The interaction of cytosolic components of neutrophil NADPH oxidase with phosphoinositides

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The superoxide-generating NADPH oxidase of neutrophils can be activated in a cell-free system consisting of cell membranes, cytosol and an activating detergent (e.g. arachidonate or SDS). It has previously been reported [Aviram and Sharabani (1989) *Biochem. Biophys. Res. Commun.* **161**, 712–719] that a mixture of phosphoinositides (PPIs), as well as the individual inositol lipids, interfere with the activation process. In the present study it is shown that exposure of the cytosol to PPI results in a progressive ($t_{1/2} = 30$ s) loss of its oxidase-supporting activity and

that Mg^{2+} ions eliminate this inactivation. Neomycin, previously described as an inhibitor of cell-free activation, counteracted the effect of PPI and vice versa. Fractionation experiments implicated the p67-phox cytosolic component of the oxidase in the association with PPI. PPI blocked activity of recombinant p67-phox also and quenched the fluorescence intensity of its tryptophan residues. It is suggested that PPIs may mediate the interaction of the oxidase with the cytoskeleton and/or with the membrane.

INTRODUCTION

Specific association with polyphosphoinositides has been documented for gelsolin [1], profilin [2,3], erythrocyte glycophorin and protein 4.1 [4] and for other cellular proteins. The interaction induces alterations in the functional properties of the proteins: it interferes with the actin-severing activity of gelsolin [1] and with the inhibition of actin polymerization and phospholipase C-catalysed hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) by profilin [2,3]. Reduction of the enzymic activity of casein kinase I and protein kinase C through association with PIP_2 has also been demonstrated [5,6].

We have previously described inhibition of cell-free activation of neutrophil superoxide-generating NADPH oxidase by a mixture of phosphoinositides (PPIs) or by the individual lipids, PIP_2 , phosphatidylinositol 4-phosphate (PIP) and phosphatidylinositol (PI) [7]. NADPH oxidase (reviewed in references [8–10]) is a multicomponent enzymic complex assembled and activated in membranes of stimulated phagocytes. The active enzyme generates superoxide anions which participate in the killing of micro-organisms. NADPH oxidase can also be activated in cell fragments, consisting of neutrophil plasma membranes supplemented with several cytosolic proteins and a suitable detergent (e.g. arachidonate or SDS) [11,12]. The heterodimeric cytochrome b_{558} acting as an electron carrier is localized to the membranes [13], while the cytosol provides the three essential proteins linked to the oxidase, p47-phox, p67-phox [14,15] and the recently identified low-molecular-mass GTP-binding protein [16–19]. Recent studies demonstrated that the assembly of the enzyme involves translocation of a fraction of p47-phox and p67-phox to the cell membrane [20,21] and that active NADPH oxidase is associated with the cytoskeleton of neutrophils [22]. Association of p67-phox with the cytoskeleton of resting and activated cells and of p47-phox with the cytoskeleton of activated cells has also been suggested [21].

The previously described inhibition of SDS-mediated cell-free NADPH oxidase activation by a mixture of PPIs but not

by cardiolipin, phosphatidylserine (PS) or didodecanoyl phosphatidate was ascribed by us to a specific interaction of an essential cytosolic component of the oxidase with the lipid [7]. The possibility that this interaction with PPI might bear relevance to the assembly of active NADPH oxidase at the cell membrane prompted us to investigate the reaction further. In the present study we show that a cytosolic component of the oxidase is inactivated by association with PPI and that this inactivation is abrogated by Mg^{2+} ions. We suggest that p67-phox is the PPI-binding component of neutrophil cytosol.

MATERIALS AND METHODS

Materials

PPIs were obtained from Sigma as a mixture of PIP_2 and PIP (15–20 %) with PI and PS (30–35 %). Dextran and Ficoll-Paque were products of Pharmacia. All other phospholipids, chemicals, immunochemicals and resins were purchased from Sigma. Goat antisera to p47-phox and p67-phox were a gift from Dr. H. L. Malech and Dr. T. L. Leto. The recombinant p67-phox (rec-p67-phox) was a gift from Dr. F. B. Wientjes and Dr. A. W. Segal.

