Phospholipase A$_2$ (PLA$_2$)\textsuperscript{1} is an enzyme that cleaves sn-2 ester linkage of glycerophospholipids thereby releasing fatty acids and 2-lysophospholipids (1–3). The secreted type of PLA$_2$, sPLA$_2$, is a small (13–20 kDa), Ca$^{2+}$-dependent, disulfide-rich protein composed of extremely diverse members present in venoms, digestive exudates, inflammation sites, various mammalian tissues, and in microorganisms. In mammals, 11 genes encoding distinct sPLA$_2$ isozymes that display overlapping yet distinct tissue distributions have been identified through the extensive genomic search, but the precise roles for each individual isozyme largely remain to be specified. Group IB (sPLA$_2$-IB) and group IIA (sPLA$_2$-IIA) sPLA$_2$s are the two well characterized sPLA$_2$s, known as pancreatic and non-pancreatic/inflammatory sPLA$_2$s, respectively. sPLA$_2$-IB has been thought to be involved in the digestion of dietary phospholipids in the gastrointestinal tract (4), whereas sPLA$_2$-IIA is present in high levels in rheumatoid synovial fluid, and its expression is induced or repressed by pro- or anti-inflammatory stimuli, respectively (5–7). sPLA$_2$-IIA is also enriched in human tears (8, 9). Recently, these sPLA$_2$ isoforms have been implicated in neuronal apoptosis both in vitro and in vivo through the generation of reactive oxygen species generated in the course of arachidonic acid metabolism (10–13). Furthermore, in addition to these biological functions, which are dependent on their enzymatic activity, receptor-mediated actions of sPLA$_2$-IB and sPLA$_2$-IIA have also been proposed (14–16).

Groups X sPLA$_2$ (sPLA$_2$-X) is unique in that it has the prominent ability to liberate free fatty acids, including arachidonic acid, when added exogenously to the culture media of adherent mammalian cells, whereas other groups of sPLA$_2$ do not, except for group V sPLA$_2$ (17, 18). This difference apparently results from the distinct interfacial binding affinity of sPLA$_2$s toward lysophosphatidylcholine (PC)-rich outer leaflet of mammalian plasma membrane, because sPLA$_2$-X binds efficiently to vesicles rich in PC, and sPLA$_2$-IIA exhibits very poor binding affinity for charge-neutral PC-rich vesicles in marked contrast to anionic vesicles and thus displays virtually no enzymatic activity to PC-enriched vesicles (18, 19). The distinct tissue distribution of sPLA$_2$ enzymes, together with the variability in the substrate specificity, further argues for the existence of different physiological functions for each sPLA$_2$ enzyme.

The OGR1 subfamily of G-protein-coupled receptors (GPCRs), OGR1, G2A, GPR4, and TDAG8, displays unique ligand specificity in that they were initially proposed to recognize lysolipid molecules as ligands. OGR1 was shown to bind with high affinity to sphingosylphosphorylcholine (SPC) (20), and G2A and GPR4 were later reported to bind to SPC and lysophosphatidylcholine (LPC) with distinct affinities (21, 22). Galactosylsphingosine (psychosine) was identified as a ligand for TDAG8 (23). Upon binding to the respective receptors, these lysolipid ligands activate various second messenger pathways, including inositol phosphate accumulation, intracellular Ca$^{2+}$ mobilization, and increase or decrease of cAMP content. More recently, however, these receptors have been shown to respond to changes in extracellular pH; Ludwig et al. (24) reported that OGR1 and GPR4 are proton-sensing receptors that accumulate inositol phosphate and cAMP, respectively, in response to acidic pH of the extracellular milieu. Furthermore, they described that SPC and LPC do not exert any effects on the generation of second messengers. Subsequently, pH-dependent activation of G2A (25) and TDAG8 (26, 27) was reported; Mu-
LPC and G2A Mediate sPLA₂-induced Neurites

PC12 Cell Culture and Neurite Outgrowth Assay—Rat pheochromocytoma PC12 cells were maintained in DMEM (Dulbecco’s modified Eagle’s medium, high glucose type, Invitrogen) supplemented with 5% fetal calf serum and 5% horse serum. Cells were passaged every 3–4 days and maintained at 37 °C in 10% CO₂ in humidified air. In a typical neurite-induction experiment, PC12 cells were seeded in the growth medium at 4.5 × 10⁵ cells/cm² in collagen type I-coated 24-well culture plates (BD Biosciences), allowed to grow for 24 h, and then supplemented with each of the various protein and/or non-protein additives specified in the text. When neuritogenesis in G2A-EGFP stable transfectants was examined, DMEM containing 1% fetal calf serum (FCS) and indicated amount of LPC were used. After 24 h, neurite outgrowth was quantified by taking four random photographs/well; cells bearing neurites were counted, and dividing the total counts present in the medium by the sum of the counts measured in the medium and in the corresponding cell lysate; background radioactivity, measured in phospholipase-unsupplemented, control incubations was subtracted from each data point.

