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Activation of the $O_2^{\cdot-}$-Generating Oxidase in Plasma Membrane from Bovine Polymorphonuclear Neutrophils by Arachidonic Acid, a Cytosolic Factor of Protein Nature, and Nonhydrolyzable Analogues of GTP†

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ABSTRACT: A reconstitution system for activation of the $O_2^{\cdot-}$-generating oxidase from bovine polymorphonuclear neutrophils (PMN) is described. This system consisted of three components, namely, a particulate fraction enriched in plasma membrane, a supernatant fluid (cytosolic fraction) recovered by high-speed centrifugation from sonicated resting bovine PMN, and arachidonic acid. The pH optimum (7.8) and the K_M value for NADPH (45 μM) of the activated oxidase were virtually the same as those found in the purified enzyme. All three components had to be present during the preincubation for elicitation of oxidase activity. A further enhancement of oxidase activity was observed with the addition of nonhydrolyzable GTP analogues, such as guanosine 5'-*O*-(3-thiotriphosphate) (GTP-γ-S) and guanosine 5'-(β,γ-imidotriphosphate) (GMP-PNP), to the preincubation medium. In contrast, GDP-β-S drastically decreased oxidase activation. In a two-stage experiment, a 9-min preincubation of PMN membranes with arachidonic acid and GTP-γ-S followed by a 1-min contact with the cytosolic fraction led to a more marked activation than did preincubation of the cytosol with arachidonic acid and GTP-γ-S for 9 min followed by a 1-min contact with membranes, suggesting the presence of a G-protein in the membrane fraction. In the absence of added cations, the reconstitution system exhibited a substantial oxidase activity which was totally prevented by ethylenediaminetetraacetic acid (EDTA). Mg^{2+} added at a concentration of 0.5-1 mM enhanced oxidase activation by about 30%, indicating that endogenous Mg^{2+} or other activating cations were sufficient to ensure 70% of maximal activation. ATP was not required, and all assays carried out to check whether protein phosphorylation occurred during the activation process were negative. In particular, protein kinase C did not appear to be directly involved. The ratio of arachidonic acid to the amount of membrane material was the critical factor in the activation process. Maximal activation occurred with a ratio of 1 mol of arachidonic acid per 4 mol of membrane phospholipids. Long-chain unsaturated fatty acids of the cis and trans conformation were as effective as arachidonic acid. The protein nature of the factor present in the cytosolic fraction was demonstrated by loss of activity upon trypsin and proteinase K treatment. The cytosolic factor was found in PMN and not in other tissues, such as brain and heart; in contrast, its species specificities were rather broad, as cytosol from bovine PMN could replace cytosol from rabbit PMN to activate the oxidase from rabbit PMN membranes. The maximal rate of $O_2^{\cdot-}$ production depended on the amount of cytosol protein in contact with the membrane fraction, indicating that activation was not a catalytic process but involved a stoichiometric interaction between the cytosolic factor and a strategic component of the membrane fraction. Oxidase activation was temperature-dependent. The plateau of activation was attained in about 5 min at room temperature (22-25 °C) and in more than 30 min at 0 °C.

The NADPH-specific oxidase producing superoxide ions, $O_2^{\cdot-}$, is a characteristic enzyme of the phagocytosing blood cells. In the last few years increasing interest has been paid to this intriguing enzyme, but neither its structure nor its function or regulation has yet been unambiguously clarified [see Rossi (1986)]. The discovery of a technique for the in vitro activation of the NADPH oxidase in isolated membranes, originally described by Bromberg and Pick (1983, 1984) for guinea pig macrophages, was an important step in the investigation of the mechanism of activation. This cell-free activation system, consisting of arachidonic acid and cytosol, has been successfully applied to neutrophil granulocytes (Heyneman & Vercauteren, 1984; McPhail et al., 1985; Curnutte, 1985), and some properties of this system have been

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recently explored by several groups (Seifert et al., 1986; Seifert & Schultz, 1987; Gabig et al., 1987; Clark et al., 1987).

The molecular mechanism of in vitro activation is unclear. The data of two groups (Seifert et al., 1986; Gabig et al., 1987) suggest the participation of a G-protein in the process. For arachidonic acid, a second messenger function has been proposed by Bromberg and Pick (1983, 1984), and the possibility of a physicochemical effect on the membrane structure has been raised by Badwey et al. (1984). Protein phosphorylation has also been implicated in the mechanism of activation of NADPH oxidase. During studies of $O_2^{\bullet-}$ production by intact PMN,[1] a great number of phosphorylated proteins have been detected, and various authors have attributed special significance to one or another of them (Irita et al., 1984; Cooke & Hallett, 1985; Gennaro et al., 1985; Papini et al., 1985; Hayakawa et al., 1986; Blackburn et al., 1987). In this context, experiments have been reported which suggest that activation of protein kinase C induces activation of the NADPH oxidase (Dewald, 1984; Papini et al., 1985). A direct involvement of protein kinase C in the activation of a dormant NADPH oxidase has been postulated; however, it was pointed out that this was not the sole pathway for oxidase activation (Cox et al., 1987). Although careful examination by Seifert and Schultz (1987) did not lead to support for the hypothesis about the participation of protein kinase C, the inclusion of hexokinase and glucose in the incubation mixture to decrease the level of ATP diminished the stimulatory effect of arachidonic acid plus cytosol (Seifert & Schultz, 1987; Clark et al., 1987).

In view of these controversies, we decided to carry out a detailed characterization of the in vitro activation process in membranes of bovine PMN. Preference for bovine PMN can be justified by the easy availability of large amounts of material, which is necessary for the purification of the cytosolic factors involved in the oxidase activation, and the existence of a purified oxidase preparation (Doussière & Vignais, 1985).

