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Activation of NADPH-dependent Superoxide Production in Plasma Membrane Extracts of Pig Neutrophils by Phosphatidic Acid*

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Phosphatidic acid (PA), a molecule that is rapidly produced by the stimulated turnover of phospholipids in a variety of cells including blood neutrophils, elicited NADPH-dependent superoxide anion (O_2^-) production in detergent extracts from membranes of resting pig neutrophils. The stimulatory effect of PA was independent of cytosolic factors, differing from arachidonic acid and sodium dodecyl sulfate which, on the contrary, absolutely required the presence of cytosol to elicit the same result. The O_2^--forming activity of the detergent extract activable by PA, as that by sodium dodecyl sulfate and arachidonic acid plus cytosol, was found in the chromatographic fractions containing cytochrome b_{558} and presented a chromatographic profile identical to that of the activated NADPH oxidase, which was obtained from neutrophils prestimulated with phorbol 12-myristate 13-acetate.

The PA-induced NADPH-dependent O_2^--forming activity showed kinetic properties and sensitivity to the inhibitors similar to the classical ones of the activated neutrophil NADPH oxidase.

The data suggest that, in this cell-free system, PA may stimulate O_2^- formation by direct interaction with latent NADPH oxidase of neutrophils or with some of its regulatory components.

The generation of oxygen-derived free radicals by phagocytes, induced by a wide variety of stimulatory agents, is due to the activation of a membrane-bound NADPH oxidase, which may consist of at least two components, the low potential cytochrome b_{558} (1-4) and a flavoprotein (4-7). The mechanism that regulates the conversion of the enzyme system from an inactive to an active form is poorly understood. When the problem has been investigated in cell-free systems, several laboratories have reported that NADPH-dependent O_2^- production can be activated in subcellular fractions (membranes and specific granules) by SDS[1] (8, 9), arachidonic acid (10-14), and other fatty acids (15). All these *in vitro* activation systems exhibited an absolute requirement for a cytosolic factor, whose nature remains to be clarified, and probably involve the participation of GTP-binding proteins (16, 17).

Since it is known that the respiratory burst of neutrophils is associated with increased hydrolysis of phospholipids and that phosphatidic acid (PA) is a central intermediate in the activated phospholipid metabolism of stimulated cells (18-23), we have investigated the effect of PA on NADPH-dependent O_2^- production in a cell-free system.

This paper shows that this acidic phospholipid is able to activate NADPH-dependent O_2^- formation by membrane extracts of resting pig neutrophils. The effect of PA does not require cytosolic factors and differs in this aspect from arachidonic acid- and SDS-induced activation.

MATERIALS AND METHODS

Reagents—Ultrogel AcA 34 was purchased from LKB Produkter (Bromma, Sweden). Arachidonic acid (Behring Diagnostics) was dissolved in ethanol and stored under N_2. Human Cu,Zn-superoxide dismutase was a gift from Dr. J. V. Bannister (Cranfield Biotechnology Centre, Bedford, United Kingdom) Diphenylene iodonium was kindly provided by Dr. A. R. Cross (University of Bristol, Bristol, United Kingdom). All other reagents, including PA and phospholipids, were purchased from Sigma. PA and phospholipids were dissolved in chloroform, dried under N_2, and resuspended in water by sonication.

