P2Y₂ Receptor-G_{q/11} Signaling at Lipid Rafts Is Required for UTP-Induced Cell Migration in NG 108-15 Cells

Koji Ando, Yutaro Obara, Jun Sugama, Atsushi Kotani, Nobuyuki Koike, Satoko Ohkubo, and Norimichi Nakahata

Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University, Sendai, Japan (K.A., Y.O., J.S., A.K., N.K., S.O., N.N.); and Division of Pharmacology, National Institute of Health Sciences, Tokyo, Japan (S.O.)

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ABSTRACT
Lipid rafts, formed by sphingolipids and cholesterol within the membrane bilayer, are believed to have a critical role in signal transduction. P2Y₂ receptors are known to couple with Gq family G proteins, causing the activation of phospholipase C (PLC) and an increase in intracellular Ca^{2+} ([Ca^{2+}]_i) levels. In the present study, we investigated the involvement of lipid rafts in P2Y₂ receptor-mediated signaling and cell migration in NG 108-15 cells. When NG 108-15 cell lysates were fractionated by sucrose density gradient centrifugation, a part of P2Y₂ receptors was distributed in a fraction where the lipid raft markers, cholesterol, flotillin-1, and ganglioside GM1 were abundant. Methyl-β-cyclodextrin (CD) disrupted not only lipid raft markers but also G_{q/11} and P2Y₂ receptors in this fraction. In the presence of CD, P2Y₂ receptor-mediated phosphoinositide hydrolysis and [Ca^{2+}]_i elevation were inhibited. It is noteworthy that UTP-induced cell migration was inhibited by CD or the G_{q/11}-selective inhibitor YM254890 [(1R)-1-{(3S,6S,9S,12S,18R,21S,22R)-21-acetamido-18-benzyl-3-[(1R)-1-methoxymethyl]-4,9,10,12,16,22-hexamethyl-15-methylene-2,5,8,11,14,17,20-heptaoxo-1,19-dioxa-4,7,10,13,16-pentaazacyclodocosan-6-yl}-2-methylpropyl rel-(2S,3R)2-acetamido-3-hydroxy-4-methylpentanoate]. Moreover CD and YM254890 completely inhibited Rho-A activation. Downstream of Rho-A signaling, stress fiber formation and phosphorylation of cofilin were also inhibited by CD or YM254890. However, UTP-induced phosphorylation of cofilin was not affected by the expression of p115-regulator of G protein signaling, which inhibits the G_{12/13} signaling pathway. This implies that UTP-induced Rho-A activation was relatively regulated by the G_{q/11} signaling pathway. These results suggest that lipid rafts are critical for P2Y₂ receptor-mediated G_{q/11}–PLC–Ca^{2+} signaling and this cascade is important for cell migration in NG 108-15 cells.

Introduction
Recent studies indicate the existence of microdomains composed of sphingomyelin, glycosphingolipids, and cholesterol on the cell surface. These microdomains are called “lipid rafts” because they are considered to float as liquid-ordered microdomains within the lipid-disordered glycerophospholipid membrane bilayer (Simons and Ikonen, 1997). Caveolae is also a well known microdomain, which is observed as the flask-shaped small pit on the cell surface, and has recently been considered as one of the lipid raft subtypes whose structure is stabilized by caveolin, an integral plasma membrane protein. It has been reported that lipid rafts are important in diverse cellular responses, such as polarized protein sorting (Simons and Ikonen, 1997), cholesterol homeostasis (Schlegel et al., 2000), and cellular signaling (Vainio et al., 2002). Biochemically, lipid rafts are isolated as a nonionic detergent-insoluble fraction (detergent-resistant membrane) (Brown and London, 2000). A wide range of proteins, such as glycosylphosphatidylinositol-anchored proteins (Varma and Mayor, 1998) and fatty acid-modified (myristoylated or palmitoylated) proteins (Neumann-Giesen et al., 2004), have been reported to be partitioned in lipid rafts. Furthermore, many

ABBREVIATIONS: [Ca^{2+}], intracellular Ca^{2+}; CD, methyl-β-cyclodextrin; YM254890, [(1R)-1-{(3S,6S,9S,12S,18R,21S,22R)-21-acetamido-18-benzyl-3-[(1R)-1-methoxymethyl]-4,9,10,12,16,22-hexamethyl-15-methylene-2,5,8,11,14,17,20-heptaoxo-1,19-dioxa-4,7,10,13,16-pentaazacyclodocosan-6-yl}-2-methylpropyl rel-(2S,3R)-2-acetamido-3-hydroxy-4-methylpentanoate; DMEM, Dulbecco’s modified Eagle’s medium; GPCR, G protein-coupled receptor; HRP, horseradish peroxidase; LPA, lysophosphatidic acid; PLC, phospholipase C; RGS, regulator of G protein signaling; Y27632, (2R,3R,4R,5R,7R,8S,9R,10S,13S,14R,16R,18S,19R,20R,21R,22R)-trans-N-(4-4-pyridyl)-4-(1-aminoethyl)cyclohexane carboxamide; LDH, lactate dehydrogenase; BzATP, benzoyl benzoi amino ATP; tBuBHQ, tert-butyl hydroquinone; Fura-2/AM, fura-2/acetoxyethyl ester; MT, 3-(4,5-dimethylthiazol-2-y)2,5-diphenyterazolium bromide; PTX, pertussis toxin; PBS, phosphate-buffered saline; TBST, 10 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4; ER, endoplasmic reticulum; SH3, Src homology 3; MOI, multiplicity of infection; GEF, guanine nucleotide exchange factor.
G protein-coupled receptor (GPCR)-mediated signaling molecules, such as heterotrimeric G proteins and their effectors (e.g., adenyl cyclase, protein kinase C, Src family tyrosine kinase), are incorporated into lipid rafts (Foster et al., 2003). Therefore, the lipid rafts are now considered as a platform in GPCR-mediated cellular signaling that enables cells to transmit signals from external to internal spaces efficiently. Several pharmacological tools are useful for analyzing the involvement of lipid rafts in the cellular response. For example, methyl-β-cyclodextrin (CD) disrupts lipid raft structures because of its ability to bind and extract cholesterol from the cell membrane (Keller and Simons, 1998). Indeed, depletion of cholesterol by CD results in inhibition of bradykinin-induced phosphoinositide hydrolysis in A431 cells (Pike and Miller, 1998) or enhancement of β-adrenergic receptor or forskolin-stimulated cAMP accumulation in rat cardiomyocytes (Rybin et al., 2000), suggesting the involvement of lipid rafts in the regulation of GPCR-mediated cellular signaling.

