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Demonstration of calcium-dependent phospholipase A_2 activity in membrane preparation of rabbit neutrophils

Absence of activation by fMet-Leu-Phe, phorbol 12-myristate 13-acetate and A-kinase

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The presence of a phospholipase A_2 (PLA_2) activity in rabbit neutrophil membrane preparation that is able to release [1-^{14}C]oleic acid from labelled *Escherichia coli* has been demonstrated. The activity is critically dependent on the free calcium concentration and marginally stimulated by GTPγS. More than 80% of maximal activity is reached at 10 μM-Ca^{2+}. The chemotactic factor, fMet-Leu-Phe, does not stimulate the PLA_2 activity in this membrane preparation. Pretreatment of the membrane preparation, under various experimental conditions, or intact cells, before isolation of the membrane with phorbol 12-myristate 13-acetate (PMA), does not affect PLA_2 activity. Addition of the catalytic unit of cyclic AMP-dependent kinase to membrane preparation has no effect on PLA_2 activity. Pretreatment of the intact neutrophil with dibutyryl-cAMP before isolation of the membrane produces a small but consistent increase in PLA_2 activity. The activity of PLA_2 in membrane isolated from cells treated with the protein kinase inhibitor 1-(5-isoquinolinesulphonyl)-2-methyl piperazine dihydrochloride (H-7) is significantly decreased. Furthermore, although the addition of PMA to intact rabbit neutrophils has no effect on the release of [^{3}H]arachidonic acid from prelabelled cells, it potentiates significantly the release produced by the calcium ionophore A23187. This potentiation is not due to an inhibition of the acyltransferase activity. H-7 inhibits the basal release of arachidonic acid but does not inhibit the potentiation by PMA. These results suggest several points. (1) fMet-Leu-Phe does not stimulate PLA_2 directly, and its ability to release arachidonic acid in intact neutrophils is mediated through its action on phospholipase C. (2) The potentiating effect of PMA on A23187-induced arachidonic acid release is most likely due to PMA affecting either the environment of PLA_2 and/or altering the organization of membrane phospholipids in such a way as to increase their susceptibility to hydrolysis. (3) The intracellular level of cyclic AMP probably does not directly affect the activity of PLA_2.

INTRODUCTION

In response to stimuli, many different cell types, including neutrophils, release arachidonic acid, which is then metabolized to generate one or more biologically active eicosanoids such as leukotrienes, prostaglandins and thromboxane (Lands & Samuelsson, 1968; Hirata *et al.*, 1979; Billah *et al.*, 1980; Corey *et al.*, 1980; Rubin *et al.*, 1981; Feinstein & Sha'afi, 1983; Flower, 1985; Alonso *et al.*, 1986; Burch *et al.*, 1986; Pollock *et al.*, 1986; Smith & Waite, 1986; Godfrey *et al.*, 1987). Since the level of free arachidonic acid in the cytoplasm is low, the rate-limiting step in eicosanoid biosynthesis is the liberation of arachidonic acid (Irvine, 1982). This, and the importance of the released eicosanoids in inflammatory reactions, has led to phospholipase A_2 (PLA_2) being thought of as a key enzyme in eicosanoid production and neutrophil physiology. Although it is generally agreed that activation of PLA_2 is closely involved in the release of arachidonic acid from membrane phospholipids following stimulation, the various parameters that activate and regulate PLA_2 activity are not well understood.

Recently Becker and his associates (Bormann *et al.*, 1984) have reported that the addition of the chemotactic factor fMet-Leu-Phe to membrane preparation isolated from rabbit neutrophils stimulates PLA_2 activity (as measured by the release of oleic acid from [1-^{14}C]oleic acid-labelled *E. coli*). This indicates that chemotactic factors release arachidonic acid in intact cells by activating PLA_2 directly, and not through increasing the concentration of intracellular free calcium by the generation of inositol 1,4,5-trisphosphate. If it is true, this finding is quite important.

In neutrophils, Volpi *et al.* (1985) have shown that the active phorbol esters potentiate arachidonic acid release produced by the calcium ionophore A23187. Similar results were found in platelets (Halenda *et al.*, 1985; Mobley & Tai, 1985). Although other explanations are possible (Pollock *et al.*, 1986), this potentiation has been interpreted in terms of activating PLA_2 by the protein kinase C system.