Cell Isolation and Fractionation—Pig neutrophils were isolated from 8 liters of blood by dextran sedimentation and purification over Ficoll (24) and suspended (10×10^8 cells/ml) in Krebs-Ringer phosphate buffer, pH 7.0, containing 2 mM EDTA, 1 mM NaN_3, 5 mM glucose, 0.5 unit/ml heparin. In order to minimize proteolysis, cells were treated at 0 °C with 3 mM diisopropyl fluorophosphate for 10 min. Neutrophils were then pelleted at $400 \times g$ for 10 min and resuspended (10×10^8 cells/ml) in ice-cold 40 mM Tris-HCl, pH 7.4, 0.34 M sucrose, 10 mM EDTA, 1 mM $MgSO_4$, and 1 mM phenylmethylsulfonyl fluoride. The cells were disrupted by sonication (three or four bursts of 100 watts). This and all the subsequent steps were performed at 0-4 °C. Nuclei and unbroken cells were discarded by centrifugation at $800 \times g$ for 10 min. 15 ml of the postnuclear supernatant were layered on discontinous gradients formed by 10 ml of 50% sucrose and 5 ml of 20% sucrose in 40 mM Tris-HCl, pH 7.4, 10 mM EDTA, 1 mM $MgSO_4$, and 2 mM NaN_3 and centrifuged at $100,000 \times g$ for 90 min. The band between 20 and 50% sucrose (membrane fraction) was collected, washed with 2 volumes of 0.45 M NaCl (final concentration = 0.3 M), and centrifuged at $10,000 \times g$ for 10 min. The pellet was discarded, and the supernatant was centrifuged at $100,000 \times g$ for 60 min. The NaCl-washed membranes pellet was suspended in 50 mM sodium/sodium phosphate buffer, pH 8.0, containing 20% (v/v) glycerol, 2 mM EGTA, 1 mM $MgSO_4$, 2 mM NaN_3, and 1 mM phenylmethylsulfonyl fluoride (glycerol/phosphate buffer) at a protein concentration of about 4 mg/ml.

Preparation of Cytosol—To obtain large amounts of cytosol, resting pig polymorphonuclear leukocytes were suspended (15×10^7 cells/ml) in 10 mM potassium/potassium phosphate buffer, pH 7.0, containing 0.13 M NaCl, 1 mM EGTA, 0.34 M sucrose, 1 mM phenylmethylsulfonyl fluoride; sonicated for three or four bursts of 10 s (100 watts); and centrifuged at $800 \times g$ for 10 min to discard nuclei and unbroken cells. The postnuclear supernatant was centrifuged at $100,000 \times g$ for 90 min. The $100,000 \times g$ supernatant (cytosolic fraction) was aliquoted and stored at −195 °C until use.

Preactivation of Neutrophils—In some experiments, preactivation of cells with PMA was required. After treatment of cells with diiso-

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; PA, phosphatidic acid; EGTA, [ethylene bis(oxyethylenenitrilo)]tetraacetic acid; PMA, phorbol 12-myristate 13-acetate.

propyl fluorophosphate, the cells were suspended (5×10^7 cells/ml) in Krebs-Ringer phosphate buffer, pH 7.4, containing 2 mM EDTA, 1 mM NaN_3, 5 mM glucose, 0.5 unit/ml heparin and incubated with PMA (3 μg/ml) for 10 min at 37 °C. The activation was stopped by addition of 10-fold ice-cold buffer, and the suspension was centrifuged at 400 × *g* for 10 min. All the subsequent steps were performed as described for the resting cells.

Solubilization of Membranes—The membranes from resting and PMA-activated neutrophils, obtained by centrifugation on a sucrose gradient, were solubilized with 0.4% (v/v) sodium deoxycholate and 0.4% (v/v) Lubrol PX with constant stirring for 20 min. After centrifugation at 100,000 × *g* for 60 min, the pellet was discarded, and the supernatant was kept and stored at −195 °C before use.

Ultrogel Chromatography—The solubilized membranes (8 ml) were loaded on an Ultrogel AcA 34 column (1.6 × 30 cm) equilibrated with glycerol/phosphate buffer containing 0.15% (v/v) Lubrol PX and 0.15% (v/v) sodium deoxycholate and eluted with the same buffer. Fractions of 2.5 ml were collected at a flow rate of 30 ml/h. The fractions were tested for the cytochrome b_{558} contents (24) and NADPH-dependent O_2^- formation.

