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Charge-dependent regulation of NADPH oxidase activities in intact and subcellular systems of polymorphonuclear leukocytes

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It has been reported that respiratory bursts with *N*-formylmethionylleucylphenylalanine, A23187, phorbol ester and fatty acids are switched off and on by modulating the net charges of plasma membranes in guinea-pig neutrophils (Miyahara, M. et al. (1987), Biochim. Biophys. Acta, 929, 253–262). In the present study, this was further extended in cells treated with protein kinase C inhibitors which completely suppressed the phorbol ester-dependent respiratory burst. This suggested that the initiation of the respiratory burst, which is generally accepted as linked to protein kinase C activation, might also be implicated in the net charge changes of plasma membranes. The above results were also supported by data obtained with a cell-free system reconstituted with plasma membranes and cytosolic fractions from unstimulated neutrophils, guanosine 5′-[γ-thio]triphosphate and NADPH. Arachidonate stimulated NADPH oxidase activity accompanied by a marked phoshorylation of membrane proteins. The phosphorylation was sensitive to H-7, but it did not appear to be essential for the respiratory burst, because the oxidase activation was insensitive to H-7. Pretreating the plasma membranes with positively charged cetylamine inhibited the oxidase activation by arachidonate. These results suggest that a charge-dependent process, which does not use protein kinase C, may play an important role in the reaction leading to NADPH oxidase activation, and this may be related to the interaction of plasma membranes with the cytosolic activation factor.

Introduction

In neutrophils, NADPH oxidase is a plasma membrane-bound enzyme and its activation by an appropriate stimulant is accompanied by a complex series of signal transduction events in which the translocation and activation of protein kinase C has been reported to play a central role in triggering respiratory bursts [1–8]. However, it is not yet clear whether the respiratory burst is triggered through a reaction that uses protein kinase C, because recent studies on the *N*-formylmethionylleucylphenylalanine (fMet-Leu-Phe)

Abbreviations: fMet-Leu-Phe, *N*-formylmethionylleucylphenylalanine; O_2^-, superoxide anion; PMA, phorbol 12-myristate 13-acetate; PMSF, phenylmethylsulfonyl fluoride; H-7, 1-(5-isoquinolinesulfonyl)methylpiperazine dihydrochloride; $C_{20:4}$, sodium arachidonate; $C_{14:0}$, sodium myristate; C-I, 1-(5-isoquinolinesulfonyl)piperazine; SDS, sodium dodecyl sulfate; LBS, laurylbenzenesulfonate; $C_{16}NH_2$, cetylamine; $C_{16}OH$, cetylalcohol; CTAB, cetyltrimethylammonium bromide; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; GTP[S], guanosine 5′-[γ-thio]triphosphate. H-8, *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride;

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-dependent respiratory burst indicate that the phosphoinositide turnover [9,10], intracellular movement of calcium ions [9,10], translocation and activation of protein kinase C [11–13] are not always necessary for the respiratory burst to occur. In addition to this, recent studies on the mechanism of the NADPH oxidase activation have been facilitated by the development of cell-free systems in which the enzyme can be activated by detergents in a manner insensitive to H-7 [14–17]. In addition to these complicated reports, Bromberg and Pick [14] and we [18] have suggested that a membrane charge also regulates the respiratory burst. The respiratory burst was reversibly switched off and on by modulating membrane charges [18]. The present study was aimed at extending this charge-dependent regulation of the respiratory burst, by focusing on the question of whether the protein kinase C activation is implicated in the initiation of the respiratory burst in guinea-pig polymorphonuclear leukocytes. We report that the charge-dependent regulation could be observed in cells treated with protein kinase C inhibitors and that the regulation might be involved in the interaction of cytosolic activation factor with plasma membranes.

Materials and Methods

Materials

The reagents used throughout the experiments were described previously [18]. [^{32}P]NaH_3PO_4 (spec. act. 1 Ci/mmol) and [γ-^{32}P]ATP tetra(triethylammonium) salt (spec. act. 28.9 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA, U.S.A.), and trifluoperazine and palmitoylcarnitine were from Sigma (St. Louis, MO, U.S.A.)