Materials and Methods

Materials. Fatty acids, superoxide dismutase, ferricytochrome *c* (horse heart, grade VI), trypsin (type III), soybean trypsin inhibitor (type I-S), proteinase K, and phorbol myristate acetate were purchased from Sigma; NADPH and NADH were from Boehringer; and Percoll was from Pharmacia. Free fatty acids were dissolved in absolute ethanol and stored at −80 °C under N_2 until used. H-7 (Seikagaku Kogyo, Japan) was dissolved in dimethyl sulfoxide. [γ-^{32}P]ATP was obtained from Amersham.

Preparation of Cytosol and Membrane Fractions. Polymorphonuclear neutrophils (PMN) were prepared from calf blood according to the procedure described by Doussière and Vignais (1985). The cells obtained from 5 L of blood were resuspended in 10 mL of phosphate-buffered saline (PBS) consisting of 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4. The suspension was then incubated for 5 min at 37 °C either in the absence (nonactivated cells) or in the presence of 1 μg/mL PMA (activated cells). In both cases, disruption was achieved by ultrasonic treatment for 4 × 15 s at 0 °C with a Branson sonicator at 40-W output. Nondisrupted cells, as well as nuclei and granular material, were eliminated by centrifugation at 10000*g* for 10 min in a Sorvall rotor at 4 °C. The resulting supernatant was spun for 90 min at 130000*g* in a Beckman 40 rotor. The supernatant of this high-speed centrifugation containing 15–30 mg of protein/mL was called "cytosol" and stored at −20 °C. The pellets were resuspended at a final concentration of 10–15 mg of protein/mL in the following medium: 0.1 M mannitol, 10 mM sodium phosphate, and 20% ethylene glycol, pH 7.4. According to previous investigations (Morel et al., 1985) this high-speed pellet contains the majority of both alkaline phosphatase and $O_2^{\bullet-}$-generating oxidase activity.

Enzyme Assays. The rate of $O_2^{\bullet-}$ production was determined as the superoxide dismutase sensitive portion of ferricytochrome *c* reduction. The assay was carried out routinely at 22–25 °C in 2 mL of PBS medium supplemented with 100 μM ferricytochrome *c*, 1 mM $MgCl_2$, and 50–150 μg of membrane protein. The reaction was initiated by addition of 250 μM NADPH. The rate of absorbance change was recorded at 550-nm wavelength in a Leres S28 spectrophotometer. After a linear phase of 1–4 min, 50 μg of superoxide dismutase enzyme was added and the recording continued for a further 2–4 min. The difference between the rate before and after addition of SOD was regarded as the rate of $O_2^{\bullet-}$ production.

Protein kinase C was assayed by measuring the transfer activity of the terminal phosphate of [γ-^{32}P]ATP to histone H_1 in the presence of Ca^{2+}, phosphatidylserine, and diacylglycerol (Kikkava et al., 1982). Correction was made for the activity observed in the absence of Ca^{2+} and lipids.

Protein content was determined either by the biuret or the Lowry procedure. SDS–polyacrylamide gels were prepared according to Laemmli and Favre (1973).

Results

Conditions of in Vitro Activation of NADPH Oxidase. The light membrane fraction of resting bovine PMN (sedimented at 130000*g*) produced on average 4 nmol of $O_2^{\bullet-}$/(min·mg of membrane protein). This value represents less than 10% of the $O_2^{\bullet-}$ generation observed with membranes prepared in an identical way from PMN stimulated by supramaximal doses of PMA (Table I). Production of $O_2^{\bullet-}$ by the isolated membranes of resting bovine PMN can be elicited in vitro by means of cytosol and arachidonic acid. For full expression of activation, all three components of the activation system, i.e., membranes, cytosol, and arachidonic acid, were required, and a nonhydrolyzable analogue of GTP had to be added (the effect of guanine nucleotides is detailed below). Cytosol was equally effective whether isolated from resting or PMA-stimulated cells. In the following experiments, preincubation and incubation were carried out at room temperature (22–25 °C), except when otherwise indicated.

The results in Table I show that widely different rates of oxidase activation could be obtained, depending on the conditions of incubation. In one series of experiments, membranes, cytosol, and arachidonic acid were successively added to the photometric cuvette; i.e., activation proceeded in the presence of the substrate (NADPH) and the electron acceptor (cytochrome *c*), as described by Seifert et al. (1986) and Seifert and Schultz (1987). By this method, rates of $O_2^{\bullet-}$ production of 24 ± 10 nmol/(min·mg of membrane protein) could be achieved. Although the activation attained was almost 6-fold relative to the oxidase activity of membranes alone, the mean rate value represented only about 40% of that observed in membranes prepared from PMA-stimulated cells [24 versus 62 nmol/(min·mg of membrane protein)]. An increase in the amount of added cytosol did not augment the production of $O_2^{\bullet-}$ (i.e., the SOD-sensitive reduction of cytochrome *c*)

[1] Abbreviations: PMN, polymorphonuclear neutrophil(s); PMA, 4β-phorbol 12-myristate 13-acetate; PKC, protein kinase C; H-7, 1-(5-isoquinolinesulfonyl)-2-methylpiperazine; GTP-γ-S, guanosine 5′-*O*-(3-thiotriphosphate); SDS, sodium dodecyl sulfate; SOD, superoxide dismutase; GMP-PNP, guanosine 5′-(β,γ-imidotriphosphate); EDTA, ethylenediaminetetraacetic acid.