Assays—Superoxide generation was measured at 37 °C with a double-beam spectrophotometer by the rate of superoxide dismutase-inhibitable cytochrome *c* reduction as described by Bromberg and Pick (8). Both reference and sample cuvettes contained 65 mM sodium/potassium phosphate buffer, pH 7.0, 0.17 M sucrose, 2 mM NaN_3, 1 mM $MgCl_2$, 1 mM EGTA, 10 μM FAD, the enzyme, and, where indicated, the stimulant. The reference cuvette contained 50 μg of superoxide dismutase. The reaction was started by the addition of 0.2 mM NADPH or the stimulants, as indicated; and the increase of absorbance was monitored. The reduction of cytochrome *c* was calculated using an extinction coefficient of 21.1 mM^{-1} cm^{-1}. Cytochrome b_{558} and protein were determined as previously described (24). Phospholipids were analyzed by thin-layer chromatography (26). ATP content was measured by bioluminescence assay (27). Protein kinase C activity was measured as Ca^{2+}/phospholipid-dependent incorporation of ^{32}P from [γ-^{32}P]ATP into type H1 histone (28).

RESULTS

Activation of NADPH-dependent O_2^- Production in Detergent Extracts of Plasma Membranes—The results reported in Fig. 1 and Table IA show that the addition of arachidonic acid and SDS to the detergent extract of plasma membranes of resting neutrophils stimulated NADPH-dependent O_2^- production. The effect of these activators was absolutely dependent on the presence of cytosol. The addition of PA to the extract resulted in stimulation of NADPH-dependent O_2^- generation independently of the presence of cytosol, and the extent of the activation of O_2^- generation by this phospholipid was similar to that by SDS and arachidonic acid in the presence of cytosol. The data reported in Fig. 1 and Table I show that the addition of cytosol did not potentiate the stimulation of O_2^- generation by PA. Some preparations of cytosol caused a slight inhibition of O_2^- production by PA, but these were discarded because they also decreased the measurement of O_2^- in the xanthine-xanthine oxidase system, probably due to the presence of a small amount of superoxide dismutase or other scavengers.

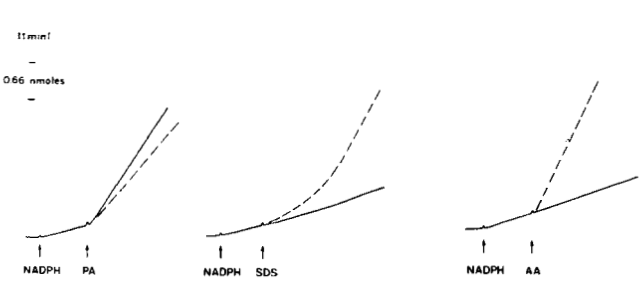

FIG. 1. **Spectrophotometric records of NADPH-dependent O_2^- production on detergent extract.** The assays were performed on plasma membrane extracts (18 μg of protein) in the presence (– – –) or absence (——) of cytosol (10^7 cell equivalents, about 350 μg of protein). The concentration of the stimulant was as follows: PA, 50 μM; SDS, 100 μM; and arachidonic acid (*AA*), 100 μM. A representative experiment is shown.

The effect of these stimulants was also tested on plasma membrane extracts prepared from pig neutrophils pretreated with 3 μg/ml PMA for 10 min, where the NADPH oxidase was present in a maximally activated state (149.2 ± 40.0 nmol of O_2^-/min/mg of protein). Both PA alone and SDS and arachidonic acid in the presence of cytosol from resting neutrophils did not cause any increase in NADPH-dependent O_2^- generation. The finding that these stimulants were ineffective on the extracts where the NADPH oxidase was in the active state indicates that, when resting preparations were used, the activation by PA, SDS, and arachidonic acid involved the "true" oxidase or some of its components.

