Calcium-dependent biosynthesis of platelet-activating factor by submandibular gland cells

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Isolated dog submandibular gland cells synthesize platelet-activating factor (PAF) when stimulated with acetylcholine (ACh). This production of PAF was concentration- and time-dependent, and was inhibited by pretreatment with anticholinergic agents. PAF that had accumulated in cells through prior stimulation with ACh vanished rapidly on addition of atropine. Phenylmethanesulphonyl fluoride produced an accumulation of PAF in non-stimulated cells and greatly potentiated further ACh-induced accumulation. PAF production and [14 C]arachidonic acid (AA) liberation induced by ACh were increased by higher concentrations of extracellular Ca^{2+} , and ACh failed to stimulate PAF formation in the absence of Ca^{2+} , although ACh still stimulated the liberation of [14 C]AA without Ca^{2+} . Both the Ca^{2+} ionophore ionomycin in intact cells and Ca^{2+} (at concentrations ≥ 300 nM) in digitonin-permeabilized cells facilitated PAF formation. 1-*O*-Alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF):acetyl-CoA acetyltransferase activity rapidly increased in cells incubated with ACh or ionomycin. These results suggest, at least, that the stimulation of a remodelling pathway is involved in the increased PAF synthesis induced by ACh. Dithiothreitol-insensitive cholinephosphotransferase activity was also activated by ACh. However, the activation of both enzymes by ACh was transient, in spite of the fact that ACh-stimulated PAF formation was continuous. This may suggest that additional mechanism(s) other than the activation of these enzymes play an important role in controlling PAF synthesis. The present study provides further evidence that the exocrine submandibular gland cells of dogs have the capacity to increase PAF turnover upon stimulation in a Ca^{2+} -dependent manner and retain PAF within the cells partly associated with the membrane and partly released into the cytosol.

INTRODUCTION

Many of the breakdown products and metabolites of membrane lipids function predominantly in signal transduction as agonists or as second messengers, which remain in the plane of the membrane, are released into the cytoplasm, or are released out of the cells. These include diacylglycerol [1,2], inositol phosphates [3,4] and arachidonic acid (AA) metabolites [5]. A further addition to this growing family of biologically and physiologically active lipids has emerged from studies of alkyletherglycerolipid metabolites. Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) was originally described as a product released from activated basophils, and was then detected in a variety of inflammatory and immune-related cells, e.g. polymorphonuclear leucocytes (PMN), monocytes, macrophages and platelets [6]. PAF is also produced by isolated rat kidney cells [7], cultured human endothelial cells [8,9], rabbit lung [10] and perfused guinea pig heart [11]. PAF is considered to be a mediator in anaphylaxis and inflammatory responses, and possesses the capacity to regulate the cardiovascular and haemodynamic system [12,13]. More recently, PAF generation has been demonstrated in a wider variety of cells and tissues, and various physiological and pathophysiological roles have been suggested. For example, PAF is synthesized in and released from Kupffer cells, and might play an important role in hepatic metabolism [14]. Endothelial-cell-associated PAF mediates the adhesion of PMN [15]. Neuronal tissues contain biosynthetic activity and specific receptors for PAF. Neurotransmitters stimulate PAF formation

in the chick retina [16], and PAF exerts electrophysiological effects in that tissue [17]. PAF inhibits the release of hypothalamic hormones from the median eminence [18], and it also stimulates prolactin release from anterior pituitary cells [19]. In addition to being produced by these cell types, PAF was shown to be present in the saliva [20], blood and urine [21] of normal humans. Söling and his colleagues [22] reported that, in exocrine glands such as the parotid glands and the pancreas of guinea pig, the incorporation of radioactive acetate or 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF) into PAF increased on stimulation of the acinar cells. PAF also stimulated amylase release from lobules of both exocrine glands [23]. In our laboratory, the role of cyclic nucleotides, prostaglandins, peptides and γ -aminobutyric acid as second messengers or modulators in stimulus-secretion coupling have been studied in adrenal chromaffin cells and salivary glands [24–26]. To ascertain whether PAF may function as a messenger for signal transduction in these secretory cells, the present study investigated the capacity for PAF synthesis and degradation and the regulatory role of Ca^{2+} in isolated dog submandibular gland cells. Some of these results have been presented in abstract form [27].

MATERIALS AND METHODS

Reagents

The following reagents were obtained as indicated: [3 H]CoA (92.5–122.1 GBq/mmol), [14 C]AA (1953.6 GBq/mmol) and 1-*O*-hexadecyl-2- 3 H]acetyl-*sn*-GPC (glycero-3-phos-

Abbreviations used: PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); lyso-PAF, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; GPC, glycero-3-phosphocholine; PMSF, phenylmethanesulphonyl fluoride; AA, arachidonic acid; DTT, dithiothreitol; PMN, polymorphonuclear leucocytes; ACh, acetylcholine; LDH, lactate dehydrogenase.

