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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

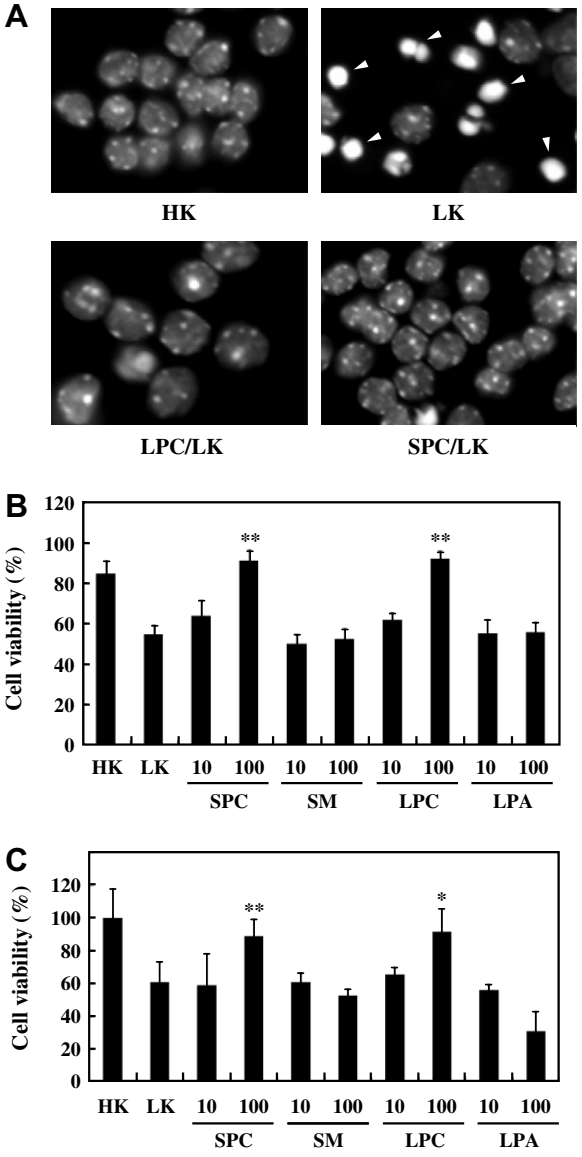

Fig. 1. SPC rescues CGNs from apoptosis. (A) CGNs isolated from 7-day old mice cerebella were cultured for 5 days in the HK medium containing 25 mM KCl. Cells were then switched to the HK medium (HK) or LK medium (LK) without or with added LPC (100 μM; LPC/LK) or SPC (100 μM; SPC/LK) for 24 h. Nuclei were then stained and live/dead cell numbers were counted. Apoptotic nuclei in LK are shown by arrowheads. (B) CGNs displaying live nuclear phenotype were counted and expressed as the percentages of the total number of cells. SPC, LPC, and lysophosphatidic acid (LPA) were added to LK at 10 and 100 μM. Sphingomyelin (SM) was added at 10 and 100 μg/ml. (C) Cell viability was measured by the MTT assay. The MTT-reducing activity of CGNs grown in HK was set at 100%. $^{**}p < 0.01$, $^{*}p < 0.05$, compared with LK by t test.

