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Phosphatidic acid induces the respiratory burst of electropermeabilized human neutrophils by acting on a downstream step of protein kinase C

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Phosphatidic acid (PA) dose-dependently induced superoxide (O_2^-) production of electropermeabilized human neutrophils but not of intact neutrophils, indicating that PA induces the activation of NADPH oxidase by acting on an intracellular target. The O_2^- production by PA was not inhibited by protein kinase C (PKC) inhibitors, such as staurosporine and calphostin C, and an inhibitor of PA phosphohydrolase, propranolol. These observations suggest that the activation of the oxidase by PA is independent of the activity of PKC and may dominate the activation by diacylglycerol which is formed from PA via the action of PA phosphohydrolase. Furthermore, the production by PA, as well as that by phorbol myristate acetate, was inhibited by cyclic AMP and GDPβS. Therefore, PA seems to act at a site downstream of PKC.

Human neutrophil; Respiratory burst; Electropermeabilization; Signal transduction; Phosphatidic acid; Propranolol

1. INTRODUCTION

Neutrophils are important in the host defense against microbial infection [1] and for this purpose are equipped with an enzymatic complex, NADPH oxidase, which is able to catalyze the one-electron reduction of molecular oxygen to superoxide (O_2^-). The oxidase is dormant in non-activated neutrophils and the signal transduction process leading to the activation of the oxidase has been extensively studied [2,3]. It is generally accepted that binding of agonists to their receptors stimulates phospholipase C (PLC) through the activation of a GTP-binding protein. The PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to the calcium ion mobilizer, inositol 1,4,5-trisphosphate, and the protein kinase C (PKC) activator, 1,2-diacylglycerol (DG). The activation of PKC leads to the conversion of the dormant oxidase into its active form in an unknown manner. Phospholipase D (PLD) has been reported to be involved in the signaling pathway for the respiratory burst [4–15]. The activated PLD hydrolyzes phospholipid, preferentially phosphatidylcholine, into phosphatidic acid (PA). Although PA is further degraded to DG, a PKC activator, by the action of PA phosphohydrolase, it has also been reported that PA itself might be a messenger for the respiratory burst [7,12,14,15].

Permeabilized neutrophils have been used to investigate the signaling pathway for the respiratory burst because they have pores on the plasma membrane which allow the permeation of agents with molecular weights below 1,000 Da, and produce O_2^- on stimulation by *N*-formyl-methionyl-leucyl-phenylalanine, vanadate, PKC activators such as phorbol myristate acetate (PMA) and L-α-1-oleoyl-2-acetyl-*sn*-3-glycerol, arachidonic acid and SDS [16–21]. We have recently reported that PKC is followed by a GTP-binding protein which may be distinct from *rac* p21 in the signaling pathway for the respiratory burst, and cyclic AMP inhibits the pathway between the GTP-binding protein and NADPH oxidase, and that the process for activating the oxidase by SDS is different from that involving PKC [20].

In the present study, we characterized the O_2^- production induced by PA and investigated a step where PA acts in the signaling pathway for the respiratory burst using electropermeabilized human neutrophils. PA dose-dependently induced the O_2^- production of permeabilized cells and this production was not inhibited by PKC inhibitors or an inhibitor of PA phosphohydrolase, propranolol, indicating that the production is independent of PKC activity, and that PA itself may act as a messenger for the respiratory burst. PA seems to act at a site downstream of PKC because PA-induced O_2 production, as well as PMA-induced production, was inhibited by cyclic AMP and GDPβS. The results also suggest that the process for activating the oxidase by PA is different from that by SDS.

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Abbreviations: DG, 1,2-diacylglycerol; PKC, protein kinase C; PLD, phospholipase D; PA, phosphatidic acid.

2. MATERIALS AND METHODS

2.1. *Reagents*

Staurosporine and calphostin C were purchased from Kyowa Medex, Tokyo, Japan. NADPH and ATP were from Oriental Yeasts, Tokyo, Japan. Phosphatidic acid (PA) prepared from egg yolk lecithin and L-α-1-oleoyl-2-acetyl-*sn*-3-glycerol were from Funakoshi Chemical, Tokyo, Japan. SDS was from Wako Pure Chemical Industries, Osaka, Japan. The following materials were obtained from Sigma Chemical Co., St. Louis, MO, USA: ferricytochrome *c*, superoxide dismutase (SOD), phorbol myristate acetate (PMA), propranolol hydrochloride, cyclic AMP, guanosine-5′-*O*-[2-thiodiphosphate] (GDPβS) All other reagents were of analytical grade. PA was dissolved in chloroform, dried under N_2, and resuspended in water by sonication

2.2. *Preparation of human neutrophils*

Human neutrophils were isolated from a healthy donor as described previously [22]. In short, after elimination of erythrocytes by dextran sedimentation followed by a brief hypotonic lysis, the cell suspension was centrifuged in a Ficoll–sodium isothalamate gradient to separate the polymorphonuclear leukocytes from lymphocytes, monocytes and platelets. Isolated cells were suspended in a HEPES-buffered salt solution, containing 135 mM NaCl, 5 mM KCl, 5 mM glucose and 20 mM HEPES (pH 7 4), and stored on ice until use For electropermeabilization, cells were suspended in an ice-cold permeabilization medium containing 140 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0 193 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH 6 7).

