Gymnopilin—a substance produced by the hallucinogenic mushroom, *Gymnopilus junonius*—mobilizes intracellular Ca^{2+} in dorsal root ganglion cells

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ABSTRACT

Gymnopilus junonius is a widely spread mushroom in Japan and well known as a hallucinogenic mushroom. Gymnopilin was purified from the fruiting body of *G. junonius* and was reported to act on the spinal cord and depolarize motoneurons. This is the only evidence that gymnopilin has a biological effect on animals and no mechanism of the action has been determined at all. In this study, we examined effects of gymnopilin on intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) of cultured cells isolated from the dorsal root ganglion (DRG) of the rat. The cell culture consisted of neurons and non-neuronal cells. Gymnopilin increased $[\text{Ca}^{2+}]_i$ in both the types of cells. The gymnopilin-evoked $[\text{Ca}^{2+}]_i$ rise in the non-neuronal cells was inhibited by cyclopiazonic acid and U-73122, inhibitors of Ca^{2+} -ATPase of the intracellular Ca^{2+} store and phospholipase C, respectively, but not by removal of extracellular Ca^{2+} . These results indicate that gymnopilin activated phospholipase C and mobilize Ca^{2+} from the intracellular Ca^{2+} store in non-neuronal cells from the DRG. This is the first report to show that gymnopilin directly acts on cells isolated from the mammalian nervous system.

Gymnopilus junonius is a widely spread wild mushroom in Japan and well known as a hallucinogenic mushroom. It is called “Ohwaraitake”, which means a big laughter mushroom in Japanese, and people who accidentally ingest them show some symptoms of the mushroom poisoning such as visual and auditory hallucinations. Psilocin and psilocybin are well known as hallucinogenic substances produced in some kinds of poisonous mushrooms (14). Although psilocybin was identified as a hallucinogenic substance in the other *Gymnopilus* species grown in America and Europe (5), psilocybin was not detected in the fruiting body of *G. junonius* grown in Ja-

pan (11). Therefore, other substances produced in the fruiting body of *G. junonius* are considered to be hallucinogenic. Gymnopilin was purified from the fruiting body of *G. junonius* (1) and its structure was determined (12). It was reported that gymnopilin showed a depolarizing effect on the ventral root from the lumbar spinal cord (15). Although this is the only study to show a biological effect of gymnopilin in animal tissues and underlying mechanisms of the action of gymnopilin are not determined at all, it is believed that gymnopilin is a cause of the *G. junonius* poisoning. Therefore, we examined effects of gymnopilin on the activity of cells isolated from the central and peripheral nervous systems of the rat. We measured intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) as an index of the cellular activity, to investigate mechanisms of the action of gymnopilin using the dorsal root ganglion cells of the rat.

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MATERIALS AND METHODS

Isolation and purification of gymnopilin. Gymnopilin was purified according to the method described in the report by Aoyagi *et al.* (1) from the wild fruiting bodies of *Gymnopilus junonius* collected in Japan. In brief, wild fruiting bodies of *G. junonius* were lyophilized, crunched and stored at -20°C . Six hundred mL of methanol was added to 20 g of the *G. junonius* powder and stirred for 1 h at room temperature. After subsequent filtration of the supernatant, the precipitate was washed with methanol. The combined supernatants were concentrated by an evaporator to give 7.45 g of methanol extract. The residue was dissolved in H_2O and washed with ethyl acetate to remove polar compounds (mono- and oligosaccharides, amino acids and so on). The pH of the aqueous phase was adjusted to 3 with 1-M HCl and extracted with ethyl acetate three times. The combined organic layer was dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and concentrated *in vacuo* to give 558 mg of crude gymnopilins. It was purified on C18 cartridge (5%, 25%, 50%, 75% and 100% methanol as an eluent). Gymnopilins were eluted in 75% methanol fractions. They were combined and concentrated to give 127 mg of gymnopilins. The ^1H NMR and ^{13}C NMR spectra of the product were compared with that reported by Aoyagi to assign it to gymnopilins. Figure 1 shows the molecular structural formulas of 3 typical types of gymnopilins. The concentration of gymnopilin in solutions used in this study was calculated on the assumption that an average molecular weight of purified gymnopilin was 1,000.

