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Cell-free activation of neutrophil NADPH oxidase by a phosphatidic acid-regulated protein kinase

(phospholipase D/diacylglycerol/phosphorylation/signaling)

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ABSTRACT **The phosphorylation-dependent mechanisms regulating activation of the human neutrophil respiratory-burst enzyme, NADPH oxidase, have not been elucidated. We have shown that phosphatidic acid (PA) and diacylglycerol (DG), products of phospholipase activation, synergize to activate NADPH oxidase in a cell-free system. We now report that activation by PA plus DG involves protein kinase activity, unlike other cell-free system activators. NADPH oxidase activation by PA plus DG is reduced ≈70% by several protein kinase inhibitors [1-(5-isoquinolinesulfonyl)piperazine, staurosporine, GF-109203X]. Similarly, depletion of ATP by dialysis reduces PA plus DG-mediated NADPH oxidase activation by ≈70%. Addition of ATP, but not a nonhydrolyzable ATP analog, to the dialyzed system restores activation levels to normal. In contrast, these treatments have little effect on NADPH oxidase activation by arachidonic acid or SDS plus DG. PA plus DG induces the phosphorylation of a number of endogenous proteins. Phosphorylation is largely mediated by PA, not DG. A predominant substrate is p47-*phox*, a phosphoprotein component of NADPH oxidase. Phosphorylation of p47-*phox* precedes activation of NADPH oxidase and is markedly reduced by the protein kinase inhibitors. In contrast, arachidonic acid alone or SDS plus DG is a poor activator of protein phosphorylation in the cell-free system. Thus, PA induces activation of one or more protein kinases that regulate NADPH oxidase activation in a cell-free system. This cell-free system will be useful for identifying a functionally important PA-activated protein kinase(s) and for dissecting the phosphorylation-dependent mechanisms responsible for NADPH oxidase activation.**

Phagocytic cells undergo a respiratory burst in response to infection or inflammation, and the oxygen products produced are toxic to microbes and surrounding tissue (1–4). The enzyme responsible is a multicomponent NADPH oxidase, consisting of a membrane-bound heterodimeric flavocytochrome, cytochrome b_{558}, and two cytosolic proteins termed p47-*phox* (*ph*agocyte NADPH *ox*idase component migrating at 47 kDa on SDS/PAGE) and p67-*phox* (1–4). At least three other proteins are also involved: a cytosolic protein termed p40-*phox* and two small GTP-binding proteins—membrane-associated Rap1a and cytosolic Rac1 or Rac2 (5–9). Activation involves translocation of the cytosolic proteins to assemble with cytochrome b_{558} in the membrane (2, 4, 10), mediated in part by Src homology 3 regions in p47-*phox* and p67-*phox* (10).

Physiological stimuli induce activation of NADPH oxidase by binding to receptors on the cell surface, triggering intracellular signaling events (11, 12). Key intermediates are phospholipases A_2, C, and D and their products: arachidonic acid (AA), inositol trisphosphate, diacylglycerol (DG), and phosphatidic acid (PA) (11, 12). These second messengers lead to protein kinase activation (11–18), and studies suggest that protein phosphorylation regulates NADPH oxidase activation (4, 11). A prominent substrate is p47-*phox*, which is phosphorylated on seven to nine serine residues by stimulation of intact cells (19). However, direct evidence for regulation of NADPH oxidase activation by phosphorylation is lacking.

We and others (see ref. 12) have linked PA to triggering of the respiratory burst in human neutrophils. Enzymes downstream of PA leading to NADPH oxidase activation have not been identified. We developed a cell-free system for activation of NADPH oxidase by PA (20). Activation occurs in mixtures of membrane and cytosolic fractions from unstimulated neutrophils and is synergistic in the presence of DG. Since this system contains many cellular enzymes, targets of PA could be present.

PA activates several protein kinases, including isoforms of protein kinase C (PKC) and purified fatty acid-regulated protein kinases (14–17). Here, we investigate whether the cell-free activation of NADPH oxidase by PA is mediated by a protein kinase intermediate. Our results indicate that PA activates a protein kinase that phosphorylates the enzyme component p47-*phox* and leads to NADPH oxidase activation. This cell-free system will be a powerful tool for dissecting the role of protein phosphorylation in NADPH oxidase activation and for characterizing a second-messenger function of PA.

