Arachidonic acid release in rabbit neutrophils

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[³H]Arachidonic acid is released after stimulation of rabbit neutrophils with fMet-Leu-Phe or platelet-activating factor (PAF). The release is rapid and dose-dependent, and is inhibited in phorbol 12-myristate 13-acetate (PMA)-treated rabbit neutrophils. The protein kinase C (PKC) inhibitor 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) prevents this inhibition. In addition, PMA increases arachidonic acid release in H-7-treated cells stimulated with fMet-Leu-Phe. [³H]Arachidonic acid release, but not the rise in the concentration of intracellular Ca²⁺, is inhibited in pertussis-toxin-treated neutrophils stimulated with PAF. The diacylglycerol kinase inhibitor R59022 increases the concentration of diacylglycerol and potentiates [³H]arachidonic acid release in neutrophils stimulated with fMet-Leu-Phe. This potentiation is not inhibited by H-7. These results suggest several points. (1) A rise in the intracellular concentration of free Ca²⁺ is not sufficient for arachidonic acid release in rabbit neutrophils stimulated by physiological stimuli. (2) A functional pertussis-toxin-sensitive guanine nucleotide regulatory protein and/or one or more of the changes produced by phospholipase C activation are necessary for arachidonic acid release produced by physiological stimuli. (3) Agents that stimulate PKC potentiate arachidonic acid release, and this potentiation is not inhibited by H-7. These agents produce their actions in part by direct membrane perturbation.

INTRODUCTION

Many different cell types, including the neutrophilic polymorphonuclear leucocytes (neutrophils), release arachidonic acid in response to stimulation. The fatty acid is then metabolized to generate one or more biologically active eicosanoids such as prostaglandins, thromboxanes and leukotrienes (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). Although the steps involved in the release of arachidonic acid from membrane phospholipids after cell stimulation are complex and not fully understood, the main step involves the action of phospholipase A₂ (PLA₂). Because the concentration of free arachidonic acid is low, the rate-limiting step in eicosanoid biosynthesis is the liberation of arachidonic acid (Irvine, 1982).

The various parameters that activate and regulate the PLA₂ activity are not well understood. PLA₂ activity has been assessed directly, and the evidence obtained from these studies suggests that Ca²⁺ is necessary for the activities of the various cellular PLA₂s. The concentration of Ca²⁺ required for optimal activation of PLA₂ *in vitro* is significantly higher than that obtained in cells after stimulation (Feinstein & Sha'afi, 1983).

The present studies were undertaken to examine three main questions. First, is the rise in the intracellular concentration of free Ca²⁺ produced by physiological stimuli such as platelet-activating factor (PAF) sufficient to release arachidonic acid? Second, do compounds that activate protein kinase C (PKC) potentiate the stimulated

release of arachidonic acid? Third, is the potentiation, if any, mediated through PKC?

MATERIALS AND METHODS

Preparation of cells

Rabbit neutrophils (4 h exudate) (neutrophils obtained from 12–14 h exudate were much less responsive) were collected, washed and suspended in Hanks' Balanced Salt Solution buffered with 10 mM-Hepes (pH 7.4) as previously described (Matsumoto *et al.*, 1988). Unless specified, the cells were incubated with 0.5 µg of pertussis toxin/ml for 1 h, 20 ng of phorbol 12-myristate 13-acetate (PMA)/ml for 3 min, or 25 µM-1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H-7) for 5 min; the cells were stimulated with 50 nM-fMet-Leu-Phe for 1 min and with PAF (0.1 µM) for 30 s. In the Figures, each point represents the mean ± S.E.M. for at least three separate experiments.

Labelling of cells

Labelling was carried out as previously described (Volpi *et al.*, 1984). Briefly, [³H]arachidonic acid was sampled into a round-bottom flask, the ethanol was evaporated with a stream of N₂, and immediately a known volume of a cell suspension (10⁸ cells/ml) was added. The cell suspension was incubated at 37 °C for 30 min, and then they were pelleted by centrifugation (750 g for 5 min), and the packed cells were washed twice and resuspended in Hanks' buffer (10⁸ cells/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/propan-2-ol (3:2, v/v).

Abbreviations used: PKC, protein kinase C; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine; PMA, phorbol 12-myristate 13-acetate; R59022, 6-(2-{4-[(4-fluorophenyl)phenylmethylene]-1-piperidinyl}ethyl)-7-methyl-5H-thiazolo-[3,2-a]pyrimidin-5-one; fMet-Leu-Phe, formylmethionyl-leucyl-phenylalanine; PAF, platelet-activating factor; PLA₂, phospholipase A₂.

