Involvement of G protein βγ-subunits in diverse signaling induced by G_{i/o}-coupled receptors: study using the Xenopus oocyte expression system

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Submitted 5 March 2004; accepted in final form 13 May 2004

Uezono, Yasuhito, Muneshige Kaibara, Osamu Murasaki, and Kohtaro Taniyama. Involvement of G protein βγ-subunits in diverse signaling induced by G_{i/o}-coupled receptors: study using the Xenopus oocyte expression system. Am J Physiol Cell Physiol 287: C885–C894, 2004. First published May 19, 2004; 10.1152/ajpcell.00125.2004.—We studied the functions of βγ-subunits of G_{i/o} protein using the Xenopus oocyte expression system. Isoproterenol (ISO) elicited cAMP production and slowly activating Cl⁻ currents in oocytes expressing β₂-adrenoceptor and the protein kinase A-dependent Cl⁻ channel encoded by the cystic fibrosis transmembrane conductance regulator (CFTR) gene. 5-Hydroxytryptamine (5-HT), [d-Ala², D-Leu⁵]-enkephalin (DADLE), and baclofen enhanced ISO-induced cAMP levels and CFTR currents in oocytes expressing β₂-adrenoceptor-CFTR and 5-HT₁A receptor (5-HT₁A R), δ-opioid receptor, or GABA_B receptor, respectively. 5-HT also enhanced pituitary adenylate cyclase activating peptide (PACAP) 38-induced cAMP levels and CFTR currents in oocytes expressing PACAP receptor, CFTR and 5-HT₁A R. The 5-HT-induced enhancement of G_i/G_o-coupled receptor-mediated responses was abrogated by pretreatment with pertussis toxin (PTX) and coexpression of G transducin α (G_{oα}). The 5-HT-induced enhancement was further augmented by coexpression of the Gβγ-activated form of adenylate cyclase (AC) type II but not AC type III. Thus βγ-subunits of G_{i/o} protein contribute to the enhancement of G_i/G_o-coupled receptor-mediated responses. 5-HT and DADLE did not elicit any currents in oocytes expressing 5-HT₁A R or δ-opioid receptor alone. They elicited Ca²⁺-activated Cl⁻ currents in oocytes coexpressing these receptors with the Gβγ-activated form of phospholipase C (PLC)-β2 but not with PLC-β1. These currents were inhibited by pretreatment with PTX and coexpression of G_{oα}, suggesting that βγ-subunits of G_{i/o} protein activate PLC-β2 and then cause intracellular Ca²⁺ mobilization. Our results indicate that βγ-subunits of G_{i/o} protein participate in diverse intracellular signals, enhancement of G_i/G_o-coupled receptor-mediated responses, and intracellular Ca²⁺ mobilization.

G protein-coupled receptor; cystic fibrosis transmembrane conductance regulator gene; cross talk; electrophysiology

STIMULATION OF G PROTEIN-COUPLED RECEPTORS activates diverse intracellular signaling pathways and a variety of cellular functions that are dependent on the coupling of a set of G protein families, among which G_{i/o}-coupled receptors are known to activate a variety of effectors (20, 21). Although stimulation of G_{i/o}-coupled receptors is generally considered to inhibit the accumulation of cAMP in most cells, activation of these receptors, including GABA_B receptor (12, 14, 30), dopamine D₂ receptor (9), and α₂-adrenoceptor (23), enhance cAMP production mediated by G_{i}-coupled receptors such as β₂-adrenoceptor and vasoactive intestinal peptide receptor. Using a protein kinase A-mediated Cl⁻ channel encoded by the cystic fibrosis transmembrane conductance regulator (CFTR) gene as an electrophysiological sensor for cAMP changes, investigators at our laboratory (29) previously demonstrated that activation of GABA_B receptors enhances cAMP production induced by G_{i}-coupled receptors. It has been established that increased Cl⁻ current through CFTR is correlated to increased cAMP level in Xenopus oocytes (28).

It is well known that the βγ-subunits of G protein regulate the activities of several types of effectors, including phospholipase C (PLC), adenylate cyclases (AC), phosphatidylinositol 3-kinases, G protein-coupled receptor kinases, and some ion channels (4, 20, 25). Although Gβγ-subunits are members of all trimeric G proteins, it is generally accepted that high concentrations of free Gβγ-subunits in the cells arise from G_{i/o} proteins, which are abundantly expressed in the cells compared with other G proteins (20, 25). Thus it is important to elucidate whether the Gβγ-subunits released from G_{i/o} proteins regulate the activity of various effectors.