Extracellular purine/pyrimidine compounds mediate diverse physiological responses via activation of purine/pyrimidine receptors (Ralevic and Burnstock, 1998). P2 receptors, which preferentially bind adenine and uridine nucleotides, have been classified into P2X (P2X1–7) and P2Y (P2Y1, 2, 4, 6, 11, 12, 13, 14) receptors (North, 2002; Sak and Webb, 2002). Whereas P2X receptor subtypes form the nonselective cation channels, P2Y receptor subtypes couple with heterotrimeric G proteins to regulate the effector systems including phospholipase C (PLC) and adenylyl cyclase. The neuroblastoma × glioma hybrid NG 108-15 cell line shows diverse cellular responses to purine/pyrimidine compounds, such as elevations in intracellular [Ca2+]i via Gq-PLC (Matsumo et al., 1995). Among P2Y receptors, the P2Y2 receptor has been shown to couple with Gαs family G proteins, causing the activation of PLC-β isoforms and an increase in [Ca2+]i (Lustig et al., 1993). Recently, the P2Y2 receptor has been shown to couple with not only Gα11 but also Gα13 and G12/13 when the receptor is associated with αβ2/β5 integrins through the Arg-Gly-Asp (RGD) domain in its first extracellular loop in human 1321N1 astrocytoma cells (Erb et al., 2003; Liao et al., 2007). By interacting with αβ2/β5 integrins, the P2Y2 receptor effectively mediates migration of 1321N1 human astrocytoma cells. In addition to this cell line, the P2Y2 receptor regulates cell migration in epidermal keratinocytes, lung epithelial carcinoma cells, and smooth muscle cells (Greig et al., 2003). Studies on phagocytic removal of apoptotic cells revealed that nucleotides released by apoptotic cells act as a “find-me” signal and the P2Y2 receptor acts as a sensor of the nucleotides to promote migration of cells such as monocytes (Elliott et al., 2009). Moreover, chemotaxis is important for many physiological and pathological processes, and nucleotides play important roles as chemoattractants in many cells (Elliott et al., 2009).

Cell migration via the P2Y2 receptor has been reported; however, the detailed regulatory mechanism is not well known. As mentioned above, lipid rafts are involved in GPCR-mediated cellular signaling as a platform. In the present study, we examined the role of lipid rafts in P2Y2 receptor-mediated signaling in detail, focusing on cell migration signaling, in NG 108-15 cells.

Materials and Methods

Materials. DMEM, fetal calf serum, ATP, UTP, benzoyl benzoic ATP (BzATP), lysophosphatidic acid (LPA), cholesterol, and anti-β-tubulin antibody were obtained from Sigma-Aldrich (St. Louis, MO). Hypoxanthine, aminopterin, and thymidine (HAT) supplement was purchased from Invitrogen (Carlsbad, CA). Tert-butyl-hydroquinone (tBuBHQ) and fura-2/acetoxymethyl ester (Fura-2/AM) were obtained from Wako Pure Chemicals (Tokyo, Japan). (R)-(+)-trans-N-(4-pyridyl)-4-(1-aminoethyl)-cyclohexanecarboxamide (Y27632) was obtained from Calbiochem (San Diego, CA). YM254890 [(1R)-1-(3S,6S,9S,12S,18R,21R,22R)-21-acetamido-18-benzyl-3-[(1R)-1-methoxyethyl]-4-9, 10, 12, 16, 22-hexamethyl-15-methylene-2, 5, 8, 11, 14, 17, 20-heptaoxa-1, 19-dioxo-4, 7, 10, 13, 16-pentaaazacyclodocosan-6-yl]-2-methylpropylrel-(2S,3R)-2-acetamido-3-hydroxy-4-methylpentanoate] was kindly provided by Astellas Pharma Inc. (Tokyo, Japan). [3H]Myo-inositol was obtained from American Radiochemicals, Inc. (St. Louis, MO). Antibodies for integrin αv, blocking antibody (P2W7) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies for Gαq/11 and P2Y2 receptor were purchased from Daichi Pure Chemicals Co. Ltd. (Tokyo, Japan) and Alomone Labs (Jerusalem, Israel), respectively. Antibodies for phospho-cofilin (Ser3) (7702) was purchased from Cell Signaling Technology (Danvers, MA). Horseradish peroxidase (HRP)-conjugated cholera toxin B subunit and HRP-conjugated anti-rabbit IgG were obtained from Calbiochem and Cell Signaling Technology, respectively. HRP-conjugated anti-mouse IgG, Immunop. P. ECL, and Hyperfilm ECL were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Adenovirus encoding the amino terminal region containing the regulator of G protein signaling (RGS) domain of p115–RhoGEF (p115–RGS; amino acids 1–252) was kindly provided by Dr. H. Kurose (Kyushu University, Fukuoka, Japan). All other chemicals and drugs were of reagent grade or the highest quality available.

Cell Culture. NG 108-15 cells were grown in high-glucose DMEM supplemented with 10% (v/v) fetal calf serum and HAT supplement (100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine) and maintained in a humidified atmosphere of 5% CO2 in air at 37°C.

