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Mastoparan Changes the Cellular Localization of $G\alpha_{q/11}$ and $G\beta$ through Its Binding to Ganglioside in Lipid Rafts

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ABSTRACT

Although it is known that mastoparan, a wasp venom toxin, directly activates $G_{i/o}$, mastoparan-induced biological responses are not always explained by this mechanism. For instance, we have demonstrated previously that mastoparan suppressed phosphoinositide hydrolysis induced by carbachol in human astrocytoma cells (*FEBS Lett* **206:**91–94, 1990). In the present study, we examined whether mastoparan affected phosphoinositide hydrolysis by interacting with lipid rafts in PC-12 cells. Mastoparan inhibited UTP-induced increase in $[Ca^{2+}]_i$ and phosphoinositide hydrolysis in a concentration-dependent manner. UTP-induced phosphoinositide hydrolysis occurred in lipid rafts, because methyl-β-cyclodextrin, a disrupting regent of lipid rafts, inhibited the hydrolysis. Mastoparan changed the localization of $G\alpha_{q/11}$ and $G\beta$ together with cholesterol from lipid rafts to nonraft fractions or cytosol. These changes were inhibited by ganglioside mixtures, suggesting that mastoparan interacts with gangliosides in lipid rafts. In fact, ganglioside mixtures and neuraminidase, but not sialic acid, attenuated the inhibitory effect of mastoparan on phosphoinositide hydrolysis. Furthermore, fluorescence intensity of tyrosine residue of [Tyr^3]mastoparan was potentiated by ganglioside mixtures, suggesting the direct binding of mastoparan to gangliosides. Mastoparan caused cytotoxicity of PC-12 cells in a concentration-dependent manner, determined by LDH release. The mastoparan-induced cytotoxicity was significantly inhibited by neuraminidase or gangliosides. The order of inhibitory potency of gangliosides was GT1b $\approx$ GD1b $>$ GD1a $>$ GM1 $\gg$ GQ1b, but asialo-GM1 and sialic acid were inactive. These results suggest that mastoparan initially binds to gangliosides in lipid rafts and then it inhibits phosphoinositide hydrolysis by changing the localization of $G\alpha_{q/11}$ and $G\beta$ in lipid rafts.

Lipid rafts in plasma membranes are formed by accumulation of cholesterol and sphingoglycolipids principally and are assumed to be a dynamic domain that is produced and disrupted repeatedly (Simons and Ikonen, 1997). It has been revealed that a variety of signaling molecules are accumulated in lipid rafts, where signal transduction occurs more effectively. In addition, lipid rafts play important roles in intracellular vesicle transports (Nabi and Le, 2003), the homeostasis of cholesterol (Ikonen and Parton, 2000), the regulation of immunoreaction (Viola et al., 1999; Janes et al., 2000), and so on. Cholesterol is essential for the retention of lipid raft structures, because removing cholesterol from plasma membrane by treatment with methyl β-cyclodextrin (MβCD) disturbs the functions of lipid rafts (Shadan et al., 2004).

Because lipid rafts are detergent-insoluble at low temperature, they are collected as a low density fraction by sucrose density gradient centrifugation of detergent-treated cell lysates (Brown and Rose, 1992). This fraction is called detergent-resistant membrane (DRM), and various signaling molecules, such as G-protein-coupled receptors (Foster et al., 2003), G-proteins (Oh and Schnitzer, 2001), protein kinases (Wu et al., 1997), and ion channels (Martens et al., 2000) are enriched in DRM.

Mastoparan is a wasp venom toxin with a structure of 14 amino acid residues, Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2. As a structural feature, the C-terminal Leu of mastoparan is amidated. Mastoparan has various pharmacological activities, such as release of histamine from mast cells (Hirai et al., 1979), secretion of insulin from pancreatic islet cells (Yokokawa et al., 1989), regulation of sarcoplasmic reticular Ca^{2+} release by binding to glycogen phosphorylase (Hirata et al., 2000, 2003), and activation of arachidonic acid release (Gil et al., 1991). Because it has been revealed that mastoparan directly activates $G_{i/o}$ in a pertussis toxin-sensitive manner, mastoparan has been assumed to

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ABBREVIATIONS: MβCD, methyl β-cyclodextrin; DRM, detergent-resistant membrane; DMEM, Dulbecco's modified Eagle's medium; PTX, pertussis toxin; TCA, trichloroacetic acid; TBST, Tris-buffered saline containing 0.1% Tween 20; LDH, lactate dehydrogenase.

be a G-protein activator (Higashijima et al., 1988). We have demonstrated previously that mastoparan suppressed phosphoinositide hydrolysis induced by the muscarinic receptor agonist carbachol in 1321N1 human astrocytoma cells (Nakahata et al., 1990). Likewise, Wojcikiewicz and Nahorski (1989) showed that mastoparan inhibited phosphoinositide hydrolysis induced by guanosine 5′-*O*-(3-thio)triphosphate in permeabilized SH-SY5Y human neuroblastoma cells. It is difficult to explain why the inhibitory effect of mastoparan on phosphoinositide hydrolysis is mediated by its activation of $G_{i/o}$. Therefore, it seems likely that there is another mechanism of mastoparan-induced inhibition of phosphoinositide hydrolysis besides the activation of $G_{i/o}$. Because mastoparan affects various G-protein-coupled receptor signaling, mastoparan may not be a receptor antagonist. In addition, the suppression of phosphoinositide hydrolysis by mastoparan suggests that the site of action is upstream of phospholipase C. On the other hand, it has been reported that the expression level of sphingoglycolipids affects the composition of lipid rafts and changes signal transduction (Nishio et al., 2004). Thus, it is assumed that lipid rafts are important as a place for the regulation of signal transduction. Furthermore, recent investigations have revealed that a number of pathogens (microorganisms, parasites, and viruses) and toxins take advantage of lipid rafts as infection routes owing to enrichment of their receptor molecules. For instance, cholera toxin and melittin are associated with GM1 (Chatterjee and Mukhopadhyay, 2002) and *Vibrio cholerae* cytolysin requires sphingoglycolipids and cholesterol for its polymerization (Zitzer et al., 1999).

In the present study, therefore, we investigated whether mastoparan affected phosphoinositide hydrolysis by interacting with lipid rafts. The results obtained suggest that mastoparan initially binds to gangliosides, especially GD1b and GT1b on the cell surface, and then inhibits phosphoinositide hydrolysis by changing the localization of $G\alpha_{q/11}$ and $G\beta$ in lipid rafts.