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Fig. 1. Production and characterization of platelet-activating activity in ACh-stimulated dog submandibular gland cells

(a) T.l.c. of platelet-aggregating activity (PAF activity) produced in cells stimulated with ACh (100 μ M). PAF activity was estimated by measuring aggregation of washed rabbit platelets. (b) H.p.l.c. elution pattern of t.l.c.-purified submandibular PAF (fraction A on t.l.c.). Continuous lines represent the absorbance or PAF activity of standard lipids and the broken line represents the PAF activity of fraction A. PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; SM, sphingomyelin. (c) Effects of various treatments on PAF activity. a–i are platelet aggregations induced by h.p.l.c.-purified submandibular PAF, and j and k are the effects of fraction B (see a). Platelet aggregation was recorded with the following treatments: a, control; b, indomethacin (15 μ M), phosphocreatine (1.2 mM) and creatine kinase (15 units/ml); c and d, BN 52021 (0.4 μ M and 0.8 μ M respectively); e, methanol/0.5 M-NaOH; f, phospholipase A_2 ; g, phospholipase C; h and i, acetylation of e and f respectively; j, control; k, indomethacin.

phocholine) ($[^3\text{H}]\text{PAF}$, 370 GBq/mmol) were from New England Nuclear, Boston, MA, U.S.A. Cytidine 5'-diphospho[methyl- $^{14}\text{C}]\text{choline}$ (CDP-methyl $^{14}\text{C}]\text{choline}$ ammonium salt, 1.7 GBq/mmol) was from Amersham International, Amersham, Bucks., U.K. *p*-Bromophenacyl bromide, creatine kinase, hyaluronidase, indomethacin, mepacrine, 1-*O*-hexadecyl-2-acetyl-*sn*-GPC, phenylmethanesulphonyl fluoride (PMSF; dissolved in dimethyl sulphoxide), phosphatidylethanolamine, phosphocreatine, phospholipase C and physostigmine were from Sigma, St. Louis, MO, U.S.A. Acetyl-CoA trilithium salt, deoxyribonuclease, fatty-acid-free BSA (fraction V) and pig pancreas phospholipase A_2 were from Boehringer, Mannheim, Germany. Dipalmitoyl phosphatidylcholine, 1-*O*-hexadecyl-*sn*-GPC (lyso-PAF), phosphatidylinositol and phosphatidylserine were from Serdary Research Laboratories, Ontario, Canada; collagenase (type 2) and trypsin inhibitor (soya bean) were from Worthington Bio-

chemical Co., Freehold, NJ, U.S.A.; bisbenzimidazole (Hoechst 33258), 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol and ionomycin were from Calbiochem, La Jolla, CA, U.S.A.; and digitonin was from Wako Chemicals, Osaka, Japan. BN 52021 was a gift from Dr. Braquet (Institute Henri Beaufour, Le Plessis Robinson, France).

Cell preparation

Mongrel dogs of either sex weighing 7–18 kg were anaesthetized with pentobarbital, blood was drawn from the abdominal aorta, and the submandibular glands were removed. Isolated cells were prepared by the procedure described by McPherson & Dormer [28] with some modifications. Tissues (10–18 g) were minced with a McIlwain tissue chopper and rinsed with Ca^{2+} - and Mg^{2+} -free modified Hanks solution containing 5 mM-Hepes, pH 7.4. The minced tissues were suspended in 60 ml of dispersion solution and shaken vigorously (190 oscillations/min) in a baffled flask for about 60 min at 37 $^{\circ}\text{C}$ under O_2 . The dispersion solution consisted of Ca^{2+} - and Mg^{2+} -free Hanks solution (pH 7.4) containing collagenase (100 units/ml), hyaluronidase (1000 units/ml), deoxyribonuclease (20 units/ml) and Hepes (5 mM). After dispersion, the cells were filtered through a nylon mesh and washed by centrifugation through the oxygenated incubation medium [mm: NaCl 136, KCl 4.7, MgCl_2 1.1, Na_2HPO_4 1.0, CaCl_2 1.3, Hepes 10 and glucose 5.5, plus minimal essential medium amino acids, fatty-acid-free BSA (0.25 %) and trypsin inhibitor (0.01 %), pH 7.4.] The cell viability was determined by Trypan Blue exclusion or by measuring the release of cellular lactate dehydrogenase (LDH).

Cells were suspended in an incubation medium containing deoxyribonuclease and incubated with gentle shaking for 3 h at 37 $^{\circ}\text{C}$ under O_2 . Cells were then washed and suspended in a normal incubation medium or in a medium without CaCl_2 (Ca^{2+} -free) to a cell suspension corresponding to 40–110 μg of DNA/ml. The cell suspension was divided into plastic tubes (3 ml in each). The cells were preincubated for 10 min, and then for an additional 10 min with stimulants in an atmosphere of O_2 except where indicated. When acetylcholine (ACh) was used, physostigmine (1 μM) was added 2 min before addition of ACh. [$^{14}\text{C}]\text{AA}$ was incorporated into some cells by incubating 50 ml of cell suspension with [$^{14}\text{C}]\text{AA}$ (185 Kbpq, 4 μM) at 37 $^{\circ}\text{C}$ for 3 h. The cells were then washed three times before use. In some experiments, cells were rendered leaky by incubating them with 20 μM -digitonin for 15 min. Digitonin-permeabilized cells were incubated in glutamate buffer consisting of 138.7 mM-potassium glutamate, 20 mM-potassium Pipes, 5 mM-glucose, 5 mM-Tris/ATP, 7 mM-magnesium acetate and 0.5 mM EGTA, pH 7.0 [29] and a known concentration of free ionized Ca^{2+} . Ca^{2+} was measured using a Ca^{2+} electrode.

Lipid extraction, purification by t.l.c. and h.p.l.c. and characterization

Incubations were terminated by adding 30 μl of 2.6 M-formic acid and 3 ml of chloroform/methanol (1:2, v/v) and homogenizing using a Polytron homogenizer. The chloroform phase was separated according to the procedure of Bligh & Dyer [30] and dried under N_2 . The extracted lipids and standard lipids were subjected to t.l.c. on silica gel plates (Silica Gel 60; Merk, Darmstadt, Germany). Chromatography was performed using a solvent system of chloroform/methanol/water (65:35:6, by vol.). The zone for standards was cut off from the sample zones, and the standard lipids were stained by exposure to iodine vapour. Silica gel areas corresponding to the areas of migration of authentic PAF were scraped off, and lipids were extracted with chloroform/methanol/water (1:2:0.8, by vol.) three times. 1-*O*-Hexadecyl-2-acetyl-*sn*-GPC was used as a PAF standard

throughout the experiments. Phase separation was performed by the technique of Bligh & Dyer [30]. The chloroform layer was removed and dried under N_2 . The recovery of PAF was assayed using [3H]PAF.

