Production of platelet-activating factor in slugs

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Abstract The land slug, *Incilaria bilineata*, was shown to contain a large amount of 1-*O*-alkyl-2-acyl (long chain)-*sn*-glycero-3-phosphocholine, which accounts for as much as 47% of the choline glycerophospholipid fraction. Since this unique ether phospholipid has been regarded as a stored precursor form of platelet-activating factor (PAF) in mammalian inflammatory cells, we examined the possibility of the presence of PAF in this animal. We obtained the evidence for the occurrence of significant amounts of PAF in two species of slugs, *Incilaria bilineta* and *Incilaria fruhstorferi*. Gas chromatography–mass spectrometry analysis revealed that the alkyl fatty chain of PAF principally consists of 16:0. We confirmed the presence of both enzyme activities catalyzing the formation of PAF, one for the remodeling pathway and the other for the de novo pathway. We also found the occurrence of other enzyme activities involved in PAF metabolism: acetylhydrolase activity which inactivates PAF and phospholipase A_2 activity toward alkylacylglycerophosphocholine. However, we failed to detect cofactor-independent transacylation activity in this animal. The amounts of PAF in slugs were markedly increased when slugs were administered several treatments which are considered to induce shock, such as the injection of dimethyl sulfoxide or injuries. ■ These results suggest that PAF is produced and may have certain physiological and pathological roles in the land slug, as in the case of mammals.—**Sugiura, T., T. Ojima, T. Fukuda, K. Satouchi, K. Saito, and K. Waku.** Production of platelet-activating factor in slugs. *J. Lipid Res.* 1991. **32:** 1795–1803.

Supplementary key words alkylacylglycerophosphocholine • ether phospholipid • acetyltransferase • cholinephosphotransferase • acetylhydrolase • transacylase • phospholipase A_2 • invertebrate

Platelet-activating factor (PAF) (1) was initially described as a novel chemical mediator of anaphylaxis released from IgE-sensitized rabbit basophils. In 1979, its chemical structure was elucidated to be a unique ether phospholipid, 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (2–4). PAF is now known to possess a wide spectrum of biological activities in addition to platelet-stimulating activity. For instance, the administration of PAF in vitro or in vivo induces the activation of polymorphonuclear leukocytes and monocytes/macrophages, increased vascular permeability, smooth muscle contraction, hepatic glycogenolysis, systemic hypotension, gastric ulcer, and bronchial hyperreactivity (5–7). It has generally been assumed that PAF is implicated in various allergic reactions, shock, and other inflammatory processes in mammals.

Various types of mammalian tissues and cells, especially leukocytes, are known to produce PAF upon appropriate stimulation (8). Concerning the biosynthetic routes for PAF, dual synthetic pathways have been proposed (8, 9). One involves the hydrolysis of pre-existing membranous 1-alkyl-2-acyl(long chain)-*sn*-glycero-3-phosphocholine (GPC) by phospholipase A_2 followed by the acetylation of the resultant lyso-compound through the action of 1-alkyl-GPC:acetyl-CoA acetyltransferase, and the other involves de novo synthesis, in which the final step is catalyzed by 1-alkyl-2-acetyl-*sn*-glycerol:CDP-choline cholinephosphotransferase. The biosynthesis of PAF in various types of leukocytes after stimulation is presumed to proceed mainly via the former pathway (8, 9), since the activity of acetyltransferase was shown to be increased upon stimulation (8, 9) and these types of cells are known to be enriched in alkylacyl-GPC, a stored precursor form of PAF (10).

It is noteworthy that considerable amounts of alkylacyl-GPC are also present in several lower animals. For instance, Thompson and Hanahan (11) demonstrated that 49% of choline glycerophospholipids (CGP) was accounted for by alkylacyl-GPC in slugs. This value is quite similar to that observed for human peripheral polymorphonuclear leukocytes (12) and rabbit alveolar macrophages (13) and peritoneal polymorphonuclear leukocytes (14). It is, therefore, tempting to speculate that a consider-

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Abbreviations: PAF, platelet-activating factor; GPC, *sn*-glycero-3-phosphocholine; GPE, *sn*-glycero-3-phosphoethanolamine; CGP, choline glycerophospholipids; EGP, ethanolamine glycerophospholipids; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); TLC, thin-layer chromatography. Fatty acids are designated by number of carbon atoms:number of double bonds.

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able amount of PAF can be produced in slugs under certain circumstances, as in leukocytes in mammals. Numerous studies have been carried out on the analysis, metabolism, and bioaction of PAF in mammalian tissues and cells (5–9). However, not much attention has been paid to animals other than mammals. Especially, little information is so far available concerning multicellular invertebrates, although the occurrence of PAF-like lipid in unicellular organisms such as *Tetrahymena pyriformis* (15) and *Escherichia coli* (16) has been reported.

In the present study, we explored the possibility of the presence of PAF in invertebrates such as slugs. We found that significant amounts of PAF were actually present in the land slugs, *Incilaria bilineata* and *Incilaria fruhstorferi*. The amount of PAF was dramatically increased when several irritants such as organic solvents were injected into the body. We also found that enzyme activities responsible for the synthesis and catabolism of PAF are operative. These observations suggest that PAF may play certain physiological and pathological roles even in lower animals such as Mollusca.

