YM-254890, which was isolated from the culture broth of Chromobacterium sp., inhibits ADP-induced platelet aggregation and has antithrombotic and thrombolytic effects. YM-254890 blocks Gaq/11-coupled ADP receptor P2Y1-mediated Ca2+ mobilization. Here we report that YM-254890 is a selective Gaq/11 inhibitor. YM-254890 blocked Ca2+ mobilization mediated by several Gaq/11-coupled receptors but not by Gαq- or Gα12/13-coupled receptor, indicating that phospholipase Cβ activation and subsequent signaling molecules are not the target of YM-254890. YM-254890 completely prevented the serum response factor (SRF)-mediated gene transcription induced by Gaq/R183C, which is constitutively active in a receptor-dependent manner because of its reduced Kcat of GTP hydrolysis. Conversely, YM-254890 had only a modest effect on the SRF-mediated gene transcription by Gαq/Q209L, which is GTase-deficient (activated) Gαq. These suggested that the acting point of YM-254890 is receptor-Gαq interaction or the subsequent guanine nucleotide exchange step. The fact that YM-254890 (i) inhibited the SRF-mediated gene transcription by Gαq/Q209L, which interacts with Gαq-coupled receptor and possesses the effector function of Gαq, and (ii) had no effect on the Kaq value of high affinity [3H]MeSADP binding to P2Y1, which reflects the agonist-receptor-Gαq ternary complex, suggested that receptor-Gαq interaction is not the target of YM-254890. On the other hand, specific [35S]GTPγS binding to Goq/11 stimulated by the M1 muscarinic acetylcholine receptor and P2Y1 were inhibited by YM-254890. These data indicate that YM-254890 blocks the exchange of GDP for GTP in Gaq/11 activation. This novel Gaq/11-selective inhibitor is a promising and powerful tool for studying Gaq/11 protein activation, Gaq/11-coupled receptor signaling, and Gaq/11-mediated biological events.

G protein-coupled receptors (GPCRs) and heterotrimeric G proteins, consisting of Gα, Gβ, and Gγ, transduce extracellular stimuli, such as hormones, neurotransmitters, chemokines, and other local mediators, into appropriate intracellular responses (1, 2). The activation of Gα proteins is related to conformational change by guanine nucleotide interaction. The GPCRs, activated by the agonist, induced exchanges of GDP for GTP on the coupled Ga subunit. The resultant Ga-GTP complex dissociates from the Gβγ subunit and activates its downstream effectors, which in turn regulate various functions such as gene transcription, mitogenesis, metabolism, muscle contractile state, and ion channel regulation. The GTPase activity of α-subunit turns off effector signals by hydrolyzing Ga-GTP to Ga-GDP, which re-associates with Gβγ.

There are over 20 Ga subunits classified into subfamilies by their major four families, Gaq/11, Gai, Gαo, and Gα12/13. Main effector molecules of Gaq/11, Gai, Gαo, and Gα12/13 are thought to be phospholipase C (PLC), adenylyl cyclase (activation), adenylyl cyclase (inhibition), and small GTPase families, respectively (3). Transient intracellular Ca2+ mobilization is led by PLCβ activation via the Gaq/11, Gai, or Gα12/13 subunit with Gaq. PLCβ hydrolyzes phosphatidylinositol bisphosphate in the plasma membrane, and the generated inositol 1,4,5-trisphosphate (IP3) activates the IP3 receptor/Ca2+ channel in the endoplasmic reticulum (ER). Then Ca2+ is released into the cytoplasm from the Ca2+ store in the ER (4). Cyclic adenosine 3′,5′-monophosphate (cAMP) synthesized by adenylyl cyclase from ATP also represents an important second messenger. Generation of intracellular cAMP is regulated by activation and inactivation of adenylyl cyclase via Gaq and Gai.

Compounds that interact selectively to individual Gα proteins and inhibit their activation would be very useful in clarifying the regulation of Gα protein activation and the function of Gα protein and G protein-coupled receptors in vitro and in vivo. In addition, those compounds may be applicable to the therapy of several human diseases attributable to the activation of Gα protein.