Methods

Preparation of cells and their subcellular fractions. Polymorphonuclear leukocytes were prepared from peritoneal exudate of guinea-pigs [18]. The subcellular fractions were prepared using the method of Borregaard et al. [19], except that the cell disruption medium contained an additional 0.5 mM PMSF, 0.5 mM EGTA, 10 mM Hepes (pH 7.8) and that the cytosolic fraction was prepared by centrifuging the $45000 \times g$ supernatant at $180000 \times g$ for 2 h. The protein content was determined using the method of Lowry et al. [20], using bovine serum albumin as a standard. Cell counts were performed microscopically using a hemocytometer.

Assay of O_2^- generation. NADPH oxidase activities were assayed as previously described [18]. In brief, it was carried out by recording cytochrome *c* reduction with the absorbance changes at 550–540 nm ($\Delta A_{550-540}$) with $(1–5) \cdot 10^6$ cells in a total volume of 2 ml at 37 °C, using a dual beam spectrophotometer (Shimadzu UV300) equipped with a water-jacketed cell holder and a magnetic stirrer. The reaction medium contained 140 mM NaCl, 20 mM KCl, 5 mM $MgCl_2$, 250 μM $CaCl_2$, 5 mM glucose, 1.5 mM NaN_3, 80 μM cytochrome *c* (type VI) and 5 mM Tris-HCl (pH 7.4).

In the cell-free system, plasma membranes (7–10 μg protein) and the cytosolic fraction (150–200 μg protein) were used in a medium similar to that described above, supplemented with 250 μM EGTA, 10 mM NaF, 20 μM GTP[S], 250 μM NADPH and 5 mM Hepes buffer (pH 7.8), but omitting $CaCl_2$. O_2^- generation was calculated from superoxide dismutase-inhibitable cytochrome *c* reduction in the linear phase of the progression curves, using the absorption coefficient of 21.0 $mM^{-1} \cdot cm^{-1}$ [21]. O_2^- generation was also assayed polarographically by measuring oxygen consumption using a Clark type oxygen electrode with $(5–10) \cdot 10^6$ cells in a medium similar to that described above, but omitting cytochrome *c* [22].

Phosphorylation studies. In the cell-free systems, the cytosolic fraction (600 μg protein) pretreated with various concentrations of H-7 for 2–5 min at 37 °C was incubated for an additional 5 min by adding plasma membranes (40 μg protein) and 20 μM [γ-^{32}P]ATP (1000 cpm/pmol) successively. 100 μM arachidonate was added to the incubation mixture immediately after [^{32}P]ATP addition. The total volume of the reaction mixture was 1.0 ml and the components were similar to those used for the assay of superoxide generation in the cell free system. After incubation, the plasma membranes were collected on a glass filter (GF/C, Whatmann) previously impregnated with 1% serum albumin. The filter was washed three times with an ice-cold incubation medium (total 12 ml) contain-

ing an additional 100 μM pyrophosphate instead of labelled ATP, immersed in the solubilizing cocktail, treated for 3 min in boiling water and the exudate (equivalent to 10 μg membrane protein) was applied to a 10% SDS-polyacrylamide slab gel and electrophoresis was performed. The radioautograph was performed using X-ray film (Kodak, AR5) at −70°C for 40–45 h [13]. In preliminary experiments, the efficiency of the glass filter in trapping plasma membranes was determined to be 87–92% and was nearly linear to the loaded amounts.

For phosphorylation studies with intact cells, the cells ($8 \cdot 10^7$ cells) were preincubated with 0.5 mCi [^{32}P]Na_2HPO_4 in a medium similar to that used for the respiratory burst assay, in a final volume of 5 ml for 90 min at 37°C with gentle shaking. The cells were washed once and an aliquot ($4 \cdot 10^6$ cells) was treated with 100 μM H-7 for 2–5 min in a similar incubation medium except for the absence of labelled Na_2HPO_4 incubated for an additional 6 min with PMA, and then myristate was added at selected time intervals. After incubation, the cells were washed twice by centrifuging with an ice-cold incubation medium without stimulant, but supplemented with 100 μM non-labelled sodium phosphate and solubilized. The aliquot (equivalent to 30 μg protein) was used in SDS-polyacrylamide gel electrophoresis and radioautography as above [13]. The data represent typical results from four or five different preparations.