Animals. Male Wistar rats (3–7 weeks old) were used. Rats were provided *ad libitum* access to water and food pellets (CE-2; CLEA, Tokyo, Japan), and housed under the artificial lighting for 12 h a day

Fig. 1 The structure of gymnopilin. The structural formulas of 3 typical types of gymnopilins are shown. The calculated molecular weight is 1089.55 ($m = 1, n = 7$), 1175.68 ($m = 1, n = 8$), 1261.81 ($m = 1, n = 9$), 985.41 ($m = 2, n = 5$), 1071.54 ($m = 2, n = 6$), 1157.67 ($m = 2, n = 7$), 1053.53 ($m = 3, n = 5$) and 1139.66 ($m = 3, n = 6$).

(from 7:00 AM to 7:00 PM) in a temperature-controlled room ($22 \pm 2^{\circ}\text{C}$). All experiments were made according to the guidelines stipulated by the ethical committee of Tottori University. We made the maximum effort to reduce the number and the suffering of the rats used.

Cell isolation and culture. Dorsal root ganglion (DRG) cells were isolated from adult male Wistar rats (7–12 weeks old), using procedures reported previously (8, 9). In brief, rats were killed by the decollation under anesthesia by pentobarbital sodium (50 mg/kg B.W., i.p.). Ganglia were dissected from the entire length of the vertebral column. Axons extending from the ganglia were removed under a stereoscopic microscope. The ganglia were incubated at 37°C in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline (PBS) containing collagenase type IV (500 U/mL; Worthington Biochemicals, Lakewood, NJ, USA), DNase I (0.12 $\mu\text{g}/\text{mL}$; Sigma, St Louis, MO, USA) and BSA (1 mg/mL; Sigma) for 2 h, and subsequently in PBS containing trypsin (0.25% w/v; Invitrogen, Carlsbad, CA, USA) and BSA (1 mg/mL) for 15 min. After the enzymatic digestion, the cells were gently triturated with a silicon-coated Pasteur pipette. The isolated cells were suspended in Dulbecco's modified Eagle medium (DMEM; Invitrogen) containing 4.5 g/L of glucose and cultured on coverslips coated with poly-D-lysine (Sigma). The cells were kept at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and cultured until use. DMEM was supplemented with 10% fetal bovine serum (MP Biochemicals, Irvine, CA, USA), 100 U/mL of penicillin (Invitrogen), 100 ng/mL of streptomycin (Invitrogen), and 5 μM of cytosine- β -D-arabinofuranoside (Sigma). A half volume of the culture medium was replaced with a fresh medium every 2 days. $[\text{Ca}^{2+}]_i$ measurements were performed in cells cultured for 4–10 days.

Measurement of intracellular Ca^{2+} concentrations. $[\text{Ca}^{2+}]_i$ in cultured rat DRG cells was measured with the fluorescent Ca^{2+} indicator fura-2, according to a procedure reported previously (13). Cultured cells attached to round coverslips (11 mm in diameter) were incubated in HEPES-buffered normal solution (in mM: 154 NaCl, 6 KCl, 2 CaCl_2 , 5 MgCl_2 , 10 Glucose, 10 HEPES, pH 7.4 adjusted with tris) containing 3- μM Fura-2/AM (Merck, Whitehouse Station, NJ, USA) and 0.01% Pluronic F-127 (Invitrogen) for 60 min at room temperature (22 – 24°C). After the incubation, a coverslip was mounted in a chamber fixed on the stage of an inverted fluores-

cence microscope (IX71; Olympus, Tokyo, Japan). Cells were continuously perfused with various experimental solutions through polyethylene tubes connected to a peristaltic pump (Minipulse 3; Gilson, Middleton, WI, USA) at a flow rate of 1.4 mL/min. In this system, the solution around the cells could be changed rapidly within a few seconds. Changes in $[\text{Ca}^{2+}]_i$ were measured by dual excitation microfluorometry using a digital image analyzer (Aqua Cosmos/Ratio; Hamamatsu Photonics, Hamamatsu, Japan). The fluorescent signal was detected using a UV objective lens (UApo 20×3/340; Olympus), and the emitted light passing through a band pass filter (500 ± 10 nm) was detected by a cooled CCD camera (ORCA-ER; Hamamatsu Photonics). All experiments were performed at room temperature ($22\text{--}24^\circ\text{C}$).