YM-254890, which is a cyclic depsipeptide discovered in our laboratories from culture broth of Chromobacterium sp. QS3666, is a potent inhibitor of ADP-induced platelet aggregation with an IC50 value below 0.4 μM (5). The compound also has antithrombotic and thrombolytic effects in an electrically induced carotid artery thrombosis model in rats (6). At least three receptors are known as receptors related to purified nucleotide-induced platelet aggregation, which are Gaq/11-coupled ADP receptor, P2Y1, Goq-coupled ADP receptor, P2Y12, and ligand-gated ion channel, P2X1 (7, 8). This compound inhibits Ca2+ mobilization in platelet and in P2Y1-expressing cells via ADP (5). We demonstrate here that YM-254890 inhibits Ca2+ mobilization stimulated by several Gaq/11-coupled receptors but not by Gαq- or Gα12/13-coupled receptor, suggesting that YM-254890 is a modulator of Goq/11 activation. Also we show that
YM-254890 is a selective inhibitor of Goq11, and its acting point is the exchange step of GDP for GTP in Goq11 activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—YM-254890 (M = 959) was isolated from the culture broth of Chromobacterium sp. QS38666 (5). The pEF-BOS vector was a gift from Prof. S. Nagata (Osaka University Medical School, Osaka, Japan). The pSRE (response element-luc) and pCRE (cAMP response element-luc) were obtained from Stratagene (La Jolla, CA). The phRL-TK was purchased from Promega (Madison, WI). The Fluo-3-acetoxyethyl ester (Fura-3-AM) was obtained from Dojin Laboratories (Kumamoto, Japan). The CAMP homogenous time-resolved fluorescence (HTRF) kit was purchased from CIS biointernational (Bagnols-sur-Ceze, France). The LipofectAMINE 2000 and Dulbecco’s modified Eagle’s medium were purchased from Invitrogen (Carlsbad, CA). The 2-methylthioadenosine diphosphate (2MeSADP), isoproterenol, UTP, acetyl-β-methylcholine (methacholine), N-formyl-Met-Leu-Phe (fMLP), α,β-methylene ATP, nifedipin, 3-isobutyl-1-methylxanthine (IBMX), A23187, dibutyryl cAMP, pertussis toxin, apyrase, monoclonal anti-influenza virus hemagglutinin (HA), clone HA-7, and anti-mouse IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Other reagents were commercially obtained.

**DNA Construction and Preparation of GPCR-expressing Cell**—The open reading frame of the human Goq was amplified with 5′-primer containing the HA tag (5′-atggctgcaatactgagaagatc-3′) by PCR and subcloned into the expression vector pBluescript, pCMV-Myc, or pCMV-HA. Two Goq mutants were obtained by PCR using genomic primer 5′-gtgctcc-3′ to generate R183C and 5′-ggcggctagcagaga-3′ to generate Q209L and subcloned into pcDNA3. Receptor-Gos interaction inhibitory “minigene” (Goq-1), which correspond to the C-terminal peptide sequence of Goq residues 305–359, was also amplified by PCR and subcloned into pcDNA3 (9). The cDNA of chimeric G protein, Goqα, was obtained by PCR using Goq DNA as a template with two pairs of the primer: 5′-atgccctgcaatactgagaagatc-3′ and 5′-gacagcctgcaatactgagaagatc-3′. The cDNA of Goqα was subcloned into pEF-BOS-dhfr (11). ORFs of GPCR and P2X2 were also amplified by PCR and subcloned into pEF-BOS-dhfr or pEF-BOS-neo. The P2Y1, P2Y2, or P2Y12-stably expressing C6-15 cells, cysteine leukotriene receptor (CysLT-R-1), CysLT-R-2, M1 muscarinic acetylcholine receptor stably expressing CHO cells, FMRP receptor (FRP) or FPR1, and Goqα were also expressed in the plasmids HA-Q209L, HA-R183C, or HA-R183G. The plasmids HA-Q209L, HA-R183C, or HA-R183G in pcDNA3 (20 ng/gal) or pcDNA3 (20 ng/gal), and phRL-TK (5 ng/gal). After transfection, YM-254890, in the desired concentration, was also added to each well. After 18 h, the cell lysates were analyzed using the Dual-Luciferase Reporter assay system (Promega) and a model ML3000 luminometer (Dynatech Laboratories, Chantilly, VA). HEK293 cells were also co-transfected with receptor plasmids (pCAG-G, 10 ng/well, pCAG-M, 5 ng/well, pCAG-H, 20 ng/well), Goqα-I in pcDNA3 (20 ng/gal), Goqα-A in pcDNA3 (1 ng/gal), pSRE-luc (10 ng/gal), and phRL-TK (5 ng/gal). After 18 h, cells were stimulated by agonists with or without YM-254890. After 5 h, luciferase activities of the cell lysates were measured.

