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Kinetics of cell-free activation of neutrophil NADPH oxidase

Effects of neomycin and guanine nucleotides

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The effects of neomycin, fluoride and the non-hydrolysable guanine nucleotide analogue GTPγS on the kinetics of cell-free activation of NADPH oxidase in membranes of resting human neutrophils were investigated. Arachidonate-mediated activation of the oxidase followed a first-order reaction course ($k_{obs.}$ = 0.39 min^{-1} at 26 °C). In the presence of NaF during the activation process, activity was enhanced while the activation rate was slightly reduced ($k_{obs.}$ = 0.25 min^{-1} at 26 °C). Neomycin blocked activation (half-maximal effect at 25 μM) without affecting rates of superoxide release by preactivated enzyme *in vitro* or *in vivo*. In spite of reduced specific activity neither the first-order rate constant of the activation nor the K_m of the oxidase were altered by neomycin. Oxidase activated in the presence of GTPγS exhibited increased specific activity and unchanged K_m; the course of the reaction deviated from first-order kinetics. Kinetic evidence is presented for two separate activation reactions: a GTPγS-independent, basal, first-order process and a GTPγS-dependent sigmoid activation process. The results are compatible with the existence in neutrophil membranes of two separate pools of dormant oxidase. An alternative scheme of the formation of two active forms of NADPH oxidase is also presented.

INTRODUCTION

The superoxide-producing, membrane-bound NADPH oxidase of neutrophils and other phagocytes plays an important role in their microbicidal activity (Curnutte & Babior, 1986; Rossi, 1986). The enzymic complex, consisting of at least two protein components [a flavoprotein (Babior & Kipnes, 1977) and a cytochrome *b*558 (Segal & Jones, 1978)] undergoes activation upon exposure of the cell to appropriate stimuli. The exact molecular mechanism underlying this process is unknown. For certain stimuli, e.g. the chemotactic peptide, the involvement of G-proteins known to couple surface receptors to phospholipase C-catalysed hydrolysis of polyphosphoinositides has been demonstrated (Ohta *et al.*, 1985; Volpi *et al.*, 1985).

A procedure for the cell-free activation of the NADPH oxidase complex has been described (Heyneman & Vercauteren, 1984; Bromberg & Pick, 1984; Curnutte, 1985; McPhail *et al.*, 1985). The components necessary for activation are plasma membranes, cytoplasmic proteins of resting neutrophils or macrophages, and low concentrations of amphipathic anionic compounds represented by long-chain unsaturated fatty acids (e.g. arachidonic acid) or anionic detergents (e.g. SDS) (Bromberg & Pick, 1985). Non-hydrolysable analogues of guanine nucleotides (GTPγS or GppNHp) were shown to enhance activity of the oxidase activated *in vitro* (Gabig *et al.*, 1987; Seifert & Schultz, 1987; Clark *et al.*, 1987). The mode of action as well as the target of the guanine nucleotide analogues have not yet been elucidated, and it is unclear whether their participation bears any resemblance to the G-protein-mediated, phospholipase C-catalysed hydrolysis of polyphosphoinositides *in vivo* (Ohta *et al.*, 1985; Volpi *et al.*, 1985).

In an attempt to characterize the cell-free activation of the NADPH oxidase in membranes of human neutrophils and to define its relationship with signal transduction pathways *in vivo*, we investigated the kinetics of guanine nucleotide effects on the process *in vitro*. In addition, we tested the NADPH oxidase system for possible inhibition by the aminoglycosidic antibiotic neomycin. Neomycin has been implicated in a specific interaction with polyphosphoinositides (Lodhi *et al.*, 1979) and in subsequent inhibition of polyphosphoinositides phospholipase C activity in various semi-permeabilized cells and isolated membranes (Cockcroft & Gomperts, 1985; Burch *et al.*, 1986). The inhibition by neomycin of NADPH oxidase activation *in vitro* and the kinetic effects of fluoride and GTPγS on the process are described.

MATERIALS AND METHODS

Materials

Chemicals were purchased from Sigma. Dextran and Ficoll-Paque were products of Pharmacia.

