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Phosphatidic Acid and Diacylglycerol Directly Activate NADPH Oxidase by Interacting with Enzyme Components*

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The enzyme NADPH oxidase is regulated by phospholipase D in intact neutrophils and is activated by phosphatidic acid (PA) plus diacylglycerol (DG) in cell-free systems. We showed previously that cell-free NADPH oxidase activation by these lipids involves both protein kinase-dependent and -independent pathways. Here we demonstrate that only the protein kinase-independent pathway is operative in a cell-free system of purified and recombinant NADPH oxidase components. Activation by PA + DG was ATP-independent and unaffected by the protein kinase inhibitor staurosporine, indicating the lack of protein kinase involvement. Both PA and DG were required for optimal activation to occur. The drug R59949 reduced activation of NADPH oxidase by either arachidonic acid or PA + DG, with IC_{50} values of 46 and 25 μM, respectively. The optimal concentration of arachidonic acid or PA + DG for oxidase activation was shifted to the right with R59949, indicating interference of the drug with the interaction of lipid activators and enzyme components. R59949 inhibited the lipid-induced aggregation/sedimentation of oxidase components $p47^{phox}$ and $p67^{phox}$, suggesting a disruption of the lipid-mediated assembly process. The direct effects of R59949 on NADPH oxidase activation complicate its use as a "specific" inhibitor of DG kinase. We conclude that the protein kinase-independent pathway of NADPH oxidase activation by PA and DG involves direct interaction with NADPH oxidase components. Thus, NADPH oxidase proteins are functional targets for these lipid messengers in the neutrophil.

The NADPH oxidase (the respiratory burst enzyme) in phagocytic cells produces superoxide (O_2^-)[1] by catalyzing electron transfer from NADPH to molecular oxygen upon cell stimulation (1–3). This enzyme plays important roles in host defense against infection and in tissue damage due to inflammation (1–5). In addition, NADPH oxidase-like enzymes are present in a variety of other cell types, where the oxygen radicals formed may have signaling roles (5, 6). The enzyme in phagocytes consists of the membrane-bound heterodimeric flavocytochrome b_{558} ($gp91^{phox}$ and $p22^{phox}$) and four cytosolic proteins ($p47^{phox}$, $p67^{phox}$, $p40^{phox}$, Rac1/2) (2–4, 7). Components must assemble in the membrane for the enzyme to become active (2–4, 7). The activation of NADPH oxidase is initiated by receptor-ligand interaction and involves complex intracellular signaling events. These include the activation of protein kinases to phosphorylate cellular proteins and NADPH oxidase components (2, 8) and the generation of various lipid second messengers (AA by phospholipase A_2 (9); DG by phospholipase C or PA phosphohydrolase; PA by phospholipase D or DG kinase (10, 11)). In cell-free systems, these lipids can induce activation of the enzyme (12–17). AA exerts its effect by directly acting on enzyme components (18–22). PA has been shown to partially activate purified flavocytochrome b_{558} (23), suggesting it interacts with this protein. It is not known whether DG has any direct effect(s) on NADPH oxidase components.

Previous studies examined NADPH oxidase activation by PA plus DG in a cell-free system consisting of membrane and cytosolic fractions from human neutrophils (16, 17, 24). Both lipids are required for optimal activation (16, 17), but the individual roles of each are not clear. We showed (24) that activation in this system is dependent on both protein kinase activity and other undefined phosphorylation-independent mechanisms. The protein kinase-dependent mechanism may involve the phosphorylation of NADPH oxidase components $p47^{phox}$ and $p22^{phox}$ by a novel PA-activated protein kinase (10, 25, 26). Alternatively, Erickson *et al.* (17) postulated that the phosphorylation-dependent mechanism involved the conversion of DG to PA by DG kinase. They found that the DG kinase inhibitor R59949 blocked the formation of PA from DG as well as the activation of NADPH oxidase by DG.

R59949 inhibits DG kinase with an IC_{50} value of 1.25×10^{-7} M in isolated platelet membranes and in intact platelets (27). However, at concentrations above 10^{-5} M, the drug has nonspecific effects on overall lipid and protein metabolism (28). Since then, R59949 has been widely used (29, 30). Jiang *et al.* showed (31) that R59949 is selective for Ca^{2+}-activated DG kinases and that the drug interacts with the catalytic subunit of the enzyme. These observations raise the possibility that the effect of R59949 on cell-free NADPH oxidase activation (17) is unrelated to inhibition of DG kinase, since high concentrations were used, and Ca^{2+} was not present in the activation system.

Here, we further analyzed the individual roles of PA and DG on NADPH oxidase activation using purified and recombinant

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[1] The abbreviations used are: O_2^-, superoxide; AA, arachidonic acid; DG, diacylglycerol; DTT, dithiothreitol; OG, *N*-octyl-β-D-glucopyranoside; PA, phosphatidic acid; PC, phosphatidylcholine; *phox*, phagocyte oxidase; PMSF, phenylmethylsulfonyl fluoride.

NADPH oxidase components. Optimal activation still required both PA and DG, and activation was independent of ATP and protein kinase activity. We used this system to further study the mechanism of activation of NADPH oxidase. We found that R59949 acts in a DG kinase-independent manner on this process through competition between R59949 and lipids during the activation process. This characteristic of R59949 should be considered before it is used at higher concentrations (>10 μM) in either *in vivo* or *in vitro* assays. These results strongly suggest that both PA and DG interact directly with NADPH oxidase components and that this interaction is responsible for the protein kinase-independent mechanism of oxidase activation by these lipid second messengers.

EXPERIMENTAL PROCEDURES

Materials—Phosphatidylcholine (PC) (porcine liver, 99% pure) was from Doosan-Serdary Research Laboratories (Englewood Cliffs, NJ), and Type IV-S PC (soybean, 40% pure) was from Sigma. The PA used was 1,2-dicapryl-*sn*-glycero-3-phosphate, and the DG used was 1-oleoyl-2-acetyl-*sn*-glycerol; both were from Avanti Polar Lipids, Inc. (Alabaster, AL). Lipids were freshly prepared by sonication in water (16). Arachidonic acid was from Nu Chek Prep. Inc. (Elysian, MN), and was prepared in 25% ethanol (16, 32). R59949 [3-[2-[4-[bis(4-fluorophenyl) methylene]-1-piperidinyl]ethyl]-2,3-dihydro-2-thioxo-4(1H)-quinazolinone] was from Alexis Corp. (San Diego, CA) and was dissolved in Me_2SO. *N*-Octyl-β-D-glucopyranoside (OG) was from Calbiochem-Novabiochem. SDS-polyacrylamide gel electrophoresis reagents were from Bio-Rad. NADPH and superoxide dismutase were from Roche Molecular Chemicals. Glutathione beads, dextran T-500, and Q-Sepharose Fast Flow were from Amersham Pharmacia Biotech. Isolymph was from Gallard-Schlesinger Industries (Carle Place, NY). The ATP determination kit was from Molecular Probes (Eugene, OR). The $p47^{phox}$ and $p67^{phox}$ vector-containing baculoviruses were generous gifts from Dr. David Lambeth (Emory University, Atlanta, GA). The $p47^{phox}$ and $p67^{phox}$ proteins were produced in Sf9 cells and partially purified on CM-cellulose ($p47^{phox}$) or Q-Sepharose Fast Flow ($p67^{phox}$) (33). The goat anti-$p47^{phox}$ and anti-$p67^{phox}$ antibodies were generous gifts from Dr. Tom Leto (NIAID, NIH, Bethesda, MD). The Q61L active conformational mutant of Rac1 (a generous gift from Dr. Tom Leto) was produced in *Escherichia coli* as a glutathione *S*-transferase fusion protein and purified on glutathione-Sepharose beads by thrombin cleavage (34). All other reagents were from Sigma.