MATERIALS AND METHODS

Chemicals

[1-^{14}C]Acetyl-CoA (50 mCi/mmol) was purchased from ICN Radiochemicals (Irvine, CA). 1-O-[^{3}H]Hexadecyl-2-acetyl-GPC (PAF) (40 mCi/mmol), cytidine diphosphate (CDP)-[methyl-^{14}C]choline (42 mCi/mmol), and 1-O-alkyl-2-[5,6,8,9,11,12,14,15-^{3}H]arachidonoyl-GPC (126 Ci/mmol) were obtained from Du Pont-New England Nuclear (Boston, MA). Acetyl-CoA, 1-O-hexadecyl-GPC (lyso-PAF), CDP-choline, essentially fatty acid-free bovine serum albumin (BSA), lipase (*Rhizopus arrhizus*), and zymosan were purchased from Sigma (St. Louis, MO). 1-O-Hexadecyl-2-arachidonoyl-GPC was from Biomol (Plymouth Meeting, PA). A23187 was from Calbiochem (La Jolla, CA). 1-O-Hexadecyl-2-acetyl-GPC (C16:0 PAF) was obtained from Bachem (Bubendorf, Switzerland). 1-O-Hexadecyl-2-acetyl-sn-glycerol was from Novabiochem (Laeufelfingen, Switzerland). Dimethyl sulfoxide (DMSO) was purchased from Wako Pure Chemical Ind. (Osaka, Japan). 16:0-CoA and 18:2-CoA were provided by Dr. A. Yamashita (Teikyo University). A specific PAF antagonist, CV 6209, was a generous gift from Takeda Chemical Ind. (Osaka, Japan). In order to obtain 1-O-[^{3}H]hexadecyl-GPC, 1-O-[^{3}H]hexadecyl-2-acetyl-GPC was hydrolyzed by a brief treatment with 0.5 M sodium methoxide. The resultant lyso-compound (1-O-[^{3}H]hexadecyl-GPC) was purified by TLC on plates developed with chloroform–methanol–water 65:35:6 (v/v) before use. 1-O-Hexadecyl-2-[^{3}H]acetyl-GPC was prepared from 1-O-hexadecyl-GPC and [^{3}H]acetic anhydride (ICN, Ir-

vine, CA) in the presence of pyridine. All other chemicals were of reagent grade.

Animals

Japanese native land slugs, *Incilaria bilineata* and *Incilaria fruhstorferi*, were collected in the areas of Utsunomiya (Tochigi Prefecture), Yugawara (Kanagawa Prefecture), and Tottori (Tottori Prefecture) in Japan. They were kept at 20–22°C in a box containing moist papers and unglazed pots and were fed with carrots and cabbages. They were starved for 24 h before use in experiments. In order to examine the effects of several treatments on PAF production, slugs (*Incilaria bilineata*) were injected, dorsally through the body wall, with organic solvents or zymosan-water solution (4 mg/ml) (50 μl/g body weight) or cut in two with scissors or given an electric shock (150 V, 30 mA for 1 sec). After standing for 15 min, they were killed by soaking in chloroform–methanol 1:2 (v/v) followed immediately by homogenizing with a Waring blender.

Phospholipid analysis

Total lipids were extracted by the method of Bligh and Dyer (17). Individual phospholipids were separated from each other by two-dimensional TLC using the solvent system chloroform–methanol–28% NH$_4$OH 65:35:5 (v/v) for the first development and chloroform–acetone–methanol–acetic acid–water 5:2:1:1.3:0.5 (v/v) for the second development (13). Lipid spots were visualized with iodine vapor or under ultraviolet light after spraying the TLC plates with primuline (13). Lipid phosphorus was estimated after digestion with HClO$_4$ as described (18), except that the TLC plates were sprayed in advance with ninhydrin and then heated at 120°C before being scraped in order to ensure the cleavage of the C–P bond in ceramide 2-aminoethylphosphonate (19). Alkenylacyl, alkylacyl, and diacyl subclasses of CGP and ethanolamine glycerophospholipids (EGP) were separated as 1,2-diradyl-3-acetyl-glycerol derivatives by TLC developed first with petroleum ether–diethyl ether–acetic acid 90:10:1 (v/v) and then with toluene as described earlier (13). The amounts of these three lipid classes were estimated by measuring their fatty acyl moieties with a capillary column (Supelco, SP2330) on a gas chromatograph (Shimadzu, GC-8A) using 17:0 methyl ester as an internal standard (13).

PAF bioassay

The area corresponding to authentic PAF was scraped from TLC plates that had been developed with chloroform–methanol–water 65:35:6 (v/v). PAF was extracted from silica gel by the method of Bligh and Dyer and dissolved in 0.25% bovine serum albumin (BSA)-containing HEPES-buffered Tyrode's solution (pH 7.4). Recovery of PAF through the extraction procedure was usually more

than 95%. The amount of PAF was estimated by measuring its ability to aggregate washed rabbit platelets suspended in HEPES-buffered Tyrode's gelatin solution containing 1.3 mM Ca²⁺, in an aggregometer (Niko Hematracer, PAT-2M) as described earlier (20). PAF concentrations were calculated from a calibration curve obtained with C16:0-PAF added at a final concentration of 20–200 pM.

