HIGH LEVEL OF 1-O-ALKYL-LINKED GLYCEROPHOSPHOCHOLINE (PLATELET-ACTIVATING FACTOR PRECURSOR) IN A MARINE GASTROPOD, *APLYSIA KURODAI*

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**ABSTRACT**

A water-insoluble lipid (SM-1) containing phosphocholine was isolated from the skin of a marine gastropod, *Aplysia kurodai*. SM-1 was purified from Folch’s lower-phase material of the skin of *A. kurodai* using two solvent systems of column chromatography on silicic acid. Based on the results of thin-layer chromatography, chemical analysis, IR absorption spectrometry, and fast atom bombardment-mass spectrometry, the structure of SM-1 was identified as 1-O-alkyl-2-acyl (long chain)-sn-glycero-3-phosphocholine (alkylacyl-GPC), which is regarded as a precursor of the platelet-activating factor (PAF). Fatty chains at positions 1 and 2 of SM-1 were analyzed by capillary gas-liquid chromatography. The 1-O-alkyl fatty chain consisted principally of 16:0, whereas the 2-acyl fatty acid chain consisted of 16:0, 18:1, 20:1, 20:4 and 22:4 as major components and 16:1, 17:1 and 18:0 as minor components. These data provide evidence that SM-1 is present in the skin, nervous tissue and hemolymph of *A. kurodai*, and suggest these tissues contain a precursor for the synthesis of PAF.

We found more than 30 water-soluble lipids in the nervous tissues of a marine gastropod, *Aplysia kurodai* (1). They were phosphoryl-[2-aminoethylphosphonic acid (AEPn)] and phosphoryl-(phosphoethanolamine) glycosphingolipids (2–8).

In the present study, we purified a considerable amount of phosphocholine-containing ether glycerophospholipid (SM-1) from the water-insoluble lipid fraction comprising Folch’s lower-phase material, extracted from the skin of *Aplysia*, and identified it to be 1-O-alkyl-2-acyl (long chain)-sn-glycero-3-phosphocholine. This ether phospholipid is often overlooked because its functions and biological roles are not known and its content is relatively low in normal tissues except for platelets, testis (10) and inflammatory cells (macrophages, neutrophils and eosinophils) (23, 26, 31, 39) in mammals. However, observations that the stimulation of phagocytic cells can lead to the formation of the platelet-activating factor (PAF) and proof that this ether phospholipid is a precursor of PAF in mammalian inflammatory cells (9, 25, 27) stimulated further studies on ether-containing glycerophospholipids (16). PAF is now known to be involved in various biological and pathological processes in addition to its platelet-stimulating activity (13, 15). Although the content of alkyl ether phospholipids, in particular, *sn*-glycero-3-phosphocholine (GPC), is usually not high in mammals, it is noteworthy that a considerable amount of alkylacyl-GPC is present in several lower animals (38). For instance, Sugiura et al. (38) reported that animals classified under phyla other than Arthropoda possess large amounts of alkylacyl-GPC and PAF. They analyzed the contents of subclasses of choline glycerophospholipids
(CGP) (alkylacetyl-, alkenylacetyl- and diacyl-GPC) in various invertebrates. The content of the alkylacetyl subclass was high in lower animals (19, 21, 30, 38, 40, 42, 43). Thompson and Hanahan (43) and Sugiuara et al. (40) demonstrated that 47–49% of CGP in slugs were alkylacetyl-GPC. These results suggest that alkyl-linked phospholipids including PAF are physiologically important molecules, especially in invertebrates. In the present study, we found considerable amounts of alkylacetyl-GPC (SM-1) in the skin, nervous tissue and hemolymph of A. kurodai, analyzed the composition of the fatty chain at positions 1 and 2 of SM-1, and elucidated its structure.