Measurement of Lactate Dehydrogenase Activity. NG 108-15 cells in the 12-well plates were washed twice with modified Tyrode’s solution (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl2, 0.18 mM CaCl2, 5.6 mM glucose, 10 mM HEPES, pH 7.4) and treated with various concentrations of CD for 30 min at 37°C. The incubation medium was collected, and lactate dehydrogenase (LDH) activity was assayed with a commercially available kit (Wako Pure Chemicals).

Measurement of Cholesterol Content. The cholesterol content was examined with a commercially available kit (Wako Pure Chemicals). NG 108-15 cells were detached from 150-mm-diameter dishes, washed twice, and suspended with modified Tyrode’s solution. The cellular response. For example, methyl-β-cyclodextrin (CD) disrupts lipid raft structures because of its ability to bind and extract cholesterol from the cell membrane (Keller and Simons, 1998). Indeed, depletion of cholesterol by CD results in inhibition of bradykinin-induced phosphoinositide hydrolysis in A431 cells (Pike and Miller, 1998) or enhancement of β-adrenergic receptor or forskolin-stimulated cAMP accumulation in rat cardiomyocytes (Rybin et al., 2000), suggesting the involvement of lipid rafts in the regulation of GPCR-mediated cellular signaling.

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Role of Lipid Rafts in P2Y2 Receptor-G_{q,11}, Signaling

Measurement of [Ca^{2+}]_{i}. Increases in [Ca^{2+}]_{i}, were examined by monitoring the intensity of Fura-2 fluorescence as described previously (Sugama et al., 2005). Cells were loaded with 1 μM Fura-2/AM for 15 min at 37°C, washed twice, and suspended at 0.5 to 1 × 10^{6} cells/ml in modified Tyrode’s solution. The cell suspension was placed in a 1.5-ml quartz cell and constantly stirred at 37°C. The increase in [Ca^{2+}]_{i}, was determined by measuring the fluorescence intensity of Fura-2 (excitation wavelength at 340 and 380 nm and emission wavelength at 510 nm) with a fluorescence spectrophotometer (Hitachi (Tokyo, Japan) F-2000). [Ca^{2+}]_{i}, levels were calculated by using the K_{d} value of Fura-2 to Ca^{2+} at 24°C.

Preparation of CD–Cholesterol Complex. CD–cholesterol complex was prepared by modifying the method described by Zidovetzki and Levitan (2007). CD (10 mM) solution in serum-free DMEM was added to the dried cholesterol equivalent of 100 mM (CD/cholesterol molar ratios of 1:10). They were vortexed, sonicated in bath sonicator for 2 min, and incubated overnight in a shaking bath at 37°C. Immediately before using this solution, excess cholesterol crystals were removed by 0.45-μm syringe filter (Millipore Corporation, Billerica, MA). This CD solution was considered to be 100% saturated with cholesterol. To prepare different concentrations of cholesterol combined with 10 mM CD, this 100% saturated 10 mM CD–cholesterol solution was mixed with various amounts of 10 mM CD without cholesterol. In brief, 75% saturated CD–cholesterol solution was prepared by mixing with 3:1 (100% saturated 10 mM CD–cholesterol solution/10 mM CD alone), and 50% saturated CD–cholesterol solution was prepared by mixing with 1:1 (100% saturated 10 mM CD–cholesterol solution/10 mM CD alone).

MTT Assay. NG 108-15 cells grown in the 96-well plates coated with poly-L-lysine (100 ng/ml) were treated with various concentrations of CD for 30 min or 18 h at 37°C. After removal of the incubation medium, 100 μl of Eagle’s minimum essential medium-HEPES or MTT solution was added, and cells were incubated for 4 h at 37°C. After removal of the medium, 100 μl of dimethyl sulfoxide was added, and the absorbance was measured with a spectrophotometer (Sunrise, Tecan, Austria).

Cell Migration Assay. Cell migration assays were performed with 8-μm pore size cell culture inserts (BD Bioscience, San Jose, CA) by the method described previously (Wang et al., 2005) with a slight modification. The cells (5 × 10^{5}) were pretreated with or without 10 mM CD for 30 min or 1 μM YM254890 for 30 min, suspended in 100 μl of serum-free DMEM supplemented with 100 μM hypoxanthine, 1 μM aminopterin, and 16 μM thymidine, and placed in the inserts (upper chamber). CD was washed out, and the medium was replaced with a new medium. YM254890 was kept in the medium during the following 18-h incubation. The lower chamber was filled with 600 μl of serum-free medium with or without 100 μM UTP. Cells were allowed to migrate under the incubation for 18 h at 37°C. Cells migrating to the lower side of the membrane were fixed with 4% (v/v) paraformaldehyde and stained with 1 ng/ml Hoechst dye 33258. Photographs of all fields were taken, and the number of migrating cells was counted.

Measurement of Phosphoinositide Hydrolysis. Phosphoinositide hydrolysis was determined as described previously with slight modifications (Nakahata et al., 1990). Cells grown in 12-well plates were labeled with [3H]inositol (2 μCi/ml) in DMEM for 18 to 24 h before experimentation. Cells were washed twice and incubated in modified Tyrode’s solution with or without the various concentrations of CD for 30 min at 37°C. After incubation, cells were washed twice and incubated in modified Tyrode’s solution containing 10 mM LiCl for 10 min at 37°C. Cells were stimulated with agonists for 10 min at 37°C, and the reaction was terminated by the addition of 5% (v/v) trichloroacetic acid after aspiration of the incubation medium. Total amounts of [3H]inositol phosphates in the ether-washed trichloroacetic acid extract were separated by using an anion exchange column (AG 1X-8, formate form). The [3H]radioactivity in the eluates was measured with a liquid scintillation counter.