Isolation of Neutrophils and Subcellular Fractions—Heparinized venous blood was obtained from healthy donors, and neutrophils were isolated by dextran sedimentation and centrifugation through Isolymph (35). Isolated neutrophils were treated with diisopropyl fluorophosphate (26), resuspended in sonication buffer (11% sucrose, 50 mM Na_xPO_4, pH 7.0, 120 mM NaCl, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM benzamidine, 1 μg/ml leupeptin, 1 μM pepstatin, 1 μg/ml aprotinin), and broken by sonication (36). Sonicates were centrifuged (200 × *g*, 10 min) to remove unbroken cells and nuclei, layered onto a 15/40% (w/v) discontinuous sucrose gradient, and centrifuged (150,000 × *g*, 30 min) (36). Cytosolic fractions were collected from the top layer down to the 15% interface, and membrane fractions were collected from the 15/40% interface. Fractions were stored at −70 °C. Protein concentration was determined using the Coomassie Plus Protein protocol from Pierce using bovine serum albumin as a standard.

Purification and Relipidation of Flavocytochrome b_{558}—Human neutrophil membrane fractions were prepared as described (37). NaCl was added to the suspended membranes to a final concentration of 1 M, and membranes were centrifuged at 100,000 × *g* for 30 min at 4 °C. The pellet was resuspended by transferring to a cell homogenizer and grinding until milky in 1–2 ml of solubilization buffer (10 mM HEPES, 1 mM EDTA, 1 mM $MgCl_2$, 40 mM OG, 100 mM KCl, 10 mM NaCl, and 2 mM NaN_3, pH 7.4, with 10 μg/ml chymostatin, 1 mM dithiothreitol (DTT), 10 μM FAD, 100 μM NADPH, and 1 mM PMSF added just before use). The homogenate was reconstituted to its original volume in solubilization buffer, sonicated briefly on ice using a probe-style sonicator, stirred on ice for 1–2 h, and centrifuged at 100,000 × *g* for 45 min at 4 °C. The supernatant was diluted to a salt concentration of 75 mM with dilution buffer (10 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 40 mM OG, 2 mM NaN_3, pH 7.4, supplemented with 10 μg/ml chymostatin, 0.1 mM DTT, 10 μM FAD, 100 μM NADPH, and 1 mM PMSF just before use). The diluted supernatant was introduced to a heparin-agarose affinity column (Sigma catalog number H0402) that had previously been rinsed with 30 bed volumes of dilution buffer with 2 M NaCl added and equilibrated with ~50 bed volumes of wash buffer (10 mM HEPES, 1 mM EGTA, 1 mM $MgCl_2$, 40 mM OG, 75 mM NaCl, 2 mM NaN_3, pH 7.4, supplemented with 10 μg/ml chymostatin, 0.1 mM DTT, 10 μM FAD, 100 μM NADPH, and 0.2 mM PMSF just before use); the flow rate was 20 ml/h. The cytochrome was eluted using a 0.075–2 M NaCl gradient in elution buffer (50 mM NaH_2PO_4, 1 mM EGTA, 1 mM $MgCl_2$, 40 mM OG, 2 mM NaN_3, pH 7.4, supplemented with 10 μg/ml chymostatin, 0.1 mM DTT, 10 μM FAD, 100 μM NADPH, and 0.2 mM PMSF just before use). One-ml fractions were collected, and those containing flavocytochrome *b* absorbance were pooled and concentrated to a final volume of 1 ml using a 50-kDa nominal molecular mass centrifugal concentration device (Millipore). The sample was passed through a 0.2-μm pore size syringe filter and introduced to a gel filtration column (Amersham Pharmacia Biotech Superdex 200 HR, 10–30) maintained at 4 °C and previously equilibrated in high performance liquid chromatography column buffer (50 mM NaH_2PO_4, 1 mM EGTA, 1 mM $MgCl_2$, 40 mM OG, 150 mM NaCl, 2 mM NaN_3, pH 7.4, supplemented with 10 μg/ml chymostatin, 0.1 mM DTT, 10 μM FAD, and 0.2 mM PMSF just before use). Peak fractions containing flavocytochrome *b* were collected in 500-μl aliquots, and cytochrome concentrations were determined using ϵ_{414} = 130.8 mM^{-1} cm^{-1} (38). The preparation was greater than 90% pure cytochrome, the major contaminant being CD11b/CD18. Glycerol was added to each fraction to a final concentration of 20%, and fractions were stored at 4 °C, wrapped in foil, and relipidated within 2 days. The entire purification process, beginning at the salt wash, was completed in 1 day.

Stored fractions were relipidated as described (19, 23), with modifications. Briefly, PC (soybean, Type IV-S, 7 mg/ml), was prepared by sonication in buffer A (50 mM Na_3PO_4, pH 7.0, 1 mM EGTA, 1 mM $MgCl_2$, 50 mM Na_3N, 20% glycerol, 40 mM OG, 0.05 mM DTT, 1 μM FAD). The lipid solution (0.7 mg/ml final) was added to the purified flavocytochrome b_{558} (600 μl, 0.85–1.2 μM). The mixture was kept on ice for 20 min, diluted 6-fold with phosphate buffer (50 mM Na_3PO_4, pH 7.0, 1 mM EGTA, 1 mM $MgCl_2$) and stored at −70 °C. The flavocytochrome b_{558} concentration in the relipidated mixture was determined spectrophotometrically from the reduced minus oxidized spectrum using $\epsilon_{428\ nm}$=106 mM^{-1} cm^{-1} (39).

