Kinase-dependent Activation of the Leukocyte NADPH Oxidase in a Cell-free System

PHOSPHORYLATION OF MEMBRANES AND p47^{PHOX} DURING OXIDASE ACTIVATION*

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The leukocyte NADPH oxidase catalyzes the 1-electron reduction of oxygen to O$_2^-$ at the expense of NADPH: 2 O$_2$ + NADPH $\rightarrow$ 2 O$_2^- +$ NAD$^+$ + H$. The oxidase is dormant in resting cells but acquires activity when the cells are stimulated with a suitable agent. Activation in whole cells is accompanied by extensive phosphorylation of p47$^{PHOX}$, an oxidase subunit located in the cytosol of resting cells that during oxidase activation migrates to the plasma membrane to complex with cytochrome b$_558$, an oxidase-specific flavohemoprotein. Oxidase activation can be mimicked in a cell-free system using an anionic amphiphile as activating agent. We now report a cell-free system in which the oxidase can be activated in two stages using phosphorylated p47$^{PHOX}$. The first stage, which effects a change in the membrane, requires ATP and GTP and is blocked by the protein kinase inhibitor GF-109203X, suggesting a protein kinase requirement. The second stage requires phosphorylated p47$^{PHOX}$ and GTP, but no ATP, and is unaffected by GF-109203X; assembly of the oxidase may take place during this stage. Activation is accomplished by p47$^{PHOX}$ phosphorylated by protein kinase C but not protein kinase A or mitogen-activated protein kinase. We believe that activation by phosphorylated p47$^{PHOX}$ is more physiological than activation by amphiphiles, because the mutant p47$^{PHOX}$ S379A, which is inactive in whole cells, is also inactive in this system but works in systems activated by amphiphiles.

The leukocyte NADPH oxidase is an enzyme found in neutrophils and certain other leukocytes that catalyzes the one-electron reduction of oxygen to O$_2^-$ at the expense of NADPH: 2 O$_2^- +$ NADPH $\rightarrow$ 2 O$_2 +$ NAD$^+$ + H$. The oxidase is dormant in resting cells but acquires catalytic activity when the cells are exposed to any of a variety of stimuli. Activation involves the transfer to the plasma membrane of cytochrome b$_558$, a flavohemoprotein located in the membranes of the secretory vesicles and specific granules in the resting neutrophil (8–10). A cytosolic complex consisting of the oxidase components p47$^{PHOX}$, p67$^{PHOX}$, and p40$^{PHOX}$ then associates with the cytochrome to assemble the active oxidase (6, 11–15).

The mechanism of activation of the oxidase is unclear. The phosphorylation of p47$^{PHOX}$ is a well-recognized concomitant of oxidase activation in whole cells (16–22), but to date a cause-and-effect relationship between the phosphorylation of this oxidase component and the activation of the enzyme has not been definitively established. The oxidase can be activated in a cell-free system, but the activating agent usually employed is an anionic amphiphile such as arachidonic acid or SDS (23, 24). At least two examples of phosphorylation-mediated oxidase activation in a cell-free system have been reported, however. In 1985, Cox et al. (25, 26) reported that the phosphorylation of resting neutrophil membranes with protein kinase C led to a low level of oxidase activity. More recently, McPhail and associates (27) showed that ATP increased by a factor of $\approx 2.5$ the rate of O$_2$ production in a cell-free system that had been activated by 0.1 mM each of phosphatidic acid and diacylglycerol, suggesting the possibility that a phosphatidic acid-activated protein kinase participated in oxidase activation in their system. We describe here the activation of the leukocyte NADPH oxidase in a cell-free system by p47$^{PHOX}$ that had been pre-phosphorylated by protein kinase C.

**EXPERIMENTAL PROCEDURES**

**Materials**

Chemicals and enzymes were obtained from the following sources: dextran and Ficoll-Hypaque from Pharmacia; luminol, phosphatidylserine, diacetylceramide, sucrose, isopropyl $\beta$-D-thiogalactoside, NADPH, ATP, guanosine 5'-O-(3-thiotriphosphate) (GTP$\cdot$S), guanosine 5'-O-(2-thiotriphosphate) (GDP$\cdot$S), guanosine 5'-O-(2-thiotriphosphate) (Gpp$\cdot$N$\cdot$H); glutathione-agarose, phenylmethylsulfonyl fluoride, cytochrome c, and hexokinase from Sigma; rat brain protein kinase C (PKC), rat brain protein kinase C catalytic subunit (PKM), bovine heart protein kinase A catalytic subunit (PKA), horseradish peroxidase, ca-

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1 The abbreviations used are: GTP$\cdot$S, guanosine 5'-O-(3-thiotriphosphate); GDP$\cdot$S, guanosine 5'-O-(2-thiotriphosphate); Gpp$\cdot$N$\cdot$H; $\gamma$-imidodiguanosine 5'-triphosphate (Gpp$\cdot$N$\cdot$H$\cdot$p); PAGE, polyacrylamide gel electrophoresis; PKA, protein kinase A; PKC, protein kinase C; PKM, protein kinase C catalytic subunit; MAP, mitogen-activated protein; PIPES, 1,4-piperazinediethanesulfonic acid; GST, glutathione S-transferase; GFX, GF-109203X; RLU, relative light units; GNPB, guanine nucleotide binding protein.
lysin A, okadic acid, GF-109203X (GFX, H-7, and horseradish peroxidase were from Calbiochem; mitogen-activated protein kinase p42 (MAP kinase) was from Santa Cruz Biotechnology; anti-pan PKC from Upstate Biotechnology Inc.; and the Bio-Rad protein assay kit and electrophoresis and immunoblotting reagents were from Bio-Rad.

