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Secretory Phospholipases A_2 Induce Neurite Outgrowth in PC12 Cells through Lysophosphatidylcholine Generation and Activation of G2A Receptor*

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We previously demonstrated that secretory phospholipase A_2 ($sPLA_2$) and lysophosphatidylcholine (LPC) exhibit neurotrophin-like neuritogenic activity in the rat pheochromocytoma cell line PC12. In this study, we further analyzed the mechanism whereby $sPLA_2$ displays neurite-inducing activity. Exogenously added mammalian group X $sPLA_2$ ($sPLA_2$-X), but not group IB and IIA $sPLA_2$s, induced neuritogenesis, which correlated with the ability of $sPLA_2$-X to liberate LPC into the culture media. In accordance, blocking the effect of LPC by supplementation of bovine serum albumin or phospholipase B attenuated neuritogenesis by $sPLA_2$ or LPC. Overproduction or suppression of G2A, a G-protein-coupled receptor involved in LPC signaling, resulted in the enhancement or reduction of neuritogenesis induced by $sPLA_2$ treatment. These results indicate that the neuritogenic effect of $sPLA_2$ is mediated by generation of LPC and subsequent activation of G2A.

Phospholipase A_2 (PLA_2)[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 and is the principal bactericidal factor against Gram-positive bacteria (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 phosphatidylcholine (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-

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[1] The abbreviations used are: PLA_2, phospholipase A_2; $sPLA_2$, secretory PLA_2; BSA, bovine serum albumin; FCS, fetal calf serum; GPCR, G-protein-coupled receptor; LPC, lysophosphatidylcholine; NGF, nerve growth factor; PC, phosphatidylcholine; PLB, phospholipase B; shRNA, short hairpin RNA; SPC, sphingosylphosphorylcholine; DMEM, Dulbecco's modified Eagle's medium; GFP, green fluorescent protein; EGFP, enhanced GFP; RT, reverse transcription.

rakami *et al.* (25) showed that previously proposed ligand of G2A, LPC, inhibited pH-dependent accumulation of inositol phosphate in cells expressing G2A, whereas Wang *et al.* (26) described that cAMP generation in TDAG8-expressing cells was inhibited by psychosine. The latter authors also demonstrated that psychosine acts antagonistically to the pH-dependent generation of second messengers in GPR4- or OGR1-expressing cells. Thus, although the controversial results remain to be reconciled, they raise the possibility that OGR1 subfamily receptors can respond to proton in addition to lysolipid ligands, which act either agonistically or antagonistically depending on the experimental systems employed.

Our previous work with fungal, bacterial, and bee venom sPLA$_2$s has demonstrated that sPLA$_2$s display neurotrophin-like neurite-inducing activity in PC12 cells (28–31). Unlike nerve growth factor (NGF), neuritogenesis of PC12 cells by sPLA$_2$s was insensitive to tyrosine kinase inhibitor K-252a. Conversely, inhibition of L-type Ca^{2+} channel or depletion of extracellular Ca^{2+}, which were ineffective in blocking NGF-induced neuritogenesis, inhibited sPLA$_2$-induced neurite outgrowth. Our subsequent work demonstrated that sPLA$_2$-induced neuritogenesis requires the activation of src and ras proteins and is accompanied by the activation of mitogen-activated protein kinase cascade (28). The enzymatic activity of sPLA$_2$s was required for induction of neurites, although direct addition of arachidonic acid (as well as oleic acid) failed to induce neurites. Also, inhibitors of cyclooxygenase and lipoxygenase did not attenuate the neuritogenic activity of sPLA$_2$. These results suggest that arachidonic acid release and subsequent conversion to eicosanoids is not involved in the neuritogenesis of PC12 cells. In contrast, we found that lysophosphatidylcholine (LPC), but not other lysophospholipids, induced neurite formation, suggesting that sPLA$_2$ induces neurites via LPC generation (32).

In this study, we first compared the neurite-inducing activity of a subset of sPLA$_2$s and found that sPLA$_2$-X exhibits potent neuritogenic activity as did the sPLA$_2$s from other species. We then demonstrated that LPC was actually generated by sPLA$_2$ treatment. To elucidate the mode of action of LPC, we modified the expression of G2A, a GPCR involved in LPC signaling, and found that the expression level of G2A correlates with the sensitivity of PC12 cells toward sPLA$_2$ or LPC treatments. Collectively, these results strongly suggest that neuritogenic action of sPLA$_2$ is mediated by LPC generation and subsequent activation of G2A.

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 (C18:0; P1418), lysophosphatidylinositol (L7635), lysophosphatidylethanolamine (L4754), lysophosphatidylserine (L3401), and 1-oleyl-*sn*-glycero-3-phosphate (L7260), sphingosylphosphorylcholine (SPC; S4257), phospholipase B from *Vibrio* sp. (PLB; P8914), and nicardipine (N7510) were purchased from Sigma. 1-Lauroyl-*sn*-glycero-3-phosphocholine (C12:0) was from Avanti Polar Lipids (855475P). Methylcarbamyl platelet activating factor C-16 was from Cayman (catalog no. 60908). Fatty acid-free bovine serum albumin (BSA) was from Wako (017-15416). [^{3}H]Oleic acid (9,10-^{3}H-labeled, 15 Ci/mmol) was from PerkinElmer Life Sciences (NET-289). [*methyl*-^{14}C]Choline chloride (50 mCi/mmol) was from Amersham Biosciences (CFA424). Silica 60 TLC plates (8 × 8 cm) were from Merck.

Production of carboxyl-terminally hemagglutinin- and hexahistidine-tagged mouse sPLA$_2$-IB and sPLA$_2$-IIA (32) was conducted by using the baculovirus expression system. Proteins were purified with the nickel-nitrilotriacetic acid-agarose chromatography. Recombinant human group X sPLA$_2$ was a generous gift from Dr. M. Gelb at the University of Washington (17). Preparation of recombinant p15 and phospholipase activity assay were described previously (32).