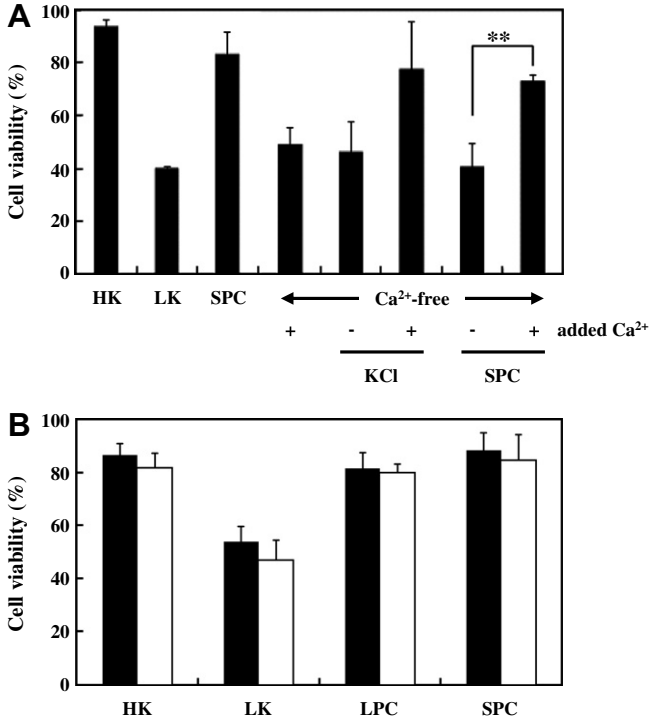

Fig. 2. SPC-mediated CGN survival requires extracellular Ca^{2+}, but is insensitive to PTX. (A) Survival of CGNs were examined by Hoechst staining. From the left: HK, high potassium medium; LK, low potassium medium; SPC, LK containing 50 μM SPC; '+' and '−', Ca^{2+}-free media with or without the supplementation of $CaCl_2$ (2 mM), respectively; KCl and SPC, 25 mM KCl and 50 μM SPC added to the Ca^{2+}-free media, respectively. $^{**}p < 0.01$ by t test. (B) Cell viability was examined in the absence (solid bars) or presence (open bars) of PTX (500 ng/ml). Survival of CGNs were examined by Hoechst staining. LPC and SPC were used at 100 μM.

as was seen in LPC-treated PC12 cells, although the effect less potent than that of LPC. The neurites in SPC- or LPC-treated cells were morphologically straight and less branched compared with those in NGF-treated cells (Fig. 3B). Furthermore, neuritogenesis by SPC was observed at earlier time points than that induced by NGF, at as early as 4 h post-treatment (Fig. 3C). The neurite-inducing effect of SPC was temporal and gradually declined after 10 h, as was observed in LPC-induced neuritogenesis (not shown). This was in contrast to NGF-induced neuritogenesis which continued during the period of examination (24 h).

We next examined the Ca^{2+} requirement of the neuritogenic action of SPC. As shown in Fig. 4A, Ca^{2+}-deprivation from the culture medium abolished the neuritogenic activity of SPC, as was observed in LPC-induced neurite formation (data not shown). Furthermore, nicardipine, an inhibitor of L-type Ca^{2+} channel, attenuated neuritogenesis by SPC, which was also the case with $sPLA_2$- or LPC-induced neuritogenesis (Fig. 4B) [14,17]. Taken together, our results show that SPC acts in a similar manner to LPC and plays neurotrophic roles in CGNs and in PC12, most likely in an L-type Ca^{2+} channel-dependent manner.

SPC has been implicated in a variety of cellular processes, including mitogenic action, intracellular Ca^{2+} mobilization, cytoskeletal reorganization, and chemotactic cell migration [1]. Among these, intracellular increase in $[Ca^{2+}]$ seems to be most relevant to this study. It has been shown that extracellular application of SPC induced cytosolic $[Ca^{2+}]$ rise in various cell types, which was blocked by PTX [3–6]. Removal of extracellular Ca^{2+} did not affect $[Ca^{2+}]$ increase [3,4], but depletion of intracellular Ca^{2+} pool by thapsigargin treatment abolished the $[Ca^{2+}]$ rise