**Purification of cAMP 3′-kinase**—The cell pellets were lysed with 200 μl of SDS sample buffer (5% w/v glycerol/1% w/v SDS/30 mM Tris-HCl/pH 6.8/2.5% w/v 2-mercaptoethanol), sonicated, and boiled. Two microliters of these samples were electrophoresed on 10–20% polyacrylamide gel in the presence of SDS and transferred to a polyvinylidene difluoride membrane. The blot was blocked with Block-Ace (Yukijirushi Co., Sapporo, Japan) and incubated with monoclonal anti-Gos antibody. HRP-labeled secondary antibody was added and incubated. The blot was washed, the antigens were visualized by the ECL plus Western blotting detection system.

**Binding Assay**—P2Y1 stably expressing C6-15 cells, M1 stably expressing CHO cells or P2Y12 stably expressing C6-15 cells were pooled and centrifuged at about 200 × g for 5 min. The pellets were resuspended in 0.1 mM EDTA and 10 mM HEPES (pH 7.4). The binding of [3H]2MeSADP to the membrane of P2Y1-expressing cells was carried out as described previously (13). To detect [3H]GTPγS binding to Goq11, the membrane aliquots of M1 or P2Y12 were diluted in assay buffer (100 mM NaCl, 10 mM MgCl2, and 10 mM HEPES (pH 7.4)) to give a final protein concentration of 50 μg/ml (M1-CHO) or 50 μg/ml (P2Y1-CHO-C6–15). Membranes were added to 50 μl of assay buffer containing 2 mM [35S]GTPγS (1,000 Ci/mmol), 2 mM GDP, and 2 mM acetyl-β-methylcholine for M1 or 200 mM 2MeSADP for P2Y1 with or without YM-254890 and then incubated at 37 °C for 5 min. The reactions were terminated by the addition of 900 μl of ice-cold assay buffer. The samples were then centrifuged at 20,000 × g for 10 min. Pellets were solubilized by the addition of 50 μl of ice-cold solubilization buffer (100 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA, and 1.25% Igepal CA 630 (pH 7.4) containing 0.2% SDS). Once pellets were completely solubilized, an equal volume of solubilization buffer without SDS was added to each tube. The solubilized pellets were precleared with 50 μl of protein G-Sepharose (100 μl of TE buffer (10 mM Tris/HCl and 10 mM EDTA, pH 8.0) for 30 min at 4 °C. After centrifugation at 20,000 × g for 5 min, 100 μl of the supernatant was transferred to a fresh tube containing 1 μl of anti-Goqα, -E, and -Goqα-A, and 37% samples were allowed to stand for 60 min at 4 °C, then added to 70 μl of protein A-Sepharose suspension and rotated for 60 min at 4 °C. Protein A-Sepharose beads were then pelleted at 20,000 × g and washed three times with 500 μl of Gq/11-selective Inhibitor

47439

The activity of the N-type Ca2+ channel was measured by the maximal intracellular Ca2+ concentration after stimulation with high K HBS plus nifedipin (140 mM NaCl, 50 mM KCl, 1.8 mM CaCl2, and 10 mM HEPES NaOH (pH 7.4)). The activity of the N-type Ca2+ channel was measured by the maximal intracellular Ca2+ concentration after stimulation with high K HBS plus nifedipin (140 mM NaCl, 50 mM KCl, 1.8 mM CaCl2, and 10 mM HEPES NaOH (pH 7.4)).
RESULTS AND DISCUSSION

