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Reconstitution of GTPγS-Induced NADPH Oxidase Activity in Streptolysin-O-Permeabilized Neutrophils by Specific Cytosol Fractions

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GTPγS activates the NADPH oxidase and this activity declines rapidly with time after preexposure to streptolysin O. This was not due to loss of $p47^{phox}$, $p67^{phox}$, or Rac. To identify the component(s) leaking out of the permeabilized cell responsible for loss of activity, a GTPγS-dependent reconstitution assay was established. Neutrophil cytosol was subjected to chromatographic fractionation steps for purification of the minimum fraction required to restore activity. The reconstitution of the GTPγS-stimulated activity was dependent on ATP. The inhibitors staurosporine and calphostin C greatly reduced the activity in the reconstitution assay, implicating the involvement of a protein kinase C (PKC) pathway. PKC isoforms β and δ were eliminated as the active factors in the most pure reconstitution fraction. With this novel cell-based reconstitution assay, we have identified the requirement for a protein kinase, or its substrate, for the restoration of GTPγS activation of the NADPH oxidase.

The NADPH oxidase enzyme found mainly in phagocytic leukocytes catalyzes the production of oxygen radicals. It utilizes electrons derived from NADPH to reduce molecular oxygen to superoxide, which is subsequently dismutated to hydrogen peroxide and can lead to formation of other radicals species. These are important for optimal killing and digestion of ingested bacteria in the phagocytic vacuole but their uncontrolled formation could cause tissue damage. The NADPH oxidase is a complex enzyme consisting of five unique subunits: $gp91^{phox}$ (*phox*, phagocyte oxidase) and $gp22^{phox}$, which together comprise the membrane associated flavo-hemoprotein cytochrome b_{558}, and the cytosolic components $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ [1]. In addition, a small guanine triphosphate (GTP)-binding protein, Rac, is required for a functional NAPDH-oxidase [2]. Another small GTP-binding protein, Rap1A, is associated with the flavocytochrome in the plasma membrane [3].

The NADPH oxidase is dormant in intact resting cells but becomes active when neutrophils are exposed to a number of stimuli. These include soluble chemoattractants and opsonized particles that interact with surface receptors, and activators of PKC, like phorbol myristate acetate. Many agonists (such as fMLP, C5a, LTB_4, IL-8) are capable of activating the oxidase enzyme interact with receptors that are coupled to the heterotrimeric G-proteins. These receptors, via G-proteins, mediate the stimulation of several effector enzymes, such as phosphatidylinositol bisphosphate (PIP_2) 3-kinase and phospholipases C, D and A_2, which regulate the levels of some important intracellular second messengers; phosphatidylinositol lipids, Ca^{2+}, phosphatidic acid, diacyl glycerol, and arachidonic acid [4]. It is now clear that the effector enzymes are under the control of several upstream factors. For example, the activity of PLD is regulated by the small cytosolic G-proteins ARF and, to a lesser extent, RhoA and Rac [5]. There are data strongly supporting the idea that phosphatidylinositol lipids, especially PIP_2, are required for PLD activity [6]. Also PLC seems to require a cytosolic factor for its full activity. This recently identified 35-kDa protein, phosphatidylinositol transfer protein (PITP), participate in the supply of PIP_2, the PLC substrate, at the plasma membrane [7, 8].

There is a substantial amount of data demonstrating the importance of the above mentioned pathways in the initial steps of NADPH oxidase activation in neutrophils. It is becoming apparent that the initial signal transduction pathways for the oxidase activation can

Abbreviations used: ARF, ADP-ribosylation factor; GTPγS, guanosine 5′-(γ-thio)triphosphate; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; Pipes, 1,4-piperazine diethane sulfonic acid; PKC, protein kinase C; PLC, phospholipase C; PLD, phospholipase D.

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vary depending on the stimulus, but they converge at a certain point to trigger common effectors. Little is known about the downstream components of the activation pathway. Kinases are certainly involved because the NADPH oxidase activation is associated with phosphorylation of *phox* proteins. It has been suggested that the p21 activated kinases (PAKs), which were identified as targets of Rac in human neutrophils, may play an important role [9]. In addition, activation of the members of the mitogen-activated protein kinase (MAPK) superfamily is believed to provide potential signaling pathways in neutrophils. There is evidence that kinases of the ERK (extracellular regulated kinases) family and p38 MAP kinase family become rapidly activated upon receptor-mediated stimulation and that they contribute to the activation of the NADPH oxidase in human neutrophils [10]. Finally, phosphatases are likely to be involved, because the superoxide production can be modified by phosphatase 1 and 2A inhibitors [11].

The current knowledge of the NADPH oxidase and its activation pathways has mainly come from studies with intact neutrophils or a cell-free system. Although both systems have provided valuable information, they also suffer from severe limitations. Intact neutrophils are difficult to manipulate at the molecular level, requiring uptake of pharmacological compounds for manipulation of signaling pathways. The cell-free system, although successfully used to study protein-protein interactions, is artificial and therefore less suitable for elucidation of physiological activation pathways. Some of these limitations can be circumvented by using a semi-intact cell system by permeabilization of cells. Electroporation has been used to study phosphorylation in neutrophils [12, 13], and recently the effect of some inhibitors [14]. Permeabilization by electroporation is transient and makes very small lesions in the membrane. Streptolysin O (SLO), a bacterial cytolysin, makes pores in the plasma membrane of cells that permit the efflux of free cytosolic proteins within a few minutes. Proteins, which are associated with membranes or the cytoskeleton, take longer to leak out of the cells. By SLO permeabilization, direct access is also gained for influx of chemicals and proteins to the cell interior from the extracellular medium.

In this report, we have studied the NADPH oxidase activity in SLO-treated human neutrophils. The purpose of the study was to first produce NADPH oxidase activity-depleted cells, and then to try to identify, the molecular requirements for the reconstitution of the oxidase activity.

