Biosynthesis of platelet-activating factor in glandular gastric mucosa

Evidence for the involvement of the 'de novo' pathway and modulation by fatty acids

Sagrario FERNANDEZ-GALLARDO, Miguel Angel GIJON, Maria del Carmen GARCIA, Ernesto CANO and Mariano SANCHEZ CRESPO*

Laboratorio de Nefrologia, Instituto de Investigaciones Medicas de la Fundacion Jimenez Diaz, Centro Asociado al Consejo Superior de Investigaciones Cientificas (CSIC), Av. Reyes Catolicos 2, 28040 Madrid, Spain

The biosynthesis of platelet-activating factor (PAF), a phospholipid autocoid with potent ulcerogenic properties that is produced in secretory exocrine glands by physiological secretagogues, was assessed in microsomal preparations of glandular gastric mucosa. For this purpose, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF):acetyl-CoA acetyltransferase (EC 2.3.1.67); the enzymes of the 'de novo' pathway: 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate (alkyl-lyso-GP):acetyl-CoA acetyltransferase and 1-*O*-alkyl-2-acetyl-*sn*-glycerol (alkylacetyl-G):CDP-choline cholinephosphotransferase (EC 2.7.8.16); and some enzymes involved in the catabolism of PAF and lyso-PAF were assayed. Only the enzymes of the 'de novo' pathway and small amounts of PAF acetylhydrolase, phospholipase A₂ and a lysophospholipase D acting on ether lipids could be detected in the gastric preparations, whereas lyso-PAF:acetyl-CoA acetyltransferase activity was undetectable. The specific activity of alkyl-lyso-GP:acetyl-CoA acetyltransferase in the gastric mucosa was about one-tenth of that found in spleen microsomes and its apparent *K_m* for acetyl-CoA was 454 μ M compared with 277 μ M in spleen microsomes. Glandular mucosa homogenates contained preformed PAF at a concentration of 2.7 ± 0.7 ng equivalents of PAF (hexadecyl)/mg of protein. When gastric microsomes were incubated with micromolar concentrations of fatty acids (arachidonic, palmitic and oleic) prior to the assay of dithiothreitol (DTT)-insensitive cholinephosphotransferase, a dose-dependent reduction in the formation of PAF was observed, arachidonic acid being the most potent inhibitor, followed by linoleic acid (only tested on spleen microsomes) and oleic acid. By contrast, 1,2-diolein and phosphatidylcholine (dipalmitoyl) showed no or little effect. These results indicate that glandular gastric mucosa can produce PAF through the 'de novo' pathway, and that fatty acids, especially unsaturated, can reduce that synthesis by modulating the expression of DTT-insensitive cholinephosphotransferase.

INTRODUCTION

It has recently been shown that the lipid mediator platelet-activating factor (PAF, paf-acether, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) can cause gastric ulcerations when infused intravenously into experimental animals [1]. Hitherto, some studies have been carried out showing the inhibition by PAF antagonists of different types of gastric ulcerations [2], but data are lacking as to the potential of the gastric mucosa to generate the mediator. This pathophysiological approach to understanding the role of PAF in gastric mucosa is encouraged by the recent finding that short-term stimulation of PAF synthesis occurs during the stimulation of exocrine glands, i.e. isolated acini from guinea pig parotid gland and pancreas [3–5], and which suggests a widespread role for this highly active biological lipid in tissues containing exocrine secretory glands such as the gastric mucosa.

Biosynthesis of PAF may occur through two pathways. One has as a limiting step the acetylation of lyso-PAF [6] and the other includes among its final steps the acetylation

of 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate (alkyl-lyso-GP) [7] and the transfer of phosphocholine to 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphate (alkylacetyl-GP) via a 'de novo' pathway involving a dithiothreitol (DTT)-insensitive cholinephosphotransferase [8]. In this study we report: (1) the presence of enzymes of the 'de novo' pathway and the absence of 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine (lyso-PAF):acetyl-CoA acetyltransferase (EC 2.3.1.67) in the glandular mucosa; (2) the presence of significant amounts of PAF in the glandular mucosa; (3) the possible modulation of DTT-insensitive cholinephosphotransferase by low concentrations of fatty acids; (4) the presence of small amounts of other enzymes acting on PAF metabolism.

MATERIALS AND METHODS

Reagents

1-*O*-Hexadecyl-2-acetyl-*sn*-glycerol was from Nova-biochem AG, Laufelfingen, Switzerland. 1-*O*-Hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (synthetic PAF) and 1-*O*-hexadecyl-2-lyso-*sn*-glycero-3-phosphocholine

Abbreviations used: alkylacetyl-G, 1-*O*-alkyl-2-acetyl-*sn*-glycerol; alkyl-lyso-G, 1-*O*-alkyl-2-lyso-*sn*-glycerol; alkylacetyl-GP, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphate; alkyl-lyso-GP, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphate; PAF, platelet-activating factor (1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine); lyso-PAF, 1-*O*-alkyl-2-lyso-*sn*-glycero-3-phosphocholine; DTT, dithiothreitol.

* To whom correspondence should be addressed.