YM-254890 Inhibits P2Y1-mediated Intracellular Ca\textsuperscript{2+} Mobilization—YM-254890 is a type of cyclic peptide containing the uncommon amino acids \(3\)-hydroxyisoleucine, \(N\)-O-dimethylthreonine, and \(N\)-methyldehydroalanine (17). Its molecular weight is 959 (Fig. 1A). The compound was first identified as an inhibitor of ADP-induced platelet aggregation (5). Pharmacological studies have shown that purine nucleotide-induced platelet aggregation requires intracellular signaling from three distinct receptors: intracellular Ca\textsuperscript{2+} mobilization mediated by \(G_{q11}\)-coupled ADP receptor (P2Y1) and ATP-sensitive ligand-gated P2X1 cation channel, and inhibition by \(G_{q5}\)-coupled ADP receptor (P2Y12) of adenyl cyclase activation (7, 8, 18). To determine whether YM-254890 inhibits P2Y1- or P2X1-mediated signaling, we used C6–15 cells, a rat glioma cell line, which stably express human P2Y1 or human P2Y12. YM-254890 blocked 2MeSADP-induced intracellular Ca\textsuperscript{2+} mobilization in P2Y1-expressing cells with IC\textsubscript{50} values of 0.18 \(\mu\)M (Fig. 1B). The compound was more potent than MRS2179, the most potent and selective P2Y1 antagonist to date (19), which has an IC\textsubscript{50} value of 0.86 \(\mu\)M (Fig. 1B). However, YM-254890 did not block the 2MeSADP-induced inhibition of cAMP accumulation in P2Y12-expressing cells, which was inhibited by AR-C69931MX, a potent selective P2Y12 antagonist (13) with an IC\textsubscript{50} value of 2.9 \(\mu\)M (Fig. 1C). The compound also had no effect on \(\alpha,\beta\)-methylene ATP-induced intracellular Ca\textsuperscript{2+} mobilization in HEK293 cells, in which P2X1 was transiently transfected (Fig. 1D). It was inhibited by PPADS, a non-selective P2 receptor antagonist (20), with an IC\textsubscript{50} value of 0.25 \(\mu\)M. These data suggested that YM-254890 was a strong and selective P2Y1 antagonist.

YM-254890 Preferably Inhibits \(G_{q}\)-coupled GPCR Signaling—In addition to the ADP-induced platelet aggregation, TRAP (a thrombin receptor agonist) or U46619 (a thromboxane A2 receptor agonist)-induced platelet aggregation was also suppressed by YM-254890. \(2\) Thrombin receptor and thromboxan A2 receptor are coupled with \(G_{q}\) in human platelets (21, 22). Those observations suggest that YM-254890 may attenuate a common signaling pathway mediated by \(G_{q}\)-coupled receptors. We first confirmed the effect of this compound on Ca\textsuperscript{2+} mobilization mediated by various \(G_{q11}\)-coupled receptors. As shown in Table I, Ca\textsuperscript{2+} mobilization in P2Y2-expressing C6–15 cells stimulated by UTP, CysLT-R1, or CysLT-R2-expressing CHO cells by leukotriene D4, and M1 muscarinic acetylcholine receptor-expressing CHO cells by methacholine were all inhibited by YM-254890 with IC\textsubscript{50} values of about 0.1–0.2 \(\mu\)M. However, the compound (up to 10 \(\mu\)M) did not prevent cAMP accumulation by isoproterenol in HEK293 cells, in which \(\beta\)2 adrenoreceptor was transiently transfected (Table I).

The Ca\textsuperscript{2+} signal generated by GPCRs involves the activation of phospholipase C, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to inositol 1,4,5-trisphosphate and diacylglycerol (19). Inositol 1,4,5-trisphosphate releases intracellular Ca\textsuperscript{2+} from stores, thereby allowing entry of extracellular Ca\textsuperscript{2+} through 