Materials and Methods

Materials. Mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2) was obtained from Peptide Institute Inc. (Osaka, Japan). [Tyr^3]Mastoparan (Ile-Asn-Tyr-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2) was synthesized by a peptide synthesizer. Dulbecco's modified Eagle's medium (DMEM) was purchased from Nissui Pharmaceuticals (Tokyo, Japan). GM1 and GQ1b were from Calbiochem (Darmstadt, Germany). GD1a and GD1b were from Toyobo (Osaka, Japan). Uridine 5′-triphosphate (UTP), sialic acid, ganglioside mixtures, GT1b, asialoganglioside-GM1 and neuraminidase III were from Sigma (St. Louis, MO). Lactate dehydrogenase CII-test and cholesterol E-test were from Wako Pure Chemicals (Osaka, Japan). [*myo*-1,2-^{3}H]Inositol was from American Radiolabeled Chemicals (St. Louis, MO). Fura 2-AM was from Dojindo Laboratories (Kumamoto, Japan). Pertussis toxin (PTX) was from Funakoshi Pharmaceutical (Tokyo, Japan). Other chemicals and drugs were of regent grade or of the highest quality available.

Cell Culture. PC-12 cells were grown in DMEM containing 10% fetal bovine serum, 5% horse serum, 50 U/ml penicillin, and 50 U/ml streptomycin in a 37°C humidified incubator in an atmosphere of 95% air/5% CO_2.

Measurement of Intracellular Free Ca^{2+} Concentrations. The changes in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) was monitored by the intensity of fura 2 fluorescence at 37°C by spectrofluorometer (F-2000; Hitachi, Osaka, Japan), as described previously (Nakahata et al., 1994). PC-12 cells cultured on a 150-mm dish were collected by gentle pipetting and were washed twice with modified Tyrode's solution (137 mM NaCl, 2.7 mM KCl, 1.0 mM $MgCl_2$, 0.18 mM $CaCl_2$, 20 mM HEPES, and 5.6 mM glucose, pH 7.4). After centrifugation at 250*g* for 2 min, cells were treated with 1 mM fura 2-AM at 37°C for 15 min. Then cells were centrifuged at 250*g* for 2 min and washed twice with buffer. The cells were suspended in the buffer at a concentration of 10^6/ml, and 1.5 ml of the cell suspension was used for fura 2 assays. UTP-induced $[Ca^{2+}]_i$ elevation was monitored in the absence of extracellular Ca^{2+} by addition of 3.3 mM EGTA. After 100-s incubation with mastoparan, the response to UTP was observed. The minimum ratio of fluorescence was obtained by addition of 0.1% Triton X-100 in the presence of EGTA, and the maximum ratio of fluorescence was obtained in the presence of 10 mM $CaCl_2$.

Assay of [^{3}H]Inositol Phosphates. Phosphoinositide hydrolysis was monitored by measuring [^{3}H]inositol phosphates as described previously (Nakahata et al., 1990). In brief, PC-12 cells were cultured in 12-well plates at the density of 2.0×10^5 cells/well. Two days after seeding, cells were labeled overnight with DMEM containing 2 μCi/ml [^{3}H]inositol. Cells treated with PTX were cultured in DMEM containing 2 μCi/ml [^{3}H]inositol with 100 ng/ml PTX for 20 h. The cells were preincubated with modified Tyrode's solution, pH 7.4, containing 10 mM LiCl and 2 mM EGTA for 6 min after cells were washed twice with the solution. Then cells were incubated with drugs for 6 min, and they were further incubated with 100 μM UTP for an additional 6 min. The reaction was terminated by addition of equal volume of 10% trichloroacetic acid (TCA). The TCA extract was centrifuged at 1800*g* for 5 min, and the supernatant was washed three times with diethyl ether. Total inositol phosphates were separated by an anion exchange column (AG 1X-8) and counted by liquid scintillation counting.

Sucrose Density Gradient Centrifugation. PC-12 cells cultured on a 150-mm dish were washed twice with modified Tyrode's solution. Then cells detached from a dish by pipetting were incubated in 14 ml of modified Tyrode's solution containing 30 μM mastoparan for 1 h. After washing twice with the ice-cold solution, cells were centrifuged at 250*g* for 2 min, and the pellet was solubilized in 1 ml of lysis buffer containing 50 mM Tris-HCl, 50 mM NaCl, 0.1% Triton X-100, 5 mM EDTA, 1.0 mM Na_3VO_4, 5.0 mM $Na_4P_2O_7$, 1.0 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin/antipain, pH 7.6. The lysates were sonicated on ice and were incubated at 4°C for 1 h with frequent agitation. Lysates (1 ml) were mixed with 3 ml of 60% sucrose in STE buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, and 1.0 mM Na_3VO_4, pH 7.6) in a centrifuge tube, and they were overlaid with 4 ml of 35% sucrose and 4 ml of 5% sucrose. Centrifugation was performed at 200,000*g* for 16 h at 4°C with a Beckman SW41Ti rotor. Fractions of 1.0 ml were collected from the top of the gradients (Yamashita et al., 2001).

Immunoblotting. Each fraction was solubilized in Laemmli sample buffer (75 mM Tris-HCl, 2% SDS, 10% glycerol, 3% 2-mercaptoethanol, and 0.003% bromphenol blue) and resolved by SDS/PAGE on 11% acrylamide gels. Separated proteins were transferred to polyvinylidene difluoride membrane (Millipore, Bedford, MA) by a semidry blot apparatus. The blots were immersed in 3% skim milk in Tris-buffered saline, pH 7.4, containing 0.1% Tween 20 (TBST) for 2 h at room temperature. The blots were washed with TBST and incubated with the appropriate primary antibody for 2 h at room temperature. Then the blots were washed with TBST and incubated with a horseradish peroxidase-conjugated secondary antibody for 2 h. After washing, immnoreactive proteins were visualized with an enhanced chemiluminescence system.

$G\alpha_{q/11}$ Release from Membrane Preparations. PC-12 cells cultured on a 150-mm dish were washed twice with modified Tyrode's solution. Then cells detached from a dish by pipetting were lysed on ice with the lysis buffer (20 mM KCl, 2.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, and 20 mM HEPES, pH 7.4), and the lysates were sonicated on ice and centrifuged at 600*g* for 10 min at

4°C. Membrane preparations were obtained by centrifuging the supernatant at 15,000*g* for 30 min at 4°C. Membrane preparations were incubated with 30 μM mastoparan at 4°C for 1 h in 5 ml of buffer (110 mM KCl, 10 mM NaCl, 1 mM KH_2PO_4, 0.4 mM $MgCl_2$, 1 mM EGTA, 0.2 mM $CaCl_2$, pCa 7.0, 0.1 mM ATP, and 20 mM HEPES, pH 7.0), and the samples were centrifuged at 30,000*g* for 30 min at 4°C. The proteins in supernatant were precipitated by addition of equal volume of 10% TCA and were centrifuged at 10,000*g* for 10 min at 4°C. After washing three times with ethanol, the pellets were solubilized in the sample buffer (0.3 M Tris, 4.12% SDS, 16.7% glycerol, 16.7 mM Tris-HCl, 0.025% bromphenol blue, and 0.07 M dithiothreitol), and $G\alpha_{q/11}$ in the samples was detected as described under *Immunoblotting*. The density of the bands corresponding to $G\alpha_{q/11}$ was analyzed by densitometry (NIH Image 1.62), and the data were expressed as percentage of control.