Fractionation of neutrophils

Human neutrophils were isolated from fresh buffy coats by standard procedures. Cells (10^8 /ml) in 10 mM potassium phosphate-buffered saline (pH 7.0) (PBS) containing 1 mM EGTA, 3.5 mM phenylmethanesulphonyl fluoride (PMSF) and leupeptin (15 μ g/ml) were disrupted by sonication and fractionated into soluble cytosolic and light membrane fractions as described [23].

NADPH oxidase activation and assay

Arachidonate-dependent activation was performed in two successive steps [23]. In the first step, membranes and cytosol

Abbreviations used: PPI, phosphoinositide; HBS, Hepes-buffered saline; PPI-cyt, cytosol preincubated with PPI; PIP, phosphatidylinositol 4-phosphate; PIP_2 , phosphatidylinositol 4,5-bisphosphate; PMSF, phenylmethanesulphonyl fluoride; rec-p67-phox, recombinant p67-phox; PI, phosphatidylinositol; PS, phosphatidylserine; GTP γ S, guanosine 5'-[γ -thio]triphosphate; CGD, chronic granulomatous disease.

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($1-2 \times 10^6$ and $2-4 \times 10^6$ cell equivalents respectively) were incubated with arachidonate ($50-150 \mu\text{M}$) in 0.2 ml of 0.34 M sucrose/50 mM PBS, pH 7.0 (sucrose-PBS) containing 1 mM EGTA and 5 mM MgCl_2 . After 6 min at 30°C NADPH and cytochrome *c* in 0.5 ml of sucrose-PBS were added and reduction rates of cytochrome *c* were measured. The optimal arachidonate concentration was determined for each experiment.

SDS-dependent activation was carried out in a 0.8 ml vol. of 10 mM Hepes-buffered saline (HBS) (pH 6.7)/10 μM flavin adenine dinucleotide/80 μM cytochrome *c*/1 mM EGTA using 2×10^6 and 4×10^6 cell equivalents of membranes and cytosol respectively at a concentration of approx. 60 μM SDS, as described [7].

Preparation of PPI vesicles

A solution of PPI in chloroform/methanol (2:1, v/v) was stored at -70°C . Before use the solvent was dried under a stream of N_2 and the lipid suspended in HBS at 0.5 mg/ml. For NADPH oxidase assays vesicles were submitted to a short period of sonication (10 s).

Preparation of PPI-pretreated cytosol

The suspension of PPI vesicles was enriched in heavy vesicles by two successive 1 h centrifugations at 200000 *g* and resuspension in the original volume of HBS. A sample (0.2 ml) of cytosol was mixed with 0.12 ml of the vesicles and with 0.18 ml of PBS. After 20 min incubation at room temperature the vesicles were pelleted by centrifugation at 200000 *g* for 1 h. The concentration of the PPI-treated cytosol obtained as the supernatant was 4×10^7 cell equivalents/ml.

Fractionation of cytosolic proteins on an S-Sepharose column

Cytosol (1 ml) was fractionated as described previously [23]. Oxidase-linked p47-phox and p67-phox were identified in column fractions by immunoblot analysis with specific antibodies. NADPH oxidase activity was determined in a two-step arachidonate-mediated system using 40 μl of each one of the indicated column fractions and neutrophil membranes supplemented with 10 μM guanosine 5'-[γ -thio]triphosphate ($\text{GTP}\gamma\text{S}$).