MATERIALS AND METHODS

Preparation of cells

Rabbit peritoneal neutrophils were obtained 12–14 h after intraperitoneal injection of 0.1% glycogen saline. Cells were washed and resuspended in Hanks balanced

Abbreviations used: PLA_2, phospholipase A_2; PMA, phorbol 12-myristate 13-acetate; H-7, 1-(5-isoquinolinesulphonyl)-2-methyl piperazine dihydrochloride; GTPγS, guanosine 5'-thiotriphosphate.

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salt solution buffered with 10 mM-Hepes as previously described (Matsumoto *et al.*, 1987). In the experiment where the agents were added to the intact cells, neutrophil suspensions (10^8 cells/ml) were incubated with either fMet-Leu-Phe (10^{-9} M, 30 s), PMA (50 ng/ml, 3 min), dibutyryl cyclic AMP (2 mM, 10 min), H-7 (25 μM, 5 min) or dimethyl sulphoxide (0.05%), at 37 °C.

Membrane preparation

The suspensions were centrifuged, washed once, and then resuspended in ice-cold sucrose-buffered solution [0.25 M-sucrose/10 mM-Hepes (pH 7.5)/1 mM-EGTA] containing 1 mM-GDP, 1 mM-di-isopropyl fluorophosphate and 50 μg of leupeptin/ml. Suspensions were kept at 4 °C. The cells were homogenized by nitrogen cavitation (13.8 kPa) for 20 min. The homogenate was centrifuged at 8200 *g* for 30 min and the supernatants were then centrifuged at 100000 *g* for 60 min. The pellets were resuspended in sucrose buffer and used for measuring the PLA_2 activity. Protein concentration was measured using Bio-Rad protein assay (Bradford, 1976).

In the experiments for membrane treatment, the membrane was incubated with or without either PMA (50 ng/ml) or the catalytic unit of cyclic AMP-dependent kinase (45 units/ml), in the presence of ATP (100 μM)/Tris/HCl (pH 7.5, 50 mM)/$MgCl_2$ (10 mM)/ouabain (250 μM)/Ca^{2+} (0.5 mM only when PMA was used) at 37 °C for 30 min. The membranes were washed once and resuspended in sucrose-buffered solution.

Measurement of PLA_2 activity

[1-^{14}C]Oleic acid-labelled *E. coli* substrate was prepared as previously reported (Victor *et al.*, 1981). *E. coli* substrate (40000 c.p.m.) was incubated with membrane (25 μg of protein, unless otherwise stated) in 100 mM-Tris/HCl (pH 7.5) containing the desired concentration of $CaCl_2$ and 250 μM-EGTA at 37 °C for 30 min. Free Ca^{2+} concentration was calculated using the apparent stability constant for Ca^{2+}–EGTA (2.2×10^6 M^{-1}) determined by Schwarzenbach *et al.* (1957) and Owen (1976). The reactions were stopped by extraction of the lipids with 0.75 ml of methanol/chloroform (2:1, v/v), followed by 0.25 ml each of chloroform and water as described by Bligh & Dyer (1959) and Volpi *et al.* (1985). The recovered lipids in the chloroform layer were separated by t.l.c. in hexane/ether/acetic acid (40:60:1, by vol.) on precoated silica-gel plates (Volpi *et al.*, 1985). The hydrolysed [1-^{14}C]oleic acid was scraped and its radioactivity was determined in a Searly Delta 300 scintillation counting system.

Cell labelling

Labelling was carried out as previously described (Volpi *et al.*, 1984). Briefly [^{3}H]arachidonic acid (final concentration 2.9 μCi/ml) was sampled into a round-bottom flask, the ethanol was evaporated with a stream of nitrogen, and immediately a known volume of a cell suspension (10^8 cells/ml) was added. The cell suspension was incubated at 37 °C for 30 min. At the end of the incubation period, the cells were pelleted by centrifugation (800 *g*), and the packed cells were resuspended with Hanks buffer (10^8 cell/ml). The reaction was initiated by adding a known volume of the stimulus, and it was stopped by the addition of 5 ml of hexane/isopropanol (3:2, v/v).