Fractionation of neutrophils

Human neutrophils were isolated from fresh buffy coats by the standard procedure of dextran sedimentation and Ficoll density-gradient centrifugation. Cells (2×10^8/ml) in 10 mM-potassium phosphate-buffered saline (PBS; pH 7.0) containing 1 mM-EGTA, 7 mM-phenylmethanesulphonyl fluoride (PMSF) and 15 μg of leupeptin/ml were disrupted by sonication (3 × 20 s) with the microprobe of a Microson sonicator (Heat Systems Ultrasonics Inc., New York, NY, U.S.A.) set at 30% power output. Fractionation was carried out essentially as described by Fujita *et al.* (1987). First, the postnuclear

Abbreviations used: GTPγS, guanosine 5-[γ-thio]triphosphate; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethanesulphonyl fluoride; PBS, phosphate-buffered saline.

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sonicates were centrifuged for 10 min at 10000 *g* to sediment granular material; supernatants were then recentrifuged for 45 min at 100000 *g* to separate low-density membranes from cytosolic fractions employed in the NADPH oxidase activation experiments. Membranes were resuspended in PBS/0.34 M-sucrose (sucrose/PBS).

NADPH oxidase activation and assay

The activation and assay were performed in two successive steps, using a modification of the procedure of Gabig *et al.* (1987). First, membranes (8–20 μg), cytosol (140–300 μg), sucrose/PBS buffer supplemented with 1 mM-EGTA and arachidonate (240–300 μM) were mixed in a final volume of 0.1 ml in the presence of 5 mM-magnesium acetate. After 6 min at 26 °C (unless otherwise stated), the activation mixture was diluted with 0.8 ml of sucrose/PBS/EGTA buffer, and 0.2 mM-NADPH plus 900 μg of cytochrome *c* were added to give a final volume of 1.0 ml. Cytochrome *c* reduction rates were measured at 550 nm before and after the addition of 30 μg of superoxide dismutase. The difference between the rates represents superoxide-dependent reduction of cytochrome *c* (Babior & Kipnes, 1977). For estimation of the K_m for NADPH of the NADPH oxidase activated *in vitro*, essentially the same procedure was employed. The activation was carried out in aliquots as described above, and the activity of the preactivated enzyme was determined after addition of different concentrations of NADPH to each aliquot. Results were calculated using a non-linear regression computer program.

Activation of the oxidase with SDS (50 μM) was carried out in a final volume of 1.0 ml of sucrose/PBS, pH 6.7/10 μM-FAD/70 μM-cytochrome *c*/1 mM-EGTA (Fujita *et al.*, 1987). NADPH (0.2 mM) was added after 5 min of preincubation. In experiments conducted in the presence of GTPγS, Mg^{2+} was included in the activating medium.

Preparation of particulate NADPH oxidase from prestimulated cells

Neutrophils suspended in Krebs–Ringer phosphate-glucose medium (10^8/ml) were first incubated at 37 °C for 3 min with 1 μg of phorbol 12-myristate 13-acetate (PMA)/ml and disrupted by sonication. Total cell membranes sedimented from postnuclear sonicates by centrifugation at 100000 *g* were used as a source of the oxidase.

Results reported in the text are mean values from representative experiments performed in duplicate and repeated at least four times.

RESULTS

Kinetic course of arachidonate-promoted activation of NADPH oxidase

Addition of arachidonate or SDS to a mixture of plasma membranes and cytosolic fractions of resting neutrophils induces production of superoxide ions due to activation of the dormant NADPH oxidase (Heyneman & Vercauteren, 1984; Bromberg & Pick, 1984; Curnutte, 1985; McPhail *et al.*, 1985). To permit direct assessment of the course of activation, a two-step experimental procedure was employed. In the first, or activation, step, membranes, cytosol and arachidonate were preincubated in 0.1 ml of Mg^{2+}-containing buffer in the absence of both the substrate (NADPH) and the superoxide detection system, cytochrome *c*. At various intervals, a 10-fold dilution of the activation mixture was made, with a subsequent addition of NADPH and cytochrome *c*. In this second step of the reaction, superoxide dismutase-inhibitable reduction of cytochrome *c*, reflecting the rate of oxidase-catalysed superoxide generation, was measured. Since in the first step enzyme turnover was prevented by the absence of the substrate, while in the second, the low arachidonate and Mg^{2+} concentrations were unsuitable for significant activation (Gabig *et al.*, 1987; Ligeti *et al.*, 1987), a clear distinction between the activation and activity phases was possible, permitting evaluation of the kinetics of the activation process. Representative results are shown in Fig. 1. The activity of the enzyme determined in the second step exhibited an exponential dependence on the duration of the activation; the results were consistent with a first-order activation of the NADPH oxidase undergoing completion during the routine 6 min period of preincubation (at 26 °C, $k_{obs.} = 0.39 \pm 0.04\ min^{-1}$). Rates of activation determined between 20–34 °C increased with temperature. An activation energy of 50 kJ/mol was estimated for this variation (results not shown).