Results

Fig. 1 shows that both the fMet-Leu-Phe- and PMA-induced respiratory burst were reversibly and were rapidly switched off and on by modulating net charges of membrane surfaces with positively and/or negatively charged liophilic alkyl compounds. Regulation also occurred in the respiratory burst using other stimulants, such as A23187, myristate and arachidonate [18]. These results suggest that the triggering reaction leading to the respiratory burst involvs a common regulation site responsible for the membrane charges, and that the site might be located at the protein kinase C and/or a more terminal site of the reaction using protein kinase C. This was further examined by studying whether the charge-dependent regulation was affected by protein kinase C inhibitors (Table I). To monitor the extent of the inhibition of protein kinase C activities, the cells

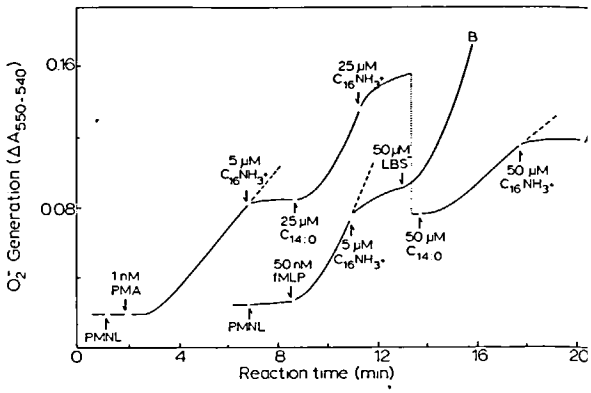

Fig. 1. Charge-dependent regulation of the respiratory burst induced by fMet-Leu-Phe and/or phorbol ester in guinea-pig polymorphonuclear leukocytes. The respiratory burst was measured spectrophotometrically by monitoring cytochrome *c* reduction with $1 \cdot 10^6$ cells for curve A and $3 \cdot 10^6$ cells for curve B. The order of addition and concentration of the agents are shown. PMNL, polymorphonuclear leukocytes; fMLP, fMet-Leu-Phe.

TABLE I

INHIBITION OF THE PMA-DEPENDENT RESPIRATORY BURST BY PROTEIN KINASE C INHIBITORS AND THE RELIEF BY MYRISTATE

$4 \cdot 10^6$ cells were preincubated with the indicated concentrations of protein kinase C inhibitors for 2 min and 2 nM PMA were added. 50 μM myristate were added 6 min after PMA addition.

Protein kinase C inhibitor	Stimulant	O_2^- generation (nmol per min per 10^7 cells)
Control	none	0.2
	PMA	16.0
	myristate	26.6
	PMA + myristate	29.7
H-7 (150 μM)	PMA	1.6
	PMA + myristate	29.4
Palmitoyl carnitine (2 μM)	PMA	1.9
	PMA + myristate	31.9
H-8 (200 μM)	PMA	3.5
	PMA + myristate	29.3
Trifluoperazine (5 μM)	PMA	2.6
	PMA + myristate	22.0