Isolation and separation of lipids

Lipids were isolated and separated as previously described (Volpi *et al.*, 1984). After the reaction was terminated, the samples were mixed with a vortex and kept overnight at 4 °C. The samples were again mixed with a vortex for 1 min and centrifuged at 800 *g* for 5 min to separate the two phases. The upper organic phase was removed and transferred to another set of test tubes. Portions of 2 ml of hexane were added to the remaining water phase, and the tubes were again mixed with a vortex for 15 s and centrifuged as before. The top hexane layer was removed and pooled with the previous one. The organic phase was dried under a stream of nitrogen and the lipids were dissolved in 100 μl of hexane/isopropanol (3:2, v/v).

To separate neutral lipids, portions of 20 μl were used to spot silica-gel 60 precoated t.l.c. plates. Arachidonic acid (1.75 μg) was added at the origin with the samples to help in the visualization of the lipids with I_2 vapour. The plates were developed in chloroform/acetone (96:4, v/v). After exposure to the I_2 vapour, the arachidonic acid was outlined with a pencil and the plate was sprayed lightly with water. The corresponding silica was carefully scraped from the plate with a razor blade, collected, and assayed for radioactivity.

Lysolecithin acyltransferase

The activity of lysolecithin acyltransferase was measured in a total volume of 200 μl containing 20 μl of neutrophil homogenate (5 μg of protein), 50 μM-1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine (2 mCi/mmol), 30 μM-arachidonoyl-CoA, 140 mM-KCl and 20 mM-Hepes (pH 7.4). The reaction was carried out at 37 °C for 10 min and then terminated by extraction of lipids with 0.75 ml of methanol/chloroform (2:1, v/v) and 0.25 ml each of chloroform and water as described above. The synthesized phosphatidylcholine in the chloroform layer was separated by t.l.c. on precoated silica plates in chloroform/methanol/acetic acid/0.9% NaCl (50:25:8:4, by vol.). The spot of phosphatidylcholine visualized by iodine vapour was cut and counted in the scintillation counter.

Chemicals

[1-^{14}C]Oleic acid was purchased from Amersham and [^{3}H]arachidonic acid was obtained from New England Nuclear. Dibutyryl cyclic AMP, arachidonoyl-CoA, 1-[1-^{14}C]palmitoyl-*sn*-glycero-3-phosphocholine ([^{14}C]-lysolecithin) and guanylylimidodiphosphate were obtained from Sigma and fMet-Leu-Phe from Peninsula Labs (Belmont, CA, U.S.A). H-7 was obtained from Seikagaku America (St. Petersburg, FL, U.S.A.) and GTPγS from Boehringer Mannheim Biochemicals. All other chemicals were of analytical grade.

RESULTS

PLA_2 activity in neutrophil membrane

Incubation of the labelled *E. coli* with neutrophil membrane resulted in the release of [^{14}C]oleic acid from *E. coli*. The position of the label was determined by hydrolysing the substrate with purified PLA_2 (*Naja naja* venom). More than 90% of the label was in the 2-acyl position of the phospholipids (results not shown). In rabbit neutrophils, and under identical experimental

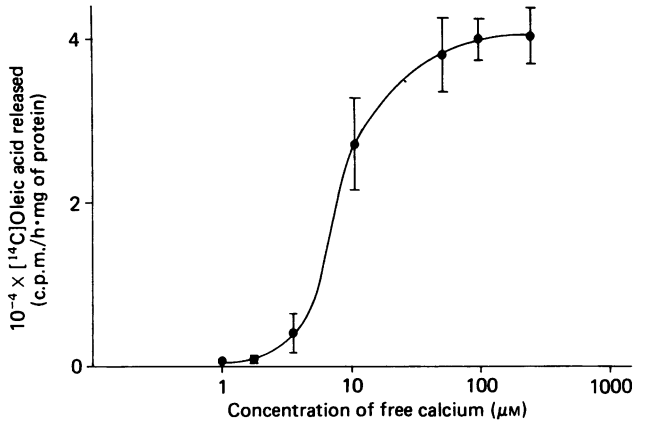

Fig. 1. Effect of free calcium concentration on fatty acid release from labelled *E. coli* activity

The reaction mixtures contained membrane (25 μg), ^{14}C-labelled *E. coli* (40000 c.p.m.) and various concentrations of Ca^{2+}. The reaction was carried out for 30 min. Free calcium concentrations were calculated as described in the Materials and methods section and no Mg^{2+} was present. The values are means ± S.E.M. of at least five experiments.

