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Novel neurotrophic effects of sphingosylphosphorylcholine in cerebellar granule neurons and in PC12 cells

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Abstract

Sphingosylphosphorylcholine (SPC) is a choline-containing naturally occurring derivative of sphingolipid involved in various biological processes. Here we show that SPC displays neurotrophic effects in cerebellar granule neurons (CGNs) and in PC12 cells. When CGNs were cultured under non-depolarizing condition, they exhibited condensed and fragmented nuclei typical of apoptotic phenotype. SPC added to the culture medium rescued cells from undergoing apoptosis. The anti-apoptotic effect of SPC was dependent on the presence of extracellular Ca^{2+}, suggesting that Ca^{2+} influx occurs upon SPC treatment. In PC12 cells, SPC displayed nerve growth factor-like neuritogenic effect which was sensitive to the presence of Ca^{2+} channel blocker and Ca^{2+} withdrawal from the medium. These results suggest that SPC plays novel neurotrophic effects in the nervous system.

Keywords: Ca^{2+} channel; Cerebellar granule neurons; Lysophosphatidylcholine; PC12; Sphingosylphosphorylcholine

Sphingosylphosphorylcholine (SPC), a choline-containing naturally occurring lysosphingolipid, has been known to be present in normal as well as in pathogenic tissues, although less studied as a signaling molecule compared to related lysolipids such as sphingosine-1-phosphate (S-1-P) and lysophosphatidic acid (LPA) [1]. Initial study has shown that SPC acts as a mitogen in a variety of cell types [2], which was followed by works demonstrating that SPC caused increase in intracellular Ca^{2+} concentration [3–6], cytoskeletal rearrangement [7], and chemotactic cell migration [8–10]. However, unlike structurally related S-1-P, for which the Edg family of G protein-coupled receptor (GPCR) has been identified and well-studied, the signaling pathway elicited by SPC remains largely obscure.

In the nervous system, both physiological and pathological roles of SPC have been suggested. SPC has been shown to increase the synaptic contacts in the cultures of embryonal cerebral cortical neurons [11]. Furthermore, hippocampal cell line HT22 overexpressing an orphan GPCR GPR12 reacted to SPC with an increase in cell proliferation and cell clustering. On the other hand, accumulation of SPC in brains has been reported in Niemann-Pick type A disease [12] which is known to be caused by the deficiency of acid sphingomyelinase. Although the mechanism of accumulation of SPC in the brains of Niemann-Pick disease as well as its relevance to the massive brain dysfunction in the disease remain to be elucidated, it might be due to the toxic effect of high level of SPC in the mitochondrial respiration.

In the course of analyzing the mechanism of neurite-inducing and neuroprotective effects of secretory phospholipase A_2 ($sPLA_2$) in PC12 cells and in cerebellar granule neurons (CGNs), respectively [13–15], we found that

Abbreviations: CGNs, cerebellar granule neurons; HK, high potassium; FCS, fetal calf serum; GPCR, G protein-coupled receptor; LK, low potassium; LPA, lysophosphatidic acid; LPC, lysophosphatidylcholine; NGF, nerve growth factor; PTX, pertussis toxin; S-1-P, sphingosine-1-phosphate; SPC, sphingosylphosphorylcholine; $sPLA_2$, secretory phospholipase A_2.

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lysophosphatidylcholine (LPC), a choline-containing lysophospholipid with structural similarity to SPC, generated by the action of $sPLA_2$ toward the plasma membrane, displays neurotrophic activity [16,17] (Y.I. et al., submitted for publication). Here we show that SPC displays similar neurotrophic effects in both PC12 and CGNs.

Experimental procedures

Materials. SPC (Sigma S4257) and sphingomyelin (SM; Sigma S7004) were dissolved in methanol. LPA (Sigma L7260) and LPC (palmitoyl; Sigma L5254) were dissolved in sterile water. Nicardipine was purchased from Sigma (N7510). Fetal calf serum (FCS) was obtained from Invitrogen.

CGN culture and assessment of cell viability. Dulbecco's modified Eagle's medium (DMEM, high glucose type; Nissui Pharmaceutical, Japan; 05915) was used for the culture of CGNs. Liquid medium was prepared by adding 0.15% $NaHCO_3$, 50 U/ml penicillin, and 100 μg/ml streptomycin sulfate (Meiji Seika, Japan), and the pH was adjusted to 7.3 by 1 N HCl. This medium was referred to as low potassium (LK) medium in this article. High potassium (HK) medium was prepared by addition of KCl to the final concentration of 25 mM. In some cases, Ca^{2+}-free DMEM (Invitrogen 21068) was used.

Cerebellar granule neurons were cultured as follows. Cerebella from 7-day-old mice were dissected by trypsinization and mechanical dissociation and cultured in HK medium containing 10% FCS on the coverslips coated with 0.2% polyethylenimine. Cells were maintained at 37 °C in 10% CO_2 in humidified air. On the next day, the culture medium was replaced with the HK medium containing FCS and 10 μM cytosine arabinoside, and cells were cultured for 48 h to remove proliferating cells. Cells were incubated for another 48 h before being switched to the LK media containing indicated reagents. After the treatment for 24 h, the cells were processed for examination of apoptosis.