Table I: Rate of $O_2^{\cdot-}$ Production by Bovine PMN Membrane Bound Oxidase Activated under Different Experimental Conditions[a]

state of PMN	components in preincubation medium	components in assay medium	$O_2^{\cdot-}$ production [nmol/(min·mg of membrane protein)]
resting PMN	no preincubation	membranes	4 ± 2[b] (11)[c]
	no preincubation	membranes + cytosol + arachidonic acid + GTP-γ-S	24 ± 10 (21)
	membranes + cytosol + arachidonic acid + $MgCl_2$	transfer of preincubation medium to assay medium	67 ± 30 (8)
	membranes + cytosol + $MgCl_2$ + arachidonic acid + GTP-γ-S	transfer of preincubation medium to assay medium	287 ± 103 (26)
PMA-activated PMN	no preincubation	membranes	62 ± 26 (24)
	no preincubation	membranes aged for 2–3 weeks at −20 °C	14 ± 4 (3)
	aged membranes + cytosol + arachidonic acid + $MgCl_2$	transfer of preincubation medium to assay medium	94 ± 13 (3)

[a] An amount of about 100 μg of membrane protein from bovine PMN, either resting PMN or PMN activated by PMA, was routinely used. When indicated, 400 μg of cytosolic protein, 1 mM $MgCl_2$, 80 nmol of arachidonic acid, and 10 μM GTP-γ-S were added. The basic medium for preincubation or the enzyme assay was PBS. Preincubation was carried out for 5 min at 22 °C, with final volume 135 μL. Then, the whole preincubation medium was transferred to the photometric cuvette containing the assay medium. Rates of $O_2^{\cdot-}$ production were determined at the same temperature as described under Materials and Methods. [b] Mean ± SEM. [c] Number of experiments in parentheses.

whereas it clearly enhanced the rate of SOD-insensitive cytochrome *c* reduction.

A more effective activation was achieved when the procedure of Gabig et al. (1987) was followed, i.e., when the essential components of the activation system, namely, membranes, cytosol, and arachidonic acid, were preincubated in a small volume (100 μL) in the presence of 1 mM $MgCl_2$ at room temperature and then transferred to the photometric cuvette containing the assay medium (cf. Materials and Methods) where generation of $O_2^{\cdot-}$ was started by the addition of the substrate. The rate of $O_2^{\cdot-}$ production was 67 ± 30 nmol/(min·mg of membrane protein). A further enhancement of oxidase activity to a value of 287 ± 103 nmol/(min·mg protein) was observed when a GTP analogue like GTP-γ-S was added to the preincubation medium.

In vitro activation using the preincubation step resulted in membranes that exhibited specific oxidase activities several times higher than those obtained by PMA stimulation of intact cells, i.e., 62 ± 26 nmol/(min·mg of membrane protein). Moreover, three preparations of membrane fractions from PMA-triggered cells aged for 3 weeks at −20 °C, which revealed weak residual $O_2^{\cdot-}$ production [14 ± 4 nmol/(min·mg of membrane protein)], could be activated in vitro to generate $O_2^{\cdot-}$ at higher rates [average 94 ± 13 nmol/(min·mg of membrane protein)] than were observed just after preparation. These observations suggest that activation is a reversible phenomenon; a deactivated oxidase can be reactivated. For all experiments carried out with preincubation of the reconstitution system, the rate of $O_2^{\cdot-}$ generation was constant from the onset of the reaction, without a detectable lag phase.

Properties of NADPH Oxidase Activated in Vitro. The oxidase activity stimulated in vitro by the addition of cytosol, arachidonic acid, and GTP-γ-S was specific for the substrate NADPH, as was the purified enzyme (Doussière & Vignais, 1985). The membrane fraction prepared from resting PMN reduced cytochrome *c* at a high rate in the presence of NADH, but this activity was strongly inhibited by arachidonic acid and insensitive to SOD, suggesting that it belonged to a diaphorase-like enzyme. As previously reported (Morel et al., 1985), this diaphorase activity was not stimulated upon activation of intact PMN with PMA.

The $O_2^{\cdot-}$-generating oxidase activated in vitro exhibited a K_M for NADPH of 45 μM and a pH optimum of 7.8 (Figure 1). The corresponding values for the purified enzyme isolated from PMA-stimulated bovine PMN were 33 μM and 7.6 (Doussière & Vignais, 1985).

Tissue and Species Specificity of Cytosolic Factor. Tissue specificity of the cytosolic factor was tested by substitution of the PMN cytosol by high-speed supernatants obtained from homogenates of various tissues. The data of Table II demonstrate that cytosol from bovine brain and rat brain or rat heart (added in equal amount as PMN cytosolic protein) induces only negligible $O_2^{\cdot-}$ production. In contrast, the cytosol from bovine PMN was able to activate efficiently NADPH

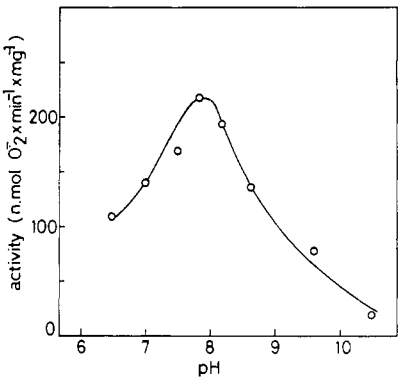

FIGURE 1: pH dependence of in vitro activated NADPH oxidase enzyme from bovine PMN. Membranes isolated from resting bovine PMN were activated under the following conditions: 70 μg of membrane protein plus 400 μg of cytosol protein plus 60 nmol of arachidonic acid in PBS supplemented with 1 mM $MgCl_2$ and 10 μM GTP-γ-S, final volume 135 μL. After 5 min of preincubation at 22 °C the whole reaction mixture was transferred to the photometric cuvette containing 2 mL of the assay medium (cf. Materials and Methods) with pH adjusted at different values between 6.5 and 10.5, and $O_2^{\cdot-}$ production was measured.

Table II: Tissue and Species Specificity of the in Vitro Oxidase System[a]

origin of membranes	origin of cytosol	$O_2^{\cdot-}$ production [nmol/(min·mg of membrane protein)]
bovine PMN	no cytosol	2
bovine PMN	bovine PMN	197
bovine PMN	bovine brain	2
bovine PMN	rat brain	8
bovine PMN	rat heart	7
rabbit PMN	no cytosol	4
rabbit PMN	bovine PMN	40

[a] All tests were carried out by preincubation of 120–130 μg of membrane protein with 400 μg of cytosolic protein in the presence of 10 μM GTP-γ-S, 1 mM $MgCl_2$, and 80 nmol of arachidonic acid in PBS for 5 min at 22 °C; final volume was 135 μL. The whole preincubation medium was transferred to the photometric cuvette for assay of $O_2^{\cdot-}$ production.