MATERIALS AND METHODS

Materials. Protease inhibitors, cytochrome c, superoxide dismutase, PMA, wortmannin, staurosporine, genistein, and HA-Ultrogel were from Sigma Chemical Co. (St. Louis, MO). PD098059, SB203580, and calphostin C were obtained from Calbiochem–Novabiochem Corp. (San Diego, CA). ATP, GTPγS and NADPH were purchased from Boehringer-Mannheim UK (Lewes, England). SLO was from Murex (Dagenham, England). EGTA and 1 mM $CaCl_2$ AnalR volumetric solution for Ca^{2+}-buffers were obtained from Fluka Chemie AG (Buchs, Switzerland) and BDH Limited (Poole, England), respectively. Protein concentration was determined with Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Recombinant $p47^{phox}$, $p67^{phox}$ were prepared as described [15]. Recombinant ARF and PITP were kind gifts of Dr D. Jones and Mr P. Swigart (Department of Physiology, University College London), and the rat brain PKC isolate was from Dr S. Moss (Laboratory for Molecular Cell Biology, University College London). Rabbit polyclonal antisera raised against recombinant $p47^{phox}$, $p67^{phox}$ and Rac were affinity-purified using antigen columns prepared with recombinant protein and Hi-Trap NHS-activated affinity columns (Pharmacia). The commercially prepared rabbit affinity-purified polyclonal antibodies against cPKCβI (c-16) and nPKCδ (c-20) were from Santa Cruz Biotechnology (Santa Cruz, CA).

Preparation of neutrophils. Neutrophils were prepared from buffy coat residues or fresh peripheral blood obtained from healthy blood donors [16]. Briefly, leukocytes were separated by dextran sedimentation and then centrifuged through a layer of Ficoll/Hypaque. After hypotonic lysis of contaminating erythrocytes, the neutrophil pellet was washed twice with PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM NaH_2PO_4, 5.6 mM glucose, pH 7.4) supplemented with 1 mg/ml bovine serum albumin (BSA). The final cell suspension was counted with a hemocytometer. All steps in neutrophil isolation were done at room temperature.

Measurement of superoxide production. Superoxide production was measured with superoxide dismutase inhibitable cytochrome c reduction assay using a multichannel, 96-well microplate reader (Multiscan MS controlled by the Genesis software, Labsystems, Espoo, Finland). The absorbance was read at 550 nm at 15 s intervals using a kinetic assay mode monitoring the maximum rate of superoxide production. An extinction coefficient of 22.95 mM^{-1} cm^{-1} was used for calculations. The path length in the microplate wells containing 100 μl of fluid was 0.31 cm. In intact cells (5×10^6 cells/ml) the superoxide generation was measured in 100 μl of PBS buffer prewarmed at 37°C, containing 20 mM Hepes, 1 mg/ml BSA, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1 mM cytochrome c and 1 μM fMLP. For acute permeabilization, the superoxide production assay was performed in Pipes buffer (20 mM Pipes, 137 mM NaCl, 2.7 mM KCl, 5.6 mM glucose, pH 6.8), containing 1 mg/ml BSA, 0.1 mM cytochrome c, 1 mM NADPH, 1 mM MgATP, 100 nM Ca^{2+} buffered with 3 mM EGTA (buffer pCa7 [17]), 0.4 units/ml SLO and the activator, 10 μM GTPγS. This reaction mixture was prewarmed to 37°C in 80-μl aliquots in a 96-well plate, and 5×10^5 (20 μl) neutrophils were added to each well just prior to putting the plate on the spectrophotometer. Each assay was done in triplicate, both in the absence and presence of 50 μg/ml superoxide dismutase. The response in the presence of superoxide dismutase was subtracted from the value obtained in absence of superoxide dismutase before data analysis.

Reconstitution assay for NADPH oxidase in permeabilized neutrophils. A solution of recombinant protein or a chromatographic fraction of cytosol was added to the reaction mixture in the 96-well plate in place of the appropriate volume of Pipes buffer. Concentrations of NaCl exceeding 150 mM impaired the assay. Permeabilized neutrophils were prepared by incubation with 0.4 units/ml SLO in Pipes buffer/glucose/BSA/pCa7 at 37°C for a predetermined period such that no more than 75% of the maximal NADPH oxidase activity was lost. After this time, the cell suspension was briefly centrifuged to give a cell pellet and the supernatant discarded. The cells were then resuspended in fresh 37°C Pipes buffer/glucose/BSA/pCa7 and immediately aliquoted to the assay plate. The time course for the depletion of activity varied between experiments and therefore needed to be established each time. A concentration of neutrophils

between 1 and 6×10^7 cells/ml was routinely used. Good reproducibility was established by quenching the SLO permeabilization with the addition of one volume of ice cold buffer, followed by centrifugation at 4°C. The reaction mixture was prewarmed in the assay plate, to which the cells were added, followed immediately by GTPγS.

Permeabilization of cells by SLO has been thoroughly established in previous studies. Release of the entire cellular store of proteins such as PITP, lactate dehydrogenase, and ARF constitutes evidence that all cells incubated in suspension with SLO are permeabilized [7, 18, 19]. Granules remain intact during permeabilization so loss of proteins by proteolysis is not a problem [20].

As a measure of NADPH oxidase activity by acute permeabilization (for 100% activity reference), a method of SLO capture was employed. This avoided the interference effect that SLO had on the spectrophotometric assay. For SLO capture, cells were incubated in Pipes buffer/glucose/BSA/pCa7 with 4 units/ml SLO, on ice for 10 min. The excess SLO was washed away from the cells by centrifugation in a large volume of buffer at 4°C, and the cells resuspended in 37°C buffer just prior to aliquoting to the assay plate. This method ensured that the permeabilization occurred in the presence of the reaction mixture and activator.