Assay of Phospholipase-mediated LPC Release from Cells—PC12 cells grown at 1.0 × 10⁵ cells/cm² in 24-well culture plates were incubated in the presence of [³⁵S]Choline chloride (SPC; S4257), phospholipase B from Vibrio sp. (PLB; P8914), and nicardipine (N7510) were purchased from Sigma. Fatty acid-free bovine serum albumin (FRA424). Silica 60 TLC plates (8 × 1/H9262/H9252/H9242), phospholipase B from Vibrio sp. (PLB; P8914), and nicardipine (N7510) were purchased from Sigma. Lipids (855475P). Methylcarbamyl platelet activating factor C-16 was used as LPC throughout this study unless specified otherwise.

EXPERIMENTAL PROCEDURES

Materials—1-Palmitoyl-sn-glycero-3-phosphocholine (Sigma, L5254) was used as LPC throughout this study unless specified otherwise. 1-Myristoyl-sn-glycero-3-phosphocholine (C14:0; L6629), 1-stearoyl-sn-glycero-3-phosphocholine (Sigma, L5254), lysophosphatidylcholine (LPC), phosphatidylcholine (C18:0; P1418), lysophosphatidylinositol (PLB; P8914), and nicardipine (N7510) were purchased from Sigma. 1-Myristoyl-sn-glycero-3-phosphocholine from Avanti Polar Lipids (855475P). Methylcarbamyl platelet activating factor (PLA₂) from Cayman (catalog no. 60908). Fatty acid-free bovine serum albumin (FRA424). Silica 60 TLC plates (8 × 1/H9262/H9252/H9242), phosphatidylcholine (C18:0; P1418), phospholipase B from Vibrio sp. (PLB; P8914), and nicardipine (N7510) were purchased from Sigma. 1-Myristoyl-sn-glycero-3-phosphocholine from Avanti Polar Lipids (855475P). Methylcarbamyl platelet activating factor C-16 was used as LPC throughout this study unless specified otherwise.

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To generate stable PC12 cell transfectants, pEGFP-N1, pEGFP-G2A, pEGFP-GPR4, and pEGFP-TDAG8 were transfected into PC12 cells using Lipofectamine 2000. At 24 h after transfection, cells were detached and re-plated at a 1:50 dilution into medium containing FCS/DMEM. After 24 h, G418-resistant clones were isolated, amplified, and tested for G2A-EGFP expression and for neurite outgrowth assay.

**Immunoblotting**—PC12 cells were scraped and suspended in SDS sample buffer (125 mM Tris-HCl, pH 6.9, 10% glycerol, 5% mercaptoethanol, 2% SDS, 0.05% bromphenol blue). An equal volume of cell lysate was subjected to SDS-PAGE immediately. SDS-PAGE was performed on 12.5% acrylamide gels under reducing conditions. Western blotting with anti-GFP antibody (1:5,000 dilution, Invitrogen, catalog no. R970-01) was performed according to the standard procedure.

**RNA Interference Experiment**—The oligonucleotides used for expression of short hairpin (sh) RNA were (i) sense 5′-ttgTTCTCCTACAAGCATGACCTTGCTTTCTTTCTGACAGCTGAGCAAGCGTTCTCCTTGATAGA-3′; (ii) sense 5′-ttttGTCGAGCGTCTCCTGTTGTTTCACTTGGTCAGACAGCTGAGCAAGCGTTCTCCTTGATAGA-3′; (iii) sense 5′-ttctggatccaaaaaGTCCTACAGAGAAACGTGCtgacaggaag-AGATGCAGGCTGTCA-3′; and (iv) sense 5′-ttttGTCGAGCGTCTCCTGTTGTTTCACTTGGTCAGACAGCTGAGCAAGCGTTCTCCTTGATAGA-3′. Three independent experiments were performed.

**Statistical Analysis**—The results shown were from one experiment representative of at least two independent experiments, each done in triplicate. Data are presented as means (±S.D.). Differences were analyzed by Student’s t test, and the values of p < 0.05 were taken as significant. The experiments with TLC plates, gels, and blots were carried out at least twice with duplicates, and one representative result is shown.