Preparation of CD–Cholesterol Complex. NG 108-15 cells grown on 150-mm dishes were suspended, washed twice, and suspended in modified Tyrode’s solution. The cells were incubated with or without 10 mM CD for 30 min at 37°C, washed twice, and lysed with 1 ml of ice-cold lytic buffer [0.1% (v/v) Triton X-100, 50 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM NaN_{3}VO_{4}, 5 mM Na_{2}P_{2}O_{7}, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin/antipain, pH 7.6]. Lysates were sonicated on ice and incubated with constant rotation at 4°C for 1 h. Lysates (1 ml) were mixed with 3 ml of 60% (w/v) sucrose in STE buffer (50 mM Tris-HCl, 50 mM NaCl, 5 mM EDTA, and 1 mM NaN_{3}VO_{4}, pH 7.6) and overlaid with 4 ml of 35% (w/v) sucrose and 4 ml of 5% (w/v) sucrose. Centrifugation was performed at 200,000g for 16 h at 4°C with a Beckman Coulter (Fullerton, CA) SW41Ti rotor. Fractions of 1 ml were collected from the top of the gradients, and 12 fractions were kept at −80°C for subsequent studies.

Samples were mixed with 3 × Laemml sample buffer and denatured by heating at 95°C for 5 min. In the case of the P2Y_{2} receptors, the samples were denatured at room temperature overnight. Standard immunoblotting was performed, and blots were probed for flotillin-1 (1:300), G_{q,11} (1:2000), and P2Y_{2} receptor (1:200). For the detection of GMI, dot blotting was performed by using a HRP-conjugated cholera toxin B subunit (Sigma-Aldrich; final 10 ng/ml).

MTT Assay. NG 108-15 cells grown in the 96-well plates coated with poly-L-lysine (100 ng/ml) were treated with various concentrations of CD for 30 min or 18 h at 37°C. After removal of the incubation medium, 100 μl of Eagle’s minimum essential medium-HEPES or MTT solution was added, and cells were incubated for 4 h at 37°C. After removal of the medium, 100 μl of dimethyl sulfoxide was added, and the absorbance was measured with a spectrophotometer.


TBST (10 mM Tris, 100 mM NaCl, 0.1% Tween 20, pH 7.4) for 1 h at room temperature, membranes were incubated with primary antibodies against phospho-cofilin (1:5000) in TBST containing 3% nonfat milk overnight at 4°C. After several washes with TBST, membranes were incubated with HRP-linked rabbit IgG (1:50,000). Bands were visualized by using the ECL system (GE Healthcare) by exposing its chemiluminescence to Hyperfilm ECL (GE Healthcare). The density of the band was analyzed by densitometry (Image J 1.36; National Institutes of Health).

Statistical Analysis. All results are expressed as the mean ± S.E.M., and statistical differences of values were determined by one-way analysis of variance with Dunnett’s or Tukey’s Kramer post hoc tests for multiple comparisons.

Results

\( \gamma_{11} \) and a Part of P2Y\(_2\) Receptors Are Localized in Lipid Rafts. First, we tried to determine the suitable concentration of CD to deplete cholesterol in plasma membranes. When NG 108-15 cells were incubated with various concentrations of CD for 30 min at 37°C, CD extracted cholesterol from the plasma membranes into the incubation medium in a concentration-dependent manner with an EC\(_{50}\) value of approximately 5.6 mM (Fig. 1A). Because cholesterol is one of the major components in plasma membranes, the reduction of cholesterol from plasma membranes by CD would cause a decrease in cell viability. In fact, the severe depletion of cholesterol is reported to cause cytotoxicity (Iwabuchi et al., 1998). Therefore, we examined cell viability after CD treatment by determining the release of LDH activity from cells into the incubation medium. CD at a concentration of 20 mM resulted in a 56.6% decrease in cell viability (Fig. 1B). For this reason, we used CD at a concentration of 10 mM or lower in all subsequent experiments. To evaluate the distribution of the signaling molecules underlying P2Y\(_2\) receptor signaling, we tried to isolate the raft fraction by sucrose-density gradient centrifugation (Fig. 2). The majority of cholesterol was concentrated in fraction 4/5, which disappeared upon pretreatment of cells with CD (Fig. 2A). Furthermore, flotillin-1 and GM1 were also concentrated in fraction 4/5, and they were moved to the higher-sucrose density fraction by CD treatment at 10 mM for 30 min (Fig. 2B and C). We therefore defined fraction 4/5 as the raft fraction. We next investigated the localization of a heterotrimeric G protein \( \gamma_{11} \) and the P2Y\(_2\) receptor. While \( \gamma_{11} \) was enriched in the raft fraction from control cells, CD disrupted the localization of \( \gamma_{11} \) and moved it into the higher-sucrose density fractions (Fig. 2D). P2Y\(_2\) receptors were partially distributed in the raft fraction, and the lipid raft-associated P2Y\(_2\) receptor moved to the higher-sucrose density fraction after CD treatment (Fig. 2E). These results are shown from three independent experiments.
suggest that G\(_{\alpha_{q/11}}\) and a part of P2Y\(_2\) receptors are localized in lipid rafts in NG 108-15 cells.

**Inhibitory Effect of CD on UTP-Induced Cell Migration.** First, we investigated the optimal condition of CD treatment for migration assays. When we examined cytotoxicity by using the MTT assay, treatment with CD for 18 h caused a decrease in cell viability at all concentrations used (Fig. 3A). In contrast, the treatment with CD for 30 min and incubation for an additional 18 h after removal of CD resulted in no cytotoxicity and a long-term reduction in cholesterol content of the plasma membranes (Fig. 3, A and B).

Next, we investigated whether lipid rafts participated in UTP-induced cell migration, which is the unique physiological function of P2Y\(_2\) receptors. Because it is considered that this migration assay reflects accumulative reaction and the inhibitory effect is more obvious when cells are incubated for a long time, the migration assay was also performed for 18-h incubation as described previously by Wang et al. (2005). After NG 108-15 cells were pretreated with or without 10 mM CD for 30 min at 37°C, they were collected and incubated in the upper chamber in the presence or absence of UTP in the lower chamber. After 18 h, cells migrating to the lower side of the membrane were visualized by staining with Hoechst dye 33258 (C) and the number of migrating cells was counted (D).