Gas chromatography–mass spectrometry analysis of PAF

Slugs (*Incilaria bilineata*) (total body weight 30–35 g) were injected with DMSO (50 μl/g body weight). They were killed 15–20 min later in chloroform–methanol 1:2 (v/v) with a Waring blender. Total lipids were extracted by the method of Bligh and Dyer and passed through a DEAE-cellulose column to remove acidic phospholipids (21). Neutral phospholipids were then separated from each other by TLC developed with chloroform–methanol–water 65:35:6 (v/v) using preparative TLC plates (Merck, 5717). The area containing PAF fraction was scraped off, extracted, and rechromatographed using the same solvent system and conventional TLC plates (Merck, 5721). The PAF fraction was further purified by TLC on plates developed with methanol–water 2:1 (v/v) (22) to remove contaminating phospholipids. After the addition of 100 ng of 1-hexadecyl-2-acetyl-d₃-GPC as an internal standard, the purified PAF fraction was hydrolyzed with phospholipase C from *Bacillus cereus*, and the dephosphorylated product was converted to a *tert*-butyldimethylsilyl derivative (23, 24). The derivative was further purified by TLC developed with hexane–diethyl ether 9:1 (v/v) and analyzed as described earlier (23, 24). A JEOL JMS-DX 300 apparatus equipped with a JMA 3100 computer was used for selected ion monitoring, with the two ions $[CH_3CO+74]^+$ and $[M-57]^+$ being monitored.

Subcellular fractionation

Slugs (*Incilaria bilineata*) were homogenized with a Potter-Elvehjem glass–Teflon homogenizer in 0.25 M sucrose-containing 1 mM EDTA. The homogenate was centrifuged at 700 g for 10 min twice to remove undisrupted tissues, cell debris, and nuclei. The supernatant was then centrifuged at 7000 g for 10 min. After centrifugation a second time at 7000 g for 10 min, the supernatant was further centrifuged at 105000 g for 60 min. The microsomal fraction was washed once with 0.25 M sucrose followed by centrifugation at 105000 g for 60 min. The pellet was resuspended in 0.25 M sucrose-containing 1 mM EDTA and stored at −80°C. In some experiments, samples were stored at 4°C and used within 24 h. Protein content was determined by the method of Lowry et al. (25).

Enzyme activities involved in PAF metabolism

The incubation mixture for the measurement of acetyltransferase activity consisted of lyso-PAF (final 20 μM),

[¹⁴C]acetyl-CoA (final 100 μM, 100000 dpm), and subcellular fractions (500 μg protein) in 0.1 M Tris-HCl buffer (pH 7.4) (final 500 μl) containing 2 mM CaCl₂. The incubation was carried out at 25°C for 15 min and terminated by adding chloroform–methanol 1:2 (v/v). After the addition of chloroform and water, the chloroform layer was washed twice with methanol–water 1:1 (v/v) as described earlier (20). The chloroform layer was then evaporated to dryness; the residue was dissolved in chloroform–methanol 1:2 (v/v) and subjected to TLC developed with chloroform–methanol–water 65:35:6 (v/v). The area comigrating with authentic PAF was scraped off into a counting vial. The radioactivity was estimated in a scintillation counter (Aloka, Model LSC 900).

The assay system for acetylhydrolase activity consisted of [³H]PAF (final 20 μM, 400000 dpm) which was sonicated before use and membrane fractions (500 μg protein) in 0.1 M Tris-HCl buffer (pH 7.4) (final 500 μl) containing 2 mM EDTA. The incubation was carried out at 25°C for 15 min and terminated by adding chloroform–methanol 1:2 (v/v). After the addition of chloroform and water containing NaHCO₃ (26), the water layer was washed three times with chloroform and transferred to a counting vial.

The activity of cholinephosphotransferase was estimated according to the method of Woodard, Lee, and Snyder (27) with slight modifications. Briefly, the incubation mixture consisted of 1-hexadecyl-2-acetyl-glycerol or dioleoylglycerol dissolved in ethanol (final 200 μM; final concentration of ethanol was 2.5%), CDP-[¹⁴C]choline (final 100 μM, 300000 dpm), and microsomal fractions (500 μg) in 0.1 M Tris-HCl buffer (pH 8.0) (final 500 μl) containing 0.5 mM EGTA, 10 mM MgCl₂, and 0.5 mg BSA. The incubation was carried out at 25°C for 7.5 min in the presence or absence of dithiothreitol (final 10 mM). The incubation was terminated by adding chloroform–methanol 1:2 (v/v). The subsequent procedures for the estimation of radioactivity were the same as those for acetyltransferase as described above.

The incubation systems for the measurement of CoA-independent or -dependent transacylation activity and lyso-PAF:acyl-CoA acyltransferase activity consisted of [³H]lyso-PAF (final 20 μM, 150000 dpm), acyl-CoA (final 30 μM, only for acyltransferase) or CoA (final 30 μM, only for CoA-dependent transacylation) and microsomal fractions (250 μg) in 0.1 M Tris-HCl buffer (pH 7.4) (final 250 μl) containing 2 mM EDTA or 2 mM CaCl₂. The incubation was carried out at 25°C for 5 min and terminated by adding chloroform–methanol 1:2 (v/v). After the extraction of total lipids, individual phospholipids were separated by TLC developed with chloroform–methanol–water 65:35:6 (v/v) and the radioactivities in neutral lipids, CGP, and lyso-PAF were estimated.