MATERIALS AND METHODS

Materials. Staurosporine was from Biomol (Plymouth Meeting, PA), GF-109203X was from LC Laboratories (Woburn, MA), and 1-(5-isoquinolinesulfonyl)piperazine (C-I) was synthesized by Mike Thomas (Wake Forest University Medical Center) (21). Recombinant p47-*phox* (Rp47) was from Tom Leto (National Institutes of Health) (22). ATP, adenosine 5′-[β,γ-imido]triphosphate (ATP[β,γ-NH]), and guanosine 5′-[γ-thio]triphosphate (GTP[γ-S]) tetralithium were from Sigma. SDS and other SDS/PAGE reagents were from Bio-Rad. The PA was 1,2-didecanoyl-*sn*-glycerol-3-phosphate and the DG was 1-oleoyl-2-acetylglycerol (Serdary Research Laboratories, Port Huron, MI); both were prepared by sonication (20). AA was from Nu Chek Prep (Elysian, MN); [γ-^{32}P]ATP was from DuPont/NEN. Other reagents have been described (20, 23).

Isolation and Fractionation of Neutrophils. Neutrophils were isolated from heparinized venous blood of several normal individuals and two patients with the p47-*phox*-deficient form of chronic granulomatous disease (CGD) (2), as described (24). Neutrophils were disrupted and membrane and cytosolic fractions were obtained by discontinuous sucrose density gradient centrifugation (20, 23). Protein was determined with

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Abbreviations: AA, arachidonic acid; ATP[β,γ-NH], adenosine 5′-[β,γ-imido]triphosphate; C-I, 1-(5-isoquinolinesulfonyl)piperazine; CGD, chronic granulomatous disease; DG, diacylglycerol; GTP[γ-S], guanosine 5′-[γ-thio]triphosphate; PA, phosphatidic acid; Rp47, recombinant p47-*phox*; PKC, protein kinase C.

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the bicinchoninic acid assay (Pierce) (25). Unless otherwise indicated, each repeat of an experiment used fractions from a separate donor.

Depletion and Measurement of Endogenous Nucleotides. Neutrophils were sonicated in 11% (wt/vol) sucrose/100 mM KCl/3 mM NaCl/3.5 mM $MgCl_2$/0.5 mM phenylmethylsulfonyl fluoride/10 mM Pipes, pH 7.0 (26) and fractionated as above. Cytosol was dialyzed for 18 hr at 4°C against three changes of sonication buffer without sucrose (26).

ATP levels in subcellular fractions were determined by luciferin–luciferase assay (27, 28), using an ATP standard curve. Samples were deproteinized with cold 2.88% perchloric acid, neutralized with 2.8 M K_2CO_3, and diluted in luminometer buffer (0.1 M Tris, pH 7.75/2 mM EDTA). After addition of Chrono-Lume reagent (Chrono-Log, Havertown, PA), light emission was read in a Chrono-Log aggregometer.

Cell-Free Activation and Assay of NADPH Oxidase. Mixtures (0.12 ml) contained 50 mM KP_i (pH 7.0), 100 μM cytochrome *c*, 10 μM FAD, 1 mM EGTA, 5 mM $MgCl_2$, 1 μM GTP[γ-S], a 1:25 ratio of membrane (0.5–1.5 μg) to cytosolic fraction (12.5–37.5 μg), and various activators and/or protein kinase inhibitors (20). After desired times at 25°C, 200 μM NADPH was added, and the reaction was stopped at 2 min by adding 1% (vol/vol) Triton X-100. Variables are described in figure legends. Absorbance of cytochrome *c* was read at 550 nm and corrected for background observed in the presence of superoxide dismutase (20). Activity is expressed as μmol of O_2^- per min per mg of membrane protein (29).