Data acquisition and analysis. Data acquisition was performed using a procedure reported previously (7, 10). Data were obtained at a sampling frequency of 0.2 Hz using Aqua Cosmos Software (Hamamatsu Photonics). The fluorescence intensity at the excitation wavelengths of 340 nm (F340) and 380 nm (F380) was determined by the analyzing software (Aqua Cosmos; Hamamatsu Photonics). To calculate $[\text{Ca}^{2+}]_i$ in individual cells, regions of interest (ROIs) were chosen to include the soma of each cell, and the average values of $[\text{Ca}^{2+}]_i$ in all pixels contained in each ROI were calculated. In this study, $[\text{Ca}^{2+}]_i$ responses larger than 30 nM to various stimulations were considered to be significant. Changes in the calculated $[\text{Ca}^{2+}]_i$ were analyzed by IGOR Pro (Wavemetrics, Lake Oswego, OR, USA) and Excel (Microsoft, Redmond, WA, USA). Data are presented as the means $\pm$ SEM. The statistical significance was assessed by Student's *t* test or the analysis of variance (ANOVA). Differences were considered statistically significant if $P < 0.05$.

Solutions and drugs. During the $[\text{Ca}^{2+}]_i$ measurement, cells were continuously perfused with the HEPES-buffered normal solution (in mM: 154 NaCl, 6 KCl, 2 CaCl_2 , 5 MgCl_2 , 10 Glucose, 10 HEPES, pH 7.4 adjusted with tris). A solution containing K^+ at 60 mM was made by the isotonic replacement of Na^+ with K^+ . A Ca^{2+} -free solution was made by simply omitting CaCl_2 . The stock solutions of acetylcholine (ACh, 0.1 mM; Sigma), ATP Na (50 mM; Sigma), bradykinin (1 mM; Peptide Institute), calcitonin gene-related peptide (CGRP, 100 μM ; Peptide Institute), γ -aminobutyric acid (GABA, 100 mM; Sigma), galanin (100 μM ; Peptide Insti-

tute), glutamate Na (100 mM; Sigma), UTP Na (10 mM; Sigma) and substance P (100 μM ; Peptide Institute) were prepared by dissolving in H_2O and stored at -30°C . The stock solution of 5-HT (0.1 mM; Sigma) was dissolved in the 100-mM HCl solution and stored at -30°C . The stock solutions of cyclopiazonic acid (CPA, 10 mM; Sigma), prostaglandin E_2 (PGE_2 , 1 mM; Sigma), prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$, 1 mM; Sigma), U-73122 (1 mM; Sigma) and U-73343 (1 mM; Sigma) were dissolved in dimethyl sulfoxide and stored at -30°C . All these drugs were diluted to desired concentrations by the experimental solution on the day of use. Other drugs and chemicals used are of an analytical grade and were purchased from Sigma or Wako pure chemical (Osaka, Japan).