The present study was designed to elucidate the involvement of Gβγ-subunits released from G_{i/o} protein in the diverse intracellular signaling pathways. In particular, we focused on the involvement of cAMP signaling and intracellular Ca²⁺ signaling pathways. To this end, using an electrophysiological assay as well as a cAMP assay with the Xenopus oocyte expression system, we examined whether a set of cloned G_{i/o}-coupled receptors regulate such signaling pathways.

MATERIALS AND METHODS

Drugs and chemicals. Isoproterenol (ISO), 5-hydroxytryptamine (5-HT), baclofen, gentamicin, pertussis toxin (PTX), sodium pyruvate, and niflumic acid were purchased from Sigma (St. Louis, MO). [d-Ala², D-Leu⁵]-enkephalin (DADLE) and pituitary adenylate cyclase activating peptide (PACAP) 38 were obtained from Osaka Peptide Institute (Osaka, Japan). All other chemicals used were of analytical grade and were obtained from Nacalai Tesque (Kyoto, Japan).

Preparation of cRNA. cDNA from the human CFTR gene was provided by Dr. J. R. Riordan (Mayo Clinic, Scottsdale, AZ). AC type II was obtained from Dr. R. Reed (The Johns Hopkins University, Baltimore, MD), and AC type III was supplied by Dr. K. G. Zinn (California Institute of Technology, Pasadena, CA). Rat PACAP receptor was obtained from Dr. A. Arimura (Tulane University, New Orleans, LA). Rat 5-HT₁A receptor (5-HT₁A R) and 5-HT₂C receptor, β₂-adrenoceptor, and δ-opioid receptor were kindly provided by Dr. H. A. Lester (California Institute of Technology). Rat phospholipase C-β1 (PLC-β1) and PLC-β2 were obtained from Dr. S. G. Rhee (National Heart, Lung, and Blood Institute, Bethesda, MD). GABA_B1 (GABA_B1) and GABA_B2 receptors were supplied by Dr. N. J. Fraser (GlaxoWellcome Research and Development, Stevenage, UK).

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cRNA was synthesized from the respective linearized cDNA using a T7 or SP6 RNA polymerase kit (Ambion, Austin, TX).

**Oocyte preparation and cRNA injection.** Immature V and VI stage *Xenopus* oocytes were enzymatically dissociated as reported previously (28). Isolated oocytes were incubated at 18°C in ND-96 medium (in mM: 96 NaCl, 2 KCl, 1 CaCl₂, 1 MgCl₂, and 5 HEPES, pH 7.4) containing 2.5 mM sodium pyruvate and 50 μg/ml gentamicin. For measurement of CFTR currents induced by stimulation of Gₛ-coupled receptors, cRNA for CFTR (4 ng) and β₂-adrenoceptor (0.1 ng) or PACAP receptor (1 ng) were injected into the oocytes together with or without 5-HT₁₄R (10 ng), δ-opioid receptor (10 ng), GABA₁ receptor (10 ng), and GABA₂ receptor (10 ng). In some oocytes, cRNA for 5-HT₂C (1 ng), G transducin α (Gaα; 5 ng), AC type II (5 ng), AC type III (5 ng), PLC-β1 (5 ng), or PLC-β2 (25 ng) was injected. The final injection volume was <50 nl in all cases. Oocytes were incubated in ND-96 medium and used 3–8 days after injection.

**Electrophysiological recordings.** Electrophysiological recordings were performed using the two-electrode voltage-clamp method with a GeneClamp 500 amplifier (Axon Instruments, Foster, CA) at room temperature. Oocytes were clamped at −60 mV and continuously superfused with ND-96 medium in a 0.25-ml chamber at a flow rate of 5 ml/min, and test compounds were added to the superfusion solution. In experiments with niflumic acid, the compound was added 10 min before agonist stimulation and maintained throughout the experiment. Voltage-recording microelectrodes were filled with 3 M KCl and had a tip resistance of 1.0–2.5 MΩ. Currents were continuously recorded and stored with Mac Lab software (AD Instruments, Castle Hill, Australia) on a Macintosh computer. The ramp protocol was performed as described previously (28).

