Metabolic fate of platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) and lyso-PAF (1-O-alkyl-2-lyso-sn-glycero-3-phosphocholine) in FRTL5 cells

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Abstract The metabolism of platelet-activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) and lyso-PAF (1-O-alkyl-2-lyso-sn-glycero-3-phosphocholine) was investigated in FRTL5 cells, a normal rat thyroid cell line. FRTL5 cells incorporated [³H]PAF and deacetylated this compound to the corresponding [³H]lyso-PAF which was not accumulated or secreted but converted mainly to alkyl-acyl-phosphocholine indicating that this acylation process was particularly active in these cells. Among metabolic products of both [³H]PAF and [³H]lyso-PAF were alkylglycerol as well as its mono- and diacyl derivatives. [³H]alkylglycerol could be the intermediate compound for the production of [³H]alkyl- and [³H]alkenyl-phosphoethanolamine (plasmalogen) which were also metabolic products. FRTL5 cells were able to convert lyso-PAF to PAF especially when they were stimulated by ionophore A23187 in the presence of [³H]lyso-PAF and phenylmethylsulfonyl fluoride. The amount of PAF increased for the first 30 min and declined thereafter. PAF resting levels were found low in the same cells. Furthermore, PAF-acetylhydrolase activity was determined in cell homogenates. The presence of metabolic products such as alkyl-phosphatidylcholine, alkyl- and alkenyl-phosphatidylethanolamine and alkyl-glycerol, as well as, its mono- and diacyl derivatives, indicates that FRTL5 cells and probably other thyroid cells, are very active in metabolizing PAF and lyso-PAF and suggests the co-operation of the corresponding metabolic pathways in these cells.—Botitsi, E., M. Mavri-Vavayanni, and A. Siafaka-Kapadai. **Metabolic fate of platelet activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) and lyso-PAF (1-O-alkyl-2-lyso-sn-glycero-3-phosphocholine) in FRTL5 cells.** *J. Lipid Res.* **1998.** 39: **1295–1304.**

Supplementary key words PAF metabolism • PAF biosynthesis • PAF-acetylhydrolase • plasmalogens • alkylglycerol • FRTL5 cells • thyroid

Platelet activating factor (PAF, 1-O-alkyl-2-acetyl-sn-glycero-3-phosphocholine) is a family of naturally occurring and structurally related acetylated phospholipid mediators. PAF serves as an extracellular mediator in the process of inflammation, has antihypertensive activity, increases vascular permeability and generally has been implicated in several pathological and physiological processes involving various tissues and cell types (1–6). The involvement of PAF in the endocrine function is also under investigation. PAF decreases the release of two hypothalamic releasing hormones, LHRH and SRIF, in rat hypothalamus (7), acts on the rat anterior pituitary to stimulate prolactin release (8), and increases the release of prolactin, by the GH4C1-rat pituitary cells (9). In addition, PAF has been reported to be involved in a number of processes associated with reproductive biology (10, 11). PAF evoked a moderate but significant inhibition of cAMP accumulation, promoted by acute stimulation by TSH on porcine thyroid cells, and affected phospholipid metabolism and protein iodination of these cells (12). It has been reported that PAF significantly augments GEJ cell numbers (human thyroid hybrid cells) and the number of binding sites for [¹²⁵I]-labeled TSH while it induces high affinity TSH-binding sites on these cells (13).

It is well established that PAF is produced by a variety of cells including platelets, neutrophilic polymorphonuclear leukocytes, mast cells, lymphocytes, and macrophages. PAF is synthesized by a relatively simple process of phospholipid deacylation–reacetylation, i.e., by the remodelling pathway (14) or by the de novo pathway, which consists of acetylation, dephosphorylation, and phosphocholine transfer steps of the 1-O-alkyl-2-lyso-sn-glycero-3-phosphate, an important intermediate of the ether-linked phospholipids. The specific enzymes involved in this pathway have not yet been purified (2, 3). Removal of the acetyl moiety

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at the sn-2 position of the glycerol backbone of PAF produces biologically inactive lyso-PAF, this reaction being catalyzed by a Ca^{2+}-independent PAF-acetylhydrolase (PAF-AH), which has been shown to be present in both extracellular (mammalian blood or plasma) (15–18) and intracellular form (blood cells, other tissues) (15, 19, 20). Additionally, investigations conducted on PAF and lyso-PAF metabolism revealed the presence of phosphatidylethanolamine mainly in the form of plasmalogens (21, 22) and neutral lipids (22, 23) among their metabolic products.

Although effects of PAF on various thyroid cells functions have been reported, PAF production and metabolism by thyroid cells have not been investigated. In previous studies, performed in our laboratory, we found that PAF levels were altered in thyroid diseases. In the present study the metabolism of PAF and lyso-PAF, as well as PAF production, was investigated in FRTL5 cells. FRTL5 cells are a cloned normal rat thyroid cell line, shown to maintain the functional characteristics of iodide uptake and thyroglobulin synthesis, widely applicable in studies of thyroid endocrine physiology and biochemistry (24, 25). The results of these studies revealed that both PAF and lyso-PAF are readily metabolized by FRTL5 cells, producing structurally diverse metabolites. The ability of these thyroid cells to incorporate and metabolize this autacoid family of phosphoglycerides may be involved in the maintenance of thyroid homeostasis.

MATERIALS AND METHODS

Materials

1-O-[1′,2′-³H]hexadecyl-2-acetyl-sn-glycero-3-phosphocholine ([³H] alkyl-PAF, 60 Ci/mmol), 1-O-[1′,2′-³H]hexadecyl-2-lyso-sn-glycero-3-phosphocholine ([³H]lyso-PAF, 36 Ci/mmol), and 1-O-hexadecyl-2[³H]acetyl-sn-glycero-3-phosphocholine ([³H]acetyl-PAF, 7.10 Ci/mmol) were purchased from New England Nuclear, (Boston, MA). The purity of [³H]alkyl-PAF was greater than 85% as determined by TLC ([³H]lyso-PAF was also present) while the purity of [³H]lyso-PAF was more than 93% based on TLC analysis. All other chemicals, as phospholipid standards including phosphatidylcholine (PC, egg yolk), lysophosphatidylcholine (lyso-PC, egg yolk), sphingomyelin (SPH, bovine brain), L-α-phosphatidylethanolamine (PE, ethanolamine phospholipids containing 60% plasmalogen, bovine brain), phosphatidylethanolamine (PE, egg yolk), l-α-lysophosphatidylethanolamine (lyso-PE, ethanolamine lysophospholipids containing 60% plasmalogen, bovine brain), lyso-phosphatidylethanolamine (lyso-PE, bovine liver) and neutral lipid standards were obtained from Sigma (St. Louis, MO). Coon's modified Ham's F12 medium and hormones (TSH, insulin, hydrocortisone, transferrin, somatostatin, glycyl-l-histidyl-l-lysine-acetate) were also obtained from Sigma (St. Louis, MO).