Preparation of Neutrophil Fractions

Neutrophil cytosol and membrane were prepared as described previously (8). Briefly, neutrophils were obtained from normal subjects by dextran sedimentation and Ficoll-Hypaque fractionation of freshly drawn citrate-coagulated blood. The neutrophils were suspended at a concentration of 10^6 cells/ml in a modified relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl_2, 10 mM PIPEs buffer, pH 7.3), and plasma membrane and cytosol were prepared by nitrogen cavitation and centrifugation through Percoll. Both cytosol and membrane were divided into aliquots and stored at −70 °C until use. Cytosol deficient in p47\(^{PHOX}\) was obtained from neutrophils isolated from p47\(^{PHOX}\)-deficient chronic granulomatous disease patients as described above.

Preparation of Recombinant GST-p47\(^{PHOX}\) Fusion Proteins

Recombinant fusion proteins composed of an upstream glutathione S-transferase (GST) linked to a downstream p47\(^{PHOX}\), either the wild type protein or the inactive mutant p47\(^{S379A}\), were isolated from Escherichia coli that had been transformed with pGEX-1T plasmids containing cDNA inserts encoding the downstream proteins, as previously reported (11). The fusion proteins were purified by affinity chromatography on glutathione-agarose as described elsewhere. Before use, excess glutathione was removed from the solution of purified recombinant protein by dialysis against relaxation buffer. The concentration of proteins was determined with the Bio-Rad assay kit using bovine serum albumin as a standard.

In Vitro Phosphorylation and Measurement of 32P Incorporation

Labeling of p47\(^{PHOX}\) with PKM, PKA, MAP kinase, or combinations of kinases was performed by incubating a reaction mixture containing 1 µg of recombinant p47\(^{PHOX}\), 1 mM ATP, 5 µCi of [γ-32P]ATP (Amer sham Corp.), 10 mM MgCl_2, 0.1 µg of the indicated kinase(s), and relaxation buffer, pH 7.3, in a total volume of 30 µl for 30 min at 37 °C. To label with PKC, 1 µg of recombinant p47\(^{PHOX}\) was incubated for 30 min at 37 °C with 0.1 µg of PKC in 10 mM magnesium acetate, 1 mM ATP, 5 µCi [γ-32P]ATP, 0.5 mM CaCl_2, 1.5 µg/ml phosphatidylinositol, and 5 µg/ml dodecyl in a total volume of 30 µl. After terminating the phosphorylation reactions by the addition of 10 µl of 4x SDS-sample buffer, the samples were subjected to SDS-PAGE using an 8% running gel according to the method of Laemmli (28). 32P-Labeled proteins on the dried gels were detected by autoradiography, and 32P was quantified by excising the labeled bands from the dried gel and measuring their radioactivity using Cerenkov counting. To determine background, a piece of nitrocellulose of similar size was excised from a 32P-free portion of each of the two proteins were phosphorylated as described above.

Preparation of Phosphorylated GST-p47\(^{PHOX}\)

Phosphorylation of recombinant GST-p47\(^{PHOX}\) was typically carried out as described above, except that radioactive ATP was omitted and 10–50 µg of fusion protein and a reaction volume of 100 µl were used. Incubations were carried out in Eppendorf tubes for 30 min at 37 °C. Each incubation was terminated by the addition of 1.0 ml of ice-cold MTPBS (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, pH 7.3) and 100 µl of packed MTPBS-washed GSH-agarose beads. The tubes were then rotated end-over-end for 1 h at 4 °C and then spun for a few seconds at maximum speed in an Eppendorf centrifuge to sediment the GSH-agarose beads. After washing the beads with four 1-ml portions of ice-cold MTPBS, the bound phosphorylated p47\(^{PHOX}\) fusion protein was eluted by incubating for 30 min at 4 °C with 200 µl of 50 mM Tris-HCl, pH 8.0, 5 mM GSH, 0.2 mM NaCl. Before use, the eluted fusion protein was dialyzed against relaxation buffer. To compare the phosphorylation of wild type GST-p47\(^{PHOX}\) and GST-p47\(^{S379A}\), 50 µg of each of the two proteins were phosphorylated as described above except that the presence of 1 µCi of [γ-32P]ATP, then analyzed by SDS-PAGE, transferred to nitrocellulose and detected and quantified by autoradiography. The labeled bands were then excised from the blot and analyzed by two-dimensional peptide mapping as described (29, 30). The autoradiogram indicated that the levels of phosphorylation of the wild type and mutant proteins were similar, and the two-dimensional peptide maps of the wild type and mutant proteins were virtually identical (Fig. 1).

Cell-free Activation of the Respiratory Burst Oxidase

The activity of protein kinase-activated NADPH oxidase was measured by chemiluminescence (31). Two types of assays were used as follows: a one-stage assay in which recombinant GST-p47\(^{PHOX}\) was added at the start of the incubation, and a two-stage assay in which the oxidase was partly activated in an initial incubation carried out in the absence of added GST-p47\(^{PHOX}\) and then fully activated and assayed for activity in a second incubation initiated by adding phosphorylated GST-p47\(^{PHOX}\), NADPH, and the detection system to the initial incubation mixture. The assays were conducted by the following procedures.