Immunoblotting analyses. Protein samples were prepared for electrophoresis by heating at 95°C for 3 min in Laemmli SDS sample buffer, resolved on 12.5% SDS–PAGE gels and transferred to nitrocellulose membranes with a semidry blotter. Membranes were stained with Ponceau solution (Sigma) to locate molecular weight markers, destained and blocked in 4% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween. The blots were then incubated with the desired dilution of antibody in 4% nonfat milk in TBS buffer, washed, and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody. After washing, the blots were developed using an enhanced chemiluminescence detection system (Amersham, UK).

Preparation of cytosol. Neutrophils were isolated as described above with the exception that the PBS buffer was prepared without BSA. After the second wash the neutrophil pellet (approx. volume 20 ml) was incubated on a roller for 15 min at room temperature with 1 mM diisopropyl fluorophosphate. Then, 40 ml of ice-cold lysis buffer (10 mM Pipes, 10 mM KCl, 3 mM NaCl, 4 mM $MgCl_2$, pH 7.0), containing 0.1 mM EDTA, 20 μM leupeptin, 1.5 μM pepstatin A, 0.5 mM PMSF, 0.1 μM microcystin, 30 μM tosyl-lysine–chloromethyl ketone, 25 mM NaF and 5 mM Na-pyrophosphate was added to the cells and the cells were disrupted by 5×5-s bursts of sonication (MSE Soniprep) on ice. The lysate was centrifuged at 400 g for 5 min at 4°C and the supernatant was then centrifuged in an ultracentrifuge (Beckman TLX) at 200,000*g* for 30 min at 4°C. The supernatant of this run provided the neutrophil cytosol. Sonication was used to lyse the cells as it is a practical method of preparing bulk cytosol for purification. The centrifugation of the lysate removes most nuclear, membrane and granule protein contaminants from the cytosolic protein supernatant. On some occasions, isolated neutrophils were stimulated with 1.6 μM PMA for 3 min at 37°C and then washed once with ice-cold PBS prior the cell lysis. This had no effect on the reconstitution activity. Neutrophil cytosol was stored at −70°C, and once thawed all subsequent handling and chromatography was conducted at 4°C.

Q Sepharose chromatography. A Pharmacia fast protein liquid chromatography system (FLPC, Pharmacia Biotech, Uppsala, Sweden) was used. Neutrophil cytosol, containing 100–150 mg protein in 10–15 ml volume, on average, was loaded at a flow rate of 1 ml/min into a 5-ml HiTrap Q anion exchange column (Pharmacia) preequilibrated with buffer A (20 mM Tris–HCl, pH 8.0). The column was washed with 5 ml of buffer A and then eluted with a 15-ml linear NaCl gradient of 0–1.0 M in buffer A, and 3-ml fractions were collected. After chromatography, the fractions were concentrated with Filtron 10K (Filtron Technology Corporation, Northborough, MA), desalted on individual gel filtration columns (Econo-Pac 10DG, Bio-Rad) by elution with Pipes buffer, pH 6.8. Each fraction was then concentrated to about 1 ml with Filtron 10K.

Heparin Sepharose affinity chromatography. 5 ml HiTrap heparin columns (Pharmacia) were used in the same way as the HiTrap Q Sepharose columns. A fast method was established such that the cytosol was applied in 137 mM NaCl, and the unbound material eluted with 10% buffer B (buffer A made up to 1 M NaCl), which removed hemoglobin from the bound fraction. The bound material could be eluted in a single step to 100% buffer B to give a concentrated volume appropriate for application to gel filtration. Fractionation was successful at flow rates up to 5 ml/min.

Gel-filtration chromatography. Both large scale conventional FPLC columns, and SMART system columns (all Pharmacia), were used for fractionation by gel filtration. These included an 80 ml Superdex 200 column, 120 ml HiLoad Superdex 75 16/60 column, 2.4 ml SMART Superdex 200 PC 3.2/30. All columns were equilibrated with Pipes buffer, pH 6.8 which contains 137 mM NaCl. The following calibration standards were used to determine the molecular weight elution profiles: blue dextran 200 kDa to mark the void volume, BSA 67 kDa, ovalbumin 43 kDa, chymotrypsin A 25 kDa. The large columns were run at flow rates of 0.5–1.5 ml/min, and fractions were concentrated with Filtron 10K before analysis in the reconstitution assay. The SMART system column was run at 40 μl/min, 1 min fractions, with multiple consecutive applications of 50 μl concentrated start material. The pooled fractions of consecutive runs did not require concentration prior to analysis.

In vitro phosphorylation assay. The phosphorylation reactions were carried out in 30 μl volumes. The reaction buffer contained 30 mM Mg acetate, 50 μM Ca acetate and 200 μM ATP, with 1.25 Ci [γ-^{32}P]ATP (>2500 Ci/mmol, Amersham, UK) per reaction. After incubation of the source of kinase (cytosol fraction or purified kinase) and substrate ($p47^{phox}$) in the reaction buffer at 30°C for 15 min, the reaction was boiled in Laemmli sample buffer and run on 12.5% SDS–PAGE. Autoradiographs of the dried gels were developed on X-ray film (Amersham, UK).