RESULTS

Effects of gymnopilin on cultured dorsal root ganglion cells

In this study, we measured $[\text{Ca}^{2+}]_i$ of cells as an index of cellular activity. DRG cell culture can be divided into two groups of cells. One is a group of cells that respond to a high concentration (50 mM) of K^+ and show rises in $[\text{Ca}^{2+}]_i$. The other is a group of cells that do not respond to the high K^+ stimulation. Cells in the former group regarded as neurons, because they possess the voltage-gated Ca^{2+} channels (the yellow cells in Fig. 2C). On the other hand, cells in the latter group regarded as non-neuronal cells (the blue cells in Fig. 2C). Since many types of non-neuronal cells in the nervous system are reported to be responsive to bradykinin (2, 3, 6), effects of bradykinin on $[\text{Ca}^{2+}]_i$ were examined. Bradykinin applied at 1 μM caused a $[\text{Ca}^{2+}]_i$ rise in the cells not responding to the high K^+ stimulation (Fig. 2D). Subsequently, gymnopilin at 100 μM was applied to the cells. Gymnopilin also increased $[\text{Ca}^{2+}]_i$ and the number of cells responding to gymnopilin was much larger than that of the cells responding to the high K^+ or bradykinin (Fig. 2E). Moreover, gymnopilin evoked a $[\text{Ca}^{2+}]_i$ rise in the cells in both two groups that responded and did not respond to the high K^+ stimulation. In 53 DRG neurons that responded to the high K^+ stimulation, 28 neurons responded to gymnopilin (53%). On the other hand, in 144 non-neuronal cells, which did not respond to the high K^+ stimulation, 120 cells responded to gymnopilin (83%). Since higher proportion of cells responded to gymnopilin in the non-neuronal cells than in the neurons, we focused on the non-neuronal cell in the DRG culture to investigate the underly-

Fig. 2 Changes in the $[Ca^{2+}]_i$ in cultured DRG cells. **A**: A bright field image of the observed area. **B–E**: Pseudo colored images of the $[Ca^{2+}]_i$ in the control condition (**B**), in the presence of 60-mM K^+ (**C**), bradykinin (1 μ M, **D**) and gymnopilin (100 μ M, **E**). Scale bars = 50 μ m.

ing mechanism of the $[Ca^{2+}]_i$ rise evoked by gymnopilin.

A Ca^{2+} source of the gymnopilin-evoked $[Ca^{2+}]_i$ rises
Cells were repetitively stimulated with gymnopilin to examine whether cells showed a $[Ca^{2+}]_i$ increase with a constant amplitude. In Fig. 3A, gymnopilin at 100 μ M was applied for 30 s three times with intervals of 15 min. The amplitude of the responses to gymnopilin decreased gradually. Although there is no significant difference between two sequential responses, the amplitude of the gymnopilin response tended to decrease during the repetitive stimulations (Fig. 3B). Because of this decrease in the amplitude of the gymnopilin response, amplitude of only the first response to gymnopilin in each cell was assessed to examine dose-dependency. To confirm whether the cells were responsive to gymnopilin or not, the second stimulation was made by gymnopilin at 100 μ M, *i.e.*, the cells were first stimulated by gymnopilin at 10, 30 or 100 μ M and subsequently stimulated by 100- μ M gymnopilin. The cells showing $[Ca^{2+}]_i$ increase larger than 30 nM to the stimulation with gymnopilin at 100 μ M were considered to be a gymnopilin-responsive cell. The amplitude of the $[Ca^{2+}]_i$ increase evoked by gymnopilin was dose dependent as shown in Fig. 3C.

To determine the Ca^{2+} source of the $[Ca^{2+}]_i$ rise evoked by gymnopilin, the cells were stimulated by gymnopilin in the absence of extracellular Ca^{2+} and in the presence of CPA, an inhibitor of Ca^{2+} -ATPase on the intracellular Ca^{2+} store. Even in the absence

of Ca^{2+} , gymnopilin applied at 100 μ M caused a $[Ca^{2+}]_i$ rise (Fig. 4A) and the amplitude was not different from that of the control response (Fig. 4B). CPA applied at 10 μ M itself caused a large increase in $[Ca^{2+}]_i$. It is well known that inhibition of the uptake of Ca^{2+} by the intracellular Ca^{2+} store results in an increase in the cytoplasmic Ca^{2+} concentration. After $[Ca^{2+}]_i$ reached a steady level in the presence of CPA, gymnopilin was applied. Under this condition, gymnopilin did not evoke a $[Ca^{2+}]_i$ rise at all in all the cells examined (Fig. 4A, B), indicating that Ca^{2+} was mobilized from the intracellular Ca^{2+} stores by the stimulation with gymnopilin.