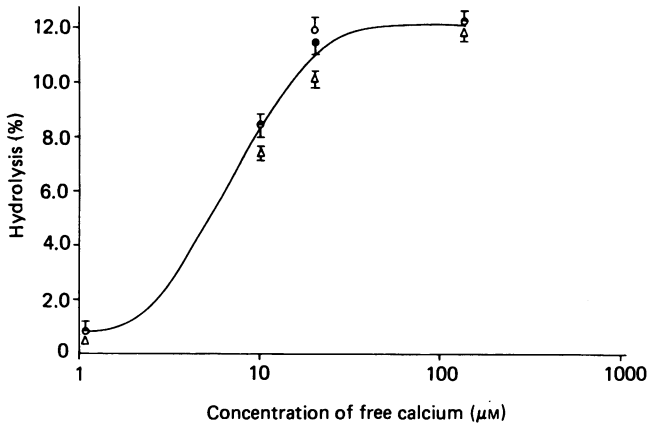

Fig. 2. Effects of fMet-Leu-Phe on [1-^{14}C]oleic acid released by 25 μg of membrane protein in 30 min

The symbols are as follows: ○, control membrane; ●, membrane treated with fMet-Leu-Phe (1 nM, 30 s); △, membrane prepared from intact neutrophils treated with fMet-Leu-Phe (1 nM, 30 s), washed and then used for isolation of the membrane. The results are means ± S.E.M. of at least three separate experiments.

conditions, it has been shown by Becker and his associates (Lanni & Becker, 1983; Bormann, 1985), using 1-[1-^{14}C]-stearyl-2,3-*sn*-glycerophosphorylethanolamine, that this activity is mostly due to PLA_2.

The time course (0–120 min) of the release of radioactivity following incubation of the labelled *E. coli* with neutrophil membrane was determined (results not shown). The amount of released radioactivity at 120 min corresponded to 30% of the total incorporated radioactivity. The amount of released free fatty acid increased linearly with time in the first 40 min. This corresponds to a range of 0–21% of the total incorporated radioactivity.

The effect of varying the membrane protein concentration in the reaction mixture on the amount of radioactivity released was also examined (results not shown). The kinetics of the release of radioactivity indicated that the reaction is linear up to 37 μg of protein. Again, this corresponds to a range of 0–20% of the total incorporated radioactivity.

Table 1. Effect of fMet-Leu-Phe added to membrane or intact neutrophils on the maximal amount of [1-^{14}C]oleate released

The treated membrane was treated with 1 nM-fMet-Leu-Phe for 30 s and was then added to the reaction mixture. The chemotactic factor was present in the reaction mixture during the entire period. Intact neutrophils were incubated with fMet-Leu-Phe for 30 s, washed and then used to prepare membrane. The maximal hydrolysis values refer to the maximal amount of [^{14}C]oleic acid released in 30 min by 25 μg of protein in the presence of 250 μM free calcium, expressed as percent of the total radioactivity incorporated in the substrate (*E. coli*). These values represent the means ± S.E.M. of at least three separate experiments.

Preparation	Maximal hydrolysis of total [1-^{14}C]-oleate-labelled *E. coli* (%)
Control membrane	12.5 ± 0.3
fMet-Leu-Phe-treated membrane	12.1 ± 0.1
Membrane prepared from fMet-Leu-Phe-treated cells	12.0 ± 0.2

Calcium requirement for PLA_2 activation

The effect of free calcium concentration on PLA_2 activity was investigated using the Ca^{2+}–EGTA buffer system. In these experiments, the free calcium concentration was calculated using the stability constant between Ca^{2+} and EGTA as described in the Materials and methods section. The results summarized in Fig. 1 clearly show that PLA_2 activity has a sharp dependence on Ca^{2+}. The activity reached a plateau at about 250 μM-Ca^{2+}. Higher concentrations of Ca^{2+} (1 and 5 mM) were also used and the results were the same.