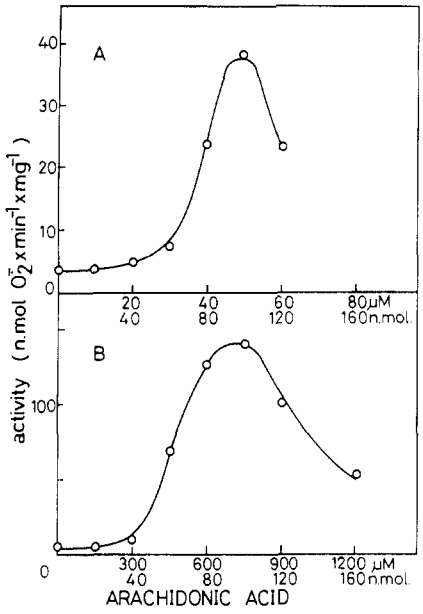

FIGURE 2: Dependence of NADPH oxidase activity on concentration or amount of arachidonic acid when stimulation was carried out. (A) Activation was directly carried out in the assay medium (2 mL) (cf. Materials and Methods) containing 180 µg of membrane protein, 500 µg of cytosolic protein, 10 µM GTP-γ-S, 1 mM $MgCl_2$, and various amounts of arachidonic acid. (B) Activation was carried in the preincubation medium with the same amounts of the same components as used in (A), final volume 130–135 µL, for 5 min at 20 °C; the whole preincubation medium was transferred to the assay medium. The amounts of arachidonic acid are given on the abscissa in nanomoles and in concentration units (micromolar) either in the incubation medium (A) or in the preincubation medium (B). The highest concentration of ethanol used in the preincubation medium was 2.5% (corresponding to the highest concentration of arachidonic acid, i.e., 1200 µM). In a control assay, it was checked that this ethanol concentration had no deleterious effect on the activation of NADPH oxidase.

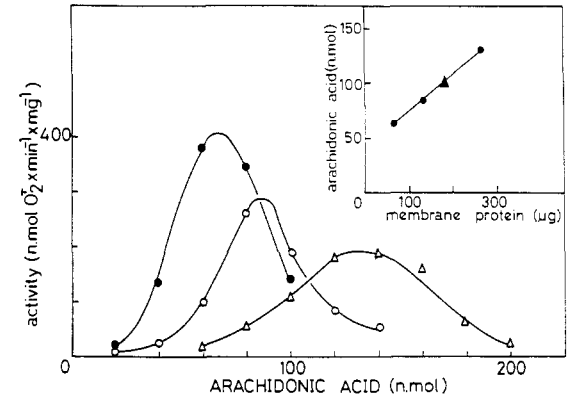

FIGURE 3: Correlation between amount of PMN membranes and amount of arachidonic acid for maximal activation of NADPH oxidase. Membranes containing either 66 (●), 132 (○), or 264 µg (▲) of protein were activated by preincubation for 5 min at 22 °C in PBS in the presence of 400 µg of cytosolic protein, 1 mM $MgCl_2$, 10 µM GTP-γ-S, and various amounts of arachidonic acid, final volume 135 µL. The entire incubation mixture was then transferred to the photometric cuvette, and $O_2^{\bullet-}$ production was measured as detailed under Materials and Methods. Insert: Amount of arachidonic acid required for optimal activation of NADPH oxidase as a function of amount of membrane protein. (●) Data from the experiment shown in this figure; (▲) this value from the experiment of Figure 2B.

oxidase in membranes prepared from rabbit peritoneal PMN. Thus, the crucial element of the in vitro activation system, the cytosolic factor, appears to be tissue-specific but not species-specific.

To determine whether the cytosolic factor was of protein nature, PMN cytosol (40 mg/mL) was treated by trypsin with a trypsin to cytosol protein ratio of 1/50 (w/v) for 1 h at 37 °C. The reaction was stopped with a 5-fold excess of soybean trypsin inhibitor. The trypsin-treated cytosol lost virtually all its stimulating activity in the production of $O_2^{\bullet-}$. Treatment by proteinase K under similar conditions also destroyed the activity of the cytosolic factor, which corroborates the protein nature of this factor.

Dependence of $O_2^{\bullet-}$ Production on Amount of Arachidonic Acid. The dependence of $O_2^{\bullet-}$ production on the amount of arachidonic acid applied was tested in both conditions detailed above. In the cuvette, optimal activation of $O_2^{\bullet-}$ production was achieved in the presence of 50 µM arachidonic acid (Figure 2A). This value corresponds to those reported for human PMN (Seifert & Schultz, 1987; Cox et al., 1987; Clark et al., 1987). However, it should be noted that this concentration of arachidonic acid brings about extensive changes in membrane suspension. In a separate experiment carried out with freshly prepared bovine PMN, 50 µM arachidonic acid was found to induce a rapid increase of the percentage of trypan blue positive cells (up to 90% in 5 min), reflecting permeabilization of the plasma membrane.

The interesting observation, shown in Figure 2, is that identical amounts of arachidonic acid (but not identical concentrations) were required for maximal activity, irrespective of whether activation of the membranes by cytosol plus arachidonic acid was carried out during the preincubation period or directly in the assay cuvette. It should be recalled that the concentration of arachidonic acid was 20 times higher in the preincubation medium than in the assay medium. In accordance with this finding, the amount of arachidonic acid allowing maximal activation of the NADPH oxidase increased with the amount of added membrane protein (Figure 3). A linear relationship was found between the amount of arachidonic acid and the amount of PMN membranes required for optimal activation (insert of Figure 3). As the amount of cytosolic protein was kept constant, the conclusion that there exists a fixed ratio of arachidonic acid to membrane protein and/or membrane lipid is inescapable. Approximate calculations based on the slope of the straight line shown in the insert of Figure 3 yielded a ratio of 1 molecule of arachidonic acid to 4 molecules of phospholipid.