(synthetic lyso-PAF) were from Bachem Feinchemikalien, Bubendorf, Switzerland. Phospholipase D from *Streptomyces chromofuscus* and acetyl-CoA were from Boehringer Mannheim GmbH, F.R.G. Analytical silica gel plates were from Merck, Darmstadt, F.R.G. [^3H]Acetyl-CoA (2 Ci/mmol), CDP-[methyl- ^{14}C]choline (58 Ci/mmol), [^3H]alkylacetyl-glycero-3-phosphocholine (80 Ci/mmol) and [^3H]alkyl-lyso-glycero-3-phosphocholine (120 Ci/mmol) were from The Radiochemical Centre, Amersham, U.K. Oleic acid, 1,2-dioleoyl-*sn*-glycerol, dipalmitoyl-*sn*-3-glycero-3-phosphocholine, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, palmitic acid, indomethacin and nordihydroguaiaretic acid were from Sigma Chemical Co., St. Louis, MO, U.S.A. Arachidonic acid was from Behring Diagnostics, La Jolla, CA, U.S.A. Alkyl-lyso-glycero-3-phosphate was prepared by treating PAF with phospholipase D according to the procedure described by Lee *et al.* [7]. The resulting alkylacetyl-glycero-3-phosphate was subsequently saponified [9] to remove the acetyl group at the *sn*-2 position of the glycerol. Both products were extracted from the reaction mixtures by a modified method of Bligh & Dyer [10], except 2% acetic acid was included in the methanol and 0.1 M-HCl was used as the aqueous phase. Purification of alkyl-lyso-glycero-3-phosphate was achieved by preparative t.l.c. on silica gel plates developed in chloroform/methanol/conc. NH_4OH (65:35:8, by vol.) (system I). To control the efficiency of this procedure for preparing the substrate, some experiments were carried out in parallel using [^3H]PAF as a tracer. This allowed us to calculate the yield of the procedure as about 30% of the initial amount of PAF. The amount of alkyl-lyso-glycero-3-phosphate was quantified by measuring phospholipid-associated P by the procedure of Rouser *et al.* [11].

Isolation of glandular gastric mucosa

Wistar rats of about 200 g were routinely used throughout the study. They were exsanguinated by heart puncture followed by perfusion with cold saline solution, and the stomachs removed, cut open along the greater curvature and flushed with cold saline solution. The mucosa was peeled off and the glandular portion separated from the fundal portion, i.e. the non-secreting part covered with a squamous epithelium, and transferred into a glass Potter tube for mechanical disruption with a motor-driven Teflon pestle. After disruption, the homogenate was mesh filtered to remove crude material and centrifuged at 900 g at 4 °C for 10 min. The supernatant was then sequentially centrifuged at 10000 g, at 27800 g and finally at 105000 g for 1 h at 4 °C. The pellet was resuspended in the medium used for enzyme activity measurements. The medium used for the isolation of the microsomal fraction was 0.1 M-Tris/HCl, pH 7.4, containing 0.3 M-sucrose, 0.5 mg of soybean trypsin inhibitor/ml and 50 μg of leupeptin/ml if lyso-PAF:acetyl-CoA acetyltransferase or DTT-insensitive cholinephosphotransferase was to be assayed. If the purpose of the experiment was to measure alkyl-lyso-GP:acetyl-CoA acetyltransferase, the isolation medium contained 0.25 M-sucrose, 20 mM-Tris/HCl, 10 mM-EDTA, 5 mM-mercaptoethanol, 50 mM-NaF and 25 μM -sodium vanadate, pH 7.4. The amount of protein contained in microsomal fractions was assayed by the procedure of Bradford [12]. Rat spleens were processed in parallel for assay of the same enzyme activities.

Assay of lyso-PAF:acetyl-CoA acetyltransferase (EC 2.3.1.67)

The acetylation reaction [6,13] was carried out in a 0.1 M-Tris/HCl buffer, pH 7.4, containing 20 μM -lyso-PAF, 0.5 μCi of [^3H]acetyl-CoA and 100 μM unlabelled acetyl-CoA, in a total volume of 1 ml. The enzyme reaction was carried out for 10 min at 37 °C and stopped by the addition of 3.7 ml of chloroform/methanol (1:2, v/v) for lipid extraction. The product extracted in the chloroform layer was separated by t.l.c. For this purpose, after lipid extraction, samples were dried under a stream of N_2 , dissolved in 100 μl of chloroform/methanol (9:1, v/v) and applied to silica gel plates, using as developer the mixture propionic acid/propanol/chloroform/water (2:2:1:1, by vol.) (system II). The areas of the plates migrating with a standard of synthetic PAF were scraped off and counted for radioactivity.

