Soluble Guanylyl Cyclase Inhibitor Prevents Sema3F-Induced Collapse of Axonal and Dendritic Growth Cones of Dentate Granule Cells

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Controlling axon and dendrite elongation is critical in developing precise neural circuits. Using isolated cultures of dentate granule neurons, we succeeded in simultaneously monitoring the behaviors of axonal and dendritic outgrowth. Our previous study shows that cAMP contributes differentially to Sema3F-induced responses of axons and dendrites, but we report here that the cGMP modulation does not have such a striking axo-dendritic difference. Treatment with Sema3F induced collapse of about 90% growth cones, and pretreatment with 1 μM LY83583, an inhibitor of soluble guanylyl cyclase, partially alleviated the collapse of both axons and dendrites. Thus, unlike cAMP, cGMP modulates axonal and dendritic extension in a similar manner.

Key words cyclic nucleotide; dentate gyrus; semaphorin; hippocampus; axon guidance; network formation

During neural development, neurites take precisely defined routes and contact with proper target nerve cells, thereby establishing the functional neural network. This process depends on proper guidance of axons and dendrites. Axons and dendrites are both tipped with motile growth cones that decide the orientation to extend by reading extracellular guidance signals.[1,2] To assess the efficacy of repulsive guidance molecules, a good index is the ratio of collapsed growth cones after bath application of these molecules[1,3]; note that repulsive molecules entirely collapse growth cones when they do not make gradients in the environment surrounding the growth cones. Growth cones have lamellipodia and filopodia; the lamellipodia contain cross-linked networks of actin filaments, whereas the filopodia contain bundled filamentous actin (F-actin). Advancement and retraction of growth cone movement depend on balanced regulation of actin polymerization and depolymerization in lamellipodia and filopodia.[2] Because of their high actin content, the high-affinity F-actin probe phalloidin can be used as a marker reagent to analyze the morphology of growth cones (Fig. 1B).[3]

Simultaneous analyses of the outgrowth of both axons and dendrites are an essential step to elucidating the mechanisms underlying the network formation, but most studies have investigated either axons or dendrites, simply because of the lack of appropriate experimental procedures to assess both simultaneously. To date, very little comprehensive information has hence been accumulated to compare axonal and dendritic outgrowth. With this respect, we recently established an experimental system to cultivate isolated granule cells prepared from the dentate gyrus, which allows simultaneous monitoring of axons and dendrites.[3]

Sema3F, a member of class 3 semaphorins that repel neurites, is expressed in the dentate gyrus *in vivo*[4,5] and efficiently repels axons arising from dentate gyrus tissues in collagen gel cultures.[6] Our previous study shows that Sema3F collapses growth cones of dendrites and axons and that cAMP contributes differentially to Sema3F signaling in axonal and dendritic responses; Sema3F-induced growth cone collapse was blocked by inhibition and activation of cAMP signaling in axons and dendrites, respectively.[3] The involvement of cGMP, however, has not so far been examined. The present work was designed to examine the effect of cGMP signaling cascade on the responses of axonal and dendritic growth cones to Sema3F.

MATERIALS AND METHODS

Primary Cultures of Dentate Granule Cells Dispersed cultures of granule cells were prepared from postnatal day 3 Wistar/ST rats (SLC, Shizuoka, Japan) as previously described,[3] according to National Institutes of Health guidelines for laboratory animal care and safety. The hippocampal formation was dissected out from hypothermized animals, and the subicular complex and the Ammon's horn were removed in ice-cold Gey's balanced salt solution. The remaining dentate gyrus was trypsinized and triturated. Neurons were plated onto 13-mm-ϕ coverslips coated with poly-L-lysine (Sigma) at a cell density of 5.0×10^3 cells/cm^2 in 50% Neurobasal/B-27 (Life Technologies, Gaithersburg, MD, U.S.A.) and 50% astrocyte-conditioned medium at 37 °C in a humidified 5% CO_2 and 95% air atmosphere. Culture medium was replaced 24 and 72 h after plating with astrocyte-conditioned medium-free Neurobasal/B-27, supplemented with 2 μM cytosine-D-arabino-furanoside (Sigma). Cultures were assayed at day 4 *in vitro*.