Contribution of phospholipase C to the gymnopilin responses

It is possible that gymnopilin would activate phospholipase C (PLC) and produce inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol to induce mobilization of Ca^{2+} from the intracellular Ca^{2+} store. To assess this possibility, an effect of an inhibitor of PLC on the gymnopilin-induced $[Ca^{2+}]_i$ rise was examined. The inhibitor of PLC, U-73122, and its non-active analog, U-73343, were utilized. As a positive control experiment, an effect of U-73122 on the bradykinin-evoked $[Ca^{2+}]_i$ rise in non-neuronal cells from the DRG was examined. Bradykinin receptors were desensitized during repetitive activation. The amplitude of the bradykinin-evoked $[Ca^{2+}]_i$ response to four sequential stimulations with intervals of 15 min decreased gradually. Since the amplitudes of responses to the 1st and 2nd stimulations with 20-nM

Fig. 3 Gymnopilin-evoked $[\text{Ca}^{2+}]_i$ rises. **A:** A typical trace of the $[\text{Ca}^{2+}]_i$ responses to the 60-mM K^+ stimulation (60K) and the repetitive stimulations with gymnopilin (100 μM , 30 s) in the non-neuronal cell from the DRG. **B:** Summarized data of the amplitude of the $[\text{Ca}^{2+}]_i$ responses ($\Delta[\text{Ca}^{2+}]_i$) to three sequential stimulations with gymnopilin ($n = 20$). N.S., not significant. **C:** The dose dependency of the $[\text{Ca}^{2+}]_i$ response to gymnopilin. The amplitudes of the $[\text{Ca}^{2+}]_i$ responses to the first stimulation with gymnopilin in each non-neuronal cell were pooled and averaged ($n = 20$ for 10 μM , $n = 14$ for 30 μM and $n = 35$ for 100 μM). N.S., not significant; ***, $P < 0.001$ by t test.

bradykinin were significantly different but there was no significant difference between those to the 2nd and 3rd stimulations, U-73122 (10 nM) and U-73343

Fig. 4 Effects of the Ca^{2+} removal and CPA on the gymnopilin-evoked $[\text{Ca}^{2+}]_i$ rise. **A:** A typical trace of the $[\text{Ca}^{2+}]_i$ responses to the 60-mM K^+ stimulation (60K) and the gymnopilin stimulation in the absence of Ca^{2+} and the presence of CPA (10 μM). **B:** Summarized data of the amplitude of the $[\text{Ca}^{2+}]_i$ responses ($\Delta[\text{Ca}^{2+}]_i$) to gymnopilin in control, in the absence of Ca^{2+} and in the presence of CPA ($n = 19$). N.S., not significant; ***, $P < 0.001$ by t test.

(10 nM) were applied before the 3rd stimulation. Compared with the control response, U-73122 but not U-73343 at 10 nM reduced the amplitude of the $[\text{Ca}^{2+}]_i$ response to the 3rd bradykinin stimulation, indicating that the inhibitory action of U-73122 can be assessed in our preparations of the non-neuronal cell from the DRG. We also examined U-73343 at higher concentrations such as 1 μM . U-73343 at such a high concentration reduced the amplitude of the bradykinin response in our preparations. Therefore we decided to apply U-73343 and U-73122 at 10 nM, which are lower than the concentration generally used to inhibit PLC. Similar to the effect on the bradykinin response, U-73122 but not U-73343 inhibited the $[\text{Ca}^{2+}]_i$ response to gymnopilin. Gymnopilin at 100 μM was applied 3 times with intervals of 15 min. U-73122 (10 nM) or U-73343 (10 nM) was applied 2 min before the 2nd stimulation with gymnopilin (Fig. 5A, B). In the presence of U-73122 but not U-73343, the amplitude of the gymnopilin-evoked $[\text{Ca}^{2+}]_i$ rise decreased significantly (Fig. 5C, D, E), suggesting that gymnopilin activated PLC in