The ability of guanine nucleotide to modulate PLA_2 activity was also examined. The reactions were carried out in the presence or absence of several guanine nucleotides and their analogues. Neither GTP nor GDP had any effect on PLA_2 activity. In addition, GTPγS and guanylylimidodiphosphate, non-hydrolysable analogues of GTP, increased the maximal activity of PLA_2 by no more than 10%.

Effect of fMet-Leu-Phe on PLA_2 activity

The effect of the chemotactic factor fMet-Leu-Phe on PLA_2 activity has been investigated. In these studies, two sets of experiments were carried out. In the first set, fMet-Leu-Phe was added to the membrane preparation before the addition of membrane to the reaction mixture. In the second set, intact neutrophils were treated with fMet-Leu-Phe (10^{-9} M) for 30 s, washed once, and then the cells used to prepare membrane; the results are summarized in Fig. 2 and Table 1. It is quite clear that the addition of fMet-Leu-Phe, either to membrane or to intact cells, does not stimulate PLA_2 activity.

Table 2. Effects of PMA and the catalytic unit of cyclic AMP-dependent kinase on PLA_2 activity of neutrophil membranes

Membranes were incubated in the presence of ATP (100 μM) with PMA (50 ng/ml) or the catalytic unit of cyclic AMP-dependent kinase (A-kinase, 45 units/ml), for 30 min at 37 °C as described in the Materials and methods section. The membranes were washed, resuspended in buffered-sucrose solution, and then added to the reaction mixture. The hydrolysis values refer to the amount of [^{14}C]oleic acid released in 30 min by 25 μg of protein of membrane preparation expressed as percent of the total radioactivity incorporated in the substrate (*E. coli*). These values represent the means ± S.E.M. of at least three separate experiments.

Concentration of free Ca^{2+} (μM)	Hydrolysis of [1-^{14}C]oleate-labelled *E. coli* (%)		
	Control	PMA	A-kinase
1	0.4±0.1	0.4±0.1	0.4±0.1
10	8.5±1.9	7.8±1.6	7.8±1.6
50	11.6±1.1	11.3±1.5	11.8±1.3
250	12.5±1.0	12.6±0.8	12.5±0.4

Table 4. Effect of H-7 and PMA on the basal and A23187-stimulated [^{3}H]arachidonic acid release from prelabelled rabbit neutrophils

H-7-treated cells were incubated with H-7 for 5 min before the addition of other reagents. A23187-treated cells were stimulated with A23187, either alone or in combination with PMA, for 2 min. The PLA_2 activity of the membrane prepared from cells treated with the same concentrations of PMA and A23187 for the same period of time was slightly reduced (< 15%) relative to the control (no addition). The [^{3}H]arachidonic acid release values represent the means ± S.E.M., and the numbers in parentheses refer to the number of separate experiments.

Condition	[^{3}H]Arachidonic acid release (c.p.m./2 × 10^7 cells)		Increase (%)
	Basal	A23187 (0.5 μM)	
No addition	3170±100	9183±2536 (11)	190
PMA (20 ng/ml)	3107±63 (6)	17435±5072 (6)	461
H-7 (25 μM)	2504±155 (10)	10778±1078 (5)	330
PMA + H-7	2409±133 (6)	20605±3487 (9)	755

Effects of PMA and the catalytic unit of cyclic AMP-dependent kinase on PLA_2 activity of membrane preparation

In order to examine the roles of protein kinase C and the cyclic AMP-dependent kinase (A-kinase) on PLA_2 activity, we have carried out two sets of experiments. In the first set, the membrane preparation was incubated in the presence of 100 μM-ATP with either PMA (50 ng/ml) or the catalytic unit of cyclic AMP-dependent kinase (45 units/ml) as described in the Materials and methods section. Then the membrane was washed, resuspended in sucrose-buffered solution, and used for measurement; the results are summarized in Table 2. It is quite clear from the Table that neither PMA nor A-kinase has any significant effect on PLA_2 activity (as measured by the amount of [1-^{14}C]oleic acid released). In separate experiments, the membrane preparation was treated with PMA as described in the Materials and methods section, except that the incubation medium contained, in addition, neutrophil cytosol (either diluted or concentrated). Again PMA under these conditions has no effect on PLA_2 activity (results not shown).