NADPH Oxidase Activation and Assay—Mixtures containing 50 mM Na_xPO_4 buffer, pH 7.0, 100 μM cytochrome *c*, 10 μM FAD, 1 mM EGTA, 5 mM $MgCl_2$, zero or 12 μM ATP, zero or 0.16 mg/ml superoxide dismutase, 0.5 μg of membrane protein (0.6 pmol of flavocytochrome b_{558}) (semi-recombinant system), or 2 pmol of relipidated flavocytochrome b_{558} (purified-recombinant system) plus 40 pmol $p47^{phox}$, 15 pmol $p67^{phox}$, and 60 pmol Q61L Rac1 (hereafter referred to as Rac1) were incubated with various concentrations of PA, DG, or PA plus DG for 90 min or with AA for 30 min in a final volume of 0.12 ml. For Figs. 4–6, R59949 or 0.1% Me_2SO (solvent control) was added into the reaction mixture before the addition of lipid activators. After the desired incubation time, 200 μM NADPH was added, and the reactions were stopped at 2 min by 1% (v/v) Triton X-100 (16, 24). In some experiments (described under "Results"), R59949 or 0.1% Me_2SO_4 (solvent control) was added with the NADPH at the end of the incubation. The O_2^- production was determined by measuring the absorbance of cytochrome *c* at 550 nm using a Thermomax® kinetic microplate reader (Molecular Devices Corp., Menlo Park, CA) and correcting for the absorbance of samples containing superoxide dismutase. Activity was linear over the 2-min assay period and was expressed as mol of O_2^-/min/mol of flavocytochrome b_{558} using an extinction coefficient of 21 mM^{-1} cm^{-1} for cytochrome *c* (40). IC_{50} values were determined in SigmaPlot (Jandel Scientific Software, San Rafael, CA) by fitting the data to the hyperbolic decay equation (competitive inhibition model) (41), activity = max activity × IC_{50}/[R59949]+ IC_{50}. EC_{50} values were determined in SigmaPlot using the single rectangular three-parameter nonlinear regression model.

ATP Determination—The ATP concentrations present in membrane fractions and stock solutions of recombinant $p47^{phox}$, $p67^{phox}$, and Rac1 were determined by using the ATP determination kit from Molecular Probes (42). Samples were boiled for 5 min before analysis. Luminescence was read on a Turner TD 20e Luminometer (Sunnyvale, CA), and values were calculated based on an ATP standard curve.

Sedimentation of $p47^{phox}$ and $p67^{phox}$ by PA + DG—Mixtures (0.12 ml) containing 50 mM Na_xPO_4 buffer, pH 7.0, 10 μM FAD, 1 mM EGTA, 5 mM $MgCl_2$, zero or 0.5 μg of membrane protein (0.6 pmol of flavocytochrome b_{558}), 40 pmol of $p47^{phox}$, 15 pmol of $p67^{phox}$, and 60 pmol of Q61L Rac1 were incubated with 20 μM PC or 10 μM PA + 10 μM DG for 90 min in the presence or absence of 50 μM R59949. Ten reaction mixtures for each condition were mixed (1.2 ml) and layered on a

15/50% (w/v) (1 ml/0.5 ml) discontinuous sucrose gradient and centrifuged in a SW55 rotor (Beckman Instruments) at 150,000 × *g* for 30 min at room temperature (43). Soluble fractions were collected from the top of the gradient (0.9 ml); the pellet fractions were collected from the 15/50% interface plus the 50% fraction (0.75 ml). Proteins from 140 μl of each soluble and pellet fraction were separated by 10% SDS-polyacrylamide gel electrophoresis (44), transferred to nitrocellulose (45), and analyzed by Western blot with a 1:500 dilution of a mixture of $p47^{phox}$ and $p67^{phox}$ antibodies. Blots were developed using SuperSignal™ enhanced chemiluminescence reagent (Pierce) and analyzed by densitometry (PDI, Huntington Station, NY). The percentage of each protein in the pellet fraction was calculated using the sum of the soluble and pellet fractions as 100%. The pmol amounts of each protein in the pellet fraction, after treatment with PA + DG, were estimated using the pmol added to the reaction mixture (calculated after correcting for the purity of the added protein) as 100%.

Membrane NADPH Oxidase Assay—Pellet fractions containing neutrophil membrane from the sedimentation assay were analyzed for their NADPH oxidase activity, as described previously (46). 80 μl of each sample was mixed and incubated for 5 min at room temperature with 20 mM K_xPO_4, pH 7.0, 1 mM EGTA, 7.6 mM $MgCl_2$, 10 μM FAD, 75 μM cytochrome *c*, and 0.16 mM SDS in a final volume of 220 μl. The mixture was divided into two cuvettes, one containing superoxide dismutase at a final concentration of 0.24 mg/ml. The reaction was started by the addition of NADPH (final concentration, 0.2 mM) to both cuvettes, and absorbance changes were monitored continuously at 550 nm by a UV-2401PC Shimadzu spectrophotometer (Columbia, MD). O_2^- production was calculated using the linear slopes and an extinction coefficient for cytochrome *c* of 21 mM^{-1} cm^{-1} (40). Activity was expressed as nmol of O_2^-/min/ml of pellet fraction, since the amount of protein and flavocytochrome b_{558} present in the fractions were too low to measure.

RESULTS

Characteristics of NADPH Oxidase Activation by PA + DG in a Cell-free System Using Purified and Recombinant Oxidase Components—Previous studies (16, 17, 24) examined cell-free NADPH oxidase activation by PA + DG using mixtures of membrane and cytosolic fractions from human neutrophils. Results indicated that oxidase activation required both PA and DG for optimal activity and involved both phosphorylation-dependent and -independent mechanisms (17, 24). Our previous data (24–26) suggested that the phosphorylation-dependent mechanism involved one or more cytosolic protein kinases. Therefore, we hypothesized that the substitution of recombinant NADPH oxidase components for the neutrophil cytosolic fraction would eliminate the phosphorylation-dependent activation pathway. To test this, we examined the ability of PA + DG to activate NADPH oxidase in a "semi-recombinant" cell-free system consisting of the recombinant proteins $p47^{phox}$, $p67^{phox}$, and Rac1 mixed with neutrophil membrane fractions. As shown in Fig. 1*A*, PA + DG induced NADPH oxidase activation in the semi-recombinant system. To test whether a protein kinase was involved in NADPH oxidase activation in this system, we examined the effect of a potent, nonselective protein kinase inhibitor, staurosporine (47). Staurosporine reduced NADPH oxidase activation by about 50% when neutrophil cytosol was used as the source of cytosolic oxidase components (Fig. 1*A*, *left panel*). In contrast, staurosporine had no effect on NADPH oxidase activation by PA + DG in the semi-recombinant system (Fig. 1*A*, *right panel*).