of PLCβ, mediated by activated Goq11. Under certain circumstances, the activation of PLCβ is also mediated by the Gβγ, which is dissociated from activated Goq. PLCβ generates IP3, which in turn activates the IP3 receptor/Ca2+ release channel (IP3R), releasing Ca2+ stored in the ER into the cytoplasm. Ca2+ release from the ER provoked the activation of Ca2+ release-activated Ca2+ channels (Icreo) or other store-operated channels (SOCs) in the plasma membrane (4). fMLP-induced Ca2+ mobilization in neutrophil-like differentiated HL60 cells is mediated by Goq (23). In our study, only faint fMLP-induced intracellular Ca2+ mobilization was observed in undifferentiated HL60 cells, while the amount of Ca2+ mobilization was dramatically enhanced in differentiated HL60 cells, which were cultured for 3 days with db-cAMP (data not shown). Intracellular Ca2+ mobilization by fMLP in the differentiated HL60 cells was almost suppressed by pretreatment with pertussis toxin for 6 h (Fig. 2A), indicating that Ca2+ mobilization by fMLP in the differentiated HL60 cells is mediated by Goq. YM-254890 at 10 μM has little effect on this Goq-mediated Ca2+ mobilization (Fig. 2A). Differentiated HL60 cells can also induce Ca2+ mobilization by UTP, which is mediated by endogenous P2Y2, and its Ca2+ mobilization is activated by both P2X-insensitive and -sensitive pathways (23). The majority of the Ca2+ mobilization by UTP in the differentiated HL60 cells was inhibited by 10 μM YM-254890 (Fig. 2B). Interestingly, in CHO cells, which expressed exogenous fMLP receptor FPR, fMLP induced Ca2+ mobilization slightly (Fig. 2C and Ref. 24). However, when FPR was co-expressed with Goq10, fMLP could increase Ca2+ mobilization. The Goq12-coupled FPR-mediated Ca2+ mobilization was not inhibited by 10 μM YM-254890 (Fig. 2C). Goq16 is one of the Goq11 family proteins, and it has the ability to activate PLCβ by non-selectively coupling with a large variety of receptors, including Goq11, Goq12, or Goqα-coupled receptors (24). YM-254890 did not inhibit Goq or Goq15-mediated Ca2+ mobilization, suggesting that the inhibitory effect of the compound on Ca2+ mobilization in Goq11-signaling is not caused by block of PLCβ enzyme activity or IP3R, Icreo, or SOCs channel activity. In addition, it was revealed that FPR and P2Y2 bring about intracellular Ca2+ mobilization by activation of distinct Go in differentiated HL60 cells.

To further assess the character of YM-254890, we examined the effect on ligand-gated or voltage-gated ion channels using the FLIPR system. The compound (up to 10 μM) did not inhibit α,β-methylene ATP-induced Ca2+ mobilization in P2X2/3-expressing cells and the activation of L- and N-type Ca2+ channels under conditions of high extracellular K+ in db-cAMP-differentiated NG108–15 cells, which were completely blocked by nifedipine and ø-conotoxin GVIA. Those observations indicated that YM-254890 preferably inhibits Goq11-mediated cellular signaling, but not Goq, Goq15-Ga12-mediated signaling, and the machinery of intracellular Ca2+ mobilization, such as PLCβ and Ca2+ channels.

The Effect of YM-254890 on Goq Active Mutant, Q209L, or R183C—To determine the direct effect of YM-254890 on Goq, we used two constitutively active Goq mutants, GoqQ209L and GoqR183C. Q209L is the activated form of Goq because of its deficient intrinsic GTPase activity and the consequent GTP-bound active conformation (25, 26, 28). R183C has a significantly reduced kcat of GTP hydrolysis (27, 28). The dissociation rate of GDP of R183C is similar to that of native Goq, but the slower rate of GTP hydrolysis is thought to be in constitutive activation because of the higher fractional occupancy of GTP. Constitutively active forms of Goqα are reported to activate serum response factor (SRF)-mediated gene transcription, which is evaluated by the c-Fos serum response element (SRE)-luciferase reporter gene assay (25). To assess the constitutive activities of the Goq mutants, SRE-luciferase reporter gene and cDNA encoding HA-tagged Goq, Q209L, or R183C were co-transfected in HEK293. Expression levels of three Gα proteins in cells were confirmed by Western blot with anti-HA antibody (Fig. 3A). Luciferase activity in cells 18 h after transfection of Q209L or R183C was 100- or 40-fold higher than in cells expressing HA-Goq, indicating that both Goq mutants have constitutive activity (Fig. 3B). Treatment with YM-254890 has only a modest effect on the constitutive activity of Q209L (Fig. 3B). Conversely, the constitutive activity of R183C was suppressed completely by treatment with the compound in a dose-dependent manner (Fig. 3B). What are the implications of differential effects of the compound on the two mutants? Besides the GTPase deficiency, we find that there is a distinction of two mutants in the receptor-stimulated guanine nucleotide exchange step. We examined the effect of co-transfection of Goq−I, which is a minigene corresponding to the C-terminal peptide sequence of Goq residues 305–359 and is shown to inhibit the receptor-Goq interaction selectively (9). Co-transfection of Goq−I resulted in a marked inhibition of SRF-mediated gene transcription induced by R183C (Fig. 3C). The data demonstrate that guanine nucleotide exchange of R183C is pro-