**RESULTS**

**Group X sPLA₂ Induces Neurite Outgrowth in PC12 Cells via L-type Ca²⁺ Channel Activity**—We previously reported that exogenously added fungal sPLA₂, p15, induces neurite outgrowth in PC12 cells via L-type Ca²⁺ channel activity (32). Interestingly, when neurotgenesis by supernatants of COS1 cells transfected with mouse sPLA₂ was examined, only the sPLA₂ activity containing sPLA₂-X, but not sPLA₂-IB nor sPLA₂-IIA, elicited neurites. To unequivocally show the neurotogenic response of PC12 cells by mammalian sPLA₂s, we prepared purified, recombinant mouse sPLA₂-IB and sPLA₂-IIA produced by the baculovirus expression system. These two sPLA₂s were used together with recombinant human sPLA₂-X (a generous gift from Dr. M. Gelb at the University of Washington) to compare the neurite-inducing activity in PC12 cells. As shown in Fig. 1A, neither sPLA₂-IB nor sPLA₂-IIA induced neurites, whereas sPLA₂-X induced neurite outgrowth to a similar extent to p15. In addition, the neurotogenic effect of sPLA₂-X was abolished by treatment with an inhibitor of L-type Ca²⁺ channel, nicardipine, which was also shown to inhibit p15-mediated neurite outgrowth (Fig. 1B). Similar inhibitory effect on sPLA₂-X-mediated neurite outgrowth was observed with nifedipine, but not with an N-type Ca²⁺ channel inhibitor ω-conotoxin GIVA (data not shown). Interestingly, PLA₂ activity of sPLA₂-X was no greater than that of sPLA₂-IB and sPLA₂-IIA when assayed using [³H]oleic acid-labeled E. coli membrane as a substrate (Fig. 1C). In contrast, oleic acid release from live PC12 cells labeled with [³H]oleic acid was hardly detected by sPLA₂-IIA, and marginally by sPLA₂-IB, in marked contrast to the potent oleic acid release by sPLA₂-X treatment (Fig. 1D). Distinct fatty acid-releasing activity of mammalian sPLA₂s from live cells has already been reported in HEK293 and RBL-28046.
Thus, these results suggest that degradation of membrane phospholipid by mammalian group X sPLA2 from live cells triggers neurite-outgrowth response in an L-type Ca\(^{2+}\)/H11545 channel-dependent manner.

**Involvement of LPC in sPLA2-mediated Neurite Outgrowth**—We previously showed that neither cyclooxygenase nor lipoxygenase inhibitors blocked sPLA2-induced neurite outgrowth (32). In addition, both exogenously added arachidonic and oleic acids failed to induce neurites (data not shown). Instead, we observed that LPC, but not other 1-acyl-lysophospholipids, including lysophosphatidylinositol, lysophosphatidylethanolamine, lysophosphatidylserine, lysophosphatidic acid, and methylcarbamyl platelet activating factor C-16, the platelet activating factor receptor agonist, induced neurite outgrowth when added to the culture medium of PC12 cells. When LPC with different saturated fatty acyl chain length was examined in this assay, we found that C14:0, C16:0, and C18:0 LPC, but not C12:0 LPC, induced neurite outgrowth to a similar extent (Fig. 2A). In addition, LPC-induced neuritogenesis was inhibited by similar doses of nicardipine that inhibit p15-induced neurite outgrowth (Fig. 2B) and by nifedipine, but not by \(\omega\)-conotoxin GIVA (Fig. 2C), suggesting that LPC mediates the neurite-inducing activity of sPLA2.

We next asked whether sPLA2s indeed liberate LPC from PC12 cells. PC12 cells prelabeled with \(^{14}\)C-choline were treated with sPLA2 for 4 h, and the total lipid fractions separately extracted from the culture media or cell lysates were analyzed by TLC. Of the total \(^{3}\)H-choline incorporated to the organic (lipid) fraction, more than 1% was released to the culture media as \(^{14}\)C-LPC upon treatment with p15 (Fig. 3A). No significant generation of \(^{14}\)C-LPC was observed by supernatants of COS1 cells expressing sPLA2-IB nor sPLA2-IIA. The COS1 supernatant containing mouse group V sPLA2 gave variable results, and the LPC release was not consistent. In addition, when the effect of purified human sPLA2-X was examined, generation of \(^{14}\)C-LPC was specifically observed in the medium, but not in the cell-associated...
B (PLB) on sPLA2- or LPC-induced neuritogenesis. BSA has inhibitory effects of bovine serum albumin (BSA) and phospholipase B PLB has been known to bind various lipids, including LPC and inhibit the effects of bioactive lipid mediators, whereas PLB nonspecifically hydrolyzes both sn-1 and sn-2 fatty acyl bonds of phospholipids and lysophospholipids. PC12 cells were incubated with p15, LPC, or NGF in the presence of BSA or PLB for 24 h, and neurite outgrowth was assessed. As shown in Fig. 4 (A and B), both BSA and PLB almost completely blocked p15- and LPC-induced neurite outgrowth. In marked contrast, no inhibition by BSA was observed in NGF or depolarization-induced neurite outgrowth, and PLB treatment also failed to suppress neuritogenesis by NGF, demonstrating that BSA and PLB did not affect cell vitality nor general machinery for neuritogenesis but are specific to signals evoked by sPLA2 and LPC. Taken together, these results demonstrate that the release of LPC fraction, which is reminiscent of the result with p15 (Fig. 3C). Therefore, the LPC-releasing activity of sPLA2 is correlated with the neurite-inducing activity, which was observed in the COS1 supernatant containing sPLA2-X (32). These results strongly suggest that membrane degradation and/or release of LPC is required for the neuritogenic effect of sPLA2.