Fractionation of cytosolic proteins on a Q-Sepharose column

Cytosol (1 ml) was loaded on to a 1-ml Q-Sepharose column pre-equilibrated with 20 mM Hepes (pH 7.5)/0.15 M NaCl. After washing with 6 ml of the column buffer, proteins were eluted stepwise with three successive volumes (5 ml) of buffer solutions containing 0.3 M, 0.5 M and 1.0 M NaCl (fractions 9–10, 14–15 and 19–20 respectively). Fractions (1 ml) were collected and their absorbance values at 280 nm were determined. Aliquots (40 μl) of indicated fractions were pooled and used for evaluation of NADPH oxidase activity in the arachidonic acid-supported two-step assay in the presence of membranes and 10 μM $\text{GTP}\gamma\text{S}$.

Interaction of rec-p67-phox with PPI

The protein (1.0 μg in 50 μl of sucrose-PBS) [24] was exposed to 5 μg of PPI. After 1 min, 5 mM MgCl_2 followed by buffer, the flowthrough of the Q-Sepharose column, membranes, $\text{GTP}\gamma\text{S}$ and arachidonate were added and activation of NADPH oxidase was determined.

Fluorescence measurements

Fluorescence emission spectra of a solution of rec-p67-phox (2.0 μg of protein) in 0.8 ml of PBS were recorded in an SLM-

Aminco 8000 spectrofluorimeter (SLM Instruments, Urbana, IL, U.S.A.). An excitation wavelength of 280 nm and slit widths of 4 nm were used. Spectra of PPI in buffer (at 0.12 mM and 5.0 mM MgCl_2 respectively) were subtracted from the final spectra of rec-p67-phox recorded in the presence of PPI (at similar MgCl_2 concentrations respectively) after stabilization of the readings. Time-dependent reduction of the fluorescence intensity after addition of PPI was measured at 340 nm.

SDS/PAGE

This was performed in a Minigel system (Hoefer) in 5–15% gradient gels [25].

Immunoblot analysis

Proteins on SDS/PAGE gels were transferred on to nitrocellulose, probed with goat antisera raised against rec-p47-phox and rec-p67-phox (1:1000) and developed as described [23].

Protein determination

The method of Lowry was employed, using BSA as a standard [26].

RESULTS

Effect of Mg^{2+} ions on the activation of NADPH oxidase and on its inhibition by PPI

In a previous report [7] we described blockade of SDS-mediated activation of NADPH oxidase by very low amounts of PPI, present during activation of the enzyme. Moreover, cytosol pretreated with PPI vesicles and then ultracentrifuged exhibited a loss of activity in SDS-mediated activation [7]. As shown in Table 1, PPI-pretreated cytosol also lost activity in arachidonate-mediated activation, suggesting that exposure to PPI depleted or inactivated cytosolic component/s essential for the generation of a functional oxidase by both detergents. Unexpectedly, however, the presence of PPI during arachidonate-supported activation did not interfere with the process (Table 1). In searching for an explanation for this finding, we compared the experimental conditions at which both activating detergents were used: the SDS-mediated assay, which exhibited a weak stimulation at low MgCl_2 concentrations and inhibition above 0.5 mM MgCl_2 .

Table 1 The effect of PPI on the activation of NADPH oxidase by SDS and arachidonate

Exposure to PPI	Activating detergent*	NADPH oxidase activity (% of control)
Pretreatment of cytosol†	SDS	18.3 ± 2.2
	Arachidonate	12.9 ± 4.8
During activation‡	SDS	29.2 ± 4.2
	Arachidonate	96.8 ± 8.6
	SDS, 0.5 mM Mg^{2+}	72.1 ± 2.1

* The activation was carried out at the optimal concentration of Mg^{2+} in each case: with SDS no Mg^{2+} was added, with arachidonate the activation medium was supplemented with 5 mM Mg^{2+} [27]. SDS-mediated activation was progressively inhibited above 0.5 mM Mg^{2+} (results not shown).

† PPI vesicles were preincubated with cytosol and sedimented by centrifugation as described in the Material and methods section.

‡ 5 μg of PPI were present during the activation.