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 vortex-mixed and kept overnight at 4 °C. The following day, the samples were again vortex-mixed for 1 min and centrifuged at 800 *g* for 5 min. The upper organic phase was removed and transferred to another set of test tubes. Portions (2 ml) of hexane were added to the remaining water phase, and the tubes were again vortex-mixed for 15 s and centrifuged as above. The top hexane layer was removed and pooled with the previous one. The organic phase was dried under a stream of N₂, and the lipids were dissolved in 100 μ l of hexane/propan-2-ol (3:2, v/v).

To separate neutral lipids, 20 μ l samples were spotted on silica-gel-60-precoated t.l.c. plates. Arachidonic acid (1.75 μ g) was added at the origin with the samples to help in the detection of the lipids with I₂ vapour. The plates were developed in chloroform/acetone (24:1, v/v). After exposure to the I₂ vapour, the arachidonic acid was circled with a pencil, the plate was sprayed lightly with water, and the corresponding silica gel was carefully scraped from the plate with a razor blade, collected, and assayed for radioactivity.

Fluorescence measurements

The cells were loaded with quin 2 as previously described (Naccache *et al.*, 1986). At the end of the loading period, the desired number of cells was washed once, and resuspended in Hanks' balanced salt solution. Fluorescence measurements were performed in an SLM (model 8000) fluorescence spectrophotometer.

Chemicals

fMet-Leu-Phe, quin 2/AM and PAF were purchased from Sigma (St. Louis, MO, U.S.A.), cytochalasin B was from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), H-7 from Seikagaku America (St. Petersburg, FL, U.S.A.), the diacylglycerol kinase inhibitor R59022 from Janssen Life Science Section (Piscataway, NJ, U.S.A.), PMA from CRC Cancer Biochemicals (Brewster, NY, U.S.A.), and the radioactive materials were from New England Nuclear. Pertussis toxin was a gift from Dr. J. J. Munoz, NIH Rocky Mountain Laboratory (Hamilton, MT, U.S.A.).

RESULTS

[³H]Arachidonic acid release in stimulated rabbit neutrophils

The [³H]arachidonic acid release from prelabelled cells stimulated with fMet-Leu-Phe and PAF has been measured, and the results are summarized in Figs. 1–4. Cytochalasin B potentiates the effects of fMet-Leu-Phe and PAF (results not shown). The increases produced by these stimuli are rapid and dose-dependent.

Is the rise in the intracellular concentration of free Ca²⁺ produced by physiological stimuli sufficient for [³H]arachidonic acid release?

Although intracellular Ca²⁺ is necessary for the activity of PLA₂ it is not known if the increase in intracellular Ca²⁺ produced by physiological stimuli is sufficient. To investigate this point, we made use of the observation that, although all the changes produced by PAF that are

Fig. 1. Stimulation of [³H]arachidonic acid release in prelabelled rabbit neutrophils stimulated with various concentrations of fMet-Leu-Phe

Fig. 2. Time course of [³H]arachidonic acid release in rabbit neutrophils stimulated with 50 nM-fMet-Leu-Phe

mediated by phospholipase C activation are inhibited in cells pretreated with pertussis toxin (Naccache *et al.*, 1985; Verghese *et al.*, 1987), the rise in the intracellular concentration of free Ca²⁺ is not. The increase in intracellular free Ca²⁺ and [³H]arachidonic acid release produced by control and pertussis-toxin-treated rabbit

Fig. 3. Time course of [³H]arachidonic acid release in neutrophils stimulated with 0.1 μM-PAF

Fig. 4. Stimulation of [³H]arachidonic acid release in neutrophils stimulated with various concentrations of PAF

The cells were stimulated with PAF for 30 s.

neutrophils stimulated with PAF were measured. The results are shown in Fig. 5 and Table 1. These data show that, although the increase in Ca²⁺ is not affected, the release of [³H]arachidonic acid is inhibited (> 85 %) in pertussis-toxin-treated cells.

Is there a role for PKC in arachidonic acid release?

In order to investigate this point, we carried out several sets of experiments. In the first set, we examined the effect of PMA on the basal and the stimulated [³H]arachidonic acid release in rabbit neutrophils stimulated with fMet-Leu-Phe or PAF. The results summarized in Table 2 clearly show that, although PMA has no effect on the basal extent of arachidonic acid release, it inhibits that produced by fMet-Leu-Phe

Fig. 5. Cell-associated quin2 fluorescence intensity in control (a) and pertussis-toxin-treated (b) rabbit neutrophils stimulated with 0.1 μM-PAF

Arrow indicates time of addition of stimulus.

Table 1. Effect of pertussis toxin on arachidonic acid release produced by PAF

Each value represents the mean ± S.E.M. of three experiments. The cells were stimulated with PAF for 30 s. For pertussis-toxin treatment, the cells were incubated with 0.5 μg of pertussis toxin/ml for 1 h before the addition of PAF. The pertussis toxin had no effect on the basal arachidonic acid release.