Partial Purification of PA-activable NADPH-dependent O_2^--forming Activity—The solubilized extract of plasma membranes from resting neutrophils was chromatographed through a gel filtration column according to a procedure previously (24, 25) utilized for partial purification of the NADPH oxidase (see "Materials and Methods"), and the fractions were challenged with PA, SDS, and arachidonic acid. The activable O_2^--forming activity was detected only in the fractions containing cytochrome b_{558} (Fig. 2). The results reported in Table IB show that the effects of all the stimulants on the fractions of the partially purified preparation containing the major peak of cytochrome b_{558} were similar to those of the crude detergent extract (Table IA), the only difference being the higher specific activity.

Extracts of plasma membranes of neutrophils preactivated with PMA were chromatographed in the same way, and the data of Fig. 2 show that the chromatographic profile of the oxidase stimulated by PA, SDS, and arachidonic acid was superimposable on that of the NADPH oxidase of PMA-activated neutrophils.

These results confirm that the NADPH-dependent O_2^--forming activity stimulated by PA, SDS, and arachidonic acid corresponds to the NADPH oxidase.

Properties of PA-induced NADPH-dependent O_2^--forming Activity from Resting Neutrophils—The main features of PA-inducible NADPH-dependent O_2^- production have been studied in the fractions of the gel filtration chromatography column containing the major peak of cytochrome b_{558} and presenting the maximum rate of O_2^- formation (Fig. 2). The O_2^--forming activity induced by PA showed an optimum pH of 7.0 and an affinity for NADPH (K_m = 0.071 mM) an order of magnitude higher than that for NADH (K_m = 0.76 mM) (Fig. 3). The activation of O_2^- production was dependent on the concentration of PA (Fig. 4). Maximal activity was found at 50 μM, and higher concentrations did not cause further stimulation.

The PA-induced O_2^--forming activity was insensitive to cyanide and was inhibited by *p*-chloromercuribenzoate, Cibacron blue, and the newly discovered inhibitor diphenylene iodonium (29) (Table II). The same results were obtained when O_2^- formation was activated by SDS and arachidonic acid in the presence of cytosol. Furthermore, these properties were similar to those of the NADPH oxidase activity obtained from PMA-activated neutrophils (29–33).

It is largely accepted that, in the NADPH oxidase activity of phagocytes, a FAD-containing flavoprotein is involved (4–7); and some authors (8, 10) have reported that FAD potentiates O_2^- formation in cell-free system whereas others (9, 17) have not confirmed this finding. In our assay system (Table II), the activation of O_2^--forming activity by PA alone and by SDS and arachidonic acid plus cytosol was dependent on the presence of FAD. Other electron acceptors such as flavine

TABLE I

NADPH-dependent O_2^- production in cell-free systems by PA, SDS, and arachidonic acid

The assays were performed as described under "Materials and Methods." The protein concentrations in the assay mixture were as follows (μg/ml): extract, 17.6 ± 10; chromatographed extract, 5.2 ± 2; and cytosol, 335.4 ± 83. The data are the mean ± S.D. from five separate experiments. Part A is the detergent extract from neutrophil plasma membrane, and B is the fraction of chromatographed detergent extract containing the major peak of cytochrome b_{558}.

	No addition	PA (50 μM)	SDS (100 μM)	AA (100 μM)[a]
	nmol/min/mg protein			
A. Extract	6.1 ± 2.3	44.9 ± 12.0	9.3 ± 3.2	7.6 ± 5.3
Extract plus cytosol	6.2 ± 6.6	35.9 ± 6.1	41.6 ± 8.9	45.2 ± 12.4
B. Chromatographed extract	29.5 ± 18.7	166.1 ± 83.0	41.7 ± 21.9	35.5 ± 4.5
Chromatographed extract plus cytosol	30.9 ± 23.8	142.6 ± 35.3	193.0 ± 91.5	180.6 ± 96.5

[a] AA, arachidonic acid.