Cell culture

The FRTL5 cell line, (ATCC CRL 8305) obtained from ECACC used in our studies, is a clone of normal rat thyroid cells (24, 25). Cells were grown in Coon's modified Ham's F12 medium supplemented with 5% newborn calf serum (GIBCO) and a mixture of six components (hormones and growth factors) consisting of insulin (10 ng/ml), hydrocortisone (10^{-8} m), trans-

ferrin (5 μg/ml), somatostatin (10 ng/ml), glycyl-l-histidyl-l-lysine acetate (10 ng/ml), and TSH (thyroid stimulating hormone) (10 mU/ml), referred as 6H-medium. Cells were grown in an atmosphere of 5% CO_2, 95% air at 37°C. Fresh medium was supplied every third day. Cells were passaged once a week as previously described (25). Cell viability was tested by the trypan blue exclusion.

Metabolism of [³H]alkyl-PAF in intact FRTL5 cells

Cells, placed onto 25-cm² flasks, maintained in the 6H-medium and grown to near 85–90% confluence, were used in our experiments. Cell viability was over 90% as tested by the trypan blue exclusion. The maintaining media were carefully removed from the cultured cells which were then rinsed twice with 5 ml of phosphate-buffered saline (PBS without Ca^{2+}, Mg^{2+}) and once with 4 ml of serum and hormone-free Coon's modified Ham's-F12 medium containing 0.1% BSA (bovine serum albumin, fraction V). One ml of the serum and hormone-free Coon's modified Ham's-F12 medium containing 0.1% BSA was added in each flask and cells were incubated for 20 min in an atmosphere of 5% CO_2, 95% air at 37°C. At the end of this preincubation period, 1-O-[³H]alkyl-PAF, dissolved in 0.25% BSA/PBS, was added in each flask (0.07 μCi/ml, 1 nm final concentration) and cells were incubated for the desired periods of time under the conditions mentioned above. The metabolic reactions were terminated by removing the media from each flask for subsequent lipid analysis and by adding methanol to the cells which were then scraped with a rubber policeman. Lipids from cells and medium were extracted and further analyzed.

Metabolism of [³H]lyso-PAF in intact FRTL5 cells

In order to study the metabolism of 1-O-[³H]-alkyl-lyso-PAF, a similar procedure was followed except that 1-O-[³H]-alkyl-lyso-PAF (0.18 μCi/ml, 5 nm final concentration) and acetylCoA, 100 μm final concentration, were added in each flask. Additionally, in one group of experiments, the metabolism of lyso-PAF was studied in the presence of 1 mm PMSF in the incubation medium.

Stimulation of FRTL5 cells by A23187

Cells were washed twice with PBS and once with serum and hormone-free culture medium containing 0.1% BSA. One ml of this medium, containing [³H]alkyl-lyso-PAF (0.18 μCi/ml, 5 nm final concentration) and acetylCoA, 100 μm final concentration, was added in each flask and incubation was carried out for 40 min in an environment of 5% CO_2, 95% air at 37°C. Cells were then stimulated with A23187 in a final concentration of 10 μm in DMSO or DMSO alone as control. In one group of experiments, stimulation was performed in the presence of 1 mm PMSF. After various incubation time intervals, the metabolic reactions were terminated as described above and lipids from either cells or media were extracted and further analyzed.

Lipid analysis

Lipids from medium and cells were extracted separately according to the Bligh-Dyer method (26). Lipids, including PAF, were present in the chloroform-rich phase of each sample which was collected and evaporated under nitrogen. The residues were dissolved in chloroform–methanol 2:1, (v/v) and small amounts of phospholipid standards (such as lysophosphatidylcholine, sphingomyelin, phosphatidylcholine) were added to each lipid extract as internal standards. All samples were subjected to thin layer chromatography (TLC) on heat-activated 0.250-mm silica gel plates (Silica Gel 60 without indicator, Merck, Darmstadt) using a solvent mixture of chloroform–methanol–water 65:35:7 (v/v) as the developing system I. Spots of separated lipids were visualized by exposure to iodine vapors. In order to perform chemical

tests on certain lipid classes, the desired areas were scraped from the plate and the lipids were extracted by the Bligh-Dyer method. The areas of the chromatographically separated lipids that were not subjected to further analysis were scraped and placed into scintillation vials after the addition of 5 ml of scintillation fluid. Radioactivity was measured in a liquid scintillation counter (Wallac 1209, Rackbeta).

Base treatment

Lipids, from cells incubated with 1-O-[³H]alkyl-PAF and/or 1-O-[³H]alkyl-lyso-PAF, for the time periods of 1 and/or 3 h, were extracted and analyzed by TLC as described above. Lipid fractions corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), and neutral lipids (NL) were extracted from silica gel and the extracts were subjected to base treatment with methanolic KOH (0.6 N), in a water bath at 60°C, for 1 h. After neutralization with 6 N HCl, lipids were isolated according to the Bligh-Dyer method. The hydrolysis products derived from PC and PE fractions were separated by TLC using solvent system I and lyso-PC, sphingomyelin (SPH) phosphatidylcholine (PC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (lyso-PE), and oleic acid as internal standards. Products derived from the hydrolysis of NL, were analyzed by TLC using as solvent system, petroleum ether–diethylether–acetic acid 70:30:1 (v/v) system II or hexane–diethylether–methanol–acetic acid 80:25:4:2, (v/v) system III with internal standards oleic acid, fatty alcohol, chimyl alcohol, fatty aldehyde, tri- and diglycerides.

Cleavage of plasmalogens

In order to cleave the 1′,2′-alkenyl bond in the *sn*-1 position of plasmalogens, one half of the TLC PC + LPE and PE fractions (solvent system I) were treated directly on a TLC plate with 10 μl of 6 N HCl added to each lane containing the sample (21). The remaining fractions were co-chromatographed on the same plate without the addition of the acid. The plate was then dried under nitrogen for 30 min. TLC with the solvent system hexane–diethyl ether–methanol–acetic acid 80:25:4:2 (v/v), system III, was used to separate the released aldehyde from phospholipids and other neutral lipids. A standard PE-plasmalogen (ethanolamine phospholipids containing 60% plasmalogen) was treated with acid on TLC plate and co-chromatographed with the samples.

PAF-acetylhydrolase activity in FRTL5 cell homogenates

PAF-acetylhydrolase (PAF-AH) activity was assayed according to the method of Pinckard and Ludwig (27) and Trapali, Mavri-Vavayanni, and Siafaka-Kapadai (28). Cells collected by trypsinization and washed twice with PBS were resuspended in PBS containing soybean trypsin inhibitor (STI 0.1 mg/ml) and homogenized by using an ultrasonic processor (vibra cell). Protein in cell homogenates was estimated by the method of Lowry et al. (29). [³H]acetyl-PAF (0.06 μCi) and cold PAF were suspended in PBS/BSA 0.1% to achieve a concentration of 20 μM. The reaction mixture contained equal volumes of cell sonicate and PAF, radioactive solution. Usually, the mixture was incubated for 10 min at 37°C in a shaking water bath. The reaction was terminated by the addition of ice-cold trichloroacetic acid (TCA 20% w/v) and tubes were placed on ice for 20 min. After centrifugation the [³H]acetate released from [³H]acetyl-PAF was determined by liquid scintillation spectrometry of the supernatant solution.

