βγ Subunits of Pertussis Toxin-sensitive G Proteins Mediate A₁ Adenosine Receptor Agonist-induced Activation of Phospholipase C in Collaboration with Thyrotropin

A NOVEL STIMULATORY MECHANISM THROUGH THE CROSS-TALK OF TWO TYPES OF RECEPTORS*  

(Received for publication, March 10, 1997, and in revised form, July 7, 1997)

Hideaki Tomura‡§, Hiroshi Itoh‡, Kimie Sho‡, Koichi Sato‡, Motoshi Nagaö‡, Michio Ui‡, Yoichi Kondo‡, and Fumikazu Okajima‡

From the ‡Laboratory of Signal Transduction, Institute for Molecular and Cellular Regulation, Gunma University, Maebashi 371, Japan, the §Faculty of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama 226, Japan, and the ¶Tokyo Metropolitan Institute of Medical Science, Hunkomagome 3-18-22, Tokyo, Japan

COS-7 cells were transiently transfected with human thyrotropin receptor and dog A₁ adenosine receptor cDNAs. An A₁ agonist, N⁶-(L-2-phenylisopropyl) adenosine (PIA), which is ineffective alone, enhanced the thyrotropin (TSH)-induced inositol phosphate production, reflecting phospholipase C (PLC) activation, but inhibited the TSH-induced cAMP accumulation, reflecting adenyl cyclase inhibition. These PIA-induced actions were completely inhibited by pertussis toxin (PTX) treatment. Moreover, in the cells expressing a PTX-insensitive mutant of G₂α or G₃α, in which a glycine residue was substituted for a cysteine residue to be ADP-ribosylated by PTX, at the fourth position of the C terminus, PIA effectively exerted both stimulatory and inhibitory effects on the TSH-induced actions although the cells were treated with the toxin. Overexpression of the βγ subunits of the G proteins enhanced the TSH-induced inositol phosphate production without any significant effect on the cAMP response; under these conditions, PIA did not further increase the elevated inositol phosphate response to TSH. On the contrary, overexpression of a constitutively active mutant of G₂α, in which the guanosine triphosphatase activity is lost, inhibited the TSH-induced cAMP accumulation but hardly affected the inositol phosphate response; under these conditions, PIA never exerted further inhibitory effects on the cAMP response to TSH. In contrast to the case of the TSH-induced inositol phosphate response, the response to a constitutively active G₁₁α mutant was not appreciably affected, and that to NaF was rather inhibited by PIA and overexpression of the βγ subunits. Taken together, these results suggest that a single type of PTX-sensitive G protein mediates the A₁ adenosine receptor-linked modulation of two signaling pathways in collaboration with an activated thyrotropin receptor; α subunits of the PTX-sensitive G proteins mediate the inhibitory action on adenyl cyclase, and the βγ subunits mediate the stimulatory action on PLC. In the case of the latter stimulatory action on PLC, the βγ subunits may not directly activate PLC. The possible mechanism by which βγ subunits enhance the TSH-induced PLC activation is discussed.

The activation of heterotrimeric guanine nucleotide-binding proteins (G proteins)¹ is involved in the stimulation of a variety of signaling pathways by hormone, neurotransmitter, and sensory receptors with seven transmembrane domains (1). Agonist-bound receptors activate G proteins by stimulating the exchange of GDP for GTP on α subunits in a trimeric form of the proteins, which, in turn, accelerates dissociation of the βγ subunits from the α subunits (2). In early studies, only the α subunits were studied as transducers, but now both the α and βγ subunits are recognized to be involved in the regulation of various effector systems such as adenyl cyclase (AC), phospholipase C (PLC), or ion channels (3, 4). At least 16, 5, and 6 species of the α, β, and γ subunits, respectively, have been identified in molecular cloning studies (5, 6). Furthermore, several isoforms of each subunit may be expressed within the same cell type (7–9). Thus, it would be reasonable to assume that a variety of G-protein-dependent actions are executed by different molecular species of the G proteins or their subunits. In this context, identification of molecular species that participate in certain signaling systems is necessary for understanding its mechanism.

