Lysophosphatidic Acid and Thrombin Receptors Require Both $G\alpha_{12}$ and $G\alpha_{13}$ to Regulate Axonal Morphology in Hippocampal Neurons

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The $G\alpha$ subunits of the G_{12} family of heterotrimeric guanine nucleotide-binding proteins (G proteins), defined by $G\alpha_{12}$ and $G\alpha_{13}$, have many cellular functions in common, including stimulation of stress fiber formation and focal adhesion assembly *via* the small GTPase RhoA activation. We and others previously showed that $G\alpha_{12}$ and $G\alpha_{13}$ mediate neurite retraction in neuronal cell lines, but their roles in primary cultured neurons have not been adequately understood. Here, we found that expression of constitutively active mutants of $G\alpha_{12}$ or $G\alpha_{13}$ caused growth cone collapse dependent on Rho-kinase activity in hippocampal neurons. The stimulation of thrombin and lysophosphatidic acid (LPA) receptors, which have been thought to selectively couple to $G\alpha_{12}$ and $G\alpha_{13}$, respectively, caused growth cone collapse and suppressed axon branching dependent on Rho-kinase activity in hippocampal neurons. Thrombin- and LPA-induced growth cone collapse was suppressed by both single knockdown of $G\alpha_{12}$ and $G\alpha_{13}$ with short hairpin RNAs and this suppression was augmented by double knockdown of both $G\alpha_{12}$ and $G\alpha_{13}$. These results suggest that thrombin and LPA receptors couple to both $G\alpha_{12}$ and $G\alpha_{13}$ for growth cone collapse.

Key words G_{12} family; G protein-coupled receptor; hippocampal neuron; growth cone collapse; thrombin; lysophosphatidic acid

Heterotrimeric guanine nucleotide-binding proteins (G proteins) act as a molecular switch that conveys signals from G protein-coupled receptors (GPCRs) in the cell membrane to intracellular downstream effectors.¹⁾ Agonist-liganded receptors interact with G proteins and catalyze the guanosine 5'-diphosphate (GDP)–guanosine 5'-triphosphate (GTP) exchange on α -subunits of G proteins ($G\alpha$), the GTP-bound active α -subunits initiating cellular responses through their intracellular effector molecules. On the other hand, the activation of α -subunits is down-regulated by regulators of G protein signalling which stimulate the intrinsic GTPase activities of α -subunits.²⁾

G proteins are divided into four subfamilies, G_s , G_i , G_q , and G_{12} , based on sequence homology of their α subunits.¹⁾ The G_{12} family is defined by $G\alpha_{12}$ and $G\alpha_{13}$, and they share 67% amino acid sequence identity.³⁾ Active forms of $G\alpha_{12}$ and $G\alpha_{13}$ induce a variety of biological responses including stimulation of stress fiber formation, focal adhesion assembly,⁴⁾ transformation of fibroblasts,⁵⁾ and induction of neurite retraction.⁶⁾ These responses are mediated by activation of Rho through the guanine nucleotide exchange factors for Rho, including p115RhoGEF, PSD-95/Dlg/ZO-1 (PDZ)-RhoGEF, and leukemia-associated Rho GEF (LARG),^{7–10)} which are major downstream targets of $G\alpha_{12}$ and $G\alpha_{13}$. On the other hand, a variety of GPCRs appear to selectively couple to $G\alpha_{12}$ and $G\alpha_{13}$.¹¹⁾ For example, the stimulation of thrombin and lysophosphatidic acid (LPA) receptors has been shown to induce stress fiber formation *via* $G\alpha_{12}$ and $G\alpha_{13}$, respectively, by using dominant negative mutants of $G\alpha_{12}$ and $G\alpha_{13}$ in fibroblast cell lines.¹¹⁾ However, the coupling of receptors with G_{12} family is not well evaluated in the context of neuronal functions.

We and other group demonstrated that expression of constitutively active mutants of $G\alpha_{12}$ and $G\alpha_{13}$ causes neurite retraction through RhoA activation in neuronal cell lines, such as rat pheochromocytoma PC12 cells and N1E-115 cells.^{6,12)} However, their roles in the regulation of morphology of primary cultured neurons remain poorly to be studied.