Determination of Cholesterol. Content of cholesterol in fractions was measured with cholesterol E-test kit. Each fraction (100 μl) was added to a 1-ml reaction mixture (1.6 units of cholesterol esterase, 0.31 units of cholesterol oxidase, 5.2 unit peroxidase, 0.95 mM sodium 3,5-dimethoxy-*N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-aniline, 0.19 mM 4-aminoantipyrine, and 4.4 units of ascorbate oxidase) and incubated at 37°C for 30 min. Then, the absorbance of the solution at 600 nm was measured by a spectrophotometer (UV mini-1240; Shimadzu, Kyoto, Japan).

Determination of Tyrosine Fluorescence of [Tyr^3]Mastoparan. The fluorescence spectra were determined with a spectrofluorometer (Hitachi F-2000) at 37°C. [Tyr^3]Mastoparan at a concentration of 10 μM in H_2O was used instead of mastoparan. For measurements of the changes of the fluorescence of tyrosine of [Tyr^3]mastoparan upon its binding to ganglioside, ganglioside mixtures in H_2O were added to a 10 μM [Tyr^3]mastoparan solution. After 1 min, [Tyr^3]mastoparan was activated at 280 nm, and the emission spectrum was recorded. Background intensities of samples were subtracted from each sample spectrum.

Measurement of Lactate Dehydrogenase Activity. The cytotoxicity of mastoparan was monitored by measuring lactate dehydrogenase (LDH) activity. For this measurement, a lactate dehydrogenase CII kit was used. In brief, PC-12 cells cultured in a 150-mm dish were collected by gentle pipetting and washed twice with modified Tyrode's solution. The cells were then suspended in the buffer at a concentration of 10^6 cells/ml. Cell suspensions (200 μl) were preincubated with gangliosides or neuraminidase in Eppendorf tubes at 37°C for 15 min, and they were incubated with 10 μM mastoparan for additional 15 min. Reactions were terminated by cooling the tubes on ice. After samples were centrifuged at 1200*g* for 2 min, 10 μl of supernatants were used for determination of released LDH.

Data Analysis. The results were expressed as mean ± S.E.M., and the statistical difference of the values was determined by one-way analysis of variance with Dunnett test for multiple comparisons.

Results

Mastoparan Inhibits the Increase in $[Ca^{2+}]_i$ and Phosphoinositide Hydrolysis Induced by UTP in PC-12 Cells. UTP increased $[Ca^{2+}]_i$ of PC-12 cells through $P2Y_2$ receptor in the absence of extracellular Ca^{2+} in a concentration-dependent manner. This $[Ca^{2+}]_i$ increase is thought to be intracellular Ca^{2+} mobilization from endoplasmic reticula by inositol 1,4,5-trisphosphate accumulated by phosphoinositide hydrolysis through the $P2Y_2$ receptor. The increase in $[Ca^{2+}]_i$ induced by UTP was inhibited by 10 μM mastoparan (Fig. 1A). And mastoparan-induced inhibition of the $[Ca^{2+}]_i$ elevation was concentration-dependent (Fig. 1B). The inhibition curve for mastoparan is similar to that for mastoparan on carbachol-induced increase in $[Ca^{2+}]_i$ in 1321N1 cells (Nakahata et al., 1994). In addition, mastoparan inhibited UTP-induced phosphoinositide hydrolysis in a concentration-dependent manner (Fig. 2A), suggesting that the suppression of $[Ca^{2+}]_i$ increase by mastoparan is due to the inhibition of phosphoinositide hydrolysis but not other $[Ca^{2+}]_i$ regulatory mechanisms connecting to Ca^{2+}pump or Ca^{2+} channel. Next, we examined whether PTX affected mastoparan-induced inhibition of the phosphoinositide hydrolysis (Fig. 2B), because $P2Y_2$ receptor has been reported to be coupled not only to $G\alpha_q$ but also to $G\alpha_i$ (Erb et al., 2001). Although UTP-induced phosphoinositide hydrolysis was decreased by PTX, mastoparan-induced inhibition of phosphoinositide hydrolysis was not attenuated by PTX. On the other hand, MβCD, a reagent extracting cholesterol from plasma membrane to disrupt lipid rafts concentration-dependently inhibited UTP-induced phosphoinositide hydrolysis as well as mastoparan did (Fig. 2C).

Mastoparan Induced Change in the Localization of Signaling Molecules in Cell Membranes. To evaluate whether mastoparan changes the localization of signaling molecules in lipid rafts, detergent-treated PC-12 cell lysates were separated into fractions by sucrose density gradient

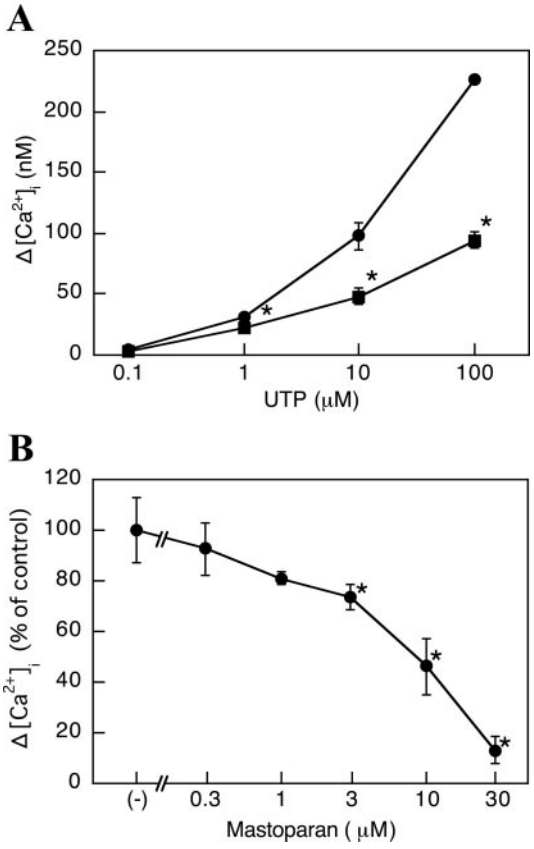

Fig. 1. Effect of mastoparan on UTP-induced $[Ca^{2+}]_i$ elevation in PC-12 cells. PC-12 cells loaded with fura 2 were suspended in the modified Tyrode's solution in a concentration of 10^6/ml as described under *Materials and Methods*. A, $[Ca^{2+}]_i$ elevation induced by 0 to 100 μM UTP in the presence (■) or absence (●) of 10 μM mastoparan under an extracellular Ca^{2+}-free condition (in the presence of 3.3 mM EGTA). *, significant difference between UTP alone and UTP plus mastoparan ($P < 0.05$). B, concentration-dependent inhibition by mastoparan of UTP-induced $[Ca^{2+}]_i$ elevation. UTP (100 μM)-induced $[Ca^{2+}]_i$ elevation was determined in the presence of various concentrations of mastoparan (0–30 μM) under an extracellular Ca^{2+} free condition. The data are mean ± S.E. of three experiments. *, significant difference from without mastoparan ($P < 0.05$).