**Involvement of G\(_{q/11}\) in UTP-Induced Cell Migration.** As reported previously, depletion of cholesterol by CD resulted in the inhibition of bradykinin-induced phosphoinositide hydrolysis in A431 cells (Pike and Miller, 1998). Because P2Y\(_2\) receptors are known to associate with G\(_{q/11}\) and cause various responses via G\(_{q/11}\), we suspected that the inhibition of UTP-induced cell migration by the disruption of lipid rafts was caused by the inhibition of G\(_{q/11}\)-mediated signaling. To examine this possibility, we investigated the effect of CD on phosphoinositide hydrolysis as an index of G\(_{q/11}\) signaling. CD itself had little effect on the total amount of \([^{3}\text{H}]\text{inositol-labeled lipids (data not shown)}\) and the resting level of \([^{3}\text{H}]\text{inositol phosphates (Fig. 4A)}\), suggesting that labeling efficiency of phosphoinositides by \([^{3}\text{H}]\text{inositol was not affected by CD treatment. However, UTP (100 } \mu\text{M})-induced phosphoinositide hydrolysis was clearly inhibited by CD treatment in a concentration-dependent manner (Fig. 4A). We next examined the effect of CD on \([\text{Ca}^{2+}]_i\) elevation. ATP (100 } \mu\text{M}) caused transient \([\text{Ca}^{2+}]_i\) elevation in Fura-2-loaded cells, which was suppressed by the pretreatment of cells with 10 mM CD for 30 min (Fig. 4, B and C). In contrast, \([\text{Ca}^{2+}]_i\) elevation induced by tBuBHQ (10 } \mu\text{M}), an inhibitor of \text{Ca}^{2+}-\text{ATPase at the endoplasmic reticulum (ER) (Moore et al., 1987), was little affected by CD treatment, suggesting that the inhibitory effect of CD on \([\text{Ca}^{2+}]_i\) increase is not caused by the reduction in \(\text{Ca}^{2+}\) uptake into the ER. Whereas the P2Y\(_2\) receptor agonist UTP (100 } \mu\text{M}) caused a transient elevation in \([\text{Ca}^{2+}]_i\), the P2X\(_2\) receptor agonist BzATP (100 } \mu\text{M}) induced a sustained elevation in \([\text{Ca}^{2+}]_i\) in normal cells. Although BzATP-induced \([\text{Ca}^{2+}]_i\) elevation was slightly facilitated by CD, UTP-induced \([\text{Ca}^{2+}]_i\) elevation was largely inhibited. These results suggest that the inhibitory effect of CD on UTP-induced \([\text{Ca}^{2+}]_i\) elevation might be caused by the suppression of the P2Y\(_2\) receptor-G\(_{q/11}\)-mediated signaling pathway. Then, we investigated whether G\(_{q/11}\) was involved in UTP-induced cell migration. As observed in the case of CD...
treatment, UTP-induced cell migration was completely suppressed to control levels in the presence of 1 mM YM254890, a specific inhibitor of G_q11 (Fig. 4D) (Takasaki et al., 2004). These results suggest that P2Y_2 receptor-mediated G_q11 signaling at lipid rafts is essential for cell migration in NG 108-15 cells.

**CD-Exogenous Cholesterol Complex Failed to Inhibit UTP-Induced Cell Migration.** To confirm that the inhibition of G_q11 signaling and cell migration by CD was caused by cholesterol depletion but not nonspecific effect, we tried to determine the cellular cholesterol level and UTP-induced phosphoinositide hydrolysis after treatment with CD alone or with CD–cholesterol complex. Whereas 10 mM CD solution without exogenous cholesterol (0% saturation) decreased cellular cholesterol content, 50% saturated 10 mM CD–cholesterol solution scarcely decreased the cellular cholesterol content in NG 108-15 cells (Fig. 5A). The inhibition of UTP-induced phosphoinositide hydrolysis by CD was attenuated by exogenous cholesterol in a concentration-dependent manner (Fig. 5B). Furthermore, 75% saturated 10 mM CD–cholesterol did not inhibit UTP-induced cell migration (Fig. 5C). These data strongly suggest that the inhibition of P2Y_2 receptor-mediated G_q11 signaling and cell migration by CD was caused by cholesterol depletion.

On the other hand, the result in Fig. 3B shows a gradual increase in cholesterol content after CD treatment. Hence, to investigate the relationship between cholesterol content and P2Y_2 receptor-mediated signaling after CD treatment we measured the UTP-induced [Ca^{2+}]_i elevation 18 h after 30-min pretreatment with CD. Although cholesterol content was recovered 18 h after 30-min pretreatment with CD, the UTP-induced [Ca^{2+}]_i elevation was not completely recovered (Fig. 5D). Therefore, it is suggested that the integrity of the lipid rafts was still disrupted 18 h after 30-min pretreatment with CD and that some factors are required to recover their function once cellular cholesterol is extracted by CD.

**G_q11-Dependent Rho-A Activation.** Figure 4 indicates the possible involvement of G_q11 in P2Y_2 receptor-mediated cell migration. On the other hand, Rho-A, which is a member of the Rho family of small GTPase, is required for cytoskeletal reorganization and cell migration (Xu et al., 2003). Therefore, to investigate this signal transduction pathway in more detail, we examined the effect of G_q11 on Rho-A activation. It is noteworthy that UTP-induced Rho-A activation was inhibited by CD or YM254890, but was weakly affected by anti-integrin α_v antibodies (Fig. 6). These data suggest that UTP-induced Rho-A activation is regulated by G_q11.

**G_q11-Dependent Stress Fiber Formation.** To assess the involvement of P2Y_2 receptor-G_q11 signaling in Rho-A activation, we investigated the effect of UTP on stress fiber formation. Actin stress fibers were observed by stimulating NG 108-15 cells with UTP (Fig. 7B), which was reversed by pretreatment with 10 mM CD or 1 mM YM254890 (Fig. 7, C and D). In addition, stress fiber formation over a population of cells was quantitated (Fig. 7F). Judging from this result, it is considered that the inhibition of stress fiber formation by CD was not partial. This is consistent with the results that CD largely blocked the UTP-induced Rho activation (Fig. 6). However, PTX, which inactivates receptor-mediated G_ia signaling, did not affect the formation (Fig. 7E). These data further support the idea that G_q11 is involved in Rho-A activation.