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% horse serum and 5% fetal calf serum. 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 (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 were 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 processes longer than the cell diameter were judged as positive. The data are mean ± S.D. of at least two independent experiments.

[^{3}H]Oleic acid release from live PC12 cells was determined as described (32). The percent oleic acid release was calculated by 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 × 10^5 cells/cm^2 in 24-well culture plates were incubated for 24 h in the presence of [^{14}C]choline chloride (0.75 μCi/ml) in 400 μl of DMEM containing sera. After washing three times with pre-warmed phosphate-buffered saline, cells were treated with sPLA$_2$ and PLB for 4 h at 37 °C. When the culture supernatants of COS1 cells transfected with each sPLA$_2$ construct were examined, they were adjusted to 5% fetal calf serum and 5% horse serum by 2-fold dilution with fresh DMEM containing 10% horse serum, and were applied to the PC12 cell culture. Culture media were then recovered, and cells were washed and suspended in 400 μl of phosphate-buffered saline. Lipids were extracted with 600 μl of chloroform/methanol (1:2, v/v), and the organic phase was separated by silica TLC, using chloroform/methanol/acetic acid/water (60:30:8:5, v/v). Radioactive lipids were visualized and quantified using a fluorescence image analyzer (FLA3000, Fuji).

RT-PCR—The expression of GPCR mRNA in the mouse brains and PC12 cells were examined by RT-PCR. Total RNA (15 μg) extracted from the mouse brains at various stages of development or PC12 cells 24 h after seeding was reverse-transcribed with oligo(dT$_{16-30}$) primer and PowerScript™ reverse transcriptase (Clontech, catalog no. 8460-1). An aliquot of cDNA corresponding to 1 μg of total RNA was used as a template for the PCR reaction (94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min; then 72 °C for 3 min) using the specific primer sets corresponding to GenBank™ sequence of mouse G2A (G2As1: 5′-GGTGACTGCTTACATCTTCTTCTGC-3′ and G2Aas1: 5′-CTGTGTGGATTCTGGACACTTCTTG-3′), OGR1 (5′-TCTGGCCCAAAGATGGGGAACATCA-3′ and 5′-AGCCCACGCTGATGTAAATGTTCTC-3′), GPR4 (5′-ATATCAGCATCGCCTTCCTGTGCTG-3′ and 5′-CAGCCACACAATTGAGGCTGGTGAA-3′), TDAG8 (5′-TGGACTTTCTCTCCCACCTTGTGCA-3′ and 5′-AGTACAGAATGGGATCGGCAACACA-3′), β-actin (5′-GTGGGCCGCTCTAGGCACCAA-3′ and 5′-CTCTTTGATGTCACGCACGATTTC-3′), and glyceraldehyde-3-phosphate dehydrogenase (5′-GACCACAGTCCATGCCATCACT-3′ and 5′-TCCACCACCCTGTTGCTGTAG-3′).

cDNA Cloning and Isolation of Stable Transfectants—Rat G2A, mouse GPR4, and TDAG8 cDNAs were amplified by PCR using *Pfx* DNA polymerase and cDNA libraries from PC12 cells (G2A) and mouse fetal brain (GPR4 and TDAG8) as templates. The following oligonucleotides were utilized as amplification primers: 5′-tcgcaagcttATGAGATCAGAACCTACCAA-3′ and 5′-tatgaattcGGCAGAGCTCGTCAGGCAGTC-3′ for G2A; 5′-atgtaagcttATGGACAACAGCACGGGCAC-3′ and 5′-tatgaattcGCTGTGCCGGGGGCAGCAGCA-3′ for GPR4; 5′-cgctaagcttATGGCGATGAACAGCATGTG-3′ and 5′-tatgaattcCGTCTATAATCTCTAATTCTA-3′ for TDAG8. Capital letters correspond to the coding regions, and lowercase underlined sequences indicate the HindIII and EcoRI sites used for cDNA cloning. Amplified cDNA fragments were digested with HindIII and EcoRI, and ligated to the pEGFP-N1 (Clontech), generating pEGFP-G2A, pEGFP-GPR4, and pEGFP-TDAG8. For localization studies, we used Neuro2A cells because the internalization of EGFP-tagged GPCRs were more easily detected in this cell line than in PC12 cells. Neuro2A cells were transfected with these plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction. After 24 h, cells were starved for additional 24 h in serum-free medium and were subjected to various treatments in the final 2 h of incubation. They were then fixed in 4% paraformaldehyde/phosphate-buffered saline and observed by fluorescence microscopy (BX52, Olympus, Tokyo, Japan).