One-stage Chemiluminescence Assay—The complete reaction mixture contained 2.5 × 10^7 cell eq cytosol, 1.5 × 10^7 cell eq membrane, 50 µM GTPγS, and the unphosphorylated GST-p47\(^{PHOX}\) mixture (5 µg) of recombinant unphosphorylated GST-p47\(^{PHOX}\), 1 mM ATP, 5 units of protein kinase C, 10 mM MgCl_2, 0.5 mM CaCl_2, 25 µM of phosphatidylserine, and 2.5 µg of diacylglycerol, adding the lipids as mixed liposomes prepared by dissolving 1.0 mg of phosphatidylserine and 0.1 mg of diacylglycerol in chloroform, removing the chloroform under a stream of nitrogen, and then sonicating the dried lipids for 2 min on ice. 0.8 ml of 20 mM Tris buffer, pH 7.4) or GST-p47\(^{PHOX}\)-S379A, 5 µg GST-p47\(^{PHOX}\) that had been phosphorylated with protein kinase C) in 0.38 ml (final volume) relaxation buffer. A chemiluminescence detection mixture containing 10 µg of horseradish peroxidase, 10 µM luminol, and 0.16 mM NADPH (final concentrations) was then added to the reaction mixtures, either immediately (for GST-p47\(^{PHOX}\)-S-containing reactions) or after incubating for 5 min at 37 °C (for reactions containing the unphosphorylated GST-p47\(^{PHOX}\) mixture). Oxidase activity was then determined by measuring chemiluminescence at room temperature in a Monolith 2010 luminometer (Analytical Luminescence Laboratories, San Diego) at successive 10-s intervals; the final volume of the assay was 0.5 ml.

One-stage Cytochrome c Assay—An identical protocol was used for the one-stage cytochrome c assay, except that the assay mixture contained 1.5 × 10^7 cell eq solubilized membrane (prepared by mixing 100 µl of 1.25 × 10^7 cell eq) of membrane suspension with 50 µl of cytochrome, 50 µl of relaxation buffer, 25 µl of octylglycoside (10%, w/v), and 25 µl of sodium deoxycholate (10%, w/v) and incubating the mixture on ice for 15 min; the detection mixture contained 0.1 mM cytochrome c and 0.16 mM NADPH (final concentrations); the final volume was 0.75 ml; and cytochrome c reduction was followed at 550 nm for 5 min at room temperature in a Uvikon 941 dual-beam recording spectrophotometer (Kontron Instruments, Milan), reading against a reference containing the same components plus 45 µg of superoxide dismutase.
To establish whether purification over GSH-agarose was variations from these general procedures are indicated in the legends to both the one-stage and two-stage chemiluminescence assays, light emission was followed until shortly past the point where it reached a maximum; this maximum, expressed in relative light units/s (RLU/s), is the luminescence value reported in the tables and figure legends. Deviations from these general procedures are indicated in the legends to the figures and tables.

The two chemiluminescence assays are diagrammed in Scheme I.

Depletion of ATP and Measurement of ATP Concentration

To deplete it of ATP, cytosol (1 × 10⁶ cell eq) was supplemented with 0.1 M glucose, 6 mM MgCl₂, and 0.1 mg of hexokinase (final concentrations) and incubated for 30 min at room temperature. ATP concentrations were measured by an ATP bioluminescent assay kit (FL-AA, Sigma), calibrating by comparison with the chemiluminescence signal from a standard curve established for known concentrations of ATP.

Separation of Membrane from Cytosol after Initial Incubation in the Two-Stage Assay

Membranes from the initial incubation were resolated by layering the incubation mixture over a discontinuous sucrose gradient composed of 1 ml of 15% sucrose layer over 0.5 ml of 50% sucrose, and centrifuging at 105,000 × g at 4 °C. After centrifugation, the contents of the centrifugation tube were carefully withdrawn from the bottom, discarding the first 250 µl and saving the next 300 µl as the preincubated membrane.

Electrophoresis and Immunoblotting

Protein samples containing GST-p47PHOX were subjected to SDS-PAGE on 8% polyacrylamide gels using the Laemmli buffer system (28). The separated proteins were electrophoretically transferred onto a nitrocellulose sheet (32) and probed with a 1:5000 dilution of partially purified rabbit polyclonal antibody raised against C-terminal decapепptide from p47PHOX and finally detected with a 1:2000 dilution of alkaline phosphatase-labeled goat anti-rabbit Ig antibody (Sigma) using 5-bromonitrophenyl phosphate/nitroblue tetrazolium as substrate (Bio-Rad). To establish whether purification over GSH-agarose was able to separate the PKC in the phosphorylation mixture from the newly phosphorylated GST-p47PHOX₁₅, antioxidant PKC antibody (Sigma) using 5-bromonitrophenyl phosphate/nitroblue tetrazolium as substrate (Bio-Rad). To establish whether purification over GSH-agarose was able to separate the PKC in the phosphorylation mixture from the newly phosphorylated GST-p47PHOX₁₅, antibody. The separated proteins were electrophoretically transferred onto a nitrocellulose sheet (32) and probed with a 1:5000 dilution of partially purified rabbit polyclonal antibody raised against C-terminal decapепptide from p47PHOX and finally detected with a 1:2000 dilution of alkaline phosphatase-labeled goat anti-rabbit Ig antibody (Sigma) using 5-bromonitrophenyl phosphate/nitroblue tetrazolium as substrate (Bio-Rad). To establish whether purification over GSH-agarose was able to separate the PKC in the phosphorylation mixture from the newly phosphorylated GST-p47PHOX₁₅, antibody.