Specificity for Different Fatty Acids. We investigated whether fatty acids other than arachidonic acid were able to participate in the in vitro activation of the NADPH oxidase of bovine origin. The results are summarized in Table III. In our system none of the long-chain saturated fatty acids tested (arachidic acid, stearic acid, palmitic acid) gave any measurable activation, and neither did the methyl and coenzyme A (CoA) esters of arachidonic acid. In contrast, the unsaturated long-chain fatty acids of both cis (oleic, linoleic, and palmitoleic acid) and trans (elaidic and palmitelaidic acid) configurations proved to be almost equally active.

Bromberg and Pick (1985) have reported that, among a number of detergents used, essentially the anionic detergent sodium dodecyl sulfate was able to elicit the production of $O_2^{\bullet-}$ by a cell-free system represented by sonically disrupted guinea pig peritoneal macrophages. They suggested that the long-chain unsaturated fatty acids that are capable of stimulating $O_2^{\bullet-}$ production in a cell-free system owe their activity to the fact that they function as anionic detergents.

Participation of a G-Protein in Oxidase Activation. The evidence presented by Seifert et al. (1986) and Gabig et al. (1987) about the participation of a pertussis toxin and cholera toxin insensitive G-protein in the process of in vitro activation of human neutrophil membranes was an interesting finding,

Table III: Fatty Acid Specificity of in Vitro Activation of Bovine NADPH Oxidase[a]

fatty acid	concn (mM)	$O_2^{\cdot-}$ production [nmol/(min·mg of membrane protein)]
arachidonic acid	0.7	195
arachidic acid	1.3	2
arachidonic acid methyl ester	1.8	6
arachidonyl-CoA	0.8	5
stearic acid	2.9	2
oleic acid	1.2	162
elaidic acid	4.1	238
linoleic acid	3.0	164
linolelaidic acid	2.7	23
palmitic acid	1.3	3
palmitoleic acid	3.3	303
palmitelaidic acid	4.5	180

[a] Activation of NADPH oxidase was carried out in PBS for 5 min at 22 °C with 180 μg of membrane protein in the presence of 500 μg of cytosolic protein, 10 μM GTP-γ-S, 1 mM $MgCl_2$, and various amounts of the different fatty acids and esters; final volume of the preincubation medium was 135 μL. The whole preincubation medium was transferred to the photometric cuvette for assay of $O_2^{\cdot-}$ production. The table indicates only those concentrations that induced the highest rate of $O_2^{\cdot-}$ production.

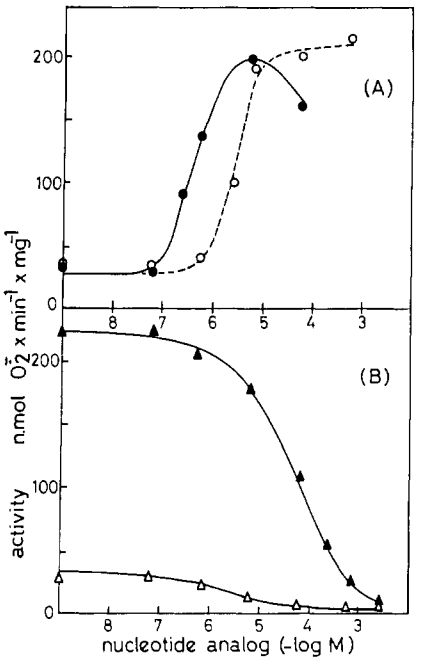

FIGURE 4: Evidence for participation of a G-protein in the in vitro activation process of NADPH oxidase from bovine PMN. Dependence of oxidase activation on the presence of guanine nucleotides. (A) GTP-γ-S (●) GMP-PNP (○) at increasing concentrations were incubated in PBS with 180 μg of membrane protein for 5 min at 22 °C in the presence of 500 μg of cytosolic protein, 600 nmol of palmitelaidic acid, and 1 mM $MgCl_2$, final volume 135 μL. The whole suspension was then transferred to the photometric assay cuvette, and $O_2^{\cdot-}$ production was measured as detailed under Materials and Methods. (B) GDP-β-S was added at increasing concentrations in the same medium as described in (A) with 10 μM GTP-γ-S (▲) or without GTP-γ-S (△).

which in our view deserved confirmation in other species. The results of our investigations on bovine PMN membranes are shown in Figure 4.

When membranes were preincubated with fatty acid and cytosol freshly isolated from resting PMN in the absence of GTP-γ-S, $O_2^{\cdot-}$ production amounted to 67 ± 30 nmol/(min·mg of membrane protein). This activity could be stimulated 4–5-fold by nonhydrolyzable GTP analogues, like GTP-γ-S and GMP-PNP (Figure 4A). NaF at a final concentration of 40 mM was as effective as the GTP analogues (not shown). However, none of these activators was able to replace either arachidonic acid or cytosol.

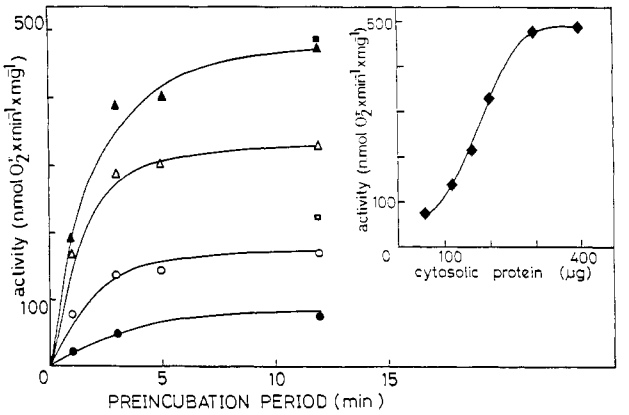

FIGURE 5: Effect of various amounts of cytosolic protein on kinetics of activation of NADPH oxidase. Seventy micrograms of membrane protein in PBS was activated for the indicated period of time at 22 °C in the presence of 10 μM GTP-γ-S, 1 mM $MgCl_2$, 60 nmol of arachidonic acid, and either 60 (●), 120 (○), 160 (□), 200 (△), 300 (▲), or 400 μg (■) of cytosolic protein, final volume 135 μL. Production of $O_2^{\cdot-}$ was measured as detailed under Materials and Methods after transfer of the preincubation medium to the photometric cuvette. Insert: Rate of $O_2^{\cdot-}$ production after 12-min preincubation as a function of amount of added cytosolic protein.