RESULTS

Spectrophotometric assay for GTPγS-induced NADPH oxidase activity in permeabilized neutrophils. A cytochrome c reduction assay was developed for the detection of NADPH oxidase activity in permeabilized neutrophils. Neutrophils were incubated at 37°C in a reaction buffer containing 0.4 units/ml SLO, which caused immediate stimulation of the NADPH oxidase in the presence of 10 μM GTPγS. This oxidase activity was inhibited by the addition of superoxide dismutase, and in the absence of the activator only a marginal amount of superoxide production occurred. The optimum reaction buffer was established under conditions of acute permeabilization, where there was simultaneous addition of SLO and GTPγS. The highest activity was detected with 100–1000 nM Ca^{2+} (Fig. 1A). For subsequent studies 100 nM Ca^{2+} (EGTA/Ca buffer pCa7) was chosen because it closely corresponds to the cytosolic free Ca^{2+} concentration in unstimulated, intact neutrophils (100–150 nM). The oxidase activity showed an absolute requirement for the enzyme substrate, NADPH (Fig. 1B). Also, the superoxide production was markedly enhanced by the presence of 1 mM MgATP, suggesting that the GTPγS-driven activation of the oxidase was dependent on phosphorylation reac-

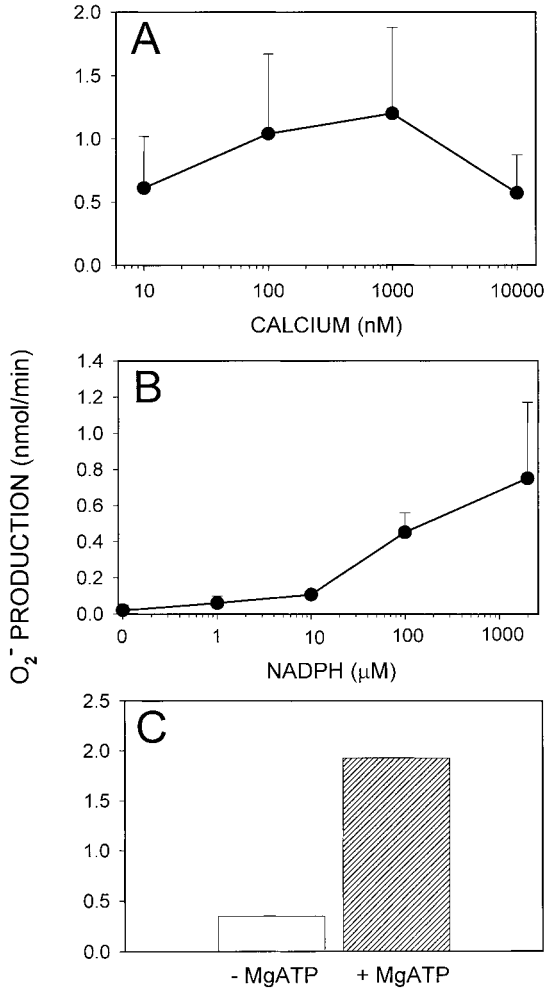

FIG. 1. Effect of calcium, NADPH and MgATP on the NADPH oxidase activity in SLO-permeabilized neutrophils. Superoxide production in response to 10 μM GTPγS stimulation in acutely permeabilized neutrophils was determined in the presence of various concentrations of (A) Ca^{2+} buffered with 3 mM EGTA and (B) NADPH, and (C) in the presence or absence of 1 mM MgATP. The y-axis shows the maximum rate of superoxide generation in 5×10^5 neutrophils measured with the cytochrome c reduction assay. Data shown A and B are means and SD of three independent experiments performed in triplicate and for C from a single experiment representative of three others.

tions in the cell (Fig. 1C). The NADPH oxidase activity of intact neutrophils was also measured by cytochrome c reduction for comparison. Typically, the maximum rate of superoxide production in GTPγS-stimulated acutely permeabilized neutrophils was slightly higher than that seen in intact cells stimulated with 1 μM fMLP.

Effect of inhibitors on the permeabilized cell NADPH oxidase assay. To further characterize the signaling pathway requirements of GTPγS-induced oxidase activation, we tested a panel of pharmacological inhibitors in acutely permeabilized neutrophils. Figure 2 illustrates the effects of seven different inhibitors which have been reported to suppress the NADPH oxidase activation in intact neutrophils with relatively well-known mechanisms of action. Ethanol, which due to the transphosphatidylation reaction blocks the PLD-dependent phosphatidic acid generation, and wortmannin, a direct inhibitor of PIP_2 3-kinase, were not able to inhibit the superoxide production in permeabilized cells. Similarly, PD098059, which indirectly blocks the activation of ERK via inhibition of MEK (MAPK or ERK kinase), had no effect on the GTPγS-induced oxidase activity. Treatment of neutrophils with genistein, a general tyrosine kinase inhibitor, did not significantly reduce the NADPH oxidase activity. Collectively, these results exclude the possibility that the involvement of PLD, PIP_2 3-kinase or MEK, or any other component directly upstream of these effector enzymes would be an absolute requirement in the NADPH oxidase activation by GTPγS. Other inhibitors tested in the assay included 10 nM okadaic acid (inhibitor of phosphatases 1 and 2A), 20 μM SB203580 (inhibitor of p38 kinase) and 10 μg/ml heparin (used as an inhibitor of casein kinase II). None of these affected the GTPγS-stimulated NADPH oxidase activity either. In contrast, staurosporine, a serine/threonine protein kinase inhibitor, strongly suppressed the oxidase activity. A marked inhibition was also observed in the presence of calphostin C which is a selective inhibitor of PKC. This implicated PKC, and possibly other serine/threonine kinases, in the GTPγS stimulation of the NADPH oxidase.

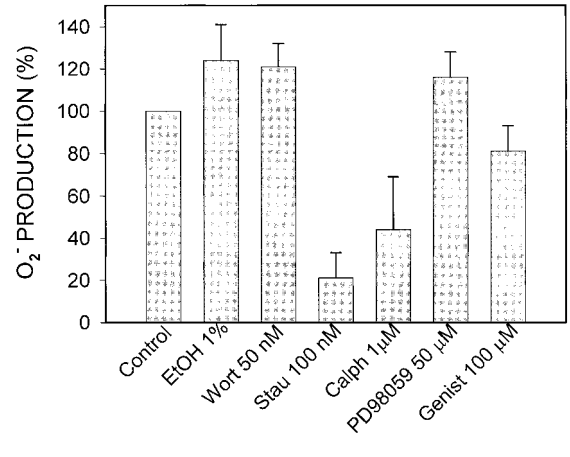

FIG. 2. Effect of inhibitors on GTPγS-induced NADPH oxidase activity in SLO-permeabilized neutrophils. Superoxide production in response to 10 μM GTPγS stimulation in acutely permeabilized neutrophils was determined in the presence of various inhibitors. In the case of calphostin C, cells were incubated for 15 min under fluorescent light in the presence of the drug prior the assay, and control cells were treated similarly in the absence of the drug. The results are shown as a percentage of the maximum rate of superoxide generation in drug-free control cells. Data presented are means and SD of 2–5 separate experiments performed in triplicate.

