Journal of Cellular Biochemistry 40:157–164 (1989)
Cell Activation and Signal Initiation: Receptor and Phospholipase Control of Inositol Phosphate, PAF and Eicosanoid Production 91–98

Phospholipase A_2-Mediated Release of Arachidonic Acid in Stimulated Guinea Pig Alveolar Macrophages: Interaction With Lipid Mediators and Cyclic AMP

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The stimulation of cultured guinea pig alveolar macrophages by the chemotactic peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine, or by the phospholipid inflammatory mediator platelet activating factor (PAF) induced an increase in arachidonic acid release and its cyclooxygenase products. This release, which was mimicked by the association of threshold concentrations of the calcium ionophore A 23187 and of the protein kinase C activator tetradecanoyl phorbol acetate arose mainly from diacyl- and alkyl-acyl-phosphatidylcholine and phosphatidylinositol. Using [1 ^{14}C]arachidonic acid-labeled membranes as an endogenous substrate as well as dioleoyl-phosphatidyl [^{14}C]ethanolamine as an exogenous substrate, we showed that phospholipase A_2 activity of stimulated macrophages increases upon stimulation. Treatment of macrophages by prostaglandin E_2 decreased the arachidonic acid release elicited by the chemotactic peptide and PAF. Furthermore, prostaglandin E_2 increased and PAF decreased the cellular content in cyclic AMP. From these results we suggest that an initial stimulation of alveolar macrophages by a bacterial signal initiates the sequential activation of a phospholipase C and of phospholipase A_2, leading to the release of PAF and eicosanoids. These mediators may in turn modulate the cell response by increasing or decreasing cyclic AMP, Ca^{2+}, or diacyglycerol macrophage content.

Key words: fMLP, PAF, prostaglandins, TPA, Ca^{2+} ionophore

Alveolar macrophages are implicated in bronchoconstriction induced by the chemotactic peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP) [1]. The phospholipids of these cells contain large amounts of arachidonic acid (AA), the precursor of eicosanoids and of 1-alkyl-2-acyl-*sn*-3-glycero-phosphocholine (alkyl-acyl PC), the precursor of platelet activating factor (PAF). Alveolar macrophages also possess phospholipase A_2 (PLA_2) activities [3] and synthetize PAF [4] and various

Received April 17, 1988; accepted August 31, 1988.

eicosanoids [5]. Free arachidonic acid and lyso-PAF are released from phospholipid stores by PLA_2; accordingly, this enzyme may be an important target for regulation. In addition, the receptors of several lipid mediators are related to transducing events, such as cyclic AMP synthesis [6] and phosphatidylinositol diphosphate (PIP_2) hydrolysis [7]. The lipid mediators may in turn induce positive or negative feedbacks on cell activation.

We previously demonstrated that the receptor-mediated stimulating agents fMLP and PAF induced AA release from phosphatidylinositol (PI), diacyl-phosphatidyl choline (DAPC), and alkyl-acyl PC of alveolar macrophages [8,9]. In the present study, we compared the effects of fMLP and PAF with those of the calcium ionophore A23187 (Io), of 12-O-tetradecanoyl phorbol-13-acetate (TPA), an agent that mimics diglyceride (DG) in stimulating protein kinase C (PKC) [10], and of prostaglandin E_2 (PGE_2), which stimulates adenylate cyclase of alveolar macrophages [11].

MATERIALS AND METHODS

Cells and Materials

Alveolar macrophages were prepared as previously described [9] from bronchoalveolar lavage of Hartley guinea pigs (300–500 g) obtained from Saint Antoine (Pleudaniel, France). Prostaglandin E_2, fMLP, bovine serum albumin (BSA, fraction V), TPA, isobutylmethylxanthine (IBMX), and the ionophore A23187 were from Sigma (St. Louis, MO); PAF was from Bachem (Switzerland).