On the other hand, both the activity observed in the absence of GTP analogues and the activity obtained in the presence of 10 μM GTP-γ-S or GMP-PNP could be completely suppressed by GDP-β-S in a dose-dependent manner (Figure 4B). Aging of the cytosol brought about a clear decline of the activity measured in the absence of the GTP analogues whereas stimulation in the presence of 10 μM GTP-γ-S or GMP-PNP remained almost unaffected. This suggests that cytosol and/or membrane contained endogenous GTP which decays with storage.

The activation system showed clear Mg^{2+} dependence. Optimal $O_2^{\cdot-}$ production was achieved between 0.5 and 1 mM Mg^{2+}, but the endogenous Mg^{2+} content of the preparation was sufficient to ensure about 70% of maximal activation. Inclusion of 5 mM EDTA in the preincubation mixture prevented NADPH oxidase activation nearly completly [$O_2^{\cdot-}$ production fell from 240 to 20 nmol/(min·mg of membrane protein)].

The possible site of action of the GTP analogues in the reconstituted system, i.e., binding to a cytosol or membrane component, was tested in the following experiment. In a first assay, membranes were incubated for 9 min with arachidonic acid and GTP-γ-S (or GMP-PNP), followed by a 1-min incubation with cytosol. The second assay consisted in incubation of cytosol with arachidonic acid and GTP-γ-S (or GMP-PNP) for 9 min followed by a 1-min incubation with membranes. In both cases, $O_2^{\cdot-}$ formation was generated by addition of NADPH. The rate of production of $O_2^{\cdot-}$ was 6-fold higher in the first assay than in the second, suggesting that the GTP analogues react primarily with a membrane component, probably of the class of the G-proteins.

Kinetics of Activation. Prolongation of the preincubation period significantly increased the specific activity of the NADPH oxidase up to a limit that was attained after 5 min (Figure 5). Thus, the process of activation comprises at least one time-dependent step. Another critical parameter in the kinetics of activation was the amount of cytosolic protein. The results of Figure 5 demonstrate clearly that in the presence of decreasing amounts of cytosolic protein both the initial rate

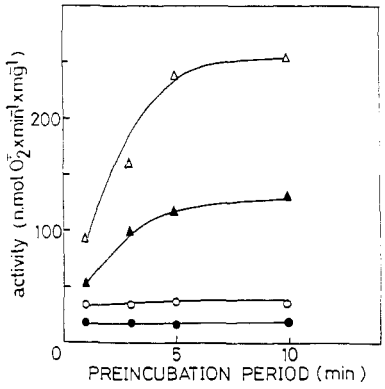

FIGURE 6: Time dependence of addition of arachidonic acid on activation of NADPH oxidase. Membrane protein (180 μg) was preincubated at 22 °C for the indicated period of time in PBS in the presence of 500 μg of cytosolic protein and 1 mM $MgCl_2$ either in the presence of 10 μM GTP-γ-S (open symbols) or in its absence (closed symbols), final volume 135 μL. Arachidonic acid (80 nmol) either was present during the whole period of preincubation (△, ▲) or was added to the photometric assay cuvette after transfer of the preincubation mixture (○, ●).

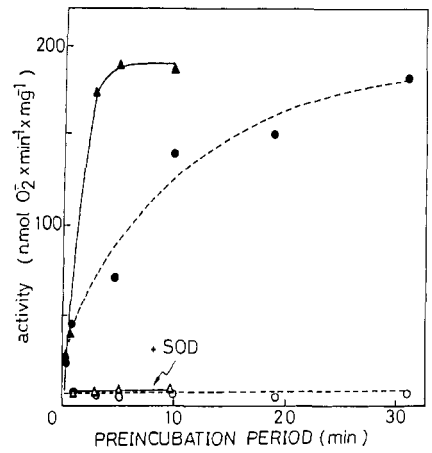

FIGURE 7: Effect of temperature on kinetics of activation of NADPH oxidase. Preincubation of 130 μg of membrane protein in PBS with 400 μg of cytosolic protein, 1 mM $MgCl_2$, 10 μM GTP-γ-S, and 60 nmol of arachidonic acid, final volume 135 μL, was performed at 25 (▲) and 0 °C (●). After transfer of the preincubation medium to the routine assay medium in the photometric cuvette, incubation was carried out at room temperature as described under Materials and Methods. The SOD sensitivity of the oxidase activated at 25 °C (△) and at 0 °C (○) is also shown.

and the end point of the kinetic curves diminished. The specific activity of the NADPH oxidase at the end point was saturable function of the amount of cytosolic protein added (insert of Figure 5); for example, with 70 μg of membrane protein, saturation was attained with 300–400 μg of cytosolic protein. In accordance with the above findings, saturation was shifted toward higher values of cytosolic protein when the amount of membrane was increased (data not shown). Taken together, these observations indicate that the reaction occurring between the membrane-bound NADPH oxidase (or eventually some other membrane component) and the cytosolic cofactor, and leading to the activation of the dormant oxidase, is governed by a stoichiometric relationship.