Cell-Free Phosphorylation Assay. Reaction mixtures contained 50 mM NaP_i (pH 7.0), 1 mM EGTA, 5 mM $MgCl_2$, 1 μM GTP[γ-S], 10 μM FAD, and membrane and cytosolic fractions at a 1:25 protein/protein ratio. Addition of [γ-^{32}P]ATP (6 μl, 47–67 μCi/ml; 1 μCi = 37 kBq) was followed by addition of activator and incubation at 25°C (see figure legends for variables). Laemmli sample buffer (30) was added, samples were boiled for 5 min, and proteins (4 μg per lane) were separated by SDS/8–15% PAGE (23, 30). Phosphorylated proteins were detected by autoradiography. Individual bands were analyzed with a Zeineh soft-laser scanning densitometer (Biomed). Rp47 (40–50 ng per lane) phosphorylated by PKC from human neutrophils (31) served as a standard.

RESULTS

Effect of Protein Kinase Inhibitors on Cell-Free Activation of NADPH Oxidase. To investigate whether protein kinases participate in NADPH oxidase activation mediated by PA plus DG, we first examined the effect of three protein kinase inhibitors that compete for the ATP-binding site in protein kinases: C-I, with specificity for protein serine/threonine kinases (21, 31); staurosporine, a potent inhibitor of protein serine/threonine and tyrosine kinases (32, 33); and GF-109203X, a more selective inhibitor of PKC (34). Concentration-dependent inhibition was observed with each inhibitor (IC_{50} values: C-I, ≈15 μM; staurosporine, ≈4 nM; GF-109203X, ≈35 nM). The maximum effect (≈70% inhibition) of each was similar (Fig. 1*A*).

Other lipids and detergents (AA, SDS, SDS plus DG) induce cell-free activation of NADPH oxidase (4, 10). These act directly on enzyme components and are not sensitive to protein kinase inhibitors (35–38). We found little effect by C-I staurosporine, or GF-109203X with SDS plus DG (Fig. 1*B*) or with AA (Fig. 1*C*) as activator. Thus, NADPH oxidase activation by PA plus DG, but not by SDS plus DG or by AA, may require a protein kinase.

ATP Dependence of NADPH Oxidase Activation Induced by PA plus DG. To provide additional support for protein kinase involvement, we examined the ATP requirement in the cell-free system. Cytosolic fractions were dialyzed to reduce the level of endogenous nucleotides. ATP levels were measured in cell fractions, and the ATP concentrations in the cell-free assay were calculated to be 2.24 and 0.015 μM (n = 2) with undialyzed and dialyzed cytosol, respectively. Activation of NADPH oxidase by PA plus DG, for the dialyzed cytosolic fraction, is shown in Fig. 2*A*. Without added nucleotides, low levels of NADPH oxidase activation occurred. Addition of 50 μM ATP slightly increased the activity, and 3 μM GTP[γ-S] alone had a larger effect (≈33% of maximal). Addition of ATP with GTP[γ-S] caused a 3-fold stimulation of activity over that seen with GTP[γ-S] alone. ATP[β,γ-NH], a nonhydrolyzable analog of ATP, did not substitute for ATP, suggesting that ATP hydrolysis was needed. Activation plateaued at 10–15 μM ATP (n = 2; data not shown).

In contrast to the results with PA plus DG, GTP[γ-S] alone supported optimal NADPH oxidase activation by AA (Fig. 2*B*). The lack of an ATP requirement for activation of NADPH oxidase by AA agrees with other reports (26, 39). Overall, our results (Figs. 1 and 2) strongly suggest that NADPH oxidase activation by PA plus DG is protein kinase-dependent.

Effect of NADPH Oxidase Activators on Cell-Free Endogenous Protein Phosphorylation. If a protein kinase is involved in NADPH oxidase activation by PA plus DG, these lipids should induce phosphorylation of endogenous proteins. When [γ-^{32}P]ATP was included in the cell-free activation mixture in the absence of lipids (Fig. 3, H_2O), only a few proteins incorporated ^{32}P. The addition of DG had little effect. In contrast, PA induced a dramatic increase in phosphorylation of a wide range of proteins. The combination of PA and DG was slightly more effective. Thus, PA is a strong activator of protein kinase activity with wide substrate specificity in human neutrophils.