MATERIALS AND METHODS

Isolation of SM-1 from the Skin

The skin with associated subcutaneous fibrous tissue of A. kurodai (caught at Sado Island, Sea of Japan) was homogenized in a Waring blender with 20 vol of cold acetone. The residue obtained after filtration was washed with 10 vol of cold acetone and dried. The dried acetone powder was extracted twice with 20 vol of chloroform/methanol/water (30:60:10, v/v/v). Then chloroform, methanol and water were added to the extracts to bring the chloroform/methanol/water ratio to 8:4:3 by volume. The aqueous upper phase containing water-soluble materials was removed. The lower phase was washed with the theoretical upper phase of Folch et al. (12), condensed and dried. The dried material (1 g) was dissolved in 10 ml of chloroform, and alkaline hydrolysis was performed with 0.5 N methanolic KOH at 10°C for 3 min (14). Alkali-stable lipids (0.67 g) obtained from Folch’s lower-phase material (1 g) were fractionated according to the modified method of Matsubara et al. (22). Briefly, the alkali-stable lipids were dissolved in chloroform/methanol (2:1, v/v), applied to a silicic acid column (1.8 × 27 cm; Iatrobeads 6RS-8060, Iatron Laboratories, Tokyo), and eluted successively with chloroform/methanol (98:2, v/v), acetone/methanol (9:1, v/v), chloroform/methanol (4:1, v/v), chloroform/methanol (3:2, v/v) and methanol. Then the methanol fraction (0.18 g) was subjected to silicic acid column chromatography (Iatrobeads; 1 × 45 cm column) with a gradient system consisting of 100 ml each of chloroform/methanol/water (60:40:10, v/v/v) and chloroform/methanol/water (40:70:15, v/v/v) in a two-chamber device. A purified lipid fraction, SM-1 (25 mg), was obtained.

Thin-Layer Chromatography

Water-insoluble lipids were separated by two-dimensional thin-layer chromatography (TLC). High performance thin-layer chromatography (HPTLC) plate (Silica Gel 60, E. Merck; 10 × 20 cm) was developed twice with chloroform/methanol/ammonia water (65:25:4, v/v/v) to a distance of 20 cm in the first dimension. Then, the plate was allowed to dry for at least 2 h, after which it was developed 4 times to a distance of 10 cm with chloroform/methanol/acetic acid/water (75:15:30:15:7.5, v/v/v/v/v) in the second dimension. The purity of SM-1 was checked on TLC using the following solvent systems: 1) chloroform/methanol/ammonia water (65:35:5, v/v/v); 2) chloroform/methanol/water (3:3:1, v/v/v); 3) n-propanol/ammonia water/water (75:5:25, v/v/v); and 4) n-propanol/water (7:3, v/v). The chromatograms were visualized with anthrone-sulfuric acid (33), Dittmer-Lester’s (11) and Dragendorff’s (36) reagents.

Mild Alkaline Treatment

To obtain the decylated SM-1 (lyso-SM-1), SM-1 was treated with 0.5 N NaOH in 90% methanol at 37°C for 90 min. The hydrolysate was neutralized with 2 N HCl, and then the fatty acid was removed with n-hexane. The resulting lower phase was dialyzed against distilled water for 2 days and lyophilized. The lyophilized sample was subjected to infrared (IR) and fast atom bombardment-mass (FAB-mass) spectrometric analyses.

Chemical Analyses

1) To determine the 1-O-alkyl chains at position 1 of SM-1, lyso-SM-1 (100–500 µg) was dissolved in 3 ml of diethyl ether and 1 ml of 0.1 M Tris-HCl buffer (pH 7.4), and 2.6 mg of phospholipase C (EC 3.1.4.3, Bacillus cereus; Sigma) was added. After continuous stirring at room temperature for 16 h, the diethyl ether layer was separated and the water layer was extracted 3 times with diethyl ether. The diethyl ether extracts were combined and evaporated to dryness. The residue was dissolved in chloroform and applied to an HPTLC plate and developed with petroleum ether/diethyl ether/acetic acid (30:70:1, v/v/v). The spot indicative of glycerol ether was scraped off and extracted with chloroform/methanol (2:1, v/v). Then glycerol ether was trimethylsilylated in a mixture of pyridine, hexa-
methylsilazane and trichloromethylsilane (5:2:1, v/v/v/v) and analyzed by gas-lipid chromatography (GLC) in a Shimadzu GC-14A apparatus with a fused-silica capillary column, ULBON HR-1 (0.32 mm × 30 m), equipped with a flame ionization detector. The column temperature was programmed to increase from 200 to 260°C at a rate of 3°C/min. Peaks were identified by comparing their retention times on GLC with those of trimethylsilyl derivatives of alkyl glyceryl ether (standard; Serdary Research Laboratories, U.S.A.) (28).