We also investigated whether the time-dependent reaction, which seems to be the key to the activation process, required the presence of arachidonic acid. In the experiment shown in Figure 6, membranes and cytosol were preincubated for the indicated period of time in the presence of arachidonic acid (plus or minus GTP-γ-S), or alternatively, preincubation proceeded without arachidonic acid (plus or minus GTP-γ-S), and the fatty acid was added subsequently to the mixture in the photometric cuvette for the assay of oxidase activity. A time-dependent increase of the specific activity of the NADPH oxidase could only be observed when arachidonic acid was present during preincubation. The presence of GTP-γ-S rendered the activation process both faster and more efficient. In contrast, when membranes were preincubated with cytosol and the fatty acid was added subsequently in the photometric cuvette, i.e., after dilution of the preincubation mixture, no time-dependent reaction could be revealed, and $O_2^{\cdot-}$ production remained low (see values of Table I). The same results as those shown in Figure 6 were obtained when 0.05% deoxycholate was included in the preincubation mixture, i.e., time-dependent activation of the oxidase when all components were present in the preincubation medium and lack of activation when arachidonic acid was omitted. Thus the failure of activation to take place when arachidonic acid was absent during the preincubation period cannot be attributed to a limitation of access of cytosolic protein to its specific target in the membrane. As the ratio of arachidonic acid to membrane remained constant under all conditions, the dilution of the fatty acid could not be the decisive factor either. It is therefore suggested that the structural change which is brought about by the insertion of the fatty acid into the membrane represents the trigger that allows reaction with the cytosolic factor and that finally results in activation of the oxidase.

Temperature had a noticeable influence on kinetics of activation. At 0 °C, full activation took about 30 min versus 5 min at 25 °C. But in the two cases, the same oxidase activity was attained (Figure 7).

Absence of Dependence of Oxidase Activation on a Phosphorylation Reaction. In attempts to determine whether a protein kinase mediated phosphorylation reaction was required for in vitro activation of bovine PMN oxidase, a number of arguments accumulated, which suggests that phosphorylation(s) dependent upon the action of protein kinase C were probably not necessary for the activation of the oxidase.

(1) Under conditions where the cytosol and arachidonic acid added together to the membrane fraction brought about strong activation of $O_2^{\cdot-}$ production, no similar effect could be achieved by the combination of either PMA, phosphatidylserine, and ATP or phosphatidylserine, diacylglycerol, ATP, and Ca^{2+}, which are known to activate protein kinase C.

(2) Both cis and trans unsaturated fatty acids were effective in the in vitro activation whereas only fatty acids of the cis configuration stimulate protein kinase C (Murakami & Routtenberg, 1985).

(3) A cytosol fraction obtained from a rat brain homogenate and exhibiting a significant protein kinase C activity (0.9 nmol/min) did not show any significant stimulatory effect on the activity of NADPH oxidase. On the other hand, aged cytosol preparations from bovine PMN which exhibited negligible protein kinase C activity were highly active in in vitro activation of NADPH oxidase.

(4) Protein phosphorylation by [γ-^{32}P]ATP and determination of $O_2^{\cdot-}$ production were carried out in parallel by preincubation of PMN membranes, cytosol, arachidonic acid, and GTP-γ-S, using a number of different combinations. In membranes alone after SDS-polyacrylamide gel electrophoresis and autoradiography, major radiolabeled bands corresponding to molecular weights between 30000 and 90000 were revealed, which were not influenced either by arachidonic acid or by GTP-γ-S. In the cytosol several protein bands were phosphorylated as well, and both their number and the intensity were significantly enhanced by arachidonic acid. However, no change in the protein phosphorylation pattern could be

detected when cytosol, arachidonic acid, and membranes were preincubated together in the absence or presence of GTP-γ-S, although these conditions were the only ones where $O_2^{\bullet-}$ production was significant. Thus, no correlation could be demonstrated between in vitro activation of NADPH oxidase and specific phosphorylation of a protein component present in sufficient amount to be detected by the conventional autoradiographic techniques. A lack of visible change in the protein phosphorylation pattern does not exclude, of course, alterations of minor constituents.

(5) Inclusion of the protein kinase inhibitor H-7 (Hidaka et al., 1984) at a final concentration of 200 μM in the preincubation mixture resulted in a 50% reduction of the intensity of all the phosphorylated bands in the autoradiography, but no decrease of $O_2^{\bullet-}$ production could be detected in the in vitro activated system.

(6) Inclusion of ATP in the complete in vitro activation system (in the presence of GTP-γ-S) or depletion of endogenous ATP by addition of F_1 ATPase isolated from beef heart mitochondria had no influence on the maximal rate of $O_2^{\bullet-}$ production. The reported stimulatory effect of ATP in achieving maximum reaction rates, in the case of NADPH oxidase of human PMN, and the decrease in oxidase activity on depletion of ATP by incubation with glucose and hexokinase (Clark et al., 1987) may have been probably due to indirect changes in the concentrations of endogenous GTP brought about by endogenous nucleoside diphosphokinase in the presence of ATP. In our hands, no requirement of NADPH oxidase activation for ATP could be demonstrated.

All these observations taken together favor the idea that the in vitro activation of NADPH oxidase dependent on cytosol and unsaturated fatty acid proceeds by another (other) mechanism(s) than phosphorylation.

Discussion

General Features of in Vitro Activation of Bovine NADPH Oxidase. Experiments reported in this paper show that the dormant NADPH oxidase present in the plasma membrane fraction isolated from resting bovine PMN could be considerably stimulated to generate $O_2^{\bullet-}$ under in vitro conditions by the addition of a mixture of cytosolic proteins and arachidonic acid. Stimulation was further enhanced by nonhydrolyzable GTP analogues like GTP-γ-S and GMP-PMP. The NADPH oxidase activated in vitro had a K_M value and an optimum pH similar to those of the isolated enzyme (Doussière & Vignais, 1985). We have carried out a detailed characterization of the in vitro activation system of bovine origin, including aspects that were studied in human PMN by other groups (Seifert & Schultz, 1987; Gabig et al., 1987; Clark et al., 1987). The fatty acid specificity and the indications for the participation of a G-protein (activation by GTP-γ-S, antagonism by GDP-β-S, and activation by NaF) were almost identical in human and bovine PMN. Thus the possibility of in vitro activation of the NADPH oxidase is not restricted to human PMN membranes but probably represents a general feature of PMN from various species. With bovine PMN, after optimization of the conditions, membranes were obtained, which produced $O_2^{\bullet-}$ at higher rates and with better reproducibility than reported for the human membranes. In addition to the large amounts of available experimental material we consider the high stability and repetitiveness as important advantages offered by bovine PMN.