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 1000 μg/ml G418 (Invitrogen). After 12 days, 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 lysates 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) r1 sense, 5′-tttGTCCTACAAAGGAACGTGCcttcctgtcaGCACGTTTCTCTGTAGGACtttttggatc-3′; r1 antisense, 5′-ctaggatccaaaaaGTCCTACAGAGAAACGTGCtgacaggaagGCACGTTCCTTTGTAGGA-3′; (ii) r2 sense, 5′-tttGTGACAGCCTGCATCTTCTcttcctgtcaAGAAGATGTAAGCTGTCACtttttggatc-3′; r2 antisense, 5′-ctaggatccaaaaaGTGACAGCTTACATCTTCTtgacaggaagAGAAGATGCAGGCTGTCA-3′; (iii) r3 sense, 5′-tttGCTCAGTAATAGTCTGAGCcttcctgtcaGCTCAGGCTATCACTGAGCtttttggatc-3′; r3 antisense, 5′-ctaggatccaaaaaGCTCAGTGATAGCCTGAGCtgacaggaagGCTCAGACTATTACTGAG-3′; and (iv) m1 sense, 5′-tttGAGTAGTTCTGATGGTAGTgtgtgctgtccACCACCACCAGGACCACTCtttttggatc-3′; m1 antisense, 5′-ctaggatccaaaaaGAGTGGTCCTGGTGGTGGTggacagcacacACTACCATCAGAACTACT-3′. The central small letters indicate the 10- to 11-bp loop sequences. Underlined sequences are substitutions introduced in the sense strand of shRNA to stabilize the double strand RNA. Italicized nucleotides in m1 indicate mismatches at the single position to the rat G2A mRNA sequence. These oligonucleotides were dissolved in TE (10 mM Tris, 1 mM EDTA, pH 8.0) at 250 μM, and 20 μl each of sense and antisense DNA was mixed with 5 μl each of distilled water and 10× annealing buffer (1 M NaCl, 100 mM Tris, pH 7.4). The mixture was heated in the boiling water for 5 min and spontaneously annealed by cooling down to the room temperature. The annealed double-stranded DNA was diluted by 4,000-fold in TE and ligated to pmU6pro vector (kindly provided by Dr. Dave Turner, University of Michigan) digested with BbsI and XbaI. The resultant plasmids were verified by DNA sequencing. These plasmids or the control plasmid pMT (which does not contain EGFP and shRNA sequences (32), 2 μg each) were co-transfected with the equal amount of pEGFP-G2A into Neuro2A cells, which were subjected to RT-PCR and flow cytometry analyses 24 h after transfection. Quantitative RT-PCR was performed using the LightCycler-FastStart DNA Master SYBR Green I (Roche Applied Science, catalog no. 3003230). The copy number of G2A transcript was quantified using the calibration curve drawn using the standard solution containing the fixed amount of the plasmid carrying G2A cDNA. Normalization was done against the amount of glyceraldehyde-3-phosphate dehydrogenase transcript.

For the expression of shRNA sequences by adenovirus vector-mediated method, a HindIII-NotI fragment containing the U6 promoter, shRNA sequence, and SV40 poly(A) sequence was blunt-ended and ligated to the cloning site (SwaI site) of the adenovirus cosmid vector, pAxCAwtit (Riken DNA bank, catalog no. 3121). The resultant cosmid DNA was processed as described below. The recombinant adenoviruses thus prepared were infected to 1.0×10^6 PC12 cells grown in a 100-mm dish for 1 h at a multiplicity of infection of 100, and 24 h after the infection, cells were processed for RT-PCR and neurite outgrowth experiments. In a single neurite outgrowth assay, more than 100 cells were counted for individual treatment. The data are mean ± S.D. of three independent experiments.

Flow Cytometry—24 h after transfection, Neuro2A cells were washed twice with phosphate-buffered saline, and cells were detached from the culture dishes by trypsin. After fixation in 4% paraformaldehyde, cells were washed twice with phosphate-buffered saline, triturated by pipetting, and filtrated through the nylon membrane to prepare dissociated cell suspension. They were then subjected to flow cytometry analysis (BD LSR, BD Biosciences) in which ~10,000 cells were counted. The areas counted for EGFP-positive and -negative cells were determined by control experiments conducted with cells transfected with pEGFP-N1 (positive control) and pMT lacking EGFP sequence (negative control), respectively.

Adenovirus Vector Construction and Infection—A PCR-based mutagenesis protocol (33) was used to generate a silent mutation at Csp45I site in G2A, which is necessary for linearization of adenoviral vector. Using the pEGFP-G2A plasmid as a template, mutated DNA fragments were amplified by PCR using the following pairs of oligonucleotides as primers: G2A antisense (used for cDNA cloning)/mutant sense (5′-GTGTGACTTCGAGAACAGGCTGTAC-3′) and G2A sense/mutant antisense (5′-GTACAGCCTGTTCTCGAAGTCACAC-3′). Underlined nucleotides correspond to the silent mutation. The two resulting PCR fragments were mixed, followed by a second amplification, carried out with the G2A sense and antisense primers. The amplified DNA fragment thus obtained was then digested with HindIII and EcoRI and ligated to the pEGFP-N1 vector. The mutated G2A-EGFP fragment was blunt-ended and ligated to the SwaI site of pAxCAwtit. The resultant cosmid DNA (1 μg) was packaged *in vitro* using 6 μl of LAMBDA INN (Nippon Gene, catalog no. 317-01741), infected to *Escherichia coli* DH5α, and amplified. Cosmid DNA (10 μg) was then purified, linearized by digestion with Csp45I, and transfected to HEK293 cells grown in a 60-mm dish using 30 μl of TransFast (Promega, catalog no. E2431). Cells were recovered 24 h later in phosphate-buffered saline, transferred to a 100-mm dish, and grown in 10 ml of 5% FCS/DMEM. Five days after, DMEM containing 10% FCS (5 ml) was added. Every 5 days, 5 ml of culture medium was removed, and the same volume of fresh 10% FCS/DMEM was added until most of the cells became detached (typically 7–30 days). Then the cells and culture medium were harvested together, freeze-thawed, and centrifuged to obtain the adenovirus-enriched supernatants. Aliquots of the supernatants were added to fresh HEK293 cells, and the recombinant adenovirus was amplified by another 3 times. The resultant adenovirus-containing media were used as virus stocks, and the titers were determined by the 50% tissue culture infectious doses method, using the plaque forming assay with 293A cells. Typically, 10^9 plaque forming units/ml viral stocks were obtained.

24 h after plating, PC12 cells were infected with either adenoviral-EGFP or adenoviral-G2A-EGFP, each at a multiplicity of infection of 50. After infection, cells were incubated for another 24 h and were subjected to the neurite outgrowth assay as described above.