2) To determine the fatty acid chain at position 2 of SM-1, SM-1 was treated with 0.1 M sodium methoxide in methanol at 37°C for 1 h. The liberated fatty acid methyl esters were analyzed by GLC (ULBON HR-1 capillary column, 0.32 mm × 30 m). The column temperature was programmed to increase from 180 to 240°C (2°C/min) (41).

**IR Absorption Spectrometry**

IR spectra were measured with a JASCO IR-700 infrared spectrometer equipped with a multifunctional ratio-recording type (Japan Spectroscopic).

**Fast Atom Bombardment-Mass Spectrometry**

FAB-mass spectra were measured by a JEOL JMS-DX304 mass spectrometer equipped with a JMA-DA 5010 computer system. Triethanolamine and nitrobenzylalcohol were used as matrices. Spectra were recorded in a negative ion mode at an accelerating voltage of 3.0 kV.

**RESULTS AND DISCUSSION**

Water-insoluble total phospholipids from the skin, nervous tissue and hemolymph of *A. kurodai* were subjected to two-dimensional thin-layer chromatography and the results are shown in Fig. 1A. The skin and nervous tissue showed similar TLC patterns, but that of hemolymph was slightly different. SM-1 was detected in these three tissues (Fig. 1B). SM-1 was purified from the total phospholipid fraction extracted from the skin, using two solvent systems of silicic acid column chromatography. Fig. 2A shows the elution profile of SM-1 using a continuous gradient of chloroform/methanol/water mixture. Purified SM-1 showed a single spot on

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**Fig. 1** (A) Two-dimensional thin-layer chromatograms of the water-insoluble phospholipid fraction from the skin, nervous tissue and hemolymph of *A. kurodai*. Sample was applied to silica Gel HPTLC plate (Merck; 10 × 20 cm), and developed twice with chloroform/methanol/ammonia water (65:25:4, v/v/v) over a distance of 20 cm in the first dimension, allowed to dry for 2 h and then developed again 4 times with chloroform/methanol/acetone/acetate acid/water (75:15:30:15:7.5, v/v/v/v/v) for 10 cm in the second dimension. PC, phosphatidylcholine; C-AEPn, ceramide 2-aminoethylphosphonate; PE, phosphatidylethanolamine. (B) Thin-layer chromatogram of the water-insoluble phospholipid fractions obtained from the skin, hemolymph and nervous tissue of *A. kurodai*. PC and purified SM-1 are used as standards. Lipids were visualized with Dittmer-Lester's reagent. Solvent system 1 (see Materials and Methods) was used for development. Lane 1, PC; lane 2, hemolymph; lane 3, SM-1; lane 4, skin; lane 5, nervous tissue.
TLC plates with the four solvent systems described in Materials and Methods (data not shown). On TLC plate, SM-1 gave a positive reaction to anthrone-sulfuric acid, Dittmer-Lester's and Dragen-dorff's reagents, but a negative reaction to ninhydrin reagent, indicating the presence of phosphate and choline (Fig. 2B) in its chemical structure. Infrared spectra of SM-1 and lyso-SM-1 in KBr method (300 μg of sample was mixed with 200 mg of KBr) are shown in Fig. 3. SM-1 showed sharp absorption peaks at 1,738 cm⁻¹ and 970 cm⁻¹, characteristic of carbonyl ester and choline, respectively (34). The peak at 1,738 cm⁻¹ disappeared from the IR spectrum when SM-1 was treated with mild alkali (lyso-SM-1). These results indicate the presence of choline phosphate and fatty acid esters in SM-1. Negative-ion-mode FAB-mass spectra of SM-1 and lyso-SM-1 are shown in Fig. 4. The mass
spectrum of lyso-SM-1 (Fig. 4A) shows three high mass ions of m/z 466, 421 and 395 which are formed as a result of the same losses of various portions of the choline moiety that phosphatidylcholine undergoes. Phosphatidylcholines do not yield (M-H)⁻ ions; instead they yield three characteristic high mass ions, (M-CH₃)⁻, [M-HN(CH₃)]⁻ and [M-HN(CH₃)⁴-C₂H₅]⁻ (17). Fragment ions of m/z 466, 421 and 395 are assigned to (M-CH₃)⁻, [M-HN(CH₃)]⁻ and [M-HN(CH₃)⁴-C₂H₅]⁻, respectively. Thus lyso-SM-1 was deduced as an 1-O-alkyl (carbon16)-2-OH-3-phosphocholine. The mass spectrum of SM-1 (Fig. 4B) shows ions attributable to 20:1 (m/z 309), 18:1 (m/z 281) and 16:0 (m/z 255) fatty acids. Four high mass ions of m/z 780, 758, 730 and 704 attributable to (M-CH₃)⁻ consisting of (sn-2; 16/22:4, 16/20:1, 16/18:1 and 16/16:0), respectively, are observed. From these FAB-mass spectral data, we elucidated the structure of SM-1 as shown in Fig. 5.