In the studies of in vitro activation carried out on human PMN membranes the rate of $O_2^{\bullet-}$ production was not compared to values obtained with membranes from stimulated cells. Our data in Table I indicate that, in the case of bovine PMN, the average $O_2^{\bullet-}$ production of membranes activated in vitro under optimal conditions was 4–5 times higher than the specific activity of membranes from PMA-stimulated cells. In addition, $O_2^{\bullet-}$ production of PMA-activated membranes could be further increased by preincubation in the presence of cytosol and fatty acids. These observations suggest that PMA, the strongest stimulatory agent of oxidase activity in PMN, does not turn on the oxidase enzyme to full capacity or, alternatively, not all the enzyme molecules are activated. In other words, even in the in vitro system described here where stimulation is not oriented and probably not receptor-mediated, either a graded or a spatially differentiated activation process has to be postulated. Another possible explanation for the extra rate of $O_2^{\bullet-}$ production by the in vitro system is the presence in intact membrane of a bound inhibitor whose release is facilitated by treatment with arachidonic acid.

Comments on the Role of Arachidonic Acid and Cytosolic Factor in the Activation of NADPH Oxidase. Investigation of the mechanism of in vitro stimulation revealed that a fixed amount of the fatty acid has to be present in the membrane in order to trigger the activation process (Figures 2 and 3). In our experiments the maximal rate of $O_2^{\bullet-}$ production was achieved when the ratio of arachidonic acid to the total phospholipid content of the membrane was about 1 to 4. It is interesting to note that, in studies of the binding of linoleic acid and its effect on the $O_2^{\bullet-}$ production by intact human neutrophils, Badwey et al. (1984) arrived at a similar value. However, the required amount of arachidonic acid is much too high, even in case of lipid compartmentalization, to account for a regulatory process in vivo PMN. On the other hand, the decrease of viability and optical density of intact cells observed in the presence of such high doses of arachidonic acid shows clearly that the fatty acid does drastically perturb the membrane structure and alter its permeability. These changes cannot be tolerated by intact cells. Although we consider the action of the fatty acid in the in vitro activation process to be completely artificial, it might represent a modification that mimics a more specific alteration occurring during the course of the physiological activation.

Our study of the dependence of the oxidase activation on the sequential addition of the different components of the in vitro system led to the conclusion that a GTP-reacting protein (G-protein) required for activation was probably located in the membrane fraction. One may imagine that the effect of arachidonic acid (or other long-chain unsaturated fatty acids) is either to facilitate the interaction of GTP-γ-S with the nucleotide-specific α subunit of this G-protein, which could directly activate the oxidase, or to facilitate the contact between the oxidase and a protein effector (possibly the cytosolic factor) controlled by the G-protein.

In contrast to the unsaturated, long-chain fatty acids, the cytosol which is essential in the in vitro activation probably contains a factor that might represent a component of the natural activation process. The cross-reactivity studies summarized in Table II suggest that this factor is strongly tissue-specific but not species-specific. Finally, in the reconstituted system reported here, evidence is provided that activation of the NADPH oxidase occurs by a protein kinase C independent mechanism. This, of course, does not rule out the possibility that protein kinase C activates the NADPH oxidase in intact cells by a separate activating mechanism (Cox et al., 1987).

Acknowledgments

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Registry No. ATP, 56-65-5; PKC, 9026-43-1; Mg, 7439-95-4; arachidonic acid, 506-32-1; NADPH-specific oxidase, 9032-22-8; superoxide, 11062-77-4; oleic acid, 112-80-1; elaidic acid, 112-79-8; linoleic acid, 60-33-3; palmitoleic acid, 373-49-9; palmitelaidic acid, 10030-73-6; guanosine 5′-*O*-(3-thiotriphosphate), 37589-80-3; guanosine 5′-(β,γ-imidotriphosphate), 34273-04-6.

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Retinal Is Not Formed in Vitro by Enzymatic Central Cleavage of β-Carotene

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ABSTRACT: Rat intestinal mucosa was prepared and incubated with β-carotene by the procedure of Goodman and Olson [Goodman, DeW. S., & Olson, J. A. (1969) *Methods Enzymol. 15*, 462–475] to determine β-carotene cleavage activity. A new detection system for the reaction products of the described enzyme β-carotene 15,15′-dioxygenase (EC 1.13.11.21) employs solvent extraction of retinoids and carotenoids followed by high-performance liquid chromatography separation and photometric detection of the pigments. It has not detected any newly formed retinal or other retinoids in the intestinal protein preparations from normal or vitamin A deficient rats. The latter were chosen as a possible source of more active enzyme preparations. With corresponding blank samples subjected to identical conditions of incubation but without added protein, small amounts of β-apocarotenals could be detected. They were previously reported as cleavage products of β-carotene [Ganguly, J., & Sastry, P. S. (1985) *World Rev. Nutr. Diet. 45*, 198–220] but are clearly not formed as a result of an enzymatic reaction. The failure to detect in vitro enzymatic central or random cleavage of the β-carotene molecule in extracts of rat intestinal mucosa emphasizes the need to reevaluate the existing theory of conversion of β-carotene into vitamin A.

Ever since it was demonstrated half a century ago by Moore (Moore, 1930) that "carotene" has to be considered a provitamin A, investigators have tried to gain insight into the nature of the conversion of β-carotene to vitamin A (Ganguly & Sastry, 1985). While it was suggested that this conversion of β-carotene takes place predominantly in the small intestine (Sexton et al., 1946), the efficiency of the conversion was always less than expected on the basis of central cleavage of β-carotene into two molecules of vitamin A aldehyde (retinal) (Brubacher & Weiser, 1985). In the late sixties, Goodman and Olson (Goodman & Huang, 1965; Goodman et al., 1966, 1967; Goodman, 1969; Fidge et al., 1969; Olson & Hayaishi,

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