were preincubated with an appropriate C kinase inhibitor, the PMA-dependent respiratory burst was measured and then charged agents were added, because it has been reported that PMA induces respiratory bursts by directly activating protein kinase C [23]. This excluded any doubt of whether the induction of respiratory bursts resulted solely from a lack of cellular penetration by the inhibitors. As shown in Table I, the cells preincubated with 150 μM H-7 showed a slight respiratory burst induced by PMA, suggesting that no protein kinase C was operating, but the subsequent addition of myristate clearly induced superoxide generation. Similar results were also observed in cells pretreated with other protein kinase C inhibitors. Furthermore, it was noted that the PMA-dependent respiratory burst was a function of increasing amounts of H-7, but its relief by myristate was independent of H-7 (Table II). Similar results were observed in cells pretreated with trifluoperazine. As the myristate is not assumed to decrease inhibition by direct removal of H-7 and/or other inhibitors from their binding sites, the results observed were assumed to show that the myristate-induced respiratory burst was independent of the reaction which was implicated in protein kinase C. Furthermore, the results shown in Table III indicate that negative charges played an effective role in the recovery of superoxide generation in cells treated with protein kinase C inhibitors. By adding neutral and positively charged compounds, even though they were similar to the above anionic lipophils in their hydrophobic tails, the oxidative burst did not reappear. Furthermore, in cells treated with cationic lipophils, H-7 inhibition was unchanged by addition of similar amounts of myristate, but the alcohol pretreatment did not arrest the reappearance. This was also the case in cells pretreated with trifluo-

TABLE II

CORRELATION BETWEEN THE PROTEIN KINASE C INHIBITOR CONCENTRATION AND MYRISTATE-INDUCED RELIEF OF THE ARRESTED RESPIRATORY BURST

$2.5 \cdot 10^6$ cells were preincubated for 2 min with the indicated concentrations of H-7 and/or trifluoperazine, and 2 nM PMA was added. 50 μM myristate was added 6 min after PMA addition. n.d., not determined. No correlation was observed.

Inhibitor	O_2^- generation (nmol per min per 10^7 cells)	
	PMA	PMA + myristate
H-7 (μM)		
0	23.4	33.0
10	12.9	32.0
50	2.2	32.4
100	0.1	35.0
Trifluoperazine (μM)		
0	27.3	33.0
1.5	8.1	n.d.
5	0.5	33.0
10	0.0	26.8

TABLE III

INHIBITION OF THE PMA-DEPENDENT RESPIRATORY BURST AND REAPPEARANCE OF THE RESPIRATORY BURST CAUSED BY ANIONIC LIPOPHILIC ALKYL COMPOUNDS IN CELLS TREATED WITH H-7 AND TRIFLUOPERAZINE

$2 \cdot 10^6$ cells were treated for 2 min with the indicated concentrations of H-7 and/or trifluoperazine, then 2 nM PMA and 50 μM agents were added in a time schedule similar to that shown in Table I.

Agent	O_2^- generation (nmol per min per 10^7 cells)
None	
None	0.1
PMA	27.1
$C_{14:0}$	37.6
H-7 (100 μM)	
PMA	0.0
$C_{14:0}$	43.2
$C_{20:4}$	29.8
SDS	39.5
LBS	35.2
$C_{16}NH_2$	0.0 (0.0 [a])
CTAB	0.0 (0.0 [a])
$C_{16}OH$	0.3 (39.3 [a])
Trifluoperazine (5 μM)	
PMA	0.7
$C_{14:0}$	38.7
$C_{20:4}$	26.1
SDS	29.6
LBS	29.6
$C_{16}NH_2$	0.0 (0.0 [a])
CTAB	0.0 (0.0 [a])
$C_{16}OH$	1.0 (31.2 [a])

[a] O_2^- generation by further addition of 50 μM myristate.

TABLE IV

RECONSTITUTION OF THE OXIDATIVE BURST IN A CELL-FREE SYSTEM

The complete system contained plasma membranes (10 μg protein), cytosolic fraction (150 μg protein), 10 mM NaF, 20 μM GTP[S] in 2 ml of the reaction mixture. The reaction was carried out by preincubating cytosol with plasma membranes for 1.5 min prior to 250 μM NADPH addition. 100 μM arachidonate was added 2 min after NADPH addition, and the rate was calculated from the linear phase of the cytochrome *c* reduction. The − and + indicate the ommission and addition respectively of corresponding components or agents.