Cell Preparation, Labeling, and Stimulation

After 1 h of differential adhesion at 37°C in 5% CO_2, the purified alveolar macrophages were labeled for 1 h with 0.5 μCi of [1 ^{14}C]arachidonic acid (60 Ci/mol, Amersham, UK) with 0.2% BSA. At the end of the incubation, the cells were washed three times with 3 ml 0.2% BSA-isotonic saline. Cells were stimulated at 37°C at the concentrations and intervals indicated in Tables I–III and Figures 1–3 in 2 ml RPMI medium (Gibco, UK), pH 7.4, which contained 0.4 mM Ca^{2+}. In time-course experiments, aliquots (100 μl) were taken and counted by liquid scintillation. At the end of the stimulation, the medium was removed, and the cells were washed three times. The medium was counted, and the cells were then treated for phospholipase A_2 assay or phospholipid extraction.

Phospholipase A_2 Assay, Lipid Extraction, and Analysis

Cells were scraped in 50 mM Tris, pH 8.5, containing 1 M NaCl and then sonicated to solubilize PLA_2 [12]. After a 30-min incubation at room temperature, the membranes were centrifuged at 30,000*g* for 20 min. The pellets obtained from non-stimulated labeled cells were resuspended in 50 mM Tris, pH 8.5, and then used as an endogenous substrate. They were almost devoid of PLA2 activity.

The PLA_2 activity found in the supernatant of 1 M NaCl-treated cell homogenates was assayed on endogenous or exogenous substrates. Aliquots of the labeled endogenous substrate were added to 1 M NaCl extracts from an equivalent number of cells and incubated 30 min at 37°C in 50 mM glycine NaOH, in optimal conditions of pH (8.5) and calcium concentration (5 mM) (final volume: 1 ml); the activity in the absence of added calcium was 50% of the activity in the presence of 5 mM calcium/pH 8.5. In some experiments, the endogenous substrate was replaced by 4 nmol of dioleoyl-phosphatidyl[^{14}C]ethanolamine (49 Ci/mol; Amersham, UK). The absence

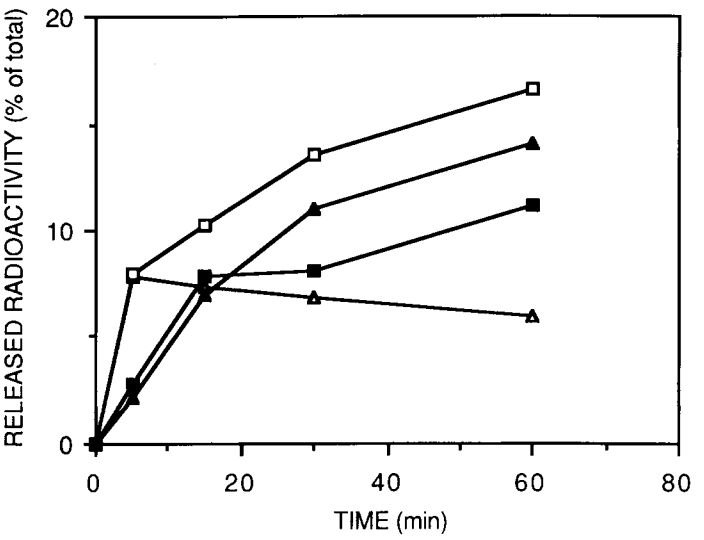

Fig. 1. Time course of arachidonic acid release by different stimuli. Adherent alveolar macrophages were prelabeled for 1 h with 0.5 μCi [1-^{14}C]AA per plate (3 × 10^6 cells). They were stimulated with 10^{-6} fMLP (□), 10^{-6} PAF (△), 10^{-6} TPA (▲), or 10^{-6} Io (■). Aliquots of the medium were taken from each incubation time and the radioactivity measured. The results are expressed as % cpm released in the medium per total cell labeling. Means of a typical measurement performed in triplicate.

of lysophospholipase activity was controlled by the lack of production of water-soluble labeled compounds, and the PLA_2 specificity was checked by the use of 1-stearoyl-2-[^{14}C]arachidonoyl phosphatidylcholine (2-arachidonoyl PC).