Conditioned Media of Sema3F-Transfected COS-7 Cells pEF mouse Sema3F-myc and pEF-myc (control) were provided by Dr. Atsushi Tamada (National Institute for Basic Biology, Okazaki, Japan). COS-7 cells were maintained in Dulbecco's modified Eagle medium (DMEM, Nacalai Tesque, Inc., Kyoto, Japan) supplemented with 10% fetal bovine serum, 50 U/ml penicillin G, and 100 μg/ml streptomycin. The medium was changed to serum-free DMEM 24 h after plating. Transfection into cells was performed with Fu-GENE6 (Roche, Indianapolis, IN, U.S.A.) according to the manufacture's instructions. After another 72 h, the supernatants of centrifugation at $100\times\boldsymbol{g}$ for 5 min at 4 °C were collected and were stored at −80 °C after filtration through filters of 0.20-μm pore size. Once thawed, the conditioned medium was used within 12 h. For every preparation, Sema3F-myc content was confirmed by Western blot analysis with anti-myc antibody,[3] which revealed that almost the same amount of Sema3F was consistently obtained in the

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conditioned media. The media were used at a 1 : 32 dilution, the least concentration at which the Sema3F effect reaches the maximum. Preliminary experiments showed that 10-min treatment with the Sema3F-containing media caused a 75% decrease in growth cones of sympathetic neurons prepared from embryonic day 17—18 Wistar/ST rats, compared to control media, which indicates that the expressed myc-Sema3F was functional (data not shown).

Growth Cone Collapse Assay After a 96-h incubation, cells were pretreated with 6-anilino-5,8-quinolinequinone (LY83583, Calbiochem, La Jolla, CA, U.S.A.) or 8-bromo guanosine cyclic monophosphate sodium salt (8-Br-cGMP, Calbiochem) for 30 min, and subsequently treated with conditioned media prepared from Sema3F-transfected or mock-transfected COS-7 cells (10 min) in the presence of the same drugs, and immediately fixed for 20 min at 37 °C in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9) containing 4% PFA, 0.25% glutaraldehyde (Nacalai Tesque, Kyoto, Japan), 0.1% Triton X-100, 10 μM Taxol (Sigma), and 1.3 μM phalloidin (Sigma).[7]

Cultures were triple-stained with rhodamine phalloidin and anti-tau-1 and anti-MAP-2 antibodies. Briefly, fixed cells were blocked with 2% goat serum for 60 min, followed by an overnight 4 °C incubation with primary antibodies against tau-1 (1 : 2000, mouse monoclonal, MAB3420, Chemicon, Temecula, CA, U.S.A.) and MAP-2 (1 : 1000, rabbit, AB5622, Chemicon). They were then incubated with rhodamine phalloidin (1 : 40, R-415, Molecular Probes, Eugene, OR, U.S.A.) and the fluorescein-conjugated secondary antibodies anti-mouse IgG Alexa-488 (1 : 400, A-11001, Molecular Probes) and anti-rabbit IgG Alexa-350 (1 : 400, A-11046, Molecular Probes) for 5 h at room temperature. Fluorescence signals were visualized with an ORCAII cooled CCD camera (Hamamatsu photonics, Hamamatsu, Japan) equipped with a Nikon ECLIPSE TE300 inverted microscope and a 40× objective. Images were analyzed with a Hamamatsu AQUACOSMOS system.