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 sPLA$_2$ Induces Neurite Outgrowth in PC12 Cells via L-type Ca^{2+} Channel Activity—We previously reported that exogenously added fungal sPLA$_2$, p15, induces neurite outgrowth in PC12 cells via L-type Ca^{2+} channel activity (32). Interestingly, when neuritogenesis by supernatants of COS1 cells transfected with mouse sPLA$_2$s was examined, only the supernatant containing sPLA$_2$-X, but not sPLA$_2$-IB nor sPLA$_2$-IIA, elicited neurites. To unequivocally show the neuritogenic response of PC12 cells by mammalian sPLA$_2$s, we prepared purified, recombinant mouse sPLA$_2$-IB and sPLA$_2$-IIA produced by the baculovirus expression system. These two sPLA$_2$s were used together with recombinant human sPLA$_2$-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. 1*A*, neither sPLA$_2$-IB nor sPLA$_2$-IIA induced neurites, whereas sPLA$_2$-X induced neurite outgrowth to a similar extent to p15. In addition, the neuritogenic effect of sPLA$_2$-X was abolished by treatment with an inhibitor of L-type Ca^{2+} channel, nicardipine, which was also shown to inhibit p15-mediated neurite outgrowth (Fig. 1*B*). Similar inhibitory effect on sPLA$_2$-X-mediated neurite outgrowth was observed with nifedipine, but not with an N-type Ca^{2+} channel inhibitor ω-conotoxin GIVA (data not shown). Interestingly, PLA$_2$ activity of sPLA$_2$-X was no greater than that of sPLA$_2$-IB and sPLA$_2$-IIA when assayed using [^{3}H]oleic acid-labeled *E. coli* membrane as a substrate (Fig. 1*C*). In contrast, oleic acid release from live PC12 cells labeled with [^{3}H]oleic acid was hardly detected by sPLA$_2$-IIA, and marginally by sPLA$_2$-IB, in marked contrast to the potent oleic acid release by sPLA$_2$-X treatment (Fig. 1*D*). Distinct fatty acid-releasing activity of mammalian sPLA$_2$s from live cells has already been reported in HEK293 and RBL-

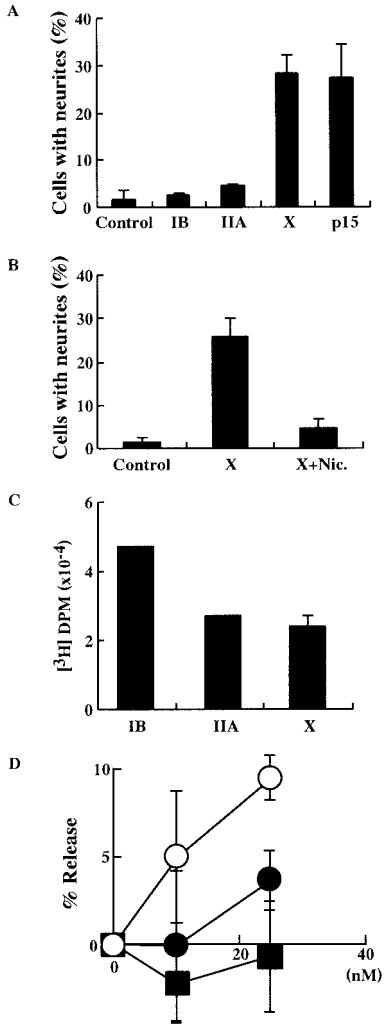

FIG. 1. **sPLA$_2$-X induces neurite outgrowth in PC12 cells by L-type Ca^{2+} channel-dependent manner.** *A*, PC12 cells were incubated for 24 h in the presence of sPLA$_2$-IB (30 nM), sPLA$_2$-IIA (100 nM), sPLA$_2$-X (100 nM) sPLA$_2$s, or p15 (100 nM) and neurite outgrowth was quantified; cells bearing processes longer than the cell diameter were judged as positive. *B*, effect of L-type Ca^{2+} channel blocker, nicardipine (*Nic.*, 10 μM) on sPLA$_2$-X-induced neurite outgrowth. *C*, enzyme activities of sPLA$_2$s (each at 25 nM) on [^{3}H]oleic acid-labeled *E. coli* membranes. *D*, [^{3}H]oleic acid release from live PC12 cells by sPLA$_2$-IB (*closed circles*), sPLA$_2$-IIA (*closed squares*), and sPLA$_2$-X (*open circles*).

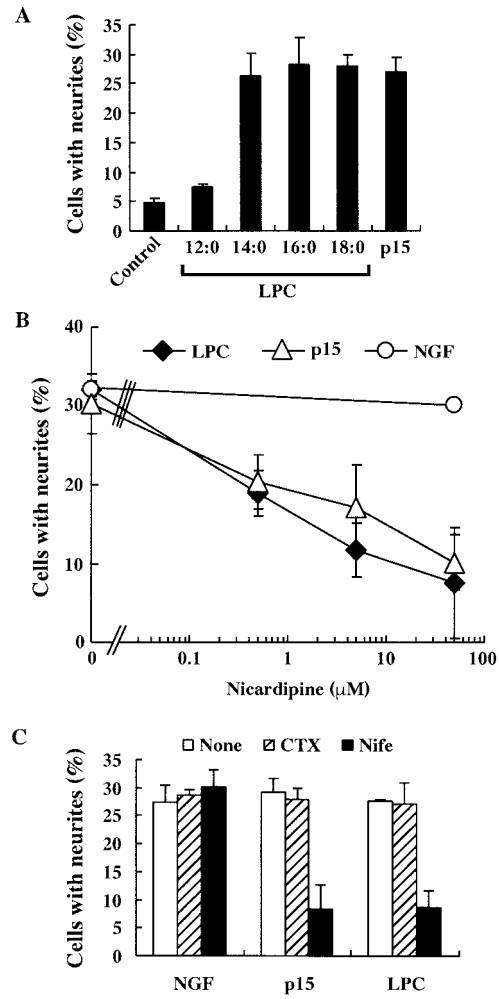

FIG. 2. **LPC induces neurite outgrowth in PC12 cells.** *A*, PC12 cells were incubated for 24 h in the presence of p15 (100 nM) or LPC (100 μM) containing various length of fatty acyl chain, and neurite outgrowth was measured. *B*, dose-dependent inhibitory effect of nicardipine on p15- and LPC-induced, but not on NGF-induced, neurite outgrowth in PC12 cells. *C*, effect of N-type (ω-conotoxin GIVA; *CTX*, 5 μg/ml) and L-type (nifedipine; *Nife*, 14.4 μM) Ca^{2+} channel blockers. Neurite outgrowth by p15 and LPC was sensitive to nifedipine but not to ω-conotoxin.

