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Hydrolysis of sphingosylphosphocholine by neutral sphingomyelinases

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Abstract Sphingosylphosphocholine (SPC), the *N*-deacylated form of sphingomyelin (SM), is a naturally occurring lipid mediator. However, little is known about the metabolism of SPC. We here report an in vitro assay system for SPC-phospholipase C (PLC). Using this assay system, we demonstrated that nSMase1 and nSMase2, human neutral sphingomyelinases (SMases), are capable of hydrolyzing SPC efficiently under detergent-free conditions. Bacterial and plasmodial neutral SMases also showed SPC-PLC activity. The substrate specificity of neutral SMases that hydrolyze SM, SPC, and monoradyl glycerophosphocholine, but not diradyl glycerophosphocholine, suggested that a hydrogen-bond donor at the C-2 or *sn*-2 position in the substrate is required for recognition by the enzymes.

Key words: Sphingosylphosphocholine; Sphingomyelin; Lysophospholipid; Phospholipase C; Sphingomyelinase

1. Introduction

Sphingosylphosphocholine (SPC), the *N*-deacylated form of sphingomyelin (SM), exists in cells and blood plasma, although its levels in normal tissues are very low [1–7]. When SPC is exogenously supplied to cells, it elicits various responses including mitogenesis, focal contact assembly, mobilization of Ca^{2+}, activation of a K^+ channel, and apoptosis, depending on concentration and cell type [8]. Recently, SPC has been shown to be a high-affinity ligand for several types of guanosine triphosphate (GTP)-binding protein-coupled receptors [9–12]. Thus, SPC has been accepted as a naturally occurring lipid mediator. However, very little is known about the metabolism of SPC.

Sphingomyelinase (SMase) or SM-phospholipase C (PLC) is the enzyme catalyzing hydrolysis of SM to release phosphocholine and ceramide. After homology searches using known sequences of bacterial SMases, two types of mammalian SMases with pH optima in a neutral pH range were identified: one was named nSMase1 [13] and the other nSMase2 [14]. Subsequently, it has been revealed that nSMase1 exhibits PLC activity toward lysophosphatidylcholine (lysoPtdCho) and lyso platelet-activating factor (lysoPAF) [15]. nSMase2 also shows lysoPAF-PLC activity under detergent-free conditions in vitro, although at a level far below the optimal level of SMase activity of nSMase2 [16]. Moreover, it has been shown that a plasmodial SMase is capable of hydrolyzing lysoPtdCho and lysoPAF, and that the lysoPtdCho-PLC activity is competed by SPC, suggesting that the plasmodial SMase recognizes SPC as well [17]. Nevertheless, it has not been determined whether these SMases are capable of hydrolyzing SPC. This is presumably because of the absence of assay systems for SPC-PLC.

In the present study, we establish an in vitro assay system for SPC-PLC, and demonstrate that various types of neutral SMases catalyze efficient hydrolysis of SPC in vitro. We also discuss the substrate specificity of neutral SMases capable of hydrolyzing SM and lysocholinephospholipids, but not PtdCho or PAF.

2. Materials and methods

2.1. Materials

[Choline-methyl-^{14}C]SM (55 mCi/mmol) and [choline-methyl-^{14}C]PtdCho L-α-dipalmitoyl (55 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. [Methyl-^{14}C]choline (55 mCi/mmol), [methyl-^{14}C]phosphocholine (55 mCi/mmol), 1-*O*-[^{3}H]octadecyl PAF (163 Ci/mmol), and 1-*O*-[^{3}H]octadecyl lysoPAF (163 Ci/mmol) were from Amersham Biosciences, and [palmitoyl-1-^{14}C]lysoPtdCho L-1-monopalmitoyl (55 mCi/mmol) was from NEN Life Science Products. When used for PLC assays, [^{3}H]PAF and [^{3}H]lysoPAF were diluted 100-fold with non-radioactive PAF and lysoPAF (Sigma), respectively.