Assay of cholinephosphotransferase activities (EC 2.7.8.16 and EC 2.7.8.2)

1-*O*-Alkyl-2-acetyl-*sn*-glycerol (alkylacetyl-G):CDP-choline cholinephosphotransferase (EC 2.7.8.16) was measured according to the procedure described by Renooij & Snyder [8] in a 0.1 M-Tris/HCl buffer, pH 8, containing 0.5 mM-EGTA, 5 mM-DTT, 10 mM- MgCl_2 , 50 μM -1-*O*-hexadecyl-2-acetyl-*sn*-glycerol, 0.5 μCi of CDP-[methyl- ^{14}C]choline and 100 μM unlabelled CDP-choline. The reaction was allowed to proceed for 10 min and the procedures of lipid extraction and t.l.c. were analogous to those described above.

Diacylglycerol:CDP-choline cholinephosphotransferase (EC 2.7.8.2) was assayed in the absence of DTT and using 50 μM -1,2-diolein, instead of 1-*O*-hexadecyl-2-acetyl-*sn*-glycerol as substrate. Samples were processed under the same conditions, except that the area migrating as a standard of phosphatidylcholine was scraped off and transferred to containers for liquid-scintillation spectrometry.

Assay of alkyl-lyso-GP:acetyl-CoA acetyltransferase

The assay was performed in a 0.1 M-Tris/HCl buffer, pH 8.4, containing 50 mM-NaF, 25 μM -sodium vanadate, 16 μM -alkyl-lyso-GP, 3 μCi of [^3H]acetyl-CoA and 600 μM unlabelled acetyl-CoA. The incubation was carried out at 23 °C for up to 5 min [7]. Reactions were stopped by addition of 0.5 ml of 20% trichloroacetic acid followed by centrifugation and lipid extraction as specified above, except that 100 μg of phosphatidylcholine was added as a lipid carrier. Products formed in the incubations were analysed by t.l.c. using as developer system I. In this system the R_f of alkylacetyl-GP is 0.11, whereas alkylacetyl-G migrates near the solvent front ($R_f = 0.94$). Further characterization of the product was performed using as developer chloroform/methanol/acetic acid (96:4:1, by vol.) (system III). In this system alkylacetyl-G migrates with an R_f of 0.43. Distribution of the label by areas was determined by zonal scraping and liquid-scintillation spectrometry.

Assay of lysophospholipase D activity

Since marked differences in the properties of lysophospholipase D have been reported, this enzyme activity was assayed under the conditions described by Wykle & Schremmer [14] in rat tissues as well as under those of Kawasaki & Snyder [15] in rabbit tissues. The

former conditions consisted of 0.1 M-Tris/HCl buffer, pH 7.2, containing 5 mM-MgCl₂, 20 μ M-lyso-PAF and 0.2 μ Ci of [³H]lyso-PAF added in 10 μ l of ethanol. The latter conditions consisted of 0.1 M-Tris/HCl buffer, pH 8.4, supplemented with 5 mM-CaCl₂. All incubations were carried out in a vol. of 1 ml of 37 °C for 10 min, and the reaction was terminated by extracting the lipids using the procedure of Bligh & Dyer [10], with the modification of the presence of 2 % glacial acetic acid in the methanol and the addition of 100 μ g of phosphatidylcholine as carrier phospholipid for t.l.c. The alkyl-lyso-G and alkyl-lyso-GP formed were assayed after separation by t.l.c. using system I as well as the developer hexane/diethyl ether/methanol (85:20:5, by vol.) (system IV).

Phospholipase A₂ activity assay

Phospholipase A₂ was determined as described by Hirohara *et al.* [16] for the gastric enzyme activity. The assay solution consisted of 100 μ M-1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 0.2 μ Ci of 1-palmitoyl-2-[1-¹⁴C]oleoyl-*sn*-glycero-3-phosphocholine and 2 mM-CaCl₂ in a Tris/HCl buffer, pH 8, in a volume of 1 ml. The reaction was incubated at 37 °C for 10 min and was stopped by Bligh & Dyer extraction [10]. The reaction products in the chloroform layer were separated by t.l.c. in the system of solvents propionic acid/propanol/chloroform/water (50:25:8:6, by vol.) (system V) and the radioactivity associated with both neutral lipid and phosphatidylcholine fractions was assayed by liquid-scintillation spectrometry.

PAF acetylhydrolase (EC 3.1.1.47)

The reaction was carried out in a 0.1 mM-Tris/HCl buffer, pH 8, containing 100 μ M-PAF and 0.2 μ Ci of [³H]PAF, in a volume of 1 ml [17]. The reaction was carried out and terminated as described for phospholipase A₂ assay, except that the radioactivity associated with the areas migrating as both PAF and lyso-PAF was quantified.

Extraction, characterization and assay of PAF

PAF was extracted from homogenates of gastric mucosa by the procedure of Bligh & Dyer [10]. The aqueous layer was washed twice with chloroform and all the chloroform extracts pooled and evaporated to dryness under N₂ stream. PAF was bioassayed on [³H]serotonin-labelled rabbit platelets and characterized as described in ref. [18], as well as by both straight- and reverse-phase h.p.l.c. Straight-phase h.p.l.c. was carried out using a dual-pump Kontron Model 420 system, fitted with a Spheri-5 Silica (220 mm $\times$ 4.6 mm, 5 μ m) column from Brownlee Laboratories, Santa Clara, CA, U.S.A. The solvent system used was: 96 % propan-2-ol:hexane (1:1), containing 0.005 % glacial acetic acid/4 % water; the percentage of water was increased linearly to 8 % over a 15-min period [19]. The chromatography proceeded for 60 min at 1 ml/min, and 1 ml fractions were collected, evaporated to dryness and used for bioassay. Isocratic reverse-phase h.p.l.c. was carried out using a Spheri-5 RP 18 (220 mm $\times$ 4.6 mm, 5 μ m) column. The mobile phase was methanol/water/acetonitrile (17:2:1, by vol.) containing 20 mM-choline chloride [20]. PAF was quantified using a standard curve constructed with synthetic PAF (hexadecyl) and expressed as ng equivalents.