	O_2^- generation (nmol per min per mg plasma membrane)	
	basal	arachidonate
Complete	22.2	229.0
−Cytosol	22.6	48.3
−Plasma membranes	0.3 [a]	4.3 [a]
−NaF, −GTP[S]	19.7	55.7
−NaF, −GTP[S], +GDP (1 mM)	14.8	41.9
+Pertussis toxin (1 μg/ml)	24.6	204.4

[a] Spec. act. of the cytosol (nmol per min per mg protein); basal activity, the NADPH-dependent activity before arachidonate addition.

perazine. These results were also confirmed by our measurements of oxygen consumption (data not shown), suggesting that the charge-dependent regulation of the respiratory burst took place in a reaction(s) after the protein kinase C and/or in a reaction(s) which does not use protein kinase C, and that the protein kinase-dependent phosphorylation was not the sole pathway leading to the initiation of the respiratory burst. This was further examined using a more simplified cell-free system.

The non-stimulated resting cells were disrupted by a nitrogen cavitation method, and a cell-free system showing a respiratory burst was reconstituted with plasma membranes, the cytosolic fraction, GTP[S], EGTA, $MgCl_2$, NADPH and arachidonate (Table IV). Other stimulants such as PMA, fMet-Leu-Phe and A23187 were unable to activate O_2^- generation even in the presence of 0.05–1 mM $CaCl_2$ and the addition of 100 μM ATP had no effect on the reaction (Table V). The activation of O_2^- generation by arachidonate in a cell-free system was, as reported previously [15,16], confirmed to require plasma membranes, cytosolic

TABLE V

COMPARISON OF THE NADPH OXIDASE ACTIVATION WITH VARIOUS STIMULANTS IN THE CELL-FREE SYSTEM

The system contained plasma membranes (8–10 μg protein), cytosolic fraction (160–250 μg protein) in a medium similar to that shown in Table IV and the text. The reaction was carried out as described in Table IV by adding the indicated amounts of stimulants. (A), (B) and (C) contained 500 μM EGTA, 100 μM $CaCl_1$ and 1 mM $CaCl_2$, respectively. The data are expressed as mean ± S.E. of four different preparations.

Stimulant	O_2^- generation (nmol per min per mg plasma membrane)
None	19.0 ± 2.5
100 μM Arachidonate (A)	232.9 ± 5.0
50 nM PMA (B)	29.7 ± 2.2
25 nM fMet-Leu-Phe (C)	22.1 ± 6.7
1 μM A23187 (C)	23.9 ± 3.9

fraction and GTP[S]. The cytosolic fraction required for maximum activation was 15 to 20-fold higher in the protein content than the plasma membranes (data not shown).

In this reconstituted system, arachidonate was shown to phosphorylate some protein species in the plasma membranes and this was completely abolished in the presence of 100 μM H-7 (Fig. 2), leading us to assume that phosphorylation is implicated in the NADPH oxidase activation. However, the activation of O_2^- generation by

TABLE VI

NO INHIBITORY EFFECT OF H-7 ON THE ARACHIDONATE-DEPENDENT NADPH OXIDASE ACTIVATION IN THE-CELL FREE SYSTEM

The cytosol fraction (150 μg protein) was treated with various amounts of H-7 for 2 min prior to plasma membrane addition (10 μg protein), stirred, and 250 μM NADPH and 80 μM arachidonate were added in a time schedule similar to that shown in Table I.

H-7 (μM)	O_2^- generation (nmol per min per mg plasma membrane)	
	basal	arachidonate
0	2.4	192.4
25	3.4	193.7
50	3.6	193.6
100	6.1	193.9
150	4.2	193.2