At the end of the incubation, total lipids were extracted by the Bligh and Dyer procedure [13] and resolved by thin-layer chromatography (TLC) as previously described [8]. The separation of the subclasses of diacyl and ether-linked PC and phosphatidylethanolamine (PE) was performed according to Blank et al. [14].

Cyclic AMP and Cyclooxygenase Products Assays

Stimulated or nonstimulated cells were incubated for 3 min in 50 mM Tris HCl, pH 7.4, in the presence of 2 × 10^{-4} M of the phosphodiesterase inhibitor 3-isobutyl-1-methyl xanthine (IBMX) alone or with 2.8 × 10^{-6} M PGE_2 at 37°C. The reaction was stopped by placing the cells at 100°C in a drying oven, and cyclic AMP (cAMP) assays were performed in the supernatants by using a high-affinity cAMP-binding protein assay [15]. Thromboxane B_2, PGE_2, and 6 keto platelet growth factor $(PFG)_{1\alpha}$ were measured by radioimmunoassay as described by Sors et al. [16], using specific antiserums from the Institut Pasteur (Paris).

RESULTS

The time course of the liberation of labeled material from stimulated adherent macrophages is shown in Figure 1. The initial rate of release was higher with fMLP and PAF than with TPA or Io (all as 10^{-6} M). After 30 min, the release of arachidonic acid by fMLP, TPA, and Io still increased, but not with PAF; it reached the same level with TPA as with fMLP. This observation agrees with that of Albert and

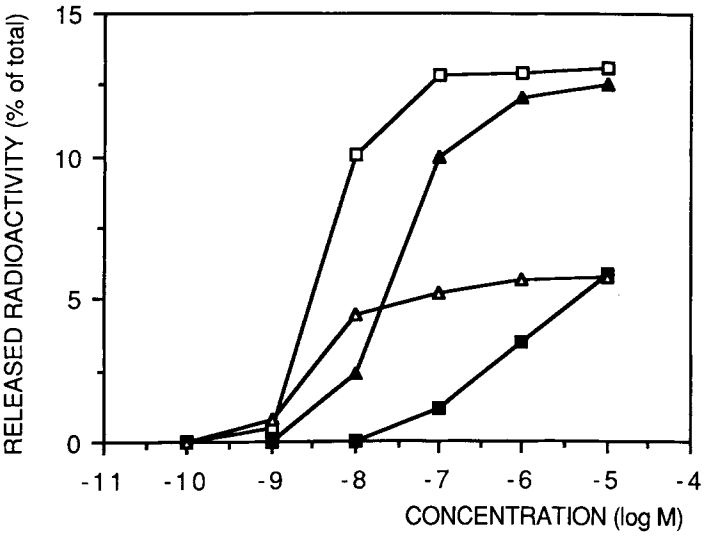

Fig. 2. Dose-dependent release of arachidonic acid. Whole cells were incubated with fMLP for 15 min (□), TPA for 30 min (▲), and PAF (△) or Io (■) for 5 min. Incubation procedure and results as in Figure 1.