(results not shown), was routinely carried out in the absence of exogenous Mg^{2+} ions, while arachidonate-dependent reaction medium was supplemented with 5 mM Mg^{2+} , resulting in an optimal activation [27]. Since phosphoinositides bind Mg^{2+} , the possibility was raised that the high Mg^{2+} concentration present during activation with arachidonate provided protection against inhibition by PPI. This hypothesis was tested by carrying out activation with SDS in the presence of 0.5 mM $MgCl_2$: indeed the blockade by PPI was significantly reduced under these conditions (Table 1).

Elimination of the inhibitory effect of PPI on the activation of NADPH oxidase by neomycin

It was of interest to establish whether blockade of NADPH oxidase activation by PPI could be relieved by the presence of neomycin, which has been implicated in a specific interaction with PPIs [28]. Elsewhere it was shown that low concentrations of neomycin interfered with cell-free activation of the oxidase [29]. Blockade of activation by PPI was indeed progressively eliminated by increasing amounts of neomycin and, vice versa, the inhibitory effect of neomycin was reduced by raising the concentration of PPI in the activation medium. In Figure 1 optimal doses of neomycin and PPI required for abrogation of the inhibition by each one of the compounds are demonstrated. The neomycin- PIP_2 complex appeared devoid of inhibitory activity which resided in the functional groups of both compounds.

Restoration of the activity of PPI-pretreated cytosol by gradient-eluted fractions from an S-Sepharose column

Chromatography on an S-Sepharose cation-exchange column permits separation of neutrophil cytosol into two main fractions, the combination of which synergistically supports cell-free activation (Table 2): the flowthrough S50 (column fractions 2 and 3) and the gradient-eluted peak S200 (column fractions 11 and 12) [23]. Immunoblot analysis identified p67-phox and p47-phox in the gradient-eluted fractions [23]. The flowthrough contains probably the third essential cytosolic component, rac1 or rac2 protein [17,18]. To establish the identity of the protein depleted or inactivated by the treatment of cytosol with PPI liposomes (Table 1), complementation experiments of the pretreated cytosol with the flowthrough and p47-phox/p67-phox-containing fractions of untreated cytosol fractionated on an S-Sepharose column were carried out. Complementation with the flowthrough did not augment activity (Table 2); conversely, complementation with the gradient-eluted fraction fully restored activity, implying that PPI-pretreated cytosol was deficient in active p67-phox or/and p47-phox. Immunoblot analysis of the PPI-pretreated cytosol with anti-(p67-phox) and anti-(p47-phox) antisera demonstrated that both proteins were present in the inactive cytosol (data not shown), suggesting that the loss of activity was not caused by sedimentation of the oxidase-linked protein(s) with the liposomes during ultracentrifugation, but rather by their inactivation.

PPI-induced inactivation of the cytosol: dose dependency and kinetic course

Inactivation was estimated by incubation of aliquots of cytosol with increasing amounts of PPI (1 min), addition of $MgCl_2$ (final concentration 5 mM), membranes, buffer and arachidonate, and subsequent determination of the residual oxidase-supporting activity in the arachidonate-mediated assay, namely at conditions at which no further PPI-elicited inactivation occurred (Table 1). The dose dependency of the effect is shown in Figure 2. The

Figure 1 Elimination of PPI-mediated inhibition of NADPH oxidase activation by neomycin and abrogation of neomycin-induced blockade by PIP_2

SDS was used as the oxidase-activating detergent. PPI concn., 5 μ g/assay; neomycin concn., 5.5 μ M. Abbreviation: neo, neomycin.

Table 2 Reconstitution of PPI-pretreated cytosol by S-Sepharose column fractions

Numbers given in subscript parentheses refer to column fractions. S50 and S200 are column fractions eluted by 50 mM and 200 mM NaCl respectively.