2H3 cells (17). Thus, these results suggest that degradation of membrane phospholipid by mammalian group X sPLA$_2$ from live cells triggers neurite-outgrowth response in an L-type Ca^{2+} channel-dependent manner.

Involvement of LPC in sPLA$_2$-mediated Neurite Outgrowth—We previously showed that neither cyclooxygenase nor lipoxygenase inhibitors blocked sPLA$_2$-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. 2*A*). In addition, LPC-induced neuritogenesis was inhibited by similar doses of nicardipine that inhibit p15-induced neurite outgrowth (Fig. 2*B*) and by nifedipine, but not by ω-conotoxin GIVA (Fig. 2*C*), suggesting that LPC mediates the neurite-inducing activity of sPLA$_2$.

We next asked whether sPLA$_2$s indeed liberate LPC from PC12 cells. PC12 cells prelabeled with [^{14}C]choline were treated with sPLA$_2$ 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. 3*A*). No significant generation of [^{14}C]LPC was observed in the cell-associated fractions. When LPC generation in PC12 cells by COS1 supernatants expressing mouse sPLA$_2$s was examined (32), we observed that only the COS1 supernatant expressing sPLA$_2$-X consistently induced [^{14}C]LPC generation (Fig. 3*B*). In contrast, no significant release of [^{14}C]LPC was observed by supernatants of COS1 cells expressing sPLA$_2$-IB nor sPLA$_2$-IIA. The COS1 supernatant containing mouse group V sPLA$_2$ gave variable results, and the LPC release was not consistent. In addition, when the effect of purified human sPLA$_2$-X was examined, generation of [^{14}C]LPC was specifically observed in the medium, but not in the cell-associated

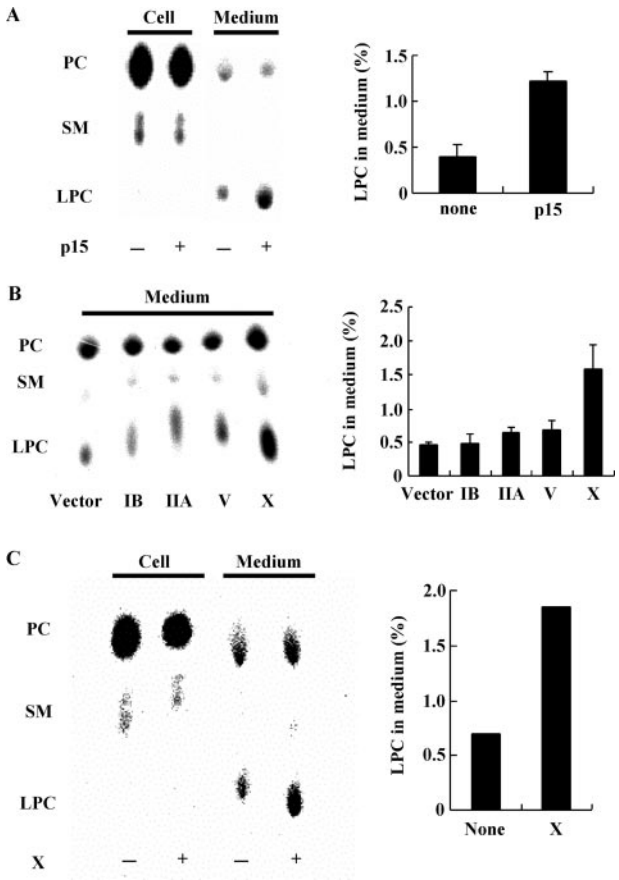

FIG. 3. **sPLA$_2$-mediated LPC release from PC12 cells.** *A*, PC12 cells prelabeled with [^{14}C]choline for 24 h were incubated with 100 nM p15 for 4 h. Total lipids in the culture medium and the cell lysate were separately extracted with organic solvents and analyzed by TLC. Radioactive lipids were visualized and quantified. At the *right*, LPC release into the medium is expressed as the percentage of total (released and cell-associated) radioactive lipids. *PC*, phosphatidylcholine; *SM*, sphingomyelin. *B*, [^{14}C]LPC release from PC12 cells by COS1 supernatants expressing group IB, IIA, V, or X mouse sPLA$_2$s. *C*, [^{14}C]LPC release from PC12 cells treated with sPLA$_2$-X (100 nM) for 4 h.

fraction, which is reminiscent of the result with p15 (Fig. 3*C*). Therefore, the LPC-releasing activity of sPLA$_2$s correlated with the neurite-inducing activity, which was observed in the COS1 supernatant containing sPLA$_2$-X (32). These results strongly suggest that membrane degradation and/or release of LPC is required for the neuritogenic effect of sPLA$_2$.