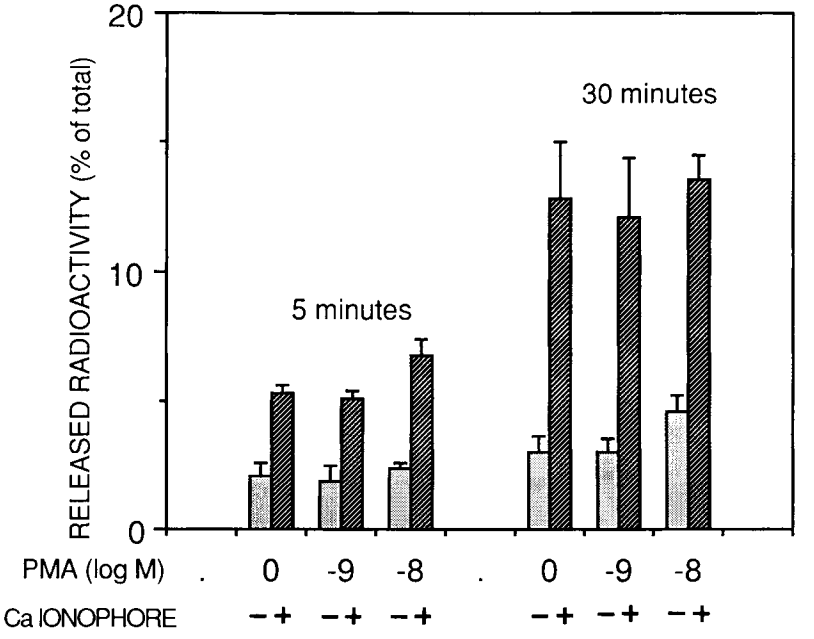

Fig. 3. Additive effects of TPA and calcium ionophore. Cells were incubated for 5 min (n = 4) or 30 min (n = 2) with (+) or without (−) 10^{-6} M Io and with 10^{-9} M, 10^{-8} M or without TPA. Each experiment (n) was performed in triplicate.

TABLE I. Phospholipase A_2 Activities in Stimulated Macrophages[a]

A. Exogenous substrate (pmol/min/mg protein)

Control	320 ± 25 [8]
10^{-7} M fMLP	358 ± 38 [8]
10^{-6} M fMLP	467 ± 42 [8]***
10^{-7} M PAF	365 ± 26 [3]
10^{-6} M PAF	390 ± 58 [3]*

B. Endogenous substrate (% radiolabel change in each phospholipid)

	PI	Alkyl-acyl PC	DAPC	DAPE	LbPA	Free AA
Control [8]	−12 ± 3	−14 ± 2	−11 ± 2	−0.7 ± 0.3	−5 ± 4	+92 ± 22
10^{-6} M fMLP [8]	−16 ± 3	−22 ± 3**	−17 ± 2***	−6 ± 2	−7 ± 1	+144 ± 19***

[a]Cells were incubated with fMLP for 15 min, with PAF for 5 min, or with their vehicle and then submitted to 1 M NaCl treatment (see Materials and Methods). For exogenous substrate (A), 4 nmol of dioleylphosphatidyl[^{14}C]ethanolamine were incubated for 15 min at 37°C with 50 μg protein of 1 M NaCl extracts (see Materials and Methods) in 50 mM glycine-NaOH, pH 8.5, containing 5 mM Ca^{2+}, final volume 1 ml. For exogenous substrate (B), aliquots of [1-^{14}C]arachidonic acid-labeled membrane (PI, 26 ± 1%; DAPC, 13 ± 1; alkyl-acyl PC, 17 ± 1; DAPE, 3.3 ± 0.3; alkeny-acyl PE, 12 ± 1; LbPA, 7.5 ± 0.5; FFA, 7.3 ± 1.5) were added to 1 M NaCl protein extract corresponding to the same number of cells incubated for 30 min, as in (A). Numbers in brackets are references.

*$P < 0.05$.

**$P < 0.01$.

***$P < 0.001$.

Snyder, who stimulated rat alveolar macrophages with Io (2×10^{-6} M) [17] and with those of Homma et al. for the stimulation of peritoneal macrophages with fMLP at 10^{-6} M [18].

Figure 2 shows that PAF and fMLP were more potent than other stimulating agents, with a threshold concentration of approximately 10^{-9} M, as already observed by Billah and Siegel in HL60 granulocytes [19]. The threshold concentration for TPA was 10^{-8} M and was even higher for Io, i.e., approximately 10^{-7} M.

The association of 10^{-8} M TPA (but not of 10^{-9} M) and 10^{-6} M Io for 5 min resulted in the release of more AA than by each agent tested separately (Fig. 3). This effect was slightly higher than additive, as is clearly noted when the increased AA release induced by the combined action of the agents (Fig. 3, 5 min) is compared with that elicited by TPA and Io alone. This effect was no longer seen after a 30-min incubation (Fig. 3).