Inhibitor Activity

The activities of inhibitors in the cell-free system were investigated by evaluating the incorporation of ³²P into 1,2-IP against proteins by gel electrophoresis and autoradiography. Reaction mixtures contained 1.5 × 10⁶ cell eq cytosol, 5 µCi of 1,2-IPATP, and where indicated 2 × 10⁶ cell eq membrane, 50 µM GTP·S, 0.25 µM calcyulin A (an inhibitor of protein kinases), and 0.5 µM GFX (an inhibitor of protein kinases), and incubated for 20 min at room temperature. After centrifugation, the centrifugation tube was subjected to SDS-PAGE on an 10% polyacrylamide gel. The gels were dried and the labeled proteins detected by autoradiography. The autoradiogram (Fig. 2) showed that in this system both inhibitors exerted their expected effects.

**RESULTS**

 Activation of the Leukocyte NADPH Oxidase by Phosphorylation—In a cell-free system, the leukocyte NADPH oxidase has customarily been activated by certain anionic amphiphiles, including arachidonic acid and SDS. In an earlier study in which we supplemented the cell-free system with extra p47PHOX (added as the GST fusion protein) (35), we found that the enzyme can also be activated by PKC. We have now employed the cytochrome c assay to obtain better quantitation of O₂ production by the PKC-activated system (36). The results obtained with this assay (Table I) were qualitatively similar to those obtained with the chemiluminescence assay. In particular, maximum rates of O₂ production were achieved only in the presence of added PKC and lipids. The sustained maximum rate of O₂ production of 3.8 ± 0.4 nmol/min/10⁷ cell eq membrane in the PKC-activated system was 15–20% of the typical rate seen in the detergent-activated system.

Activation of the cell-free oxidase by PKC may represent a more physiological process than activation by anionic amphiphiles, because in intact cells, as in the PKC-activated cell-free system, oxidase activation is associated with a PKC-dependent event (in the case of intact cells, the phosphorylation of the oxidase subunit p47PHOX). Further evidence that the PKC-activated system may mimic the physiological route of oxidase activation was provided by experiments with a p47PHOX mutant in which serine 379 was replaced by alanine (GST-p47PHOX S379A). This mutant was shown to support O₂ production by a cell-free system activated with anionic amphiphiles, but it did not restore oxidase activity to intact membranes, GTP·S, calyculin A, and GFX; lane 5, membranes, GTP·S, calyculin A, and GFX; lane 5, membranes, calyculin A and 1 mM GDP·S.

**FIG. 2. Effects of calyculin A and GFX on protein phosphorylation in the cell-free oxidase activation system.** The experiment was carried out as described under “Experimental Procedures.” Variable components in the reaction mixtures: lane 1, none; lane 2, membranes and GTP·S; lane 3, membranes, GTP·S, and calyculin A; lane 4, membranes, GTP·S, calyculin A, and GFX; lane 5, membranes, calyculin A and 1 mM GDP·S.
of protein kinase A (PKA) were able to phosphorylate p47PHOX and the catalytic subunit of protein kinase (MAP kinase) and the catalytic subunit of protein kinase C (PKC). Preliminary experiments showed that under the conditions used in our experiments, GST-p47PHOX was completely phosphorylated by a 20-min incubation with PKC and could be cleanly separated from PKC by purification over glutathione-agarose (not shown). When the phosphorylated GST-p47PHOX from PKC by purification over glutathione-agarose (not shown) was added to the assay mixture, the oxidase was activated without the need of additional PKC, whereas MAP kinase treatment resulted in the incorporation of slightly less than 1.5 phosphates/mol of protein. Pretreatment of GST-p47PHOX with PKA, whereas MAP kinase treatment resulted in the incorporation of additional phosphate into GST-p47PHOX by sub-sequent treatment with PKM. As to the abilities of GST-p47PHOX phosphorylated by various kinases to support oxidase activity in this system, the results are those expected if the protein had only been exposed to PKC (or PKM) (Fig. 6). GST-p47PHOX phosphorylated with either MAP kinase or PKA was no more active than unphosphorylated GST-p47PHOX, whereas the activity of GST-p47PHOX phosphorylated by PKM was the same as that of PKC-treated GST-p47PHOX regardless of whether or not the protein had been previously treated with MAP kinase or PKA. In summary, phosphorylation of GST-p47PHOX by PKC led to the incorporation of 6 phosphates/mol of protein. Treatment with PKM resulted in the same stoichiometry. Only 2 phosphates/mol of protein, however, were incorporated by GST-p47PHOX treated with PKA, whereas MAP kinase treatment resulted in the incorporation of slightly less than 1.5 phosphates/mol of protein. Pretreatment of GST-p47PHOX with PKA had no effect on the final amount of phosphate incorporated into the protein after subsequent treatment with PKM, consistent with earlier results showing that the serine residues phosphorylated by PKA were also phosphorylated by PKM (37). Pretreatment with MAP kinase, however, increased by ~1 mol/mol of protein the final amount of phosphate incorporated into GST-p47PHOX by subsequent treatment with PKM. As to the abilities of GST-p47PHOX phosphorylated by various kinases to support oxidase activity in this system, the results are those expected if the protein had only been exposed to PKC (or PKM) (Fig. 6). GST-p47PHOX phosphorylated with either MAP kinase or PKA was no more active than unphosphorylated GST-p47PHOX, whereas the activity of GST-p47PHOX phosphorylated by PKM was the same as that of PKC-treated GST-p47PHOX regardless of whether or not the protein had been previously treated with MAP kinase or PKA. In summary, phosphorylation of GST-p47PHOX by MAP kinase or PKA had little effect on oxidase activation in this system. Furthermore, other kinases in the incubation mixture did not appear to catalyze the incorporation of additional phosphate into GST-p47PHOX once it had been fully phosphorylated by PKM (i.e. converted to GST-p47PHOP6P6), because no radioactivity was found in GST-p47PHOP6P6 isolated on glutathione-agarose and counted after incubation for 30 min in an oxidase assay mixture containing 5 μCi of [γ-32P]ATP (data not shown).