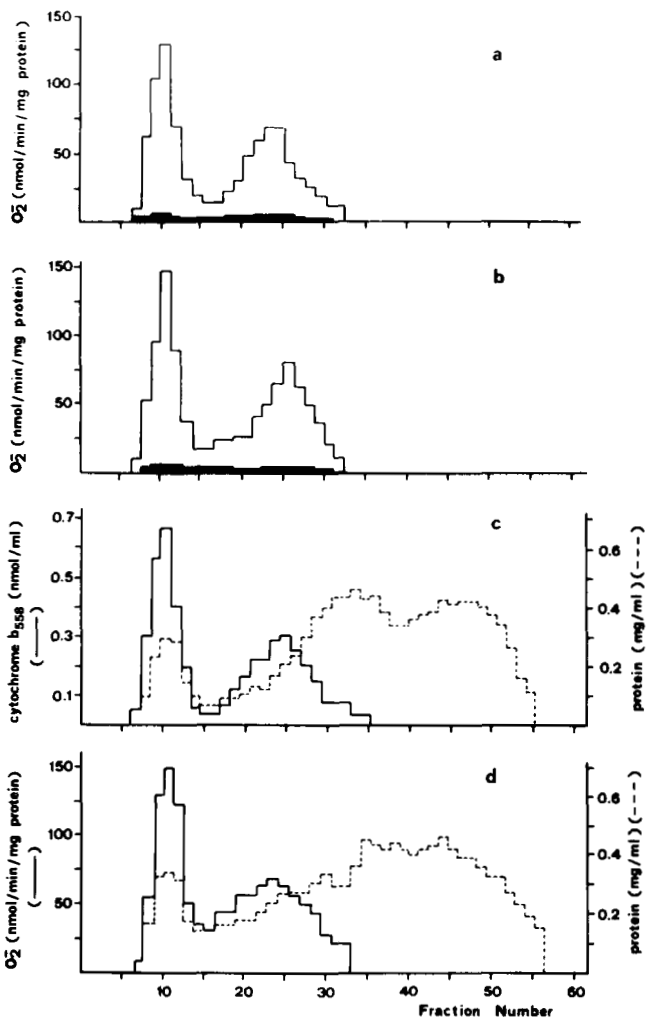

FIG. 2. **Gel filtration of extract from plasma membrane fraction of resting and PMA-activated pig neutrophils.** The Lubrol PX/deoxycholate extracts were chromatographed on an Ultrogel column, and the collected fractions were assayed as described under "Materials and Methods." *a*, NADPH-dependent O_2^--forming activity in the presence (*open bars*) and absence (*closed bars*) of 50 μM PA; *b*, NADPH-dependent O_2^--forming activity in the presence (*open bars*) and absence (*closed bars*) of 100 μM SDS plus cytosol; *c*, cytochrome b_{558} (——) and protein contents (- - -); *d*, NADPH oxidase activity (——) and protein contents (- - -) in the gel-filtrated extract from PMA-activated neutrophils. The major peak of cytochrome b_{558} of this preparation was 558 pmol/ml.

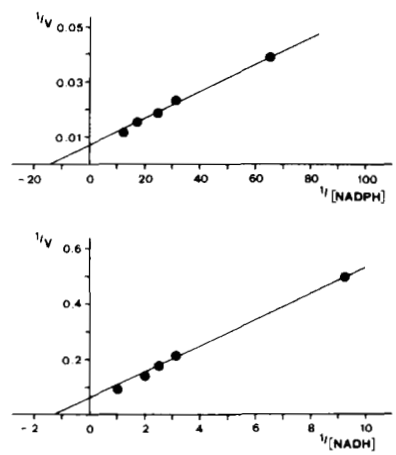

FIG. 3. **Lineweaver-Burk plots O_2^- production by PA as function of NADPH and NADH concentrations.** The assays were performed as described under "Materials and Methods." *Abscissa*, mM^{-1} NADPH or NADH; *ordinate*, (nmol of O_2^-/mg of protein/min)$^{-1}$. The concentration of PA was 50 μM. The gel-filtrated extract contained 8 μg of protein.