**cAMP assay.** The cellular cAMP level in individual oocytes was measured by conventional radioimmunoassay. Briefly, oocytes were washed three times with ND-96 buffer and preincubated with 1 mM IBMX for 30 min followed by another incubation with varying concentrations of SO or other test compounds containing 1 mM IBMX and 1 mg/ml bovine serum albumin as described previously (31). The reaction was stopped by the addition of 100 mM HCl followed by heating to 95°C for 5 min. The cAMP content of individual oocytes was assayed using a [125I]-labeled cAMP radioimmunoassay kit (Yasama, Chiba, Japan).

**Statistical analysis.** Data are expressed as means ± SE. Differences between two groups were examined for statistical significance using a paired t-test. For comparisons between multiple groups, we performed one-way analysis of variance followed by Scheffe’s test. P < 0.05 denoted the presence of a statistically significant difference.

**RESULTS**

**5-HT enhances ISO-induced CFTR currents.** ISO at the concentrations of 10⁻⁹–10⁻⁴ M elicited slowly activating inward currents in oocytes expressing a combination of β₂-adrenoceptor and CFTR, but not in noninjected oocytes, as shown previously by Uezono et al. (28). ISO at a concentration of 10⁻⁷ M, which corresponds to a 40–50% effective concentration (EC) of the maximal responses, elicited CFTR Cl⁻ currents in all oocytes expressing β₂-adrenoceptor and CFTR, regardless of the expression level of 5-HT₁₄R (Fig. 1). At a concentration of 10⁻⁷ M, 5-HT did not elicit any currents in all oocytes that expressed 5-HT₁₄R alone. Moreover, 5-HT did not elicit any currents in oocytes expressing 5-HT₁₄R and β₂-adrenoceptor-CFTR (Fig. 1). When 5-HT (10⁻⁷ M) was applied simultaneously with ISO (10⁻⁷ M), the ISO-induced CFTR currents were enhanced in oocytes that expressed β₂-adrenoceptor-CFTR and 5-HT₁₄R (Fig. 1). In noninjected oocytes, no endogenous responses to ISO, 5-HT, or both were noted, even at 10⁻⁴ M (data not shown). The 5-HT-induced enhancement was concentration dependent, and the EC₅₀ of 5-HT was 2.9 ± 0.31 × 10⁻⁸ M (Fig. 1C and Table 1), which was similar (~1.6 × 10⁻⁸ M) to that activated in inward-rectifying K⁺ channels in oocytes expressing 5-HT₁₄R and G protein-activated inward-rectifying K⁺ channels (6).

**DADLE and baclofen enhance ISO-induced CFTR currents.** In oocytes expressing β₂-adrenoceptor and CFTR, as well as δ-opioid or GABA_B receptors, ISO (10⁻⁷ M) elicited CFTR currents in oocytes regardless of expression of δ-opioid or GABA_B receptor (Fig. 2). DADLE (10⁻⁷ M) and baclofen (10⁻⁴ M) did not elicit any currents in oocytes expressing δ-opioid receptor alone (see Fig. 8) or GABA_B receptor alone...
Table 1. Comparison of EC$_{50}$ values of 5-HT$_{1A}$ receptor-, δ-opioid receptor-, or GABA$_B$ receptor-induced enhancement of cAMP production and CFTR currents activated by β$_2$-adrenoceptor in Xenopus oocytes

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligand</th>
<th>EC$_{50}$ of cAMP Production, M</th>
<th>EC$_{50}$ of CFTR Currents, M</th>
</tr>
</thead>
<tbody>
<tr>
<td>β$<em>2$R + 5-HT$</em>{1A}$R</td>
<td>5-HT</td>
<td>2.1 ± 0.10 × 10$^{-8}$</td>
<td>2.9 ± 0.31 × 10$^{-8}$</td>
</tr>
<tr>
<td>β$_2$R + δ-opioidR</td>
<td>DADLE</td>
<td>4.6 ± 0.19 × 10$^{-8}$</td>
<td>3.2 ± 0.32 × 10$^{-8}$</td>
</tr>
<tr>
<td>β$_2$R + GABA$_B$R</td>
<td>Baclofen</td>
<td>1.1 ± 0.06 × 10$^{-6}$</td>
<td>1.0 ± 0.12 × 10$^{-6}$</td>
</tr>
</tbody>
</table>