In the present study, we show that expression of constitutively active mutants of $G\alpha_{12}$ and $G\alpha_{13}$ induces growth cone collapse in hippocampal neurons, and that thrombin and LPA induce growth cone collapse and their collapse is suppressed by both $G\alpha_{12}$ and $G\alpha_{13}$ short hairpin RNAs (shRNAs), indicating that thrombin and LPA receptors couple to both $G\alpha_{12}$ and $G\alpha_{13}$ for growth cone collapse.

MATERIALS AND METHODS

Plasmid Constructions, Reagents and Antibodies The cDNAs of the constitutively active mutants of $G\alpha_{12}$ ($G\alpha_{12}$ Q229L ($G\alpha_{12}$ QL)), $G\alpha_{13}$ ($G\alpha_{13}$ Q226L ($G\alpha_{13}$ QL)), and RhoA (RhoA-V14) were obtained as described previously.¹³⁾ plasmid CAG encoding an enhanced yellow fluorescent protein (EYFP) was used as described previously.¹⁴⁾ shRNAs against $G\alpha_{12}$ and $G\alpha_{13}$ were constructed according to previous studies by Shin¹⁵⁾ and Martin,¹⁶⁾ respectively, and they were expressed using an shRNA expression vector pScilencer (Ambion).

Agents obtained and commercial sources were as follows: rabbit polyclonal antibodies against $G\alpha_{12}$ and $G\alpha_{13}$ (Santa Cruz Biotechnology), a mouse monoclonal antibody against α -tubulin (Sigma), secondary antibody conjugated to horseradish peroxidase (Dako), rhodamine-phalloidin (Invitrogen), thrombin and LPA (Sigma). A soluble form of Sema4D fused to human IgG₁-Fc was from H. Kikutani (Osaka University, Osaka, Japan), and semaphorin 4D (Sema4D) stimulation was used as described previously.¹⁷⁾

Cell Culture and Transfection Pregnant Wistar rats were purchased from Japan SLC and treated in accordance with the guidelines for the Animal Care and Use Committee of the Graduate School of Biostudies at Kyoto University. Hippocampal cultures were prepared from hippocampi of embryonic day 19 (E19) rats as described previously.¹⁴⁾ Briefly, hippocampi of Wistar rat embryos were dissected in ice-cold calcium- and magnesium-free Hank's balanced salt solution (HBSS). The hippocampi were washed in HBSS and

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incubated in HBSS with 0.25% trypsin and 0.1% DNase for 10 min at 37 °C. After incubation, the hippocampi were washed in HBSS, followed by trituration with Pasteur pipettes. The cells were seeded onto poly-D-lysine- and laminin-coated glass coverslips (circular, 13 mm in diameter) at a density of 2×10^4 cells (1×10^4 cells in Fig. 5) in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 4 mM glutamine, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin and cultured under humidified air containing 5% CO₂ at 37 °C. After 5 h, the medium was replaced with Neurobasal medium (Invitrogen) with 2% B27 supplement (Invitrogen), 0.5 mM glutamine, and 100 units/ml of penicillin and cultured under humidified air containing 5% CO₂ at 37 °C. For immunofluorescence analyses, hippocampal neurons were transfected with test plasmids at 1.5–2 d *in vitro* (DIV) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions and fixed at 3 DIV.

For Immunoblot analyses, hippocampal neurons were cultured on poly-D-lysine-coated 60-mm culture dishes and transfected with test plasmids using Rat Nucleofector kit (Amaxa Biosystems) following the manufacturer's instructions.

Separation of Membrane and Cytoplasmic Fractions

Separation of membrane and cytoplasmic fractions was performed as described previously.¹⁸⁾ Briefly, hippocampal neurons were rinsed once with HEPES buffered saline (HBS), and suspended in the ice-cold homogenization buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml of aprotinin, and 10 μ g/ml of leupeptin). Cells were frozen in liquid nitrogen and thawed at 4 °C, and then centrifuged for 10 min at 151000 *g* at 4 °C to obtain the supernatants as cytoplasmic fractions. After the pellets were washed with the ice-cold homogenization buffer, they were resuspended with Laemmli sample buffer and used as membrane fractions. The membrane and cytoplasmic fractions were analyzed by SDS-PAGE and immunoblotting.