Although further phosphorylation of GST-p47PHOP6P6 did not take place during oxidase activation, there was an additional ATP-dependent step in the activation sequence. This was shown in experiments using cytosol that had been depleted of phosphorylated p47PHOX to activate the leukocyte NADPH oxidase. The stoichiometry of phosphorylation of GST-p47PHOX by the three kinases is shown in Fig. 5. As we found previously, 2 treatment of GST-p47PHOX by PKC led to the incorporation of 6 phosphates/mol of protein. Treatment with PKM resulted in the same stoichiometry. Only 2 phosphates/mol of protein, however, were incorporated by GST-p47PHOX treated with PKA, whereas MAP kinase treatment resulted in the incorporation of slightly less than 1.5 phosphates/mol of protein. Pretreatment of GST-p47PHOX with PKA had no effect on the final amount of phosphate incorporated into the protein after subsequent treatment with PKM, consistent with earlier results showing that the serine residues phosphorylated by PKA were also phosphorylated by PKM (37). Pretreatment with MAP kinase, however, increased by ~1 mol/mol of protein the final amount of phosphate incorporated into GST-p47PHOX by subsequent treatment with PKM. As to the abilities of GST-p47PHOX phosphorylated by various kinases to support oxidase activity in this system, the results are those expected if the protein had only been exposed to PKC (or PKM) (Fig. 6). GST-p47PHOX phosphorylated with either MAP kinase or PKA was no more active than unphosphorylated GST-p47PHOX, whereas the activity of GST-p47PHOX phosphorylated by PKM was the same as that of PKC-treated GST-p47PHOX regardless of whether or not the protein had been previously treated with MAP kinase or PKA. In summary, phosphorylation of GST-p47PHOX by MAP kinase or PKA had little effect on oxidase activation in this system. Furthermore, other kinases in the incubation mixture did not appear to catalyze the incorporation of additional phosphate into GST-p47PHOX once it had been fully phosphorylated by PKM (i.e. converted to GST-p47PHOP6P6), because no radioactivity was found in GST-p47PHOP6P6 isolated on glutathione-agarose and counted after incubation for 30 min in an oxidase assay mixture containing 5 μCi of [γ-32P]ATP (data not shown).

![Graph](image1.png)

**TABLE I**

| Reaction mixture | Activity (Activity of complete system: 3.8 ± 0.4 S.D. nmol/min/10^11 cell eq of membrane) |
|------------------|---------------------------------------------------------------------|
| Complete (control) | 100 ± 14 * |
| Omit-NADPH | −0.4 ± 1.1 |
| Omit-cytosol | −4.6 ± 0.4 |
| Omit-membrane | −5.0 ± 7.1 |
| Omit-GST-p47PHOX | −4.6 ± 3.2 |
| Omit-lipids | 11.4 ± 1.4 |
| Omit-protein kinase C | 16.1 ± 1.8 |
| Omit-lipids and protein kinase C | −2.1 ± 1.4 |
| Plus 1 μM GFX | 21.1 ± 1.1 |

*a Activity of complete system: 3.8 ± 0.4 S.D. nmol/min/10^11 cell eq of membrane (n = 3).

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![Graph](image2.png)

**FIG. 4. Oxidase activity as a function of GST-p47PHOP6 concentration.** The incubations were carried out as described under "Experimental Procedures," measuring O2 production by the one-stage chemiluminescence assay. The results are presented as the mean ± range of two experiments.

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2 J.-W. Park, K. E. Scott, and B. M. Babior, submitted for publication.
ATP by hexokinase plus glucose. Treatment of cytosol with the hexokinase-glucose combination reduced the ATP concentration in the treated cytosol to 67.3 ± 12.5 μM (mean ± S.D., n = 3), a value 20–40-fold below the Kᵦ for PKC (38). This ATP-depleted cytosol was as active as untreated cytosol when used in the standard SDS-activated cell-free system (data not shown). In the GST-p47PHOXP₆-activated system, however, the depleted cytosol was only 13.0 ± 1.2% (mean ± range, n = 2) as active as untreated control. These findings suggest that at least two kinase-dependent reactions participate in protein kinase-dependent oxidase activation, one involving the phosphorylation of p47PHOX and the other the phosphorylation of a substrate (or substrates) yet to be identified.