Phosphorylation induced by PA plus DG was compared with that of other cell-free system activators of NADPH oxidase. AA, SDS, and SDS plus DG are known to induce the cell-free

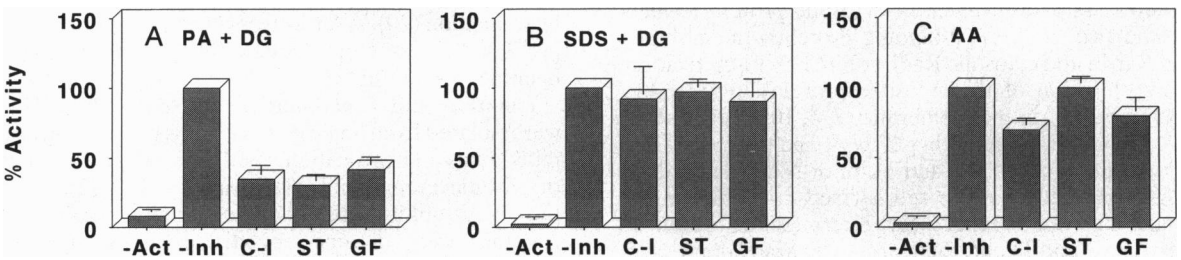

FIG. 1. Effect of protein kinase inhibitors on NADPH oxidase activation by various cell-free system activators. Cytosolic and membrane fractions were preincubated for 5 min with either solvent (−Inh), 300 μM C-I, 100 nM staurosporine (ST), or 300 nM GF-109203X (GF). Activator(s) was added and incubation was continued for the following times, before assay: 100 μM PA plus 100 μM DG, 0.5 μg of membrane, 60 min (*A*); 100 μM SDS plus 30 μM DG, 1.5 μg of membrane, 15 min (*B*); 25 μM AA, 1.0 μg of membrane, 60 min (*C*). Activities are expressed as percent of solvent control (mean ± SEM). In *A*, control activities are 4.7 ± 0.8, 4.0 ± 0.8, and 4.3 ± 0.7 μmol of O_2^- per min per mg of membrane for C-I (n = 4), staurosporine (n = 4), and GF-109203X (n = 5), respectively. In *B* and *C*, control values are 1.8 ± 0.2 (n = 4) (*B*), and 2.9 ± 0.7 (n = 4 (*C*) μmol of O_2^- per min per mg. Activity in the absence of inhibitors and activators is also presented (−Act).

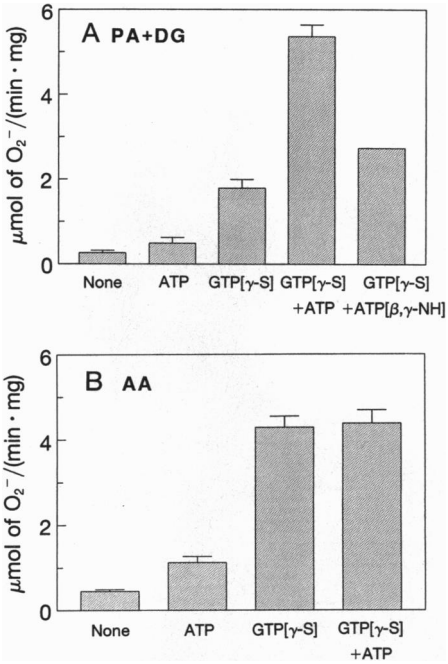

FIG. 2. GTP[γ-S] and ATP requirements for cell-free activation of NADPH oxidase by PA plus DG (*A*) or AA (*B*). Dialyzed cytosolic fractions were mixed with membrane fractions (0.5 μg in *A*, 1.0 μg in *B*), in the presence of 100 μM each PA plus DG or 25 μM AA. Incubations were for 60 min with no added nucleotides (None) or with the indicated nucleotides at the following concentrations: 50 μM ATP, 50 μM ATP[β,γ-NH], 3 μM GTP[γ-S]. Activity is expressed as mean ± SEM (n = 3–7), for cytosolic fractions from four separate donors, except that only two experiments were performed with ATP[β,γ-NH]. Undialyzed cytosolic fractions from three donors assayed in parallel (plus ATP and GTP[γ-S]) had activities of 6.3 ± 0.5 and 4.6 ± 0.3 (mean ± SEM) for PA plus DG and for AA, respectively.

phosphorylation of several proteins (23, 37, 38, 40). PA plus DG was the most effective stimulus, followed by PA alone (Fig. 3). AA induced higher levels of phosphorylation than SDS, and the inclusion of DG with SDS enhanced the response. However, these activators were much less effective than PA or PA plus DG, particularly at inducing phosphorylation of a protein migrating at 47 kDa. Preliminary experiments indicated that optimal protein phosphorylation by all activators occurred between 30 and 45 min (data not shown). We conclude that, relative to PA, other cell-free activators of NADPH oxidase (AA, SDS, and SDS plus DG) are poor inducers of protein phosphorylation.