centrifugation, and signaling molecules in the fractions were determined by immunoblotting (Fig. 3A). Flotillin-1 and GM1, lipid raft marker molecules, were mainly detected in DRM fractions (fourth to fifth fractions) in control cells without mastoparan treatment. $G\alpha_{q/11}$ was detected abundantly in DRM fractions in control cells. The treatment of cells with mastoparan resulted in a decrease in $G\alpha_{q/11}$ level in DRMs. On the other hand, the distribution of $G\alpha_{q/11}$ was not changed by UTP treatment (Fig. 3B). Mastoparan reduced the distribution levels of not only $G\alpha_{q/11}$ but also $G\beta$, $G\alpha_s$, and $G\alpha_i$ in DRMs, showing that mastoparan may affect lipid rafts fol-

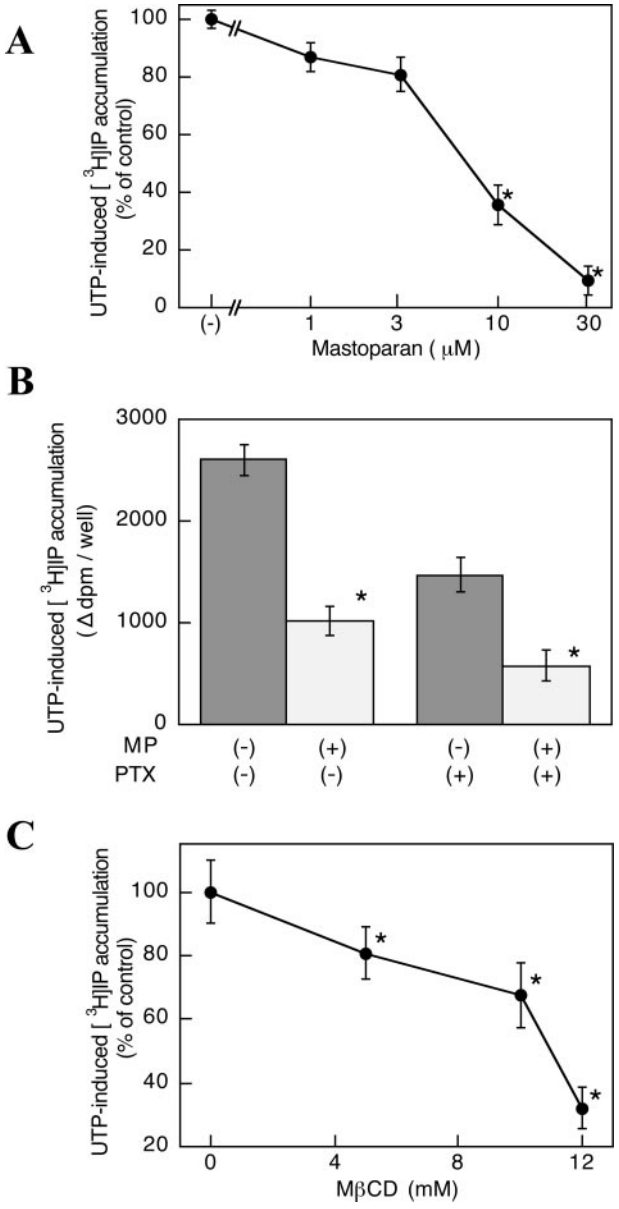

Fig. 2. Characterization of mastoparan-induced inhibition of UTP-elicited phosphoinositide hydrolysis. Phosphoinositide hydrolysis was monitored by measuring [^{3}H]inositol phosphates (IP). A, concentration-dependent inhibition of UTP-induced phosphoinositide hydrolysis by mastoparan. PC-12 cells were labeled with 2 μCi/ml [^{3}H]inositol overnight. After the cells were preincubated with mastoparan for 6 min at the indicated concentrations, they were incubated with 100 μM UTP for 6 min. Accumulated [^{3}H]IP was analyzed as described in detail under *Materials and Methods*. B, influence on the inhibition of phosphoinositide hydrolysis by mastoparan of treatment with PTX. PC-12 cells were cultured in DMEM containing 2 μCi/ml [^{3}H]inositol and 100 ng/ml PTX for 20 h. After the treatment with 10 μM mastoparan for 8 min, the cells were incubated with 100 μM UTP for 8 min. Then, accumulated [^{3}H]IP was analyzed identically. C, concentration-dependent inhibition of UTP-induced phosphoinositide hydrolysis by MβCD. [^{3}H]IP-labeled PC-12 cells were treated with MβCD for 30 min at the indicated concentrations and then incubated with 100 μM UTP for 6 min. The data are mean ± S.E. of six experiments. *, significant difference from without mastoparan (A and B) or MβCD (C) ($P < 0.05$).