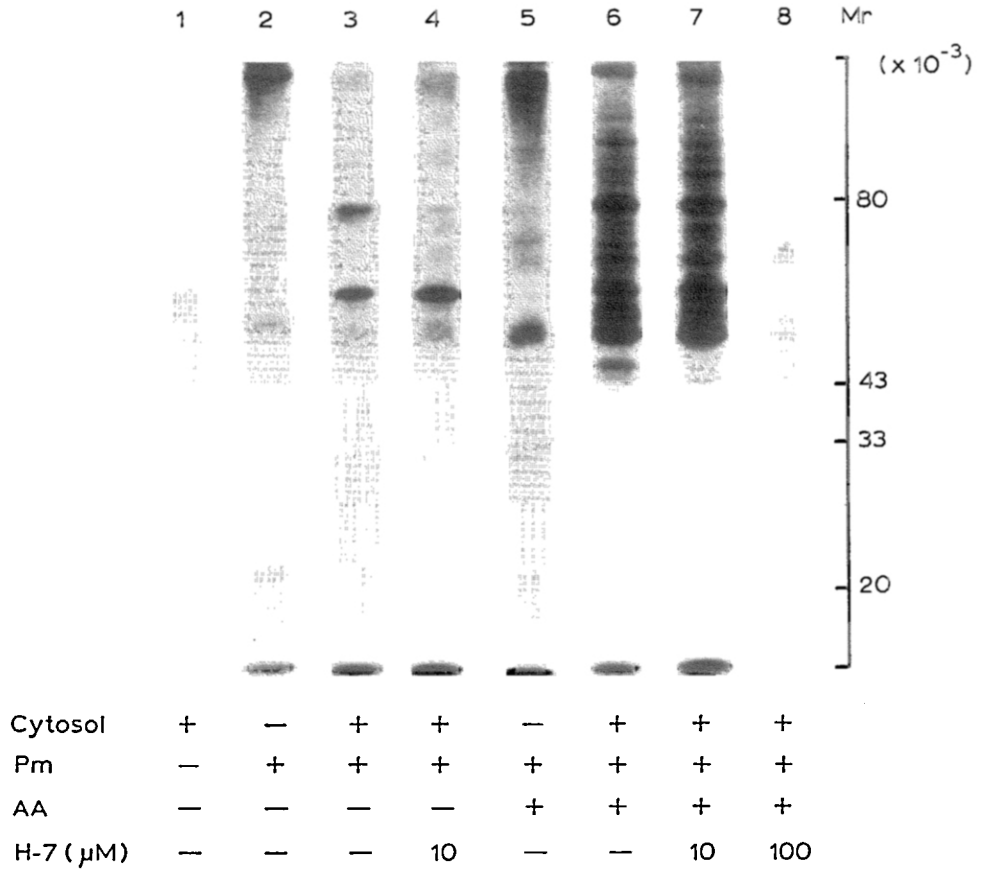

Fig. 2. Enhanced phosphorylation of plasma membrane (Pm) proteins by arachidonate and its inhibition by H-7 in the cell-free system. The phosphorylation, SDS-polyacrylamide gel electrophoresis and autoradiograph were carried out as described in the text. In lanes (4), (7) and (8), the cytosol was preincubated with the indicated amounts of H-7 for 2 min prior to mixing with plasma membranes. The final concentration of arachidonate (AA) was 100 μM. The − and + indicate the absence and the presence of the components or agents, respectively.

arachidonate was absolutely insensitive to H-7 (Table VI). The results indicated that arachidonate-induced and H-7-sensitive phosphorylation at the plasma membranes was not implicated in the activation of NADPH oxidase, and that a more direct key reaction leading to the activation of NADPH oxidase might be implicated. We then examined whether the charge dependency of the respiratory burst observed in intact cells was also the case in the above cell-free system. This was thought to be useful for determining the site of charge-dependent regulation (Fig. 3). The O_2^- generation induced by arachidonate was suppressed markedly by the addition of positively charged cetylamine (curve a), and pretreatment of the plasma membranes with the amine also inhibited the arachidonate-induced activation (curve b). This suggested that the charge-dependent regulation may be involved with the interaction of the cytosolic activation factor and the plasma membranes, as cetylamine alone had no effect on NADPH oxidase activities [18]. To confirm further this consideration, we tested the possibility that the pH value, in pretreating plasma membranes, might influence the enzyme activities (Fig. 4), because increasing pH values would accompany a relative increment in net negative charges at the membrane surfaces. As shown in the figure, the basal NADPH oxidase activity in the presence of the cytosolic fraction increased gradually with