To further address whether activation by PA + DG in the semi-recombinant system was phosphorylation-independent, we tested the effect of ATP. The endogenous ATP concentration in our reaction mixtures of membrane and recombinant oxidase proteins was determined to be 1 ± 0.9 nM (mean ± S.E., $n = 3$), which is too low to support lipid or protein phosphorylation reactions. We performed concentration curves with PA + DG in the presence or absence of 12 μM added ATP (Fig. 1*B*). The curves were virtually superimposable, indicating that ATP had no effect on the level of activity or the EC_{50} for the lipids. Taken together, these data indicate that activation of NADPH oxidase by PA + DG in the semi-recombinant system is phosphorylation- and protein kinase-independent.

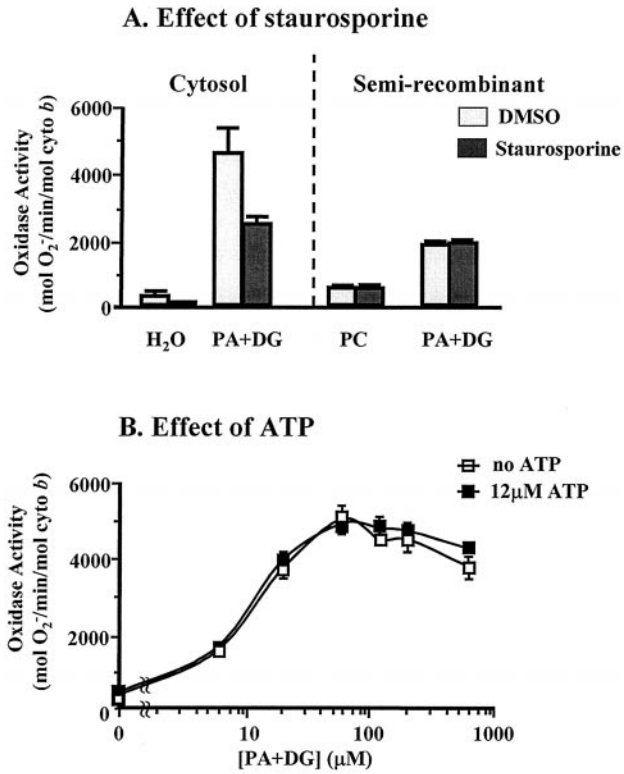


FIG. 1. **Activation of NADPH oxidase by PA + DG in a semi-recombinant cell-free system is protein kinase-independent.** *A*, effect of staurosporine. Reaction mixtures contained the following. *Left panel*, 0.25 μg of membrane protein (0.3 pmol of flavocytochrome b_{558}) and 12.5 μg of cytosolic protein and were incubated with H_2O or 100 μM PA + 100 μM DG; *right panel*, 0.5 μg of membrane protein (0.6 pmol of flavocytochrome b_{558}), 40 pmol of $p47^{phox}$, 15 pmol of $p67^{phox}$, and 60 pmol of Rac1 and were incubated with 30 μM PC or 30 μM PA + 10 μM DG. Assays were performed in the presence of 12 μM added ATP and in the presence of either Me_2SO (*DMSO*, *light gray bars*) or 100 nM staurosporine (*dark gray bars*). NADPH oxidase activity was measured as described under "Experimental Procedures" and expressed as mol of O_2^-/min/mol of flavocytochrome b_{558}. The data shown are mean ± S.E. of three (*left panel*) or seven (*right panel*) experiments. *B*, effect of ATP. Reaction mixtures contained 0.5 μg of membrane protein (0.6 pmol of flavocytochrome b_{558}), 40 pmol of $p47^{phox}$, 15 pmol of $p67^{phox}$, and 60 pmol of Rac1 and were incubated with various amounts of PA + DG in the presence (■) or absence (□) of 12 μM added ATP. PA and DG were present at equal concentrations, each representing half of the total value shown at any given concentration. NADPH oxidase activity was measured as described under "Experimental Procedures" and expressed as mol of O_2^-/min/mol of flavocytochrome b_{558}. The data shown are mean ± S.E. of three experiments.

We next addressed whether both PA and DG were required for activation to occur in the semi-recombinant system. As shown in Fig. 2*A*, oxidase activity was negligible with DG alone and was present at only low levels with PA alone. In contrast, when both lipids were added, substantial levels of oxidase activity were detected. AA induced similar levels of activation as PA + DG. Both PA and DG could have direct activating effects on one or more oxidase components. However, the neutrophil membrane fraction present in the semi-recombinant system might have other targets for one or both of these lipids, which could influence oxidase activation. Therefore, we tested the effect of PA and DG on oxidase activation when purified, relipidated (with soybean PC) flavocytochrome b_{558} replaced neutrophil membrane. Levels of oxidase activation were lower compared with the semi-recombinant system. As shown in Fig. 2*B*, DG alone was ineffective in this system, whereas PA

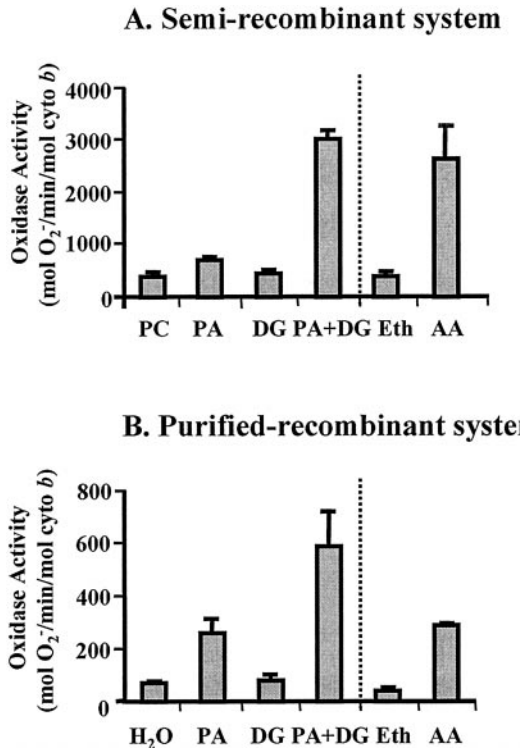


FIG. 2. **Both PA and DG are required for optimal NADPH oxidase activation in the semi-recombinant and purified-recombinant cell-free systems.** *A*, semi-recombinant system. Reaction mixtures contained 0.5 μg of membrane protein (0.6 pmol of flavocytochrome b_{558}), 40 pmol of $p47^{phox}$, 15pmol of $p67^{phox}$, and 60 pmol of Rac1 and were incubated with 30 μM PC, 10 μM PA, 10 μM DG, or PA + DG (10 μM each) for 90 min or ethanol (*Eth*) or 25 μM AA for 30 min. ATP (12 μM) was present in all reaction mixtures except those containing AA. NADPH oxidase activity was measured as described under "Experimental Procedures." Data shown are the mean ± S.E. of three experiments. *B*, purified-recombinant system. Reaction mixtures were as above, except that 2 pmol of purified, relipidated flavocytochrome b_{558} was substituted for the membrane protein. Mixtures were incubated with either H_2O, 30 μM PA, 30 μM DG, or 30 μM PA + 30 μM DG for 90 min or with ethanol (*Eth*) or 10 μM AA for 30 min. NADPH oxidase activity was measured as described under "Experimental Procedures." Data shown are mean ± S.E. (n = 3).