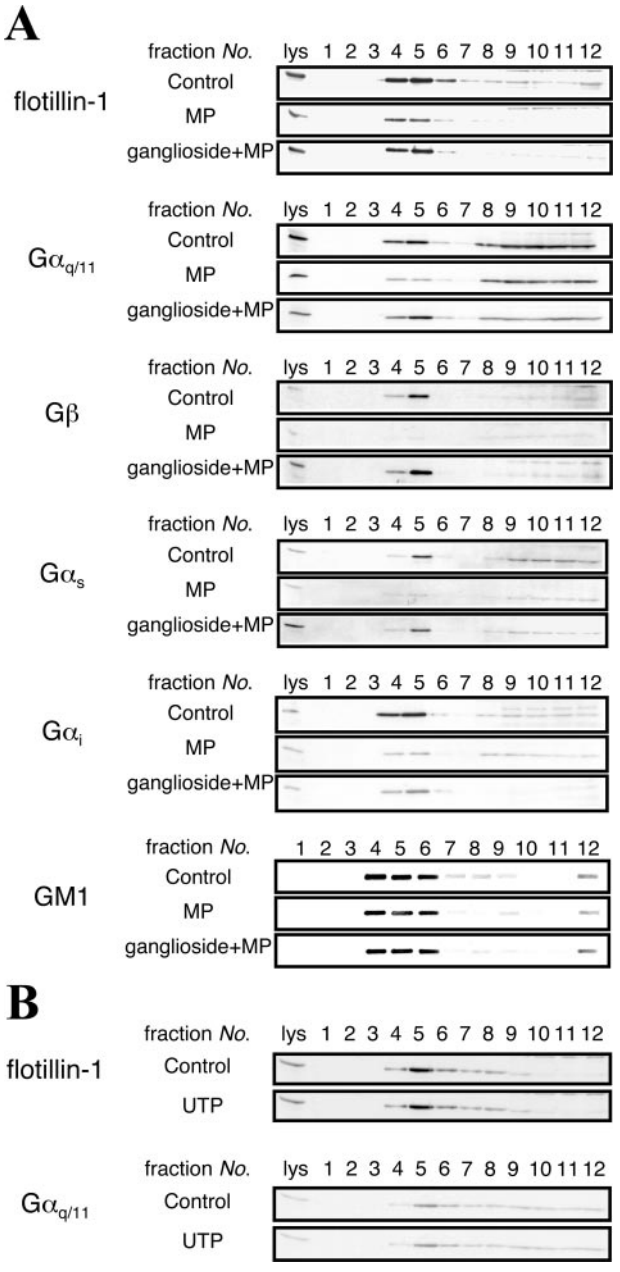

Fig. 3. Effect of mastoparan and UTP on the distribution of signaling molecules in the cells. A, effect of mastoparan (MP). PC-12 cells were incubated with 30 μM MP in the presence or absence of 300 μg/ml ganglioside mixture for 1 h in modified Tyrode's solution. After solubilization with lysis buffer containing 0.1% Triton X-100 as described under *Materials and Methods*, cell lysates were applied to sucrose density gradient centrifugation. Each sample was separated into 12 fractions, and signaling molecules such as flotillin-1, $G\alpha_{q/11}$, $G\beta$, $G\alpha_s$, and $G\alpha_i$ in the fractions were analyzed by immunoblotting. For determining GM1, dot plot analysis was used. Detergent-resistant membrane fractions were collected into fourth to fifth fractions. B, effect of UTP. PC-12 cells were incubated with 100 μM UTP for 1 min and were solubilized with lysis buffer containing 0.1% Triton X-100. After the sample was fractionated by sucrose density gradient centrifugation, $G\alpha_{q/11}$ in the fractions was analyzed by immunoblotting.

lowed by changing the distribution of signaling molecules in lipid rafts. In contrast, GM1 did not change their distributions in response to mastoparan.

The analysis of signaling molecules in DRMs suggests that mastoparan may interact with lipid rafts. We speculated that gangliosides, components of lipid rafts, have an important role as a binding site of mastoparan in lipid rafts because mastoparan has positive charges because of the three Lys residues. Negative charges of sialic acid residues of gangliosides contribute to the membrane electric charge, causing the interaction with a variety of positively charged substances (Millar et al., 1999). Therefore, we investigated the interaction of mastoparan with gangliosides by determining whether exogenously added gangliosides attenuate the effect of mastoparan. PC-12 cells were incubated with 30 μM mastoparan in the presence of 300 μg/ml ganglioside mixture in modified Tyrode's solution for 1 h, and they were fractionated by sucrose density gradient centrifugation. Mastoparan-induced decrease in signaling molecules level in DRMs was attenuated by addition of ganglioside mixture (Fig. 3A).

To examine whether the decrease in $G\alpha_{q/11}$ level in lipid rafts comes from the release of $G\alpha_{q/11}$ from lipid rafts to cytosol, we examined whether $G\alpha_{q/11}$ was released from membranes when membrane preparations were treated with mastoparan (Fig. 4). $G\alpha_{q/11}$ was barely released from membrane preparations without drug, but it was markedly released after treatment with mastoparan. Furthermore, the release of $G\alpha_{q/11}$ by mastoparan was attenuated by the incubation in the presence of ganglioside mixture.

On the other hand, mastoparan affected not only functional proteins in lipid rafts but also the content of cholesterol. Cholesterol level in five fraction was decreased from 83 ± 0.48 μg/fraction to 35 ± 0.77 μg/fraction by treatment with mastoparan (Fig. 5), and it was attenuated by the preincubation of cells with ganglioside mixture. These data show that mastoparan interacts with gangliosides in lipid rafts and decreases some signaling molecules in lipid rafts by releasing them from membranes to cytosol or by changing the localization of them in plasma membranes.

Inhibition of UTP-Induced Phosphoinositide Hydrolysis by Mastoparan Is Attenuated by the Preincubation of Neuraminidase. Next, we investigated whether exogenously added sialic acid, gangliosides, or neuraminidase, which released sialic acid from gangliosides, attenuated the inhibitory effect of mastoparan on UTP-induced phosphoinositide hydrolysis. PC-12 cells labeled with [^{3}H]inositol were preincubated for 6 min with 10 μM sialic acid or 20 μg/ml ganglioside mixture or for 20 min with 1 milliunits of neuraminidase, then they were incubated with 10 μM mastoparan for 6 min, followed by the incubation with 100 μM UTP for an additional 6 min (Fig. 6A). In sialic acid-treated cells, mastoparan inhibited UTP-induced phosphoinositide hydrolysis as well as in control cells. On the other hand, mastoparan failed to inhibit UTP-induced phosphoinositide hydrolysis in the cells treated with ganglioside mixture or neuraminidase. This attenuating effect of neuraminidase seemed to be concentration-dependent (Fig. 6B). These results suggest that mastoparan binds to sialic acid residue of gangliosides and then may affect intracellular signal transduction. The interaction of mastoparan with gangliosides is no longer observed if sialic acid residues are released from gangliosides with neuraminidase.

Fluorescence Intensity of Tyrosine Residue of [Tyr^3]Mastoparan Was Potentiated by Ganglioside Mixtures. Tyrosine fluorescence is observed at 306 nm when excited at 280 nm (Menezes et al., 1990). To examine whether

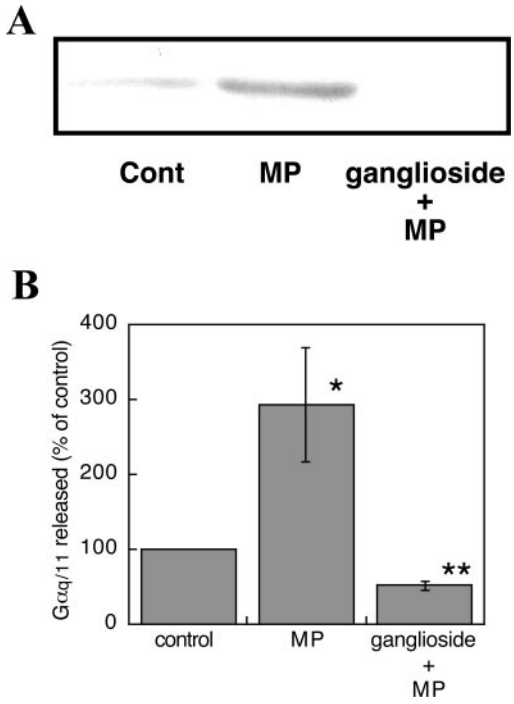

Fig. 4. Release of $G\alpha_{q/11}$ from plasma membranes by mastoparan. Membrane preparations were incubated with 30 μM mastoparan (MP) in the presence or absence of 300 μg/ml ganglioside mixture for 1 h and were concentrated by addition of the equal volume of 10% TCA. The preparations were centrifuged at 10,000*g* for 10 min at 4°C, and the pellets were washed three times with ethanol. After solubilization with the sample buffer, $G\alpha_{q/11}$ was analyzed by immunoblotting (A). The density of the bands corresponding to $G\alpha_{q/11}$ was analyzed by densitometry (NIH Image 1.62), and the data were expressed as percentage of control (B). The data are mean ± S.E. of three experiments. *, significant difference from control ($P < 0.05$). **, significant difference between MP alone and MP plus ganglioside mixtures ($P < 0.05$).