We scored cells that did not contact with adjacent cells and had one longest, tau-1-positive, MAP2-negative axon and a few tau-1-negative, MAP2-positive dendrites. Due to these strict criteria, about 50% of cells were discarded before data analysis. Growth cone collapse was judged based on phalloidin images (Fig. 1), that is, a growth cone was considered "collapsed" if it had less than two filopodia and lamellipodia smaller than 10 μm^2.[3] As for dendrites, we only analyzed terminals that were not overlapped onto neighboring protrusions within a cell of interest. Data are presented as the mean percentage (±S.E.M.) of collapsed growth cones to the total number.

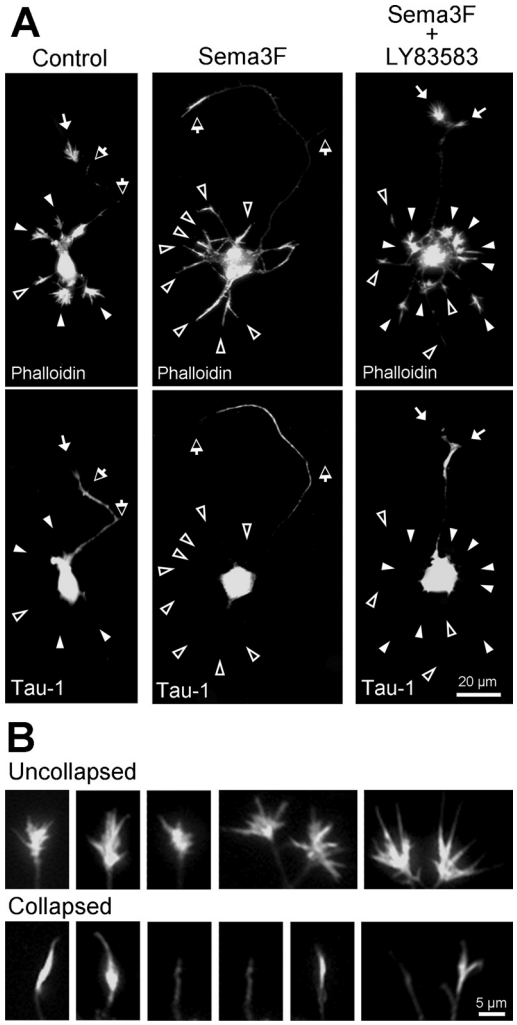

Fig. 1. Blockade of cGMP Signaling Abolishes Sema3F-Induced Collapse of Axonal and Dendritic Growth Cones

(A) Typical fluorescent microscopic images of isolated granule cells stained with rhodamine phalloidin (top) and anti-tau-1 (bottom) after 10-min treatment with control supernatant (left) or Sema3F-containing supernatant in the absence (middle) and presence of the soluble guanylyl cyclase inhibitor LY83583 (right, 1 μM) at day 4 *in vitro*. The axon was defined as the longest, tau-1-positive process, and the other tau-1-negative neurites were regarded as dendrites. Closed arrows and arrowheads indicate intact (*i.e.*, "uncollapsed") growth cones of axons and dendrites, respectively. Open arrows and arrowheads indicate "collapsed" axonal and dendritic terminals, respectively. Each top–bottom pair of images was obtained from the same microscopic field. Data are summarized in Fig. 2. (B) Representative high-magnification images of uncollapsed (top) and collapsed (bottom) growth cones of granule cells labeled with rhodamine phalloidin.

RESULTS

Using primary cultures of granule cells, we examined the response of axonal and dendritic growth cones to sema3F. Axons and dendrites were identified based on tau-1 immunoreactivity, that is, the former was tau-1-positive at day 4 *in vitro*, whereas the latter was tau-1-negative (Fig. 1). Time-lapse analysis revealed that both axonal and dendritic growth cones were highly motile and spontaneously alternated between collapsed and uncollapsed states. On average, 60—80% of growth cones were collapsed at any given time, and this value did not differ between axons and dendrites.