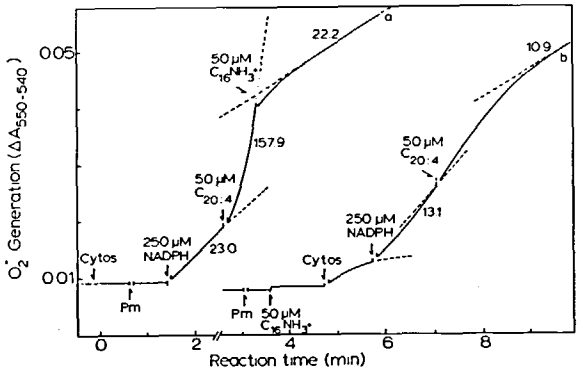

Fig. 3. Charge-dependent regulation of NADPH oxidase activity by lipophilic alkyl compounds in the cell-free system. The cytosol (cytos) and plasma membranes (Pm) were 160 and 10 μg protein, respectively. The order and the concentrations of the agents are shown. The numbers on the curves indicate the rate of O_2^- generation (nmol per min per mg membrane protein).

increasing pH. This supports the above-mentioned possibilities that negative charges at the plasma membrane surfaces may be related to the interaction of the cytosolic activation factor and that this led to NADPH oxidase activation, while an positively charged increment would suppress the event.

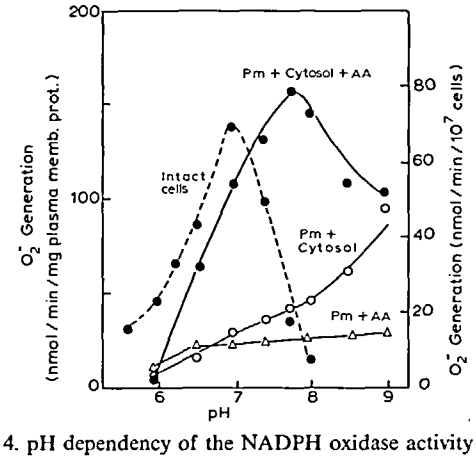

Fig. 4. pH dependency of the NADPH oxidase activity in the cell-free system. Plasma membranes (Pm) (10 μg protein) pretreated for 2 min with 50 mM $NaKP_i$ buffer solution at the indicated pH value were added to the reaction system with or without the cytosolic fraction (220 μg protein) and incubated for an additional 1.5 min. NADPH and arachidonate were added in a manner similar to that shown in Fig. 3. The dotted curve shows O_2^- generation by intact cells stimulated with 50 μM arachidonate in a manner similar to that described in Fig. 3, except that NADPH, EGTA ad GTP[S] were present in the reaction mixture. AA, 50 μM arachidonate.

Discussion

The question of whether the translocation of cytosolic protein kinase C and its activation at the plasma membranes are necessary for the onset of respiratory burst is an important one for understanding the triggering mechanism of the respiratory burst. There have been two opposite and inconsistent observations regarding this. One, as described previously, comes from the observations that the protein kinase C inhibitors such as H-7 and C-I inhibit the respiratory burst induced by fMet-Leu-Phe, A23187, diacylglycerol and PMA [3–8]. The other arises from observations that the same H-7 and C-I do not inhibit the fMet-Leu-Phe-induced respiratory burst, but that they do arrest the respiratory burst by PMA [12,24,25]. In support of the latter, fMet-Leu-Phe induces respiratory burst without associating with phosphoinositide turnover in Ca^{2+}-depleted neutrophils [9,10] and neither does it cause any translocation of cytosolic protein kinase C to the membrane fraction [26,27]. Concanavalin A has been reported to release protein kinase C from the membranes into the cytosol, accompanying the respiratory burst [28]. On the other hand, the present results, together with our previous report [18], indicate that the respiratory bursts using different stimulants, such as fMet-Leu-Phe, A23187, myristate, arachidonate and PMA, were all inhibited by modulating plasma membranes with cationic lipophilic alkyl compounds, and that the inhibition was relieved by counteracting positive charges with anionic alkyl compounds [18]. Furthermore, the inhibition of the PMA-dependent respiratory burst by H-7 and/or other inhibitors of protein kinase C was rapidly relieved by negatively charged liophilic alkyl compounds (Tables I, II and III). Cationic compounds inhibited this relief and alcohol compounds showed no effects on it. Similar results were also observed in the cell-free system (Fig. 3). Recent studies by Sakata et al. [29] are consistent with our finding in that they observed that arachidonate-induced O_2^- generation in macrophages is not inhibited by H-7. These results suggest that the fatty acid-dependent respiratory burst would be triggered in a manner similar to that described above, which is independent of the protein kinase C reaction. It