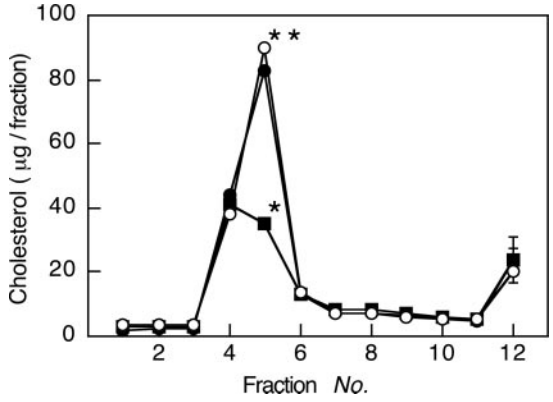

Fig. 5. Effect of mastoparan on the content of cholesterol in the cells. PC 12 cells were incubated with 30 μM mastoparan in the presence or absence of 300 μg/ml ganglioside mixture for 1 h. After solubilization with lysis buffer containing 0.1% Triton X-100, the cell lysates were applied to "sucrose density centrifugation." Cholesterol in each fraction was determined by the method described under *Materials and Methods*. ●, control (without drug); ■, mastoparan alone; ○, gangliosides plus mastoparan. The data are mean ± S.E. of three experiments. *, significant difference from without mastoparan ($P < 0.05$). **, significant difference between mastoparan alone and mastoparan plus ganglioside mixtures ($P < 0.05$).

mastoparan was directly bound to ganglioside, the changes of the intrinsic fluorescence of tyrosine residue of [Tyr^3]mastoparan by the addition of ganglioside mixtures were measured at 37°C by a spectrofluorometer. The fluorescence emission spectra of [Tyr^3]mastoparan in the presence of different concentrations of ganglioside mixtures are shown in Fig. 7A. With increasing the concentration of ganglioside mixture, the fluorescence intensity at 306 nm was increased by addition of ganglioside mixture. On the other hand, the changes of the fluorescence intensity were not observed when sialic acids were added (Fig. 7B). These results show that mastoparan binds specifically to gangliosides.

Mastoparan-Induced Cytotoxicity Was Inhibited by Exogenous Addition of Gangliosides, Especially GD1b and GT1b. The cytotoxicity of mastoparan was monitored by measuring LDH activity. Mastoparan caused cytotoxicity at the concentrations over 10 μM, and more than 60% of cells were dead at a concentration of 30 μM (Fig. 8A). The cytotoxicity was suppressed by treatment of the cells with neuraminidase in a concentration-dependent manner (Fig. 8B). Likewise, the preincubation of the cells with ganglioside mixture resulted in the attenuation of mastoparan-induced cytotoxicity (Fig. 9A). On the other hand, the mastoparan toxicity was not inhibited by treatment with sialic acid at the concentration up to 100 μM. To examine the difference in ganglioside species, several gangliosides (0.3–10 μM) were used to determine the blockade of mastoparan-induced cytotoxicity (Fig. 9B). GT1b, GD1b, GD1a, and GM1 attenuated mastoparan-induced LDH release in a concentration-dependent manner with the order of inhibitory potency of GT1b $\approx$ GD1b $>$ GD1a $>$ GM1 $\gg$ GQ1b, but asialo-GM1 was inactive. These results suggest that mastoparan binds to sialic acid residue of gangliosides, followed by induction of cell death as well as the inhibition of phosphoinositide hydrolysis. The number of sialic acid residues of gangliosides does not correlate with the inhibitory potency of mastoparan action, and the steric structure formed by ceramide and sialic acid residues may be important for mastoparan binding. Because free sialic acids in incubation solution did not suppress mastoparan-induced toxicity, not only the electrostatic interaction between mastoparan and sialic acids but also the hydrophobic interaction between the ceramide and nonpolar groups of mastoparan may participate in the binding of mastoparan to gangliosides.

Discussion

The present study demonstrated that mastoparan changed the localization of signaling molecules, such as G$\alpha_{q/11}$ and Gβ, from lipid rafts to nonlipid rafts or cytosol in PC-12 cells,

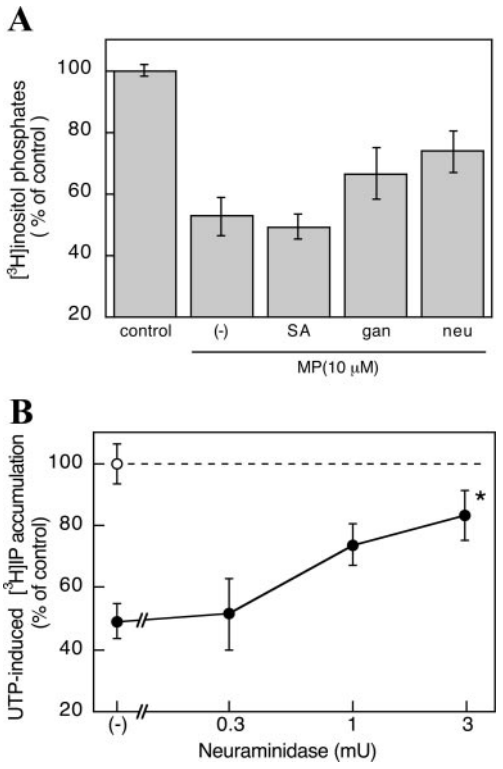

Fig. 6. Attenuation of the inhibitory effect of mastoparan on UTP-induced phosphoinositide hydrolysis by gangliosides or neuraminidase. A, [^{3}H]inositol-labeled PC-12 cells were preincubated for 6 min with 10 μM sialic acid (SA) or 20 μg/ml ganglioside mixture (gan) or for 20 min with 1 mU neuraminidase (neu), and then they were incubated with 10 μM mastoparan (MP) for 6 min followed by incubation with 100 μM UTP for 6 min. The data are mean ± S.E. of three experiments. B, the cells were pretreated with neuraminidase (0.3–3 mU) for 20 min, and they were incubated with (●) or without (○) 10 μM MP for 6 min, followed by the incubation with 100 μM UTP for 6 min. The data are mean ± S.E. of six experiments. *, significant difference from without neuraminidase ($P < 0.05$).