2.3 *Cell permeabilization*

Permeabilization was performed according to the method of Lu and Grinstein [19] except that the ice-cold permeabilization medium was adjusted to pH 6.7 (4°C). Briefly, 8×10^6 cells were transfered to a Bio-Rad Gene Pulser and permeabilized with two discharges of 5 kV/cm from a 25-μF capacitor. Between pulses, cells were rapidly sedimented and resuspended in the fresh ice-cold medium. Permeabilized cells were sedimented and finally suspended in an assay buffer containing 140 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.193 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH 6.7 at 37°C) Cells of 2×10^6 were used for each assay within 15 min of preparation.

2 4. *Acetylation of ferricytochrome* c

Ferricytochrome *c* was dissolved as previously described [23]. At 4°C, 200 mg of ferricytochrome *c* was dissolved in 10 ml of a half-saturated solution of sodium acetate After 0.4 ml of acetic anhydride was added and stirred for 30 min, the reaction mixture was dialyzed at 4°C for 12 h against the assay buffer.

2 5 *Assay of O_2^- production by intact neutrophils*

The assay mixture (1 0 ml) consisted of 50 μM ferricytochrome *c*, 1 mM $CaCl_2$ and 1×10^6 cells in the HEPES-buffered salt solution containing 135 mM NaCl, 5 mM KCl, 5 mM glucose and 20 mM HEPES (pH 7.4). Where indicated, $CaCl_2$ was omitted. Cells were incubated at 37°C for 5 min and O_2^- production was initiated by the addition of a stimulant and measured by determining the rate of superoxide dismutase (SOD)-inhibitable ferricytochrome *c* reduction at 550–540 nm using a dual-wavelength spectrophotometer (Hitachi 557), as previously described [22]. The O_2^- release was calculated using a molar absorption coefficient of 19,100 M^{-1} cm^{-1}.

2.6. *Assay of O_2^- production by electropermeabilized neutrophils*

The assay mixture (1.0 ml) consisted of 50 μM acetylated cytochrome *c* and 2×10^6 permeabilized cells in assay buffer containing 140 mM KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.193 mM $CaCl_2$, 10 mM glucose and 10 mM HEPES (pH 6.7 at 37°C). Under this condition, the Ca^{2+} concentration in the reaction mixture was about 100 nM [13] Cells were incubated at 37°C for 5 min followed by the addition of 2 mM NADPH and 1 mM ATP. The production of O_2^- was initiated by the addition of a stimulant and measured by determining the rate of SOD-inhibitable reduction of acetylated cytochrome *c* at 550–540 nm using a dual-wavelength spectrophotometer (Hitachi 557). The O_2^- release was calculated using a molar absorption coefficient of 19,100 $M^{-1} \cdot cm^{-1}$ Where indicated, permeabilized cells were replaced by intact cells.

3. RESULTS

3.1. *O_2^- production by phosphatidic acid*

We examined whether phosphatidic acid (PA), prepared from egg yolk lecithin, induces the O_2^- production of electropermeabilized and intact human neutrophils. As shown in Fig. 1, PA dose-dependently induced the O_2^- production of permeabilized neutrophils. ATP and Mg^{2+} were absolutely required for the production by PA (Table I). On the other hand, intact cells did not produce O_2^- by the addition of PA in the same assay medium (Fig. 1). This may not be due to the medium for permeabilized cells because phorbol myristate acetate (PMA) induced the production in the medium. In addition, PA did not induce the O_2^- production in intact cells when the assay medium was replaced by that for intact cells in the presence and absence of 1 mM $CaCl_2$. These results suggest that PA acts on an intracellular target.