Some PAF samples purified by t.l.c. were analysed by h.p.l.c. H.p.l.c. was performed using a TOSOH CCPE model system fitted with a silica column (4.6 mm $\times$ 25 cm) and a mobile phase of acetonitrile/methanol/water (6:3:1, by vol.) at a flow rate of 1.4 ml/min. Samples were collected every 15 s, and each fraction was lyophilized. Lyso-PAF was acetylated by treatment with acetic anhydride in pyridine and extracted as described above. H.p.l.c.-purified PAF was dissolved in methanol. Samples were mixed with methanol/0.5 M-NaOH (1:1, v/v) and stirred at room temperature for 60 min or mixed with Tris-buffered saline (pH 8.0) and incubated with phospholipase A_2 or phospholipase C at 37 °C for 30 min as reported [31]. The samples were acidified and extracted as described above and a portion of the sample was acetylated. For the PAF assay, samples were dissolved in saline supplemented with 0.25 % BSA and 4 mM-Hepes (pH 7.4) and submitted to bioassay.

PAF assay

Rabbit blood (9 vol.) was collected in 0.5 % NaCl (1 vol.) containing 60 mM-EDTA (pH 7.4). The washed platelets were prepared according to reported procedures [32]. Platelet aggregation was assayed in the presence of indomethacin (15 μ M), phosphocreatine (1.2 mM) and creatine kinase (15 units/ml) using a dual-channel aggregometer. The amount of PAF equivalents generated by dog submandibular glands was measured by aggregation assay and was expressed in fmol or pmol/mg of DNA. A standard curve for aggregation versus standard PAF concentration was prepared for each experiment. DNA concentrations were measured according to Labarca & Paigen [33].

Determination of lyso-PAF acetyltransferase and dithiothreitol (DTT)-insensitive cholinephosphotransferase

Incubations were terminated at the time points indicated by the addition of 10 vol. of an ice-cold sucrose medium (0.25 M-sucrose solution containing 1 mM-DTT, 2 mM-EDTA and 10 mM-Tris/HCl, pH 7.4). Cells were settled by a brief centrifugation (700 g for 50 s) and added to 500 μ l of sucrose medium, and then immersed in liquid N_2 until all incubations were terminated. After thawing, the cells were homogenized and centrifuged at 12000 g for 10 min. The resultant supernatants were used for the enzyme assays. The acetyltransferase assay was carried out as reported [34] in 200 μ l of the medium containing 30 μ M-lyso-PAF, 200 μ M-[3H]acetyl-CoA (7.4 KBq), 50–70 μ g of protein and 0.1 M-Tris/HCl, pH 7.4. The reaction was started by adding acetyl-CoA and stopped 10 min thereafter by adding 3 ml of chloroform/methanol (1:2, v/v). In preliminary experiments, the reaction was linear during the incubation period. Phase separation was achieved by the addition of chloroform and water, and the organic phase was removed and dried under N_2 . [3H]PAF was quantified by scraping of the appropriate area of the t.l.c. gel and liquid scintillation counting. To assay cholinephosphotransferase activity, enzyme samples were incubated for 10 min in 100 μ l of medium comprising 0.5 mM-EGTA, 10 mM-MgCl₂, 5 mM-DTT, 20 μ M-CDP-methyl[^{14}C]choline (3.7 KBq) and 5 μ M-1-*O*-hexadecyl-2-acetyl-*sn*-glycerol in 1 μ l of ethanol and 0.1 M-Tris-HCl, pH 8.0 [35]. The subsequent processing was performed in the same way as for acetyltransferase.

Protein concentrations were measured by the method of Lowry *et al.* [36].

RESULTS

Characterization of PAF from submandibular gland cells

After incubating the cells with ACh (100 μ M) for 10 min, lipids were extracted into chloroform/methanol (1:2, v/v) as described in the Materials and methods section. Cell viability was checked by Trypan Blue exclusion or by measuring the release of cellular LDH. After the incubation period, cells were more than 80 % free of Trypan Blue, and less than 6 % of the total of LDH was present in the medium. The responsiveness of the cells to neurotransmitters was also shown as follows: [^{14}C]glucosamine-labelled mucin release was increased from 2.3 ± 0.1 % to 5.5 ± 0.3 % and to 11.9 ± 0.8 % of the total cellular mucin by 10 μ M-noradrenaline and 10 μ M-ACh respectively. T.l.c. of the extract revealed the presence of platelet-aggregating activity in two fractions (Fig. 1). The platelet aggregation of one fraction was due to AA, since the R_f value of the fraction corresponded to that of authentic AA, and platelet aggregation was eliminated by treating the platelets with indomethacin. Activity in another fraction corresponding to the R_f of authentic PAF was eluted from the h.p.l.c. column as a peak with a similar retention time to that of authentic PAF (Fig. 1). Platelet aggregation induced by the h.p.l.c.-purified sample was not halted by the treatment of the platelets with indomethacin or an ADP-scavenger system, but was antagonized with the PAF antagonist BN 52021 (Fig. 1). The samples were further characterized by a base-catalysed methanolysis or by enzymic hydrolysis with phospholipases. The platelet-aggregating activities were lost in the samples treated with methanol/NaOH, phospholipase A_2 and phospholipase C, and the activities of the two former samples were restored by acetylation (Fig. 1). Similar results were obtained using authentic PAF (results not shown).