Immunoblotting Immunoblotting was performed as described previously.¹⁸⁾ Briefly, proteins were separated by 12.5% SDS-PAGE and electrophoretically transferred onto a PVDF membrane (Millipore Corp.). The membrane was blocked with 3% (w/w) low-fat milk in Tris-buffered saline and then incubated with primary antibodies. The primary antibodies were detected by using horseradish peroxidase-conjugated secondary antibodies (Dako) and an ECL detection kit (Amersham Biosciences).

Immunofluorescence Microscopy Immunocytochemistry was performed as described previously.¹⁴⁾ Briefly, all steps were carried out at room temperature, and cells were rinsed with phosphate-buffered saline (PBS) between each step. After transfection, cells on coverslips were fixed with 3.7% formaldehyde in PBS for 15 min. After residual formaldehyde had been quenched with 50 mM NH₄Cl in PBS for 10 min, cells were permeabilized with 0.2% Triton X-100 in PBS for 10 min and incubated with 10% fetal bovine serum in PBS for 30 min to block non-specific antibody binding. To visualize F-actin, fixed cells were stained with rhodamine-phalloidin (Invitrogen). Cells on coverslips were mounted in 90% glycerol containing 0.1% *p*-phenylenediamine dihydrochloride in PBS. The cells were photographed with Leica DC350F digital camera system equipped with

Nikon Eclipse E800 microscope and analyzed with Image-Pro Plus image analysis software (Media Cybernetics).

For quantification of growth cone-positive cells, hippocampal neurons with growth cones were defined as cells that have lamellipodia and filopodia. Axon length and branching in hippocampal neurons were quantified manually as described previously¹⁴⁾ using Image-Pro Plus image analysis software (Media Cybernetics). Only processes longer than 20 μ m from axon branch points were counted as axon branches.

RESULTS AND DISCUSSION

Immunohistochemical analysis of the expression of G α_{12} and G α_{13} in adult mouse brain was performed by Kuner *et al.*, and G α_{12} and G α_{13} have been shown to be expressed in a wide variety of neuronal tissues.¹⁹⁾ However, the expression of G α_{12} and G α_{13} in hippocampal neurons during the development has not been revealed. We then analysed the expression of G α_{12} and G α_{13} in cell lysates prepared from rat hippocampal neurons at 1, 3, 6 and 10 DIV by immunoblotting with G α_{12} - and G α_{13} -specific antibodies, respectively. As shown in Fig. 1A, significant amounts of G α_{12} and G α_{13} proteins were already detected at 1 DIV, and these expression continued up to 10 DIV, indicating that both G proteins are expressed during the stage of axon formation and elongation.

We recently found a distinct localization of G α_{12} and G α_{13} in various cell lines; G α_{12} and G α_{13} localize in membrane and cytoplasmic fractions, respectively.¹⁸⁾ We then examined subcellular localization of two G₁₂ family G proteins in hippocampal neurons by immunoblotting. As shown in Fig. 1B, G α_{12} localized in the membrane fraction, while G α_{13} localized in the cytoplasmic fraction in hippocampal neurons at 3 DIV. This different subcellular localization of G α_{12} and G α_{13} provides the possibility that they may contribute to somehow different systems for RhoA activation.

Both G α_{12} and G α_{13} were expressed in hippocampal neurons during the stage of axon development. Growth cones,

Fig. 1. Expression of G α_{12} and G α_{13} in Rat Hippocampal Neurons

(A) Lysates from cultured hippocampal neurons at 1, 3, 6, and 10 DIV were analyzed by SDS-PAGE and immunoblotting with antibodies against G α_{12} , G α_{13} and α -tubulin (control). (B) Homogenates of cultured hippocampal neurons at 3 DIV were separated into membrane and cytoplasmic fractions. The fractionated samples were analyzed by immunoblotting with anti-G α_{12} and anti-G α_{13} antibodies. The results shown are representative of three independent experiments that yielded similar results.