**ATP-dependent Activation of the Leukocyte NADPH Oxidase Is a Multistep Process**—Measurements of activity as a function of time showed that the activation of the oxidase by phosphorylation occurs with a very long lag, peak activity not being achieved until half an hour has passed (Fig. 7). This is in contrast to oxidase activation in whole cells and in the amphi-}

**Fig. 5. Stoichiometry of phosphorylation of GST-p47PHOX by various kinases and kinase combinations.** Phosphorylation of GST-p47PHOX was carried out, and stoichiometry of phosphorylation was determined as described in the text. Values shown represent the mean ± range of two to four separate determinations.

**Fig. 6. Activation of the leukocyte NADPH oxidase by recombinant GST-p47PHOX phosphorylated by various kinases and combinations of kinases.** The incubations were carried out as described under “Experimental Procedures,” using the one-stage chemiluminescence assay. The reaction mixtures contained recombinant GST-p47PHOX phosphorylated with various kinases and kinase combinations as indicated. Results are shown as the percent ± 1 S.D. of control activity (n = 3), where the control activity was that seen with GST-p47PHOXP₆ (i.e., GST-p47PHOX phosphorylated with protein kinase C). The absolute value for the control activity, representing the maximum rate of light emission observed during the course of a 40-min incubation, was 45,800 ± 4100 RLU/s (mean ± 1 S.D., n = 3).

To determine which of the two fractions, membrane or cytosol, is altered by the initial incubation, experiments were carried out in which membranes and cytosol were incubated together in the presence of GTPγS, then separated, combined with their unincubated complementary fractions plus GST-p47PHOXP₆ and NADPH, and immediately assayed for O₂⁻ production. Activity was only seen in assay mixtures containing preincubated membranes (Table II). An assay mixture containing preincubated cytosol plus unincubated membranes showed no O₂⁻ production. The initial incubation therefore causes a modification of some kind affecting the membranes.

Earlier results (40, 41) have indicated that a guanine nucleo...
The two-stage chemiluminescence assay was conducted as described in the text, using the one-stage or the two-stage incubations as indicated in the figure. Where shown, calyculin A was added to the first stage of the two-stage incubation at a concentration of 0.25 μM. Results are representative of three separate experiments.

**Table II**

| Conditions for second incubation | Membranes | Cytosol | Activity % control |
|----------------------------------|-----------|---------|--------------------|
| Never separated                  |           |         | 100                |
| Preincubated                     | Preincubated |         | 96.7 ± 1.5         |
| Preincubated                     | Fresh     |         | 99.4 ± 5.0         |
| Fresh                            | Preincubated |         | 8.1 ± 0.3          |
| Preincubated                     | None      |         | 3.4 ± 0.2          |

**Table III**

| Guanine nucleotide added to | Initial incubation | Second incubation | Activity % control |
|-----------------------------|------------------|------------------|--------------------|
| 50 μM GTPγS                 | None             | 50 μM GTPγS      | 100                |
| 50 μM GTPγS                 | 50 μM GTPγS      | 50 μM GTPγS      | 22.3 ± 1.1         |
| 50 μM GTPγS                 | 1 mM GDPβS       | 1 mM GDPβS       | 79.4 ± 1.0         |
| 1 mM GDPβS                  | None             | 1 mM GDPβS       | 0                  |
| 50 μM GppNHp                 | None             | 50 μM GppNHp      | 40.0 ± 1.5         |
| 250 μM GppNHp                | None             | 250 μM GppNHp     | 84.3 ± 2.9         |
| None                         | None             | None             | 1.0 ± 0.1          |

a Reisolated membranes and fresh cytosol used for the second incubation.

Activation of the Leukocyte NADPH Oxidase

FIG. 7. O₂⁻ production by GST-p47phox.P₆-activated leukocyte NADPH oxidase as a function of time.

Table II: Expediting oxidase activation by preincubation of membranes with cytosol plus GTPγS

- The two-stage chemiluminescence assay was conducted as described in the text, except that the initial incubations contained 0.25 μM calyculin A, and membranes and cytosol were separated after the initial incubation as described under “Experimental Procedures,” with the exception noted in the table. Fresh or preincubated materials were used in the second incubations as indicated in the table. Results are expressed as % of oxidase activity in a control experiment in which the membranes and cytosol were not separated after the initial incubation. The absolute value for the control experiment was 112,200 ± 6,900 RLU/s. The results are expressed as the mean ± range for two experiments.

Table III: Requirement for guanine nucleotides in the protein kinase-dependent activation of the NADPH oxidase

- The two-stage chemiluminescence assay was conducted as described in the text, adding various guanine nucleotides at the times indicated in the table. In every case, the initial incubation contained 0.25 μM calyculin A. Where indicated, membrane was reisolated from the initial incubation as described in the text and then mixed with fresh cytosol for the second incubation. Results are expressed as % of oxidase activity in a control experiment in which GTPγS was added to the initial incubation. The absolute value for the control experiment was 117,700 ± 400 RLU/s. The results are expressed as the mean ± range for two experiments.