PAF biogical assay

The biological activities of PAF fractions from FRTL5 cells were assayed by measuring their ability to aggregate washed rabbit platelets. Fractions from cell extracts were first separated by TLC using solvent system I. The phospholipid fractions were isolated from the TLC plate and subsequently purified by HPLC, us-

ing a cation-exchange column 5 cm × 4.6 mm i.d. (Partisil 10 SCX Whatman) eluting with a mixture of acetonitrile–methanol–water 60:30:7 (v/v) at a flow rate of 1.5 ml/min with UV detection at 206 nm (30, 31). Washed rabbit platelets were prepared essentially as previously described (28, 31, 32) and resuspended in Ca^{2+}-free Tyrode–gelatin buffer, pH 6.5 (1.25×10^9 plts/ml). PAF activity was tested by the addition of samples, dissolved either in ethanol or in 0.25% BSA, to 500 μl of platelet suspension (2.5×10^8 plts/ml) in Tyrode–gelatin buffer (pH 7.2) containing 1.3 mm Ca^{2+}, with constant stirring at 37°C. Aggregation was recorded as the change in light transmission monitored by an aggregometer (Chronolog). The estimation of PAF content in the samples was accomplished from standard curves, obtained by assaying standard PAF solutions at final concentrations ranging from 5 to 0.5×10^{-10} m.

Chemical acetylation of lyso-PE

Lyso-PE standard (2–3 mg) containing 60% plasmalogen and an equal amount of a lyso-PE standard (which contained both alkyl- and acyl-lyso-PE analogues) were dissolved in 0.2 ml of chloroform and acetylated with the addition of acetic anhydride in the presence of perchloric acid (33). Samples were placed on ice and the acetylated phospholipids were extracted by the Bligh-Dyer method. Lipids were chromatographed on a TLC plate with the solvent system I, in the presence of appropriate standards as PC, PE, SPH, lyso-PC, and lyso-PE. Lipids were located on the TLC plate by exposure to iodine vapors.

Statistical analysis

Results are expressed as the mean ± SEM of n experiments and statistically significant differences were evaluated by *t*-tests, where *P* values less than 0.05 were considered significant. GraphPad PRISM software (GraphPad Software Inc.) was used for graphics and all statistical analyses performed.

RESULTS

Uptake and metabolism of [³H]-alkyl-PAF

1-O-[³H]alkyl-PAF was taken up very quickly by FRTL5 cells, as shown in **Fig. 1a**. After 5 min of incubation, 25% of the added radioactivity was found associated with the cells. The uptake tended to reach a plateau slowly, i.e., 40% within an hour and 55% after 3 h of incubation.

Subsequently, the metabolism of the incorporated [³H]alkyl-PAF in the FRTL5 cells was studied. Metabolic products formed by the cells as a function of time are illustrated in Fig. 1b. Phosphatidylcholine was the main metabolic product, as 17% of the added radioactivity was converted into PC at the first 15 minutes. After 3 h the PC fraction comprised 47% of the total incorporated radioactivity. Lyso-PAF derived from PAF was not accumulated; obviously it was rapidly acylated mainly to PC. Surprisingly, we found on TLC that a relatively high percentage of radioactivity was incorporated in a fraction, with R_f corresponding to authentic PE, and the course of incorporation was time-dependent (2.5% after 5 min of incubation, 8% in 1 h, 12% after 3 h). Finally, a considerably high amount of radioactivity was incorporated into the TLC fraction corresponding to neutral lipids (NL). The incorporation reached a plateau (25% within 15 min of incubation, 27% in 30 min, and 23% after 3 h). We have also extracted lipids from the corresponding media and the
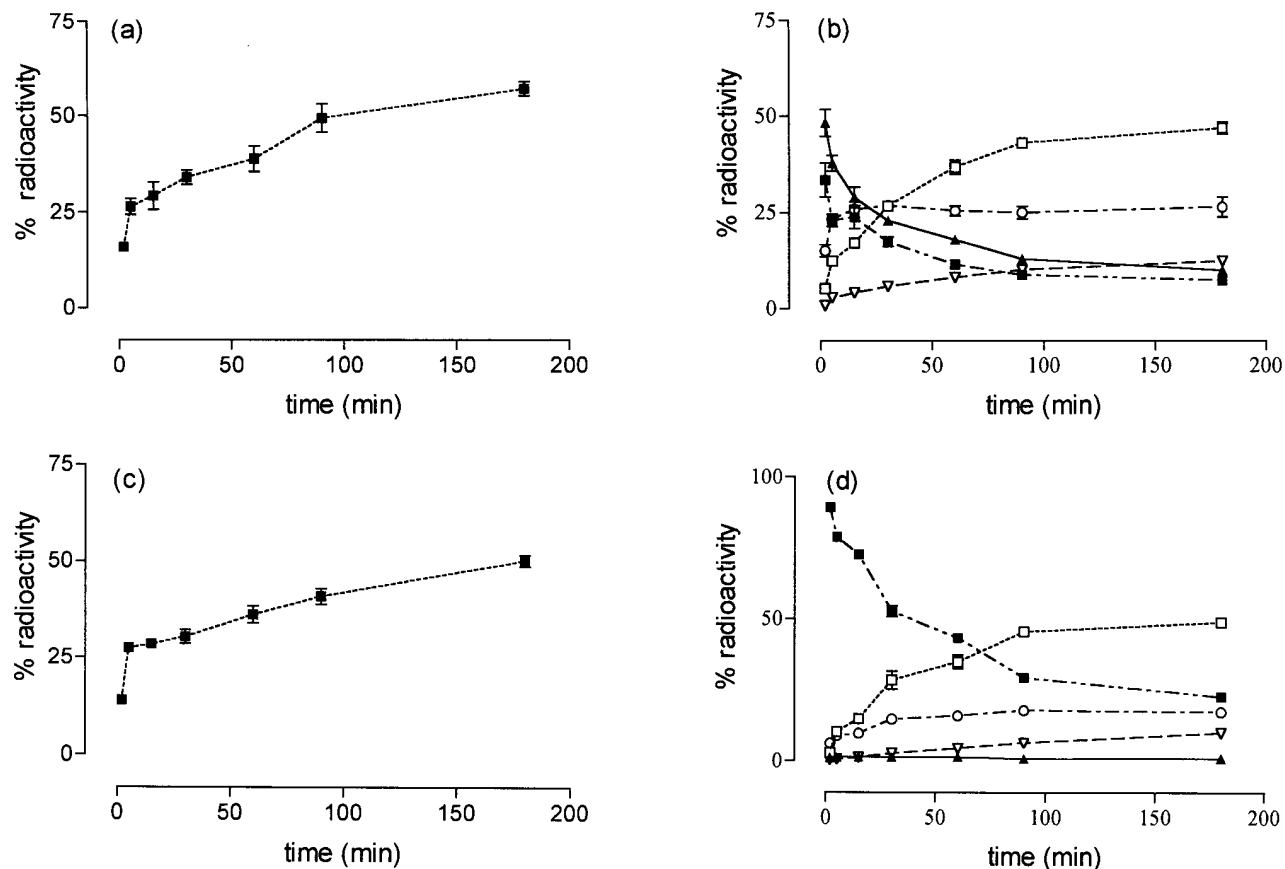
Fig. 1. Uptake and metabolism of [³H]alkyl-PAF and 1-O-[³H]alkyl-2-lyso-PAF by FRTL5 cells. Confluent cultures of FRTL5 cells were incubated with 0.07 μCi of 1-O-[³H]alkyl-2-acetyl-*sn*-glycero-3-phosphocholine, (Figs. 1a, 1b) or with 0.18 μCi of 1-O-[³H]alkyl-2-lyso-*sn*-glycero-3-phosphocholine, (Figs. 1c, 1d). At the times indicated, cells were collected and radioactivity was determined in both cells and media (Figs. 1a, 1c). The [³H]-labeled metabolites (Figs. 1b, 1d) in cellular fraction were extracted and analyzed on TLC plates with solvent system I. Lyso-PAF (■), PAF (▲), PC (□), PE (▽), and NL (○) fractions were identified with appropriate standards and spots were scraped off the gel and counted for radioactivity in a scintillation mixture. Details under methods. Data represent mean ± SEM from four independent experiments, (Fig. 1a, 1b) or from two independent experiments (Fig. 1c, 1d). Where standard error bars are not visible they are smaller than the corresponding symbol.