If sPLA2-induced neuritogenesis of PC12 cells is mediated by LPC release into the culture medium, then it is expected that the sequestration or degradation of LPC would block neurite outgrowth by sPLA2 or LPC. We therefore examined the inhibitory effects of bovine serum albumin (BSA) and phospholipase B (PLB) on sPLA2- or LPC-induced neuritogenesis. BSA has been known to bind various lipids, including LPC and inhibit the effects of bioactive lipid mediators, whereas PLB nonspecifically hydrolyzes both sn-1 and sn-2 fatty acyl bonds of phospholipids and lysophospholipids. PC12 cells were incubated with p15, LPC, or NGF in the presence of BSA or PLB for 24 h, and neurite outgrowth was assessed. As shown in Fig. 4 (A and B), both BSA and PLB almost completely blocked p15- and LPC-induced neurite outgrowth. In marked contrast, no inhibition by BSA was observed in NGF or depolarization-induced neurite outgrowth, and PLB treatment also failed to suppress neuritogenesis by NGF, demonstrating that BSA and PLB did not affect cell vitality nor general machinery for neuritogenesis but are specific to signals evoked by sPLA2 and LPC. Taken together, these results demonstrate that the release of LPC into the culture medium is required for the neuritogenic response of PC12 cells by sPLA2.

G2A Is Involved in Neuritogenic Response of PC12 Cells by sPLA2 and LPC—In the next set of experiments, we analyzed the mode of action of LPC in the neuritogenic response of PC12 cells. It has been reported that the chemotactic effects of LPC in immune cells is mediated by a G-protein-coupled receptor (GPCR), G2A (34, 35), although whether or not LPC acts as a ligand for G2A is controversial. When the expression of G2A and other structurally and functionally related putative receptors for lysolipids, OGR1, GPR4, and TDAG8, was examined by RT-PCR in the mouse brains and PC12 cells, we found that only G2A was expressed in PC12 cells (Fig. 5A, right panel). No amplification product was detected when the reverse transcription reaction was omitted (data not shown). This result led us to hypothesize that G2A mediates the neuritogenic response of sPLA2-s. To test this possibility, we first examined whether LPC, generated by sPLA2 treatment or added directly to the culture medium, activate G2A. Because GPCRs are internalized and down-regulated upon ligand treatment, we tested whether internalization of G2A occurs when cells are treated with sPLA2 or LPC. We transfected G2A-EGFP, in which enhanced green fluorescent protein (EGFP) was fused to the carboxyl terminus of G2A, into Neuro2A cells, which do not express G2A endogenously, and examined its localization after sPLA2 or LPC treatments. As shown in Fig. 5B, fluorescence of G2A-EGFP was mainly observed at the cell periphery, indicating that it localized to the plasma membrane. In contrast, EGFP fluorescence was detected in the intracellular compartment(s), presumably endosomes, when cells were treated with p15 or LPC, but not with lysophosphatidic acid, suggesting that G2A-EGFP was internalized (Fig. 5B, panels c–e). p15- and LPC-induced internalization was not observed with TDAG8-EGFP, another OGR1 family GPCR with distinct ligand specificity (Fig. 5B, panels f–h). This indicates that G2A-EGFP responded to LPC added to the culture medium and LPC generated by membrane degradation by sPLA2.

Next we examined whether the increase in G2A expression in PC12 cells results in enhanced neuritogenic response to sPLA2 and LPC. We established stable transfectants of PC12 cells expressing G2A-EGFP (Fig. 6). Increased expression of G2A was verified by both the RT-PCR (Fig. 6A) and Western blot with an anti-GFP antibody (Fig. 6B). When the transfectants as well as the wild type and EGFP-overexpressing PC12
cells were stimulated with various concentrations of p15 and LPC, we found that neurite outgrowth in cells overexpressing G2A-EGFP was elicited at lower concentrations of p15 (Fig. 6C) and LPC (Fig. 6D) than in control cells (WT and EGFP-overexpressing cells). This effect was G2A-specific, because neither GPR4-EGFP nor TDAG8-EGFP-overexpressing cells exhibited enhanced neuritogenic response to LPC (Fig. 6D). Furthermore, as has been shown for wild type PC12 cells, enhanced neurite-outgrowth response to LPC in G2A-EGFP-overexpressing cells was blocked by nicardipine (Fig. 6E), suggesting that G2A activation is linked to L-type Ca²⁺ channel activation.