RESULTS

Lyso-PAF:acetyl-CoA acetyltransferase activity

Gastric microsomes incubated under the conditions of the assay incorporated the label into a lipid compound with an *R_F* of 0.78 in system II, whereas no incorporation of label was seen in the area migrating as PAF (*R_F* = 0.32). This incorporation of label was also observed in the absence of lyso-PAF, but was not found when microsomes were boiled for 2 min prior to the enzyme assay. When the lipid extract was chromatographed in system III, the label was found in the area migrating with an *R_F* analogous to that of alkylacetyl-G, i.e. 0.43. These findings suggest that either lyso-PAF:acetyl-CoA acetyltransferase is absent from glandular stomach preparations or it is strongly inhibited by an as yet unknown substance. To test the hypothesis of a possible inhibitor, the effect of gastric microsomes on lyso-PAF:acetyl-CoA acetyltransferase activity of rat spleens, a known source of this enzyme activity, was tested. Addition of gastric mucosa microsomes to spleen microsomes at different ratios (up to 3 mg of protein from gastric mucosa microsomes per mg of protein from spleen microsomes) did not modify lyso-PAF:acetyl-CoA acetyltransferase, and this was seen as an argument against the existence of an inhibitor. However, a caution should be mentioned before ruling out a possible inhibitor, since the effect of a lipophilic compound needing inter-membrane transfer has not been fully discarded.

Alkyl-lyso-GP:acetyl-CoA acetyltransferase activity

This enzyme activity was assayed in spleen and gastric microsomes. Gastric microsomes contained about one-tenth of the activity assayed in the spleen. Interestingly, in the absence of NaF and sodium vanadate all the label migrated as alkylacetyl-G, whereas in the presence of both inhibitors of phosphohydrolase about a half of the label migrated as alkylacetyl-GP. This is strong evidence for the existence of the enzyme which cleaves the phosphate moiety from alkyl-lyso-GP and provides the substrate for the cholinephosphotransferase. Alkyl-lyso-GP:acetyl-CoA acetyltransferase activity was also detected in the absence of exogenous alkyl-lyso-GP which suggests that endogenous substrate was present. Kinetic analysis of the enzyme activity showed an apparent *K_m* for acetyl-CoA of 454 μ M, a value slightly higher than that found in spleen microsomes (277 μ M), which is in keeping with the value of 226 μ M reported by Lee *et al.* in spleen microsomes [7]. The *V_{max}* was 0.1 nmol/min $\cdot$ mg of protein for the gastric enzyme and 1 nmol/min $\cdot$ mg of protein for the splenic enzyme (Fig. 1).

Alkylacetyl-G:CDP-choline cholinephosphotransferase activity

The mean *V_{max}* for this enzyme from gastric microsomes was 3.12 $\pm$ 0.05 nmol/min $\cdot$ mg of protein when the assay medium contained 50 μ M-alkylacetyl-G (*n* = 5) (Fig. 2). In the absence of this co-substrate, negligible incorporation of the label into PAF was observed. The apparent *K_m* value for CDP-choline was 67 μ M. In parallel experiments using spleen microsomes from the same animals, the specific activity of DTT-insensitive cholinephosphotransferase was 31 $\pm$ 3 nmol/min $\cdot$ mg of protein. To assess a possible mechanism of

Fig. 1. Double-reciprocal plot for alkyl-lyso-GP:acetyl-CoA acetyltransferase activity

Microsomes from gastric mucosa (a) and spleen (b) containing 50 μg of protein were incubated with 16 μM -alkyl-lyso-GP and different concentrations of acetyl-CoA as described in the Materials and methods section. Each point represents the average of triplicate samples.

modulation of this enzyme activity, microsomes were incubated with different concentrations of fatty acids (arachidonic, oleic and palmitic), 1,2-diolein or phosphatidylcholine for 10 min prior to the addition of the substrates. Arachidonic and oleic acids inhibited the formation of [^3H]PAF in a dose-dependent manner (results not shown), whereas palmitic acid was less active. Half-maximal effective doses are given in Table 1. The effect of arachidonic acid on the formation of PAF with time was studied at two different concentrations of arachidonic acid and is shown in Fig. 3. To determine whether the action of arachidonic acid could be traced to its metabolic transformation into another product, experiments were performed which included a period of incubation with indomethacin, an inhibitor of cyclo-oxygenase, and with nordihydroguaiaretic acid, a known inhibitor of both cyclo-oxygenase and lipoxygenase, prior to the addition of arachidonate. Under these conditions, the effect of arachidonic acid was still observed, with a percentage of variation of less than 5% in three independent experiments. Phosphatidylcholine (dipalmitoyl) caused a small increase in DTT-insensitive cholinephosphotransferase activity at 10 μM and a 16% reduction at 100 μM (Table 2). 1,2-Diolein caused a modest 7% increase in enzyme activity at all concentrations tested. Some experiments were carried