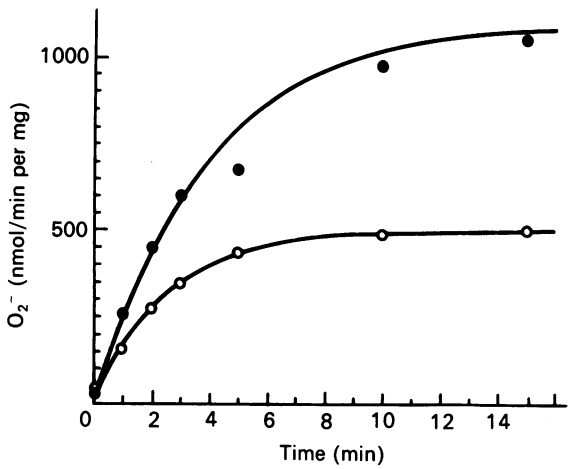

Fig. 1. Dependence of NADPH oxidase activity on preincubation time

Activation mixture (0.1 ml total vol.) consisted of neutrophil plasma membranes (9 μg), cytosolic protein (180 μg) and 310 μM-arachidonate. At various times the activation mixture was diluted with PBS/sucrose buffer supplemented with NADPH and cytochrome *c*. ○, No additions; ●, 20 mM-NaF was present during the preincubation. Activation and activity measurements were carried out at 26 °C. The lines are the theoretical first-order computer-fitted curves to the experimental points.

Effect of GTPγS on the course of NADPH oxidase activation

In agreement with several recent reports (Gabig *et al.*, 1987; Seifert & Schultz, 1987; Clark *et al.*, 1987), the presence of GTPγS during the first phase of the reaction markedly augmented the activity of the arachidonate-activated NADPH oxidase. The dose dependency of this effect determined using the standard 6 min activation time is shown in Fig. 2. The effect of GTPγS on the kinetics of activation was examined as summarized in Fig. 3. While activation of the oxidase in the absence of the nucleotide displayed clear first-order reaction

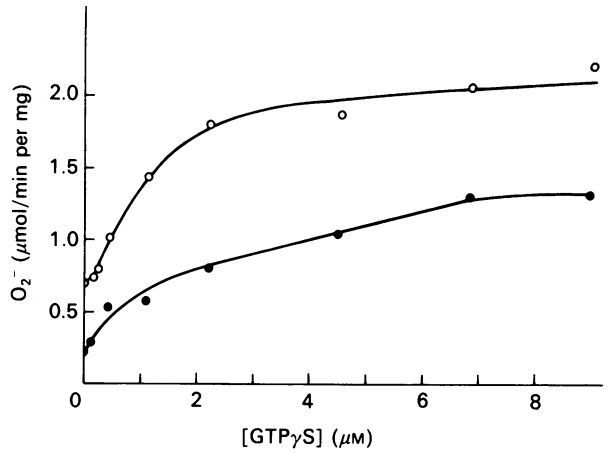

Fig. 2. Effect of GTPγS present in the NADPH oxidase activation mixture on the final activity of the enzyme

Activation was assessed in the absence (○) or presence (●) of 25 μM-neomycin during the activation phase. The assay was carried out at 34 °C; other conditions were as described in Fig. 1 legend.

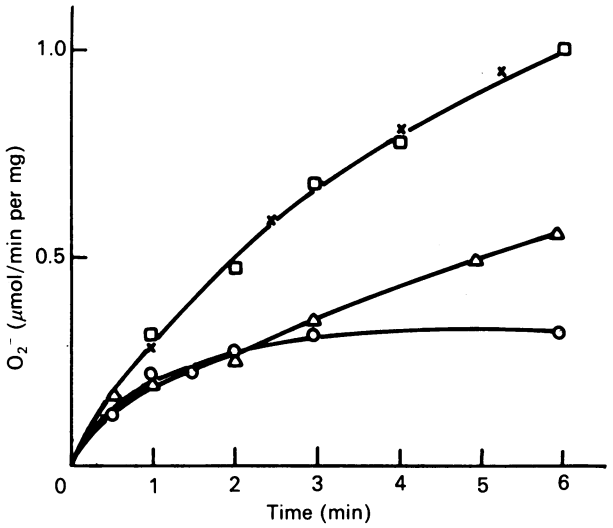

Fig. 3. Dependence of NADPH oxidase activation time course on the concentration of GTPγS in the activation medium