3.2. *Effects of protein kinase C inhibitors and propranolol on O_2^- production of permeabilized neutrophils*

Because it has been proposed that PA is degraded to diacylglycerol (DG), which in turn activates PKC, by the action of PA phosphohydrolase, we examined the effect of protein kinase C (PKC) inhibitors such as staurosporine and calphostin C on the O_2^- production to show whether PA induces the O_2^- production via the action of PKC. As shown in Table I, 10 nM staurosporine and 5 μM calphostin C completely inhibited PMA-induced O_2^- production, but did not inhibit the PA-induced production. These results suggest that the O_2^- production by PA is independent of the PKC activ-

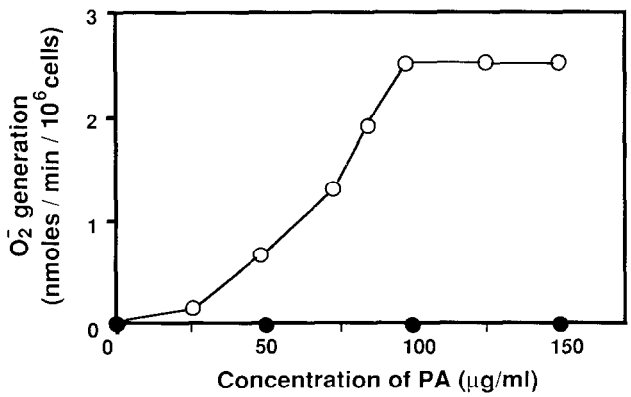

Fig 1. The O_2^- production of permeabilized and intact human neutrophils induced by phosphatidic acid The O_2^- production was measured by the assay method for permeabilized cells which is described in section 2. The results are representative of three independent experiments. Open circles (○) and closed circles (●) represent the O_2^- production by permeabilized and intact cells, respectively

ity. In the present study, we used staurosporine and calphostin C because they inhibit PKC by different mechanisms [24–26].

In connection with the above observations, we also examined the effect of propranolol on the O_2^- production induced by PA because propranolol has been reported to inhibit PA phosphohydrolase [8,9,12,14,27–30]. As shown in Fig. 2, the O_2^- production by 100 μg/ml PA, the concentration at which the maximal response was induced, was not affected by propranolol, but the production by 50 μg/ml PA, the concentration at which a quarter of maximal response was induced, was markedly enhanced by propranolol. These results suggest that PA is rapidly degraded by PA phosphohydrolase and the inhibition of PA phosphohydrolase by propranolol results in the retention of PA. The rapid degradation of PA by PA phosphohydrolase may be one of the reasons why a relatively high concentration of PA was required to induce the maximal response (Fig. 1), while L-α-1- oleoyl-2-acetyl-*sn*-3-glycerol at the low concentration of 4 μg/ml induced the maximal response of permeabilized cells (results not shown). Furthermore, the result that propranolol had no inhibitory effect on the O_2^- production by PA may suggest that DG formed by PA phosphohydrolase does not play a significant role in the O_2^- production when the activation of the oxidase is induced by PA. Therefore, the activation by PA may dominate the activation by DG generated through the action of PA phosphohydrolase. Sozzani et al. [31] have recently reported that propranolol inhibits not only PA phosphohydrolase but PKC. In fact, 200 μM propranolol inhibited PMA-induced O_2^- production of permeabilized cells by 47.5 ± 9.3% (n = 4), but did not show inhibitory effects on PA-induced O_2^- production (Fig. 2). These observations are compatible with the hypothesis that propranolol inhibits PKC, and with our result that the O_2^- production by PA was not inhibited by PKC inhibitors (Table I).

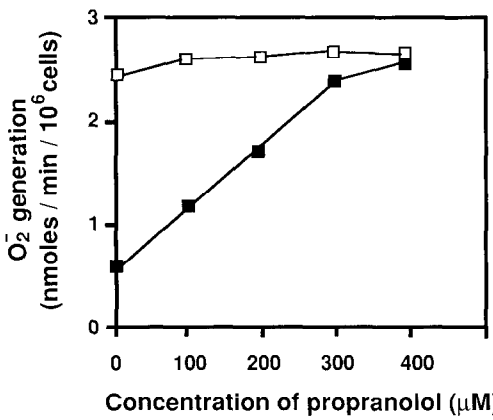

Fig. 2. Effect of propranolol on the O_2^- production of permeabilized neutrophils by phosphatidic acid. The O_2^- production was measured as described in Fig. 1 except that propranolol at various concentrations was added to the assay mixture 1 min before the addition of a stimulant. The results are representative of three independent experiments. Open squares (□) and closed squares (■) represent the O_2^- production induced by phosphatidic acid at the concentration of 100 μg/ml and 50 μg/ml, respectively.