**G_q11-Dependent Phosphorylation of Cofilin.** To further access the involvement of G_q11 in Rho-A signaling pathway, we examined the influence of the phosphorylation of cofilin, which regulates stress fiber formation (Ohashi et al., 2000). UTP induced robust cofilin phosphorylation, which was inhibited by YM254890 and CD, but not by PTX (Fig. 8, A–D), as was the case in stress fiber formation. Phosphorylation of cofilin was partially inhibited by anti-integrin α_v antibodies (Fig. 8, C and D). Because YM254890 did not inhibit phosphorylation of cofilin when NG 108-15 cells

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**Figure 4. Involvement of G_q11 in UTP-induced cell migration.**

A, concentration-dependent effect of CD on the UTP-induced phosphoinositide hydrolysis. [3H]inositol-prelabeled NG 108-15 cells were incubated with the indicated concentrations of CD for 30 min at 37°C. After washing, cells were stimulated with ATP or without UTP (100 μM UTP in the presence of 10 mM LiCl) for 10 min at 37°C. Each point represents the mean ± S.E.M. of three determinations. * indicates a significant difference compared with values without CD treatment (p < 0.05). B, effect of CD on [Ca^{2+}]_i elevation. Cells were pretreated with or without 10 mM CD for 30 min at 37°C and loaded with 1 μM Fura-2/AM for 15 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP (each 100 μM), or tBuBHQ (10 μM). C, the summarized results of [Ca^{2+}]_i elevation in the cells pretreated with CD (hatched columns) or without CD (open columns). Each column represents the mean ± S.E.M. of nine experiments. * indicates a significant difference compared with control (p < 0.05).

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**Figure 5.**

A, concentration-dependent effect of CD on UTP-induced phosphoinositide hydrolysis. [3H]inositol-prelabeled NG 108-15 cells were incubated with the indicated concentrations of CD for 30 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP or tBuBHQ (100 μM). B, effect of CD on [Ca^{2+}]_i elevation. Cells were pretreated with or without 10 mM CD for 30 min at 37°C and loaded with 1 μM Fura-2/AM for 15 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP (each 100 μM), or tBuBHQ (10 μM). C, the summarized results of [Ca^{2+}]_i elevation in the cells pretreated with CD (hatched columns) or without CD (open columns). Each column represents the mean ± S.E.M. of nine experiments. * indicates a significant difference compared with control (p < 0.05).

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**Figure 6.**

A, concentration-dependent effect of CD on UTP-induced phosphoinositide hydrolysis. [3H]inositol-prelabeled NG 108-15 cells were incubated with the indicated concentrations of CD for 30 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP or tBuBHQ (100 μM). B, effect of CD on [Ca^{2+}]_i elevation. Cells were pretreated with or without 10 mM CD for 30 min at 37°C and loaded with 1 μM Fura-2/AM for 15 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP (each 100 μM), or tBuBHQ (10 μM). C, the summarized results of [Ca^{2+}]_i elevation in the cells pretreated with CD (hatched columns) or without CD (open columns). Each column represents the mean ± S.E.M. of nine experiments. * indicates a significant difference compared with control (p < 0.05).

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**Figure 7.**

A, concentration-dependent effect of CD on UTP-induced phosphoinositide hydrolysis. [3H]inositol-prelabeled NG 108-15 cells were incubated with the indicated concentrations of CD for 30 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP or tBuBHQ (100 μM). B, effect of CD on [Ca^{2+}]_i elevation. Cells were pretreated with or without 10 mM CD for 30 min at 37°C and loaded with 1 μM Fura-2/AM for 15 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP (each 100 μM), or tBuBHQ (10 μM). C, the summarized results of [Ca^{2+}]_i elevation in the cells pretreated with CD (hatched columns) or without CD (open columns). Each column represents the mean ± S.E.M. of nine experiments. * indicates a significant difference compared with control (p < 0.05).

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**Figure 8.**

A, concentration-dependent effect of CD on UTP-induced phosphoinositide hydrolysis. [3H]inositol-prelabeled NG 108-15 cells were incubated with the indicated concentrations of CD for 30 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP or tBuBHQ (100 μM). B, effect of CD on [Ca^{2+}]_i elevation. Cells were pretreated with or without 10 mM CD for 30 min at 37°C and loaded with 1 μM Fura-2/AM for 15 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP (each 100 μM), or tBuBHQ (10 μM). C, the summarized results of [Ca^{2+}]_i elevation in the cells pretreated with CD (hatched columns) or without CD (open columns). Each column represents the mean ± S.E.M. of nine experiments. * indicates a significant difference compared with control (p < 0.05).

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**Figure 9.**

A, concentration-dependent effect of CD on UTP-induced phosphoinositide hydrolysis. [3H]inositol-prelabeled NG 108-15 cells were incubated with the indicated concentrations of CD for 30 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP or tBuBHQ (100 μM). B, effect of CD on [Ca^{2+}]_i elevation. Cells were pretreated with or without 10 mM CD for 30 min at 37°C and loaded with 1 μM Fura-2/AM for 15 min at 37°C. After washing, cells were stimulated with ATP, UTP, BzATP (each 100 μM), or tBuBHQ (10 μM). C, the summarized results of [Ca^{2+}]_i elevation in the cells pretreated with CD (hatched columns) or without CD (open columns). Each column represents the mean ± S.E.M. of nine experiments. * indicates a significant difference compared with control (p < 0.05).
were stimulated by LPA (Fig. 8, E and F), which is considered to induce Rho-A activation via G12/13, it is suggested that YM254890 specifically inhibits Gq/11 signaling. To further clarify the G proteins involved in UTP-induced phosphorylation of cofilin, we investigated the involvement of G12/13 in UTP-induced Rho-A signaling by using adenovirus encoding the amino-terminal regions containing the RGS domain of p115–RhoGEF (p115–RGS; amino acids 1–252), which specifically inhibits Gq/11 function (Honma et al., 2006). Although the expression of p115–RGS resulted in a significant attenuation of LPA-induced phosphorylation of cofilin, it did not affect UTP-induced phosphorylation of cofilin (Fig. 8, G–I). These results (Fig. 8, A–I) suggest that UTP-induced phosphorylation of cofilin is regulated by Gq/11 rather than G12/13.