should be noted that sphinganine, a positively charged phospholipid, inhibits the oxidative burst induced by a variety of stimulants, including fMet-Leu-Phe, arachidonate, diacylglycerol and PMA [30]. This is similar to results obtained using cetylamine and other lipophilic alkylamines in the previous report [18]. Pittet et al. [35] reported that sphinganine was cytotoxic and it nonspecifically inhibited various cellular responses.

Some investigators have stated that the fusion of specific and/or tirtiary granules to plasma membranes leads to the translocation of cytochrome *b* into the plasma membranes and that this leads to NADPH oxidase activation [19,32]. The present results, however, do not confirm this, because the activation of superoxide generation in the cell-free system was reconstituted by plasma membranes, cytosolic fraction and NADPH, and no other subcellular components were required when using arachidonate as a stimulant. This is also supported by a report that the deoxycholate extract of plasma membranes from resting human neutrophils showed NADPH oxidase activation by sodium dodecyl sulfate in the system containing NADPH and cytosolic fraction [17].

The majority of the phosphorylation in neutrophils has been reported to account for the protein kinase C reaction [33]. Here, the questions remains of whether the phosphorylation shown in Fig. 2 resulted from the activation of protein kinase C, because our experimental conditions were devoid of $CaCl_2$ and contained 250 μM EGTA to effect the arachidonate-dependent oxidative burst. This raises the possibility that the oxidative burst might be induced in a more marked manner if the system were supplemented with $CaCl_2$ and ATP. However, we failed to obtain any positive evidence to support this possibility in a reaction system which was reconstituted with additional components such as 50–500 μM $CaCl_2$, ATP and PMA (Table IV). Furthermore, NADPH oxidase activation by arachidonate in the cell-free system was insensitive to H-7 (Table VI). These results suggest that the arachidonate-dependent NADPH oxidase activation is triggered by a reaction which is not implicated in the phosphorylation reaction under the conditions used here. The cytosolic factor which is required for the activation is reported to be cell-specific proteins, but not protein kinase C [16,34,35]. None of this evidence supports the concept that NADPH oxidase activation is linked to the protein kinase C reaction, although Cox et al. [7] have reported that the addition of purified protein kinase C to the plasma membranes in the cell-free system activates the NADPH oxidase. Our conclusion is also confirmed by the fact that arachidonate did not phosphorylate any particular membrane proteins treated with 100 μM H-7 (Fig. 2), and evoked a marked O_2^- generation (Table VI). As a possible site for the charge-dependent regulation, the O_2^- generation can be activated by increasing net negative charges of the plasma membranes and this may regulate the interaction of the cytosolic activation factor to the membrane-bound NADPH oxidase, as indicated in Figs. 3 and 4.

In summary, the present data indicate that alteration of the membrane charges would function as one of the triggers of respiratory bursts in neutrophils. Quite recently, Babior et al. [36] reported that sodium dodecyl sulfate converted the inactive form of solubilized NADPH oxidase to its active form which was capable of binding the cytosolic activation factor. This may explain the charge dependency of the respiratory burst and may offer a new approach to studies on the mechanism of the respiratory burst. At present, it remains unclear how this charge-dependent regulation is associated with the trigger of respiratory bursts using fMet-Leu-Phe and concanavalin A.

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