Fig. 2. Double-reciprocal plot for alkylacetyl-G:CDP-choline cholinephosphotransferase activity

Microsomes from gastric mucosa containing 50 μg of protein were incubated with 50 μM -alkylacetyl-G and different concentrations of CDP-choline for 10 min in the presence (○) and absence (●) of 50 μM -arachidonic acid. Enzyme activity was assayed as described in the Materials and methods section. Each point represents the average of triplicate samples.

Table 1. Inhibition of DTT-insensitive cholinephosphotransferase by fatty acids

Microsomal fractions from glandular gastric mucosa and spleen containing 50 μg of protein were incubated for 10 min in the presence of fatty acid (1–100) μM prior to the addition of the substrates. DTT-insensitive cholinephosphotransferase was assayed as described in the Materials and methods. IC_{50} indicates the concentration of fatty acid producing a 50% inhibition of the enzyme activity measured in the absence of fatty acid. Results represent mean values of two experiments in duplicate. N.D., not done.

Fatty acid	IC_{50} (μM)	
	Spleen	Stomach
Palmitic	78	59
Oleic	30	35
Arachidonic	25	18
Linoleic	22	N.D.

out in the presence of 0.13 M-KCl, a concentration of salt which reduces the concentration of lipids required for the formation of micelles, and which reduced enzyme activity by 35%, although fatty acids still showed the same degree of inhibitory action (results not shown). These findings, together with the action of oleic acid and the reduced effect of palmitic acid, surely indicate an inhibitory effect by fatty acids by themselves rather than an action linked to metabolic conversion. Double-

Fig. 3. Kinetics of alkylacetyl-G:CDP-choline cholinephosphotransferase activity

Microsomes from gastric mucosa were incubated with 50 μ M-alkylacetyl-G and 100 μ M-CDP-choline, in the absence (○) and presence of 50 μ M- (◐) and 100 μ M- (●) arachidonic acid. At the times indicated aliquots were taken and the amount of [3 H]PAF formed assessed by scintillation spectrometry after t.l.c.

Table 2. Effect of phosphatidylcholine and diolein on stomach DTT-insensitive cholinephosphotransferase (EC 2.7.8.16)

Microsomal fractions from glandular gastric mucosa containing 50 μ g of protein per sample were incubated with the indicated concentrations of phosphatidylcholine (dipalmitoyl) or 1,2-diolein for 10 min prior to the addition of the reaction substrates. DTT-insensitive cholinephosphotransferase was assayed as indicated in the Materials and methods section. Results are mean values of two experiments with duplicate samples.

Addition	Specific activity (nmol/min · mg of protein)
None	1.2
Phosphatidylcholine (10 μ M)	1.4
Phosphatidylcholine (50 μ M)	1.1
Phosphatidylcholine (100 μ M)	1.0
1,2-Diolein (10 μ M)	1.2
1,2-Diolein (50 μ M)	1.2
1,2-Diolein (100 μ M)	1.2

reciprocal representation of the effect of arachidonic acid on the formation of PAF through DTT-insensitive cholinephosphotransferase decreased the $V_{\max}$ from 3.38 to 0.51 nmol/min · mg of protein in the presence of 50 μ M-arachidonic acid (Fig. 2). To ascertain whether the effect of arachidonic acid was selective for DTT-insensitive cholinephosphotransferase (EC 2.7.8.16), arachidonic acid was also tested in the assay of diacylglycerol:CDP-choline cholinephosphotransferase (EC 2.7.8.2). Again a dose-dependent inhibition of activity was observed, which indicates that the effect

Table 3. Effect of arachidonic acid on diacylglycerol:CDP-choline cholinephosphotransferase (EC 2.7.8.2)

Microsomes from glandular gastric mucosa were preincubated with arachidonic acid prior to triggering the cholinephosphotransferase reaction. The reaction was carried out in the presence and absence of exogenous 1,2-diolein as a co-substrate. Results represent means $\pm$ s.d. of three experiments.

Addition	Specific activity (nmol/min · mg of protein)
Boiled microsomes + 1,2-diolein	0
1,2-Diolein	4.0 $\pm$ 0.5
1,2-Diolein + arachidonic acid (50 μ M)	2.3 $\pm$ 0.2
1,2-Diolein + arachidonic acid (100 μ M)	1.6 $\pm$ 0.1
No exogenous 1,2-diolein	3.9 $\pm$ 0.1

Table 4. Lysophospholipase D activity in glandular gastric mucosa microsomes

Gastric microsomes were incubated with lyso-PAF in a Tris/HCl-buffered medium (0.1 M, pH 7.2) in the presence of different additions. At the end of the incubation period, lipids were extracted and separated by t.l.c. Results represent means $\pm$ s.d. of four experiments in duplicate.