distribution of radioactivity into the same lipid classes was analyzed by TLC and we found that the labeled metabolites remained almost unchanged up to 3 h, i.e., radioactivity incorporated to PC and PE fractions was less than 4% and 1%, respectively, while a small amount of radioactivity, near 5%, was present in the NL fraction (data not shown). Based on these data, we hypothesized that PAF metabolites were derived from lyso-PAF which is known to be the first intermediate product of PAF. Thus we proceeded with the investigation of [³H]lyso-PAF uptake and metabolism by FRTL5 cells.

Uptake and metabolism of 1-O-[³H]alkyl-lyso-PAF

The time course of incorporation of [³H]lyso-PAF in FRTL5 cells is shown in Fig. 1c. After 15 min of incubation, almost 30% of the radioactivity added was incorporated and then reached a plateau (50% after 3 h of incubation). As shown in Fig. 1d, 1-O-[³H]alkyl-lyso-PAF was rapidly metabolized (from 90% in 2 min to 55% in 30 min and 22% after 3 h of incubation). The main metabolic product was again PC as approximately 50% of the radioactivity incorporated in cells was found in the PC fraction

after 1 h of incubation. [³H]PAF was produced in minute amounts ranging from 1 to 3% of the cell radioactivity, while total neutral lipids (NL fraction) was near 8% in 5 min and increased, in a time-dependent manner, up to 15% in 30 min and then reached a plateau (16%, 18%, 17% in 60 min, 90 min, and 180 min, respectively). [³H]PE was also found in metabolic products and the incorporated radioactivity was also increased in a time-dependent manner, from 0.6% in 5 min to 3.5% in 30 min to 10% after 3 h of incubation and did not reach a plateau. These results in combination with the results of [³H]alkyl-PAF metabolism in FRTL5 cells suggest that PAF was converted to PC, PE, and NL through lyso-PAF.

Base treatment of [³H]-PAF metabolites

In order to get more information about the structure of [³H]-labeled metabolites derived from [³H]alkyl- PAF or [³H]alkyl-lyso-PAF (after 1 and/or 3 h of incubation), the total lipid extract was separated to known lipid classes by TLC with solvent system I. Fractions corresponding to PC + LPE, PE, and NL were isolated from the silica gel and half of each fraction was subjected to base treatment while the

remaining half was used as control. Because LPE co-chromatographs in the PC area, as shown by the TLC of appropriate standards, the PC fraction will subsequently be referred to as the PC + LPE fraction. Hydrolyzed products of the initially isolated fractions of PC + LPE and PE and their controls were further analyzed by TLC with solvent system I, while the respective fractions of the NL sample were separated by TLC with solvent systems II and/or III.

Results obtained before and after base treatment of PC + LPE fraction are shown in **Fig. 2a**. When the isolated PC + LPE fraction was rechromatographed, the majority of the radioactivity (~70%) was found in the fraction corresponding to PC + LPE while the rest was dispersed to other areas on the TLC plate (probably due to further purification of the PC fraction with the second TLC). After base treatment, $32 \pm 4\%$ remained in the same TLC area, suggesting the presence of $[^3H]$alkyl- and/or alkenyl-lyso-PE between PAF metabolites, while $42.3 \pm 2.9\%$ migrated to the fraction corresponding to lyso-PC, suggesting the presence of $[^3H]$alkyl-acyl-PC converted to $[^3H]$alkyl-lyso-PC. It should be mentioned that $[^3H]$lyso-PE could also be derived from PAF-PE which, if present, can be found in the original PC + LPE fraction as shown by the TLC analysis of acetylated PE standards. In order to determine the R_f of PAF-PE we synthesized it by acetylation of alkyl/alkenyl-lyso-PE, and we confirmed that the R_f was very similar to lyso-PE and PC in the solvent system I (data not shown). Finally, about $13.3 \pm 2.7\%$ of radioactivity was found in the NL fraction, suggesting the presence of a radioactive acyl-group in the original sample.

Results obtained before and after base treatment of the PE fraction are illustrated in Fig. 2b. The majority of radioactivity, almost 80%, was located in the fraction corresponding to PE before base treatment as expected; after treatment, only $7.05 \pm 2.90\%$ remained in this area instead of $65.6 \pm 2.3\%$, which migrated to the fraction corresponding to PC + LPE suggesting the presence of $[^3H]$alkyl/alkenyl-acyl-PE hydrolyzed to $[^3H]$alkyl/alkenyl-lyso-PE. Additionally, $17.0 \pm 1.0\%$ of the radioactivity after base treatment was transferred to the NL fraction, indicating that labeled-acyl-PE was associated to the initially isolated PE fraction.

Finally, results obtained before and after base treatment of the NL fraction are depicted in Fig. 2c. Interestingly, 25% of radioactivity was located in the fraction corresponding to free alkyl- and/or alkenyl-glycerol which after base treatment increased to 70%, while an amount of 50% present in the fraction corresponding to FAME + TG decreased to 20%. These results indicate that metabolites, originally characterized as NL, consist mainly of free and esterified neutral glycerylethers. The presence of 20% of the radioactivity in the FAME fraction after base treatment suggests that these methylesters could have been derived from $[^3H]$-labeled fatty acid free and/or mono-, di-, and triglycerides esterified with labeled acyl-moieties apparently produced after the cleavage of the ether bond. The coexistence of a labeled fatty aldehyde in this TLC area cannot be excluded.