In oocytes expressing the above combinations of receptors with or without cystic fibrosis transmembrane conductance regulator (CFTR), varying concentrations of 5-hydroxytryptamine (5-HT), [d-Ala$^2$, d-Leu$^5$]-enkephalin (DADLE), or baclofen together with isoproterenol (10$^{-7}$ M) were applied and then cAMP levels and CFTR currents were measured as described in Figs. 1C and 3. Data from 3–6 oocytes at each concentration are shown as means ± SE. EC$_{50}$ of CFTR are not significant compared with EC$_{50}$ of cAMP production. β$_2$R + 5-HT$_{1A}$R, β$_2$-adrenoceptor-CFTR combined with 5-HT$_{1A}$R receptor; β$_2$R + δ-opioidR, β$_2$-adrenoceptor-CFTR combined with δ-opioid receptor; β$_2$R-GABA$_B$R, β$_2$-adrenoceptor-CFTR combined with GABA$_B$ receptor.

(data not shown). These compounds also did not elicit any currents in oocytes expressing δ-opioid or GABA$_B$ receptor and β$_2$-adrenoceptor-CFTR (Fig. 2). When DADLE (10$^{-7}$ M) or baclofen (10$^{-4}$ M) was applied simultaneously with ISO (10$^{-7}$ M), the ISO-induced cAMP currents were enhanced in oocytes that expressed β$_2$-adrenoceptor-CFTR and δ-opioid or GABA$_B$ receptor (Fig. 2), respectively. Baclofen (10$^{-4}$ M)-induced enhancement was noted in oocytes that expressed β$_2$-adrenoceptor-CFTR and a heterodimer of GABA$_B$ receptor [GABA$_B$A$_{1}(b)$ and GABA$_B$B$_{2}$], but not in oocytes that expressed β$_2$-adrenoceptor-CFTR and GABA$_B$A$_{1}(b)$ receptor alone or GABA$_B$A$_{2}$ receptor alone (Fig. 2), confirming that functional GABA$_B$ receptors need heterodimerization (16).

G$_G$-coupled receptor agonists enhance ISO-induced cAMP production. ISO (10$^{-7}$ M) elevated cAMP production in oocytes expressing β$_2$-adrenoceptor, but not in the noninjected oocytes or in oocytes not expressing β$_2$-adrenoceptor (Fig. 3, A–C). 5-HT, DADLE, and baclofen did not elevate cAMP production in oocytes expressing 5-HT$_{1A}$R, δ-opioid receptor, and heterodimeric GABA$_B$ receptor together with or without β$_2$-adrenoceptor, respectively (Fig. 3, A–C). In the oocytes expressing β$_2$-adrenoceptor together with 5-HT$_{1A}$R, δ-opioid receptor, or heterodimeric GABA$_B$ receptor, simultaneous application of 5-HT, DADLE, or baclofen with ISO, but not individual application, enhanced ISO-induced cAMP production (Fig. 3, A–C). The EC$_{50}$ values of 5-HT, DADLE, and baclofen in the enhancement of ISO-induced cAMP production were almost similar to those for the enhancement of ISO-induced CFTR currents (Table 1).