Although PA plus DG induces the phosphorylation of many endogenous proteins, of particular interest are the predominant phosphoproteins migrating at 47 and 40 kDa (arrows in Fig. 3). The protein at 47 kDa is p47-*phox*, based first on comigration with prephosphorylated Rp47 (Fig. 3, Std). Also, a phosphorylated protein at 47 kDa is absent in cell-free mixtures containing cytosolic fractions from patients with CGD that are missing p47-*phox* (Fig. 4). Finally, the inclusion of unphosphorylated Rp47 with the cytosolic fraction from a patient with CGD caused the band at 47 kDa to reappear (Fig. 4). The 40-kDa phosphoprotein may be p40-*phox* (5), although this must be proven. Thus, at least one NADPH oxidase component, p47-*phox*, is a substrate for the PA-activated protein kinase.

Correlation Between Phosphorylation and NADPH Oxidase Activation Induced by PA plus DG. We examined the relationship between phosphorylation of p47-*phox* and activation of NADPH oxidase. First, p47-*phox* was required for activation of NADPH oxidase by PA plus DG. NADPH oxidase activation was not induced by PA plus DG when CGD cytosolic

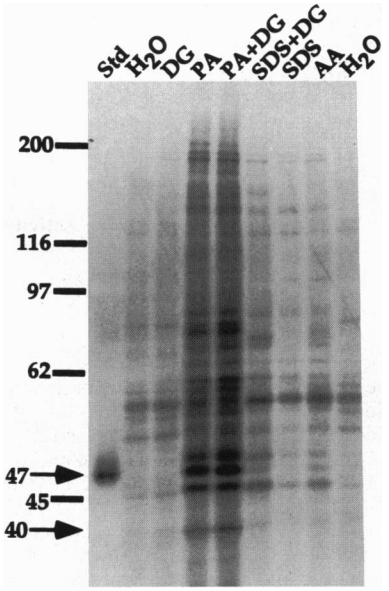

FIG. 3. Cell-free endogenous protein phosphorylation induced by various NADPH oxidase activators. Membrane (1 μg) and cytosolic (25 μg) fractions were incubated for 30 min in the presence of H_2O or activator and analyzed by SDS/PAGE (4 μg per lane) and autoradiography. Activator concentrations were 100 μM PA, 30 μM (+ SDS) or 100 μM (± PA) DG, 100 μM SDS, and 25 μM AA. Migration of molecular size (kDa) standards is indicated at left. Arrows indicate phosphoproteins migrating at 47 and 40 kDa. The autoradiogram shown represents 11 experiments with H_2O, DG, PA, or PA plus DG and 7, 3, and 5 experiments with SDS plus DG, SDS, and AA, respectively. Lane marked Std contained 50 ng of phosphorylated Rp47.

fractions lacking p47-*phox* were mixed with normal membrane fractions (CGD, 0.6 ± 0.4; normal = 4.2 ± 0.7 μmol of O_2^- per min per mg, mean ± SEM, n = 3). Rp47 (200 ng) restored activity (CGD, 3.1 ± 0.7; normal, 4.2 ± 0.9), and the recombinant protein was heavily phosphorylated (Fig. 4).

Second, the time courses for phosphorylation and NADPH oxidase activation by PA plus DG were compared (Fig. 5). Phosphorylation of p47-*phox* proceeded linearly between 1 and 15 min, reached maximum by 30–45 min, and decreased at 60 min. NADPH oxidase activation had a distinct lag and was still increasing slightly at 30–60 min. Clearly, phosphorylation of p47-*phox* preceded NADPH oxidase activation, consistent with a transducing role for the phosphorylation event.