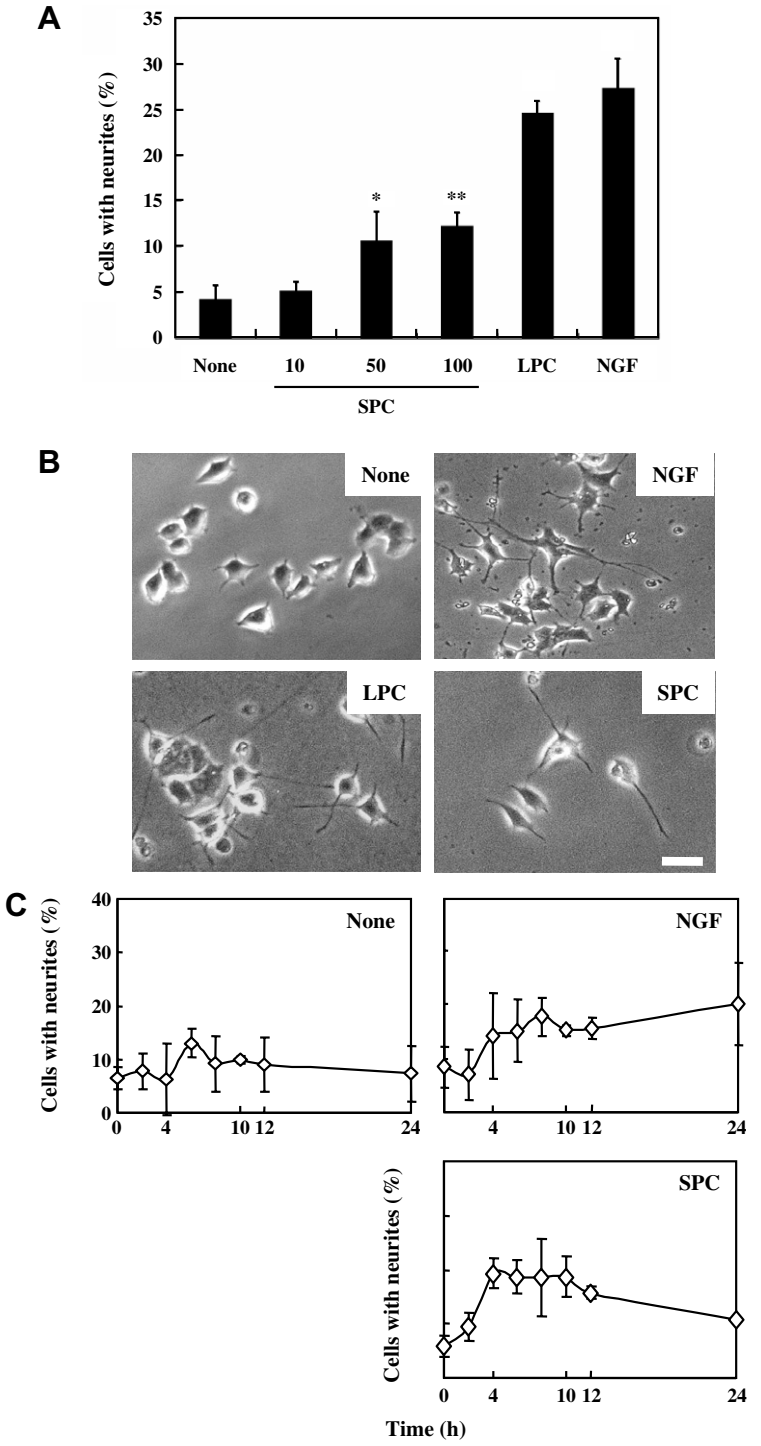

Fig. 3. SPC induces neurite outgrowth in PC12 cells. (A) PC12 cells were incubated for 24 h in the absence ('None') or presence of SPC (10, 50, and 100 μM), LPC (100 μM), or NGF (100 ng/ml) and neurite outgrowth was quantified; cells bearing processes longer than the cell diameter were judged as positive and were expressed as the proportion of the total number of cells. $^{**}p < 0.01$, $^{*}p < 0.05$, compared to 'None' by t test. (B) Morphology of PC12 cells treated for 24 h without ('None') or with NGF (100 ng/ml), LPC (100 μM), or SPC (100 μM). Bar, 50 μm. (C) Time course of neurite induction in the control ('None'), NGF (100 ng/ml)-, or SPC (100 μM)-treated cells. Note that neurite induction by SPC was faster than that by NGF.

response and mitogen-activated protein kinase activation [5]. Thus the origin of increased [Ca^{2+}] in response to SPC treatment is predominantly attributable to G protein-mediated release from the intracellular Ca^{2+} stores. In a different mode of action, experiments using permeabilized cells and microsomes prepared from diverse cell types have shown that SPC has unknown intracellular target(s) and causes release of Ca^{2+} from the internal stores [1]. Both extracellular and intracellular modes of action described above seem to differ from the mechanism of SPC-induced