If sPLA$_2$-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 sPLA$_2$ or LPC. We therefore examined the inhibitory effects of bovine serum albumin (BSA) and phospholipase B (PLB) on sPLA$_2$- 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 sPLA$_2$ and LPC. Taken together, these results demonstrate that the release of LPC

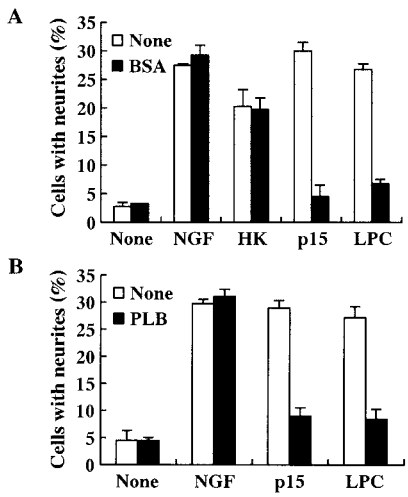

FIG. 4. **BSA and PLB inhibit sPLA$_2$- and LPC-induced neuritogenesis.** *A*, effect of 1% BSA on NGF (50 ng/ml), high potassium (*HK*; 40 mM), p15 (100 nM), and LPC (100 μM)-induced neurite outgrowth. *B*, inhibitory effect of PLB (2.5 mg/ml) on p15- and LPC-induced neurite outgrowth.

into the culture medium is required for the neuritogenic response of PC12 cells by sPLA$_2$.

G2A Is Involved in Neuritogenic Response of PC12 Cells by sPLA$_2$ 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. 5*A*, *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 sPLA$_2$s. To test this possibility, we first examined whether LPC, generated by sPLA$_2$ 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 sPLA$_2$ 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 sPLA$_2$ or LPC treatments. As shown in Fig. 5*B*, 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. 5*B*, *panels c–e*). p15- and LPC-induced internalization was not observed with TDAG8-EGFP, another OGR1 family GPCR with distinct ligand specificity (Fig. 5*B*, *panels f–h*). This indicates that G2A-EGFP responded to LPC added to the culture medium and LPC generated by membrane degradation by sPLA$_2$.

Next we examined whether the increase in G2A expression in PC12 cells results in enhanced neuritogenic response to sPLA$_2$ 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. 6*A*) and Western blot with an anti-GFP antibody (Fig. 6*B*). When the transfectants as well as the wild type and EGFP-overexpressing PC12

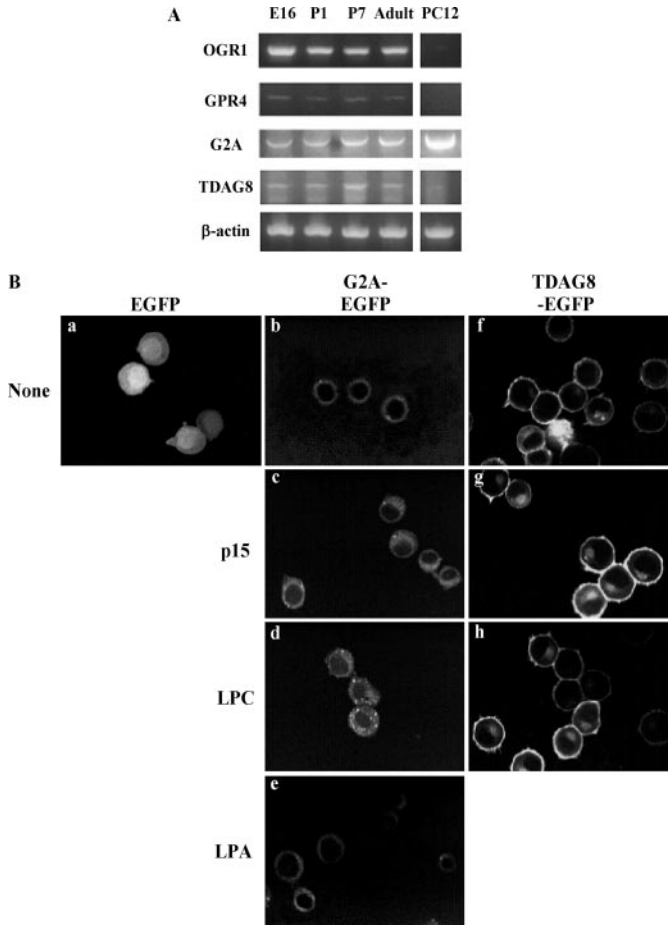

FIG. 5. **G2A is expressed in PC12 cells and is internalized upon treatments with sPLA$_2$ or LPC.** *A*, the expression of OGR1 subfamily receptors, OGR1, GPR4, G2A, and TDAG8, was examined in the mouse cerebra (*E*, embryonic days; *P*, postnatal days) and in PC12 cells. Expression of β-actin was used as a standard. Note that only G2A was expressed in PC12 cells. *B*, Neuro2A cells were transfected with plasmids for expression of EGFP (*a*), G2A-EGFP (*b–e*), or TDAG8-EGFP (*f–h*). Cells were cultured for 24 h in FCS (10%)-containing medium and additional 24 h in serum-free medium. In the final 2 h of incubation, cells were treated without (*a*, *b*, and *f*) or with p15 (*c* and *g*, 100 nM), LPC (*d* and *h*, 100 μM), or lysophosphatidic acid (*LPA*) (*e*, 100 μM), and fixed.

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. 6*C*) and LPC (Fig. 6*D*) 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. 6*D*). 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. 6*E*), suggesting that G2A activation is linked to L-type Ca^{2+} 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. 7*A*) also elicited enhanced neurite outgrowth response at lower doses of p15, as observed in the experiments with the stable transfectants (Fig. 7*B*). LPC-induced neurite outgrowth was also strengthened (Fig. 7*C*). In addition, depolarization-induced, but not NGF-induced, neuritogenesis was enhanced (Fig. 7*C*). These results suggest that production of LPC and subsequent activation of G2A is responsible for the neurotrophic action of sPLA$_2$ in PC12 cells.

RNA Interference-mediated Suppression of G2A Expression Inhibits Neurite Outgrowth by sPLA$_2$s—We next examined whether RNA interference-mediated chronic inhibition of G2A expression in PC12 cells suppresses sPLA$_2$-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. 8*A*, *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. 8*B*).