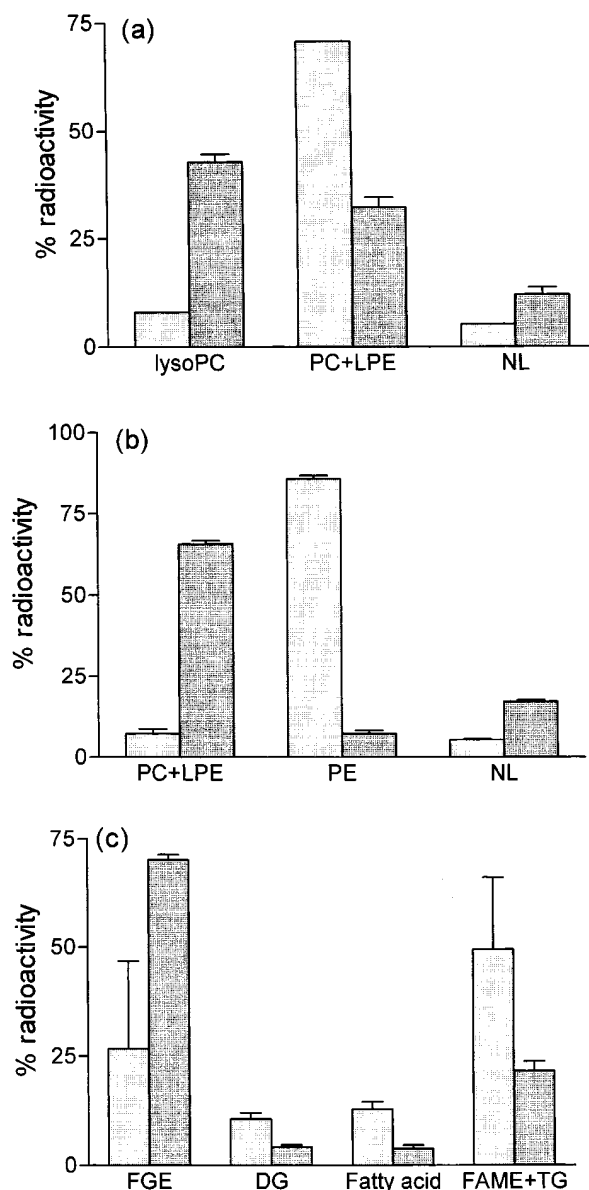The distribution of the radioactivity incorporated in the

Fig. 2. Base treatment of $[^3H]$alkyl-PAF metabolites. Labeled lipids, derived from cells incubated with $[^3H]$alkyl-PAF, were separated by TLC with solvent system I. Aliquots of the identified fractions were subjected to base treatment. Hydrolysis products and the respective untreated samples were resolved simultaneously on TLC with system I, for the PC + LPE and PE fractions and with system II and/or III for the NL fraction. (a) PC + LPE fraction, (b) PE fraction and (c) NL fraction, before (light bars) and after (dark bars) base treatment. Error bars within the limits of the symbols are not shown.

NL fraction as a function of the incubation time has also been investigated. In one representative experiment, cells incubated with $[^3H]$alkyl-lyso-PAF for the time periods of 1 and 3 h were extracted and analyzed in order to isolate the respective NL fractions. Aliquots of these samples were subjected to base treatment while the remaining amounts were used as reference samples, and both were resolved by TLC. An amount of 20% of the radioactivity, incorporated in the 1-h sample, was located with free GE while 42.5% migrated to the region of FAME + TG. The

respective TLC fractions when the 3 h sample was resolved showed 7% of the radioactivity in the GE area and 66% in the FAME + TG area, while after base treatment the GE fraction was increased to 73% and the FAME + TG area was decreased to 17%. The latter product was found to consist predominantly of the 1-O-[3H]alkyl-2,3-acyl-glycerol as saponification yielded 1-O-[3H]alkyl-glycerol. As the amount of free GE was diminished from 1 h to 3 h of incubation and the esterified GE was at the same time increased, we concluded that free GE (1-O-[3H]alkyl-glycerol) was converted to 1-O-[3H]alkyl-2,3-acyl-glycerol.

Plasmalogen content of etherglycerophospholipids and neutral lipid fractions

The contribution of plasmalogens content to the total radioactivity incorporated in the PC + LPE, PE and neutral glycerylether fractions was evaluated by chromatographing aliquots of these samples on TLC plates, using solvent system III, after treating them with HCl in order to cleave the alkenyl-bonds. Untreated controls were run simultaneously on the same TLC plate.

Results obtained are shown in **Fig. 3a** and **3b**. When the

[3H] PC + LPE fraction, obtained after incubation of FRTL5 with [3H]alkyl-lyso-PAF, was treated with acid, 34% of the radioactivity migrated to the chromatographic region corresponding to aldehydes. Based on our data, we conclude that the radioactivity incorporated in this glycerophospholipid fraction was present in the forms of 1-alkyl and 1-alkenyl derivatives at $55.5 \pm 3.9\%$ and $44.5 \pm 3.9\%$, respectively, as may be seen in Fig. 3a. A substantial amount of the radioactivity associated with the PE fraction, approximately 50%, was transferred to long chain fatty aldehydes, after treatment with acid. As seen in Fig. 3b, the [3H]PE fraction was [3H]alkenyl-linked at $62.5 \pm 4.2\%$ and [3H]alkyl-linked at $37.5 \pm 4.2\%$, respectively. The data obtained from this investigation are consistent with the concept that PAF and lyso-PAF can be metabolized in FRTL5 cells by a pathway that will lead to the production of PE and LPE/PC containing a high proportion of plasmalogens.

The extracted material corresponding to neutral alkyl/alkenyl-GE, obtained after TLC with solvent system I to separate the NL fraction and its subsequent analysis on TLC with solvent system III to isolate the different neutral lipid classes, was exposed to 6 N HCl for 30 min and subsequently analyzed by TLC on the neutral lipid system III. All the label, nearly 90%, remained at the origin and no evidence for the formation of radioactive aldehyde was found. It was concluded, therefore, that the GE fraction was devoid of plasmalogens in FRTL5 cells incubated with [3H]alkyl-lyso-PAF.