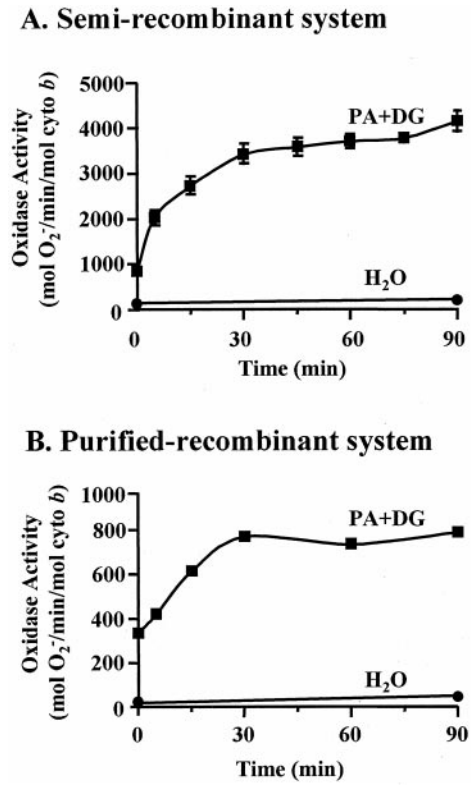


FIG. 3. **Time-dependent activation of NADPH oxidase by PA + DG in semi-recombinant and purified-recombinant cell-free systems.** *A*, reaction mixtures as in Fig. 2*A* were incubated with 30 μM PA + 30 μM DG (■) or H_2O (●) for 0–90 min. *B*, reaction mixtures as in Fig. 2*B* were incubated with 30 μM PA + 30 μM DG (■) or H_2O (●) for 0–90 min. ATP was omitted from all reaction mixtures. NADPH oxidase activity was measured as described under "Experimental Procedures." Activity is expressed as mol of O_2^-/min/mol of flavocytochrome b_{558}. Values with PA + DG are the mean ± S.E. of three experiments (*A*) and the average of two closely agreeing experiments (*B*). H_2O control values are the average of two experiments.

showed slight activation of the enzyme. However the combination of the two lipids induced a greater than additive response, similar to that observed in the semi-recombinant system. AA also could activate NADPH oxidase in this purified-recombinant system, similar to observations by others (18, 19). These results strongly suggest that one or more NADPH oxidase components is a direct target(s) of PA and DG for the activation of the enzyme.

As shown in Fig. 3, the activation of NADPH oxidase by PA + DG in both the semi-recombinant and purified-recombinant cell-free systems reached a maximum by 30 min and did not decline for up to 90 min. The rate of activation was faster in the purified-recombinant system, since ~40% of maximum activity was achieved without preincubation (Fig. 3*B*) compared with ~25% of maximum in the semi-recombinant system at zero time (Fig. 3*A*). The zero time point includes a 2-min incubation for measurement of NADPH oxidase activity, indicating that the rate of activation in the first 2 min is almost twice as fast in the purified-recombinant cell-free system.

Effect of R59949 on the Activation of NADPH Oxidase in the Semi-recombinant Cell-free System—The above data indicated that oxidase activation in the semi-recombinant system was independent of phosphorylation reactions and suggested that both PA and DG were required for the activation process. Previously, Erickson *et al.* (17) used the DG kinase inhibitor R59949 to suggest that a phosphorylation-dependent mechanism of oxidase activation by PA + DG involved the conversion of DG to PA by DG kinase. Since our results suggest that DG has direct effects on NADPH oxidase component(s), we hypothesized that R59949 might exert its inhibitory effect in a DG kinase-independent way. Thus, we studied the effect of R59949 on oxidase activation in the semi-recombinant system, where phosphorylation-dependent reactions are not involved. NADPH oxidase activation was examined in the presence of various concentrations of R59949 using either AA or PA + DG as lipid activators. Assays were performed in the presence of 12 μM ATP to maximize the ability of any DG kinase present in the membrane to convert DG to PA. As shown in Fig. 4*A*, R59949 had no inhibitory effect on oxidase activation until the concentration was above 10 μM, and inhibition was nearly complete at 100 μM. The IC_{50} for inhibition by R59949 was 25 μM with PA + DG and 46 μM with AA. R59949 (50 μM) was not inhibitory when added after the preincubation with lipids (PA + DG: 2608 ± 260 *versus* PA + DG + R59949: 2521 ± 260; AA: 3130 ± 434 *versus* AA + R59949: 4196 ± 262 mol O_2^-/min/mol of flavocytochrome b_{558}, mean ± S.E., n = 3). AA is documented to induce NADPH oxidase activation through direct interaction with NADPH oxidase components (18, 19). Our results in Figs. 1–3 indicate that PA and DG do the same in the semi-recombinant cell-free system. Therefore, these data suggest that R59949 directly interferes with the ability of lipids to induce activation of NADPH oxidase.

R59949 Shifts to the Right the Optimal Concentration of

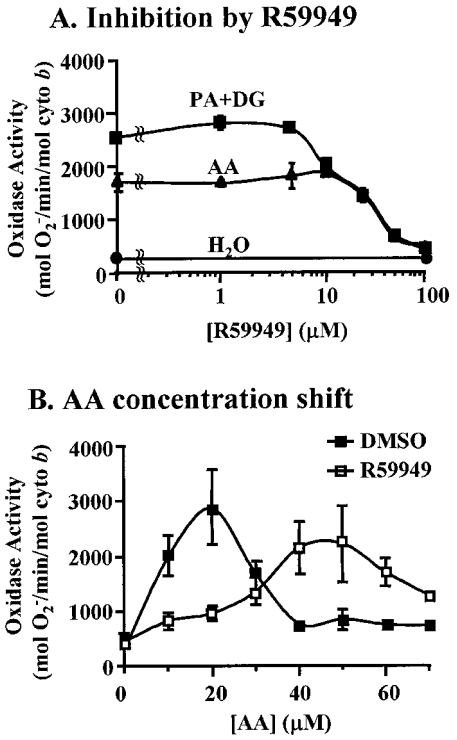


FIG. 4. **R59949 inhibits the activation of NADPH oxidase in the semi-recombinant cell-free system.** *A*, inhibition by R59949 is concentration-dependent. Reaction mixtures as in Fig. 2*A* were incubated with 15 μM AA for 30 min (▲), 10 μM PA + 10 μM DG for 90 min (■), or H_2O (●) in the presence of 0–100 μM R59949. 12 μM ATP was present in the PA + DG-activated system. *B*, R59949 shifts the AA concentration curve to the right. Reaction mixtures as in Fig. 2*A* were incubated with 0–70 μM AA for 30 min in the presence (□) or absence (■) of 50 μM R59949. For both *panels*, NADPH oxidase activity was measured as described under "Experimental Procedures" and expressed as mol of O_2^-/min/mol of flavocytochrome b_{558}. Values with lipid activators are the mean ± S.E. of three experiments. Water controls are the average of two experiments. *DMSO*, Me_2SO.