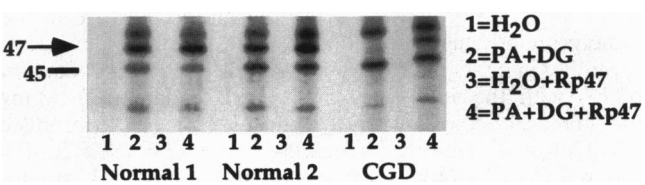

FIG. 4. Comparison of cell-free endogenous protein phosphorylation induced by PA plus DG for cytosolic fractions isolated from normal neutrophils or neutrophils from a patient with p47-*phox*-deficient CGD. The indicated cytosolic fractions (12.5 μg each) were each mixed with a membrane fraction (0.5 μg) from a normal donor in the presence of H_2O (lanes 1 and 3) or PA plus DG (100 μM each, lanes 2 and 4) and in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 200 ng of Rp47. After 45 min, samples were analyzed by SDS/PAGE (4 μg per lane) and autoradiography. Migration of a 45-kDa standard is indicated at left. Arrow indicates the position of p47-*phox*. The autoradiogram shown is representative of two experiments with the same patient. A similar experiment was performed with a separate patient, confirming the absence of a phosphorylated protein at 47 kDa.

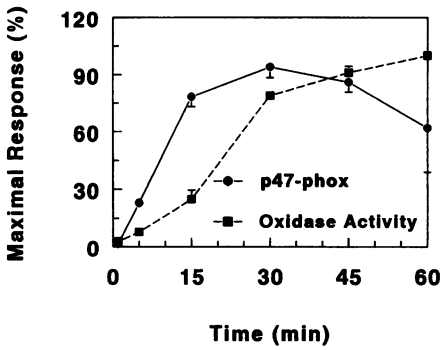

FIG. 5. Time courses of p47-*phox* phosphorylation and NADPH oxidase activation by PA plus DG. Membrane (0.5 μg) and cytosolic (12.5 μg) fractions were incubated with PA plus DG (100 μM each) for the indicated times under conditions used for phosphorylation (see *Materials and Methods*), except that 10 μM nonradioactive ATP replaced [γ-^{32}P]ATP, and cytochrome *c* and NADPH were included when NADPH oxidase activity was assessed. Samples were assayed for NADPH oxidase activity (■) or analyzed by SDS/PAGE (4 μg per lane), autoradiography, and densitometry of the p47-*phox* band (●). For p47-*phox*, the highest densitometry value in each experiment was used as 100%. NADPH oxidase activity at 60 min (100%) was 3.0 ± 0.2 μmol of O_2^- per min per mg. Values are mean ± SEM, n = 3.

Finally, the inhibitors that decreased NADPH oxidase activation by PA plus DG in Fig. 1 were tested for their effects on protein phosphorylation induced by PA plus DG. Each inhibitor diminished the overall level of protein phosphorylation, including phosphorylation of p47-*phox* and the protein migrating at 40 kDa (Fig. 6). Staurosporine (lane 5) was the most effective, followed by C-I (lane 4) and GF-109203X (lane 6). Thus, the protein kinase(s) activated by PA plus DG is sensitive to the same inhibitors that reduce NADPH oxidase activation. Overall, these results (Figs. 3–6) suggest that phosphorylation of p47-*phox* by a PA-activated protein kinase regulates the cell-free activation of NADPH oxidase.

DISCUSSION

We find, in a cell-free system from human neutrophils, that PA activates one or more protein kinases that participate in the activation of the multicomponent enzyme NADPH oxidase. Three lines of evidence support this conclusion. (*i*) The synergistic activation of NADPH oxidase by PA plus DG is sensitive to several protein kinase inhibitors and is ATP-dependent. (*ii*) PA induces the phosphorylation of a wide array of endogenous proteins, including p47-*phox*, a phosphoprotein component of NADPH oxidase. (*iii*) Phosphorylation precedes induction of enzyme activation and is sensitive to the same protein kinase inhibitors.

Studies in intact neutrophils have correlated the production of PA with the activation of NADPH oxidase (12). Many physiological agonists activated phospholipase D to produce PA (12, 41–44). However, a second-messenger function for PA was not proven. Our cell-free system for NADPH oxidase activation by PA plus DG (20) allows us to address this issue.