Because transgene-independent phenotypes often occur when stable transfectants are generated, we also examined the effect of adenoviral vector-mediated transient expression of G2A-EGFP. PC12 cells were infected with adenoviral vectors containing G2A-EGFP or EGFP alone, and 24 h after infection, cells were treated with or without p15, LPC, depolarization, or NGF (Fig. 7). The transient expression of G2A-EGFP (Fig. 7A) also elicited enhanced neurite outgrowth response at lower doses of p15, as observed in the experiments with the stable transfectants (Fig. 7B). LPC-induced neurite outgrowth was also strengthened (Fig. 7C). In addition, depolarization-induced, but not NGF-induced, neuritogenesis was enhanced (Fig. 7C). These results suggest that production of LPC and subsequent activation of G2A is responsible for the neurotrophic action of sPLA₂ in PC12 cells.

RNA Interference-mediated Suppression of G2A Expression Inhibits Neurite Outgrowth by sPLA₂—We next examined whether RNA interference-mediated chronic inhibition of G2A expression in PC12 cells suppresses sPLA₂-induced neurite outgrowth. We selected three sequences from the rat G2A cDNA (r1, r2, and r3), and generated plasmids that express short hairpin (sh) RNA under the control of mouse U6 promoter. To test the efficacy of these shRNAs in suppressing the expression of G2A, these plasmids was co-transfected with pEGFP-G2A, the expression plasmid for G2A-EGFP, into Neuro2A cells. When the expression of G2A-EGFP was evaluated by semi-quantitative and quantitative RT-PCR experiments (Fig. 8A, upper and lower panels, respectively), we found that r2 displayed the strongest inhibitory effect on the expression of G2A-EGFP, whereas the effects of r1 and r3 were slightly weaker. As a control, we used m1 sequence, which contains single base pair mutation against rat G2A, and found that this shRNA sequence did not show any inhibition. Consistent with the results of RT-PCR experiments, the fluorescence-activated cell-sorting analysis of the same set of Neuro2A cells for EGFP fluorescence demonstrated that r2 exhibited the strong inhibition, whereas r1 and r3 were less effective, and m1 displayed no inhibition (Fig. 8B).

We then tested the inhibitory effect of shRNA sequences on G2A endogenously expressed in PC12 cells. To this end, we employed adenovirus vector-mediated transfer of shRNA sequence, because the transfection efficiency of PC12 cells by available techniques are relatively low (typically <10%). The DNA fragment encoding the U6 promoter, shRNA sequences, and the polyadenylation signal was introduced into the adenovirus vector system (36), and PC12 cells infected with shRNA-expressing adenoviruses were assayed for G2A expression and sPLA₂-induced neurite outgrowth. Consistent with the results obtained with Neuro2A cells, infection of r2 adenovirus resulted in suppression of endogenously expressed G2A, whereas r3 and m1 adenoviruses were ineffective in the RT-PCR experiment (Fig. 8C). Finally, we tested the effect of shRNA-mediated G2A suppression on sPLA₂- and LPC-induced neurite outgrowth. As shown in Fig. 8D, infection of r2 adenovirus resulted in inhibition of p15- and sPLA₂-X-induced neurite outgrowth in PC12 cells. Also, when cells were treated with LPC, r2 adenovirus infection displayed a tendency to reduce the number of neurite-positive cells. This was not due to the nonspecific effect of adenoviral infection, because infection of m1- and GFP-expressing adenoviruses did not cause any recognizable inhibition on neuritogenesis, and depolarization-induced neuritogenesis was not blocked by r2 adenovirus. Interestingly, infection of r2 adenovirus slightly inhibited NGF-induced neuritogenesis, implying that it partially depends on G2A activation.