In thyroid cells, thyrotropin (TSH) activates PLC through the G₁₁/G₂₁ protein as well as AC through the G₄ protein, resulting in mobilization of Ca²⁺ and accumulation of cAMP in the cells (10). We have shown that in rat FRTL-5 thyroid cells, adenosine and its derivatives such as phenylisopropyl adenosine (PIA) inhibited TSH-induced AC activation and, in contrast, enhanced TSH-induced PLC activation and subsequent Ca²⁺ mobilization through the A₁ type receptor (A₁R) and PTX-sensitive G protein (11). This PTX-sensitive G protein-mediated PLC activation is not restricted to the cross-talk between the TSH and adenosine signaling mechanisms. In FRTL-5 thyroid cells, PIA, through A₁R, also enhanced PLC activation induced by α₁-adrenergic receptor agonists (12) and P₂₁-purinergic receptor agonists (13, 14) as well as TSH. Likewise, A₁R agonists enhance PLC activation induced by IgE in RBL2H3 cells and those induced by ATP and bradykinin in the

¹ The abbreviations used are: G protein, heterotrimeric guanine nucleotide-binding protein; PTX, pertussis toxin; TSH, thyrotropin; TSHB, TSH receptor; A₁R, A₁ adenosine receptor; PLC, phospholipase C; AC, adenyl cyclase; Ga, Gb, and Gγ, G protein α, β, and γ subunits, respectively; IP, inositol phosphate; PIA, N⁶-(L-2-phenylisopropyl) adenosine.

---

* This work was supported in part by a research grant from the Ministry of Education, Science, and Culture of Japan and by a research grant from Taisho Pharmaceuticals. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Laboratory of Signal Transduction, Institute for Molecular and Cellular Regulation, Gunma University, 3-39-15 Showa-machi, Maebashi 371, Japan. Tel.: 81-27-220-8833; Fax: 81-27-220-8889.
smooth muscle cell line (15–17). All of these A1R-mediated actions were abolished by PTX treatment. In addition, this cross-talk is not specific to the A1R agonist; in NG108–15 cells, enkephalin, somatostatin, α1-adrenergic agonist, and carbachol have also been shown to enhance P2Y receptor agonist- or bradykinin-induced PLC activation and Ca2+ mobilization, in a PTX-sensitive manner (18–20). In a previous study, we also showed that the PTX-sensitive G protein-mediated modulation of the TSH and muscarinic acetylcholine actions by adenosine are reconstituted by expressing both the TSH receptor (TSHR) and A1R in COS-7 cells (21) and both the m3 muscarinic acetylcholine receptor and A1R in CHO cells (22), respectively. Thus, our findings in concert with those of others have suggested the presence of a universal cross-talk mechanism mediated by a PTX-sensitive G protein(s) between AC-inhibitory and PLC-stimulatory signaling mechanisms resulting in the enhancement of PLC activation.

Two receptors involved in this cross-talk regulation of PLC are characterized as one type of receptor that couples to PTX-sensitive Gi/Gi proteins whose stimulation inhibits AC but exhibits only a small or undetectable effect on PLC, and the other type of receptor, the so-called Ca2+ mobilizer, couples to Gq/G11 proteins whose stimulation leads to activation of PLC. Thus, AC-inhibiting receptor agonists, through Gi/Gi proteins, permissively or synergistically enhance Ca2+ mobilizing receptor agonist-induced PLC activation. The molecular mechanism by which the respective receptor agonists induce stimulation of each signaling pathway leading to AC inhibition and PLC activation through Gi2/Go and Gq/G11, respectively, has been well characterized (6). However, it has not been well elucidated how a single type of AC-inhibiting receptor simultaneously links to two signaling pathways with the aid of the PTX-sensitive G protein(s).

In this study, we aimed to further define the role of PTX-sensitive G proteins in the cross-talk phenomena in COS-7 cells where TSHR and A1R were expressed. The specific objectives were to determine (a) whether a single type of PTX-sensitive G protein mediates the modulation of the two signaling pathways and (b) how the G protein participates in the two signaling pathways. In the reconstituted cross-talk systems constructed by recombiantin receptors and manipulated G-protein pools in the cells, we found that at least a single molecular species of G2 or G3 can mediate the modulation of two signaling pathways; enhancement of PLC is mediated by βγ subunits, and inhibition of AC is mediated by a single species of the Gα subunit. Furthermore, our results suggest that the primary target of the βγ subunits may not be PLC itself but may instead be located upstream of the enzyme in the signaling pathway, possibly at the level of the α subunits of the Gα11 proteins.