The current studies suggest that a target for PA in this cell-free system is one or more protein kinases. PA, but not DG, AA, SDS, or SDS plus DG, strongly induces the phosphorylation of many endogenous proteins (Fig. 3). The inability of DG to induce protein phosphorylation and the small enhancing effect of DG on phosphorylation induced by PA imply that the role of DG in the activation of NADPH oxidase by PA plus DG is not protein kinase-dependent. Similar conclusions have been reached by others (40, 45, 46), using SDS plus DG to induce NADPH oxidase activation. The function of DG in the activation mechanism remains to be elucidated.

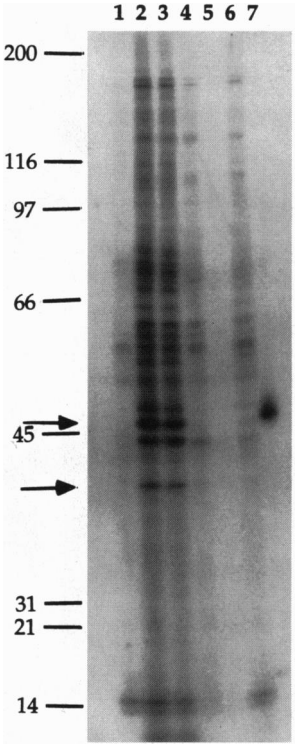

FIG. 6. Effect of protein kinase inhibitors on protein phosphorylation induced by PA plus DG. Membrane (0.5 μg) and cytosolic (12.5 μg) fractions were incubated for 5 min with solvent or inhibitor and then for 45 min with H_2O or PA plus DG (100 μM each) and analyzed by SDS/PAGE and autoradiography. Lanes: 1, H_2O; 2, PA plus DG; 3, PA plus DG plus dimethyl sulfoxide; 4, PA plus DG plus 300 μM C-I; 5, PA plus DG plus 100 nM staurosporine; 6, PA plus DG plus 300 nM GF-109203X; 7, 40 ng of phosphorylated Rp47 standard. Migration of molecular size (kDa) standards is shown at left. Arrows indicate p47-*phox* and a 40-kDa phosphoprotein. The autoradiogram shown represents three experiments.

The protein kinase(s) activated by PA in this cell-free system is not yet identified. The inhibitor profile (Fig. 6) is consistent with a protein serine/threonine kinase related to PKC. However, staurosporine, with specificity for both serine/threonine and tyrosine kinases, was a more effective inhibitor than C-I or GF-109203X, suggesting that phosphorylation of some proteins on tyrosine residues contributes to the pattern obtained. This has not been investigated. Since the three inhibitors were about equally effective for inhibition of NADPH oxidase activation (Fig. 1*A*), it is likely that the protein kinase(s) critical for this process is a serine/threonine kinase.

PA is known to activate some isoforms of PKC (14, 17, 47), as well as one or more purified fatty acid-regulated protein kinases (13, 15, 16, 48). The activation of phospholipase D has been linked to the activation of several unidentified protein kinases in neutrophils from guinea pigs (49). Thus, several protein kinases are candidate targets for PA.

Our results suggest that the activation of NADPH oxidase by PA plus DG represents a different cell-free system for activation of this enzyme. Activation by PA plus DG, but not that by AA or by SDS plus DG, is largely ATP-dependent and sensitive to protein kinase inhibitors (Figs. 1 and 2) (26, 35–39). The basis for the low level of protein kinase-independent activation is unknown. Nevertheless, the PA plus DG-dependent cell-free system should provide insight into the phosphorylation-dependent mechanisms of NADPH oxidase activation. The protein kinase(s) involved and its relevant substrates can be identified, purified, and functionally characterized. Phosphorylation-dependent mechanisms identified

in the cell-free system can be further assessed in an intact cell context.

A prominent endogenous substrate for the PA-activated protein kinase is the NADPH oxidase component of p47-*phox* (Figs. 3–6). The dynamic state of p47-*phox* phosphorylation may modulate NADPH oxidase activation (50–55), but the mechanisms involved are unclear. The PA plus DG-dependent cell-free system is an important tool to address this issue.

In conclusion, we have demonstrated the involvement of a PA-activated protein kinase in a cell-free system for the activation of the neutrophil NADPH oxidase enzyme system. A functional role for a PA-activated protein kinase has not been reported. This system provides a powerful tool for structurally identifying the protein kinase(s), dissecting its functional effects, and determining the structural features of components important for the phosphorylation-dependent activation of the NADPH oxidase.

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