The formation of PAF induced by ACh was dose-dependent and the effect of ACh was antagonized by atropine and to a lesser extent by pirenzepine (Fig. 2). Other muscarinic cholinergic agents, muscarine and carbachol, also stimulated PAF formation, but isoprenaline did not (results not shown). The time course of PAF formation induced by ACh in dog submandibular gland cells is shown in Fig. 3(a). A negligible amount of PAF was found in unstimulated cells, but ACh caused a rapid increase in PAF formation with a peak effect at 2 min after the addition. The increased levels of PAF lasted throughout the 30 min incubation, and then declined. When atropine was added to the incubation medium at 10 min after the addition of ACh, the

Fig. 2. Dose-response relationships of ACh and PAF production (a) and of anti-cholinergic agents and ACh-induced PAF production (b) in dog submandibular gland cells

(b) Atropine (○) and pirenzepine (●) were added 2 min before the addition of ACh (30 μ M). Values represent the means $\pm$ S.E.M. of measurements from five incubations.

Fig. 3. Turnover of PAF in non-stimulated and ACh-stimulated cells from dog submandibular glands

(a) Time course of the increase in PAF concentration with or without ACh and the effects of atropine (Atr.) or PMSF. ○, Without ACh, ●, ■, ▲, with ACh (30 μ M). Atropine (6 μ M, ▲) or PMSF (0.25 mM, ■) were added 10 min after the addition of ACh (●, control). (b) Dose-response relationship of PMSF to PAF accumulation in the absence (○) or the presence (■) of ACh (30 μ M). Values represent the means $\pm$ S.E.M. ($n = 5$).

amount of PAF in the cells dropped sharply to the unstimulated level within 2 min. On the other hand, the addition of PMSF after the levels of PAF in ACh-stimulated cells had reached a plateau caused the further accumulation of PAF. Increasing the PMSF concentration increased levels of PAF in non-stimulated cells and markedly enhanced ACh-induced accumulation of PAF (Fig. 3b).

Ca^{2+} -dependency of PAF formation

When cells were incubated in Ca^{2+} -free medium, ACh stimulated the liberation of [^{14}C]AA from cells prelabelled with [^{14}C]AA. However, ACh failed to stimulate PAF formation in the absence of Ca^{2+} . Both [^{14}C]AA liberation and PAF formation stimulated by ACh increased with increasing concentrations of Ca^{2+} in the medium (Table 1). To test the direct stimulation of PAF synthesis by Ca^{2+} , the effects of Ca^{2+} ionophore ionomycin on PAF formation were examined. The addition of ionomycin to the incubation medium at concentrations of 1, 3 and 8 μM increased PAF formation by 229 ± 65 , 492 ± 170 and 2375 ± 214 fmol/mg of DNA respectively. In other experiments, the effects of changes in the Ca^{2+} concentration on PAF formation were examined in leaky cell preparations (Table 2). Cells were subjected to permeabilization by digitonin. When cells were incubated with 20 μM -digitonin for 15 min, $24.4 \pm 1.5\%$ of total LDH activity was found in the medium, in contrast with $5.6 \pm 0.6\%$ in control medium. In the digitonin-permeabilized cells, PAF was formed by increasing the free Ca^{2+} concentration from 300 nM to 3.0 μM . No further increase in PAF synthesis was observed with higher concentrations of Ca^{2+} . The stimulation of PAF synthesis by Ca^{2+} was inhibited by the inhibitors of phospholipase A_2 , p -bromophenacyl bromide and mepacrine.

Localization of PAF

PAF formed by the stimulation of cells with ACh was not released into the incubation medium, even if a large amount of PAF accumulated in the cells because of inhibition of its hydrolysis by PMSF (Table 3). The cells were then homogenized

Table 1. Effects of external Ca^{2+} concentration on the production of PAF and the liberation of [^{14}C]AA induced by ACh in dog submandibular gland cells

After being labelled with [^{14}C]AA as described in the Materials and methods section, the cells were washed three times with Ca^{2+} -free medium containing 4 mM-EGTA. Then the cells were suspended in Ca^{2+} -free medium without EGTA, divided into incubation tubes and incubated for 10 min with or without ACh (30 μM) in the presence of CaCl_2 at the concentrations indicated. After the incubations, lipids were extracted and separated on t.l.c. Areas of the gel corresponding to PAF and AA were extracted and submitted to assay of platelet activity and radioactivity respectively. Values represent the means $\pm$ S.E.M. ($n = 5$). N.D., not detectable. * $P < 0.05$, ** $P < 0.01$ versus cells in the absence of ACh.

CaCl_2 (mM)	PAF production (pmol/mg of DNA) (+ ACh)	$10^{-3} \times$ Release of [^{14}C]AA (d.p.m.)	
		– ACh	+ ACh
0	N.D.	1.98 ± 0.15	$3.04 \pm 0.16^*$
0.03	0.02 ± 0.01	1.99 ± 0.14	$3.15 \pm 0.17^*$
0.10	0.04 ± 0.02	2.11 ± 0.18	$3.33 \pm 0.17^*$
0.30	0.13 ± 0.03	2.22 ± 0.22	$3.56 \pm 0.25^*$
1.30	0.95 ± 0.15	2.23 ± 0.23	$4.28 \pm 0.35^{**}$
2.60	1.22 ± 0.19	2.48 ± 0.21	$4.60 \pm 0.36^{**}$

and fractionated into a 105000 g particulate fraction and the supernatant, PAF was distributed in both particulate and soluble fractions. When PAF synthesis was stimulated by 3 μM - Ca^{2+} in digitonin-permeabilized cells, PAF was released into the incubation medium.