Alveolar macrophages stimulated by 10^{-6} M fMLP or PAF exhibited a higher PLA_2 activity in 1 M NaCl extracts than controls (Table IA). Experiments with endogenous substrate showed that the increased AA release paralleled a higher breakdown of alkyl-acyl PC and diacyl PC than PI. The radioactivity decreased minimally in diacyl PE (DAPE), and there was no increase in the slight hydrolysis of lyso bis phosphatidic acid (LbPA) (Table IB).

The release of cyclooxygenase products by alveolar macrophages was strongly enhanced by fMLP (Table II). Thromboxane B_2 was secreted in a higher amount than PGE_2 and 6 keto $PGF_{1\alpha}$. Basal levels of intracellular cAMP of adherent alveolar macrophages were doubled in the presence of 2×10^{-4} M IBMX and increased three times in the presence of PGE_2 associated with IBMX (Table III). These agents strongly decreased the AA release induced by fMLP and PAF (Table III). The phos-

TABLE II. Cyclooxygenase Products Released After fMLP Stimulation*

	Control cells	fMLP-treated cells
Prostaglandin E_2	0.24 ± 0.07	3.4 ± 0.2**
Thromboxane B_2	17.0 ± 4.5	290 ± 30**
6 Keto prostaglandin $F_{1\alpha}$	0.10 ± 0.02	1.4 ± 0.1**

*10^{-6} M fMLP. Results in ng/ml, means of 12 experiments ± SD.
**$P < 0.001$.

phodiesterase inhibitor IBMX was more potent than PGE_2 in inhibiting macrophage activation, as already observed by Godfrey et al. in human monocytes [20].

DISCUSSION

Release of AA from alveolar macrophages may result either from PLA_2 activation or from the combined effect of activation of phospholipase C (PLC) and DG lipase. In this paper we demonstrate that the chemotactic peptide fMLP induces in 15 min an increase in 1 M NaCl-extracted PLA_2 activity directed mainly toward alkyl-acyl PC and diacyl PC and to a lesser extent toward PI (Table IB). We cannot, however, rule out a release from ethanolamine phospholipids as well, since the 1 M NaCl extract hydrolyzes exogenous dioleoyl PE (Table IA) and since the incorporation of radiolabeled AA did not reach a steady state after a 1-h incubation [2,9]. Indeed, a release of AA from a poorly labeled pool of PE might easily be masked by a transfer from DAPC into ethanolamine phospholipids by a two-step mechanism, as suggested by Colard for rat platelets (personal communication) or by Wynkoop et al. for fMLP-stimulated human neutrophils [21].

The initial event occuring in fMLP stimulation is reported to be the hydrolysis of PIP_2 by a specific PLC, leading to a transient rise in inositol triphosphate (ITP) and DG in peritoneal macrophages [18]. These compounds increase the cytosolic Ca^{2+} concentration and activate PKC, as suggested by the additive effect of a 5-min treatment by threshold concentrations of TPA and Io on AA release (Fig. 3). A synergistic effect was already reported for rabbit neutrophils [22], mast cells [23], or platelets [24]. Synergism tends to disappear for longer stimulation periods (Fig. 3, 30 min), probably because of the long-term cellular responses induced by TPA and Io, which can interfere with PLC- and PKC-mediated events. TPA is said to act as a long-living DG analogue, releasing AA in a dose-dependent manner in parallel to the activation of PKC [25] and also to activate other pathways of the lipid metabolism such as a PC-specific PLC in Madin Darbin Canine Kidney (MDCK) cells [26]. High concentrations of Io ($>10^{-5}$ M) elicited not only a PLA_2-mediated breakdown, but also a PLC-mediated breakdown of PC [27] and PI [28] in peritoneal macrophages.

In our experimental conditions, we failed to show a significant decrease in the labeling of LbPA, in contrast to the results of Cochran et al. in rabbit alveolar macrophages [29], but guinea pig alveolar macrophages contained less LbPA than those from the rat [9], and the labeling conditions were completely different (1 h vs. 24 h).