Cells fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) were stained with Hoechst 33258 dye (1 μg/ml) for 15 min, washed with PBS, and then observed under the fluorescent microscope (Model BX52; Olympus, Japan) equipped with an SenSys-1401E cooled CCD camera (Roper Scientific, USA). Cells were scored as apoptotic if their nuclei were condensed or fragmented. In general, more than 400 cells in total from four wells were counted for each condition, and the percentages of viable cells were calculated by dividing the number of live cells with the total number of live and apoptotic cells. The data are the mean ± standard deviation of three independent experiments.

MTT-reducing activity of CGNs was measured as follows. After various treatments for 24 h in 96-well plates, cells were further incubated for 4 h in 60 μl of DMEM containing 0.5 mg/ml of MTT. Then cells were lysed by adding 50 μl of lysis buffer (20% SDS, 50% *N*,*N*-dimethylformamide, adjusted to pH 4.7 with 0.5 N HCl, 40% acetate) and were left overnight. Absorbance at 590 nm was measured, and each value is given as the percentage of HK-treated culture. The data are the mean ± standard deviation of three independent experiments.

PC12 cell culture and neurite outgrowth assay. Rat pheochromocytoma PC12 cells were maintained in DMEM supplemented with 5% horse serum and 5% FCS. Cells were passaged every 3–4 days and maintained at 37 °C in 10% CO_2 in humidified air. In a typical neurite-induction experiment, PC12 cells were seeded in the growth medium at 4.5×10^3 cells/cm^2 in collagen type I-coated 24-well culture plates (Becton–Dickinson), allowed to grow for 24 h, and then supplemented with each of the various protein and/or non-protein additives specified in the text. After 24 h, neurite outgrowth was quantified by taking four random photographs/well; cells bearing processes longer than the cell diameter were judged as positive. In the experiment using the Ca^{2+}-free medium, cells were grown in the medium containing 1% FCS and no horse serum was used. The data are the mean ± standard deviation of three independent experiments.

Statistical anaylsis. The data shown are representative of three independent experiments with similar results. Each value is the mean ± standard deviation of triplicate experiments.

Results and discussion

SPC rescues CGNs from apoptosis

When CGNs maintained in the high potassium (HK) medium are shifted to the low potassium (LK) medium, they undergo apoptosis characterized by condensed and fragmented nuclei [18]. Since we found that LPC, a choline-containing lysophospholipid, rescued CGNs from LK-induced cell death (Y.I. et al., submitted), we examined whether structurally related lysophospholipid, SPC, displays a similar neurotrophic activity. As shown in Fig. 1A and 1B, when added at 100 μM to LK, SPC markedly reduced the proportion of cells displaying the apoptotic phenotype and increased cell survival, as assessed by the nuclear morphology. Prevention of apoptosis was further confirmed by an independent assay, MTT assay, in which the mitochondrial activity of live cells was measured (Fig. 1C). Promotion of cell survival by SPC was comparable to that by depolarization (HK) or by LPC, and reached nearly to 90%. In contrast, neither sphingomyelin nor LPA affected the survival of CGNs in LK, indicating that these choline-containing lysophospholipids specifically supported the survival of CGNs.

To examine whether SPC-induced survival of CGNs was dependent on the presence of extracellular Ca^{2+}, as was observed in LPC-induced CGN survival (Y.I. et al., submitted), the effect of SPC was tested in the Ca^{2+}-free medium. As shown in Fig. 2A, in the absence of extracellular Ca^{2+}, depolarization-induced survival of CGNs was significantly reduced (fourth bar from the right). Similarly, SPC-induced cell survival was also compromised in the absence of extracellular Ca^{2+} (second bar from the right). In both cases, cell survival was restored when Ca^{2+} was added back to the Ca^{2+}-free medium (third and first bars from the right), suggesting that SPC induces the influx of Ca^{2+} and activates Ca^{2+} signaling pathway, thereby supports the survival of CGNs.

Since rapid Ca^{2+} mobilization induced by SPC in HL60 leukemia cells or porcine aortic smooth muscle cells was inhibited by treatment of the cells with pertussis toxin (PTX) [4,5], we tested whether SPC-induced survival of CGNs is also sensitive to PTX. As shown in Fig. 2B, potentiation of CGN survival by SPC was not affected by PTX, indicating that the response of CGNs to SPC is mediated through a distinct mechanism.

SPC induces neurite outgrowth in PC12 cells

We then tested whether SPC exhibits neurite-inducing activity in PC12 cells to test whether SPC acts as a neurotrophic factor in different cellular system. As shown in Fig. 3A, SPC added at 50 and 100 μM induced neurites