**EXPERIMENTAL PROCEDURES**

**Materials**—PIA was purchased from Sigma; staurosporine was from Kyowa Medex Co. (Tokyo, Japan); and myo-[2-3H]inositol (23 Ci/mmol) was from NEN Life Science Products. Human TSHR cDNA in the pSVL expression vector (23) and dog A1R (24) cDNA were generously provided by Dr. G. Vassart (Université Libre de Bruxelles, Belgium), bovine Gβ1 cDNA by Dr. M. I. Simon (California Institute of Technology), bovine Gγ2 cDNA by Dr. T. Tukada (Tokyo Institute of Psychiatry, Tokyo, Japan), and pCDL-SRα 296 vector (25) by Dr. Y. Takebe (National Institute of Health, Tokyo, Japan). Rabbit antiseraum specific to G2a was generously provided by Dr. Y. Kanano (Tokyo Institute of Technology, Yokohama, Japan), and the β subunit of the G protein was supplied by Dr. T. Katada (Tokyo University, Tokyo, Japan). The radioimmunoassay of cAMP used a Yamasa cAMP assay kit, which was a gift from Yamasa Shoyu Co. (Choshi, Chiba, Japan). The sources of all other reagents were the same as described previously (11, 14, 26).

**Plasmid Construction**—The PTX-insensitive mutant G2a(C352G) expression plasmid was obtained by polymerase chain reaction mutagenesis using wild type rat G2a cDNA (27) as a template. The 5′-primer, 5′-gggggaattcCACCATTGCGTACCGGCTGAG-3′, contains the EcoRI site, Kozak sequence (CCACC) (28), and the first six amino acids of the G2a. The 3′-primer, 5′-ggggggggtcTCAGAAGGACGCGCGGCTGGCTCTTCGGTTGTTGATGAGCT-3′, contains the NotI site, a stop codon, and 14 amino acids in the C-terminal region of G2a, except for GCC (underlined), which encodes glycine instead of cysteine at position 352 from the N terminus. The PTX-insensitive mutant G3α(c351g) expression plasmid was obtained by polymerase chain reaction mutagenesis using wild type rat G3α cDNA (29) as a template. The 5′-primer, 5′-gggggaattcCACCATTGCGTCGCGCTGGCTGAG-3′, contains the EcoRI site, Kozak sequence (CCACC), and the first 11 amino acids of G3α. The 3′-primer, 5′-ggggggggtcTCAGAAGGACGCGCGGCGGCTGGCTCTTCGGTTGTTGATGAGCT-3′, contains the NotI site, a stop codon, and 14 amino acids in the C-terminal region of G3α, except for GCC (underlined), which encodes glycine instead of cysteine at position 351 from the N terminus. The amplified fragment was digested with EcoRI and NotI and then inserted into the EcoRINotI site of the pcDNA/AMP expression plasmids (Invitrogen, CA). The constitutively active mutant Gα(c209l) expression plasmid was obtained by polymerase chain reaction mutagenesis using wild type mouse Gα1 cDNA from mouse S49 lymphoma cells as a template. The 5′-primer, 5′-tgagaaggactTATGCTTGGACGGCTGGTTT-3′, contains the HindIII site and the first eight amino acids of Gα1. The 3′-primer, 5′-ctagagattaTCTTGGACGGCTGGTTT-3′, contains the BamHI site and 14 amino acid rat enkephalin, somatostatin, and G11 cDNA (29) as a template.