Fig. 5 Effects of the PLC inhibitor on the gymnopilin-evoked $[Ca^{2+}]_i$ rise. **A, B:** Typical responses to the 60-mM K^+ stimulation (60K) and the gymnopilin stimulation in the absence (control) and presence of U-73122 (10 nM, A) or U-73343 (10 nM, B). Gymnopilin at 100 μ M was applied three times at intervals of 15 min. The responses to the 1st and 2nd stimulations are shown. U-73122 and U-73343 were applied 2 min before the 2nd stimulation with gymnopilin. **C, D:** Summarized data of the amplitude of the gymnopilin responses in the absence (control) and presence of U-73122 (10 nM, C) or U-73343 (10 nM, D). The amplitude of the responses to the 3rd stimulation applied after the washout of the drugs is also shown (W.O.). N.S., not significant; **, $P < 0.01$; ***, $P < 0.001$ by t test. **E:** Relative amplitudes of the responses to the 3rd stimulation with gymnopilin in the presence of U-73122 or U-73343 to those to the 2nd stimulation. N.S., not significant; **, $P < 0.01$ by ANOVA

the non-neuronal cells from the DRG. After removal of U-73122, both the bradykinin- and gymnopilin-evoked responses recovered (Fig. 5C). However, after removal of U-73343, the amplitudes of responses to both bradykinin and gymnopilin decreased significantly (Fig. 5D).

Correlation between the gymnopilin response and the responses to the other G protein-coupled receptor agonists

The DRG contains various types of cells such as neurons and glial cells. Since various sizes of neurons with a diameter of the soma ranging 10 to 50 μ m exist in the DRG and it is not necessary that neurons extend long and axons and dendrites under

the primary culture conditions, it is difficult to distinguish neurons and the other types of cells based on their morphology. However, since most of the cultured non-neuronal cells from the DRG of the rat had immunoreactivity to the anti-S-100 and anti-GFAP antibody (our unpublished observation), it is thought that these non-neuronal cells would be derived from the astroglial cells. Since the DRG contains satellite cells and Schwann cells, most of the non-neuronal cells in the DRG culture may be originated from these types of the astroglial cells. It was reported that the glial cells expressed many kinds of Gq protein-coupled receptors (4). Since it was unclear what kinds of receptors were activated directly by gymnopilin, we examined correlation between

Fig. 6 Comparison between the amplitudes of the responses to gymnopilin and the agonists of the other G protein-coupled receptors. **A:** The amplitudes of the gymnopilin response ($\Delta[\text{Ca}^{2+}]_i$ of the evoked response) in the 45 non-neuronal cells are plotted against the amplitude of the responses to bradykinin at 20 nM in each cell. **B:** The amplitudes of the gymnopilin response in the 40 non-neuronal cells are plotted against the amplitude of the responses to ATP at 30 μM in each cell.

the gymnopilin responses and the responses to 12 agonists binding to the corresponding G protein-coupled receptors (GPCRs) coupling with Gq protein. In Fig. 6, the amplitudes of the gymnopilin response were plotted against the amplitudes of the bradykinin- and ATP-evoked responses in each cell. We also examined responses to the other agonists including glutamate (30 μM), GABA (10 μM), UTP (10 μM), PGE_2 (1 μM), $\text{PGF}_{2\alpha}$ (1 μM), ACh (30 μM), 5-HT (30 μM), substance P (1 μM), CGRP (1 μM) and galanin (1 μM). In this series of experiments, the agonists were applied at high enough concentrations to evoke the maximum response. However, we did not find any significant correlation between the amplitudes of the responses to gymnopilin and the other agonists.