The assay system for phospholipase A₂ activity consisted of 1-O-hexadecyl-2-[³H]arachidonoyl-GPC (final 40 μM, 30000 dpm) which was sonicated before use and

subcellular fractions (500 μg protein) in 0.1 M Tris-HCl buffer (pH 7.4) (final 500 μl) containing 2 mM EDTA or 2 mM CaCl₂. The enzyme reaction was carried out at 25°C for 30 min and stopped by adding chloroform-methanol 1:2 (v/v). After the extraction of total lipids by the method of Bligh and Dyer (17), phospholipids and liberated free fatty acid were separated from each other by TLC developed with petroleum ether–diethyl ether–acetic acid 70:30:1 (v/v) and their radioactivities were estimated.

RESULTS

Slugs, *Incilaria bilineata*, contain phospholipids at the level of 175 μg P/g body weight. The major constituents are CGP and EGP, accounting for 52.8% and 27.9% of total phospholipids, respectively. Serine glycerophospholipids (4.9%), inositol glycerophospholipids (4.4%), cardiolipin (2.4%), phosphatidic acid (1.4%), ceramide 2-aminoethylphosphonate (2.3%), as well as lyso-CGP (1.1%) and lyso-EGP (0.8%) were also found. The lipid fractions from slugs do not, however, contain sphingomyelin, as has already been reported (11).

Alkenylacyl, alkylacyl, and diacyl subclasses of CGP and EGP are shown in **Table 1**. A large portion of CGP was accounted for by alkylacyl-GPC, which is known to be a stored precursor form of PAF in mammalian tissues. Alkylacyl-GPC comprised as much as 25% of total phospholipids in this animal. The proportion of alkenylacyl subclass in CGP was, on the other hand, shown to be low. In contrast, EGP contained a large amount of alkenylacyl compounds, which accounted for 11% of total phospholipids.

Predominant fatty acids found at the 2-position of alkylacyl-GPC were 18:1 (48%), 18:2 (16%), 20:4 (11%), and 22:4 (5%). On the other hand, the main fatty acids esterified in diacyl-GPC (at the 1- and 2-positions) were 16:0 (11%), 18:1 (13%), 18:2 (17%), 20:2 (25%), 20:4 (12%), and 22:5 (8%). Alkenylacyl-GPE and diacyl-GPE also contained various types of fatty acids. The major constituents were 16:0 (7%), 18:1 (9%), 18:2 (7%), 20:4
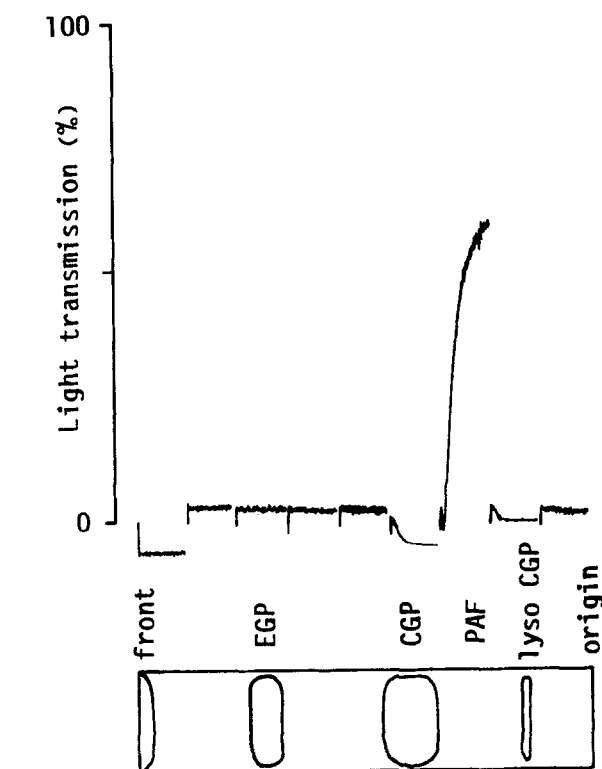

Fig. 1. Ability of individual phospholipid fractions to aggregate washed rabbit platelets. The total lipid fraction was subjected to TLC developed with chloroform–methanol–water 65:35:6 (v/v). After the development, silica gel was zonally scraped off (2 cm width) the plate, and lipid was extracted and dispersed in 0.25 ml of 0.25% BSA-containing Tyrode's solution. An aliquot (12.5 μl) was added to 237.5 μl of platelet suspension and the change in light transmission was recorded.

(15%), 22:2 (14%), and 22:4 (28%) for alkenylacyl-GPE and 16:0 (8%), 18:0 (17%), 18:1 (5%), 18:2 (8%), 20:2 (15%), and 20:4 (28%) for diacyl-GPE.

We then examined the possibility of the presence of platelet-activating factor in slugs. As shown in **Fig. 1**, the lipid fraction obtained from slugs (*Incilaria bilineata*) contained a platelet-aggregating lipid that comigrated with authentic PAF on TLC plates. We could not detect platelet-aggregating activity in other lipid fractions. The biologically active material was identified as PAF on the basis of the following criteria. *1)* The R_f value for the biologically active material was the same as that for PAF. *2)* The treatment of the sample either with phospholipase A₂ (*Naja naja atra*) (0.2 mg protein in 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.4) containing 10 mM CaCl₂ and 0.5 ml of diethyl ether, 15 min) or with phospholipase C (*Bacillus cereus*) (0.2 mg protein in 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.4) and 0.5 ml of diethyl ether, 15 min) completely abolished the biological activity, whereas the biological activity was resistant to lipase (*Rhizopus arrhizus*) (0.2 mg protein in 0.5 ml of PIPES buffer (pH 6.5) containing 10 mM CaCl₂ and 0.1% deoxycholate, 15 min). *3)* The biological activity was completely lost after alkaline treatment (0.2 M sodium methoxide, 5 min) but not after acid