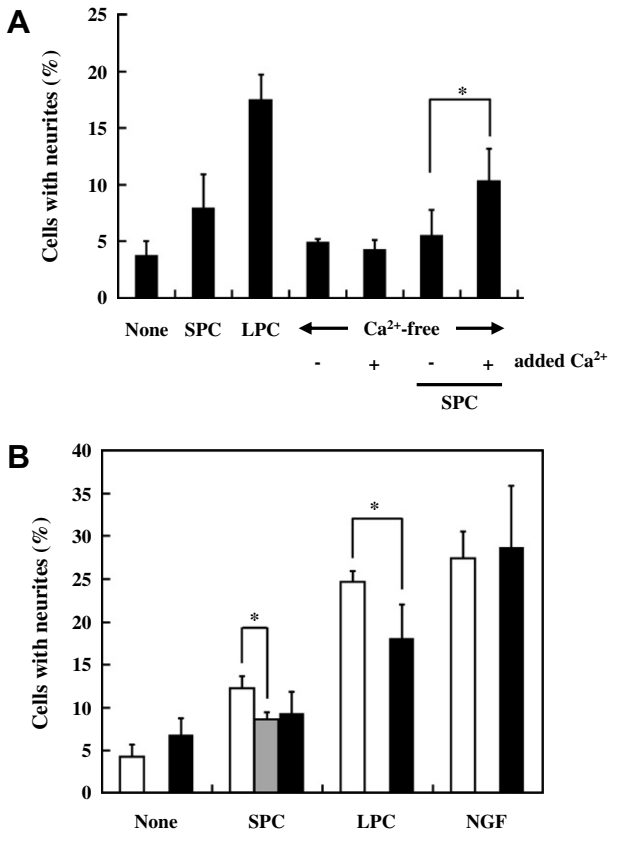

Fig. 4. Requirement of extracellular Ca^{2+} and L-type Ca^{2+} channel activity for SPC-induced neuritogenesis. (A) PC12 cells were treated as indicated for 24 h in the medium containing 1% FCS. From the left; none, control; SPC, 10 μM SPC; LPC, 10 μM LPC; '+' and '−', Ca^{2+}-free media with or without the supplementation of $CaCl_2$ (2 mM), respectively. (B) Nicardipine sensitivity of neuritogenesis by SPC and LPC. 'None', control; SPC, 100 μM SPC; LPC, 100 μM LPC; NGF, 100 ng/ml NGF. Open bars, no addition; gray bar, 5 μM nicardipine addition; solid bars, 50 μM nicardipine addition. $^*p < 0.05$ by t test.

CGN survival and PC12 neuritogenesis, since the latter is sensitive to Ca^{2+}-withdrawal from the medium and, in at least in CGNs, is resistant to PTX treatment (Fig. 2).

The precise action of SPC toward CGNs and PC12 cells remains unclear. The action of SPC shown in this study is in many ways reminiscent of that of LPC. We previously showed that LPC generated by the action of $sPLA_2$ induced neurite outgrowth in PC12 cells [14,16,17]. The neuritogenic response was dependent on the presence of extracellular Ca^{2+} and sensitive to the inhibition of L-type Ca^{2+} channel activity. Although the neuritogenic response to SPC was much weaker compared to that to LPC, the response was reproducible. In addition, the responses by SPC and LPC were both quicker than that by NGF. In contrast, both SPC (this study) and LPC (Y.I. et al., submitted) supported the survival of CGNs to a similar extent in an extracellular Ca^{2+}-dependent manner. These results suggest that SPC and LPC act through a similar mechanism. One possible mechanism of action by SPC and LPC involves OGR1 family of G protein-coupled receptors. In PC12 cells, the neuritogenesis induced by $sPLA_2$ or LPC treatment was dependent on G2A, a member of OGR1 family [17]. In addition, both SPC and LPC induced cell chemotaxis in Swiss 3T3 cells overexpressing GPR4, another member of OGR1 family [10]. Thus, although it is unlikely that these receptors directly interact with SPC and/or LPC, circumstantial evidence supports the possibility that they are indirectly involved in the cellular responses elicited by SPC and LPC. Further studies will be needed to clarify the cellular pathways that lead to the novel neurotrophic role of SPC in the nervous system.

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