- **Table II** shows that the addition of calyculin A to the first stage of the two-stage incubation at a concentration of 0.25 μM results in a much lower activity than when calyculin A was added to the second stage of the two-stage incubation. This result suggests that calyculin A inhibited oxidase activation primarily by preventing the phosphorylation of p47phox, an event that probably does not occur in this experimental system until GST-p47phox is activated through the activation of the oxidase.

- **Table III** shows that GFX inhibited oxidase activation in a control experiment in which GTPγS was added to the initial incubation. The absolute value for the control experiment was 117,700 ± 400 RLU/s. The results are expressed as the mean ± range for two experiments.

- **Table IV** shows that GFX inhibited oxidase activation in a control experiment in which GTPγS was added to the initial incubation. The absolute value for the control experiment was 117,700 ± 400 RLU/s. The results are expressed as the mean ± range for two experiments.
is required during the initial incubation but that if phosphorylated p47PHOX is supplied, further phosphorylation during the second incubation is unnecessary.

The kinase requirement during the initial incubation reflected more than just the preliminary phosphorylation of the p47PHOX already present in the cytosol. This is indicated by the finding that cytosol deficient in p47PHOX was able to function in the initial incubation, as indicated by the short lag and the high level of oxidase activity seen during the second incubation in experiments using the deficient cytosol (Fig. 8).

The use of GFX made it possible to ask whether unphosphorylated p47PHOX could function in the second stage of the oxidase activation reaction. For this experiment, the initial incubation was carried out as described under “Experimental Procedures,” but the second incubation received either no protein, unphosphorylated GST-p47PHOX, or GST-p47PHOX P6 as indicated in Table V. GFX was added to the second incubations as indicated, to inhibit protein phosphorylation during the second stage of the reaction. The results showed that as compared with O2 production in a reaction mixture containing no added p47PHOX, O2 production was more than doubled by the addition of unphosphorylated GST-p47PHOX to the second incubation. This effect of unphosphorylated GST-p47PHOX, however, was abolished by the simultaneous presence of GFX in the second incubation, suggesting that before it could stimulate O2 production, GST-p47PHOX had to be phosphorylated. Confirming this conclusion were the results obtained with GST-p47PHOX P6 (Table V). These results showed that the fully phosphorylated protein led to an even greater augmentation of O2 production than was seen with the unphosphorylated protein. Furthermore, in contrast to the results with unphosphorylated p47PHOX, the increase seen with p47PHOX P6 was unaffected by the protein kinase inhibitor. These results indicate that 1) the form of p47PHOX that was active in the protein kinase-dependent oxidase activating system was the phosphorylated protein, and 2) the phosphorylation of p47PHOX was the only second stage phosphorylation required for the activity of that system.

Activity of Endogenous p47PHOX—In most of the work described above, recombinant p47PHOX P6 was used in the assays. Neutrophil cytosol, however, contains an amount of p47PHOX comparable to the amount of recombinant protein added to the incubation mixtures in the foregoing experiments, so to further evaluate the physiological significance of the protein kinase-dependent oxidase activation system, it was necessary to determine whether this endogenous p47PHOX could serve as an element of this system. For this purpose, cell-free oxidase activation by endogenous p47PHOX phosphorylated with PKC was compared with cell-free oxidase activation by recombinant p47PHOX P6. The detergent-activated cell-free system was also examined. The results (Fig. 9) showed that the peak rate of O2 production by the unsupplemented kinase-activated system was 60% greater than the rate seen in the GST-p47PHOX-supplemented system, confirming that endogenous p47PHOX could participate in the kinase-dependent oxidase activation system. O2 production by the detergent-activated system, however, was 5-fold greater than O2 production by the PKC-activated system (not shown), suggesting that additional facets of the kinase-dependent oxidase activation system remain to be investigated.

DISCUSSION

It has been known for many years that p47PHOX one of the cytosolic subunits of the leukocyte NADPH oxidase, becomes heavily phosphorylated on serine when the oxidase is activated (16–22). More recent studies showed that the phosphates are confined to the C-terminal quarter of the molecule, identified many of the phosphorylation sites (37, 45), and suggested a heavily phosphorylated on serine when the oxidase is activated (16–22). More recent studies showed that the phosphates are confined to the C-terminal quarter of the molecule, identified many of the phosphorylation sites (37, 45), and suggested a heavy

![Figure 8](image)

**FIG. 8. Activation of NADPH oxidase using p47PHOX-deficient cytosol in the initial incubation.** Incubations were carried out using a two-stage chemiluminescence assay as described in the text, with the following modifications. In the first stage, normal cytosol was replaced by p47PHOX-deficient cytosol, and 0.25 μM calyculin A was present. In the second stage, wild-type GST-p47PHOX P6 or the inactive mutant GST-p47PHOX P6, S379A was added or GST-p47PHOX P6 was omitted, as indicated in the figure. Results are representative of two separate experiments.