Fig. 2. Expression of Constitutively Active Mutants of $G\alpha_{12}$, $G\alpha_{13}$, and RhoA Caused Growth Cone Collapse in Rat Hippocampal Neurons

Primary cultured hippocampal neurons were transiently cotransfected with $G\alpha_{12}$ QL, $G\alpha_{13}$ QL, and RhoA-V14 together with EYFP. Then cells were treated with or without 10 μ M Y27632 for 24 h at 2 DIV and fixed at 3 DIV. F-actin was stained with rhodamine-conjugated phalloidin to visualize the growth cone structure. Quantitative analyses of effects of overexpression of constitutively active mutants of $G\alpha_{12}$, $G\alpha_{13}$, and RhoA on axonal morphology in hippocampal neurons were also shown. Results shown are the means $\pm$ S.E.M. of three independent experiments ($n=60$). Statistically significant differences from the value of control cells are indicated by * ($p<0.005$). Scale bar, 10 μ m.

located at the tip of axons, act as a sensor for a variety of axon guidance factors and various regulatory molecules for axon development, and regulation of morphology of growth cones are thought to be critical for axon development for neuronal network formation. We then examined the effect of expression of constitutively active mutants of $G\alpha_{12}$ and $G\alpha_{13}$ on the morphology of axonal growth cones of hippocampal neurons. We transfected hippocampal neurons with $G\alpha_{12}$ QL or $G\alpha_{13}$ QL together with EYFP at 2 DIV, and fixed the cells at 3 DIV. As shown in Fig. 2, $G\alpha_{12}$ QL and $G\alpha_{13}$ QL strongly induced growth cone collapse in hippocampal neurons. RhoA-V14 also strongly induced growth cone collapse. Quantitative analyses showed that $G\alpha_{12}$ QL, $G\alpha_{13}$ QL and RhoA-V14 significantly decreased the number of growth-cone-positive cells ($G\alpha_{12}$ QL, $11.67 \pm 3.33\%$, $p<0.005$; $G\alpha_{13}$ QL, $15.00 \pm 2.89\%$, $p<0.005$; RhoA-V14, $10.00 \pm 2.89\%$, $p<0.005$) compared to control (pcDNA3 (empty vector), $78.33 \pm 3.33\%$; Fig. 2). In contrast, growth cone collapse induced by these active mutants was abolished by Y27632, an inhibitor of Rho-kinase (Fig. 2). Quantitative analyses showed that Y27632 suppressed the growth cone collapse caused by expression of $G\alpha_{12}$ QL, $G\alpha_{13}$ QL or RhoA-V14 (pcDNA3/Y27632, $85.00 \pm 5.77\%$; $G\alpha_{12}$ QL/Y27632, $58.33 \pm 1.17\%$; $G\alpha_{13}$ QL/Y27632, $61.67 \pm 3.33\%$; RhoA-V14/Y27632, $60.00 \pm 2.89\%$; Fig. 2). We next examined the effect of expression of $G\alpha_{12}$ QL, $G\alpha_{13}$ QL, and RhoA-V14 on the axon length of hippocampal neurons. Three active mutants caused axonal retraction in hippocampal neurons (Fig. 3). Quantitative analyses showed that $G\alpha_{12}$ QL, $G\alpha_{13}$ QL and RhoA-V14 significantly decreased the total axon length ($G\alpha_{12}$ QL, $64.23 \pm 10.54 \mu$ m, $p<0.005$; $G\alpha_{13}$ QL, $69.86 \pm 6.37 \mu$ m, $p<0.005$; RhoA-V14, $57.77 \pm 7.86 \mu$ m, $p<0.005$) compared to

control (pcDNA3, $324.87 \pm 23.29 \mu$ m; Fig. 3). Our results demonstrate that $G\alpha_{12}$ and $G\alpha_{13}$ regulate axon development through RhoA-Rho kinase signalling pathway in hippocampal neurons. Growth cone collapse is linked to increase in actin bundle contractility in growth cones, and RhoA-Rho-kinase activation has been shown to promote this actin bundle contractility.²⁰ It is inferred that activation of $G\alpha_{12}$ and $G\alpha_{13}$ induces growth cone collapse in part through promotion of actin bundle contractility by activation of RhoA and Rho-kinase.