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 sPLA$_2$-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. 8*C*). Finally, we tested the effect of shRNA-mediated G2A suppression on sPLA$_2$- and LPC-induced neurite outgrowth. As shown in Fig. 8*D*, infection of r2 adenovirus resulted in inhibition of p15- and sPLA$_2$-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 sPLA$_2$ 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 sPLA$_2$s and found that sPLA$_2$-X, but not sPLA$_2$-IB and sPLA$_2$-IIA, induced neurites in PC12 cells. The known substrate preference of these sPLA$_2$s for PC-rich vesicles (X≫IB, IIA) is in line with the notion that LPC generation is linked to the neuritogenic effect of sPLA$_2$. Indeed, a significant amount of [^{14}C]LPC was produced only by the treatments with sPLA$_2$s with neuritogenic activity (sPLA$_2$-X and p15).

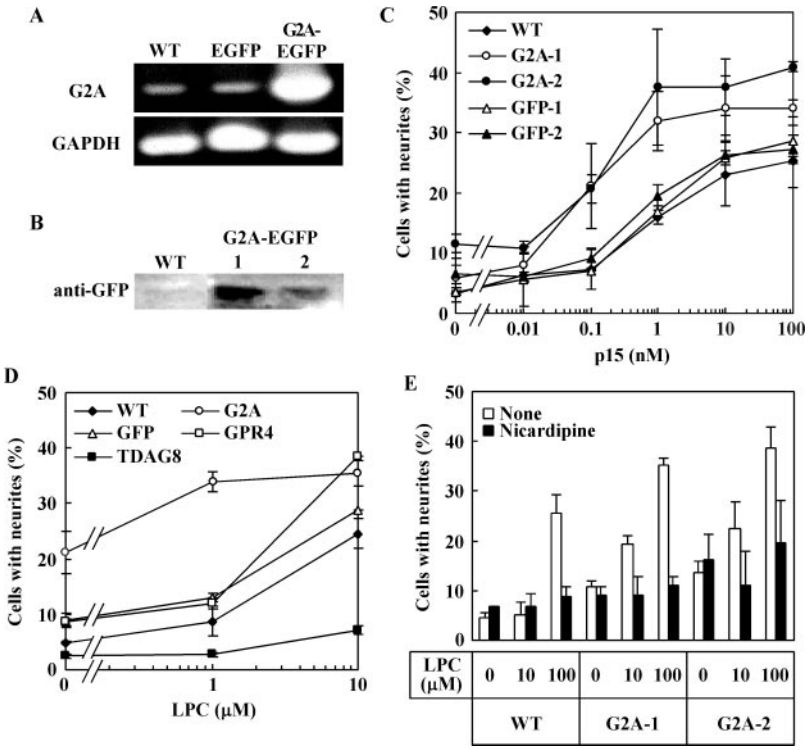

FIG. 6. **sPLA$_2$- and LPC-induced neurite outgrowth is potentiated in PC12 cells stably transfected with G2A-EGFP.** *A*, the expression of G2A in wild type PC12 (*WT*), stable transfectants of PC12 expressing EGFP or G2A-EGFP (clone no. 1) was examined by RT-PCR. *B*, the expression of G2A-EGFP fusion protein (67 kDa) in the stable transfectants (clone nos. 1 and 2) was examined using anti-GFP antibody. *C*, WT, stable transfectants of PC12 expressing EGFP or G2A-EGFP were treated with indicated concentrations of p15. Neurite outgrowth was assessed 24 h later. *D*, WT, EGFP-, G2A-EGFP-, GPR4-EGFP-, or TDAG8-EGFP-expressing stable transfectants (clone no. 1) were incubated for 24 h in the low serum medium (1% FCS) containing indicated concentrations of LPC. *E*, effect of L-type Ca^{2+} channel blocker, nicardipine (20 μM), on LPC-induced neurite outgrowth in G2A-EGFP stable transfectants (clone nos. 1 and 2).

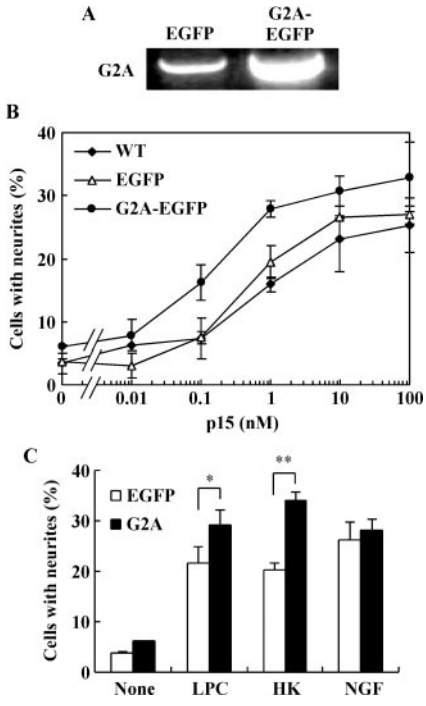

FIG. 7. **Adenoviral vector-mediated transient expression of G2A-EGFP enhances sPLA$_2$- and LPC-induced neurite outgrowth in PC12 cells.** *A*, the expression of G2A in PC12 cells infected with EGFP- or G2A-EGFP-expressing adenovirus was examined by RT-PCR. *B*, PC12 cells were infected with EGFP- or G2A-EGFP-expressing recombinant adenovirus. After 24 h, infected cells were incubated for another 24 h in the presence of indicated concentration of p15. *C*, the effect of adenoviral transient expression of G2A-EGFP on LPC (100 μM)-, high potassium (*HK*; 40 mM)-, and NGF (50 ng/ml)-induced neurite outgrowth. *, $p < 0.05$; **, $p < 0.001$ (*t* test; $n = 3$).