It is known that cofilin is phosphorylated by the LIM domain kinase that is activated downstream of Rho-A, Rac1, or Cdc42 (Edwards et al., 1999; Ohashi et al., 2000). The activity of the LIM domain kinase is regulated by ROCK, which is an effector molecule of Rho-A, or PAK1, which is an effector molecule of Rac1 and Cdc42 (Edwards et al., 1999; Ohashi et al., 2000). When NG 108-15 cells were stimulated with UTP, the phosphorylation of cofilin was potently inhibited by Y27632, a ROCK inhibitor (Fig. 8, J and K). Because phosphorylation of cofilin was regulated by ROCK and Gq/11, it was speculated that UTP-induced Rho-A activation was regulated by the P2Y2 receptor-Gq/11 signaling pathway.

**Discussion**

In the present study, we have clearly demonstrated that the P2Y2 receptor and Gq/11 are present in the cholesterol-rich lipid rafts that are required for effective signal transduction, and this signal transduction via Gq/11 is essential for...
cell migration in NG 108-15 cells. Many GPCRs and their effector systems have been shown to exist in microdomains, including caveolae. This is the first direct demonstration that the P2Y2 receptors are localized in lipid rafts where their physiological function is regulated.

It has been shown that ATP interacts with P2X7 and P2Y2 receptors expressed in NG 108-15 cells (Watano et al., 2002). CD is reported to extract the cholesterol followed by disruption of the cholesterol-dependent lipid rafts on the plasma membrane in NG 108-15 cells. In this study, CD treatment inhibited the UTP-induced, but not BzATP-induced, \([\text{Ca}^{2+}]_i\) elevation, suggesting that CD treatment inhibits the P2Y2 receptor-mediated, but not the P2X7-mediated, signaling pathway. On the other hand, tBuBHQ-induced \([\text{Ca}^{2+}]_i\) elevation was unaffected by CD treatment, suggesting that CD did not decrease the \([\text{Ca}^{2+}]_i\) levels in the ER and CD-induced inhibition of the P2Y2 receptor-mediated signaling pathway occurred upstream of \([\text{Ca}^{2+}]_i\) release from the ER. In fact, CD suppressed UTP-induced phosphoinositide hydrolysis in a concentration-dependent manner, indicating that P2Y2 receptor-mediated \(G_{\alpha q/11}\)-PLC activation would be interrupted by CD. It is noteworthy that we have also shown that CD inhibited P2Y2 receptor-mediated PLC activation in PC12 cells (Sugama et al., 2005).

Toselli et al. (2001) reported that NG 108-15 cells possess lipid rafts, but not caveolae. The results in Fig. 2 provide strong evidence that the signaling molecule complex for the P2Y2 receptor exists in lipid rafts. The lipid raft marker molecules, ganglioside GM1 and flotillin-1, were colocalized in fraction 4/5. CD reduced the levels of these molecules in the fraction, suggesting that the depletion of cholesterol from the plasma membranes would effectively disrupt the lipid raft composition followed by the redistribution of these molecules to nonraft compartments. On the other hand, \(G_{\alpha q/11}\) and a part of the P2Y2 receptor were found in a raft fraction derived from CD-untreated cells. This may suggest the effective coupling between P2Y2 receptors and \(G_{\alpha q/11}\) in the cholesterol-rich lipid raft compartment. Consistent with our results, \(G_{\alpha q/11}\) is reported to be localized in lipid rafts including caveolae (Oh and Schnitzer, 2001), because the \(G_{\alpha q/11}\) subunit interacts with membrane lipids via saturated acyl chains (typically myristate and/or palmitate) covalently attached at the amino terminus of this molecule (Morris and Malbon, 1999). In contrast to \(G_{\alpha q/11}\), which was detected in the raft fraction, P2Y2 receptor showed more broad distribution in both raft and nonraft fractions (Fig. 2E). CD treatment fully inhibited P2Y2 receptor-mediated PLC activation and \([\text{Ca}^{2+}]_i\) mobilization accompanied by reduced P2Y2 receptor levels in raft fractions, indicating that only lipid raft-associated P2Y2 receptors would be functional to couple with the \(G_{\alpha q/11}\)-PLC-\([\text{Ca}^{2+}]_i\) pathway.