○, Standard activation mixture; △, 1 μM-; □, 5 μM-; and ×, 10 μM-GTPγS. Assays were performed at 34 °C; other conditions were as specified in Fig. 1 legend.

kinetics, in the presence of GTPγS a different activation course was observed and longer incubation times were required to reach maximal activity. Increasing concentrations of the nucleotide analogue augmented the activity of the oxidase, without shortening the time required for maximal activation. At saturating doses of GTPγS (above 5 μM), a further increase in its concentration had no effect on the activation profile, indicating that the slow activation process reflected a rate-limiting step taking place after the initial binding of GTPγS to a component of the system (Fig. 3). This slow activation required the simultaneous presence of all components of the system, including arachidonate, and was not altered by a preincubation of GTPγS with membranes, cytosol or membranes plus cytosol (results not shown). Surprisingly, when SDS was substituted for arachidonate, the enhancing effect of GTPγS was not observed (results not shown).

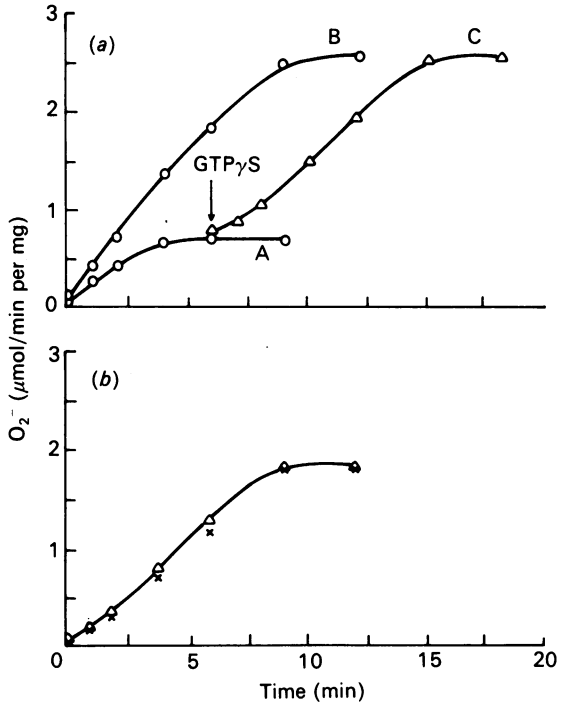

Fig. 4. Time course of NADPH oxidase activation

(*a*) Curve A, standard activation mixture; curve B, same plus 5 μM-GTPγS; curve C, at the indicated time, 5 μM-GTPγS was added to the undiluted activation mixture and the preincubation continued as indicated. (*b*) △, Net-GTPγS-induced activation curve calculated by subtraction of basal NADPH oxidase activity from activity values depicted in curve C; ×, activity values obtained by subtraction of basal activity (curve A) from the appropriate activity values shown in curve B. The activation mixture consisted of 10.5 μg of plasma membrane protein, 140 μg of cytosolic protein and 240 μM-arachidonate. Assays were carried out at 26 °C.

The mode of action of GTPγS was further investigated in the experiment summarized in Fig. 4. Activation courses of NADPH oxidase in the absence (Fig. 4*a*, curve A) or presence (curve B) of GTPγS were determined by the regular procedure. In a separate experiment the nucleotide was added to the non-diluted activation mixture at 6 min, i.e. after the basal, first-order activation process has been completed. The incubation was then continued for various time intervals and the activity of the oxidase was determined. The results indicated the presence of a second, GTPγS-elicited, sigmoid activation step (Fig. 4*a*; curve C); each experimental point of curve C represents the basal activity of the enzyme at the time of GTPγS addition plus activity induced by the nucleotide. Fig. 4(*b*) depicts the net course of GTPγS-induced activation obtained by subtraction of the basal activity from the experimentally obtained values in curve C. Included in the Figure are activity values obtained by subtraction of the basal activation curve (A) from the activation curve determined in the presence of GTPγS introduced at the beginning of the activation (B). The good correspondence between the two sets of data

suggests that activation of NADPH oxidase measured in the presence of GTPγS may consist of two separate reactions, experimentally resolved by the addition of the nucleotide after the initial basal activation has been completed.