In the second set of experiments, intact neutrophils were treated either with PMA (50 ng/ml) for 3 min, 2 mM-dibutyryl cyclic AMP for 10 min or H-7 (25 μM) for 5 min, washed once and then used to prepare membrane; the results are summarized in Table 3. As in the case for membrane, PMA added to the intact cell fails to produce any significant effect on PLA_2 activity. The addition of dibutyryl cyclic AMP to intact cells produces a small increase in PLA_2 activity. On the other hand, pretreatment of the intact neutrophils with H-7 produces significant ($p < 0.01$) inhibition.

Basal and A23187-induced [^{3}H]-arachidonic acid release in control, PMA, H-7, and PMA plus H-7-treated neutrophils

The effects of PMA, H-7 and PMA plus H-7 on the basal and calcium ionophore (A23187)-induced [^{3}H]-

Table 3. [1-^{14}C]Oleic acid release by membrane preparations isolated from control, PMA-, dibutyryl cyclic AMP- and H-7-treated rabbit neutrophils

Prior to the isolation of the membrane preparation, the cells were treated with PMA (50 μg/ml) for 3 min or dibutyryl cyclic AMP (2 mM) for 10 min or H-7 (25 μM) for 5 min, washed once and then used for membrane preparation. The hydrolysis values refer to the amount of [^{14}C]oleic acid released in 30 min by 25 μg of membrane protein expressed as percent of the total radioactivity incorporated in the substrate (*E. coli*). The values represent the means ± S.E.M. of at least three experiments.

Concentration of free Ca^{2+} (μM)	Hydrolysis of [1-^{14}C]oleate-labelled *E. coli* (%)			
	Control	PMA-treated	Dibutyryl cyclic AMP-treated	H-7-treated
1.0	0.3±0.1	0.4±0.1	0.3±0.1	0.3±0.1
10	7.9±1.8	6.3±3.0	9.5±1.3	6.6±1.2
50	11.4±0.6	12.1±0.1	12.6±0.6	9.3±0.7
250	12.0±0.5	13.4±0.2	13.7±1.0	9.5±0.2

Table 5. Effect of PMA treatment on lysolecithin acyltransferase activity in neutrophil homogenate

Neutrophils were incubated with or without PMA at 37 °C for 3 min, washed once, resuspended in buffered sucrose solution and then homogenized with a motor-driven Teflon glass homogenizer. Supernatant of 800 *g* centrifugation was used for acyltransferase assay. Activity of acyltransferase was calculated from the amount of radioactivity of synthesized phosphatidylcholine and the specific activity of [^{14}C]lysolecithin in reaction mixture. The values are means ± S.E.M. of at least four separate experiments.

Condition	Acyltransferase activity (nmol/h·mg)
Control	1527 ± 131
PMA (10 ng/ml)	1558 ± 296
(50 ng/ml)	1316 ± 150

arachidonic acid release in rabbit neutrophils were investigated (Table 4). These results show that while PMA has no effect on the basal release, it potentiates significantly ($P < 0.001$) the release produced by the calcium ionophore. In addition, H-7 inhibits the basal release but not the potentiation by PMA.

This potentiation by PMA of arachidonic acid release produced by the ionophore could be mediated through an inhibition of the re-incorporation of the released arachidonic acid into phospholipids. To examine this, we have measured the activity of lysolecithin acyltransferase in neutrophil homogenates of control and PMA-treated cells (Table 5). In these studies, the time course (0–20 min) of the activity of lysolecithin acyltransferase was determined (results not shown). Phosphatidylcholine production increased linearly with time in the first 10 min. The effect of varying the membrane protein concentration in the reaction mixture on the amount of phosphatidylcholine produced was also examined. The kinetics of production indicated that the reaction is linear with up to 10 μg of protein. It is clear from the results in Table 5 that pretreatment with PMA does not inhibit significantly lysolecithin acyltransferase activity.