**Assay of Inositol Phosphate and cAMP**—The [3H]inositol-labeled cells were washed twice with Hepes-buffered medium, which consisted of 105 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4, 2 mM CaCl2, 2.5 mM NaHCO3, 5 mM glucose, and 0.1% BSA. Samples were then incubated at 37 °C for 10 min with [3H]inositol-labeled cells in the presence of 0.3 kV, 500 microfarads, Gene Pulser II, Bio-Rad) with dog A1R expression plasmids. After transfection, the cells (about 2 × 105) were harvested at 12 days on well plates (Costar) in isoinitol-free Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, KS) in a 5% CO2 atmosphere at 37 °C. For the transfection experiments, the cells were harvested with 0.05% trypsin and 0.5 mM EDTA (Life Technologies, Inc.), washed once with Mg2+ and Ca2+-free phosphate-buffered saline, and suspended in the same phosphate-buffered saline solution. The cell suspensions (about 10 cells in 0.8 ml) were transfected by electroporation using Gene Pulser II (Bio-Rad) with dog A1R cDNA in pCDL-SRα 296 vector (20 μg) and human TSHR cDNA in pSVL (20 μg) expression plasmids in combination with cDNAs encoding bovine Gβ1 (40 μg), bovine Gδ2 (40 μg), Gα2(c352g) (50 μg), G3α(c351g) (50 μg), and Gα1(c209l) (1, 2, or 4 μg) in pCMV5 (p1, Y2, and Gα1(c209l)) or pcDNA/AMP (Gα2(c352g) and G3α(c351g)) expression plasmids, unless otherwise specified. The total amount of DNA for each transfection was adjusted by the empty pcDNA/AMP expression plasmids. After transfection, the cells (about 2 × 105) were cultured on 2-mm wells of Costar in isoinitol-free Dulbecco’s modified Eagle’s medium (Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum and myo-[2-3H]inositol (2 μCi/ml), unless otherwise specified.

**Immunoblot Analysis**—Crude plasma membranes and their cholate extracts were prepared as described previously (33, 34). The cholate extract (25 μg of protein) was resolved on SDS-12.5% polyacrylamide slab gel electrophoresis and then electrophoretically transferred to a...
**RESULTS**

A Single Type of PTX-sensitive G protein, $G_2$ or $G_3$, Mediates $\alpha_1R$-linked Stimulation of Two Signaling Pathways Leading to Inhibition of AC and Enhancement of PLC in Collaboration with an Activated TSHR—In accordance with our previous study (21), when both TSHR and $\alpha_1R$ were expressed in COS-7 cells, although PIA, an $\alpha_1$ agonist, alone hardly affected the basal activities of PLC and AC, this $\alpha_1$ agonist enhanced TSH-stimulated PLC but inhibited the TSH-stimulated AC in a way similar to those in FRTL-5 thyroid cells (11) (Fig. 1, A and C). These PIA actions were completely abolished by a PTX treatment without an appreciable effect on intrinsic TSH actions (Figs. 1, B and D).

In COS cells, several types of PTX-sensitive G proteins are expressed (36-38). To ascertain the role of the PTX-sensitive G protein subtype in the bidirectional $\alpha_1R$ agonist action, we planned to use the cells where only a species of PTX-insensitive and active $G_2\alpha$ mutant is expressed under the conditions where native $G_2\alpha$ is inactive by the PTX treatment. We constructed a mutant cDNA, $G_2\alpha(C352G)$, in which a glycine residue substitutes for a cysteine residue at position 352 of $G_2\alpha$. COS-7 cells were transfected with this PTX-insensitive $G_2\alpha$ cDNA together with cDNAs of the $\beta1$ and $\gamma2$ subunits of the G protein, expecting the expression of the PTX-insensitive $G_2\alpha(C352G)$, which was expressed in COS-7 cells, also coupled to $\alpha_1R$ and modulated the TSH-induced actions even in the PTX-treated cells; the TSH (100 nM)-induced levels were 857 ± 83 or 1162 ± 45 dpm for IP production and 2.42 ± 0.05 or 1.75 ± 0.06 nmol/mg for cAMP accumulation in the absence or presence of PIA, respectively. In all of the cases using or not using either the $G_2\alpha$ or $G_3\alpha$ mutant, PIA alone exerted no significant effect on either the AC or PLC activities.