**DISCUSSION**

In this study, we analyzed the mechanism whereby sPLA₂ induces neurite outgrowth and found that generation of LPC and subsequent activation of G2A were involved based on the following results. First, we compared the neuritogenic activity of mammalian sPLA₂-s and found that sPLA₂-X, but not sPLA₂-IB and sPLA₂-IIA, induced neurites in PC12 cells. The known substrate preference of these sPLA₂-s for PC-rich vesicles (X>>IB, IIA) is in line with the notion that LPC generation is linked to the neuritogenic effect of sPLA₂. Indeed, a significant amount of [14C]LPC was produced only by the treatments with sPLA₂-s with neuritogenic activity (sPLA₂-X and p15).
Second, in the previous and current studies, we showed that LPC added to the culture media induced neurites, but other lysophospholipids and arachidonic/oleic acids were ineffective (32). Third, both sPLA2- and LPC-induced neurite outgrowth was inhibited by similar doses of nicardipine. Fourth, sequestration or degradation of LPC by addition of BSA or PLB, respectively, reduced neuritogenic activity of both sPLA2 and LPC. Fifth, neuritogenic response of sPLA2 and LPC is likely to be mediated by G2A, a GPCR involved in chemotactic response of immune cells toward LPC, because overproduction of G2A by transfection or adenoviral infection of sPLA2 and LPC. Conversely, suppression of endogenous expression of G2A by shRNA caused decreased neuritogenic response by sPLA2 and LPC. Collectively, these results are consistent with the hypothesis that sPLA2-LPC-induced neurite outgrowth involves LPC release and subsequent signal elicited through G2A.

It should be noted, however, that LPC is abundantly present in the blood (37), although no significant neuritogenesis was observed in our “ordinary” culture medium containing 5% FCS and 5% horse serum (without added sPLA2). This apparent discrepancy might be because LPC in the plasma is present mainly in BSA- and lipoprotein-bound forms (38), and these forms of LPC are unavailable for activation of cellular processes that lead to neurite formation. In line with this, the presence of molar excess of fatty acid-free BSA inhibited sPLA2- and LPC-induced, but not NGF-induced, neuritogenesis (Fig. 4), suggesting that carrier-free LPC is required for induction of neurites.

A similar explanation can be given with regard to the considerably high concentration of LPC (100 μM) used to induce neurites in PC12 cells. At 10 μM LPC a small number of neurites was formed, but the response was significantly weaker than that by 100 μM LPC (data not shown). Lowering the serum content of the medium to 1% FCS (with no added horse serum) considerably enhanced neurite formation at 10 μM LPC, which was close to the extent of neuritogenesis by 100 μM LPC in the medium routinely used for PC12 cell culture (Fig. 6D). This suggests that carrier-free LPC is required for induction of neurites.

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The decrease in LPC content; neurite outgrowth typically takes 8–12 h to be detected, whereas in other studies were exposed to LPC for the maximum period of 2 h (in chemotaxis assays) in the serum-free medium. Although we do not know the actual concentration of LPC released from cells by sPLA₂ treatment, ~1% of total [¹⁴C]choline in the organic fraction was converted to [¹⁴C]LPC (Fig. 3); we speculate that this amount is much smaller than expected from the concentration of exogenously added LPC (100 µM) required to induce neurites. When PC12 cells were treated with sPLA₂, however, continuous generation of LPC at low but sufficient levels to activate G2A (Fig. 5B) and stimulate neuritogenesis might have occurred in proximity to the plasma membrane. This speculation is supported by our observation² that fungal p15 was fully active even after 24-h incubation in the ordinary culture medium of PC12 cells. Thus, although the possibility that nonspecific or detergent-like effects of LPC (40), especially at high doses, have induced neuritogenesis cannot be ruled out, our results showing that sPLA₂ treatment and LPC addition exhibited similar effects, including the sensitivity to nicardipine, BSA, and PLB, along with the inhibitory effect of G2A suppression on responsiveness to both sPLA₂ and LPC, strongly argue in favor of the specific neuritogenic effect of LPC.

Although it is unlikely that LPC directly binds to G2A, previous studies have shown that G2A mediates the actions of LPC. For example, G2A is required for the chemotaxis of immune cells and peritoneal macrophages toward LPC (34, 35). In these cells, genetic ablation of G2A or RNA interference-mediated suppression of G2A expression resulted in the loss of chemotactic capability to LPC, indicating that G2A is involved in this process. G2A-dependent chemotactic response of macrophage cell line was prominent toward C16:0, C18:0, and C18:1 LPC, whereas 50% less number of cells migrated toward C14:0 LPC (35). We observed similar (but not identical) tendency in the neuritogenic ability of LPC (Fig. 2A): LPC with acyl chains longer than C14:0 induced neurites, whereas C12:0 LPC displayed no neuritogenic activity. This similarity further supports the hypothesis that LPC-induced neurite outgrowth is dependent on G2A activation.