When granule cells were treated for 10 min with the culture medium harvested from COS-7 cells transfected with myc-tagged Sema3F cDNA, the percentage of collapsed growth cones was increased (Figs. 1A, 2). To investigate whether Sema3F-induced growth cone collapse requires cGMP signaling pathway, we applied LY83583, an inhibitor of soluble guanylyl cyclase, for 30 min and subsequently together with Sema3F for 10 min. LY83583 prevented Sema3F-induced collapses of both axons and dendrites (Fig. 2). Treatment for 40 min with 5mM 8-Br-cGMP, cGMP analog, alone induced growth cone collapses of both axons and dendrites (Fig. 2).

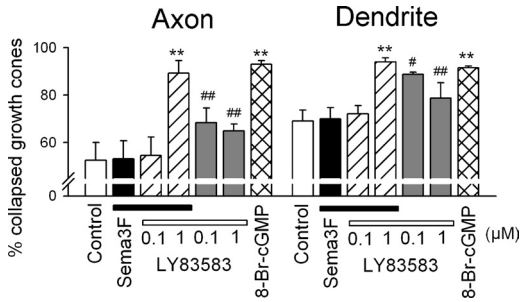

Fig. 2. cGMP Signaling is Required for Sema3F-Induced Growth Cone Collapse of Both Axons and Dendrites

Sema3F-induced growth cone collapse was assessed 10 min after co-treatment with LY83583. Cultures were treated with LY83583 (hatched columns) for 40 min, during the last 10-min period of which Sema3F was co-applied. 8-Br-cGMP (5mM, cross-hatched bars) was applied for 40 min. Data represent means±S.E.M. of four independent experiments (32—48 neurons). $^{**}p<0.01$ *versus* control, $^{\#}p<0.05$, $^{\#\#}p<0.01$ *versus* Sema3F; Duncan's test after one-way ANOVA.

DISCUSSION

We previously found that within identical neurons, activation of cAMP signaling enhances and prevents Sema3F-induced collapse of axonal and dendritic growth cones, respectively.[3] As for cGMP, it is reported that growth cones of chick dorsal root ganglion neurons are collapsed by increasing cellular cGMP levels,[8] whereas apical dendrites of cortical pyramidal cells are attracted to Sema3A *via* cGMP signaling.[9] Therefore, it was possible that the cGMP modulation also differed between the axon and dendrites of a granule cell. We have shown that the blockade of cGMP signaling inhibits sema3F-induced neurite collapse, but failed to find evidence for any axo-dendritic difference.

Ayoob *et al.*[10] recently demonstrated that the receptor guanylyl cyclase *Gyc76C* genetically interacts with the molecules involving semaphoring-1a (Sema-1a) signaling. This interaction is necessary for the action of Sema-1a in the developing *Drosophila* nervous system. Our previous study using organotypically cultured hippocampal slices demonstrated that blockade of cGMP signaling disturbs target-specific innervation of mossy fibers.[11] Thus, the cGMP signaling pathway is likely to serve to lead the mossy fibers to the limited area, *i.e.*, stratum lucidum. We speculate that Sema3F may exist in CA3 subfields other than the stratum lucidum, thereby preventing mossy fibers from straying out of the stratum lucidum *via* intracellular cGMP signaling.

In conclusion, axonal and dendritic growth cones of dentate granule cells collapse when exposed to Sema3F, and this Sema3F effect requires the activation of cGMP signaling. It is a significant finding that, unlike cAMP, the cGMP action did not show a marked contrast between axons and dendrites. Given that in *Xenopus* spinal neurons, the ratio of cAMP to cGMP activities determines the turning behavior of extending axons,[12] a combination of the complex cAMP action and the monotonic cGMP action on axonal and dendritic outgrowth of granule cells could embody a fine tuning of the dentate circuit development.

Acknowledgements The authors thank Dr. Atsushi Tamada for providing us with Sema3F-myc cDNA. This work was supported in part by Grant-in-Aid for Science Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (No. 17023015, No. 17650090, No. 17689004) and by the Sumitomo Foundation (No. 050038).

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