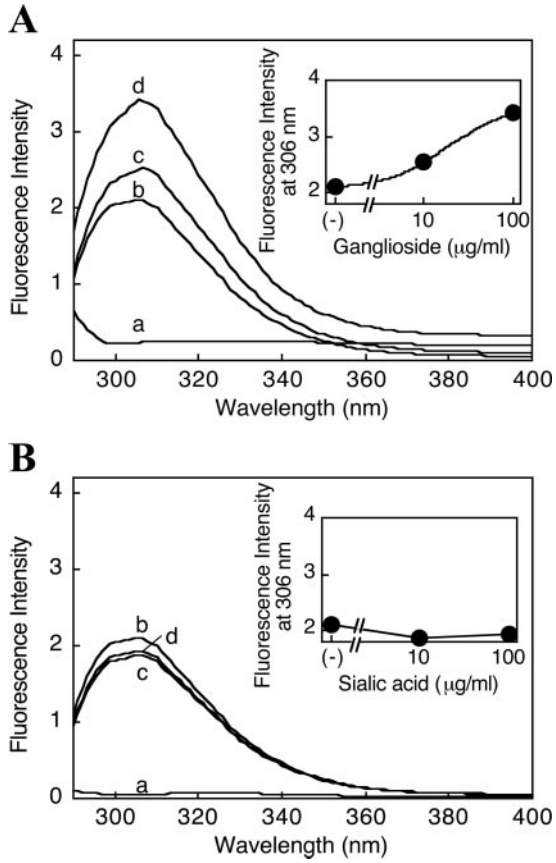

Fig. 7. The changes in fluorescence of [Tyr^3]mastoparan by ganglioside mixtures. The fluorescence emission spectra of [Tyr^3]mastoparan was recorded at 37°C in the presence or absence of ganglioside mixture (A) or sialic acids (B). The fluorescence emission spectrum of 10 μM [Tyr^3]mastoparan was monitored in the absence (b) or presence of 10 μg/ml (c) and 100 μg/ml (d) of ganglioside mixtures or sialic acids. The fluorescence emission spectrum of 100 μg/ml ganglioside mixtures or sialic acids without [Tyr^3]mastoparan is shown in a.

and that this change might result in the inhibition of $G\alpha_{q/11}$-mediated phosphoinositide hydrolysis (Figs. 2 and 3A). Furthermore, it was revealed that $G\alpha_{q/11}$ was released from plasma membrane by mastoparan in membrane preparations (Fig. 4). Bhatnagar et al. (2004) showed that G_q-coupled P2Y signaling were decreased in caveolin-1 knockdown cells by RNA interference. Caveolin-1 is a scaffolding protein of caveolae, promoting interactions between $G\alpha_q$ and G-protein coupled receptors. Therefore, mastoparan-induced inhibition of G_q-signaling is similar to the case in knockdown of caveolin-1. The similar observations was reported when lipid rafts were disrupted by treatment of cells with MβCD (Pike and Miller, 1998). As shown in Fig. 3A, mastoparan also affected the localization of $G\alpha_s$ and $G\alpha_i$ as well as $G\alpha_{q/11}$, suggesting that the effect of mastoparan on lipid rafts has not been limited to a specific G-protein. Thus, these actions of mastoparan are independent of the direct activation of $G\alpha_i$. Several reports indicate that mastoparan shows biological activities without the direct activation of $G_{i/o}$ reported by Higashijima et al. (1988), such as the inhibition of [^{3}H]inositol phosphate accumulation by bradykinin (Nakahata et al., 1996), the activation of phospholipase D (Mizuno et al., 1995), and the inhibition of the action of calmodulin (Ohki et al., 1991). The elucidation of the molecular mechanism of mastoparan action in lipid rafts may enable us to explain the mechanisms of diverse actions of mastoparan that have not yet been understood in detail. A modification of proteins by the covalent attachment of myristic acid and palmitic acid is one of the mechanisms that proteins accumulate in lipid rafts (Melkonian et al., 1999). It is expected that long-chain saturated fatty acids are inserted into lipid rafts with high affinity, allowing the accumulation of the protein in lipid rafts. For the example, acylated Gα mainly localizes in lipid rafts (Okamoto et al., 1998). Thus, the lipid rafts, where sphingoglycolipids and cholesterols are accumulated by van der Waals force and hydrogen bond, are important for sorting of signaling molecules (Brown, 1998; Rietveld and Simons, 1998). The treatment of cells with mastoparan resulted in the change of the localization of some proteins, such as $G\alpha_{q/11}$ and Gβ, decreasing the content of cholesterol in lipid rafts (Fig. 3A and 5), suggesting that the change in the component of lipid rafts by mastoparan may cause the decrease of hydrophobic interactions within lipid raft molecules followed by the change in the localization of signaling molecules. The influence of mastoparan on the localization of molecules in lipid rafts was different in each molecule. This difference might possibly be due to the manner of each protein in its interaction with lipid rafts. Hughes et al. (2001) showed that mem-

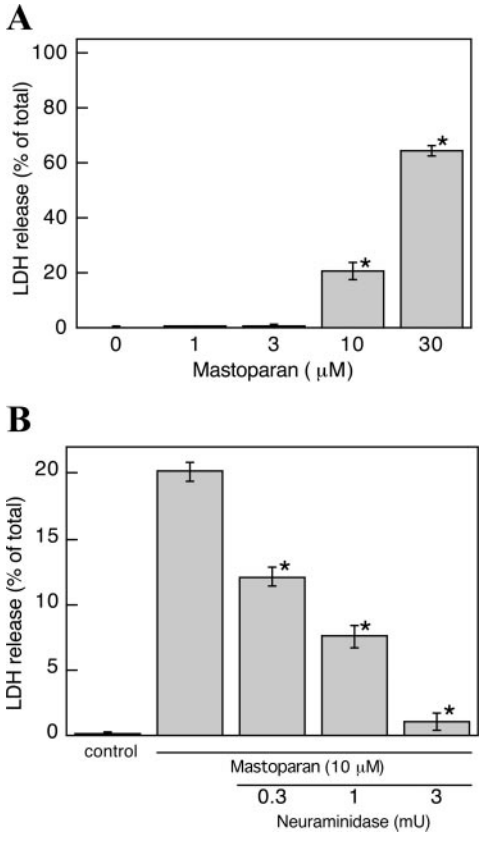

Fig. 8. Mastoparan-induced cytotoxicity and its attenuation by neuraminidase. PC-12 cells were suspended in the modified Tyrode's solution at the concentration of 10^6/ml. A, cells were incubated with mastoparan at the indicated concentration for 15 min and centrifuged. LDH activity in supernatants after centrifugation was measured as described under *Materials and Methods*. *, significant difference from without mastoparan ($P < 0.05$). B, cells were preincubated with 0.3–3 mU of neuraminidase at 37°C for 15 min, then incubated with 10 μM mastoparan for 15 min. The data are mean ± S.E. of six experiments. *, significant difference between mastoparan alone and mastoparan plus neuraminidase ($P < 0.05$).