5-HT enhances PACAP-induced cAMP production and CFTR currents. We next examined whether cAMP production and CFTR currents activated by another G$_G$-coupled receptor were also enhanced by G$_G$-coupled receptor activation. PACAP$_38$ (10$^{-7}$ M), an agonist of PACAP receptor, elicited cAMP production and CFTR currents in oocytes that expressed PACAP receptor and CFTR together with or without 5-HT$_{1A}$R (Fig. 4, A and B). 5-HT did not elicit cAMP production or any currents in oocytes expressing PACAP-CFTR and 5-HT$_{1A}$R (Fig. 4). Simultaneous application of 5-HT (10$^{-7}$ M) and PACAP$_38$ (10$^{-7}$ M) enhanced the PACAP-induced responses in oocytes expressing PACAP-CFTR and 5-HT$_{1A}$R (Fig. 4).
activated by Gβγ-subunits in oocytes. As a control, we expressed Gα-coupled 5-HT₂R, known to elevate [Ca²⁺], which in turn activates Ca²⁺-activated Cl⁻ channels endogenously expressed in Xenopus oocytes (24). Application of 5-HT (10⁻⁷ M) elicited Ca²⁺-activated Cl⁻ currents in oocytes expressing 5-HT₂R, as reported previously (24) (Fig. 8). 5-HT and DADLE at concentrations up to 10⁻³ or 10⁻⁵ M, respectively, did not elicit any currents in oocytes expressing 5-HT₁A or δ-opioid receptor alone. On the other hand, in oocytes expressing 5-HT₁A or δ-opioid receptor together with PLC-β2, both 5-HT (10⁻⁷ M) and DADLE (10⁻⁷ M) elicited inward currents. The current displayed a reversal potential of approximately −25 mV, which is composed of Cl⁻ currents, in terms of ramp protocol from −80 to +80 mV [Fig. 8C and Uezono et al. (28)]. Furthermore, the currents elicited by 10⁻⁷ M 5-HT in oocytes expressing 5-HT₁A and PLC-β2 were significantly inhibited by the Ca²⁺-activated Cl⁻ channel blocker niflumic acid (100 μM) (3), to 10% [5-HT, 340.1 ± 50.1 nA; 5-HT + niflumic acid, 38.0 ± 5.4 nA (n = 6)], indicating that the currents were elicited through Ca²⁺-activated Cl⁻ channels. When PLC-β1, an isotype insensitive to Gβγ-subunits (15), was expressed instead of PLC-β2 together with 5-HT₁A or δ-opioid receptor, neither 5-HT nor DADLE at concentrations up to 10⁻³ or 10⁻⁵ M, respectively, elicited any currents (Fig.
Fig. 3. Effects of 5-HT, DADLE, and baclofen on isoproterenol-induced cAMP production in oocytes expressing a combination of β₂-adrenoceptor with 5-HT₁A R (A), δ-opioid receptor (B), or heterodimeric GABA₉ receptor (C). Isoproterenol (10⁻⁷ M) with or without 5-HT (10⁻⁷ M), DADLE (10⁻⁷ M), or baclofen (10⁻⁴ M) was applied for 10 min as indicated. A: 5-HT enhances isoproterenol-induced cAMP production in oocytes expressing both β₂-adrenoceptor and 5-HT₁A R. B: DADLE enhances isoproterenol-induced cAMP production in oocytes expressing both β₂-adrenoceptor and δ-opioid receptors. C: baclofen enhances isoproterenol-induced cAMP production in oocytes expressing both β₂-adrenoceptor and heterodimeric GABA₉ receptor. Each bar represents the mean ± SE of cAMP levels in the oocytes. *P < 0.05. n.s., Not significant.

Fig. 4. Effects of 5-HT on pituitary adenylate cyclase activating peptide (PACAP) 38-induced cAMP production and CFTR currents. A: 5-HT enhances PACAP38 (10⁻⁷ M)-induced cAMP production in oocytes expressing a combination of PACAP receptor-CFTR and 5-HT₁A R (PACAPR + 5-HT₁A R). PACAP38 (10⁻⁷ M) and 5-HT (10⁻⁷ M) were applied for 10 min. Data are means ± SE. B: representative traces of CFTR currents from oocytes expressing a combination of PACAP receptor-CFTR and 5-HT₁A R (PACAPR + 5-HT₁A R). C: summary of CFTR currents. Each bar represents the mean ± SE of peak CFTR current expressed as %PACAP38-induced peak currents from each of 6 oocytes. *P < 0.05.

8), although 5-HT (10⁻⁷ M) caused significantly greater Cl⁻ currents in oocytes expressing 5-HT₂A R and PLC-β1 than in those expressing 5-HT₂A R alone (Fig. 8), possibly because of G₁₉α-mediated activation of PLC-β1. To further investigate the role of Gβγ-subunit released from G₁₉α proteins, oocytes expressing 5-HT₁A R or δ-opioid receptor with PLC-β2 were
pretreated with PTX or coexpressed with G\textsubscript{t} as described in Figs. 5 and 6. As expected, the currents caused by 5-HT or DADLE were completely inhibited by PTX pretreatment or suppressed by coexpression of G\textsubscript{t} (Fig. 9).