Stimulation of FRTL5 cells by ionophore A23187

The aim of this group of experiments was to test the ability of FRTL5 cells to synthesize PAF by incubating them with precursors such as [3H]alkyl-lyso-PAF and ace-
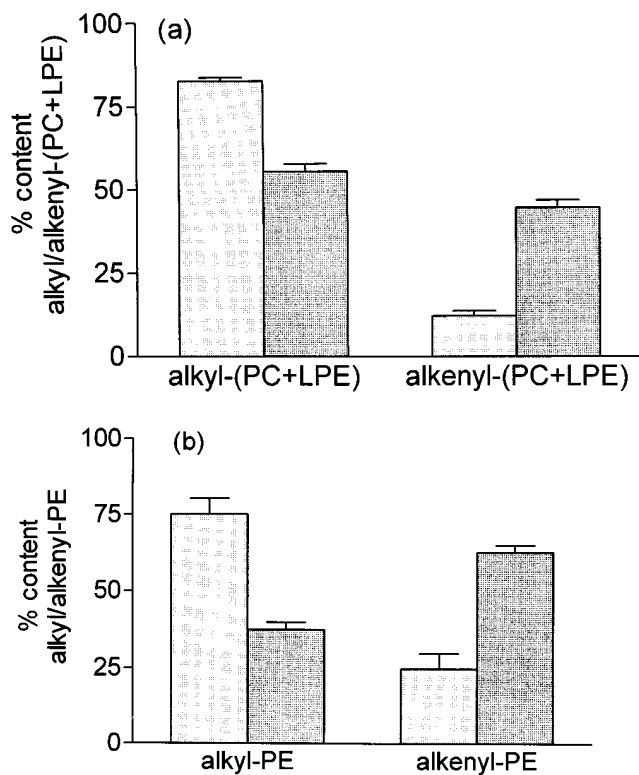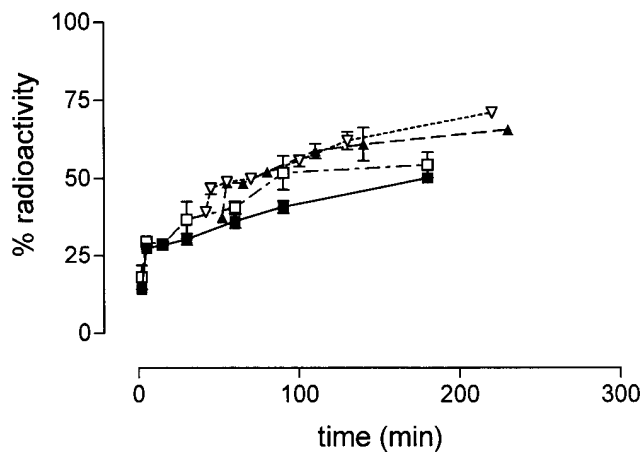

Fig. 3. Plasmalogen content of [3H]alkyl-lyso-PAF metabolites. Labeled lipids, derived from cells incubated with [3H]alkyl-lyso-PAF, were separated by TLC with solvent system I. Aliquots of the PC + LPE fraction and of the PE fraction and PE identified fractions were subjected to acid treatment, as described under Methods and the remaining samples were used as reference, as they were both analyzed on TLC with system III. Results, based on the distribution of the incorporated radioactivity before and after acid treatment, show (a) the percentage of plasmalogen associated within PC + LPE fraction and (b) the percentage of plasmalogen associated within PE fraction, before (light bars) and after (dark bars) cleavage of plasmalogens. Error bars within the limits of the symbols are not shown.

Fig. 4. Uptake of 1-O-[3H]alkyl-2-lyso-PAF by stimulated and non-stimulated FRTL5 cells. Confluent cultures of FRTL5 cells were incubated with 0.18 μCi of 1-O-[3H]alkyl-2-lyso-sn-glycero-3-phosphocholine and 100 μm acetylCoA in the presence or the absence of 1 mm PMSF. Stimulation of cells was performed with the addition of 10 μm A23187. At the times indicated, cells were collected and radioactivity was determined in both cells and media. Data represent mean ± SEM from two or three independent experiments. Experiments were performed in the presence of A23187 and PMSF (▽), A23187 (▲), PMSF (□), and no addition (■). Differences were considered significant when P was less than 0.05.

tylCoA and stimulating by A23187. [3H]alkyl-lyso-PAF metabolism was studied as follows. *a*) FRTL5 cells were incubated with [3H]alkyl-lyso-PAF and acetylCoA in the absence and in the presence of PMSF for the times indicated. *b*) FRTL5 cells were pre-incubated with [3H]alkyl-lyso-PAF and acetylCoA in the absence and in the presence of PMSF for 40 min and then stimulated by A23187 for the designated times. Results concerning the rate of the incorporation of the radioactivity from [3H]alkyl-lyso-PAF under the conditions described are shown in **Fig. 4**. We may conclude that the stimulation of cells by A23187 increased the rate of radioactivity uptake as compared to non-stimulated cells, both in the presence or the absence of PMSF ($P < 0.05$, *t*-tests).

The distribution of the incorporated radioactivity, for the times indicated, for each lipid class tested under the four different experimental conditions described above is depicted in **Fig. 5**. As it is evaluated in Fig. 5a, the rate of [3H]PAF production was differentiated between stimulated and non-stimulated cells in the presence of PMSF ($P = 0.04$, *t*-test), and in non-stimulated cells in the absence and in the presence of PMSF ($P = 0.003$, *t*-test), but

the overall estimation derived is that PAF is synthesized in very small amounts even after stimulation in the presence of PMSF. More specifically, the level of PAF synthesized from cells treated with precursors (i.e., [3H]alkyl-lyso-PAF and acetylCoA) was calculated based on the specific radioactivity, 20 fmol/flask at 30 min of incubation, which increased to 60 fmol/flask, when PMSF was added to the incubation medium. Additionally, cells treated with precursors and stimulated by A23187 in the absence of PMSF gave a PAF amount equal to 30 fmol/flask at 30 min of stimulation, which increased to 104 fmol/flask when the experiment was performed in the presence of PMSF.