TABLE 1. Subclass composition of choline and ethanolamine glycerophospholipids in the slug, *Incilaria bilineata*

Subclass	CGP	EGP
	%	%
Alkenylacyl	4.1 ± 1.7	38.3 ± 3.1
Alkylacyl	47.4 ± 5.0	12.8 ± 2.5
Diacyl	48.5 ± 3.4	48.9 ± 0.8

Choline and ethanolamine glycerophospholipids were converted to 1,2-diradyl-3-acetylglycerol derivatives as described in Materials and Methods. Three subclasses were separated by TLC and the quantities of their fatty acyl moieties were estimated by GLC using 17:0 methyl ester as an internal standard. Values are means ± SD from three determinations.

treatment (0.2 M HCl in 90% methanol, 5 min). 4) The pretreatment of platelets for 1 min with a specific PAF antagonist, CV6209 (0.1 μM) inhibited the aggregation of platelets upon the addition of the sample. The amount of PAF in *Incilaria bilineata* was calculated to be 2.1 ± 0.9 pmol/g body weight (mean ± SD from seven determinations) (370 nmol/mol total phospholipids) and that in *Incilaria fruhstorferi* was 1.7 ± 0.7 pmol/g body weight (mean ± SD from four determinations) (540 nmol/mol total phospholipids).

The presence of PAF was further confirmed by a gas chromatography–mass spectrometry analysis. **Fig. 2** illustrates the trace of selected ion monitoring of *tert*-butyldimethylsilyl derivatives of PAF and 1-acyl-PAF obtained from slugs, *Incilaria bilineata*. Upon monitoring at *m/z* 117, one main peak and three minor peaks were seen. Peak no. 2 and peak no. 3 were identified as C16:0 PAF and C16:0 1-acyl-PAF, respectively, on the basis of comparison of their retention times with those of standard PAF and standard 1-acyl-PAF. Two minor components, peak no. 1 and peak no. 4, remain unidentified as yet. On the other hand, we could not find peaks with retention times equal to those of C18:0 PAF and C18:1 PAF. The identification and quantitation of PAF were also performed by monitoring at [M–57]⁺ (data not shown). The PAF content estimated by gas chromatography–mass spectrometry analysis was in reasonable agreement with that quantified by bioassay. These results clearly indicate that the lipid fraction from slugs actually contains PAF besides a small amount of 1-acyl PAF analogue and that C16:0 PAF is the predominant species of PAF.

Fig. 3 shows the effects of several treatments of slugs on the amounts of PAF. The injection of distilled water did not affect the PAF levels significantly. On the other hand, the injection of zymosan particles, suspended in distilled water, caused an increase of PAF levels (3-fold). Interestingly, the amounts of PAF were dramatically increased (4-
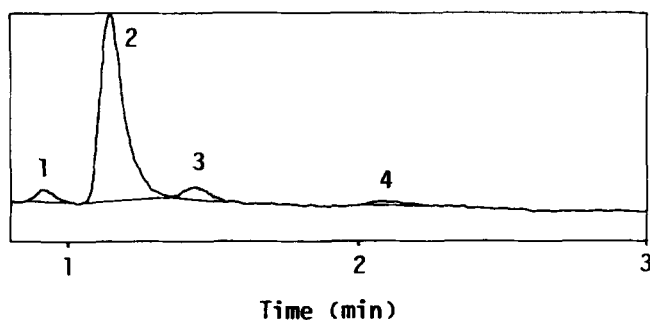

Fig. 2. A trace of selected ion monitoring (*m/z* 117) of *tert*-butyldimethylsilyl derivative of PAF from DMSO-injected slugs, *Incilaria bilineata*. Purified PAF was converted to the *tert*-butyldimethylsilyl derivative and analyzed by gas chromatography–mass spectrometry as described in Materials and Methods. The percentages of peak areas were 5.0% (peak 1), 85.9% (peak 2), 6.3% (peak 3), and 2.8% (peak 4). The trace shown here is a representative of three separate experiments with similar results.
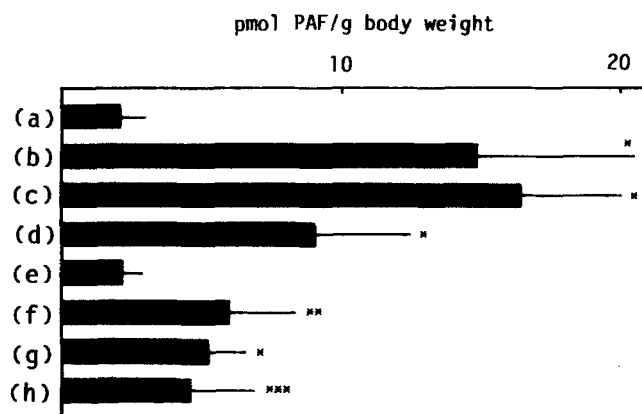