2.2. Preparation of enzyme sources

Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml of penicillin, and 100 μg/ml of streptomycin at 37°C in a humidified 5% CO_2 incubator. pHisNSM, in which human nSMase1 cDNA was cloned on pcDNA3.1/HisC [15], was generously provided by Dr. Yusuf A. Hannun (Department of Biochemistry and Molecular Biology, Medical University of South Carolina, SC, USA). The human nSMase2 cDNA sequence (GenBank accession number XM007890) was amplified by polymerase chain reactions using a human cDNA library as template, and inserted into *Bam*HI/*Not*I cloning sites of the mammalian expression vector pcDNA3.1(+) (Invitrogen). The resultant plasmid was named phNSM2. HEK293 cells were transiently transfected with pHisNSM, phNSM2, or pcDNA3.1(+) using FuGENE6 reagent (Roche Applied Science). Briefly, on day 1, 1.5×10^6 HEK293 cells were seeded in a 100-mm dish containing 10 ml of culture medium, and cultured overnight. On day 2, the cells were incubated with an appropriate plasmid (4 μg) complexed with 12 μl of FuGENE6 reagent in 8 ml of culture medium overnight. On day 3, the transfected cells were reseeded in a 150-mm dish containing 25 ml of culture medium, and cultured overnight. Then, microsomes were prepared from the cells as described previously [18]. In brief, after washing with phosphate-buffered saline (PBS), the transfected

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Abbreviations: SM, sphingomyelin; SPC, sphingosylphosphocholine; SMase, sphingomyelinase; PLC, phospholipase C; PtdCho, phosphatidylcholine; PAF, platelet-activating factor; HEK, human embryonic kidney; PtdSer, phosphatidylserine; TLC, thin-layer chromatography

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cells were collected in PBS by scraping, and centrifuged (150×*g*, 5 min). The precipitated cells were suspended in 3 ml of HSEI buffer (10 mM HEPES–NaOH, pH 7.5, containing 0.25 M sucrose, 1 mM ethylenediamine tetraacetic acid (EDTA) and a protease inhibitor cocktail), and sonicated three times with a probe-type sonicator at 20 W for 10 s. After centrifugation (1400×*g*, 15 min) of the lysate, the supernatant fluid was subjected to high-speed centrifugation (100 000×*g*, 1 h). The particulate as the microsome fraction was suspended in 600 μl of HSEI buffer at a protein concentration of 3–4 mg/ml. For assays of PLC activity, the microsome fraction was diluted at 50 μg/ml with HSEI buffer. As an enzyme source for plasmodial SMase, membranes of *Escherichia coli* cells expressing the plasmodial SMase as a recombinant fusion protein with glutathione transferase were prepared as described previously [17]. Recombinant *Bacillus cereus* SMase (Higeta Shoyu Co., Tokyo, Japan) was dissolved in 10 mM HEPES–NaOH (pH 7.5) containing 0.25 M sucrose at 1 μg/ml. The prepared samples were stored at −80°C. Protein concentrations of the preparations were determined with the Pierce bicinchoninic acid protein assay kit with bovine serum albumin as the standard.

2.3. Preparation of [choline-methyl-^{14}C]SPC

[Choline-methyl-^{14}C]SM was deacylated as described previously [19] with several modifications. On 20 μl of 50 mM sodium acetate buffer (pH 5.5) containing 40 nmol of [choline-methyl-^{14}C]SM (55 mCi/mmol), 0.2% sodium cholate (Sigma) and 12 milliunits of sphingolipid ceramide *N*-deacylase (Takara Bio Inc., Shiga, Japan), 200 μl of dodecane was layered, and the solution was incubated for 17 h at 37°C. The aqueous phase was evaporated under vacuum, dissolved in chloroform/methanol (2/1, by volume), and subjected to thin-layer chromatography (TLC) (silica gel 60 TLC plate, Merck) with chloroform/methanol/10% acetic acid (5/4/1, by volume) as the developing solvent. After detection of radioactive lipids separated on the TLC plate with a BAS2000 image analyzer (Fuji, Tokyo, Japan), the gel containing radioactive SPC was collected in a glass tube from the plate by scraping. Then, radioactive SPC was extracted from the gel with chloroform/methanol/deionized water (5/4/1, by volume). After centrifugation (1400×*g*, 3 min) to precipitate the gel, the supernatant fraction containing purified radioactive SPC was evaporated under vacuum. The dried sample was dissolved in methanol, and stored at −20°C until use. The radioisotopic specific activity of purified [choline-methyl-^{14}C]SPC was assumed to be identical to that of the precursor [choline-methyl-^{14}C]SM. If necessary, non-radioactive standard SPC separated on a TLC plate was visualized by brilliant blue staining [20]. When used for the SPC-PLC assay, the purified radioactive SPC was diluted two-fold with non-radioactive SPC (Matreya Inc., Pleasant Gap, PA, USA). On the day of assays, appropriate volume of methanolic solution of [choline-methyl-^{14}C]SPC (23 mCi/mmol) was put in a polypropylene tube, and dried by rotary evaporation. Then, dried [^{14}C]SPC was dissolved in deionized water at 20–50 μM by vortex mixing and bath-type sonication (∼5 min at room temperature). Note that SPC is water-soluble.

