this transduction cascade. In contrast to the granule NADPH oxidase, activation of the plasma membrane oxidase appears to require additional factors beside an intracellular Ca^{2+} rise.

Although neutrophils express many different receptors coupled to distinct responses, the signaling pathways described are restricted to a few that operate in most eukaryotic cells. One of these involves a pertussis toxin-sensitive heterotrimeric G protein that can activate different signal transduction pathways downstream of an occupied receptor. The cleavage mediated by the action of phospholipases (A_2, C, and D) generates a number of different signaling molecules, including inositol 1,4,5-trisphosphate (which releases intracellular Ca^{2+}) and diacylglycerol (which activates protein kinase C). There are probable "cross-talking" links between these signals and other signaling systems, including those involving tyrosine kinases and cyclic AMP. So far, the signal transduction mechanisms directly involved in the assembly and activation of the neutrophil NADPH oxidase are largely unknown, and the suggested pathways have not been shown to involve any protease activity. However, the dramatic effect of pretreatment with DFP on the subsequent cellular response to ionomycin provides evidence for a novel component of the signaling pathway leading to activation of the respiratory burst, possibly related to the subcellular localization of the activated oxidase. It is obvious that the suggested protease is located after branching of the signal transduction pathway leading to NADPH oxidase activation and other cellular responses induced by ionomycin. The protease inhibitor DFP could thus prove very useful for the evaluation of the mechanisms involved in the activation of the NADPH oxidase.

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ABBREVIATIONS

CR3, complement receptor 3; DFP, diisopropyl fluorophosphate; fMLF, formylmethionyl-leucyl-phenylalanine; HRP, horseradish peroxidase; KRG, Krebs–Ringer phosphate buffer containing glucose, Ca^{2+}, and Mg^{2+}; MPO, myeloperoxidase; PHPA, *p*-hydroxyphenylacetate; SOD, superoxide dismutase.

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