Cytosolic fraction	Oxidase activity (nmol of O_2 /min per mg of protein)
PPI-pretreated cytosol (PPI-cyt)*	70
Flowthrough of S-Sepharose column S50 ₍₂₊₃₎ †	0
Gradient fractions S200 ₍₁₁₊₁₂₎ †	0
S50 ₍₂₊₃₎ + S200 ₍₁₁₊₁₂₎ †	350
PPI-cyt + S50 ₍₂₊₃₎	73
PPI-cyt + S200 ₍₁₁₊₁₂₎	472

* Cytosol was preincubated with PPI vesicles and centrifuged as described in the Materials and methods section.

† NADPH oxidase activation mixture contained 40 μ l of each indicated fraction. The results are expressed as mean values of a representative experiment repeated four times with essentially similar results.

Figure 2 Dose dependency of the inhibition of the NADPH oxidase-supporting activity of cytosol by PPI

Aliquots (40 μ l) of cytosol were preincubated with PPI in a final volume of 50 μ l; after 1 min $MgCl_2$ was added (to give a final concentration of 5 mM) and the residual cytosolic activity was determined in arachidonate-mediated assay. The data are mean values of a representative experiment repeated in duplicate four times.

Figure 3 Time course of inactivation of cytosol by 5 µg of PPI (○)

At indicated times after mixing PPI with cytosol, Mg^{2+} was added followed by other components of the arachidonate-mediated NADPH oxidase activation assay. ●, 5 mM $MgCl_2$ was present during the preincubation with PPI.

inhibition by 5 µg of PPI was time dependent and completed within a few minutes ($t_{1/2}$ = approx. 30 s) (Figure 3). When 5 mM Mg^{2+} was present during the period of preincubation with PPI, full cytosolic activity was retained (Figure 3). Complete protection required the presence of Mg^{2+} ions at concentrations above 1 mM (results not shown). In a few experiments in which purified PIP_2 and PIP were used, a dose-dependent inhibition of cytosolic NADPH oxidase-activating capacity was also observed: doses required for 50% inhibition were 2.0 µg for PIP_2 and 5.5 µg for PIP.

Identification of the PPI-inactivated component on Q-Sepharose

Identification of the inactivated component required separation of p67-phox and p47-phox. This was accomplished by chromatography of cytosol on a Q-Sepharose anion-exchange column equilibrated with 0.15 M NaCl and eluted stepwise with NaCl (Figure 4a) [30]. Immunoblot analysis revealed that the p47-phox component eluted in the flowthrough of the column (fractions 2–4, Q150), while p67-phox bound to the resin and was eluted by 0.3 M NaCl (fractions 9 and 10, Q300) (Figure 4b). The low-molecular-mass G-protein [16–19], was probably present in the flowthrough of the column [16]. While both the flowthrough (Q150) and the 0.3 M NaCl-eluted (Q300) fractions were inactive when tested separately in the NADPH oxidase assay, combination of both reconstituted oxidase activity (Table 3). Oxidase activity could also be reconstituted by the addition of recombinant p67 to the flowthrough of the Q-Sepharose column (Table 3). Column fractions eluted by higher concentrations of NaCl (0.5 M and 1.0 M) did not contain p47-phox/p67-phox (Figure 4b) or exhibit oxidase-supporting activity (results not shown).

When cytosol premixed with PPI (cytosol–PPI) was fractionated on Q-Sepharose, the combination of the flowthrough with the 0.3 M NaCl-eluted fraction (Q300–PPI) exhibited, as expected, a significantly reduced activity (Table 3). The activity was not augmented by the addition of fractions eluted by 0.5 M and 1.0 M NaCl (results not shown). Supplementation of the 0.3 M NaCl-eluted fraction of the cytosol–PPI-treated column (Q300–PPI) with the flowthrough of the control cytosol-treated column (Q150–c) was unable to restore activity; the reciprocal combination of the flowthrough of the cytosol–PPI column (Q150–PPI) with the 0.3 M–NaCl-eluted fraction of the control column (Q300–c) restored full superoxide-generating activity (Table 3). Immunoblot analysis of cytosol–PPI fractions from the Q-

Figure 4 Fractionation of cytosol (1 ml) on a Q-Sepharose column

(a) The pattern of protein distribution; cytosol–PPI (1 ml of cytosol mixed with 125 µg of PPI) exhibited a similar distribution of proteins. (b) and (c) Immunoblot analysis of the Q-Sepharose column fractions of control cytosol (b) and cytosol–PPI (c) with a mixture of anti-(p47-phox) and anti-(p67-phox) antibodies. Lanes 1, flowthrough; lanes 2, 3 and 4, fractions eluted with 0.3 M, 0.5 M and 1.0 M NaCl respectively.