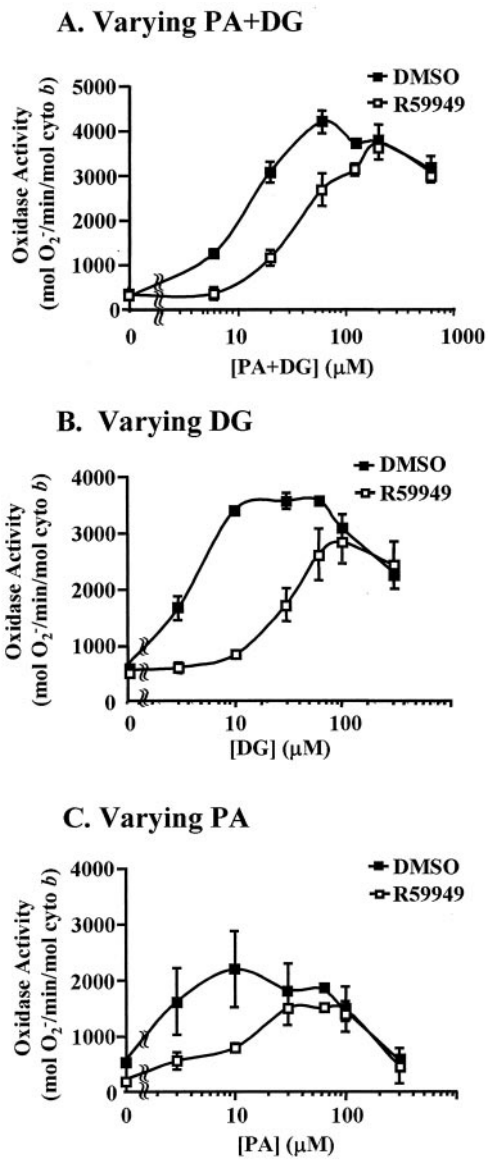


FIG. 5. **R59949 shifts the concentration curves of PA and DG for NADPH oxidase activation to the right in the semi-recombinant cell-free system.** *A*, varying PA + DG. Reaction mixtures as in Fig. 2*A* were incubated with various amounts of PA + DG in the presence (□) or absence (■) of 50 μM R59949. PA and DG were present at equal concentrations, each representing half of the total value shown at any given concentration. *B*, varying DG. Reaction mixtures as in *panel A* were incubated with 10 μM PA plus 0–300 μM DG. *C*, varying PA. Reaction mixtures as in *panel A* were incubated with 10 μM DG plus 0–300 μM PA. NADPH oxidase activity was measured as described under "Experimental Procedures" and expressed as mol of O_2^-/min/mol of flavocytochrome b_{558}. Values shown are the mean ± S.E. of three experiments. *DMSO*, Me_2SO.

Lipid Activators for the Activation of NADPH Oxidase in the Semi-recombinant Cell-free System—To address the mechanism of inhibition by R59949, we further characterized the effect of R59949 on the concentration of AA (0–70 μM) needed to induce NADPH oxidase activation (Fig. 4*B*). The presence of 50 μM R59949 shifted the optimal concentration of AA to the right, from 15–25 to 40–50 μM AA, and slightly reduced the maximal level of activation. These data indicate that inhibition by R59949 of NADPH oxidase activation is likely due to competition with AA for binding to and/or interaction with components of the enzyme.

Next we addressed whether R59949 might exert a similar effect on the amount of PA + DG needed for NADPH oxidase activation. As observed with AA, 50 μM R59949 shifted the optimal concentration of PA plus DG to the right (Fig. 5*A*). R59949 increased the EC_{50} of PA plus DG from 17 ± 7 to 72 ± 22 μM (mean ± S.E., $n = 3$). We next asked whether R59949 exerted its effect on both PA and DG or on just one of these lipids. The concentration of DG (Fig. 5*B*) or PA (Fig. 5*C*) was varied, keeping the other lipid concentration at 10 μM. R59949 (50 μM) shifted the optimal concentration of DG to the right, increasing the EC_{50} of DG from 3 ± 2 to 24 ± 15 μM (mean ± S.E., $n = 3$). The drug also slightly shifted the optimal concentration of PA, and it also decreased the maximal NADPH oxidase activation under these conditions. Taken together, these results indicate that the effect of R59949 on PA + DG-mediated oxidase activation is similar to that in the AA-activated system, with a shift to the right in the lipid concentration required for optimal NADPH oxidase activation. Furthermore, increasing the concentration of DG was better able to overcome the inhibitory effect of the drug compared with PA. These data suggest that R59949 is acting in a competitive manner with the lipid activators, with selectivity for DG over PA.

R59949 Inhibits NADPH Oxidase Activation in the Purified-recombinant Cell-free System—We tested whether R59949 would have similar inhibitory effects on NADPH oxidase activation in the purified-recombinant cell-free system. R59949 was slightly less effective in this series of studies, inhibiting NADPH oxidase activation by PA + DG in the semi-recombinant system by 45% at 100 μM (30 μM PA + 30 μM DG, data not shown). As illustrated in Fig. 6*A* for the purified-recombinant

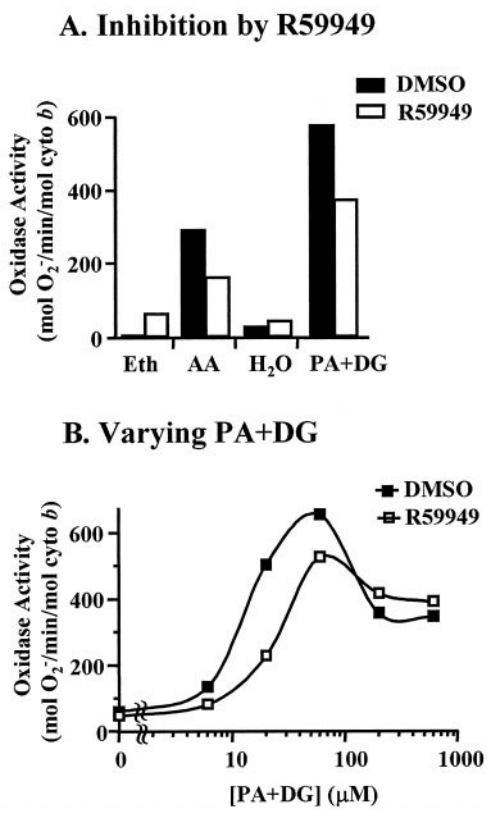
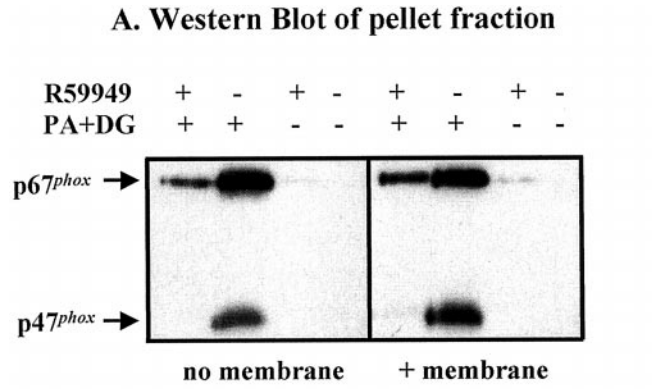