The rate of [3H]PC production is shown in Fig. 5b. Differences statistically significant were observed in the next cases: *a*) FRTL5 cells stimulated by A23187, in the absence and in the presence of PMSF ($P = 0.04$, *t*-test); *b*) FRTL5 cells stimulated and non-stimulated in the presence of PMSF ($P = 0.04$, *t*-test). *c*) FRTl5 cells non-stimulated in the absence and in the presence of PMSF ($P = 0.003$, *t*-test). Results concerning the rate of [3H]PE production are shown in Fig. 5c, where statistically significant differences were as follows: *a*) FRTL5 cells stimulated by A23187
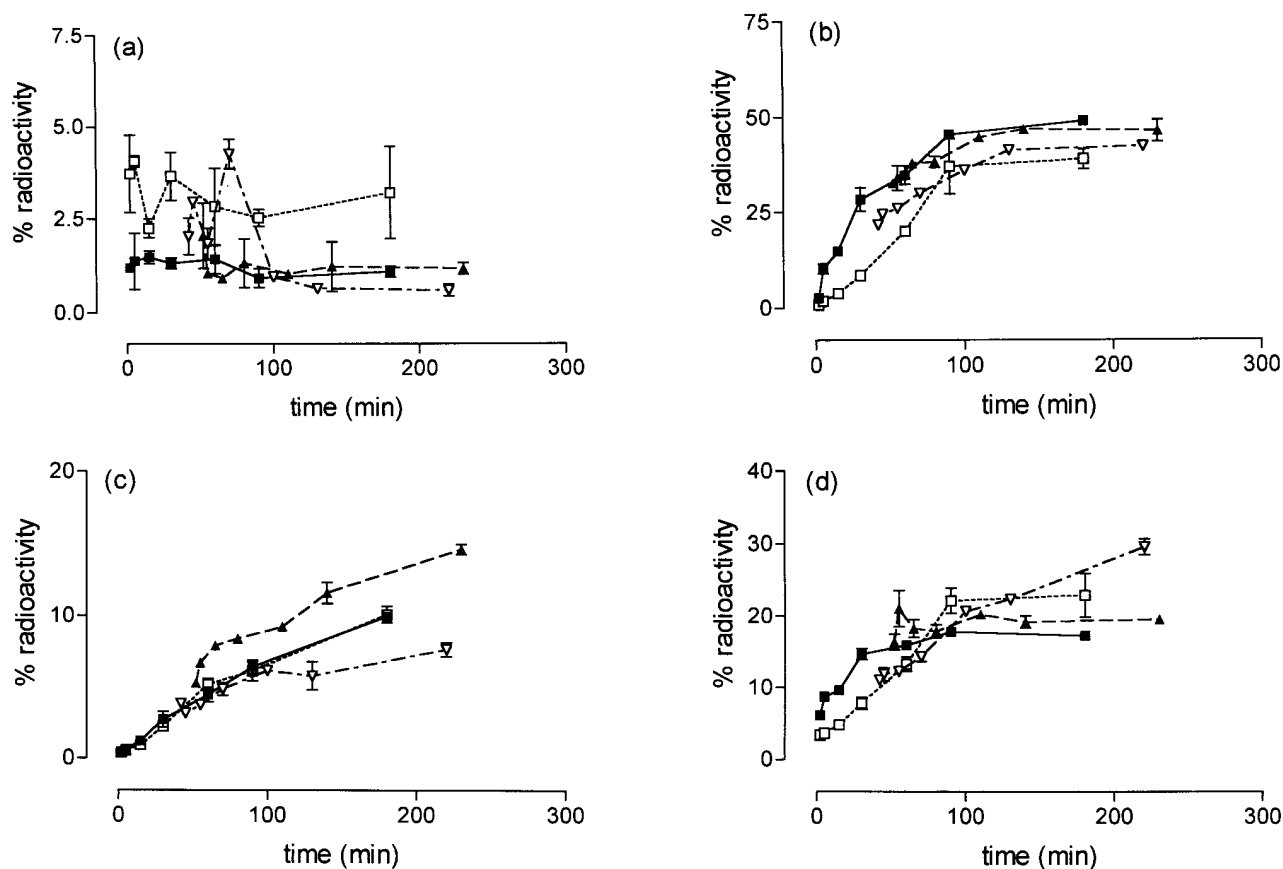

Fig. 5. Effect of stimulation on [3H]alkyl-lyso-PAF metabolism in the presence or the absence of PMSF. Confluent cultures of FRTL5 cells were incubated with 0.18 μCi of 1-O-[3H]alkyl-2-lyso-*sn*-glycero-3-phosphocholine, 100 μm acetylCoA in the presence or the absence of 1 mm PMSF. Stimulation of cells was performed with the addition of 10 μm A23187. At the times indicated, cells were collected and lipids were extracted for subsequent separation on TLC. (a), (b), (c), (d) represent the % percentage of the radioactivity incorporated into PAF, PC, PE, and NL, respectively. Experiments were performed in the presence of A23187 and PMSF (▽), A23187 (▲), PMSF (□) and no addition (■). Data represent mean ± SEM from three independent experiments, in each case. Differences were considered significant when *P* was less than 0.05.

versus non-stimulated in the absence of PMSF ($P = 0.01$, t-test); b) FRTL5 cells stimulated by A23187 in the absence and in the presence of PMSF ($P = 0.01$, t-test). The rate of [^{3}H]NL production was also studied, Fig. 5d, where no statistically significant difference was observed.

PAF-acetylhydrolase (PAF-AH) activity in FRTL5 cells

Results reported above indicate that PAF is rapidly metabolized by FRTL5 cells obviously through its conversion to lyso-PAF, with which they probably share the same metabolic pathways as their metabolic products are similar. As PAF levels even after stimulation remain low, we assumed that PAF-acetylhydrolase (PAF-AH) activity must be present in FRTL5 cells. When we assayed FRTL5 cell homogenates for PAF-AH, we found a PAF-AH-like enzymatic activity with a specific activity reaching the level of 0.8–1.0 nmol/mg protein per min and an apparent K$_m$ of 24 μm. The presence of PC (0.4 mm, final concentration in the reaction mixture) and CaCl$_2$ (2 mm) did not alter the enzyme's activity while PMSF, (2 mm) caused an almost complete inhibition (results shown in **Table 1**).

PAF Resting Levels

Our data indicate that resting levels of PAF in FRTL5 cells should be very low, taking into consideration that the concentration calculated on the basis of incorporated radioactivity in the presence of precursors and A23187 was very low: 104 fmol/flask or 104 fmol/2 × 10^7 cells was the maximum level obtained after 30 min of stimulation with A23187 in the presence of PMSF (Fig. 5a). In order to further confirm our suggestion, we determined PAF levels in non-stimulated FRTL5 cells by fractionating lipids from cells in 30 flasks (25 cm^2), each containing approximately 8 × 10^8 confluent cells, by TLC using solvent system I. The fractions corresponding to LPC, PC, and PAF were further purified by HPLC and assayed for their ability to induce platelet aggregation of washed rabbit platelets. In this way, the amount of PAF purified by HPLC was estimated to be 1.5 fmol/flask or 1.5 fmol/2 × 10^7 cells (mean value from two independent experiments). This explains why our previous attempts to determine PAF levels from confluent cells collected from 8–10 flasks (25 cm^2) were unsuccessful as the amount of PAF isolated was below the detection limit of biological assay.

DISCUSSION

The results of the current study document that [^{3}H]PAF and [^{3}H]lyso-PAF were readily incorporated and rapidly metabolized by FRTL5 cells, as has already been reviewed for a number of cells and tissues (1–3). The rapid but transient effect of PAF on the inhibition of cAMP accumulation under TSH action in porcine thyroid cells suggests the degradation of PAF in thyroid cells (12). Specific binding of PAF on FRTL5 cells was not found under our experimental conditions due probably to the intense PAF metabolism (E. Botitsi, M. Mavri-Vavayanni, A. Siafaka-Kapadai, unpublished data).

Our findings demonstrate that most of the intracellular label derived from alkyl-labeled PAF was associated with a compound that migrated into the area of the phosphatidylcholine fraction (Fig. 1b) apparently through the conversion of PAF to lyso-PAF by the action of a PAF-AH. No intracellular accumulation or secretion of lyso-PAF was detected as it was probably metabolized by the action of a CoA-independent transacylase (34–36) and converted mainly to alkyl-acyl-GPC. As has been well established, PC is the main metabolic product of PAF in a variety of cells and tissues (23, 37). Identical results were obtained when alkyl-labeled lyso-PAF was incorporated to FRTL5 cells (Fig. 1d) where lyso-PAF was converted mainly to alkyl-acyl-GPC. Interestingly, [^{3}H]PE was detected among the metabolic products of both PAF and lyso-PAF and its concentration was found to gradually increase in a 3 h period (Fig. 1b, 1d). It has recently been reported that a CoA-independent transacetylase (38) can use PAF as the acetate donor and a variety of lyso-phospholipids including radyl-lyso-glycerophosphoethanolamine as the acetate acceptor molecule. As the PAF and lyso-PAF used were labeled at the 1-O-alkyl moiety, we were not able to determine whether this pathway was applicable to the case of FRTL5 cells. Our data also indicate the presence of alkyl-glycerol as well as its mono- and diacyl derivatives among PAF and lyso-PAF metabolic products. Thus, the presence of radioactivity at the *sn*-1 position of PE and PE-plasmalogen (Fig. 3b) in combination with the existence of [^{3}H]alkyl-glycerol between metabolites apparently show that [^{3}H] PAF was hydrolyzed to [^{3}H]lyso-PAF which was subsequently cleaved to [^{3}H]alkyl-glycerol by a reaction sequence involving a lyso-phospholipase D and a phosphohydrolase, enzymes common in several cell types (23, 39–41). It has been demonstrated that alkyl-glycerol is an important intermediate of PAF metabolism in isolated rat intestinal epithelial cells (23, 36, 37).