### Table 1

<table>
<thead>
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<th>Target</th>
<th>Stimuli</th>
<th>Assay</th>
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<td>P2Y1</td>
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<td>Ca2+ mobilization</td>
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<tr>
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<td>100 nM UTP</td>
<td>Ca2+ mobilization</td>
<td>0.19</td>
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<tr>
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<td>Ionophore</td>
<td>1 μM 2MeSADP</td>
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*The data represent the mean of two separate experiments performed in triplicate.

b 2MeSADP, 2-methylthio-ADP.

c LTDs, leukotriene D4.
d MCh, methacholine.
e ISO, isoprotrocol.
f 2MeSADP, 2-methylthio-ADP.
g Inhibition of forskolin-induced cAMP accumulation.
promoted by endogenous Gαi-coupled receptors. In contrast, SRF-mediated gene transcription induced by Q209L was not prevented by co-transfection of Gαq-I (Fig. 3C). The fact that Q209L has been activated without receptor stimulation suggests that GTP-occupied Q209L is post-translationally generated in cytoplasm with receptor-independent manner because Q209L may have a higher affinity for the transition state of GTP. The fact that YM-254890 inhibits the activation of R183C suggests the target of the compound might be receptor-Gαq interaction or guanine nucleotide exchange step. And the fact that YM-254890 does not inhibit the constitutive activity of Q209L supports PLCβ activation, and subsequent signaling molecules are not the target of the compound.

The Effect of YM-254890 on Receptor-Gαq Interaction—We examined the effect of co-expression of receptor-Gαq interaction inhibitory minigene, Gαq-I, on the signaling mediated by Gαq, Gαi, and Gαs (Fig. 4, A–C). Co-transfection of Gαq-I prevented LTD4-induced SRF-mediated gene transcription in CysLT-R2-expressing cells (Fig. 4A). Transcription of cAMP response element (CRE) reporter gene was induced by stimulation of Gαs-coupled receptors and was not suppressed by
In the case of Gq/H9251i-coupled P2Y12, 2MeSADP-dependent SRF-mediated gene transcription could be detected when Gq/H9251qi5 was co-expressed with P2Y12 (Fig. 4C). The Gq/H9251qi5 is a chimeric Gq/H9251 protein where the five C-terminal amino acids are replaced with that of Gq/i, which interacts with Gq/i-coupled receptor and possesses the effector function through the Gq region. Interestingly, co-expression of Gq/I has little effect on the P2Y12/Gq/H9251qi5-mediated gene transcription (Fig. 4C). The data point out the five C-terminal amino acids of Gq/H9251 is required for receptor-Gq/H9251 interaction and guanine nucleotide exchange is not inhibited by Gq/I. If the target of YM-254890 is receptor-Gq/H9251 interaction, P2Y12/Gq/H9251qi5-mediated gene transcription would be not prevented by the compound like Gq/I. As shown in Fig. 4, D and E, 10 μM YM-254890 inhibited CysLT-R2-mediated but not β2-mediated gene transcription. However, P2Y12/Gq/H9251qi5-mediated gene transcription was suppressed completely by treatment with the compound (Fig. 4F). These results propose the target of YM-254890 is not receptor-Gq interaction.

In addition, we examined the effect of the compound on high affinity agonist binding, which reflects the agonist-receptor-Gq ternary complex. [3H]2MeSADP bound to P2Y1-expressing cell membrane (Kd = 38 ± 1.0 nM, Bmax = 11 ± 0.4 pmol/mg, the mean ± S.D. of three separate experiments, Fig. 5). Addition of 10 μM GTPγS to the reaction mixture reduced the Kd value of [3H]2MeSADP binding (Kd = 103 ± 22 nM, Bmax = 12 ± 2 pmol/mg), suggesting that GTPγS allows Gq/11 activation, which leads to dissociation of the activated Gq/11 from ternary complex and formation of Gq/11-uncoupled low affinity agonist binding site. However, addition of 10 μM YM-254890 did not affect the high affinity [3H]2MeSADP binding (Kd = 38 ± 17 nm, Bmax = 10 ± 2 pmol/mg). These data also indicate that YM-254890 does not induce uncoupling of Gq/11 with receptors.