Lyso-PAF produced from alkyl-acyl PC by PLA_2 action may be converted into PAF by fMLP-activated macrophages, since the increase of intracellular Ca^{2+} is known to stimulate acetyl transferase in rat alveolar macrophages [4]. The production of PAF provides a positive feedback, since PAF itself can increase the PLA_2 activity (Table I) and release AA (Fig. 1) from cellular phospholipids [9] through a mecha-

TABLE III. Arachidonic Acid Release and Cyclic AMP Content Under Various Treatments*

	Arachidonic acid release (% total dpm release)	Cyclic AMP content (pmol/10^{-6} cells)
Control cells	ND	7.1 ± 0.8[g]
IBMX	ND	12.4 ± 1.9[h]
IBMX + PGE_2	ND	31.3 ± 3.9[i]
PAF	5.0 ± 0.5[a]	ND
PAF + IBMX	2.0 ± 0.2[b]	11.2 ± 2.3[h]
PAF + IBMX + PGE_2	1.2 ± 0.5[c]	17.3 ± 2.6[j]
fMLP	13.5 ± 1.5[d]	ND
fMLP + IBMX	4.5 ± 0.2[e]	ND
fMLP + IBMX + PGE_2	3.7 ± 1.5[f]	ND

*Cells were preincubated for 3 min with 2×10^{-4} M IBMX, IBMX + 2.8×10^{-6} M PGE_2 or their vehicle and then stimulated or not by 10^{-7} M PAF for 5 min or 10^{-6} M fMLP for 15 min. For arachidonic acid release, controls have been deducted. ND, not determined. Means of six experiments.
$P < 0.001$:a vs. b or c; d vs. e or f; g vs. h, i, or j; j vs. h or i; i vs. h.
$P < 0.05$:b vs. c; f vs. e.

nism involving PLC activation [7]. The time course of AA release induced by PAF remains puzzling, as the response plateaued within 5 min (Fig. 1). This finding cannot be fully explained by the metabolism of PAF, because its metabolites appeared only after 30 min in rat alveolar macrophages and because less than one-quarter was recovered as lyso-PAF in the incubation medium at that time [30]. Furthermore, only 40% of lyso-PAF was reacylated after 10 min when added to the culture medium [31].

Guinea pig alveolar macrophages can also convert AA into various eicosanoids (Table II). The thromboxane pathway was predominant, but it is not known whether macrophages possess TxA_2 receptors, which would provide another positive loop. However, this mediator might activate the myocytes of the bronchi or other alveolar cells. The amounts of prostaglandin E_2 released by stimulated alveolar macrophages was rather low (Table II), but AA released upon activation may be taken up by other alveolar cells, which might then transform it into PGE_2. The latter increased the cAMP content of the alveolar cells and decreased the release of AA upon stimulation with fMLP or PAF (Table III), providing a negative feedback.

The extract mechanism responsible for PLA_2 activation remains so far unexplained. Some authors suggested that PKC might phosphorylate and inactivate lipocortin [32,33], but the lipocortin effect has been related to substrate interaction rather than to a direct effect on the enzyme itself [34]. Phospholipase A_2 is Ca^{2+}-dependent [35], and PKC might also increase the calcium influx in the cytosol, as suggested by Nasmith and Grinstein [36], and, conversely, cAMP-activated protein kinase A might decrease the calcium influx [37]. However, these mechanisms cannot account for the sustained PLA_2 activation that we observed in our experiments after cell disruption and 1 M NaCl extraction (Table I).

In conclusion, our findings indicate that bacterial signals such as fMLP can trigger PLA_2 activation through a receptor-PLC-mediated mechanism and that there is a clear interaction between the lipid mediators whose synthesis is at least in part controled by PLA_2 and by the stimulating process itself. These relationships might involve various cell types, which should have taken up the precursors lyso-PAF and free AA and should lead to positive or negative feedbacks, depending on the ability of these

cells to synthesize PAF, PGE_2, TxA_2 or leukotrienes. In the macrophage, these interactions may occur through the complex network of second messengers: Ca^{2+}, cAMP, DG, and their enzymatic targets.

ACKNOWLEDGMENTS

These studies were supported by the scientific committee of the Faculty of Medicine Saint Antoine (grant 1987) and grant INSERM 87460.

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