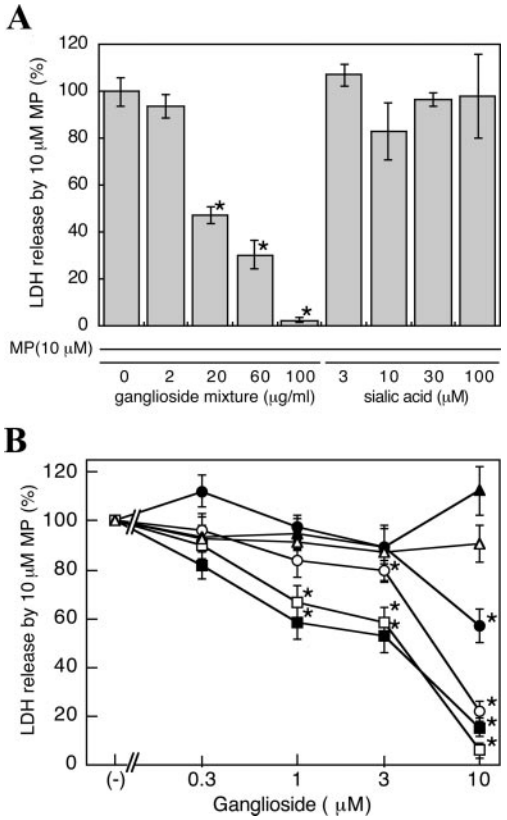

Fig. 9. Inhibition of mastoparan-induced cytotoxicity by gangliosides. A, PC-12 cells at the concentration of 10^6/ml in modified Tyrode's solution were preincubated with various concentrations of ganglioside mixtures or sialic acid for 15 min, and they were incubated with mastoparan (MP, 10 μM) for 15 min. *, significant difference between MP alone and MP plus ganglioside ($P < 0.05$). B, inhibition of MP (10 μM)-induced cytotoxicity by GM1 (●), GD1a (○), GD1b (■), GT1b (□), asialo-GM1 (▲), and GQ1b (△). The data are mean ± S.E. of six experiments. *, significant difference from without gangliosides ($P < 0.05$).

brane-associated $G\alpha_q$ did not change the distribution upon activation. In fact, the stimulation of $P2Y_2$ receptor by UTP had no influence on $G\alpha_q$ distribution (Fig. 3B). Although we have shown that mastoparan releases $G\alpha_q$ from lipid rafts in the plasma membranes, the kinds of mechanisms involved in the release remain unknown. Thus, because we do not yet know the exact mechanism by which mastoparan changes the localization of these molecules, more detailed study is required for the elucidation of this mechanism.

Gangliosides are glycolipids composed of sphingolipid ceramide and oligosaccharide with one or more sialic acid residues, and they are highly expressed in membranes of neuronal cells of brain. Gangliosides are also known to participate in various physiological functions, such as cell proliferation, differentiation, and signal transduction (Mitsuda et al., 2002). Because the pretreatment of cells with gangliosides or neuraminidase attenuated the cytotoxicity of mastoparan in the present study, it is suggested that mastoparan initially binds to gangliosides in lipid rafts and then causes a variety of actions (Figs. 7 and 8). It has been reported previously that histamine release induced by mastoparan in mast cells was inhibited by the pretreatment of cells with neuraminidase (Mousli et al., 1989), although it is believed that mastoparan-induced histamine release is mediated via pertussis toxin-sensitive G protein ($G_{i/o}$). Because MβCD causes cytotoxicity by removing cholesterol from plasma membranes, mastoparan-induced cytotoxicity might be also caused by a decrease of cholesterol in lipid rafts. Cholesterol is important in the formation of lipid rafts, and the decrease of cholesterol in lipid rafts is one of the reasons for mastoparan-induced change in the localization of signaling molecules. Although mastoparan caused cytotoxicity and inhibition of PI hydrolysis through separate mechanisms, the binding of mastoparan to gangliosides might cause a decrease of cholesterol in lipid rafts, resulting in the cytotoxicity as well as the inhibition of PI hydrolysis through the release of $G\alpha_q$ protein.

Mastoparan is an amphipathic peptide with four positive charges, three Lys residues and a terminal amino group. When mastoparan is bound to a phospholipid bilayer, the C-terminal 12 residues of mastoparan form α-helix structure with three Lys residues' positively charged hydrophilic side chains located on one side and with hydrophobic side chains on the other side (Wakamatsu et al., 1992). Taken in the light of the conformation of mastoparan in lipid bilayers, the electrostatic interaction between Lys residue side of mastoparan and gangliosides on cell surface is suggested as the first step of cell recognition, and then the hydrophobic side enters into the phospholipid bilayer. The analysis of mastoparan analogs showed that the change of amphiphilicity also changed the activity of GDP/GTP exchange activation (Jones and Howl, 2004), showing that amphipathic character of mastoparan may be essential for binding to cell membranes by means of the mechanism described above. Furthermore, we examined the difference in inhibitory effects of various kinds of gangliosides on cytotoxicity of mastoparan. The order of inhibitory potency of gangliosides was GT1b $\approx$ GD1b $>$ GD1a $>$ GM1 $\gg$ GQ1b (Fig. 8B). Asialo-GM1 and sialic acid were inactive. GQ1b, which has four sialic acid residues, showed a lower inhibitory potency than other gangliosides that have one to three sialic acid residues, suggesting that the number of sialic acid of gangliosides does not correlate with the inhibitory potency of gangliosides to mastoparan action. Gangliosides have the structure of ceramide-Glc-Gal-Gal-NAc-Gal, and sialic acid binds to Gal, which is the second or fourth sugar residue from ceramide. In comparing the binding manners of sialic acid residues to ceramide with the inhibitory potency, the increased number of sialic acids that bind to second Gal are likely to enhance the inhibitory potency, whereas the excessive number of sialic acids bound to the fourth Gal may decline the inhibitory potency. Thus, it is possible that the Lys residue side of mastoparan interacts with sialic acids bound to the second Gal. If a number of sialic acids that bind to the second Gal are increased, the electrostatic interaction between mastoparan and gangliosides is increased and mastoparan binds strongly to gangliosides. On the other hand, when the number of sialic acids that bind to the fourth Gal is increased, mastoparan binds to gangliosides weakly because of the repulsion between sialic acid residues (hydrophilicity) and hydrophobic side of mastoparan. To prove this hypothesis, further study is required using several kinds of gangliosides. We examined mastoparan-induced cell cytotoxicity not only in PC-12 cells but also in 1321N1 human astrocytoma cells, showing that the cytotoxic sensitivity of 1321N1 cells to mastoparan is approximately one third that of PC-12 cells (J. Sugama and N. Nakahata, unpublished observations). It is necessary to examine the composition of gangliosides between PC-12 cells and 1321N1 cells to clarify the reasons for the different sensitivity of mastoparan to both cell lines. Because both of mastoparan's effects, the inhibition of G_q signaling and the cytotoxicity, are attenuated by gangliosides and neuraminidase, it is interesting to clarify the molecular mechanisms of these effects in the future.

In conclusion, mastoparan initially binds to sialic acids of gangliosides in lipid rafts; then it changes the components of lipid rafts followed by the decrease of some signaling molecules in lipid rafts, such as $G\alpha_{q/11}$ and $G\beta$, by changing their localization in lipid rafts. It is predicted that mastoparan, using such a mechanism, inhibits $G\alpha_q$-mediated phosphoinositide hydrolysis. This is the first demonstration of the lipid raft-mediated mechanism of mastoparan action.

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