Thrombin and LPA receptors have been shown to activate RhoA through selective coupling to $G\alpha_{12}$ and $G\alpha_{13}$, respectively in fibroblast cells and HEK293T cells.^{11,13} Thrombin and LPA have been reported to induce neurite retraction through RhoA activation in N1E-115 cells and PC12 cells.^{21,22} However, coupling of G_{12} family with thrombin and LPA receptors for regulation of neuronal morphology has not been investigated. Therefore, we first examined whether thrombin and LPA regulate axonal morphology in hippocampal neurons. Rat hippocampal neurons were transfected with EYFP at 2 DIV to visualize the morphology and treated with either thrombin or LPA at 3 DIV. As shown in Fig. 4, both thrombin and LPA induced growth cone collapse in hippocampal neurons expressing EYFP, but the growth cone collapse induced by thrombin and LPA was abolished by Y27632, suggesting that the RhoA-Rho kinase signalling pathway is involved in the collapse. Quantitative analyses showed that thrombin and LPA significantly decreased the number of growth-cone-positive cells (thrombin, $36.67 \pm 1.67\%$ (mean $\pm$ S.E.M.), $p<0.005$; LPA, $36.67 \pm 6.67\%$, $p<0.005$) compared to control (no stimulation, $80.00 \pm 2.89\%$; Fig. 4), and Y27632 suppressed the thrombin- and LPA-in-

Fig. 3. Expression of Constitutively Active Mutants of $G\alpha_{12}$, $G\alpha_{13}$, and RhoA Caused Axonal Retraction in Rat Hippocampal Neurons

Primary cultured hippocampal neurons were transiently cotransfected with $G\alpha_{12}$ QL, $G\alpha_{13}$ QL and RhoA-V14 together with EYFP at 2 DIV and fixed at 3 DIV. Transfected cells are shown by EYFP fluorescence. Quantitative analyses of effects of overexpression of constitutively active mutants of $G\alpha_{12}$, $G\alpha_{13}$, and RhoA on axonal morphology in hippocampal neurons were also shown. Results shown are the means $\pm$ S.E.M. of three independent experiments ($n=60$). Statistically significant differences from the value of control cells are indicated by * ($p<0.005$). Scale bar, 20 μ m.

duced growth cone collapse (no stimulation/Y27632, $83.33 \pm 1.17\%$; thrombin/Y27632, $71.67 \pm 4.41\%$, $p<0.005$; LPA/Y27632, $73.33 \pm 1.17\%$, $p<0.005$; Fig. 4).

We also examined the effect of thrombin or LPA stimulation on axon branching, another important morphological phenomenon during axon development. Both thrombin and LPA suppressed the axon branching in rat hippocampal neurons expressing EYFP at 3 DIV (Fig. 5). Quantitative analyses showed that thrombin and LPA significantly decreased the number of axon branch points (thrombin, 3.01 ± 0.24 , $p<0.005$; LPA, 2.81 ± 0.19 , $p<0.005$) compared to control (no stimulation, 5.15 ± 0.30 ; Fig. 5). These results demonstrated that thrombin and LPA not only induced growth cone collapse but also suppressed axon branching in hippocampal neurons.

To determine whether $G\alpha_{12}$ and $G\alpha_{13}$ are involved in the thrombin- and LPA-induced growth cone collapse in hippocampal neurons, we next generated shRNAs against $G\alpha_{12}$ and $G\alpha_{13}$, which effectively reduced the endogenous expression of $G\alpha_{12}$ and $G\alpha_{13}$ in rat hippocampal neurons (Fig. 6A), and we investigated their effects on the thrombin- or LPA-induced growth cone collapse. We previously demonstrated that a repulsive axon guidance molecule, Sema4D, induces the growth cone collapse of hippocampal neurons through RhoA activation as well as R-Ras GAP activity.¹⁷ We also examined the effects of shRNAs on the Sema4D-induced

Fig. 4. Thrombin and LPA Caused Growth Cone Collapse in Rat Hippocampal Neurons

Primary cultured hippocampal neurons were transiently transfected with EYFP and pretreated with or without 10 μ M Y27632 for 24 h at 2 DIV. Then cells were treated with either 1 unit/ml thrombin or 1 μ M LPA for 0.5 h at 3 DIV. F-actin was stained with rhodamine-conjugated phalloidin to visualize the growth cone structure. Quantitative analyses of effects of thrombin and LPA treatment on axonal morphology in hippocampal neurons were also shown. Results shown are the means $\pm$ S.E.M. of three independent experiments ($n=60$). Statistically significant differences from the value of non-stimulated cells are indicated by * ($p<0.005$). Scale bar, 10 μ m.