2.4. Assays of PLC activity with various phospholipids

PLC reactions were started by adding enzyme sources to appropriate assay buffers. For SPC-PLC assays, microsomes (0.5 μg protein) were incubated in 50 μl of 50 mM HEPES–NaOH buffer (pH 7.5) containing 10 mM $MgCl_2$, and 10 μM [choline-methyl-^{14}C]SPC (23 mCi/mmol) in 1.5-ml polypropylene tubes at 37°C for 30 min. The reaction was stopped by addition of 0.8 ml of chloroform/methanol (2/1, by volume) to the tube. After addition of 250 μl of deionized water, the sample was mixed vigorously, and centrifuged (10 000×*g*, 2 min). 400 μl of the aqueous upper phase was transferred to a new tube, and evaporated under vacuum (with heating up to 60°C, if necessary). The dried sample was dissolved in methanol, and subjected to TLC with methanol/0.5% NaCl/28% ammonia (50/50/1, by volume) as the developing solvent. The radioactivity of [^{14}C]phosphocholine produced was analyzed with the BAS2000 image analyzer, followed by radioactive measurements of the product with a liquid scintillation counter. In the experiments shown in Fig. 1B (lanes 5 and 6), standard [^{14}C]phosphocholine and [^{14}C]choline, respectively, were diluted with 400 μl of the aqueous upper phase from mock incubations without the enzyme source or substrate, dried, and applied to TLC as standards.

PLC activity toward other substrates was assayed as described previously [17,21] with minor modifications. Briefly, for assays under detergent-replete conditions, microsomes (0.5 μg protein) were incubated in 50 μl of 50 mM HEPES–NaOH buffer (pH 7.5) containing 10 mM $MgCl_2$, 0.2 mM porcine brain phosphatidylserine (PtdSer) (Avanti Polar Lipid Inc., Alabaster, AL, USA), 0.1% Triton X-100, 0.2% β-octylglucoside, and 10 μM radioactive substrate at 37°C for 30 min. For assays under detergent-free conditions, Triton X-100, β-octylglucoside, and PtdSer were omitted from the assay mixture. In some experiments, *PfNSM*-expressing or mock-transfected *E. coli* membranes (5 μg protein per assay), and recombinant *B. cereus* SMase (10 ng protein per assay) were used as enzyme sources. In all PLC assays, background activity from the enzyme-free controls, in which a vehicle buffer instead of the enzyme source was added, was subtracted from the activities of samples containing enzyme sources.

3. Results

3.1. SPC-PLC activity of nSMase1 and nSMase2

To obtain the substrate for SPC-PLC assays, we prepared [choline-methyl-^{14}C]SPC by deacylation of [choline-methyl-^{14}C]SM with a bacterial sphingolipid ceramide *N*-deacylase (Fig. 1A). Purified [^{14}C]SPC displayed one predominant radioactive spot on TLC (Fig. 1A). The doublet appearance of purified [^{14}C]SPC on TLC is possibly due to heterogeneity in the structure of natural sphingolipid bases, in which sphingosine is a major and dihydrosphingosine a minor component.