Fig. 3. Effects of various treatments on the PAF levels in slugs, *Incilaria bilineata*: (a), control; (b), injected with DMSO (50 μl/g body weight); (c), injected with 2 mM A23187 in DMSO (50 μl/g body weight); (d) injected with ethanol (50 μl/g body weight); (e), injected with distilled water (50 μl/g body weight); (f), injected with zymosan–water solution (4 mg/ml) (50 μl/g body weight); (g), given an electric shock (1 sec); (h), cut in two. After 15 min, slugs were killed and total lipids were extracted as described in Materials and Methods. PAF contents were estimated by bioassay. Values are means ± SD from six to seven determinations. *P < 0.001 versus (a). **P < 0.01 versus (a). ***P < 0.05 versus (a). Statistical significance was analyzed by Student's *t*-test.

to 7-fold) when slugs were injected with several organic solvents such as ethanol and dimethyl sulfoxide (DMSO). When slugs were given an injection of A23187 dissolved in DMSO, the PAF levels were slightly increased compared with the case of the vehicle DMSO alone, although the difference was not statistically significant. The amounts of PAF in slugs after the injection of DMSO increased with time at least up to 60 min (**Fig. 4**), indicating that PAF is continuously produced during the in vivo stimulation with such irritants. The induction of PAF synthesis was observed not only by the injection of chemicals but also by several physical treatments such as an electric shock or cutting (Fig. 3).

The activities of enzymes involved in PAF metabolism were then explored. We confirmed the occurrence of enzyme activities for the synthesis and the catabolism of PAF in slugs. **Table 2** shows the subcellular distribution of the activities of lyso-PAF:acetyl-CoA acetyltransferase and PAF acetylhydrolase. Acetyltransferase activity was found in the 105000 *g* pellet fraction, although lower activity was also observed in the 7000 *g* pellet fraction. The enzyme activity was not influenced when 2 mM EDTA was added in place of 2 mM CaCl₂ (data not shown). We could not detect appreciable acetyltransferase activity in the 105000 *g* supernatant fraction. In contrast to the case of acetyltransferase, acetylhydrolase activity was mainly distributed in the 105000 *g* supernatant fraction. The activity of acetylhydrolase in the 7000 *g* pellet fraction and that in the 105000 *g* pellet fraction were relatively low.

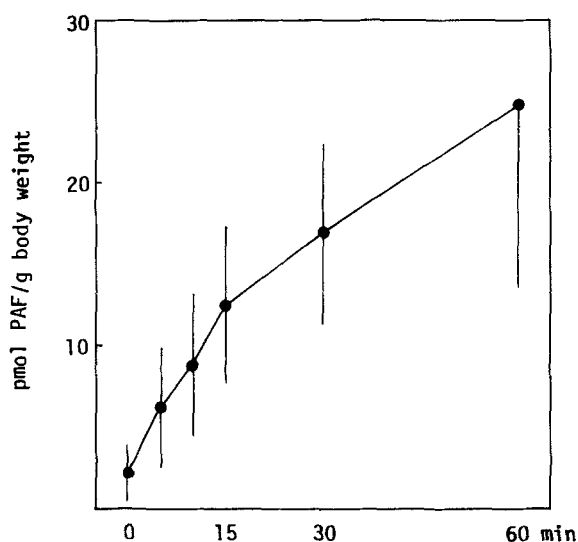The slug 105000 *g* pellet fraction also contained enzyme activity for the de novo synthesis of PAF besides that for

Fig. 4. Time-dependent increase in PAF levels in slugs, *Incilaria bilineata* after the injection of DMSO (50 μl/g body weight). Values are means ± SD from six to eight determinations.

TABLE 2. Subcellular distribution of the enzyme activities of acetyltransferase and acetylhydrolase in the slug, *Incilaria bilineata*

Fraction	Acetyltransferase	Acetylhydrolase
	pmol/min/mg protein	*pmol/min/mg protein*
700 *g* supernatant	10 ± 2	74 ± 6
7000 *g* pellet	30 ± 5	16 ± 6
105000 *g* pellet	47 ± 4	31 ± 11
105000 *g* supernatant	1 ± 1	105 ± 10

Acetyltransferase and acetylhydrolase activities were estimated as described in Materials and Methods. Values are means ± SD from three to four determinations.

DISCUSSION

Several types of lower animals are known to contain substantial amounts of alkenyl and alkyl ether phospholipids (28). Thompson and Hanahan (11) demonstrated in 1963 that two types of terrestrial slugs, *Ariolimax columbianus* and *Arion ater* contained large amounts of alkyl ether-linked phospholipids. The percentage of alkylacyl subclass in CGP from slugs *Ariolimax columbianus* was 49%, which accounts for more than 25% of total phospholipids. The values for a Japanese native species *Incilaria bilineata* (47% of CGP and 25% of total phospholipids) obtained in the present study coincide well with these previous data for a native species of North America (*Ariolimax columbianus*) and for a species introduced from Europe (*Arion ater*).

Considering the extremely large amount of alkylacyl-GPC in slugs, the occurrence of a significant level of PAF, an acetyl analogue of alkylacyl-GPC, in this animal is not so surprising. We confirmed the presence of enzyme activities responsible for the hydrolysis of alkylacyl-GPC to provide lyso-PAF and for the acetylation of lyso-PAF to yield PAF (Table 2). Hence, one can assume that the biosynthesis of PAF in this animal proceeds mainly via the direct acetylation of pre-existing lyso-PAF and/or the remodeling of membranous alkylacyl (long chain)-GPC.

the remodeling pathway (**Table 3**). The activity of 1-alkyl-2-acetyl-glycerol:CDP-choline cholinephosphotransferase was high compared with diolein used as an acceptor in the cholinephosphotransferase reaction, at least under the present experimental conditions. The presence of dithiothreitol (10 mM) did not markedly affect the enzyme reaction where either 1-alkyl-2-acetyl-glycerol or diolein was used as a substrate.