DISCUSSION

The results reported here demonstrate the presence of a PLA_2 activity in membrane preparations isolated from rabbit neutrophils. The activity of this enzyme requires the presence of calcium, and at best, can be marginally (< 10%) potentiated by non-hydrolysable GTP analogues. The activity of this enzyme is extremely sensitive to variations in the concentration of free calcium in the range 1–10 μM-Ca^{2+}.

The chemotactic factor, fMet-Leu-Phe, added either to the membrane preparation or to intact cells before the isolation of the membrane, failed to stimulate PLA_2 activity. The inability of fMet-Leu-Phe, when added to the intact neutrophils before the preparation of the membrane, to stimulate PLA_2 activity was not unexpected because of the transient nature of fMet-Leu-Phe-induced effects. On the other hand, the failure of fMet-Leu-Phe to stimulate PLA_2 activity when added directly to the membrane was quite unexpected since it was in conflict with published results (Bormann *et al.*, 1984). Using similar membrane preparation and experimental techniques, these authors have found that fMet-Leu-Phe (1 nM) stimulated PLA_2 activity 3-fold. Although the basis for this discrepancy is not known, it cannot be due to differences in the experimental methods.

We have reported previously that the addition of PMA to intact rabbit neutrophils potentiates [^{3}H]arachidonic acid release by the calcium ionophore A23187 (Volpi *et al.*, 1985). Similar results have been found in platelets (Halenda *et al.*, 1985; Mobley & Tai, 1985). Based on these findings, it has been suggested that activation of protein kinase C by PMA positively modulates the activity of PLA_2. We have reconfirmed this potentiation. In addition, we have shown that this potentiation is not inhibited by H-7. This enhancing effect is not due to PMA action on the metabolism of arachidonic acid by either the lipoxygenase or the cyclo-oxygenase pathway (Halenda *et al.*, 1985; McColl *et al.*, 1986). Furthermore, since the activity of lysolecithin acyltransferase in homogenates isolated from control or PMA-treated neutrophils is the same, the observed potentiation by PMA cannot be due to an inhibition of the re-incorporation of released arachidonic acid into phospholipids.

Since the addition of PMA, under different experimental conditions, does not stimulate PLA_2 activity, it is reasonable to conclude that its potentiating action is not mediated through a direct effect on the enzyme. In intact cells, PMA, either directly or indirectly, could potentiate the A23187-induced arachidonic acid release by affecting the environment of PLA_2. Another possibility is that PMA may alter the organization of membrane phospholipids in such a way as to increase their susceptibility to hydrolysis. Although a role for protein kinase C cannot be ruled out completely, the inability of H-7 to inhibit the PMA-induced potentiation, suggests that PMA produces its action, in part, by direct membrane perturbation. A similar hypothesis was advanced for regulating arachidonate liberation in platelets in response to collagen at low intracellular free calcium concentrations (Pollock *et al.*, 1986). Although the potentiation by PMA appears to be mediated by events other than a direct effect on the enzyme, one must not conclude that PLA_2 activity in intact cells is independent of protein kinase C activity. It is conceivable that, in these cells, the basal activity of protein kinase C is sufficient to produce the maximal influence of protein kinase C on phospholipase A_2 activity. In fact, we would like to hypothesize that the basal activity of PLA_2 is modulated to some degree by H-7-sensitive kinase (probably protein kinase C). There are two experimental observations which are consistent with this view. (1) PLA_2 activity in membrane isolated from H-7-treated neutrophils is significantly ($P < 0.01$) decreased. (2) The amount of [^{3}H]arachidonic acid released under basal conditions is significantly reduced in H-7-treated neutrophils.

Since neither the active unit of cyclic AMP-dependent kinase nor the addition of dibutyryl cyclic AMP greatly affects the activity of PLA_2, it is reasonable to conclude that intracellular variations of cyclic AMP levels, in intact cells, may not modulate, directly, PLA_2 activity. This does not rule out an indirect effect of cellular cyclic AMP on stimulated liberation of arachidonic acid. One possibility is that cyclic AMP may affect the intracellular level of free calcium, and/or it may affect one or more

steps in the sequence of events leading to the generation of arachidonic acid.

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