**DISCUSSION**

In the present study, we investigated signaling pathways activated by a set of cloned G\textsubscript{i/o}-coupled receptors using electrophysiological assays as well as cAMP assay in the *Xenopus* oocyte expression system. ISO elicited cAMP production and Cl\textsuperscript{−} currents through CFTR channels in oocytes expressing a combination of β\textsubscript{2}-adrenoceptor and CFTR. In oocytes expressing β\textsubscript{2}-adrenoceptor-CFTR and G\textsubscript{i/o}-coupled receptors such as 5-HT\textsubscript{1A}R, δ-opioid receptor, and GABA\textsubscript{B\textsubscript{2}} receptors, application of 5-HT, DADLE, or baclofen together with ISO enhanced ISO-induced cAMP production and CFTR currents. CFTR currents and cAMP production elicited by another G\textsubscript{i/o}-coupled receptor, PACAP receptor, were also enhanced by 5-HT in oocytes expressing PACAP receptor and CFTR together with 5-HT\textsubscript{1A}R. CFTR has been reported to be regulated by several intracellular second messengers, such as cGMP-dependent protein kinases, protein kinase C, or tyrosine kinases, as well as cAMP-dependent protein kinases (26). In
Stimulation of G_{i/o}-coupled receptors is generally considered to inhibit cAMP accumulation in most cells. On the other hand, stimulation of G_{i/o}-coupled receptors, including GABA\_B receptor, dopamine D\_2 receptor, or \( \alpha_2 \) adrenergic receptor, has been shown to enhance cAMP production induced by G\_s-coupled receptors such as \( \beta_2 \)-adrenoceptor and vasoactive intestinal peptide receptor (9, 12, 23, 30). Some G_{i/o}-coupled receptors, including opioid receptor, \( \alpha_3 \)-adrenoceptor, muscarinic M\textsubscript{4} receptor, and cannabinoid CB\textsubscript{1} receptor, are coupled not only to G\_i/o protein but also to G\_s protein and are known to stimulate cAMP production (5, 7, 8, 11). We have shown in this study that 5-HT-induced enhancement of ISO- and PACAP38-mediated responses was abrogated by pretreatment with PTX in oocytes expressing CFTR, \( \beta_2 \)-adrenoceptor, or PACAP receptor together with 5-HT\textsubscript{1A}R. Because PTX interrupts signaling pathways from G\_i/o-coupled receptors to G\_i/o proteins without affecting G\_s protein-mediated responses (9), we conclude that G\_i/o proteins, but not G\_s proteins, are involved in such enhancement.

Because \( \beta \gamma \)-subunits of G protein regulate the activities of several types of effectors, including PLC, AC, and some ion channels (4, 20), we investigated whether the \( \beta \gamma \)-subunits of G\_i/o protein contribute to the enhancement of responses mediated by G\_s-coupled receptors. G\_\alpha has been shown to inhibit downstream signaling through G\_\beta\gamma-subunits by sequestering free \( \beta \gamma \)-subunits (9). We previously demonstrated that G\_i/o-coupled cannabinoid CB\textsubscript{1} and CB\textsubcript{2} receptors activated inward rectifying K\textsuperscript{+} channels through G\_\beta\gamma-subunits by showing that these effects were inhibited by coexpression of G\_\alpha (13). Our results also showed that coexpression of G\_\alpha prevented 5-HT-induced enhancement of CFTR currents caused by ISO, suggesting that G\_\beta\gamma-subunits released by G\_i/o-coupled receptor stimulation caused the enhancement of G\_i/o-coupled receptor-mediated cAMP production. cAMP is produced by activation of AC, and to date, eight types of AC genes have been cloned, each of which is differentially regulated by G\_\alpha, G\_\beta\gamma, Ca\textsuperscript{2+}/calmodulin, protein kinase C, and [Ca\textsuperscript{2+}]. (27). The \( \beta \gamma \)-subunits are known to activate AC types II and IV, but not AC type III, in the presence of activated G\_\alpha (19, 27). In agreement with these reports, our results showed that enhancement of 5-HT-induced CFTR currents was augmented by coexpression of AC type II, but not AC type III, in oocytes expressing \( \beta_2 \)-adrenoceptor-CFTR and 5-HT\textsubscript{1A}R. Thus \( \beta \gamma \)-subunits released by activation of G\_i/o-coupled receptors contribute to the enhancement of G\_i/o-coupled receptor-mediated responses, presumably by the activation of AC type II or IV present in oocytes. Collectively, these findings demonstrate that activation of G\_i/o-coupled receptors enhance the cAMP production induced by G\_i/o-coupled receptors through G\_\beta\gamma-subunits released from PTX-sensitive G\_i/o proteins.