Condition	[³ H]Arachidonic acid release (relative to control)	
	Control	Pertussis toxin
No addition	1.0	0.94 ± 0.1
PAF (0.1 μM)	2.5 ± 0.2	1.2 ± 0.13

Table 2. Effect of PMA on the basal and stimulated [³H]-arachidonic acid release from prelabelled rabbit neutrophils

The cells were stimulated with fMet-Leu-Phe for 1 min and with PAF for 30 s. Each value represents the mean ± S.E.M. of at least three experiments. The concentration of PMA used was 20 ng/ml, and it was added 3 min before addition of the agonist.

Condition	[³ H]Arachidonic acid release (c.p.m./2 × 10 ⁷ cells)	
	Control	PMA-treated
No addition	1320	1300 ± 63
+ PAF (0.1 μM)	2840 ± 180	1560 ± 80
+ fMet-Leu-Phe (50 nM)	2774 ± 200	2290 ± 60

(> 32 %) and PAF (> 80 %). PMA was effective as early as 30 s after its addition (results not shown).

In the second set of experiments, we examined the effect of the PKC inhibitor H-7 on both the basal and stimulated arachidonic acid release. The results are

Table 3. Effect of H-7 on the basal and fMet-Leu-Phe-stimulated [³H]arachidonic acid release from control and PMA-treated rabbit neutrophils

Each value represents the mean $\pm$ S.E.M. of at least three separate experiments. The concentration of PMA used was 20 ng/ml, and it was added 3 min before the agonist.

Cells*	[³ H]Arachidonic acid release (relative to control)		
	Basal	PAF†	fMet-Leu-Phe†
Control	1.0	2.2 $\pm$ 0.15	2.0 $\pm$ 0.1
PMA-treated	0.95 $\pm$ 0.06	1.16 $\pm$ 0.1	1.6 $\pm$ 0.08
H-7-treated	0.92 $\pm$ 0.1	2.1 $\pm$ 0.1	2.1 $\pm$ 0.12
H-7-and then PMA-treated	1.0 $\pm$ 0.1	1.9 $\pm$ 0.09	2.62 $\pm$ 0.11

* The cells were incubated with 25 μ M-H-7 for 5 min before the addition of PMA.

† The cells were stimulated with fMet-Leu-Phe (50 nM) for 1 min and with PAF (0.1 μ M) for 30 s.

Fig. 6. Time course of [³H]arachidonic acid-labelled diacylglycerol production in control and R59022-treated neutrophils stimulated with 50 nM-fMet-Leu-Phe

○, Control cells; ●, cells treated with 10 μ M-R59022 for 5 min.

summarized in Table 3, and show two main points. First, H-7 prevents the inhibition by PMA of arachidonic acid release produced by fMet-Leu-Phe and PAF. Second, PMA increases significantly ($P \leq 0.005$) arachidonic acid release in H-7-treated cells stimulated with fMet-Leu-Phe.

In the third set of experiments, we examined the influence of changing the stimulated concentration of diacylglycerol, by using the diacylglycerol kinase inhibitor R59022, on [³H]arachidonic acid release stimulated by fMet-Leu-Phe. In these experiments, we first examined diacylglycerol production in control and

Table 4. Effect of H-7 on the basal and fMet-Leu-Phe-stimulated arachidonic acid release in control and R59022-treated cells

The cells were treated with R59022 (10 μ M) for 5 min. They were incubated with H-7 (25 μ M) for 5 min. For fMet-Leu-Phe treatment, the cells were stimulated with fMet-Leu-Phe (50 nM) for 1 min. Each value represents the mean $\pm$ S.E.M. of at least three separate experiments.

Condition	[³ H]Arachidonic acid release (relative to control)	
	Basal	fMet-Leu-Phe
No addition	1.0	2.90 $\pm$ 0.20
+ R59022	1.05 $\pm$ 0.10	3.68 $\pm$ 0.30
+ H-7	1.02 $\pm$ 0.09	3.28 $\pm$ 0.30
+ H-7 + R59022	1.0 $\pm$ 0.15	4.72 $\pm$ 0.4

R59022-treated rabbit neutrophils stimulated with fMet-Leu-Phe. R59022 increases the concentration of diacylglycerol (Fig. 6) in neutrophils stimulated with fMet-Leu-Phe. Next, the release of arachidonic acid in control and R59022-treated cells stimulated by fMet-Leu-Phe were measured, and the results are summarized in Table 4. These data clearly show that R59022 potentiates arachidonic acid release produced by fMet-Leu-Phe. Note that this potentiation is not inhibited by H-7, and in fact it is increased in H-7-treated neutrophils ($P < 0.01$).