Stimulation of acetyltransferase and cholinephosphotransferase activities

After the incubation of the submandibular gland cells with or without stimulants for the periods indicated in Fig. 4, the cells

Table 2. Increases in PAF production produced by Ca^{2+} and the effects of *p*-bromophenacyl bromide and mepacrine in digitonin-permeabilized submandibular gland cells

Cells were rendered leaky with digitonin and incubated for 10 min in the medium mimicking intracellular conditions containing different concentrations of free Ca^{2+} adjusted with Ca/EGTA buffer as given in the Materials and methods section. Values represent the means $\pm$ S.E.M. of measurements from five incubations. * $P < 0.05$, ** $P < 0.01$, versus control (Student's *t* test).

Additions (μM)	Ca^{2+} (μM)	PAF (fmol/mg of DNA)
Expt. 1:		
0	0.06	748 $\pm$ 191
0	0.1	669 $\pm$ 98
0	0.3	1035 $\pm$ 114
0	1.0	1477 $\pm$ 221
0	3.0	1932 $\pm$ 326
0	10.0	1653 $\pm$ 307
0	30.0	1948 $\pm$ 286
Expt. 2:		
None	1.0	1072 $\pm$ 279
<i>p</i> -Bromophenacyl bromide		
3	1.0	1147 $\pm$ 132
10	1.0	363 $\pm$ 73**
30	1.0	335 $\pm$ 74**
Mepacrine		
3	1.0	530 $\pm$ 190*
10	1.0	502 $\pm$ 368*
30	1.0	274 $\pm$ 72**

Table 3. Localization of PAF produced in dog submandibular gland cells

After incubation of intact cells with 30 μM -ACh for 10 min in the presence or absence of PMSF, cells (1) and medium (2) were separated by centrifugation at 700 *g* for 50 s (a), or cells were frozen in liquid N_2 , homogenized in Hepes/saline (pH 7.4) containing 3 mM EDTA, 1 mM-PMSF and 0.25% BSA and then centrifuged at 105 000 *g* for 60 min and particulate (1) and supernatant (2) fractions were separated (b). (c) Digitonin-permeabilized cells were incubated for 10 min in the medium containing 3 μM - CaCl_2 and then cells (1) and medium (2) were separated by centrifugation at 700 *g* for 5 s. PAF was extracted from each fraction and submitted to assay. The values represent means $\pm$ S.E.M. ($n = 6$). N.D., not detectable.

Additions	PAF (pmol/mg of DNA)	
	1	2
(a) Intact cells		
ACh	0.23 $\pm$ 0.03	N.D.
PMSF + ACh	17.16 $\pm$ 2.55	N.D.
(b) Intact cells		
PMSF + ACh	0.28 $\pm$ 0.04	0.36 $\pm$ 0.06
(c) Leaky cells		
PMSF + CaCl_2	2.86 $\pm$ 0.39	0.31 $\pm$ 0.04

were homogenized and the enzyme activities were assayed as described in the Materials and methods section. The activities of acetyltransferase and cholinephosphotransferase in unstimulated cells were 0.52 ± 0.04 and 0.11 ± 0.01 nmol/10 min per mg of

protein respectively. Acetyltransferase activity rapidly increased with ACh, approaching the maximal level 15 s after stimulation. ACh activation of acetyltransferase was attenuated quickly to the non-stimulated level after a 5 min incubation. Ionomycin induced the activation of acetyltransferase, but the onset of the activation was slower, and the duration longer, than the ACh-induced changes. Cholinephosphotransferase activity was also activated by ACh to a greater extent than that of acetyltransferase. The activation was transient.

DISCUSSION

Stimulation of muscarinic cholinergic receptors of the dog submandibular gland cells provoked the production of PAF-like material. PAF was identified on the basis of the lipid natures on t.l.c. and h.p.l.c., chemical and lipase treatments, and its biological characteristics in platelet activation, though the molecular species of PAF has not been identified. PAF can be synthesized by either a remodelling or a *de novo* pathway. The former pathway is Ca^{2+} -dependent and responsible for the synthesis of PAF during stimulus-mediated cell activation. The latter pathway is present in many cell types and has the capacity to produce PAF without stimulation, and is independent of Ca^{2+} [37]. The present study indicates that dog submandibular gland cells possess the two enzymic activities able to synthesize PAF, and that both activities can be stimulated by the addition of ACh. In addition to the Ca^{2+} -dependency of the effects of ACh on PAF synthesis, the evidence of direct stimulation of PAF synthesis by the calcium ionophore ionomycin and by increasing free Ca^{2+} concentrations in the digitonin-permeabilized cell preparations suggests that ACh may stimulate PAF formation in submandibular cells by stimulating the entry of Ca^{2+} from the medium. The synthesis of PAF by the remodelling pathway is thought to first involve the activation of phospholipase A_2 , which supplies a substrate, lyso-PAF, for an acetylation reaction involving acetyl-CoA and acetyltransferase. Phospholipase A_2 and acetyltransferase appear to form the 'trunk' of the PAF biosynthesis pathway. Since certain phospholipase A_2 enzymes require Ca^{2+} for full expression of their enzymic activity [38], extracellular Ca^{2+} may be important in the generation of lyso-PAF. Although the specificity of mepacrine and *p*-bromophenacyl bromide as inhibitors of phospholipase A_2 has been questioned [39,40], the present observations that these compounds inhibited Ca^{2+} -stimulated PAF synthesis in digitonin-permeabilized cells agree with this hypothesis. However, ACh failed to stimulate PAF synthesis in the absence of extracellular Ca^{2+} , even though ACh still stimulated the liberation of [^{14}C]AA. These results may suggest that the substrate, lyso-PAF, is not available without Ca^{2+} . Another possibility is that the supply of precursor alone is not sufficient, and the concurrent stimulation of acetyltransferase is required for ACh stimulation of PAF synthesis. From the extensive studies in platelets, PMN and macrophages, the role of acetyltransferase as the limiting step has been suggested. In these cells, a phosphorylation-dephosphorylation mechanism is proposed for regulation of acetyltransferase activity [37]. The involvement of extracellular Ca^{2+} in the enhancement of acetyltransferase activity by various stimuli has also been demonstrated [41,42]. Domenech *et al.* [43] reported that carbamylcholine and A23187 both stimulated acetyltransferase in exocrine secretory glands, and that this stimulation was due to the activation of acetyltransferase by Ca^{2+} via phosphorylation by Ca^{2+} /calmodulin-dependent protein kinase. In dog submandibular gland cells, as in other cells, the regulation of acetyltransferase by Ca^{2+} may be a critical factor in the control of PAF production. In PMN, cholinephosphotransferase cannot be activated by stimuli that activate