Activation of NADPH oxidase in the presence of fluoride

Gabig *et al.* (1987), Seifert & Schultz (1987) and Clark *et al.* (1987) observed that NaF augments the activity of NADPH oxidase in isolated neutrophil membranes. Since fluoride is well recognized for its activating interaction with G-proteins (Sternweis & Gilman, 1982), the finding corroborated the hypothesis of participation of a GTP-binding protein in the cell-free activation of the oxidase. In the present study, the course of activation of the enzyme in the presence of 20 mM-NaF was followed. The results depicted in Fig. 1 indicate that in spite of the enhancement of oxidase activity, fluoride did not significantly alter the course of activation: a first-order rate constant of 0.25 ± 0.03 min^{-1} was estimated in its presence, compared with 0.39 ± 0.04 min^{-1} evaluated at identical conditions in the absence of NaF.

Inhibition of NADPH oxidase activation by neomycin

Neomycin, shown to interact specifically with polyphosphoinositides (Lodhi *et al.*, 1979), is commonly regarded as an inhibitor of polyphosphoinositide phospolipase C. If, as implied by the effects of GTPγS and fluoride, hydrolysis of polyphosphoinositides is indeed involved in cell-free activation of the oxidase, inhibition of the process by neomycin might be expected. Indeed, the presence of low doses of neomycin in the activation mixture blocked oxidase activity, as shown in Fig. 5. Inhibition by neomycin was also evident in the presence of GTPγS and vice versa: a fixed dose of neomycin did not abolish the enhancement by the nucleotide analogue (Fig. 2). Similarly to GTPγS (Gabig *et al.*, 1987), neomycin was active during the activation phase only: when present in the diluted reaction mixture after completion of the activation step, the drug failed to affect rates of superoxide production even at concentrations as high as 75 μM (Table 1). Likewise, rates of O_2^- production by particulate preparations of NADPH oxidase derived from phorbol ester-stimulated cells were not affected by neomycin, indicating that it did not interact with the active form of NADPH oxidase (Table 1). Neomycin was also unable to block the respiratory burst in phorbol ester- or chemotactic peptide-stimulated intact cells, probably due to the impermeability of their cell membranes to the drug (results not shown).

Contrary to GTPγS, neomycin interfered with activation of the enzyme without affecting its course: at 26 °C, the first-order rate constant evaluated in the presence of 25 μM-neomycin was 0.39 ± 0.1 min^{-1} (results not shown). The inhibitory effect of neomycin was retained when SDS was substituted for arachidonate (results not shown).

Kinetic parameters of NADPH oxidase

To characterize the properties of the oxidase activated in the presence and absence of neomycin or GTPγS, we determined the kinetic parameters of the enzyme obtained in each case by the routine activation procedure. As shown in Table 2, oxidase obtained in all three cases exhibited a similar K_m for NADPH and differed only in its specific activity or $V_{max.}$.

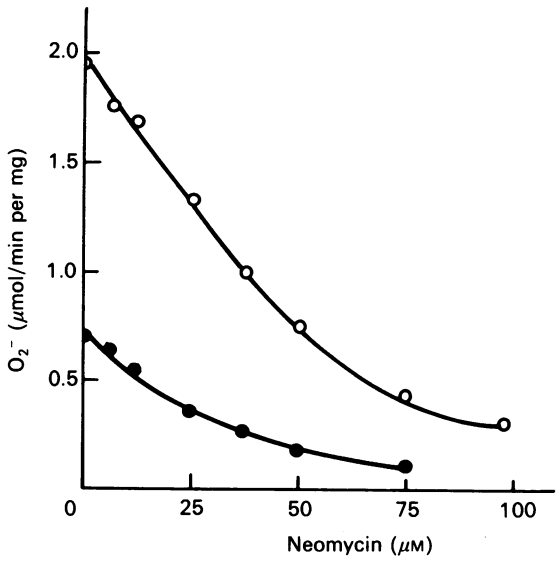

Fig. 5. Effect of neomycin on NADPH-dependent superoxide production measured in the absence (●) and presence (○) of 9.1 μM-GTPγS

Conditions were as described in Fig. 1 legend.

Table 1. Effect of neomycin on superoxide production by NADPH oxidase activated *in vitro*

These results are mean values of a representative experiment performed in triplicate.