3.3. *Effect of GDPβS and cyclic AMP on O_2^- production by phosphatidic acid*

We further investigated whether PA acts at a site downstream of PKC in the signaling pathway involving PKC or acts by different mechanisms as does SDS. Because cyclic AMP inhibits the O_2^- production of permeabilized cells induced by PMA but not by SDS, and GDPβS inhibits the production by PMA more strongly than the production by SDS [20], we examined the effects of GDPβS and cyclic AMP on the O_2^- production induced by PA (Table I). The O_2^- production by PA, as well as that by PMA, was half inhibited by 0.1 mM GDPβS and completely inhibited by 1 mM GDPβS, while the production by SDS was less sensitive to GDPβS. PA- and PMA-induced O_2^- production was inhibited about 50% by 0.75 mM cyclic AMP and by more than 80% by 3 mM cyclic AMP, but SDS-induced production was not inhibited by cyclic AMP. These results and the above observation that PKC inhibitors did not affect PA-induced O_2^- production (Table I) may suggest that PA induces the activation of NADPH oxidase at a site downstream of PKC. This suggestion was supported by the finding that the O_2^- production by PA was not enhanced by the addition of PMA (data not shown).

4. DISCUSSION

In the present study, we have directly demonstrated

Table I

Effects of various agents on the O_2^- production of permeabilized neutrophils induced by phosphatidic acid

Condition	Stimulants (% of control)		
	PA	PMA	SDS
− ATP	0.0	0.0	50.8 ± 2.9
− Mg^{2+}	0.0	0.0	39.7 ± 1.2
+ 10 nM staurosporine	93.3 ± 4.7	0.0	95.0 ± 4.1
+ 5 μM calphostin C	90.0 ± 2.4	0.0	98.0 ± 1.4
+ 0.1 mM GDPβS	50.7 ± 3.3	52.7 ± 5.7	80.7 ± 3.3
+ 1.0 mM GDPβS	0.0	0.0	29.8 ± 1.9
+ 0.75 mM cyclic AMP	51.2 ± 3 6	50.7 ± 1 9	100.0 ± 0.0
+ 3.0 mM cyclic AMP	13.0 ± 2.9	13.6 ± 2.6	100.0 ± 0.0

O_2^- production was measured as described in section 2. Where indicated, ATP or Mg^{2+} was omitted. The permeabilized neutrophils (2×10^6/ml) were preincubated with indicated agents at 37°C and then stimulated by 100 μg/ml phosphatidic acid (PA), 10 ng/ml PMA or 100 μM SDS. Staurosporine, GDPβS or cyclic AMP was added to the assay mixture 1 min before the addition of a stimulant, and calphostin C was added 5 min before the addition of a stimulant. The control activities of permeabilized cells by PA, PMA and SDS were 2.22 ± 0.06, 2.22 ± 0.05 and 3.80 ± 0.19 nmol/min/10^6 cells, respectively. The data represent the means ± S.D. of three separate experiments.

that phosphatidic acid (PA) induces the respiratory burst of electropermeabilized human neutrophils. PA seems to act on an intracellular target because PA did not induce the O_2^- production of intact cells. Ohtsuka et al. [32] have reported that 1,2-didecanoyl-3-*sn*-phosphatidic acid but not PA prepared from egg yolk lecithin induces the O_2^- production of the intact cells, which was in accord with our result.

It is generally accepted that PA, which is generated by the action of phospholipase D (PLD), is degraded to DG by the action of PA phosphohydrolase, and the DG activates PKC. Rossi et al. [14], however, have reported that the activation of the NADPH oxidase by *N*-formyl-methionyl-leucyl-phenylalanine correlates with the formation of PA but not with that of DG. It has also been reported that PA is capable of stimulating the NADPH oxidase in a cell-free system [12,15]. Because the O_2^- production by PA was not inhibited by PKC inhibitors (Table I) and by an inhibitor of PA phosphohydrolase, propranolol (Fig. 2), the activation of the NADPH oxidase by PA seems to be independent of the PKC activity, and the hypothesis that PA itself acts as a messenger for the respiratory burst may be confirmed.

The step at which PA is involved in the activation of the NADPH oxidase has not been identified. The present study indicates that PA may act at a site downstream of PKC in the signaling pathway involving PKC, on the basis of the following observations: (i) the PA-induced O_2^- production was not inhibited by PKC inhibitors (Table I), (ii) the production was not inhibited by propranolol (Fig. 2), (iii) the production was inhibited by cyclic AMP in a similar dose-dependent manner as the production induced by PMA (Table I), (iv) both productions were inhibited by GDPβS in a similar dose-dependent manner (Table I), and (v) the O_2^- production by PA was not enhanced by the addition of PMA (data not shown). The process for activating the NADPH oxidase by PA seems to be different from that by SDS, because ATP and Mg^{2+} were absolutely required for the O_2^- production by PA but not by SDS, and SDS-induced O_2^- production was not inhibited by cyclic AMP and was less sensitive to GDPβS than PA-induced production (Table I).

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