It is noteworthy that cell migration was regulated by Gq/11 in NG 108-15 cells and the number of migrated cells was completely decreased by pretreatment with YM254890 (Fig. 4D). This indicates that \(G_{\alpha q/11}\) has a critical role in cell migration in NG 108-15 cells. Indeed, Rho-A activation was totally inhibited by YM254890. Downstream of Rho-A signaling stress fiber formation (Fig. 7D) and phosphorylation of...
Fig. 8. A, Gq/11-dependent phosphorylation of cofilin. NG 108-15 cells pretreated with or without 1 μM YM254890 for 30 min, 5 μg/ml PTX for 24 h, or vehicle were stimulated with 100 μM UTP for 3 min. B, summarized data of Gq/11-dependent phosphorylation of cofilin. C, lipid raft- and integrin αv-dependent phosphorylation of cofilin. NG 108-15 cells pretreated with or without 10 mM CD or 5 μg/ml anti-integrin αv antibody for 30 min were stimulated with 100 μM UTP for 3 min. D, summarized data of Gq/11-dependent phosphorylation of cofilin by LPA. NG 108-15 cells pretreated with or without 1 μM YM254890 for 30 min were stimulated with 10 μM LPA for 3 min. E, Gq/11-independent phosphorylation of cofilin by LPA. NG 108-15 cells were infected with recombinant adenoviruses encoding control (vector-GFP) or p115-RGS at MOI of 100 or 150 for 48 h. Then, cells were stimulated with 100 μM UTP or 10 μM LPA for 3 min after 8-h starvation. F, summarized data of the effect of p115–RGS at MOI of 150 on UTP-induced phosphorylation of cofilin. G, summarized data of the effect of p115–RGS at MOI of 150 on LPA-induced phosphorylation of cofilin. H, summarized data of ROCK-dependent phosphorylation of cofilin. Phospho-cofilin (p-cofilin) was determined by immunoblotting with antiphospho cofilin antibody. β-Tubulin was used as an internal control (A, C, E, G, and J). Phosphorylation of cofilin was normalized with the corresponding β-tubulin then normalized amounts of phosphorylation of cofilin were shown as percentage of UTP stimulation (B, D, H, and K) or LPA stimulation (F and I). Each column of the summarized data represents the mean ± S.E.M. of three experiments (B, D, F, I, H, and K). * indicates a significant difference compared with the value of UTP- or LPA-treated cells (p < 0.05).
crosst density gradient centrifugation. It has been shown that existed in the broad membrane fractions separated by susphingosine 1-phosphate receptor Edg-1 existed in the caveo-rafts. A possible mechanism for regulating the localization of involvement of \( \alpha_v \beta_5 / \beta_2 \) integrins (Wang et al., 2005). Our results are consistent with that report, i.e., phosphorylation of cofilin was inhibited by pretreatment with anti-integrin \( \alpha_v \) antibody (Fig. 8, C and D), indicating the involvement of \( \alpha_v \beta_5 / \beta_2 \) integrins. Judging from these results, we assumed that the P2Y2 receptor-G\( \alpha_{q1} \) signaling pathway may activate integrins, resulting in the activation of Rho-A. Nevertheless, Rho-A activation was not affected by anti-integrin \( \alpha_v \) antibody, suggesting that \( \alpha_v \beta_5 / \beta_2 \) integrins may transmit the signal to Rac1 or Cdc42 because cofilin is regulated by not only Rho-A but also Rac1 or Cdc42 (Edwards et al., 1999; Ohashi et al., 2000). Hence, these results indicate that P2Y2 receptor-mediated activation of Rho-A is relatively G\( \alpha_{q1} \)-specific in NG 108-15 cells. This P2Y2 receptor-G\( \alpha_{q1} \) mediated Rho-A activation is a new signal transduction pathway. We suspected that G\( \alpha_q \)-dependent RhoGEF, for example LARG (Booden et al., 2002) and p63 RhoGEF (Rojas et al., 2007), mediated the UTP-induced Rho-A activation in NG 108-15 cells recently. The crystal structure of G\( \alpha_q \)-p63 RhoGEF–Rho-A was determined (Lutz et al., 2007). Moreover, G\( \alpha_q \) was found to directly activate p63 RhoGEF, resulting in the activation of Rho-A (Rojas et al., 2007).

It is not clear how the P2Y2 receptor is retained in the lipid rafts. A possible mechanism for regulating the localization of molecules is glycosylation. Kohno et al. (2002) suggested that sphingosine 1-phosphate receptor Edg-1 existed in the cavelae and the mutated form of nonglycosylated N30D-Edg-1 existed in the broad membrane fractions separated by sucrose density gradient centrifugation. It has been shown that the human P2Y2 receptor has two N-glycosylation sites at 9 and 13 residues (Lustig et al., 1993; Erb et al., 2001). Thus, further studies are necessary to clarify whether these glyco-sylation sites are essential for the distribution of the P2Y2 receptor in lipid rafts. Recent studies indicated that human P2Y2 receptors possess several functional domains, i.e., one RGD integrin-binding sequence in the first extracellular loop (Liao et al., 2007) and two proline-rich Src homology 3 (SH3) domain binding sites (PXXP motif) in the carboxyl-terminal tail (Liu et al., 2004). The putative amino acid sequence of P2Y2 receptor cloned from NG 108-15 cells (GenBank accession no. A0A89871.1) showed the presence of one RGD sequence and one SH3 domain binding site. In human P2Y2 receptors, expression of P2Y2 receptors replaced with an RGE sequence from a RGD sequence did not change G\( \alpha_q \)-mediated signaling (Liao et al., 2007). Furthermore, a mutant P2Y2 receptor lacking two PXXP motifs still stimulated [Ca\(^{2+}\)]\( _{\text{i}} \) mobilization (Liu et al., 2004), suggesting that both the RGD sequence and the SH3-binding sites would not be important for G\( \alpha_{q1} \)-mediated Ca\(^{2+}\) signaling and localization of the P2Y2 receptor in lipid rafts. It might be possible that there are unidentified motifs in the P2Y2 receptors that enable them to interact with other molecules existing in lipid rafts. Further studies will be necessary to clarify the detailed mechanism of P2Y2 receptor localization within lipid rafts.

In the present study, we have shown that P2Y2 receptor-mediated activation of the G\( \alpha_{q1} \)-PLC–Ca\(^{2+}\) pathway in lipid rafts enables effective cell migration. The P2Y2 receptor is known to work as a sensor for cell migration (Elliott et al., 2009) and has an important role in physiological and pathological processes such as brain injury (Norton et al., 1992). Analysis of the spatiotemporal regulation mechanism will be helpful for understanding the physiological and pathological processes in the future.

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References


**Address correspondence to:** Dr. Norimichi Nakahata, Department of Cellular Signaling, Graduate School of Pharmaceutical Sciences, Tohoku University, Aoba 6-3, Aramaki, Aoba-ku, Sendai 980-8578. E-mail: nakahata@mail.pharm.tohoku.ac.jp