FIG. 6. **R59949 inhibits activation of NADPH oxidase in the purified-recombinant system.** *A*, inhibition by R59949. Reaction mixtures containing purified, relipidated flavocytochrome b_{558} and recombinant cytosolic oxidase components as in Fig. 2*B* were incubated with ethanol (*Eth*) or 10 μM AA or with H_2O or 30 μM PA + 30 μM DG in the presence (*white bars*) or absence (*black bars*) of 100 μM R59949. *DMSO*, Me_2SO. *B*, varying PA + DG. Reaction mixtures as in Fig. 2*B* were incubated with various amounts of PA + DG in the presence (□) or absence (■) of 100 μM R59949. PA and DG were present at equal concentrations, each representing half of the total value shown at any given concentration. Data represent the average of two closely agreeing experiments.



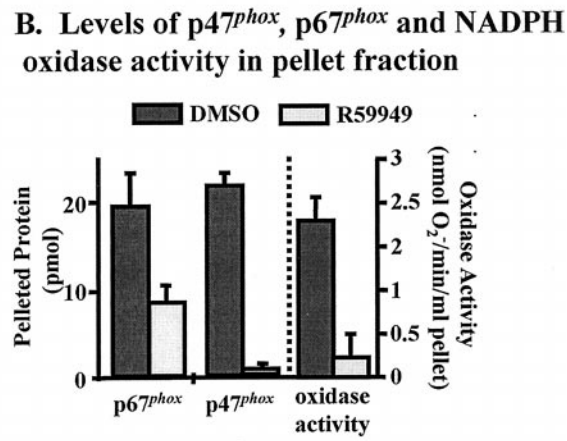


FIG. 7. **R59949 inhibits the aggregation/sedimentation of p47*phox* and p67*phox* induced by PA + DG.** Reaction mixtures containing zero or 0.5 μg of membrane protein (0.6 pmol flavocytochrome b_{558}), 40 pmol of p47*phox*, 15 pmol of p67*phox*, and 60 pmol of Rac1 were incubated with 20 μM PC (no activator control) or 10 μM PA + 10 μM DG for 90 min in the presence or absence of 50 μM R59949. Ten reaction mixtures were combined, and pellet and soluble fractions were separated as described under "Experimental Procedures." NADPH oxidase activity in the pellet fractions containing neutrophil membrane was measured as described under "Experimental Procedures." Proteins from the soluble and pellet fractions were separated on a 10% polyacrylamide gel, transferred to nitrocellulose, and analyzed by Western blot for p47*phox* and p67*phox*. *Panel A*, shown is a representative Western blot of pellet fractions. *Panel B*, *left side*, Western blots of recovered pellet and soluble fractions were analyzed by densitometry. The distributions of p47*phox* and p67*phox* were determined, and the amount (pmol) of each protein present in the pellet fraction was calculated as described under "Experimental Procedures." *Right side*, NADPH oxidase activity was expressed as nmol of O_2^-/min/ml of pellet fraction. Values shown in *panel B* are mean ± S.E. of three experiments. *DMSO*, Me_2SO.

cell-free system, 100 μM R59949 reduced NADPH oxidase activation induced by either AA or PA + DG by 66 and 40%, respectively. We also examined whether R59949 was competitive with PA + DG in this cell-free system. As shown in Fig. 6*B*, the presence of R59949 during NADPH oxidase activation shifted the optimal concentration for the lipid activators to the right. These data indicate that the mechanism of inhibition of NADPH oxidase activation by R59949 is the same when only NADPH oxidase components are present. Thus, the drug must directly interfere with the ability of lipid activators to interact with NADPH oxidase components.

*R59949 Inhibits the Lipid-induced Sedimentation of Recombinant p47*phox* and p67*phox*—*To further support the direct interference of R59949 with lipid activators, we tested the effect of the drug on the ability of PA + DG to induce aggregation and sedimentation of cytosolic NADPH oxidase components. Previously, AA was shown to induce the presumed aggregation of p47*phox* and p67*phox* in either the presence or absence of neutrophil membrane fractions, resulting in the sedimentation of these proteins during high speed centrifugation (48). In the presence of neutrophil membrane, this process aids in the assembly of the active NADPH oxidase enzyme. Here, we determined whether PA + DG induced a similar effect and whether R59949 interfered with the aggregation/sedimentation process. Recombinant oxidase proteins were incubated with or without neutrophil membrane fractions in the presence or absence of lipid activators and/or 50 μM R59949, followed by separation of pellet and soluble fractions over a discontinuous sucrose gradient. PA + DG induced the appearance of both p47*phox* and p67*phox* in the recovered pellet fraction either in the presence or absence of neutrophil membrane (Fig. 7*A*). This indicates that PA + DG, like AA (48), induces presumed aggregation of these soluble proteins. The presence of 50 μM R59949 inhibited the sedimentation of p47*phox* and p67*phox* (Fig. 7*A*). The sedimentation of p47*phox* appeared to be more sensitive to R59949, since the drug inhibited the appearance in the pellet fraction of 98 ± 2% of p47*phox* compared with 57 ± 2% of p67*phox* (Fig. 7*B*). Parallel to the inhibition of p47*phox* and p67*phox* sedimentation, R59949 also markedly reduced the level of NADPH oxidase activity appearing in the pellet fractions containing neutrophil membrane (Fig. 7*B*). These results suggest that R59949 interferes with the ability of lipid activators to bind to one or more soluble NADPH oxidase components and

induce the aggregation process, which might be an important step for transporting $p47^{phox}$ and $p67^{phox}$ to the membrane components in cell-free assays (48).

DISCUSSION

Here, we provide evidence that the second messenger lipids PA and DG interact directly with components of NADPH oxidase to induce assembly and activation of the enzyme. This evidence includes the following. 1) NADPH oxidase activation by PA plus DG in the semi-recombinant cell-free system was ATP-independent and insensitive to the nonselective protein kinase inhibitor staurosporine, indicating a phosphorylation-independent mechanism; 2) PA + DG induced NADPH oxidase activation when only oxidase components were present (the purified-recombinant system); 3) PA and DG were both required for optimal activation, suggesting that each lipid may have one or more oxidase protein targets; and 4) the drug R59949 competitively inhibited the interaction of PA and DG with oxidase components and prevented the assembly of $p47^{phox}$ and $p67^{phox}$ with flavocytochrome b_{558}.