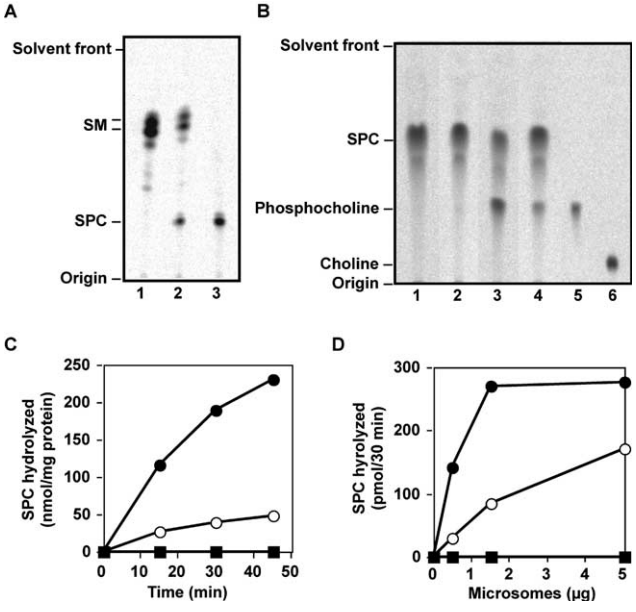

Fig. 1. SPC-PLC activity of nSMase1 and nSMase2. A: Preparation of [choline-methyl-^{14}C]SPC. Lane 1, commercially purchased [choline-methyl-^{14}C]SM; lane 2, [choline-methyl-^{14}C]SM treated with sphingolipid ceramide *N*-deacylase; lane 3, purified [choline-methyl-^{14}C]SPC. Radioactive lipids of each sample were analyzed by TLC with a solvent of chloroform/methanol/10% acetic acid (5/4/1, by volume). B: Hydrolysis of [^{14}C]SPC by nSMase1 and nSMase2. [Choline-methyl-^{14}C]SPC was incubated in 50 mM HEPES–NaOH buffer (pH 7.5) containing 10 mM $MgCl_2$ with 0.5 μg of mock (lane 2), nSMase1 (lane 3) or nSMase2 microsomes (lane 4) or without microsomes (lane 1) for 30 min. Then, water-soluble radioactive compounds were analyzed by TLC with a solvent of methanol/0.5% NaCl/28% ammonia (50/50/1, by volume). Lanes 5 and 6, standard [^{14}C]phosphocholine and [^{14}C]choline, respectively. C: Time course of SPC hydrolysis. [^{14}C]SPC was incubated with 0.5 μg of nSMase1 (closed circles), nSMase2 (open circles), and mock microsomes (closed squares) for the indicated periods of time. D: Enzyme dose dependence of SPC hydrolysis. [^{14}C]SPC was incubated with various doses of nSMase1 (closed circles), nSMase2 (open circles), and mock microsomes (closed squares) for 30 min.

For enzyme sources, we transfected HEK293 cells with a mammalian expression vector encoding human nSMase1 or nSMase2, and isolated microsomes (referred to as nSMase1 or nSMase2 microsomes, respectively). Microsomal preparations from empty vector-transfected cells, referred to as mock microsomes, were used as a control. [^{14}C]SPC was incubated with microsomes under detergent-free conditions, and the release of the radioactive head group from [^{14}C]SPC was analyzed by TLC. Both nSMase1 and nSMase2 microsomes, but not mock microsomes, hydrolyzed SPC to release [^{14}C]-phosphocholine (Fig. 1B). Hydrolysis of SPC by nSMase1 and nSMase2 microsomes was almost linear for up to 30 min (Fig. 1C), and proportional to the dose of enzyme sources up to 1.5 μg of protein (Fig. 1D). The SPC-PLC activity of nSMase1 and nSMase2 was severely inhibited by 0.1% Triton X-100, regardless of the presence of 0.2 mM PtdSer in the assay mixture (data not shown, see also Table 1). TLC analysis of chloroform-soluble lipids of the incubated samples showed that no radioactive SM was produced during the assay (data not shown), ruling out the possibility that [^{14}C]SPC was *N*-acylated to form [^{14}C]SM, from which [^{14}C]phosphocholine could be released by the SM-PLC reaction, during the incubation with microsomes.