Other studies have demonstrated distinct actions of LPC, which are dependent on G2A: LPC treatment augments the apoptosis of HeLa cells overexpressing G2A (39), and anti-G2A antibody attenuated the protective effect of LPC against sepsis-induced lethality in mice (41). In addition, a recent report (25) has demonstrated that LPC antagonizes the low pH-dependent activation of G2A. Thus, our results showing that sPLA₂ and LPC addition induced suppression of G2A expression resulted in the loss of responsiveness to both sPLA₂ and LPC, strongly argue in favor of the specific neuritogenic effect of LPC.

Recently, it has been reported that OGR1 and GPR4 are proton-sensing GPCRs that are activated at acidic pH (24). Likewise, Murakami et al. (25) reported that G2A is also an acid pH-activated GPCR that accumulates inositol phosphate upon exposure to low pH condition. Interestingly, activation of G2A at low pH was antagonized by LPC. Furthermore, structurally similar GPCR, TDAg8, has also been reported to respond to acidic pH (26, 27). Wang et al. reported that the pH response of TDAg8 was antagonized by its ligand, psychosine, which also attenuated the activation of OGR1 and GPR4 at low pH. Thus OGR1 subfamily GPCRs seem to display dual speci-
ficiency toward proton and their ligands. Distinct cellular responses of PC12 cells observed in the present and the previous studies (25) in LPC and G2A-mediated cellular effects are not mutually exclusive, because inositol phosphate generation and neuritogenesis occurred at substantially different time scales. In addition, LPC-induced neurite outgrowth demonstrated in this study was unlikely to be due to the pH-dependent activation of G2A, because no change in the pH of the culture medium was detected upon supplementation of LPC at 100 μM. In this regard, it would be of interest to examine the effect of pH on the neuritogenesis in PC12 cells, which is currently under study. The presence of OGR1 subfamily GPCRs, especially G2A, in the brain suggest yet-to-be-identified roles of sPLA2 and LPC (and possibly proton) in the neuronal development and function.