Addition	Specific activity (pmol/min · mg of protein)
None	43 $\pm$ 13
Mg $^{2+}$ (5 mM)	76 $\pm$ 24
Ca $^{2+}$ (5 mM)	33 $\pm$ 17
EDTA (2 mM)	5 $\pm$ 0.9
EDTA (2 mM) + Ca $^{2+}$ (5 mM)	26 $\pm$ 7
EDTA (2 mM) + Mg $^{2+}$ (5 mM)	63 $\pm$ 18

found was not restricted to the enzyme activity involved in PAF formation (Table 3).

Lysophospholipase D, phospholipase A₂ and PAF acetylhydrolase activities

Initial findings of the lack of lyso-PAF:acetyl-CoA acetyltransferase activity prompted a detailed analysis of the mechanism for interference with the assay by competition with either substrates or reaction products, and the above mentioned enzyme activities were assayed. Lysophospholipase D activity with specificity for ether lipids was measured in gastric microsomes (Table 4). This activity was not observed when 1-palmitoyl-2-lyso-*sn*-glycero-3-phosphocholine was used as substrate (results not shown) and required Mg $^{2+}$ ions for optimal expression. These findings, together with the lack of effect of Ca $^{2+}$ ions and the interval for optimal pH (no activity could be assayed at pH 8.4), indicate that the lysophospholipase D activity assayed in gastric microsomes is analogous to the enzyme reported by Wykle & Schremmer [14] in rat tissues, rather than to the enzyme reported by Kawasaki & Snyder [15] in rabbit

Fig. 4. H.p.l.c. behaviour of PAF extracted from gastric mucosa homogenates

An aliquot of a homogenate of two specimens of glandular gastric mucosa containing 1 mg of protein (105 mg wet wt.) was extracted according to the procedure of Bligh & Dyer [10], divided into two, evaporated to dryness and resuspended in propan-2-ol/hexane (1:1, v/v) for straight-phase h.p.l.c. (a) or in methanol for reverse-phase h.p.l.c. (b). PAF was bioassayed in the different fractions and expressed in ng equivalents as explained in the Materials and methods section. The arrows indicate the retention times for a [^3H]PAF (hexadecyl) standard.

renal medulla. Nevertheless, the small activity detected makes it unlikely that this enzyme by metabolizing lyso-PAF and therefore competing with lyso-PAF:acetyl-CoA acetyltransferase could be responsible for the inability to assay this enzyme activity in gastric microsomes. Again, since the possibility exists of an inhibitory effect on lyso-PAF:acetyl-CoA acetyltransferase linked to lipid products [21], 1-*O*-hexadecylglycerol (final product of lysophospholipase D on microsomes in the absence of phosphohydrolase inhibitors) was added to spleen microsomes at a concentration of 1–100 μM prior to the assay of lyso-PAF:acetyl-CoA acetyltransferase. Under these conditions no effect on PAF formation was observed. Phospholipase A_2 activity was also detected in gastric microsomes at a specific activity of 586 ± 30 nmol/min $\cdot$ mg of protein ($n = 3$ experiments with duplicate samples). This activity was only detected in the presence of Ca^{2+} ions and was found when 1-palmitoyl-2-oleoyl-*sn*-glycero-phosphocholine was used as substrate. By contrast, when PAF was used as substrate, a low and Ca^{2+} -independent degradation was observed (0.618 ± 0.3 in the presence of Ca^{2+} ions vs. 0.627 ± 0.2 nmol/min $\cdot$ mg of protein in the absence of Ca^{2+} ions, $n = 3$ experiments in duplicate). These results indicate that

PAF degradation in the gastric mucosa is carried out by PAF-acetylhydrolase rather than by phospholipase A_2 .

Presence of PAF in glandular gastric mucosa homogenates

PAF extracted from 10 different specimens of glandular-gastric-mucosa homogenates was assayed and found at a concentration of 2.7 ± 0.7 ng equivalents of PAF (hexadecyl)/mg of protein. Analysis of gastric PAF by straight-phase h.p.l.c. showed a retention time of 34 min (Fig. 4a). Reverse-phase h.p.l.c., in a system usually used to unmask differences in molecular species of alkyl chains, showed that most of the PAF extracted from gastric mucosa showed a retention time analogous to that of a [^3H]hexadecyl standard (Fig. 4b).