We further studied several enzyme activities implicated in the metabolism of a stored PAF precursor, alkylacyl-GPC, and of a degradation product of PAF, lyso-PAF. We first examined the subcellular distribution of phospholipase A_2 activity acting on 1-alkyl-2-arachidonoyl-GPC in the presence of Ca^{2+}. The phospholipase A_2 activity was found to occur in each subcellular fraction. The enzyme activities were 3.0 pmol/min per mg protein in the 7000 *g* pellet fraction, 5.5 pmol/min per mg protein in the 105000 *g* pellet fraction, and 7.7 pmol/min per mg protein in the 105000 *g* supernatant fraction. The presence of Ca^{2+} was required for the enzyme activity in each case. As for the acylation of lyso-PAF, we found that lyso-PAF can be acylated through the lyso-PAF:acyl(long chain)-CoA acyltransferase reaction (**Fig. 5**). Both 16:0-CoA and 18:2-CoA act as acyl donors and the presence or absence of Ca^{2+} did not influence the enzyme activity. On the other hand, we failed to detect cofactor-independent transacylation activity in slugs (Fig. 5). Furthermore, we could not find CoA-dependent transacylation activity toward lyso-PAF, suggesting that neither a cofactor-independent transacylation system nor a CoA-dependent transacylation system is involved in the metabolism of lyso-PAF in this animal.

TABLE 3. Cholinephosphotransferase activities toward 1-alkyl-2-acetyl-glycerol and 1,2-dioleoylglycerol in the 105000 *g* pellet fraction of the slug, *Incilaria bilineata*

Substrate	Without DTT	With DTT
	pmol/min/mg protein	
1-Alkyl-2-acetylglycerol	254 ± 62	227 ± 81
1,2-Dioleoylglycerol	24 ± 3	18 ± 2

Cholinephosphotransferase activities toward 1-alkyl-2-acetylglycerol and 1,2-dioleoylglycerol in the 105000 *g* pellet fraction were estimated in the presence or absence of 10 mM dithiothreitol (DTT) as described in Materials and Methods. Values are means ± SD from three determinations.
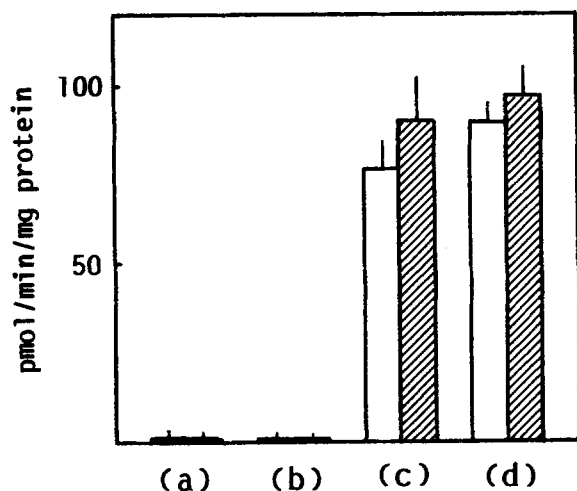
Fig. 5. Acylation of lyso-PAF by the 105000 g pellet fraction in the presence or absence of acyl-CoA or CoA. Acylation activity was estimated using the buffer containing 2 mM EDTA (open bar) or 2 mM CaCl$_2$ (hatched bar): (a), without cofactors; (b), with CoA (30 μM); (c), with 16:0-CoA (30 μM); (d), with 18:2-CoA (30 μM). Values are means ± SD from four determinations.

This pathway has also been presumed to play a central role in the biosynthesis of PAF in various types of mammalian inflammatory cells such as neutrophils and macrophages (8, 9). However, we also found the presence of enzyme activity catalyzing the formation of PAF through the de novo pathway (Table 3), although information on the level of the substrate, 1-alkyl-2-acetyl-glycerol, is not yet available. This enzyme pathway could also be effective if a sufficient amount of 1-alkyl-2-acetyl-glycerol was supplied within the cell. It is difficult, therefore, to answer at present the question of which pathway is more important in the formation of PAF in this animal. It is possible that the biosynthetic route for PAF is different among tissues and is also dependent upon the type of stimulus as has been suggested for mammals (8, 9, 29). In any event, this is the first report, to our knowledge, to describe and verify the occurrence of PAF in multicellular animals other than vertebrates.

The molecular species analysis of PAF demonstrated that the alkyl chain of PAF obtained from DMSO-injected slugs principally consisted of 16:0. This finding is in keeping with previous results for the alkyl chain composition of long chain fatty acid-containing alkylacyl-GPC in slugs, *Ariolimax* (11), i.e., 94% and 6% of alkyl ether moieties were accounted for by 16:0 and 15:0, respectively, in the latter case. Such a fatty chain composition of alkylacyl-GPC in slugs seems to be considerably different from those for rabbit alveolar macrophages (13) and rabbit (14) and human (12) neutrophils, where 18:0 and 18:1 are substantial components. Thompson and Hanahan (11) also reported that 72% and 32% of alkyl ether moieties were accounted for by 16:0 in triradylglycerol fraction and EGP fraction, respectively. Although the alkyl fatty chain

occurring in alkylacetylglycerol was not examined, it is conceivable that PAF precursor(s) as well as its stored form in this animal are enriched in 16:0 alkyl fatty chain, leading to the production of PAF enriched in C16:0 species.