System	Superoxide production (% of control)
Control	100.0*
+neomycin (75 μM) in activation mixture (0.1 ml)	22.9
+neomycin (75 μM) in reaction mixture (1.0 ml)	101.5
Membranes from PMA-stimulated cells	100.0†
+neomycin (75 μM)	103.2

* Control activity was 345 nmol of O_2^-/min per mg.
† Control activity was 50.1 nmol of O_2^-/min per mg.

Table 2. Effect of GTPγS and neomycin on kinetic parameters of NADPH oxidase

Activation and activity assays were conducted at 34 °C.

	K_m (μM)	$V_{max.}$ (nmol/min per mg)
Control	44.1 ± 10.9	1760
+GTPγS (1.5 μM)	43.8 ± 3.6	2333
+GTPγS (3.0 μM)	35.0 ± 3.2	4121
+Neomycin (25 μM)	43.7 ± 8.6	723

DISCUSSION

Enhancement of the cell-free activation of NADPH oxidase by guanine nucleotide analogues (Gabig *et al.*, 1987; Seifert & Schultz, 1987; Clark *et al.*, 1987) suggested a possible participation of G-proteins in the gener-

ation of the active enzyme. The polyphosphoinositide phospholipase C (Ohta *et al.*, 1985; Volpi *et al.*, 1985), or a component of the NADPH oxidase itself, were suggested as possible effectors acted upon by the putative G-protein (Gabig *et al.*, 1987; Seifert & Schultz, 1987; Clark *et al.*, 1987; Doussiere *et al.*, 1988). Augmentation of oxidase activity by NaF, known to activate G-proteins (Sternweis & Gilman, 1982), supported this theory.

In cases in which involvement of phosphoinositol lipid hydrolysis in cellular activities was questioned, blockade by neomycin has been used to provide supportive evidence (Cockcroft & Gomperts, 1985; Burch *et al.*, 1986). As shown in the present study (Fig. 5, Table 1), relatively low concentrations of neomycin blocked arachidonate- and SDS-promoted activation (but not activity) of NADPH oxidase, suggesting a specific interaction with the activating system. These data are consistent with a G-protein-mediated participation of phospholipase C in the NADPH oxidase activation process. Phospholipase C-catalysed hydrolysis of polyphosphoinositides is expected to affect activation of the oxidase mainly through a diacylglycerol-maintained effect on protein kinase C (Cox *et al.*, 1985, 1987). Studies aimed at the elucidation of the role of protein kinase C in the activation of the oxidase *in vitro*, including those employing specific inhibitors, failed however to confirm participation of protein kinase C in the reaction (Clark *et al.*, 1987; Ligeti *et al.*, 1987). Involvement of phospholipase C-catalysed hydrolysis inositol phospholipids bypassing protein kinase C requires the assumption of alternative pathways linking polyphosphoinositide turnover to the activation of NADPH oxidase *in vitro*, e.g. implication of an intermediate distinct from diacylglycerol, or of polyphosphoinositide-hydrolysis-induced changes in the physical properties of the microenvironment of the enzyme within the membrane (Badwey *et al.*, 1984).

While speculating on the role of phospholipase C in cell-free activation, we wish to point out that neomycin may not exhibit absolute selectivity towards inositol lipids and that studies conducted in its presence should be interpreted with caution (Nakashima *et al.*, 1987; Polascik *et al.*, 1987). It follows that although the effects of fluoride, neomycin and GTPγS on the activity of NADPH oxidase are consistent with a G-protein-mediated activation of phospholipase C, other interpretations are possible. Fluoride, guanine nucleotides and the positively charged neomycin may exert their effects through a direct specific binding to different sites on one of the oxidase regulatory or catalytic components, inducing conformational changes and alterations in their properties.

The basal arachidonate-promoted activation of NADPH oxidase followed a first-order reaction course (Figs. 1, 3 and 4). A first-order rate constant of a similar magnitude has recently been derived by Babior *et al.* (1988) from the kinetic course of superoxide generation in detergent-solubilized resting neutrophil membranes activated by SDS.