We also have demonstrated that \( \beta \gamma \)-subunits released by activation of G\_i/o-coupled receptors appear to be involved in another signaling pathway, the Ca\textsuperscript{2+} mobilization signaling pathway. We have clearly shown that 5-HT or DADLE elicited Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} currents in oocytes expressing 5-HT\textsubscript{1A}R or \( \delta \)-opioid receptor together with PLC\_B\_2. It has been shown that G\_\beta\gamma-subunits activate PLC\_B\_2, but not PLC\_B\_1, in cotransfection assays with COS-7 cells (15). As such, substitution of PLC\_B\_1 for PLC\_B\_2 in the coexpression did not elicit Ca\textsuperscript{2+}-activated Cl\textsuperscript{-} currents induced by 5-HT or DADLE in oocytes expressing 5-HT\textsubscript{1A}R or \( \delta \)-opioid receptor, respectively. In
Xenopus oocytes, Gβγ-subunit-sensitive PLC-β-like proteins, namely, PLC-βX, were present (10). Accordingly, Gβγ liberated by activation of Gi/o-coupled receptors may increase [Ca^{2+}] through activation of endogenously expressed PLC-βX in oocytes. In fact, 5-HT elicited Ca^{2+}-activated Cl^- currents without additional expression of PLC-β2 or -β3 in oocytes expressing 5-HT1A receptor in the vector, which was designed to overexpress the receptor in oocytes (22). However, we found it necessary to express PLC-β2 to elicit Gi/o-coupled receptor-mediated Ca^{2+}-activated Cl^- currents in Xenopus oocytes; extremely overexpressed receptors may liberate Gβγ-subunits sufficiently to activate endogenous PLC-βX.

We have further demonstrated that 5-HT1A- and δ-opioid receptor-mediated Ca^{2+}-activated Cl^- currents were suppressed by pretreatment with PTX and by coexpression of Gi/α. These findings suggest that Gβγ-subunits released by activation of Gi/o-coupled receptors could activate PLC-β2 and then stimulate intracellular Ca^{2+} mobilization. A recent study (34) in Xenopus oocytes showed that Gβγ-subunits caused direct activation of inositol 1,4,5-trisphosphate (IP3) receptors in addition to activation of PLC-β, and both pathways consequently increased [Ca^{2+}]. However, it may be less likely that Gβγ-subunits liberated by 5-HT1A or δ-opioid receptor stimulation directly activated IP3 receptors to increase in [Ca^{2+}]; in our experimental condition, because exogenously expressed PLC-β2 was required to elicit Ca^{2+}-activated Cl^- currents. Nonetheless, further detailed studies are necessary to clarify the involvement of this novel signaling pathway in Gi/o-coupled receptor-mediated Ca^{2+} mobilization in the Xenopus oocyte and also in the mammalian cell expression systems.

The present study demonstrates that the βγ-subunits released from Gi/o proteins participate in diverse signal pathways such as enhancement of the Gi-coupled receptor-mediated cAMP system and mobilization of [Ca^{2+}]i, provided that a set of particular effectors, such as AC type II or PLC-β2, is present in the cells. The number of G protein subunits identified to date includes 27 subunits of Ga, 5 of Gβ, and 14 of Gγ (1). As expected, various combinations of Gβ and Gγ are possible, and they appear to lead to a diversity of cellular functions. Using the Gβγ and Gγ subunit reconstitution assay, investigators in several studies have examined cellular functions mediated by particular combinations of Gβγ-subunits. For instance, the Gβ1γ2 subunit activates, but the Gβγγ2 subunit inhibits, AC type II (2); the Gβγγ subunit activates PLC-β1 and PLC-β2.
and all combinations of Gβ1.5γ2 except Gβ3γ2 activate AC type II and inhibit AC type I (19, 20). The present study demonstrates that Gβγ-subunits released by activation of Gi/o-coupled receptors activate both AC type II and PLC-β2. Because our experiments were performed with the Xenopus oocyte expression system without expression of any G protein subunits, the expressed Gi/o-coupled receptors in oocytes used endogenously expressed G proteins. Thus the combination of Gβ and Gγ subunits responsible for such signaling pathways remains to be elucidated. Further studies are necessary to determine the combinations of Gβ and Gγ that are responsible for cellular signaling pathways activated by Gi/o-coupled receptors.