The Effect of YM-254890 on Receptor-induced [35S]GTPγS Binding—At this point, it is expected that the target of YM-254890 is the guanine nucleotide exchange step. The receptor-mediated binding of [35S]GTPγS to Go was measured to evaluate the effect of the compound on the exchange of GDP for GTPγS.
Most conventional \[^{35}\text{S}]\text{GTP}\_S binding assays using the cell membrane fraction have been restricted to the analysis of the activation of \(\text{Go}_{q/11}\)-coupled receptors, although significant stimulation of \(\text{Go}_{q/11}\) is often masked by a higher basal level of \[^{35}\text{S}]\text{GTP}\_S binding (29). We could detect agonist-stimulated \[^{35}\text{S}]\text{GTP}\_S binding to \(\text{Go}_q\), using membranes of \(\text{P2Y12}\)-expressing cells, but the \(\text{P2Y12}\)-mediated \[^{35}\text{S}]\text{GTP}\_S binding was not inhibited by addition of YM-254890 (up to 1 \(\mu\text{M}\)) (Fig. 6A). In the conventional \[^{35}\text{S}]\text{GTP}\_S binding assay, we have detected no significant agonist-stimulated \[^{35}\text{S}]\text{GTP}\_S binding using membranes of \(\text{M1}\)- or \(\text{P2Y1}\)-expressing cells (data not shown). Akam et al. (30) have reported that \[^{35}\text{S}]\text{GTP}\_S-bound \(\text{Go}_q\) can be immunoprecipitated specifically, and they have observed the reproducible agonist-stimulated binding. Therefore, to isolate and enrich the \[^{35}\text{S}]\text{GTP}\_S-bound \(\text{Go}_{q/11}\), we took the experimental approach described by Akam et al. After incubating \[^{35}\text{S}]\text{GTP}\_S with the membrane fraction of \(\text{M1}\) or \(\text{P2Y1}\)-expressing cells, \(\text{Go}_{q/11}\), in the reactions were solubilized by detergents and immunoprecipitated by anti-\(\text{Go}_{q/11}\). Then their radioactivities were measured. By this approach, we could detect methacholine- and \(2\text{MeSADP}\)-stimulated \[^{35}\text{S}]\text{GTP}\_S binding using \(\text{M1}\) and \(\text{P2Y1}\) membrane, respectively (Fig. 6, B and C). The \(\text{M1}\) and \(\text{P2Y1}\)-mediated \[^{35}\text{S}]\text{GTP}\_S binding to \(\text{Go}_{q/11}\) were inhibited by addition of \(\text{YM-254890}\) in a dose-dependent manner. These indicate that \(\text{YM-254890}\) blocks the exchange step of GDP for GTP in \(\text{Go}_q\) activation. The effective dose of \(\text{YM-254890}\) is relatively lower than that for \(\text{Ca}^{2+}\) mobilization, which was probably caused by the difference between cell membrane and living cell, or the measurement of G protein activation and effector function.

Taken together, our observations indicate that \(\text{YM-254890}\) is a selective \(\text{Go}_{q/11}\) inhibitor, and the target of this compound is the exchange step of GDP for GTP in \(\text{Go}_{q/11}\) activation states. There have been reports of compounds that inhibit the activation of Go selectively. For example, the suramin analogue NF023 has been established as a selective \(\text{Go}_{\alpha_{1c}}\) inhibitor, which suppresses GTP\_S binding directly as well as \(\text{Go}_{\alpha_{1c}}\)-coupled \(\beta\)-adrenergic receptor-mediated cellular signaling (31). NF449 has also been reported as a \(\text{Go}_q\) inhibitor, suppressing GTP\_S binding and \(\text{Go}_{\alpha_{1c}}\)-coupled \(\beta\)-adrenergic receptor-mediated cellular signaling (32). It has also been reported that a substance P-related peptide, named GP\_Ant-2A, inhibits the activation of \(\text{Go}_{q/11}\) selectively by competing with the receptor and G protein (33). However, GP\_Ant-2A is useless in vitro and in vivo studies, because of inability to cross cell membrane. The acting point of \(\text{YM-254890}\) is distinct from that of GP\_Ant-2, and moreover \(\text{YM-254890}\) is functional in vitro and in vivo. \(\text{YM-254890}\) is a first discovered \(\text{Go}_{q/11}\) selective inhibitor.

![Image](http://www.jbc.org/content/early/2017/08/16/jbc.M117.791402/F5.large.jpg)
selective inhibitor, blocking the exchange step of GDP for GTP in vitro and in vivo. This novel compound is a promising and powerful tool for studying Goq/11 protein activation, Goq/11-coupled receptor signaling, and Goq/11-mediated biological events.

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