Fig. 4. Increases in lyso-PAF acetyltransferase and DTT-insensitive choline phosphotransferase activities in stimulated submandibular gland cells

After the cells were incubated with ACh or ionomycin for the times indicated, homogenization of cells, preparation of enzyme samples and the measurement of enzyme activities were performed as described in the Materials and methods section. Acetyltransferase (●, ACh 30 μ M; ○, ionomycin 8 μ M) and choline phosphotransferase (▲, ACh 30 μ M) activities were measured. Values represent the means $\pm$ S.E.M. ($n = 6$).

acetyltransferase [44]. In these cells, only phorbol esters stimulate cholinephosphotransferase without affecting acetyltransferase activity. Cholinephosphotransferase in chick embryonic retina has been shown to be activated by ACh and dopamine, and this activation was not accompanied by the activation of acetyltransferase, whereas A23187 activated only acetyltransferase in these cells [16]. Thus the transcholinergic pathway mediates neurotransmitter-stimulated, but not A23187-stimulated, PAF production in chick retina. In contrast, both acetyltransferase and cholinephosphotransferase are concurrently activated in submandibular cells stimulated with ACh. However, whether or not the transcholinergic pathway is involved in ACh-stimulated PAF synthesis in submandibular gland cells has yet to be determined, because it is known that Ca^{2+} is an inhibitor of, as opposed to being required for, cholinephosphotransferase activity [45].

PAF is inactivated by a cytosolic acetylhydrolase. PMSF, a serine hydrolase inhibitor, has been shown to inhibit acetylhydrolase [46]. The marked acceleration of PAF accumulation caused by inhibiting acetylhydrolase with PMSF and the quick removal of accumulated PAF after blockade of ACh action with atropine suggest that PAF synthesis and hydrolysis are taking place quite rapidly in stimulated cells and also in non-stimulated cells in which PAF levels are almost undetectable without inhibiting hydrolysis. It is apparent from this evidence that PAF could not be stored in the cells. Thus the amount of PAF is the balance of its synthesis and the hydrolysis. However, the time course of the increase in PAF synthesis stimulated by ACh is not parallel with the activation of acetyltransferase or cholinephosphotransferase; the former lasts throughout stimulation but the latter two events are quite transient. Therefore the stimulation of PAF synthesis could not be simply explained by the activation of acetyltransferase or cholinephosphotransferase. Ninio *et al.* [47] reported the regulation of acetyltransferase by an enzyme-substrate imbalance in PMN. Such a mechanism, one that supplies a large amount of lyso-substrate, would result on the one hand in the inhibition of acetyltransferase, and on the other hand in an increase in PAF formation. This mechanism may help to explain the present discrepancy between the time

course of the activation of the enzyme and the formation of PAF. Gomez-Cambronero *et al.* [48] reported that Ca^{2+} is necessary but not sufficient on its own for PAF release in neutrophils, and the co-ordinated actions of receptor-coupled G-proteins and other parameters are also required. On the other hand, it has been shown that the accumulation of PAF in mature macrophages is regulated by acetylhydrolase [49]. We have further addressed the question of how cellular PAF levels are controlled in such cases.

Söling *et al.* [23] have reported that PAF stimulated the secretion of amylase from isolated guinea-pig parotid glands and exocrine pancreatic lobules. PAF stimulates mucin release of explants of rodent airways in organ culture [50]. We have recently observed that the muscarinic cholinergic system, via a Ca^{2+} -dependent mechanism, plays a primary role in mucosecretion in dog submandibular glands (different from the case in other species, where mucin secretion is mostly mediated by an adrenergic β -receptor/adenylate cyclase system [51]). Thus the effect of PAF on mucin release from dog submandibular glands has been examined, but neither 1-*O*-hexadecyl- or 1-*O*-octadecyl-2-acetyl-*sn*-GPC affected basal or ACh-stimulated mucin release (T. Dohi, unpublished work). PAF is thought to be released from the cells in which PAF is produced on stimulation and exerts the autocrine or paracrine actions by interacting with its specific receptors on the cell surface. On the other hand, it has become apparent that a significant proportion of the PAF synthesized upon activation remains inside the cell in many cell types [51]. Thus different types of roles for PAF can be proposed: intracellular roles such as participation in the alkylacyl-GPC cycle, membrane-active properties, and intracellular messenger roles [52]. The present study showed that the PAF synthesized due to stimulation of intact submandibular gland cells is retained within the cell, some associated with the membrane and the rest released into the cytosol. The evidence that PAF was released into the incubation medium in digitonin-permeabilized cells further suggests that PAF can move from one site to another within the cell. The occurrence of PAF-specific transfer proteins in the cytosol [53] and receptors for PAF at intracellular sites or even on the inner leaflet of the plasma