DISCUSSION

This study shows the presence of enzymes of the 'de novo' pathway for the synthesis of PAF in gastric mucosa microsomes and the absence of lyso-PAF:acetyl-CoA acetyltransferase activity. These results together with the presence of PAF in the gastric mucosa indicate the involvement of the 'de novo' pathway in the assembly of PAF in this tissue. This study, together with the report by Woodard *et al.* [22] on the inner-renal medulla, and a recent report on the generation of PAF by human polymorphonuclears in response to phorbol esters [23], provide the first evidence of the involvement of this pathway in the assembly of PAF in animal tissues. Most cells so far studied appear preferentially to utilize the lyso-PAF:acetyl-CoA acetyltransferase pathway. Our inability to demonstrate the lyso-PAF:acetyl-CoA acetyltransferase pathway in gastric mucosa together with a number of control experiments lead to the conclusion that the enzyme activity is absent from this preparation. Our results are not consistent with the possible existence of an inhibitor of lyso-PAF:acetyl-CoA acetyltransferase in gastric mucosa microsomes. A possible interference with lyso-PAF:acetyl-CoA acetyltransferase due to the cleavage of lyso-PAF by a lysophospholipase D has been addressed by assaying this enzyme activity. Although we found measurable amounts of the enzyme described by Wykle & Schremmer [14], its specific activity was below the level reported by these authors in pulmonary tissue, i.e. the tissue with the least specific activity, and accordingly possible interference with the lyso-PAF:acetyl-CoA acetyltransferase assay is not related to lyso-PAF cleavage. Similar arguments rule out interference from phospholipase A_2 and PAF acetylhydrolase activities. The former enzyme seems not to act on PAF, and if the latter was the interfering factor, its action should also be seen in the DTT-insensitive cholinephosphotransferase assay, which it was not. All these results support our statement that gastric microsomes lack lyso-PAF:acetyl-CoA acetyltransferase but contain alkyl-lyso-GP:acetyl-CoA acetyltransferase. A possible contamination of glandular gastric mucosa microsomes by blood cells is ruled out by the absence of lyso-PAF:acetyl-CoA acetyltransferase, the enzyme primarily involved in PAF biosynthesis in leucocytes [6,13].

Opinions on the regulation of DTT-insensitive cholinephosphotransferase usually stress the role of co-substrates and modulators rather than changes in

enzyme activity linked to covalent modification [22]. As shown in this study, modulation of DTT-insensitive cholinephosphotransferase is possibly related to the presence of fatty acids, especially unsaturated ones. Arachidonic acid seems to be the most potent inhibitor, at least in gastric microsomes (IC_{50} 18 μ M vs. 35 μ M for oleic acid, Table 1). This finding prompted us to carry out further experiments using this fatty acid, even though other fatty acids could play a more relevant role in DTT-insensitive cholinephosphotransferase modulation under physiological conditions since they are much more abundant than arachidonate. For example, Bills *et al.* [24] have detected much higher concentrations of free palmitic, stearic, oleic and linoleic acid than arachidonic acid in human platelets. Furthermore, free arachidonic acid was undetectable in the absence of thrombin stimulation, whereas the reverse was true of the other fatty acids. The presence of a free carboxyl group seems to be a prerequisite for this effect, since 1,2-diolein and 1,2-dipalmitoyl-glycero-phosphocholine showed little or even a small enhancing effect. From the values for critical micelle concentration of fatty acids in the literature, it seems unlikely that the effect observed could be due to micelle formation [25], and even the modification of experimental conditions to favour the formation of micelles (i.e. a high salt concentration) did not modify the effect of fatty acids, although it did reduce the enzyme activity by 35%. Our findings are reminiscent of reports showing inhibition of prostaglandin biosynthesis in various tissues [26–29]. Furthermore, Franson *et al.* [30] and Ballou & Cheung [31] demonstrated inhibition of phospholipase A_2 by unsaturated fatty acids at concentrations somewhat higher than those shown in the present paper. Taverna & Hanahan [32] found that the liberation of < 1% of total membrane fatty acids by phospholipase A_2 treatment results in a marked stimulation of (Mg^{2+} , Ca^{2+})-ATPase activity in human erythrocyte membranes. More recently, Murakami *et al.* [33] reported the activation of protein kinase C by *cis*-fatty acids in the absence of Ca^{2+} ions and phospholipids at a concentration similar to that tested in the DTT-insensitive cholinephosphotransferase assay. Billah *et al.* [34] reported the modulation of the biosynthesis of PAF in human polymorphonuclear leucocytes by lipoxygenase products, which was related to an enhancement of phospholipase A_2 activity. Furthermore, Leach & Blumberg [35] reported the activation of protein kinase C by μ M concentrations of lipoxin A. Our results with inhibitors of both pathways of arachidonate metabolism and the effect displayed by other fatty acids militate against a specific role for eicosanoids. The effect of fatty acids was also observed on the cholinephosphotransferase acting on diacylglycerol (EC 2.7.8.2), and seems to be independent of the source of enzyme, since it has also been observed in spleen microsomes.

In summary, our results show: the formation of PAF by gastric microsomes through DTT-insensitive cholinephosphotransferase; the absence of lyso-PAF:acetyl-CoA acetyltransferase in gastric mucosa microsomes; and the possible action of fatty acids, especially unsaturated, on the formation of PAF by modulating the expression of DTT-insensitive cholinephosphotransferase. Since the effect of fatty acids is observed at concentrations likely to occur in tissues, this finding could explain why PAF is not continuously secreted at high concentrations from animal tissues containing high

amounts of DTT-insensitive cholinephosphotransferase activity.