Table 3 Effect of PPI on the activity of p67-phox and p47-phox fractionated on a Q-Sepharose column

Abbreviations used: Q150-c and Q300-c, flowthrough and active p67-phox-containing fractions respectively of a control-cytosol-fractionating column (see Figure 4); Q150-PPI and Q300-PPI, flowthrough and 0.3 M NaCl-eluted fractions respectively of Q-Sepharose column loaded with cytosol premixed with PPI; rec-p67 and rec-p67-PPI were tested at 1 µg/assay. Results are of a representative experiment repeated three times with similar results.

Cytosolic components combined	Activity (%)
Q150-c + Q300-c	100.0
Q150-PPI + Q300-PPI	24.8
Q150-c + Q300-PPI	3.3
Q300-c + Q150-PPI	98.0
Q150-c + rec-p67	77.0
Q150-c + rec-p67-PPI	17.0
Q150-c	9.1
Q300-c	1.3
Q150-PPI	11.1
Q300-PPI	1.3

Sepharose column revealed the presence of p67-phox in 0.3 M-eluted fractions, as well as in 0.5 M NaCl-eluted fractions, of the column (Figure 4c). This altered distribution of the protein suggested an increased anionic charge, resulting probably from an association of p67-phox with anionic phospholipids binding to the resin. The 0.5 M NaCl-eluted fractions were inactive in reconstitution experiments and did not enhance activity of the 0.3 M–NaCl-eluted fraction (results not shown). The results of the experiment implicated this latter fraction (Q300–PPI) in the inactivation by PPI (Table 3, Figure 4).

The capacity of rec-p67-phox to complement the flowthrough of the Q-Sepharose column was lost upon exposure to PPI (Table

Figure 5 Fluorescence emission spectra of rec-p67-phox (2.0 μ g in 0.8 ml) in the absence and presence of PPI (10 μ g)

Spectra in the presence of PPI represent the final stable spectrum obtained after the addition of PPI. Upper panel, plus 5 mM $MgCl_2$.

3). The presence of Mg^{2+} prevented this inactivation (results not shown). These results corroborated the identification of p67-phox at the PPI-susceptible oxidase component.

The effect of PPI on the fluorescence emission spectrum of rec-p67-phox

Figure 5 depicts the fluorescence emission spectrum of rec-p67-phox in the presence and absence of PPI. The addition of PPI resulted in a slow quenching of the fluorescence of the protein, which was completed within 5 min (results not shown). PPI quenched the fluorescence of rec-p67-phox in the presence of $MgCl_2$ also (Figure 5), implying interaction in conditions at which the activity of p67-phox was not affected (Table 1).

DISCUSSION

Three cytosolic proteins, p47-phox, p67-phox [14,15] and a low-molecular-mass GTP-binding protein [16–19] are essential for the formation of an active NADPH oxidase complex. Cells of patients suffering from autosomal recessive chronic granulomatous disease (CGD) are deficient in p67-phox or p47-phox and exhibit an impaired capacity to produce oxygen radicals and combat infections [14,15].

Recently we described the inhibition of cell-free activation of NADPH oxidase by individual inositol phospholipids, as well as by their mixture (PPI) [7]. This preparation contained PS also, which exhibited no effect on the activation of the oxidase [7]. In the present study we further characterized the interaction of the lipids with the cytosolic oxidase-linked component(s). As inhibition by purified phosphatidylinositol mono- and bisphosphate was confirmed, in most experiments the same preparation of PPI was used.