3.2. The levels of PLC activity toward various cholinephospholipids

We also determined the PLC activity directed toward SM, PtdCho, PAF, lysoPtdCho, and lysoPAF in the presence or absence of 0.1% Triton X-100 and 0.2 mM exogenous PtdSer (Table 1). For both nSMase1 and nSMase2, the level of activity toward SM was much higher in the presence than in the absence of detergent (Table 1). With detergent in the solution, the levels of PLC activity toward lysoPtdCho, lysoPAF, and SPC were less than 4% of the level of SM-PLC activity. In contrast, under detergent-free conditions, the levels of PLC activity of nSMase1 toward these lysocholinephospholipids were comparable to or higher than the optimal level of SM-PLC activity determined when detergent was present. For nSMase2, the levels of lysoPtdCho- and lysoPAF-PLC activity determined under detergent-free conditions were still less than 10% of the optimal level of SM-PLC activity. Nevertheless, the level of SPC-PLC activity of nSMase2 reached ~16% of the optimal level of SM-PLC activity (Table 1). Exogenous PtdSer (0.2 mM) did not enhance but rather slightly repressed the lysoPtdCho-PLC activity of both nSMase1 and nSMase2 under detergent-free conditions (data not shown), while PtdSer enhanced the SM-PLC activity in the presence of detergents (data not shown; see also [14,16]). In mock microsomes, PLC activity was negligible (Table 1). The observed patterns of SM-, lysoPtdCho-, and lysoPAF-PLC activities of nSMase1 and nSMase2 were consistent with previous studies [13–16].

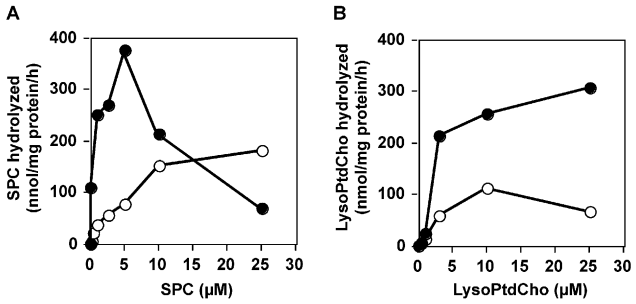

Fig. 2. Hydrolysis of SPC and lysoPtdCho at various concentrations by nSMase1 and nSMase2. Hydrolysis of SPC (A) and lysoPtdCho (B) by nSMase1 (closed circles) and nSMase2 (open circles) were assayed at various substrate concentrations under detergent-free conditions. At each substrate concentration, activity from mock-transfected microsomes was also determined as a background activity, and subtracted from the activities of nSMase1 and nSMase2 microsomes.

To examine affinity for lyso substrates, PLC activity was determined at various concentrations of SPC and lysoPtdCho. For nSMase1, the half-optimal concentrations of SPC and lysoPtdCho were estimated to be ~0.1 and ~3 μM, respectively, while those to SPC and lysoPtdCho for nSMase2 were estimated to be ~5 and ~3 μM, respectively (Fig. 2). These results showed that nSMase1, but not nSMase2, has much higher affinity for SPC than for lysoPtdCho in vitro.

3.3. SPC-PLC activity of a bacterial neutral SMase

Previous studies have shown that bacterial neutral SMases from *Staphylococcus aureus* and *B. cereus* are capable of hydrolyzing lysoPtdCho, but not PtdCho efficiently [22,23]. However, as far as we know, it has not been determined whether bacterial SMase also recognizes SPC-PLC. Because bacterial SMases do not require detergents for efficient hydrolysis of SM unlike mammalian SMases [22,23], we assayed SM-PLC and SPC-PLC activities of purified recombinant *B. cereus* SMase under detergent-free conditions. The level of SPC-PLC activity (29.2 ± 2.2 μmol/mg protein/h, $n = 3$) was comparable to the level of SM-PLC (27.3 ± 2.0 μmol/mg protein/h, $n = 3$).