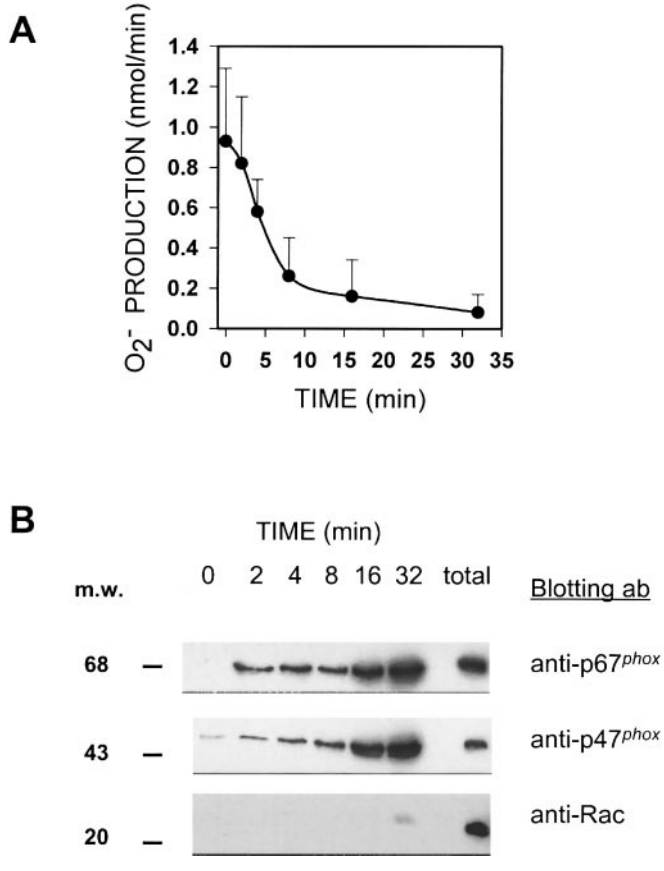

FIG. 3. Timecourse of the depletion of NADPH oxidase activity, and the release of $p47^{phox}$, $p67^{phox}$, Rac in SLO-permeabilized neutrophils. Neutrophils were permeabilized for varying times up to 32 min with 0.4 units/ml SLO at 37°C followed by addition of 10 μM GTPγS. (A) The maximum rate of superoxide production in 5×10^5 cells is shown as the mean and SD of four independent experiments performed in triplicate. (B) Equal volume samples of the supernatants from the permeabilized cells (which were sedimented by centrifugation after the incubation with SLO) were electrophoresed on 12.5% SDS–PAGE gels and transferred to nitrocellulose membrane. The membranes were probed with affinity purified antibodies and developed by the ECL method. Whole cell lysate is loaded as a total, and represents one sixth of the cell equivalent loaded in the time course lanes. The experiment shown is representative of a minimum of two experiments performed in duplicate.

Loss of GTPγS-induced NADPH oxidase activity in permeabilized neutrophils. It has been shown earlier that if neutrophils are permeabilized first and an effector function, such as secretion, is monitored subsequently, the activity is lost [20]. To test the effect of a prolonged permeabilization on the NADPH oxidase activity in neutrophils, the cells were treated with SLO for 0, 2, 4, 8, 16, and 32 min and the oxidase activity was determined in response to GTPγS stimulation (Fig. 3A). These experiments showed that the superoxide anion production decreases rapidly upon permeabilization: 75%, on average, of the initial GTPγS-dependent NADPH oxidase activity in acutely permeabilized cells was lost during the first 8 min of permeabilization and after 32 min less than 10% of the activity was left.

Leakage of cytosolic components of the NADPH oxidase from permeabilized neutrophils. As the observed loss of responsiveness may reflect the leakage of the cytosolic components essential to the NADPH oxidase, the extracellular medium was analyzed for the presence of $p67^{phox}$, $p47^{phox}$ and Rac. Figure 3B shows immunoblots of permeabilized cell supernatants over the depletion time course analyzed in Fig. 3A. These studies showed that less than 15% of the total cytosolic $p47^{phox}$ and $p67^{phox}$ were detectable in the supernatant after 8 min permeabilization and at 32 min time still less than 40%, on average, of these proteins were outside the permeabilized cells. Interestingly, Rac was almost undetectable in the supernatant even after 32 min permeabilization, suggesting that it is not freely soluble under conditions where most cellular architecture is maintained, as is the case in permeabilized cells. To check whether the minor release of $p47^{phox}$ and $p67^{phox}$ during the first 10 min of permeabilization was responsible for the decrease in superoxide production, recombinant $p47^{phox}$ and $p67^{phox}$ were added back to SLO-treated neutrophils. In two experiments, neither $p47^{phox}$ and $p67^{phox}$ alone nor their combination in final concentrations of 10 and 40 μg/ml were able to enhance the GTPγS-induced NADPH oxidase activity in 5-min and 10-min permeabilized cells (data not shown).