Fig. 5. Thrombin and LPA Suppressed Axon Branching in Rat Hippocampal Neurons

Primary cultured hippocampal neurons were transiently transfected with EYFP at 3 DIV. Then cells were treated with either 1 unit/ml thrombin or 1 μ M LPA for 12 h and fixed at 3 DIV. Transfected cells are shown by EYFP fluorescence. Quantitative analyses of effects of thrombin and LPA treatment on axon branching in hippocampal neurons were also shown. Results shown are the means $\pm$ S.E.M. of three independent experiments ($n=60$). Statistically significant differences from the value of non-stimulated cells are indicated by * ($p<0.005$). Scale bar, 20 μ m.

Fig. 6. Knockdown of $G\alpha_{12}$ and $G\alpha_{13}$ in Cultured Rat Hippocampal Neurons Suppressed the Thrombin- or LPA-Induced Growth Cone Collapse

(A) Lysates from primary cultured hippocampal neurons transfected with non-specific shRNA, $G\alpha_{12}$ shRNA, $G\alpha_{13}$ shRNA, or both were analyzed by immunoblotting with anti- $G\alpha_{12}$, anti- $G\alpha_{13}$ and anti- α -tubulin (control) antibodies. (B) Primary cultured hippocampal neurons were transiently cotransfected with EYFP and non-specific shRNA, $G\alpha_{12}$ shRNA, $G\alpha_{13}$ shRNA, or both at 1.5 DIV. Then cells were treated with either 1 unit/ml thrombin, 1 μ M LPA for 0.5 h or Sema4D-contained conditioned medium for 1.5 h, and fixed at 3 DIV. F-actin was stained with rhodamine-conjugated phalloidin to visualize the growth cone structure. Scale bar, 10 μ m.

growth cone collapse as a reference. Hippocampal neurons were transfected with EYFP and each shRNA (non-specific shRNA, $G\alpha_{12}$ shRNA and $G\alpha_{13}$ shRNA) at 1.5 DIV and treated with either thrombin, LPA or Sema4D at 3 DIV. The expression of non-specific shRNA, $G\alpha_{12}$ shRNA, $G\alpha_{13}$ shRNA, or both $G\alpha_{12}$ shRNA and $G\alpha_{13}$ shRNA, in hippocampal neurons had little effect on the growth cone morphology (percentage of the number of growth cone-positive cells: non-specific shRNA, $76.66 \pm 3.33\%$; $G\alpha_{12}$ shRNA, $86.66 \pm 3.33\%$; $G\alpha_{13}$ shRNA, $90.00 \pm 2.89\%$; both $G\alpha_{12}$ shRNA and $G\alpha_{13}$ shRNA, $83.33 \pm 4.41\%$; Figs. 6B and 7). In contrast, the expression of $G\alpha_{12}$ shRNA or $G\alpha_{13}$ shRNA significantly suppressed the thrombin-induced growth cone collapse ($G\alpha_{12}$ shRNA, $61.67 \pm 1.17\%$, $p < 0.005$; $G\alpha_{13}$ shRNA, $58.33 \pm 1.17\%$, $p < 0.005$) compared to non-specific shRNA ($36.67 \pm 1.17\%$; Figs. 6B and 7). The expression of $G\alpha_{12}$ shRNA or $G\alpha_{13}$ shRNA also significantly suppressed the LPA-induced growth cone collapse ($G\alpha_{12}$ shRNA, $60.00 \pm 2.89\%$, $p < 0.005$; $G\alpha_{13}$ shRNA, $65.00 \pm 2.89\%$, $p < 0.005$) compared to non-specific shRNA ($33.33 \pm 4.41\%$; Figs. 6B and 7). On the other hand, the expression of neither $G\alpha_{12}$ nor $G\alpha_{13}$ shRNA attenuated the Sema4D-induced growth cone