3.4. SPC-PLC activity of a plasmodial SMase

We recently identified a *Plasmodium falciparum* gene, *PfNSM*, encoding a neutral SMase having structural similarity to mammalian nSMase1 and nSMase2 [17]. Under detergent-free conditions, the *PfNSM* product hydrolyzes lysoPtdCho and lysoPAF, but not PtdCho or PAF [17]. We here

Table 1
PLC activity of nSMase1 and nSMase2 toward various substrates under detergent-replete and detergent-free conditions

Microsomes	Detergent[a]	PLC activity (nmol/mg protein/h)					
		SM	SPC	PtdCho	LysoPtdCho	PAF	LysoPAF
nSMase1	+	367 ± 25	13.9 ± 1.1	< 0.5	< 0.5	< 0.5	4.9 ± 0.8
nSMase1	−	3.4 ± 2.1	434 ± 31	6.5 ± 1.8	611 ± 9	< 0.5	349 ± 31
nSMase2	+	953 ± 25	5.9 ± 2.1	< 0.5	< 0.5	< 0.5	< 0.5
nSMase2	−	15.5 ± 3.9	152 ± 14	1.1 ± 1.0	92.2 ± 1.3	< 0.5	8.6 ± 0.5
Mock	+	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5	< 0.5
Mock	−	< 0.5	< 0.5	< 0.5	0.6 ± 0.1	1.3 ± 0.2	1.7 ± 0.5

[a]Assay buffers contain (+) or do not contain (−) 0.1% Triton X-100 and 0.2 mM PtdSer.

examined its SPC-PLC activity. The membrane fraction of bacteria expressing the *PfNSM* product exhibited significant SPC-PLC activity (1.8 ± 0.7 nmol/mg protein/h; $n = 3$) under detergent-free conditions, while SPC-PLC activity of the control membrane from mock-transfected bacteria was below the detectable level (< 0.2 nmol/mg protein/h). For the *PfNSM*-expressing membrane, SM-PLC activity (assayed under detergent-replete conditions) and lysoPtdCho-PLC activity (assayed under detergent-free conditions) were ~14 nmol/mg protein/h and ~5 nmol/mg protein/h, respectively, being consistent with our previous study [17].

4. Discussion

In the present study, we established a reliable in vitro assay for SPC-PLC with [choline-methyl-^{14}C]SPC as the substrate. This system should also be applicable to the assay of SPC-phospholipase D. Using this assay system, we demonstrated that nSMase1 and nSMase2 are capable of hydrolyzing SPC to release phosphocholine under detergent-free conditions (Fig. 1 and Table 1). The SPC-PLC activity is inhibited by the detergent Triton X-100, whereas detergent is required for the optimal SM-PLC activity (Table 1). The levels of SPC-PLC activity of nSMase1 and nSMase2 reached ~120 and ~16%, respectively, of the optimal levels of SM-PLC activity of these enzymes (Table 1), indicating that, like SM, SPC is an effective substrate for these enzymes, at least, in vitro.

Whereas nSMase1 recognizes SM but not PtdCho or PAF, it efficiently recognizes not only SPC ('lysoSM') but also lysoPtdCho and lysoPAF (Table 1). Similar substrate specificity was observed for nSMase2, although the hydrolysis of lysoPtdCho and lysoPAF by nSMase2 is poor (Table 1). Moreover, *B. cereus* and plasmodial SMases also have similar substrate specificity (this study and [17,22,23]). As depicted in Fig. 3, both amido and amino groups at the C-2 position of SM and SPC, respectively, can serve as hydrogen-bond donors. By contrast, in glycerophospholipids, the ester group at the *sn*-2 position of diradyl forms can not serve as a hydrogen-bond donor, while the hydroxyl group at this position of lyso forms can. These results suggest that a hydrogen-bond donor at this position is required for substrate recognition by the PLCs for SM/lysocholinephospholipids.

The PLC activity toward lysocholinephospholipids (i.e. SPC, lysoPtdCho, and lysoPAF), but not SM, of neutral SMases is severely inhibited by Triton X-100 even at low concentrations (this study and [15–17]). This differential effect of the detergent might be accounted by the aspect of hydrophobic interaction between the enzyme and its lipid substrates. SM has two hydrophobic long chains, while lysocholinephospholipids have one. Hence, hydrophobic interaction of SM with the catalytic site of the enzyme could be strong enough to form a reaction complex even in the presence of detergents affecting hydrophobic interaction, while affinity of the enzyme with lysocholinephospholipids might not be strong enough.