Reconstitution of the GTPγS-induced NADPH oxidase activity with cytosol preparations. An assay was established to determine whether the partially depleted permeabilized neutrophils could be restored by addition of recombinant proteins or neutrophil cytosol. It was found that neutrophils exposed to SLO for a limited time, such that greater than 25% activity was retained, could show an elevated response if incubated in certain cytosolic fractions. In initial studies, whole neutrophil cytosol was added to permeabilized neutrophils. This cytosol preparation contained an agent/agents that strongly interfered with the cytochrome c reduction assay, such that the colorimetric reaction was not superoxide dismutase-inhibitable. Therefore, cytosol was applied to fractionation by chromatography, then the fractions were assayed for their reconstituting activity in permeabilized cells. Both Q Sepharose anion exchange and heparin Sepharose affinity chromatography had the same result of effectively removing the interfering agent(s) from the cytosol. The bound material exhibited reconstitution activity and could be eluted with approx. 0.3 M NaCl solution. The activity was stable at 4°C for several days but was reduced after repeated freezing and thawing and lost after being heated for 5 min at 56°C. It is noteworthy that the active fraction had no effect on the weak superoxide production in permeabilized cells in the absence of

GTPγS. It was standard procedure to check that the reconstitution activity being purified was both GTPγS-dependent and superoxide dismutase-inhibitable.

Following either HiTrap Q or HiTrap heparin chromatography, the active cytosol material was loaded onto a gel filtration column. The reconstitution activity eluted in one major peak from a large FPLC column. Immunoblotting showed that this cytosolic material was devoid of $p47^{phox}$, $p67^{phox}$, and Rac. At this stage the reconstituting activity was lost rapidly in a few days even when stored at 4°C. When the active fractions were subjected to SDS–PAGE, at least 15 bands were visible by Coomassie-staining, indicating that the reconstituting factor was not purified to homogeneity. We tested several other chromatographic columns such as S Sepharose cation exchange, hydroxyapatite-Ultrogel and *phox* protein affinity columns, but were not successful in purifying the reconstitution factor further. Purification attempts after the first two steps of fractionation were severely hampered by the labile nature of the reconstituting activity.

The best results for the resolution of reconstitution activity from cytosol were achieved using the combination of steps outlined in Fig. 4A. Cytosol preparations were combined to give a large amount of start material (approx. 4×10^{10} cell equivalent cytosol, 235 ml). This was subjected to HiTrap heparin chromatography, yielding a total of 15 ml eluate in 1 M NaCl. This was a fast step and provided useful separation, removing both the reducing agent(s) and hemoglobin to the unbound material, both of which otherwise interfered with the colorimetric assay. The eluate was concentrated to a smaller volume, but did not require desalting, prior to applying to gel filtration. A high capacity column of Superdex 75 was used first, with consecutive loadings of 2 ml and collection of 1 ml fractions each time. As the equilibration buffer was the same as the permeabilization assay buffer, the fractions could be assayed directly but they did require concentration by 8-fold to 200 μl in order to detect activity. Protein eluted between fractions 5 and 39, and reconstitution activity was detected in a broad peak from fraction 9 to 23. This amounted to a 60-ml pool which was concentrated to <700 μl.

This semipurified material was found to contain PKC. There is considerable evidence of a role for PKC in the activation of the NADPH oxidase [21–23], and in our acutely permeabilized cells there was sensitivity to staurosporine and calphostin c (Fig. 2). Therefore we investigated whether the PKC could be the reconstitution factor. By taking the heparin Sepharose/S75 gel filtration purified activity on the further purification, we were able to resolve the PKC away from the reconstitution activity (Fig. 4B). Immunoblotting for the β and δ isoforms of PKC (predominant species in the neutrophil [23, 24]), revealed that the majority of PKC eluted in fraction 15 but was not detectable in the most active fraction 23.

To further establish that PKC was not the reconstitution factor we carried out further experiments. Interestingly, there appeared to be a correlation between the depletion of activity over time of exposure to SLO and the leakage of PKC isoforms β and δ from the cell (Fig. 5A). This suggested that while the cells were still responsive to GTPγS stimulation, there was indeed most of the PKC still present. However, the addition of either full length PKC or the constitutively active catalytic subunit, PKM, were unable to restore NADPH oxidase activity to depleted cells (data not shown). A substrate for PKC in the NADPH oxidase is the cytosolic component $p47^{phox}$, so we tested whether the reconstitution material may be carrying out this phosphorylation in our system. In an *in vitro* phosphorylation assay, under conditions that rat brain isolated PKC was able to phosphorylate $p47^{phox}$, the active fractions of heparin Sepharose/S75 gel filtration purified cytosol did indeed phosphorylate $p47^{phox}$ (Fig. 5B). This kinase activity correlated with the presence of PKC β and δ however, as the SMART system fraction 15 phosphorylated $p47^{phox}$ but fraction 23 did not. These data do not exclude the possibility that a different kinase is being delivered for which one of the *phox* proteins is the substrate, but it does suggest that the signaling pathway restored by the purified cytosol fraction is unlikely to involve the phosphorylation of $p47^{phox}$.

The identity of the protein(s) conferring the reconstitution to the permeabilized cell system was not discovered. As the purified material eluted from the SMART gel filtration column with an apparent molecular weight of approximately 40 kDa, the possibility that $p40^{phox}$ is the active factor was examined. Immunoblotting across the fractions of the S200 run showed that although $p40^{phox}$ was present in the semipurified start material, it had all eluted off the column before the purified active fraction was collected. Therefore $p40^{phox}$ cannot be the reconstitution factor. Also, because there was no correlation between any visible bands by gel electrophoresis and the most stringent resolution of activity, it was not possible to identify the active factor by protein sequencing.