DISCUSSION

In this study, we first showed that gymnopilin has an activating effect on neuronal and non-neuronal cells isolated from the peripheral nervous system. We focused on the non-neuronal cell, most of which were considered to be derived from the satellite and Schwann cells, to determine the underlying mechanisms of the gymnopilin-evoked $[\text{Ca}^{2+}]_i$ rise. The amplitude of the $[\text{Ca}^{2+}]_i$ rise evoked by gymnopilin increased with elevated concentrations of applied gymnopilin and decreased by the repetitive stimulation with gymnopilin. These results indicate that the observed effect of gymnopilin was not a physical and/or chemical artifact, but was a biological effect. The mechanism of the $[\text{Ca}^{2+}]_i$ rise by gymnopilin

has properties to be desensitized. The gymnopilin-evoked response was not affected by the removal of the extracellular Ca^{2+} and inhibited by the inhibition of Ca^{2+} -ATPase on the intracellular Ca^{2+} store, clearly indicating that gymnopilin caused the mobilization of the cytosolic Ca^{2+} from the intracellular Ca^{2+} store but did not activate the Ca^{2+} entry from the extracellular space. Moreover, since the PLC inhibitor, U-73122, inhibited the gymnopilin responses, it was suggested that PLC was activated during the stimulation with gymnopilin. We have no idea why the amplitude of bradykinin- and gymnopilin-evoked responses decreased after the removal of U-73343. It may be caused by an unidentified effect of U-73343.

We hypothesized three mechanisms of the action of gymnopilin: 1) gymnopilin activates some type of the GPCR coupling with Gq proteins and subsequently activates Gq proteins, 2) gymnopilin activates Gq proteins directly and subsequently activates PLC, and 3) gymnopilin activates PLC directly and produces IP_3 and DG. Among the non-neuronal cells tested, there are some cells that responded to bradykinin or ATP, but not to gymnopilin, and showed increase in $[\text{Ca}^{2+}]_i$. If the hypotheses 2 or 3 were true, gymnopilin should have evoked a $[\text{Ca}^{2+}]_i$ rise even in these bradykinin- or ATP-responding cells, because they have enough Gq proteins and PLC to mobilize Ca^{2+} from the intracellular Ca^{2+} stores. Therefore we concluded that gymnopilin acted on some GPCR and mobilized Ca^{2+} from the intracellular Ca^{2+} stores depending on the activation of PLC (the hypothesis 1). It has been reported that many types of GPCRs were expressed on the membrane of the glial cells (4). We tested correlations between the gymnopilin-evoked response and the responses evoked by the 12 agonists that bind and activate the corresponding GPCRs. However, no significant correlation was detected between them. This result suggested that gymnopilin acted on some GPCR other than tested GPCRs in this study.

Similar activating effects of gymnopilin on the cultured neuronal and non-neuronal cells were also observed in the cells isolated from the cerebral cortex of the rat (our unpublished observation). It is not necessary that the same mechanism of the action of gymnopilin described above contributed in the DRG neurons and the cerebral cortical cells. However, gymnopilin-sensitive mechanisms to induce a $[\text{Ca}^{2+}]_i$ rise may be generally involved in the mammalian central and peripheral nervous systems. At present, we have no idea what types of GPCRs gymnopilin acts on in the glial cells from the DRG. We are planning to clarify the gymnopilin receptor

in the future study. The extract purified as gymnopilin in this study is considered to consist of some types of gymnopilins with different molecular structure such as gymnopilin A and gymnopilin B (1, 12). It is also important to know what kinds of gymnopilins in the different groups have the biological effect on the neurons and glial cells.

It was reported that gymnopilin depolarized the motor nerve included in the ventral root from the lumbar spinal cord based on the extracellular recording of the neuronal activity (15). However, up to the present, there have been no evidence that gymnopilin directly acts on the neuron and glial cell. Here we first demonstrate gymnopilin activates the neurons and the glial cells isolated from the DRG of the rat. This effect may lead to the depolarization of the motoneurons. Moreover, it is also possible that these actions of gymnopilin would be a cause of the symptoms of the mushroom poisoning of patients who ingest *G. junonius*. It may be also possible that gymnopilin would be delivered into the brain crossing the blood-brain barrier and show an excitatory effect on the central nervous system. In the case that the serotonergic or dopaminergic systems, which are known to be excited by psilocybin, are excited by gymnopilin, we would have to take serious care of handling gymnopilin and *G. junonius*, because they can be materials to produce narcotics like psilocybin and psilocin. Therefore, it is also important to know how gymnopilin is delivered and what effect gymnopilin gives on the mammalian central nervous system.

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