Liliom et al. recently reported that SPC is present in rabbit normal plasma and serum at ~50 and ~130 nM, respectively [1]. They also showed that, in perfused heart preparations from guinea pigs, supplied SPC was rapidly removed from the circulation by an unknown mechanism [1]. SPC is accumulated in tissues of patients with Niemann–Pick disease type A, an inherited disorder characterized by a defect in lysosomal acid SMase [4]. SPC is also significantly accumulated in the skin of atopic dermatitis patients, compared to healthy controls [7], and this accumulation has been suggested to be due to the induction of endogenous or (infected bacteria-derived) exogenous SM deacylase [24]. Abnormally accumulated SPC may be involved in the pathogenicity of these diseases. Thus, the regulation of SPC levels in low concentration ranges could be an important physiological function. nSMase1 is widely expressed in various tissues [13], while nSMase2 is predominantly expressed in the brain [14]. Interestingly, nSMase1 acts as a high-affinity SPC-PLC under detergent-free conditions (Fig. 2). Collectively, we propose the previously unrecognized possibility that the SPC-PLC activity of nSMase1 and/or nSMase2 contributes to the regulation of

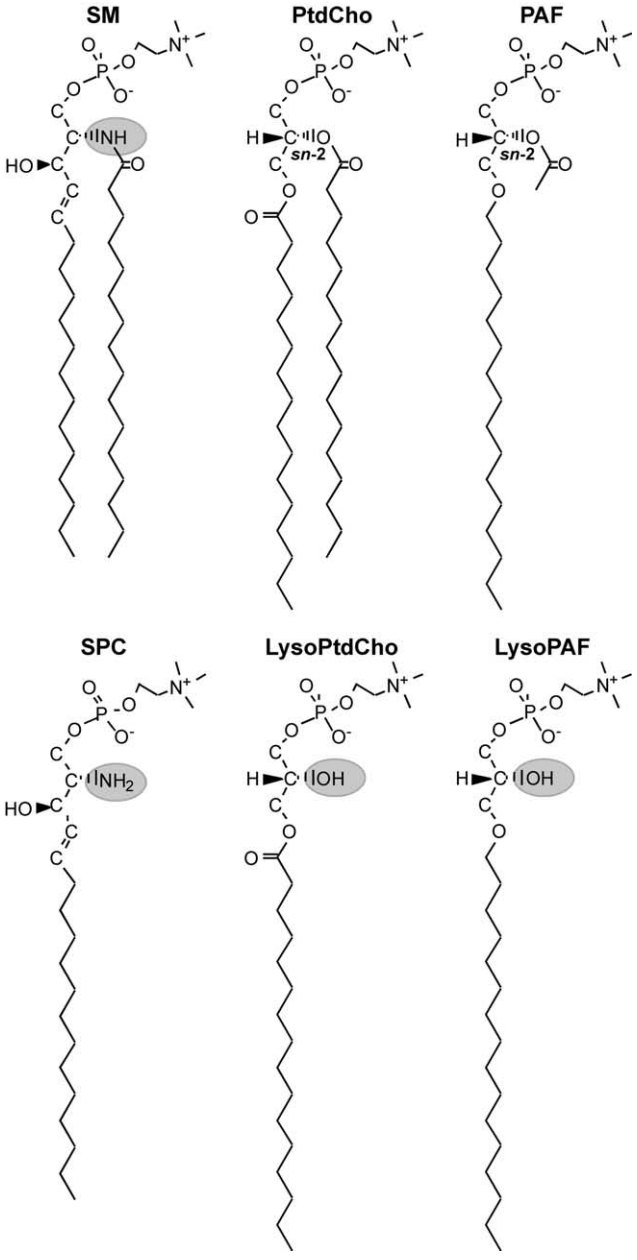

Fig. 3. Structures of choline-containing phospholipids. The groups highlighted by gray ovals are capable of acting as hydrogen-bond donors at the C-2 position of the sphingolipids or at the *sn*-2 position of the glycerolipids.

SPC levels in vivo, although more studies are needed to test this hypothesis.

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