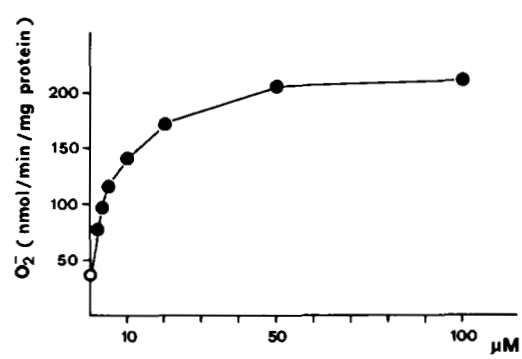

FIG. 4. **NADPH-dependent O_2^- production as function of PA concentration.** Various concentration of PA were tested on gel-filtrated extracts (2.9 μg of protein). The values are the means of three separate experiments.

mononucleotide (FMN), riboflavin, and menadione could not replace FAD. The NADPH oxidase activity from plasma membrane extracts from PMA-pretreated neutrophils did not require FAD (data not shown).

Recent reports have implicated protein kinase C in the series of events leading to the activation of phagocyte NADPH oxidase (for a review, see Ref. 36). Protein kinase C did not appear to be involved in the stimulation of NADPH-dependent O_2^- formation by PA in the cell-free assay system used here. In fact, (*a*) our assay mixture did not contain ATP, (*b*) neither ATP nor protein kinase C activity was present in the

TABLE II

Effect of different compounds on NADPH-dependent O_2^- formation by PA, SDS, and arachidonic acid

The assays were performed using the chromatographed plasma membrane extract plus cytosol when SDS and arachidonic acid (AA) were tested. The values are expressed as percentage respect the control (complete mixture) as described under "Materials and Methods."

	PA (50 μM)	SDS (100 μM)	AA (100 μM)
		% of control	
Complete mixture	100	100	100
p-Chloromercuribenzoate (100 μM)	3 ± 1	2.3 ± 1	5 ± 1
Cibacron blue (10 μM)	3 ± 2	3.7 ± 1	2 ± 1
Diphenylene iodonium (10 μM)	3.5 ± 1	1.2 ± 2	2.9 ± 1
H-7 (70 μM)	106 ± 3	112 ± 1	119 ± 3
Minus FAD	6 ± 1	8 ± 3	5 ± 3
Minus FAD plus FMN (10 μM)	5.5 ± 1	9 ± 3	5.8 ± 2
Minus FAD plus riboflavin (10 μM)	6.2 ± 2	10 ± 2	6 ± 1
Minus FAD plus menadione (10 μM)	6 ± 1	7 ± 1	6 ± 2

chromatographed enzyme used here, and (*c*) H-7 (1-(5-isoquinolinesulfonyl)-2-methylpiperazine) did not inhibit stimulation of O_2^- generation.

Effect of Different Phosphatidic Acids and Phospholipids—The data so far reported were obtained by using natural PA from egg yolk lecithin. Thin-layer chromatography of chloroform/methanol extracts of PA showed that it was essentially pure and did not contain free fatty acids and that the fatty acid composition of PA was the following: palmitic, 31%; oleic, 31.5%; linoleic, 17%; stearic, 13%; arachidonic, 2%; and lignoceric, 1%. To investigate the specificity of the stimulatory agent, we tested synthetic phosphatidic acids with different fatty acid compositions and other phospholipids. Among the compounds tested, the activating capability was also a property of two synthetic PAs, dioleoyl-PA and dilauroyl-PA, with a dose dependence similar to that of natural PA. No activity was elicited by phosphatidylcholine, phosphatidylinositol, dipalmitoyl-PA, and distearoyl-PA at concentrations between 10 and 100 μM.