membrane [52,54] have also been suggested. These possibilities favour the concept of PAF as an intracellular messenger. It has been demonstrated that protein kinase C is activated by PAF in an enzyme-translocation-independent fashion in rabbit platelets [55,56]. PAF receptor antagonists inhibited stimulation-induced eicosanoid generation by cultured endothelial cells and by macrophages [57–59], and blocked the rise in the intracellular Ca^{2+} concentration induced by opsonized particles in human neutrophils [60]. This evidence suggests that PAF can act as an intracellular messenger in these cells. Na^+/K^+ -ATPase is located on the basolateral membrane of salivary glands and controls ionic transport during secretion. PAF has been reported to inhibit the Na^+/K^+ -ATPase of the red blood cell membrane [61]. It is also reported that PAF stimulates or inhibits $\text{Na}^+/\text{Ca}^{2+}$ exchange [62,63] and affects Ca^{2+} -lipid interactions in phospholipid vesicles [64]. Although the role of PAF in dog submandibular gland cells has yet to be discovered, the possibility that PAF may have intracellular activities for controlling cellular functions remains to be determined, since Na^+/K^+ -ATPase, $\text{Na}^+/\text{Ca}^{2+}$ exchange and Ca^{2+} -lipid interactions may be important in the function of acinar cell homeostasis and stimulus-response coupling. These results provide further evidence that the dog submandibular gland has a receptor-coupled PAF-metabolizing system in which PAF undergoes rapid synthesis and hydrolysis upon stimulation of the cells. This tissue may be a useful model in studying the mechanism that regulates the synthesis and degradation of PAF, as well as its intracellular activities.