Additionally, the generation of labeled PC, PE and alkyl-glycerol, as well as its esterified analogues, can be attributed to the following pathway: [^{3}H]alkyl-PAF and/or [^{3}H]alkyl-lyso-PAF are converted to alkyl-acyl-GPC via a coenzyme-A-independent transacylation mechanism, which in turn is

TABLE 1. Influence of various agents on
PAF-AH activity of cell homogenate

Testing Agent	Final Conc. in Reaction Mixture	Enzyme Activity Compared to Respective Control
	mM	% *decrease*
CaCl$_2$	2	no effect
PC	0.4	27 ± 6
PMSF	2	100 ± 12

Cell homogenates were preincubated at 37°C for 15 min in the presence of the appropriate amount of CaCl$_2$, phosphatidylcholine (PC), or phenylmethylsulfonyl fluoride (PMSF). An equal amount of radiolabeled PAF solution (20 μm) was added and the mixture was incubated for 10 min at 37°C. Inhibition was calculated as the decrease in the enzyme activity compared to the respective controls which consisted of water (CaCl$_2$), ethanol (PC), and DMSO (PMSF). Data represent mean ± SEM of five separate determinations.

metabolized to its ethanolamine counterpart by removal of the head group by phospholipase C and formation of alkyl-glycerol and alkyl-acyl-glycerol. The transfer of ethanolamine phosphate would be mediated by a CDP- ethanolamine phosphotransferase, and desaturation between carbons $1'$ and $2'$ of the alkyl side chain in the *sn*-1 position would give rise to the final product, PE-plasmalogen. This pathway has been already proposed for the WISH cells (21) and may apply to the case of FRTL5 cells, as labeled-PE was enriched in plasmalogens (Fig. 3b) and alkyl-glycerol was detected among PAF metabolites. Another metabolic pathway that might be involved, based on our reported findings, could be the following: alkyl-acyl-GPC can generate alkyl-acyl-glycerol by the reverse reaction of choline phosphotransferase which, in equilibrium with PC, may be converted to alkyl-2,3-acyl glycerols (product identified in our case) or to alkyl/alkenyl-PE depending upon the availability of fatty acids or CDP-ethanolamine, as already suggested for MDCK cells (22). A recently identified Mg^{2+}-dependent lysophospholipase C in MDCK cell line (42), hydrolyzed either 1-alkyl or 1-alkenyl lysophosphoglycerides with the ultimate formation of alkyl- or alkenyl-glycerols, indicating a metabolic pathway applicable in FRTL5 cells but in contrast to our finding that the fraction of alkylglycerol isolated was devoid of plasmalogen.

The role of PE-plasmalogens in FRTL5 cells is not known, but the conversion of alkyl-acyl-GPC into an ethanolamine-containing glycerophospholipid would remove the ether lipid from the pool of intermediates that could produce biologically active PAF. Moreover, the activity of plasmalogenase would provide a mechanism by which the ether bond could be metabolized as in most tissues the pteridine-requiring reaction for the direct oxidation of the alkyl bond has a relatively low activity (21, 43). The labeled acyl moieties detected after base treatment of PC and PE fractions, as well as the presence of labeled fatty acids and aldehydes in the NL fraction isolated by the FRTL5 cells, may also be attributed to the action of a mono-alkyl-oxygenase responsible for the cleavage of the ether bond (44, 45).

Based on our current data, the relatively low resting levels of PAF substances in FRTL5 cells can be partially attributed to the significant PAF-AH activity present in cell homogenates. Interestingly, PAF-AH activity was found both in cytosolic and microsomal fractions from FRTL5 cells (E. Botitsi, A. Siafaka-Kapadai, M. Mavri-Vavayanni, unpublished observations) comparable to the enzyme activity of cytosolic fractions from isolated rat intestinal cells, INT 407 cells, WISH cells (23), and of whole homogenates from other rat tissues (18, 28). FRTL5 cells incubated with the appropriate precursors showed an increased rate of PAF production, further enhanced in the presence of PMSF (a PAF-AH inhibitor, Table 1) and stimulation by ionophore A23187, (Fig. 5). Another interesting aspect of PMSF action on lyso-PAF metabolism by FRTL5 cells is the decrease that occurred in the rate of alkyl-acyl-PC production in the presence of PMSF compared to similar experiments performed in the absence of PMSF. These results can be attributed to the PMSF inhibitory action on PAF-AH activity and on CoA-independent transacylase (2, 3).

It is especially of note that the stimulation of FRTL5 cells with ionophore A23187 resulted in a statistically significant increase of labeled PE and in a concomitant decrease of PC (Fig. 5). Consequently the PC/PE ratio was significantly decreased after stimulation with ionophore A23187, according to the previously reported finding that stimulation of FRTL5 cells with TSH decreases the PC/PE ratio apparently through the inhibition of the methylation pathway of PE (46, 47). Increased amounts of alkyl- and alkenyl-PE metabolites of PAF and lyso-PAF might alter the cell membrane fluidity which is considered relevant to the transduction of a variety of signals acting at the cell surface (46).

In summary, we have demonstrated that the resting levels of PAF are very low in FRTL5 cells. PAF production was significantly increased in the presence of precursors and especially when PMSF was added in the incubation medium; an increase, by 3-fold, on PAF production was observed after stimulation with ionophore A23187. For the first time, the presence of a quite significant PAF-AH activity in the whole cell homogenate was documented. Our studies on [³H]alkyl-PAF and [³H]alkyl-lyso-PAF, metabolism by FRTL5 cells, indicated that [³H]alkyl-PAF is metabolized through its conversion to [³H]alkyl-lyso-PAF, which is not accumulated but is further metabolized to a variety of structurally diverse metabolites such as 1-O-alkyl-PC, acyl-PC, 1-O-alkyl-PE, and 1-O-alkenyl-PE, free and esterified alkyl-glycerols, free fatty acids, and fatty aldehydes. Further studies will be required to elucidate the enzymes involved in the metabolic pathways of PAF and lyso-PAF described for the FRTL5 cells. Finally, the active metabolism of PAF by these cells might suggest a role for PAF and/or its metabolites on the thyroid function.

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