Previous studies reported that some Gi/o-coupled receptor-mediated effects were diverse and closely correlated to biological and biomedical effects. Analgesic μ-opioid receptors couple to Gi/o proteins to inhibit AC activity, but they stimulate AC activity under certain circumstances. In a study measuring paw withdrawal thresholds to mechanical stimuli in Sprague-Dawley rats, Khasar et al. (17) reported that preadministration of the μ-opioid receptor agonist [D-Ala2, N-Me-Phe4, Gly5-ol]-enkephalin (DAMGO) attenuated hyperalgesia induced by prostaglandin E2 (PGE2), an agonist of Gs-coupled PGE2 receptors. On the other hand, postadministration of DAMGO just after PGE2 administration enhanced PGE2-induced hyperalgesia. Khasar et al. concluded that the DAMGO-induced enhancement was mediated by Gβγ from Gi/o-coupled μ-opioid receptors and activated PGE2 receptors. Yao et al. (32) showed that ethanol consumption in the rat could be mediated by activation of cAMP-dependent protein kinases by synergistic stimulation of Gβγ-subunits released from Gi/o-coupled dopamine type 2 receptors and activation of Gs-coupled aden-

(18); and all combinations of Gβ1.5γ2 except Gβ3γ2 activate AC type II and inhibit AC type I (19, 20). The present study demonstrates that Gβγ-subunits released by activation of Gi/o-coupled receptors activate both AC type II and PLC-β2. Because our experiments were performed with the Xenopus oocyte expression system without expression of any G protein subunits, the expressed Gi/o-coupled receptors in oocytes used endogenously expressed G proteins. Thus the combination of Gβγ subunits responsible for such signaling pathways remains to be elucidated. Further studies are necessary to determine the combinations of Gβ and Gγ that are responsible for cellular signaling pathways activated by Gi/o-coupled receptors.

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Fig. 9. Pretreatment with PTX and coexpression of Gαs abrogates 5-HT- or DADLE-induced inward currents. 5-HT (10−7 M) and DADLE (10−7 M) were applied for 30 s. The currents were measured in oocytes expressing 5-HT1A-R and PLC-β2 (5-HT1A-R + PLC-β2) or δ-opioid receptor and PLC-β2 (δ-opioidR + PLC-β2). A. left: representative traces of inward currents from these oocytes pretreated with 2 μg/ml PTX (+PTX) or without PTX (−PTX) for 16 h; right: representative traces of inward currents from these oocytes coexpressing Gαs (+Gαs) or not expressing Gαs (−Gαs). B: summary of effects of PTX treatment or Gαs coexpression on the currents. Each bar represents the mean ± SE of peak Cl− currents from each of 6 oocytes.
osine type 2 receptors, both of which were induced by having the rats drink ethanol. In another study at the same laboratory, Yao et al. (33) reported that some addictive effects caused by activation of δ-opioid or cannabinoid CB1 receptors were possibly due to enhanced activation of cAMP-dependent kinases by a synergy of such G_{i/o}-coupled δ-opioid or CB1 receptors with adenosine type 2 receptors. Yao and coworkers proposed that drugs that target Gβγ-subunits or the synergy of several G_{i/o}-coupled receptors with adenosine type 2 receptors might prevent or attenuate excessive drinking of ethanol and the addictive effects of ethanol and other drugs (32, 33). The present results and further experimental studies should clarify the roles of Gβγ-subunits in such physiological phenomena.

In conclusion, using a set of cloned G_{i/o}-coupled receptors, we have demonstrated that Gβγ-subunits liberated by activation of G_{i/o}-coupled receptors are involved in diverse signaling pathways such as enhancement of cAMP pathways and intracellular Ca^{2+} mobilization.

GRANTS

This work was supported in part by grants from the Ministry of Education, Science, Sports, and Culture of Japan (to Y. Uezono, M. Kaibara, and K. Taniyama) and the Naito Foundation (to Y. Uezono).

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