On the basis of fractionation and complementation experiments cytosolic p67-phox was suggested to be the component inactivated by PPI (Figure 4, Table 3). This conclusion was further supported by the PPI-induced inactivation of rec-p67-phox at similar conditions (Table 3). The altered mobility exhibited by p67-phox on fractionation of a mixture of cytosol with PPI on a Q-Sepharose column, was consistent with an interaction of the protein with the negatively charged phospholipids bound electrostatically to the resin (Figure 4c, Table 3). Moreover, quenching of the fluorescence of rec-p67-phox upon exposure to PPI also suggested an interaction of the protein with the lipid (Figure 5). A similar quenching of fluorescence of protein kinase C and profilin by PIP_2 has recently been reported [6,31]. It is noteworthy that the high degree of exposure of tryptophan residues in rec-p67-phox, suggested by the red emission of the protein [32] (Figure 5), was not affected by the interaction with PPI. It thus appears that the PPI-binding and emitting regions of the molecule do not overlap.

While cytosol pre-exposed to PPI at low Mg^{2+} concentrations became similar in its functional properties to the p67-phox-deficient cytosol of CGD patients [14,15], adequate concentrations of Mg^{2+} protected cytosolic and rec-p67-phox from inactivation by PPI. The fluorescence of rec-p67-phox was, however, affected by PPI in a similar manner in the presence of low and high Mg^{2+} concentrations, implying interaction in both cases (Figure 5). It may be inferred that whereas at Mg^{2+} concentrations prevailing *in vivo*, inactivation of p67-phox by endogenous PPI is unlikely, association of the protein with the lipid in the intact cell can be expected. Similar findings were reported in the case of protein kinase C: fluorescence measurements indicated association with PIP_2 in the presence, as well as in the absence, of Mg^{2+} , while inactivation by the lipid was observed only in its absence [6].

It is noteworthy that p67-phox and p47-phox were retained in the supernatant of cytosol pretreated with PPI liposomes and did not associate with the pelleted lipid. As interaction of proteins with phospholipids depends on their state of aggregation [1] it may be suggested that p67-phox associates specifically with small aggregates of PPIs which remained in the supernatant after centrifugation. Binding by profilin of small clusters of PIP_2 molecules has been documented [43]. Alternatively, p67-phox might have interacted with PPI irrespective of the size of its vesicle, undergone a change in conformation linked to inactivation, and finally dissociated from the vesicle.

The mechanism by which cell-free activation of NADPH oxidase is blocked by micromolar concentrations of neomycin [29] (Figure 1) is not understood. It does not involve inhibition of phospholipase C-mediated hydrolysis of PIP_2 by the drug, since cleavage of PIP_2 is not required for detergent-mediated activation [34,35]. The described association of p67-phox with inositol phospholipids may, however, reflect a requirement for an inositol phospholipid in the course of assembly of the active oxidase in the cell-free system. Sequestration of the phospholipid by neomycin would then be expected to interfere with the activation and could be overcome by the addition of appropriate amounts of PPI (Figure 1).

Although both p47-phox and p67-phox have been purified [36,37], cloned and expressed [24,38–40], their exact function in the activation of NADPH oxidase has not been elucidated. Recent studies described activation-dependent partial translocation of p47-phox and p67-phox to the cell membrane [20] and presented evidence for the association of p67-phox with the cytoskeleton of resting [21] and activated neutrophils [22]. Cytoskeletal association of p67-phox is consistent with the presence of SH3 sequences in the molecule [41,42]. On the basis of the affinity of p67-phox for PPI it may be speculated that endogenous PIP₂ acts as a membrane- or cytoskeletal-anchor for the cytosolic oxidase component. In this respect, it is of note that the proteins implicated in structural and functional interaction with PPI are also linked to the cytoskeleton [1–3,31,43].

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