S.F.-G. and M.A.G. are fellows of the Conchita Rabago Foundation. M.C.G. is a fellow of Plan Nacional de Investigacion. E.C. has been the recipient of a grant for visiting scientists from Fondo de Investigaciones Sanitarias. This study has been supported by grants from Fondo de Investigaciones Sanitarias, CAICYT and Fundacion Alvarez de Toledo.

REFERENCES

1. Rosam, A. C., Wallace, J. L. & Whittle, B. J. R. (1986) *Nature* (London) **319**, 54–56
2. Wallace, J. H. & Whittle, B. J. R. (1986) *Eur. J. Pharmacol.* **124**, 209–210
3. Söling, H. D., Elb, H. & Fest, W. (1984) *Eur. J. Biochem.* **144**, 65–72
4. Söling, H. D. & Fest, W. (1986) *J. Biol. Chem.* **261**, 13916–13922
5. Domenech, C., Machado de Domenech, E. & Söling, H. D. (1987) *J. Biol. Chem.* **262**, 5671–5676
6. Wykle, R. L., Malone, B. & Snyder, F. (1980) *J. Biol. Chem.* **255**, 10256–10260
7. Lee, T.-C., Malone, B. & Snyder, F. (1986) *J. Biol. Chem.* **261**, 5373–5377
8. Renooij, W. & Snyder, F. (1981) *Biochim. Biophys. Acta* **663**, 545–556
9. Dawson, R. M. C. (1960) *Biochem. J.* **75**, 45–53
10. Bligh, E. C. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917
11. Rouser, G., Siakotos, A. N. & Fleischer, S. (1966) *Lipids* **1**, 85–86
12. Bradford, M. (1976) *Anal. Biochem.* **72**, 248–254
13. Alonso, F., Garcia Gil, M., Sanchez Crespo, M. & Mato, J. M. (1982) *J. Biol. Chem.* **257**, 3376–3378
14. Wykle, R. L. & Schremmer, J. M. (1974) *J. Biol. Chem.* **249**, 1742–1746
15. Kawasaki, T. & Snyder, F. (1987) *Biochim. Biophys. Acta* **920**, 85–93
16. Hirohara, J., Sugatani, J., Okumura, T., Sameshima, Y. & Saito, K. (1987) *Biochim. Biophys. Acta* **919**, 231–238
17. Blank, M. L., Lee, T.-C., Fitzgerald, V. & Snyder, F. (1981) *J. Biol. Chem.* **256**, 175–178
18. Caramelo, C., Fernandez-Gallardo, S., Marin, Cao, D., Iñarrea, P., Santo, J. C., Lopez Novoa, J. M. & Sanchez Crespo, M. (1984) *Biochem. Biophys. Res. Commun.* **120**, 789–796
19. Blank, M. L. & Snyder, F. (1983) *J. Chromatogr.* **273**, 415–420
20. Jackson, E. M., Mott, G. E., Hoppens, C., McManus, L. M., Weintraub, S. T., Ludwig, J. C. & Pinckard, R. N. (1984) *J. Lipid Res.* **25**, 753–757
21. Miwa, M., Hill, C., Kumar, R., Sugatani, J., Olson, M. L. & Hanahan, D. J. (1987) *J. Biol. Chem.* **262**, 527–530
22. Woodard, D. S., Lee, T.-C. & Snyder, F. (1987) *J. Biol. Chem.* **262**, 2520–2527
23. Nieto, M. L., Velasco, S. & Sanchez Crespo, M. (1988) *J. Biol. Chem.* **263**, 2217–2222
24. Bills, T. K., Smith, J. B. & Silver, M. J. (1977) *J. Clin. Invest.* **60**, 1–6
25. Tanford, C. (1980) *The Hydrophobic Effect: Formation of Micelles and Biological Membranes*, John Wiley and Sons, New York
26. Shen, T. Y. (1979) in *Handbook of Experimental Pharmacology* (Van, J. R. & Ferreira, S. H., eds.), vol. 50, pp. 305–347, Springer, Berlin
27. Flower, R. J. & Vane, J. R. (1974) *Biochem. Pharmacol.* **23**, 1439–1450

28. Pace-Ascak, C. & Wolfe, L. S. (1968) *Biochim. Biophys. Acta* **152**, 784–787
29. Ahern, D. G. & Downing, D. T. (1970) *Biochim. Biophys. Acta* **210**, 456–461
30. Franson, R., Patriarca, P. & Elsbach, P. (1974) *J. Lipid Res.* **15**, 380–388
31. Ballou, L. R. & Cheung, W. Y. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 5203–5207
32. Taverna, R. D. & Hanahan, D. J. (1980) *Biochem. Biophys. Res. Commun.* **94**, 652–659
33. Murakami, K., Chan, S. Y. & Routtenberg, A. (1986) *J. Biol. Chem.* **261**, 15424–15429
34. Billah, M. M., Bryant, R. W. & Siegel, M. I. (1985) *J. Biol. Chem.* **260**, 6899–6906
35. Leach, K. L. & Blumberg, P. M. (1985) *Cancer Res.* **45**, 1958–1963

Received 22 February; accepted 11 May 1988