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REFERENCES

1. Murakami, M., and Kudo, I. (2001) Adv. Immunol. 77, 163–194
2. Kudo, I., and Murakami, M. (2002) Prostaglandins Other Lipid Mediat. 68–69, 5–8
3. Murakami, M., and Kudo, I. (2004) Biol. Pharm. Bull. 27, 1158–1164
4. Richmond, B. L., Boliac, A. C., Zheng, S., Huggins, K. W., Granholm, N. A., Tse, P., and Hui, D. Y. (2001) Gastroenterology 120, 1195–1202
5. Kramer, R. M., Hessien, C., Johansen, B., Hayes, G., McClary, P., Chow, E. P., Tizard, R., and Pepinsky, R. B. (1989) J. Biol. Chem. 264, 5768–5775
6. Oka, S., and Arita, H. (1991) J. Biol. Chem. 266, 9556–9960
7. Nakano, T., Ohara, O., Terasaki, H., and Arita, H. (1990) J. Biol. Chem. 265, 12745–12748
8. Qu, X. D., and Lehrer, R. I. (1998) Infect. Immun. 66, 2791–2797
9. Foreman-Wykert, A. K., Weiss, J., and Elsabah, P. (2000) Infect. Immun. 68, 1259–1264
10. Yamagi, T., Ueda, K., Asakura, K., Hata, S., Kuroda, T., Sakaeda, T., Takasu, N., Tanaka, K., Gembba, T., and Hori, Y. (2002) Mol. Pharmacol. 61, 114–126
11. Yamagi, T., Ueda, K., Asakura, K., Hayasaka Kajiwara, Y., Nakazato, H., Sakaeda, T., Hata, S., Kuroda, T., Takasu, N., and Hori, Y. (2002) J. Neurochem. 81, 449–461
12. Yamagi, T., Ueda, K., Asakura, K., Sakaeda, T., Hata, S., Kuroda, T., Sakauchi, G., Itoh, N., Hashimoto, Y., and Hori, Y. (2003) Brain Res. 980, 71–80
13. Yamagi, T., Ueda, K., Asakura, K., Nakazato, H., Hata, S., Kuroda, T., Sakaeda, T., Sakauchi, G., Itoh, N., Hashimoto, Y., and Hori, Y. (2003) J. Neurochem. 85, 749–758
14. Lambeau, G., and Lazzunski, M. (1999) Trends Pharmacol. Sci. 20, 162–170
15. Hanasaki, K., and Arita, H. (2002) Prostaglandins Other Lipid Mediat. 68–69, 71–82
16. Hanasaki, K. (2004) Biol. Pharm. Bull. 27, 1165–1167
17. Bezzine, S., Koduri, R. S., Valentin, E., Murakami, M., Kudo, I., Ghomashchi, F., Sadilek, M., Lambeau, G., and Gelb, M. H. (2000) J. Biol. Chem. 275, 3179–3191
18. Singer, A. G., Ghomashchi, F., Le Calvez, C., Bollinger, J., Bezzine, S., Rouault, M., Sadilek, M., Nguyen, K., Lazzunski, M., Lambeau, G., and Gelb, M. H. (2002) J. Biol. Chem. 277, 48535–48549
19. Bezzine, S., Bollinger, J. G., Singer, A. G., Veatch, S. L., Keller, S. L., and Gelb, M. H. (2002) J. Biol. Chem. 277, 48523–48534
20. Xu, Y., Zhu, K., Hong, G., Wu, W., Baudhuin, L. M., Xiao, Y., and Damron, D. S. (2000) Nat. Cell Biol. 2, 261–267
21. Kabarowski, J. H., Zhu, K., Le, L. Q., Witte, O. N., and Xu, Y. (2001) J. Biol. Chem. 276, 41325–41335
22. Zhu, K., Baudhuin, L. M., Hong, G., Williams, F. S., Cristina, K. L., Kabarowski, J. H., Witte, O. N., and Xu, Y. (2001) J. Biol. Chem. 276, 702–705
23. Im, D. S., Heise, C. E., Nguyen, T., O'Dowd, B. F., and Lynch, K. R. (2001) J. Cell Biol. 153, 429–434
24. Ludwig, M. G., Yanek, M., Guerini, D., Gasser, J. A., Jones, C. E., Junker, U., Hofstetter, H., Wolf, R. M., and Seuwen, K. (2003) Nature 425, 93–98
25. Murakami, N., Yokomizo, T., Okuno, T., and Shimizu, T. (2002) J. Biol. Chem. 279, 42484–42491
26. Wang, J. Q., Kon, J., Mogi, C., Tsubo, M., Damir, A., Sato, K., Komachi, M., Malchinkhun, B., Murata, N., Kimura, T., Kowabara, A., Wakahatsu, S., Koizumi, H., Uede, T., Trujimoto, G., Kurose, H., Sato, T., Harada, A., Misawa, N., Tomura, H., and Okajima, F. (2004) J. Biol. Chem. 279, 45026–45033
27. Ishii, S., Kihara, Y., and Shimizu, T. (2005) J. Biol. Chem. 280, 9083–9087
28. Wakatsuki, S., Arioka, M., Dohmae, N., Takio, K., Yamashiki, M., and Kitamoto, K. (1999) J. Biochem. 126, 1151–1160
29. Wakatsuki, S., Yokoyama, T., Nakashima, S., Nishimura, A., Arioka, M., and Kitamoto, K. (2001) Biochim. Biophys. Acta 1522, 74–81
30. Nakashima, S., Wakatsuki, S., Yokoyama, T., Arioka, M., and Kitamoto, K. (2003) Biochim. Biophys. Acta 1623, 1193–1202
31. Nakashima, S., Kitamoto, K., and Arioka, M. (2004) Brain Res. 1015, 207–211
32. Nakashima, S., Ikemoto, Y., Yokoyama, T., Kuwana, M., Bolchi, A., Onotello, S., Kitamoto, K., and Arioka, M. (2003) Biochem. J. 376, 655–666
33. Imai, Y., Matsushima, Y., Sugimura, T., and Terrada, M. (1991) Nucleic Acids Res. 19, 2785
34. Rud, C. G., Yang, L. V., Riedinger, M., Au, M., and Witte, O. N. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 245–250
35. Yang, L. V., Rud, C. G., Wang, L., Riedinger, M., and Witte, O. N. (2005) Blood 105, 1127–1134
36. Price, L. S., Langeslag, M., ten Klooster, J. P., Hofstetter, H., Wolf, R. M., and Seuwen, K. (2003) Blood 102, 265–267
37. Croset, M., Brossard, N., Polette, A., and Lagarde, M. (2000) Biochem. J. 345, 61–67
38. Pin, P., and Ye, R. D. (2003) J. Biol. Chem. 278, 14379–14386
39. Jalink, K., van Corven, E. J., and Moolenaar, W. H. (1990) J. Biol. Chem. 265, 12232–12239
40. Yan, J. J., Jung, S. J., Lee, J. E., Lee, J., Huh, S. H., Kim, H. S., Jung, K. C., Cho, J. Y., Nam, J. S., Suh, H. W., Kim, Y. H., and Song, D. K. (2004) Nat. Med. 10, 161–167
41. Soga, T., Ohishi, T., Matsui, T., Sato, T., Matsumoto, M., Takasaki, J., Matsumoto, S., Kamohara, M., Hiyama, H., Yoshida, S., Monose, K., Ueda, Y., Matsumoto, H., Kohb, M., and Furuschi, K. (2005) Biochem. Biophys. Res. Commun. 326, 744–751