Previously (16, 24), using mixtures of membrane and cytosolic fractions from human neutrophils, we found that NADPH oxidase activation by PA + DG involved both protein kinase-dependent and -independent pathways. A novel cytosolic PA-activated protein kinase capable of phosphorylating two NADPH oxidase components ($p47^{phox}$ and $p22^{phox}$) was implicated as responsible for the phosphorylation-dependent pathway (10, 24–26). In addition, Erickson *et al.* (17) suggested that DG kinase contributes to a phosphorylation-dependent pathway in mixtures of membrane and cytosolic fractions. These pathways are clearly not operative in the semi-recombinant cell-free system, where recombinant cytosolic oxidase components replace neutrophil cytosol.

Based on the results reported here, it is evident that the phosphorylation-independent mechanism of NADPH oxidase activation by PA + DG involves direct interaction between the lipids and oxidase components. This direct mechanism is likely analogous to that used by AA, since the drug R59949 caused a similar shift to the right in the concentration curves of either AA or PA + DG. AA has been shown to directly induce conformational changes in both flavocytochrome b_{558} and $p47^{phox}$ (20–22), resulting in the SH3 domain-mediated binding of $p47^{phox}$ with the $p22^{phox}$ subunit of the flavocytochrome (49). PA has been shown to induce partial activation of purified flavocytochrome b_{558} in the absence of cytosolic components (23). The inhibition of the aggregation/sedimentation of $p47^{phox}$ and $p67^{phox}$ by R59949 implies that the lipids bind to one or both of these components. A likely target for lipid binding is $p47^{phox}$, since a change in conformation of $p47^{phox}$ appears to initiate the translocation/assembly process (49–51). Indeed, we have shown in a separate study[2] that PA selectively binds to $p47^{phox}$. DG can enhance the binding of PA to the enzyme CTP:phosphocholine cytidylyltransferase through a proposed mechanism involving effects of DG on the clustering of PA molecules in the lipid bilayer (52). The synergy between PA and DG for NADPH oxidase activation could involve a similar mechanism. Studies to address these issues are under way.

Our results also clearly show that the DG kinase inhibitor R59949 directly interferes with the ability of lipids to activate NADPH oxidase. Because of its lipophilic nature, R59949 may exert its effect by interaction with the lipids, preventing their binding to NADPH oxidase components. Alternatively, R59949 may compete for the lipid binding site(s) on oxidase proteins. Binding sites on DG kinase or other proteins for R59949 have not been identified. It is possible that R59949 competes with DG for binding to the active site of certain DG kinase isoforms; however, the catalytic binding site for DG is not known (53, 54), and kinetic studies to address this possibility have not been published. Our results show that increasing the concentration of DG was more effective than increasing the concentration of PA at overcoming the inhibition by the drug. Increasing the concentration of AA could also overcome the effect of the drug. This suggests that, in the oxidase system, R59949 is primarily competitive with DG or AA.

The direct inhibitory effect on NADPH oxidase activation by R59949 complicates interpretation of previous results (17) using this inhibitor to implicate DG kinase in cell-free NADPH oxidase activation. Erickson *et al.* (17) showed that R59949 inhibited the conversion of DG to PA and also blocked the ability of DG alone to activate NADPH oxidase. Our results suggest that the inhibition of NADPH oxidase by R59949 in those studies could be due to competition with lipids for oxidase activation. However, since in our hands DG alone cannot activate NADPH oxidase, it is likely that the conversion of some of the added DG to PA by DG kinase was involved in the activation observed by Erickson *et al.* (17). DG kinase may play a regulatory role in intact neutrophils, since a number of studies (55–57) show that DG kinase inhibitors enhance O_2^- release during agonist stimulation. These studies used drug concentrations too low to have direct effects on lipid-mediated oxidase activation. However, caution should be used when interpreting the effects of R59949 on intact cell functions when concentrations above 10–20 μM are used. NADPH oxidase-like enzymes are now being found in a variety of cell types, where the resulting O_2^- and H_2O_2 may be involved in signaling pathways (5, 6). Thus, the direct inhibition of NAD(P)H oxidase activation also may confound the use of R59949 to implicate a role for DG kinase in functional responses in cell types other than neutrophils.

Activation of NADPH oxidase in intact neutrophils is complexly regulated and incompletely understood. A wealth of data (reviewed in Refs. 2 and 10)) implicates the activation of protein kinases and phospholipases, particularly phospholipase D and phospholipase A_2, but how these signaling pathways converge on NADPH oxidase activation is unclear. The phospholipase-generated lipid messengers could activate protein kinase C or other protein kinases (58–62), contributing to the phosphorylation of NADPH oxidase components. Phosphorylation of at least one oxidase component, $p47^{phox}$, is required for oxidase activation by the nonphysiological agonist phorbol myristate acetate, which activates protein kinase C isoforms (2, 8). Phosphorylation of $p47^{phox}$ by protein kinase C can disrupt the "closed conformation" of the protein, allowing it to initiate NADPH oxidase assembly (50, 51). However, the phosphorylation-dependent mechanisms used by physiological agonists have not been elucidated with any certainty. Shiose and Sumimoto (49) recently demonstrated that AA can synergize with phosphorylation of $p47^{phox}$ to activate NADPH oxidase. This is an attractive model that may help to explain the complex regulation of oxidase activation in intact cells by physiological agonists. In addition, we recently showed (63) that phospholipase D-dependent pathways are involved in the phosphorylation of $p22^{phox}$ in intact cells induced by physiological agonists. The use of the semi-recombinant and purified-recombinant cell-free activation systems described here should allow careful dissection of the functional effects of phosphorylation (by added protein kinases) and lipids on NADPH oxidase components during the activation of the enzyme by PA and DG. Mechanisms identified in cell-free systems would then need to

[2] W.-X. Zhang, J. B. Nixon, T. L. Leto, and L. C. McPhail, manuscript in preparation.

be studied in intact cells to determine their physiological relevance.

In conclusion, we have identified a protein kinase-independent pathway for NADPH oxidase activation by the lipid second messengers PA and DG. The pathway clearly involves direct interaction of the lipids with NADPH oxidase components. Thus, PA and DG have multiple potential protein targets in human neutrophils, including a novel PA-activated protein kinase (10, 25), protein kinase C isoforms (58, 59), and now, NADPH oxidase proteins. Interaction of PA and DG with all of these proteins may contribute to the regulation of NADPH oxidase in neutrophils and other cell types.

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