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REFERENCES

- Nishizuka, Y. (1986) *Science* **233**, 305–312
- Nishizuka, Y. (1988) *Nature (London)* **334**, 661–665
- Berridge, M. J. (1984) *Biochem. J.* **220**, 345–360
- Berridge, M. J. & Irvine, R. F. (1989) *Nature (London)* **431**, 197–205
- Needleman, P., Turk, J., Jakschik, B. A., Morrison, A. R. & Lefkowitz, J. B. (1986) *Annu. Rev. Biochem.* **55**, 69–102
- Camussi, G. & Brentjens, J. R. (1987) in *Platelet-Activating Factor and Related Lipid Mediators* (Snyder, F., ed.), pp. 299–322, Plenum Press, New York
- Pirotzky, E., Ninio, E., Bidault, J., Pfister, A. & Benveniste, J. (1984) *J. Immunol.* **51**, 567–572
- Camussi, G., Aglietta, M., Malavasi, F., Tetta, C., Piacibello, W., Sanavio, F. & Bussolino, F. (1983) *J. Immunol.* **131**, 2397–2403
- Prescott, S. M., Zimmerman, G. A. & McIntyre, T. M. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3534–3538
- Kravis, T. C. & Henson, P. M. (1975) *J. Immunol.* **115**, 1677–1681
- Levi, R., Burke, J. A., Guo, Z.-G., Hattori, Y., Hoppens, C. M., McManus, L. M., Hanahan, D. J. & Pinckard, R. N. (1984) *Circ. Res.* **54**, 117–124
- Pinckard, R. N., McManus, L. M. & Hanahan, D. J. (1982) *Adv. Inflamm. Res.* **4**, 147–180
- Vargaftig, B. B. & Benveniste, J. (1983) *Trends Pharmacol. Sci.* **4**, 341–343
- Chao, W., Siafaka-Kapadai, A., Olson, M. S. & Hanahan, D. J. (1989) *Biochem. J.* **257**, 823–829
- Zimmerman, G. A., McIntyre, T. M., Mehra, M. & Prescott, S. M. (1990) *J. Cell Biol.* **110**, 529–540
- Bussolino, F., Gremo, F., Tetta, C., Pescarmona, G. P. & Camussi, G. (1986) *J. Biol. Chem.* **261**, 16502–16508
- Doly, M., Braquet, P., Bonhomme, B. & Meyniel, G. (1987) *Int. J. Tissue React.* **9**, 33–37
- Junier, M. P., Tiberghien, C., Rougeot, C., Fafeur, V. & Dray, F. (1988) *Endocrinology (Baltimore)* **123**, 72–80
- Camoratto, A. M. & Grandison, L. (1989) *Endocrinology (Baltimore)* **124**, 1502–1506
- Cox, C. P., Wardlow, M. L., Jorgensen, R. & Farr, R. S. (1981) *J. Immunol.* **127**, 46–50
- Schlondorff, D. & Neuwirth, R. (1986) *Am. J. Physiol.* **251**, F1–F11
- Söling, H.-D. & Fest, W. (1986) *J. Biol. Chem.* **261**, 13916–13922
- Söling, H.-D., Elbl, H. & Fest, W. (1984) *Eur. J. Biochem.* **144**, 65–72
- Kinjo, K., Nishikawa, T. & Tsujimoto, A. (1983) *Arch. Oral Biol.* **28**, 97–98
- Kitayama, S. & Tsujimoto, A. (1986) in *GABAergic Mechanisms in the Mammalian Periphery* (Bowery, N. G. & Erdo, S. L., eds.), pp. 249–259, Raven Press, New York
- Tsujimoto, A., Morita, K. & Dohi, T. (1991) in *Presynaptic Regulation of Neurotransmitter Release* (Feigenbaum, J. J. & Hanani, M., eds.), Freund Publishing House, London, in the press
- Dohi, T., Ohtsuki, H., Kitayama, S., Morita, K. & Tsujimoto, A. (1990) *Jpn. J. Pharmacol.* **52**, (suppl.), 115p
- McPherson, M. A. & Dormer, R. L. (1984) *Biochem. J.* **224**, 473–481
- Baldys-Waligorska, A., Pour, A., Moriarty, C. M. & Dowd, F. (1987) *Biochim. Biophys. Acta* **929**, 190–196
- Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
- Benveniste, J. & Le Couedic, J. P. (1977) *Nature (London)* **269**, 170–171
- Camussi, G., Mencia-Huerta, J. M. & Benveniste, J. (1977) *Immunology* **33**, 523–534
- Labarca, C. & Paigen, K. (1980) *Anal. Biochem.* **102**, 344–352
- Wykle, R. L., Malone, B. & Snyder, F. (1980) *J. Biol. Chem.* **255**, 10256–10260
- Renooij, W. & Snyder, F. (1981) *Biochim. Biophys. Acta* **663**, 545–556
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Snyder, F. (1988) *Proc. Soc. Exp. Biol. Med.* **190**, 125–135
- Van Den Bosch (1980) *Biochim. Biophys. Acta* **604**, 191–246
- Wykle, R. E., Malone, B. & Snyder, F. (1980) *J. Biol. Chem.* **255**, 10256–10260
- Garcia, M. d. C., Fernandez-Gallardo, S., Gijon, M. A., Garcia, C., Nieto, M. L. & Sanchez-Crespo, M. (1990) *Biochem. J.* **268**, 91–98
- Gomez-Cambronero, J., Inarrea, P., Alonso, F. & Sanchez-Crespo, M. (1984) *Biochem. J.* **219**, 419–424
- Gomez-Cambronero, J., Nieto, M. L., Mato, J. M. & Sanchez-Crespo, M. (1985) *Biochim. Biophys. Acta* **845**, 511–515
- Domenech, C., Domenech, E. M.-D. & Söling, H.-D. (1987) *J. Biol. Chem.* **262**, 5671–5676
- Nieto, M. L., Velasco, S. & Crespo, M. S. (1988) *J. Biol. Chem.* **263**, 2217–2222
- Woodard, D. S., Lee, T.-C. & Snyder, F. (1987) *J. Biol. Chem.* **262**, 2520–2527
- Touqui, L., Hatmi, M. & Vargaftig, B. B. (1985) *Biochem. J.* **229**, 811–816
- Ninio, E., Joly, F. & Bessou, G. (1988) *Biochim. Biophys. Acta* **963**, 288–294
- Gomez-Cambronero, J., Durstin, M., Molski, T. F. P., Naccache, P. H. & Sha'afi, R. I. (1989) *J. Biol. Chem.* **264**, 21699–21704
- Elstad, M. R., Stafforini, D. M., McIntyre, T. M., Prescott, S. M. & Zimmerman, G. A. (1989) *J. Biol. Chem.* **264**, 8467–8470
- Adler, K. B., Schwarz, J. E., Anderson, W. H. & Welton, A. F. (1987) *Exp. Lung Res.* **13**, 25–43
- 50a. Dohi, T., Yamuki, M., Morita, K., Kitayama, S., Tsuru, M. & Tsujimoto, A. (1991) *Arch. Oral Biol.*, in the press
- Elstad, M. R., Prescott, S. M., McIntyre, T. M. & Zimmerman, G. A. (1988) *J. Immunol.* **140**, 1618–1624
- Henson, P. M. (1987) in *Platelet-Activating Factor and Related Lipid Mediators* (Snyder, F., ed.), pp. 255–271, Plenum Press, New York
- Lumb, R., Pool, G. L., Bubacz, D. G., Blank, M. L. & Snyder, F. (1983) *Biochim. Biophys. Acta* **750**, 217–222
- Marcheselli, V. L., Rossowska, M. J., Domingo, M.-T., Braquet, P. & Bazan, N. G. (1990) *J. Biol. Chem.* **265**, 9140–9145
- Pelech, S. L., Charest, D. L., Howard, S. L., Paddon, H. B. & Salari, H. (1990) *Biochim. Biophys. Acta* **1051**, 100–107
- Salari, H., Duronio, V., Howard, S., Demos, M. & Pelech, S. L. (1990) *Biochem. J.* **267**, 689–696

57. Stewart, A. G., Dubbin, P. N., Harris, T. & Dusting, G. J. (1989) *Br. J. Pharmacol.* **96**, 503–505
58. Stewart, A. G. & Phillips, W. A. (1989) *Br. J. Pharmacol.* **98**, 141–148
59. Stewart, A. G., Dubbin, P. N., Harris, T. & Dusting, G. J. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3215–3219
60. Tool, A. T. J., Verhoeven, A. J., Roos, D. & Koenderman, L. (1989) *FEBS Lett.* **259**, 209–212
61. Hollister, A. S. & Kochhar, S. (1989) *Clin. Res.* **37**, 395A
62. Kester, M., Kumar, R. & Hanahan, D. J. (1986) *Biochim. Biophys. Acta* **888**, 306–315
63. Meng, H., Kutryk, M. J. B. & Pierce, G. N. (1989) *J. Mol. Cell. Cardiol.* **21** (Suppl. II), S. 113
64. Bratton, D. L., Harris, R. A., Clay, K. L. & Hensor, P. M. (1988) *Biochim. Biophys. Acta* **943**, 211–219

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