The fatty chain composition determined by chemical analysis of SM-1 is shown in Table 1. Almost all of the 1-O-alkyl fatty chain consists of 16:0 (15.9%), 18:1 (39.1%), 20:1 (11.2%), 20:4 (10.4%) and 22:4 (7.7%) as major components, and 16:1 (1.5%), 17:1 (3.1%) and 18:0 (3.4%) as minor components. These chemical analyses confirmed the results obtained by FAB-mass spectral data. Fatty chain composition of position 2 of alkylacyl-GPC was studied in rabbit alveolar macrophages (39), sarcoplasmic reticulum (47) and polymorphonuclear neutrophils (24), and human neutrophils (23), eosinophils (31) and platelets (26). Our chemical analysis data agree with those from human
neutrophils, although considerably more 18:2, and less 20:1 and 22:4, were found in human neutrophils. The results also suggest a close relationship between arachidonate metabolism and SM-1 in *Aplysia*, since 10% of the fatty acids are arachidonate. On the other hand, reports of the fatty chain composition of alkylacyl-GPC isolated from lower invertebrates are few; one of these deals with the Japanese oyster *Crassostrea gigas* (19). The contents of alkylacyl-GPC are low in various tissues of mammals. Alkylacyl-GPC has not been detected in the liver, brain, heart and kidney, but has been detected in high amounts in macrophages, neutrophilic and eosinophilic leukocytes, and platelets. Alkylacyl-GPC is stable to phospholipase A1, and the presence of large amounts of alkylacyl-GPC may help to protect biomembranes from lipolytic enzymes. Another possible role of alkylacyl-GPC is to serve as a precursor of PAF. PAF content markedly increased when slugs were subjected to several shock treatments, such as injection of DMSO (40), or when an earthworm, *Eisenia fetida*, was pricked with needle or cut (38). Sugiura et al. (38) found that PAF is widely distributed among various lower animals which have large amounts of alkylacyl-GPC. The presence and possible physiological importance of PAF in various mammalian tissues have been reported by sev-
Table 1  Fatty Chain Distribution of SM-1

<table>
<thead>
<tr>
<th>Fatty chain</th>
<th>Position 1a</th>
<th>Position 2b</th>
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<tr>
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<td>—</td>
<td>7.7</td>
</tr>
<tr>
<td>Others</td>
<td>—</td>
<td>7.9</td>
</tr>
</tbody>
</table>

aThis value is expressed as percentage in 1-O-alkyl fatty chain. bThese values are expressed as percentage in 2-O-acyl fatty chain.

eral investigators (18, 20, 29, 32, 35, 37, 44, 46). Recently, Hattori et al. (15) suggested that PAF and PAF acetylhydrolase may play some role in the development of the cerebral cortex; they isolated the complementary DNA for one isoform (45K subunit) of PAF acetylhydrolase present in bovine cerebral cortex. Sequence analysis revealed a striking homology (99%) of the subunit with a protein encoded by the causative gene (LIS-1) for Miller-Dieker lissencephaly, a human brain malformation characterized by a smooth cerebral surface and abnormal neural migration. This indicates that the LIS-1 gene product is a human homologue of the 45K subunit of intracellular PAF acetylhydrolase.

We observed also large amounts of alkylacyl-GPC (SM-1) in hemolymph of A. kurodai. Alkylacyl-GPC in the hemolymph may lead to the formation of PAF via a deacylation-reacylation pathway. Since the self-defense mechanisms or "immune systems" in invertebrates seem to be attributable to lectin, glycoprotein and another factor of the hemolymph (45), PAF may also be involved in the self-defense mechanisms in hemolymph of A. kurodai. Another aspect of the function and behavior of PAF in lower animals is expected to be clarified in the near future.

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