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 sPLA$_2$- 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 sPLA$_2$ and LPC. Fifth, neuritogenic response of sPLA$_2$ and LPC is likely to be mediated by G2A, a GPCR involved in chemotactic response of immune cells toward LPC, because overproduction of G2A either by transfection or adenoviral infection of G2A-EGFP resulted in enhanced neuritogenic response by sPLA$_2$ and LPC. Conversely, suppression of endogenous expression of G2A by shRNA caused decreased neuritogenic response by sPLA$_2$ and LPC. Collectively, these results are consistent with the hypothesis that sPLA$_2$-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 sPLA$_2$). 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 sPLA$_2$- 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. 6*D*). This suggests that, in view of the inhibitory effect of BSA on neurite-formation as described above, BSA and/or lipoproteins present in the serum reduced the effective concentration of LPC by binding to and sequestering it. In this regard, it should be noted that serum-free media were generally used to detect and analyze the effects of 1~20 μM LPC in cells expressing G2A (34,

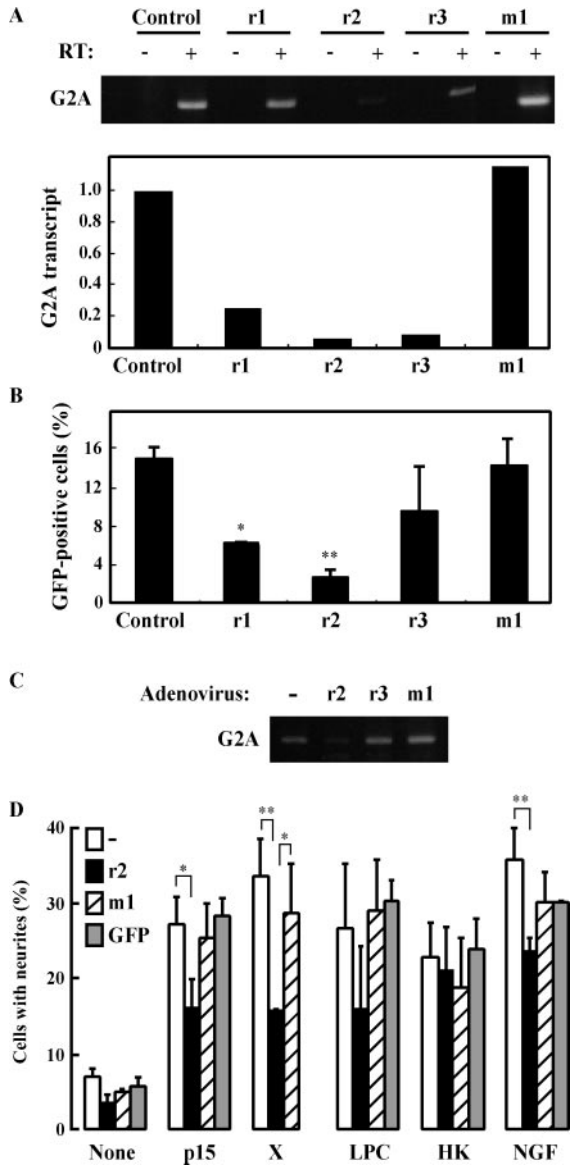

FIG. 8. **Suppression of G2A expression by shRNA inhibits neurite outgrowth induced by sPLA$_2$ and LPC in PC12 cells.** *A*, Neuro2A cells co-transfected with pEGFP-G2A and either the control plasmid pMT (*Control*), pmU6pro containing shRNA sequences (*r1–r3*), or mismatched sequence (*m1*) were examined for the expression of G2A. Semi-quantitative (*A*, *upper panel*) and quantitative (*A*, *lower panel*) PCR experiments were carried out to evaluate the efficacy of shRNA sequences on G2A mRNA expression. The absence of amplification product (629 bp) in the lanes where the reverse transcription (*RT*) reaction was omitted unequivocally shows that the bands are derived from mRNA, not genomic DNA. In the *lower panel*, the amount of G2A transcript relative to the control is shown. *B*, the same set of transfected Neuro2A cells were examined for EGFP fluorescence by fluorescence-activated cell-sorting analysis. The number of EGFP-positive cells were expressed as the percentages of total number of cells. *, $p < 0.05$; **, $p < 0.01$ (*t* test). *C*, inhibition of G2A expression in PC12 cells by infection of recombinant adenovirus expressing r2 shRNA sequence. RT-PCR was carried out with the total RNA isolated from PC12 cells infected without (−) or with adenoviruses expressing the indicated shRNA sequences. *D*, neurite outgrowth assay. PC12 cells infected without (*open bars*) or with adenoviruses expressing shRNA sequences (r2, *solid bars*; m1, *striped bars*) or GFP (*gray bars*) were examined for the neurite outgrowth response induced by the indicated stimulation (p15 and sPLA$_2$-X, 100 nM; LPC, 100 μM; HK, 40 mM; NGF, 50 ng/ml) for 24 h. *, $p < 0.05$ (*t* test).

35, 39). Another possibility for the requirement of high doses of LPC for neuritogenesis is the degradation and/or cellular incorporation of added LPC during the culture period, leading to the decrease in LPC content; neurite outgrowth typically takes 8–12 h to be detected, whereas in other studies cells 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 sPLA$_2$ treatement, ~1% of total [^{14}C]choline in the organic fraction was converted to [^{14}C]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 sPLA$_2$, however, continuous generation of LPC at low but sufficient levels to activate G2A (Fig. 5*B*) and stimulate neuritogenesis might have occurred in proximity to the plasma membrane. This speculation is supported by our observation[2] 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 sPLA$_2$ 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 sPLA$_2$ 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. 2*A*): 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 G2A is involved in sPLA$_2$-induced neurite outgrowth via LPC generation delineate a novel LPC-induced cellular response that is mediated by G2A. However, an existence of another pathway that could involve GPR119 (42), a recently identified receptor for LPC whose expression was not examined in the current study, should be considered, because the neuritogenesis was not completely inhibited by suppression of G2A expression (Fig. 8*D*).

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-

[2] Y. Ikeno and M. Arioka, unpublished observation.

ficity 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 sPLA$_2$ and LPC (and possibly proton) in the neuronal development and function.

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