Neither neomycin nor fluoride affected the course of activation of the NADPH oxidase (Fig. 1), suggesting that the mechanism of the reaction was not altered by their presence. Unlike neomycin and fluoride, introduction of the guanine nucleotide analogue GTPγS resulted in a reaction which deviated from a first-order reaction course (Fig. 3; Fig. 4*a*, curve B). Addition of GTPγS after completion of the basal activation step (Fig. 4*a*, curve A) resolved the net GTPγS-promoted activation from the basal process (Fig. 4*a*, curve C). Summation of the basal and GTPγS-enhanced reaction curves yielded an activation curve similar to the one obtained upon introduction of GTPγS at the onset of the activation. Interestingly, in membranes of bovine neutrophils at low temperatures and in the presence of GTPγS, Doussiere *et al.* (1988) have recently observed a biphasic course of activation of the NADPH oxidase. The first phase in their activation seems thus to correspond to the basal activation, while the delayed second phase might represent the GTPγS-requiring step characterized by sigmoid kinetics (Fig. 4).

This kinetic behaviour may be attributed to the existence of two forms of inactive oxidase in the neutrophil membrane preparation. One form, E_{i1}, is capable of undergoing activation in the presence of arachidonate and cytosolic factors only (eqn. 1), while activation of the other, E_{i2}, also requires GTPγS (eqn. 2).

$$E_{i1} \xrightarrow{\text{ara.}} E_a \quad (1)$$

$$E_{i2} \xrightarrow{\text{ara., GTP}\gamma\text{S}} E_a \quad (2)$$

Accordingly, the addition of arachidonate and GTPγS at zero time elicits simultaneous activation of both forms; while addition of the nucleotide after the completion of the basal reaction (eqn. 1), elicits activation of E_{i2} (Fig. 4*a*, curve C). An apparent heterogeneity of this kind can be accounted for by assuming that low residual concentration of endogenous cytosolic GTP, essential for the activation of the oxidase, permits transformation of only a fraction of the enzyme into the active oxidase. This explanation is however contradicted by the observation reported by Gabig *et al.* (1987), and confirmed by us, that in contrast with GTPγS, exogenous GTP does not affect the activity of NADPH oxidase activated by the cell-free procedure. Thus a simple deficiency in GTP is unlikely to be responsible for the kinetic results shown in Fig. 4.

The apparent heterogeneity, which may reflect variations in the degree of coupling of resting oxidase molecules to a G-protein, is thus consistent with the presence of two pools of oxidase exhibiting different mechanisms and kinetics of activation. According to this hypothesis, both pools generate the same species of active oxidase E_a, in keeping with the unaltered K_m evaluated in the absence and presence of the nucleotide (Table 2). Since plasma membranes employed in our study may also contain specific granule markers (Fujita *et al.*, 1987), it is conceivable that the two pools of kinetically distinguishable enzyme may be derived from a dual distribution of oxidase or its components. The fact that the enhancement of NADPH oxidase activity by GTPγS was also reported by investigators employing pure plasma membrane preparations fractionated on Percoll gradients (Seifert & Schultz, 1987) rules out this hypothesis. The basis of the heterogeneity of the dormant enzyme thus remains unresolved.

In the kinetic model described above, GTPγS increased the fraction of activatable NADPH oxidase molecules. According to an alternative model, the enhancement of oxidase activity by the nucleotide analogue may be attributed to a modulation of the activation process reflected in the formation of enzyme molecules exhibiting higher catalytic activity. This mode of action is

summarized in the following equations, describing the basal and nucleotide-assisted activation of a single pool of resting enzyme, generating two active oxidase species E_{a1} (eqn. 3) and E_{a2} (eqn. 4) respectively:

$$E_i \xrightarrow{\text{ara.}} E_{a1} \quad (3)$$

$$E_i \xrightarrow{\text{ara., GTP}\gamma\text{S}} E_{a2} \quad (4)$$

The particular case of addition of GTPγS after completion of the basal activation (curve C of Fig. 4*a*) involves the conversion of E_{a1} into E_{a2}:

$$E_{a1} \xrightarrow{\text{ara., GTP}\gamma\text{S}} E_{a2} \quad (5)$$

This mechanism, though less likely to account for the kinetic courses shown in Fig. 4, cannot be excluded.

It is noteworthy that the enhancement by GTPγS was observed with arachidonate-promoted activation only. Whether the lack of effect on SDS-induced activation is due to a denaturation effect of SDS on certain components of the system (Seifert & Schultz, 1987), to variations in the experimental conditions or to a difference between the mechanism and/or target of arachidonate and SDS in the cell-free activation process cannot at present be decided.

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