We confirmed the presence of various enzyme activities involved in PAF metabolism in slugs (Tables 2–3, Fig. 5). The subcellular distribution of acetyltransferase and acetylhydrolase (Table 2) resembled those observed in mammalian tissues and cells (26, 30), although the enzyme activities in slug subcellular fractions (estimated at 25°C) seemed to be lower than those in several mammalian tissues such as rat spleen (measured at 37°C). The presence of EDTA (2 mM) did not interfere with the reaction of acetyltransferase, which is also different from the case of mammals (8). DTT-insensitive cholinephosphotransferase, which catalyzes the final step of de novo synthesis of PAF, was also found to occur in this animal. Under the present experimental conditions, the activity was shown to be high when alkylacetylglycerol was used as a substrate compared with the case where diolein was added as a substrate. This may be due, at least in part, to the differences in the physicochemical condition of substrates in the incubation mixture. The addition of DTT (10 mM) did not greatly affect the activity of cholinephosphotransferase towards either diolein or alkylacetylglycerol as a substrate; this is different from the case of mammals in which the cholinephosphotransferase activity toward diolein was markedly reduced in the presence of DTT (27). A possible reason for the difference may be that the basal enzyme activity of cholinephosphotransferase acting on diolein is originally very low under the present experimental conditions.

Phospholipase A$_2$ activity toward alkylacyl(long chain)-GPC in this animal was also shown to be relatively low. In fact, the hydrolysis of alkylarachidonoyl-GPC by the 105000 g supernatant fraction proceeded slowly compared with that of alkylacetyl-GPC by acetylhydrolase in the same fraction. Phospholipase A$_2$ activities in the 105000 g pellet fraction and 7000 g pellet fraction, however, may actually be somewhat higher than those observed, since these fractions contained considerable amounts of endogenous CGP. The values of the endogenous CGP in these fractions were 25 nmol/0.5 mg protein for the 700 g supernatant fraction, 94 nmol/0.5 mg protein for the 7000 g pellet fraction, 36 nmol/0.5 mg protein for the 105000 g pellet fraction, and 4 nmol/0.5 mg protein for the 105000 g supernatant fraction.

As for the acylation of lyso-PAF, we observed the presence of lyso-PAF:acyl-CoA acyltransferase activity in the 105000 g pellet fraction using 16:0-CoA or 18:2-CoA as the acyl donor; this enzyme activity was, however, only two times higher than acetyltransferase activity. Interestingly, cofactor-independent transacylation activity, which has been assumed to play a very important role in the reacylation of ether-containing lysophospholipids in several

mammalian inflammatory cells (31–34), was not detectable in this animal despite the abundance of ether phospholipids. This observation suggests that the acylation system(s) of the ether-containing lysophospholipids in slugs is considerably different from that in mammals. The absence of cofactor-independent transacylation activity in slugs seems to be in accordance with the observation that 20:4 is not accumulated in either alkylacyl-GPC or alkenylacyl-GPE in this animal, which is in contrast to the case of rabbit alveolar macrophages (13) and human neutrophils (12) and eosinophils (35), where 20:4 is highly concentrated in ether phospholipids; we and other investigators (31–34) have already suggested that such an accumulation of 20:4 in ether phospholipids could be attributed, at least in part, to the action of a cofactor-independent transacylation system present in various inflammatory cells.

The physiological meaning of the occurrence of PAF in slugs remains unclear. In mammals, PAF has been assumed to be crucially involved in the pathogenesis of several diseases such as endotoxin shock, ischemia, nephritis, anaphylactic shock, and asthma (5–7). It should be noted that the PAF level was markedly increased in slugs subjected to several treatments that induce shock in this animal (Fig. 3). It seems likely that PAF amplifies the tissue injuries induced by such physical or chemical stress, leading to irreversible lethal damage. Alternatively, PAF may play some role in the homeostatic reactions against injuries, such as wound healing. Recent studies by Furuta and co-workers (36, 37) demonstrated the presence of three types of hemocytes in hemolymph obtained from slugs, *Incilaria fruhstorferi*. Among them, type I cells possess a high phagocytic activity and morphologically resemble granulocytes (36, 37). They further reported the occurrence of fragments of cells, termed plate-like structures, that adhere to latex beads (36, 37). It is thus tempting to speculate that PAF stimulates these cells as a defense mechanism against foreign materials in cooperation with other humoral and cellular factors as in mammals. Studies on the roles of PAF in invertebrates are currently in progress.

In conclusion, we found that slugs contain a large amount of alkylacyl-GPC, a stored precursor form of PAF. A significant level of PAF was shown to occur in slugs, and the C16:0 species was the major constituent of PAF. The PAF content was markedly increased when slugs were subjected to several treatments considered to induce shock, such as the injection of DMSO. Various enzyme activities involved in PAF metabolism were detected in slugs, although not the cofactor-independent transacylation system. Studies on PAF in invertebrates should lead to a more general understanding of the role as well as the behavior of PAF in mammals under physiological and pathological conditions. ∎

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