DISCUSSION

The addition of fMet-Leu-Phe or PAF to rabbit neutrophils causes the release of incorporated radio-labelled arachidonic acid. The release is rapid, transient and dose-dependent (Figs. 1–4), and their actions can be greatly potentiated by cytochalasin B (results not shown).

The results shown in Fig. 5 and Table 1 clearly show that [³H]arachidonic acid release, but not the increase in intracellular Ca^{2+} , is almost totally inhibited in pertussis-toxin-treated rabbit neutrophils stimulated with PAF. We and others have previously shown that several of the changes produced by phospholipase C activation are totally abolished in pertussis-toxin-treated neutrophils stimulated with PAF (Naccache *et al.*, 1985; Verghese *et al.*, 1987). On the basis of this information and the data shown in Fig. 5 and Table 1, it is reasonable to conclude that, at least for PAF, a rise in intracellular Ca^{2+} is not sufficient for arachidonic acid release. Furthermore, these results suggest that a functional pertussis-toxin-sensitive guanine nucleotide regulatory protein and/or one or more of the changes produced by phospholipase C activation are necessary for the arachidonic acid release in rabbit neutrophils stimulated by PAF.

We have reported previously that the addition of PMA to intact rabbit neutrophils potentiates [³H]arachidonic acid release by the calcium ionophore A23187 (Volpi *et al.*, 1985). This potentiation is not inhibited by H-7 (Matsumoto *et al.*, 1988). Similar results have been found in platelets (Halenda *et al.*, 1985; Mobley & Tai, 1985). These results have been confirmed in the present

studies (results not shown). 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) or an inhibition of the activity of lysophosphatidylcholine acyltransferase (EC 2.3.1.23) (Matsumoto *et al.*, 1988). Although other interpretations are possible, it has been suggested, on the basis of these findings, that activation of PKC by PMA modulates positively the activity of PLA₂.

With respect to the action of PMA on arachidonic acid release, several points can be made. First, PMA inhibits arachidonic acid release produced by either fMet-Leu-Phe (> 30%) or PAF (> 80%) (Table 2). Second, H-7 does not affect the potentiation by PMA of A23187-induced arachidonic acid release (Matsumoto *et al.*, 1988; those results were also confirmed in the present studies). Third, H-7 prevents the inhibition by PMA of arachidonic acid release in rabbit neutrophils stimulated by fMet-Leu-Phe or PAF. In fact, PMA increases significantly ($P \leq 0.005$) arachidonic acid release in H-7-treated cells stimulated with fMet-Leu-Phe. This potentiation could be due to an inhibition of protein kinase A by H-7. The inhibitory effect of PMA is at a point or points before the rise in intracellular Ca²⁺ produced by fMet-Leu-Phe and PAF (Naccache *et al.*, 1985; Molski *et al.*, 1988).

In order to obtain further information on the role of PKC in arachidonic acid release in neutrophils stimulated by physiological agonists such as fMet-Leu-Phe, we made use of the diacylglycerol kinase inhibitor R59022. Stimulation of rabbit neutrophils by fMet-Leu-Phe produced a rapid and transient increase in diacylglycerol (Fig. 6). This increase is greatly potentiated in R59022-treated rabbit neutrophils. Arachidonic acid release is significantly greater in R59022-treated than in control rabbit neutrophils stimulated with fMet-Leu-Phe, and this potentiation is not inhibited, and in fact it is increased, in H-7-treated cells.

The results presented above make several important points.

1. Although an increase in intracellular Ca²⁺ is necessary, it is not sufficient for arachidonic acid release in rabbit neutrophils stimulated by physiological stimuli such as PAF.

2. In intact cells, a functional pertussis-toxin-sensitive guanine nucleotide regulatory (G) protein and/or one or more of the changes produced by phospholipase C activation are necessary for arachidonic acid release produced by physiological stimuli. A role for a pertussis-toxin-sensitive G-protein in arachidonic acid release has been suggested by Axelrod *et al.* (1988). They have found that, when added to dark-adapted transducin-depleted rod outer segments, the $\beta\gamma$ -subunits caused a marked increase in PLA₂ activity, whereas addition of an equivalent amount of the α -subunit caused only a slight increase (Axelrod *et al.*, 1988). The stimulatory action of $\beta\gamma$ -subunits can be due to their lipophilic nature, and it may not be due to the specific effect of the complex.

3. Agents that stimulate PKC potentiate arachidonic acid release, and this potentiation is not inhibited by the PKC inhibitor H-7. Although a role for PKC cannot be ruled out completely, the inability of H-7 to inhibit the

observed potentiation by PMA or diacylglycerol suggests that these compounds produced their actions, 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 Ca²⁺ concentrations (Pollock *et al.*, 1986).

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