**TABLE IV**

| Inhibitor | Added to   | Activity | % control |
|-----------|------------|----------|-----------|
| None      | Initial incubation | 100      | 100       |
| GFX       | Initial incubation | 21.7 ± 1.3 | GFX       |
| GFX       | Second incubation | 97.2 ± 1.2 | GFX       |

**TABLE V**

| GST-p47PHOX | Calyculin A | GFX | Activity | n  |
|-------------|-------------|-----|----------|----|
| None        | –           | –   | 100      | 4  |
| Unphosphorylated | –     | –   | 228.0 ± 40.8 | 8  |
| Unphosphorylated | –     | +   | 85.2 ± 16.9 | 3  |
| GST-p47PHOX P6 | –     | –   | 312.8 ± 7.4 | 4  |
| GST-p47PHOX P6 | –     | +   | 302.5 ± 23.8 | 3  |
| Unphosphorylated | +     | –   | 262.4 ± 10.6 | 2  |
| GST-p47PHOX P6 | +     | –   | 484.0 ± 9.4  | 2  |
phosphorylation of p47^PHOX. The question is still open, however, whether the phosphorylation of p47^PHOX actually plays a role in oxidase activation or is just an epiphenomenon; the only serine in the phosphorylated portion of the molecule that has been shown to be essential for oxidase activity is Ser-379, and it is not certain that Ser-379 is a functionally significant phosphorylation target (31). The experiments reported here have shown that the oxidase can be activated in a cell-free system by the addition of phosphorylated GST-p47^PHOX but not by the unphosphorylated molecule. They show further that p47^PHOX phosphorylated by PKC is competent to activate the oxidase but that p47^PHOX phosphorylated by PKA or MAP kinase is inactive. These findings strongly suggest that the phosphorylation of p47^PHOX that occurs during the activation of the leukocyte NADPH oxidase is of functional significance and that PKC is at least one of the enzymes capable of converting p47^PHOX into a functionally active molecule by phosphorylation. They further suggest that the kinase-dependent activation mechanism described here may reflect at least in part an oxidase activating system that operates in the whole cell, because of the extensive literature implicating phosphorylation in the activation of the oxidase and because a p47^PHOX mutant that is nonfunctional in the whole cell (i.e. p47^PHOX S379A (31)) is also nonfunctional in the kinase-dependent cell-free system, even though the phosphorylation of the p47^PHOX mutant appears to be normal both in vitro and in whole cells (37).

The assay mixtures used in these experiments contained 2.5 × 10⁶ cell equivalents of cytosol. Assuming that the volume of a neutrophil is 800 fl, that its cytosol contains 20% protein (w/v), and that p47^PHOX represents 0.4% of this protein (43), it can be calculated that the cytosol contributed ~10 μg of unphosphorylated p47^PHOX to the reaction mixture. From this calculation and the above results, it appears that the oxidase activity elicted by GST-p47^PHOX-P₆ is roughly comparable to that which would be observed in a system containing a similar amount of (unphosphorylated) p47^PHOX. Therefore on a weight-for-weight basis, GST-p47^PHOX-P₆ seems to be as active as p47^PHOX that has been activated by endogenous phosphorylation. This conclusion is supported by the results in Fig. 9, which shows that the activity achieved in an assay containing only endogenous p47^PHOX is comparable to that obtained in an assay that was supplemented with GST-p47^PHOX-P₆.

The foregoing results also show that the protein kinase-dependent activation of leukocyte NADPH oxidase in a cell-free system is a multi-step process that can be divided into three distinct stages: 1) the activation of the membrane, which appears to take place during the initial incubation; 2) the phosphorylation of p47^PHOX, and finally 3) the assembly of the active oxidase on the activated membrane. The activation of the membrane seems to be the most complicated of these events. The participation of a GNBP in membrane activation is suggested by the finding that GTP is required in the initial incubation, and the involvement of one or more protein kinases is suggested by the ATP requirement in the initial incubation and by the ability of GFX and H-7, antagonists of protein kinase C, to inhibit membrane activation.

All these observations suggest the following as a possible route of activation for the oxidase: 1) processing of the membrane, a complex series of events involving a GNBP-dependent step and a protein kinase; 2) the activation of PKC, which phosphorylates p47^PHOX; and finally 3) the assembly of the active NADPH oxidase on a membrane that somehow has been rendered capable of supporting oxidase activation by virtue of its newly acquired protein phosphate. With regard to membrane processing, the transfer to the membrane of Rac2, a GNBP, is already known to participate in oxidase activation (41, 59), but many other possibilities can be considered, as for example the activation of the p21raco/cdc42 activated kinase (54), a single event that could explain the dependence of membrane processing on both a GNBP and a protein kinase; phosphorylation of a membrane-associated oxidase subunit; the participation of phosphatidic acid in ATP-dependent oxidase activation as described by McPhail and associates (27); and others. It is clear, however, that protein kinase-dependent oxidase activation requires an alteration in the membrane, at least in this system and perhaps in the intact cell as well.

In the activation of the cell-free system by amphiphiles, a GNBP (specifically, Rac1 or Rac2) is needed for the assembly of the oxidase (40, 41). The GTP requirement in the second incubation implies that a GNBP is necessary for the events taking place in that incubation, most likely the assembly of the oxidase, although the possibility of other reactions taking place during this incubation cannot be excluded on the basis of the present results. With regard to ATP, the experiments with ATP-depleted cytosol and the protein kinase inhibitor GFX suggest that, apart from its role in the phosphorylation of p47^PHOX, this nucleotide is not required in the second incubation. A requirement for ATP in the second incubation cannot be conclusively ruled out, however, because it is conceivable that the very low level of ATP in the depleted cytosol is sufficient to support any (hypothetical) ATP-dependent reactions that may take place during the second incubation and that such hypothetical reactions may not be affected by GFX.

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