collapse (non-specific shRNA, $33.33 \pm 4.41\%$; $G\alpha_{12}$ shRNA, $36.67 \pm 1.17\%$; $G\alpha_{13}$ shRNA, $31.67 \pm 1.17\%$; Figs. 6B and 7), indicating that $G\alpha_{12}$ and $G\alpha_{13}$ are not involved in Sema4D signalling pathway in hippocampal neurons. We further examined the effects of double knockdown of $G\alpha_{12}$ and $G\alpha_{13}$ with their shRNAs on the growth cone collapse. As shown in Figs. 6B and 7, the expression of both $G\alpha_{12}$ shRNA and $G\alpha_{13}$ shRNA significantly augmented the suppression of thrombin- and LPA-induced growth cone collapse by single knockdown of either $G\alpha_{12}$ or $G\alpha_{13}$ (thrombin, $75.00 \pm 2.89\%$, $p < 0.005$; LPA, $76.67 \pm 1.17\%$, $p < 0.005$). Double knockdown had also no effect on the Sema4D-induced growth cone collapse (Sema4D, $33.33 \pm 4.41\%$). We further examined the effects of single and double knockdown of $G\alpha_{12}$ and $G\alpha_{13}$ shRNAs on the growth cone collapse induced by lower concentrations of LPA and thrombin. As shown in Fig. 8, although the levels of growth cone collapse induced by lowered concentrations of LPA and thrombin were decreased, the tendency toward augmentation of the effects of single knockdown by double knockdown was observed. These results indicate that thrombin and LPA receptors induce growth cone collapse through coupling to both $G\alpha_{12}$ and $G\alpha_{13}$ in hip-

Fig. 7. Quantitative Analyses of Effects of Knockdown of $G\alpha_{12}$ or $G\alpha_{13}$ on Axonal Morphology in Rat Hippocampal Neurons

Results shown are the means $\pm$ S.E.M. of three independent experiments ($n=60$). Statistically significant differences from the value of non-specific shRNA-transfected cells are indicated by * ($p<0.005$).

pocampal neurons.

Thrombin and LPA receptors have been shown to selectively couple to $G\alpha_{12}$ and $G\alpha_{13}$ respectively in fibroblast cell lines and HEK293T cells.^{11,13} In contrast, thrombin and LPA have been known to stimulate GTP binding to both $G\alpha_{12}$ and $G\alpha_{13}$ *in vitro*, indicating that thrombin and LPA receptors have ability to couple to both $G\alpha_{12}$ and $G\alpha_{13}$.^{4,23,24} In this study, we demonstrated that thrombin and LPA receptors use both G proteins for growth cone collapse. Considering potential coupling ability of thrombin and LPA receptors to two G_{12} family G proteins, usage of G proteins by the receptors may be ascribed to cell specificity or diversity of coupling systems. In contrast to repulsive responses of Sema4D in axon guidance of various neurons, physiological roles of thrombin receptors in central nervous system have not been well understood, but the roles of LPA receptors during development of cortical neurons have been studied. Expression of LPA receptor, LPA1, is enriched in the ventricular zone, a neurogenic region of the embryonic cerebral cortex, where neuroblasts undergo neuronal morphological changes, having leading process, and extracellular LPA produced by postmitotic neurons induces process retraction of neuronal cells in the ventricular zone.²⁵ Thus, LPA may act as stop signals for leading process formation of newly developed cortical neurons in the ventricular zone.

In conclusion, we have demonstrated that the activation of $G\alpha_{12}$ or $G\alpha_{13}$ induces axonal growth cone collapse dependent on Rho-kinase activity in rat hippocampal neurons and that thrombin and LPA receptors induce the growth cone collapse through coupling to both two G_{12} family G proteins, $G\alpha_{12}$ and $G\alpha_{13}$, which are differentially localized in neurons. At present, functional significance of differential localization of two G proteins, $G\alpha_{12}$ and $G\alpha_{13}$, for RhoA activation and subsequent growth cone collapse, remains unclear. Further studies addressing this issue will contribute to understanding

Fig. 8. Concentration Dependency of the Thrombin- or LPA-Induced Growth Cone Collapse and Effects of Single and Double Knockdown of $G\alpha_{12}$ and $G\alpha_{13}$ in Cultured Rat Hippocampal Neurons

Primary cultured hippocampal neurons were transiently cotransfected with EYFP and non-specific shRNA, $G\alpha_{12}$ shRNA, $G\alpha_{13}$ shRNA, or both at 1.5 DIV. Then cells were treated with the indicated concentrations of either thrombin (A) or LPA (B) for 0.5 h, and fixed at 3 DIV. Results shown are the means $\pm$ S.E.M. of three independent experiments ($n=60$).

of regulation of neuronal morphology by GPCR- G_{12} family signalling pathways.

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