DISCUSSION

The data reported in this paper show that, in cell-free systems, PA was able to activate NADPH-dependent O_2^- production by crude and gel-filtrated detergent extracts obtained from resting pig neutrophils. Contrary to the activation by SDS and arachidonic acid, that by PA did not require the presence of cytosol. The extent of activation was similar to that by SDS and arachidonic acid plus cytosol.

A series of experimental evidences indicates that the effect of PA, like those of SDS and arachidonic acid plus cytosol, was the result of the activation of components of the latent NADPH oxidase, which is responsible for the respiratory burst of intact cells. First, the purification profile of the PA-stimulated NADPH-dependent O_2^--forming activity was superimposable both on that of cytochrome b_{558}, a putative well-known component of the oxidase system (1–4), and on that of preactivated NADPH oxidase, extracted from plasma membranes of PMA-treated neutrophils and chromatographed under the same experimental conditions (Fig. 2). Second, NADPH-dependent O_2^- formation was not stimulated by PA in extracts of plasma membranes of neutrophils preactivated with PMA, where the NADPH oxidase was present in activated state. Third, the PA-activated enzyme exhibited optimum pH, kinetic properties (k_m for NAD(P)H), substrate specificity (NADPH *versus* NADH), and a sensitivity to the inhibitors similar to those of the classical NADPH oxidase responsible for the respiratory burst (29–33).

The precise mechanism whereby exogenous stimuli result in NADPH oxidase activation in phagocytes remains largely uncharacterized, and the ability to activate the oxidase in cell-free systems by unsaturated fatty acids and anionic detergents (8–15) has provided a new approach to understand this mechanism. Several groups of investigators have shown that these cell-free systems require the simultaneous presence of particulate fractions (membranes, specific granules) or membrane extracts from unstimulated phagocytes of cytosolic factor(s) and of the activating agent (*e.g.* arachidonic acid or SDS). It has been reported that the activation is enhanced by guanine nucleotides and fluoride in a manner consistent with the involvment of a GTP-binding protein (16, 17). The mechanism of activation of NADPH-dependent O_2^- protein by PA under the conditions employed here appears to differ from that of other stimulants. In fact, the effect of PA was independent of cytosolic factor(s), and these data indicate that, with respect to arachidonic acid and SDS, PA acts at a level more proximal to the enzymatic system on a step where the cytosolic factor is not necessary. It is likely that PA modifies some component of the NADPH oxidase, resulting in an easier interaction with the NADPH or with molecular oxygen. Another possibility is that PA causes a molecular perturbation of the lipid environment, thereby allowing the functional coupling among various enzyme components. The effect of FAD, which may accelerate (or permit) the electron transport chain at some still unidentified step, is in agreement with this last hypothesis. A definite clarification of the site where PA, FAD, and the membrane-bound oxidase interact awaits further investigation on the structure of enzyme complex.

Whichever the mechanism of PA may be, the data presented here indicate that, in the crude and gel-filtrated detergent extracts from plasma membrane fractions of resting pig neutrophils, all the components of the NADPH-dependent O_2^--forming system are present in a latent state and suggest that the cytosolic factor(s) necessary for other stimulants belong to the activation machinery and not to the effector enzyme.

The physiological significance of the events presented in this paper remains to be elucidated. The problem is whether PA and other stimulants in cell-free systems, like arachidonic acid, play a direct role in the activation of the NADPH oxidase in intact cells stimulated by a variety of signals, or whether these compounds mimic the final mechanism of activation that in intact cells is due to other final messengers. Interestingly, both an increase in PA production and fatty acids liberation is associated with the induction of the respiratory burst in leukocytes (18–20, 23).

There is a series of evidences that the mechanism(s) responsible for the activation of the oxidase in intact cells involves distinct multiform pathways when different classes of stimulatory agents are used (34–39). The direct modification of the oxidase, as shown by PA in cell-free systems, provides a model for the study of the terminal step of the multiform mechanisms of transduction involving various messengers and reactions.

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