DISCUSSION

Cell-free systems of reconstitution have proved valuable in the identification of $p47^{phox}$, $p67^{phox}$, and Rac. However, these systems are in nature artificial in their means of activation, so we have developed a reconstitution method which does not override cellular mechanisms of signal transduction. SLO permits the flux of cytosolic proteins within 5–10 min and this loss of cytosolic proteins coincides with loss of signaling functions. These partially depleted cells have been used as the basis of reconstitution assays where exogenous pro-

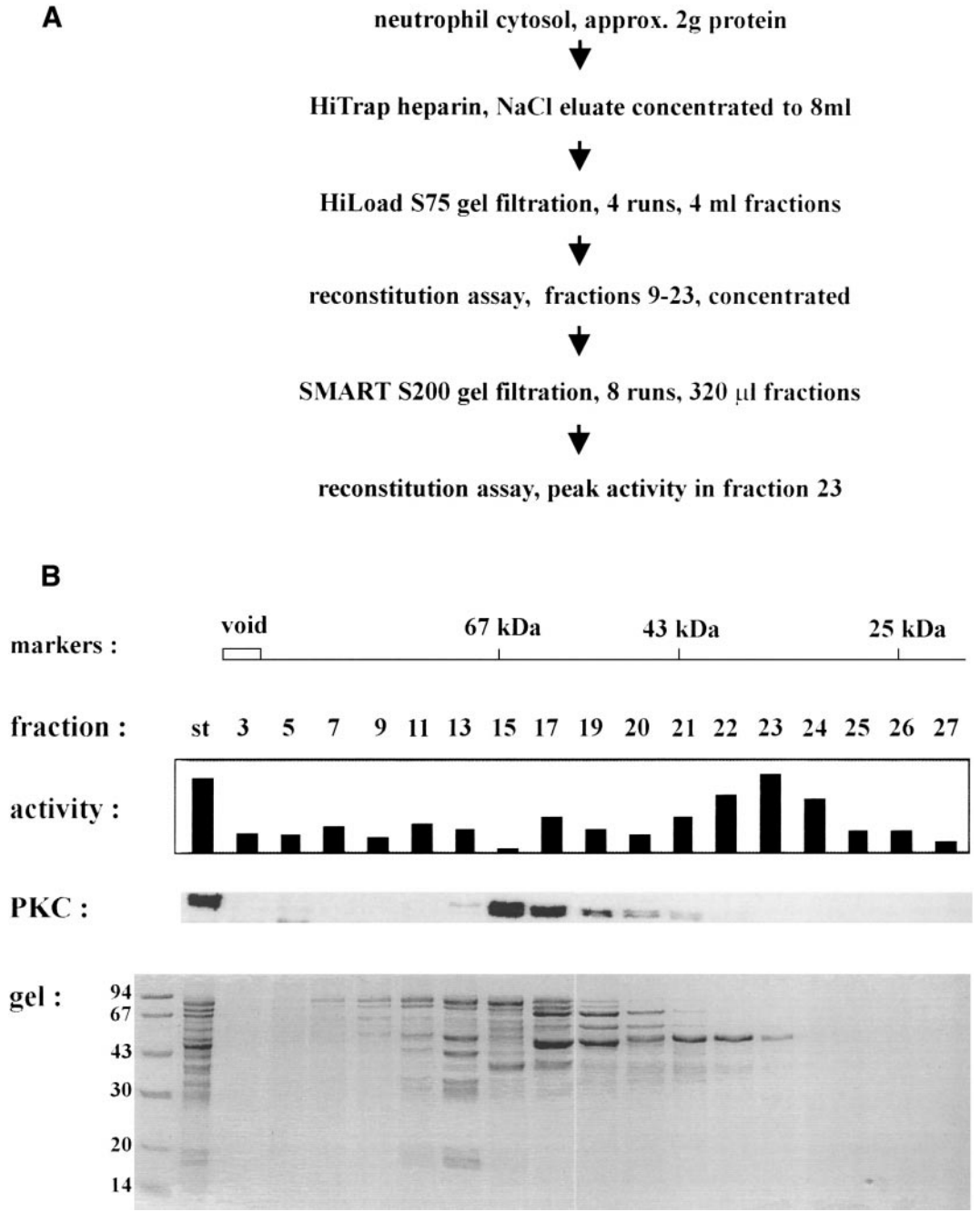

FIG. 4. Optimal purification of the reconstituting activity in neutrophil cytosol for restoration of the NADPH oxidase in SLO-permeabilized cells. (A) A flow diagram of the purification steps. After both large scale FPLC runs it was necessary to concentrate the material to smaller volumes. The fractions were tested in the permeabilization assay before the final purification by gel filtration on the SMART system. (B) Characterization of the final fractionation by SMART Superdex 200 gel filtration. The column was pre-equilibrated in the assay Pipes buffer, and calibrated with molecular weight standards (markers). The relative activity of 30 μl of the fractions in the permeabilization assay was compared with 10 μl of the semipurified start material obtained after Superdex 75 gel filtration (st). An equal volume (2.5 μl) of each 320 μl fraction was applied to 12.5% SDS–PAGE and stained with Coomassie to compare with the semipurified start material (gel). An immunoblot probed with antibodies against C-terminal βI and δ PKC, shows the elution across the fractionation at the molecular weight of 50 kDa which corresponds to the catalytic subunit (PKC).

teins are added back to regain signaling function. Using this approach, PITP was identified as an essential component for PLC signaling [7] and ARF was identified as an activator of PLD [25].

We have now applied the same principle to establishing a reconstitution assay for identifying the signaling pathways in the NADPH oxidase. The agonist that was chosen for activation of the NADPH oxidase was

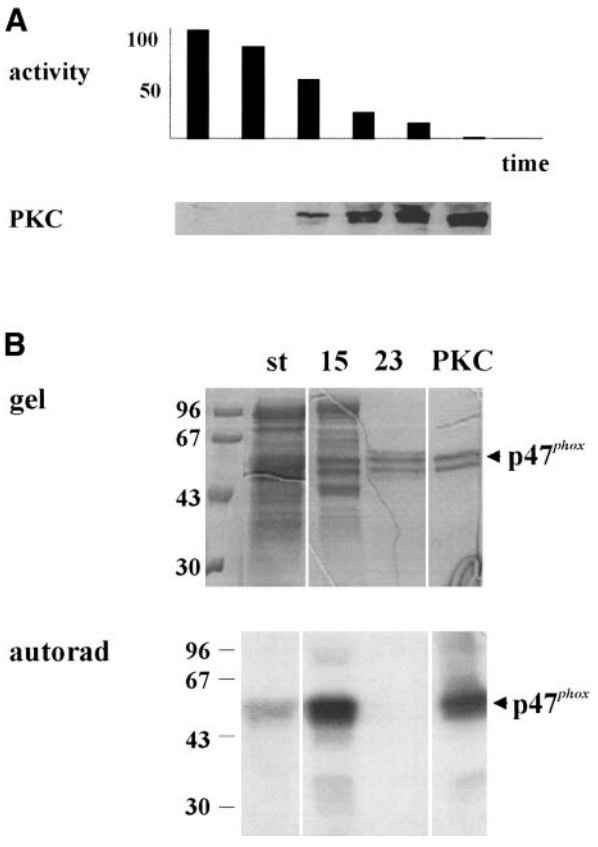

FIG. 5. Investigation of PKC as a candidate for the reconstitution factor. (A) Neutrophils were permeabilized in the same conditions as for a reconstitution assay, and the NADPH oxidase activity was measured across a time course of SLO incubation. The % relative activity is shown compared with acutely permeabilized cell activity of 100%. The leakage of PKC into the extracellular medium was determined for each time point by running an equal volume of permeabilized cell supernatant on SDS–PAGE and immunoblotting with antibodies against C-terminal βI and δ PKC. (B) An *in vitro* phosphorylation assay was carried out to test for the phosphorylation of $p47^{phox}$. Recombinant $p47^{phox}$ was prepared by thrombin cleavage of a full-length GST–$p47^{phox}$ fusion protein, and approximately 5 μg was added to each reaction and shows as a doublet (gel). The fractions tested for kinase activity were 1.5 μl semipurified start material for SMART S200 gel filtration (st), 5 μl PKC-positive but reconstitution activity-negative SMART S200 fraction (15), and 5 μl PKC-negative SMART S200 fraction with reconstitution activity (23). Rat brain isolated PKC of mixed isoforms (10 ng) was used as a positive control (PKC). The gel was dried and exposed to film at −70°C, overnight (autorad).

GTPγS, which can activate both heterotrimeric and monomeric G-proteins. This choice of agonist was led by the knowledge that G-protein-coupled receptors are responsible for triggering the NADPH oxidase by many stimuli. Experimenting with the conditions of the permeabilization bioassay showed that GTPγS stimulation was insensitive to 1% ethanol and 50 nM wortmannin. This suggests that the signaling induced by GTPγS does not require, or is downstream of PLD and PI-3-kinase. These results were supported by the observation that addition of 25 μM ARF and/or 25 μM PITP that are effective in PLC and PLD reconstitution, were unable to support NADPH oxidase activity (data not shown). The reconstitution did not require micromolar concentration of calcium, neither did exogenous arachidonic acid have an effect, eliminating a role for cytosolic PLA_2 activation.

The loss of responsiveness to GTPγS with increased exposure to SLO was not due to loss of the known NADPH oxidase components $p47^{phox}$, $p67^{phox}$, and Rac. Similar to the behavior of Rac, negligible flux of the small G-protein RhoA has been observed in other SLO-permeabilized cell systems [19], suggesting adherence to an intracellular structure such as the cytoskeleton. For identification of fractions exhibiting reconstitution activity, neutrophil cytosol was purified by consecutive chromatography runs. The SMART system proved to be advantageous for obtaining superior resolution and reproducibility. We also examined cytosol from rat brain for the presence of reconstituting activity, as this should be rich in signaling molecules and larger amounts of start material would be available. However, we were unable to detect any reconstituting activity in rat brain cytosol suggesting the activity is due to neutrophil-specific components.

Consistent with the dependence of the reconstitution system on ATP, kinase activity was detected in active fractions of cytosol. Therefore it may be that an essential kinase is the active component that is restoring the GTPγS signaling pathway. Another possibility is that the reconstitution fraction is providing the required substrate for a kinase. Inhibitor studies showed that the permeabilized cell assay was strongly inhibited by staurosporine and calphostin C, but was not affected by okadaic acid, genistein, PD098059, SB203580, or heparin.

A candidate phosphorylation pathway of importance to the activation of the NADPH oxidase is the phosphorylation of $p47^{phox}$. It is known that $p47^{phox}$ undergoes multiple phosphorylation events upon stimulation of the NADPH oxidase [26] and that PKC is responsible for at least some of the phosphorylations that are essential for activation and translocation of the protein to the membrane [27, 28]. PKC was found to rapidly leak out of the cell upon permeabilization and this raised the question whether our reconstitution fractions were delivering PKC back to the cell. Although PKC isoforms β and δ were found to be present in the semipurified reconstitution material, these were separated from the reconstitution factor(s) by the final gel filtration step. Therefore PKC β or δ are not the active components of the reconstitution fraction.

It is quite likely that the reconstitution activity is conferred by a complex of cytosolic proteins rather than a single factor. This would explain the lability of the activity after multiple purification steps. The process of identifying the reconstitution factor(s) is hindered by the lability of the purified activity, and by the practical

limits of scaling up to larger purifications. This technique will be a valuable tool in combination with other approaches for studying the signaling mechanisms. This should include investigation of the specific target molecule(s) for the activator GTPγS which would direct the identification of the downstream effector molecules. In summary, we have demonstrated a cell-based reconstitution assay that has provided insight to the signal transduction pathway involved in GTPγS-induced activation.

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