It has been reported that the C-terminal region of $\alpha$ subunits of G proteins is important for their receptor recognition (39). Therefore, we examined whether the cysteine to glycine substitution affects the ability of PTX-sensitive G proteins to enhance the TSH-induced PLC activation. As shown in Fig. 3, no significant difference was detected between mock- and $G_2\alpha(C352G)$-transfected cells in terms of their response to any PIA dose. This result indicates that $G_2\alpha(C352G)$ still retains the ability to associate with $\alpha_1R$ in COS-7 cells in a way similar to endogenous PTX-sensitive G proteins. The response of $G_2\alpha(C352G)$ transfected cells to higher than 100 nM PIA was slightly stronger than those of the mock-transfected cells. However, this feature of the mutant transfected cells is not specific for the PIA effect on the TSH-induced PLC activation, because...
The experimental conditions of cell pretreatment for control cells (○ and ●) and for the PTX-insensitive mutant-transfected cells (△ and ▲) were essentially the same as those for Figs. 1 and 2, respectively, except that the cells were not treated with PTX in the control cells but treated with the toxin in the mutant-transfected cells. The cells were incubated with the indicated doses of PIA in the presence (●) or absence (○) of 100 nM TSH. The data are expressed as percentages of the respective basal values without agonists. Normalized basal values (cpm) for IP production in each transfected cell were 461 ± 67 and 653 ± 53 for control and G2α(C352G)-expressing cells, respectively. Results are means ± S.E. of three separate experiments.

α Subunits of PTX-sensitive G Proteins Are Responsible for the Inhibition of the AC and βγ Subunits for the Enhancement of PLC—We next examined which subunits, i.e., the α subunit or βγ subunits of the PTX-sensitive G protein, mediate each A1R-mediated signaling. To clarify this point, we transfected mutant DNAs encoding GTPase-deficient G2α(Q205L), Gβ1, Gγ2, or a combination of them. G2α(Q205L) is mutated by substituting a leucine residue for a glutamine residue at position 205. This mutant is lacking in GTPase activity and thereby constitutively stimulates an effector enzyme (40). Expression of the mutated α and β subunits was verified by immunoblotting using specific antisera against the G2α (Fig. 4A) or β (Fig. 4B) subunit. Consistent with previous results (41), no significant increase in the expression of the β subunit was detected when this subunit cDNA was transfected alone. A β subunit might be unstable in an intracellular environment unless it forms a complex with a γ subunit. We measured cAMP accumulation and IP production in the absence or presence of TSH in the cells overexpressing these α or βγ subunits. As shown in Fig. 4D, TSH-induced cAMP accumulation was significantly inhibited by the expression of G2α(Q205L), whereas the transfection of β and/or γ subunit cDNAs hardly affected the cAMP response. Conversely, the overexpression of βγ subunits stimulated the TSH-induced PLC activation, whereas the expression of G2α(Q205L) did not appreciably influence it (Fig. 4C). These results suggest the potential roles of the α and βγ subunits for the inhibition of the TSH-induced AC activation and the enhancement of the TSH-induced PLC activation, respectively.

To determine whether these G protein subunits actually mediate the A1R agonist-induced modulation of TSH-induced actions, we examined the effects of PIA on the dose-dependent TSH activation of AC and PLC in the cells overexpressing G2α(Q205L) or βγ subunits (Fig. 5). As previously shown (Ref. 21 and Figs. 1 and 2), PIA enhanced the dose-dependent TSH activation of IP production (Fig. 5A) and conversely inhibited the TSH stimulation of cAMP accumulation (Fig. 5D). When the βγ subunits were overexpressed, similar to the results shown in Fig. 4, the level of TSH-induced PLC (Fig. 5B) but not cAMP accumulation (Fig. 5E) was increased from that of the control cells at any TSH dose. Under these conditions, the addition of PIA together with TSH did not change the PLC level obtained by TSH alone but reduced the cAMP accumulation induced by the hormone. On the other hand, when the constitutively active mutant of G2α was expressed in the cells, the level of the TSH-induced accumulation of cAMP, but not of PLC activation, was lower than that in the control cells. In this case, the further addition of PIA did not change the level of TSH-induced cAMP accumulation but induced a further increase in the PLC level from that obtained by TSH alone. Thus, the overexpressed βγ subunit complex mimicked the stimulatory action of PIA on the TSH-induced PLC, while the constitutively active α subunit mimicked the inhibitory action of PIA on the TSH-induced AC.

βγ Subunits May Not Directly Activate PLC—The direct in-
These results support the idea that the target of the bg (80 m) induction was as high as that in the cells stimulated by TSH.

TSH-induced activation of PLC is mediated by the TSHR activator (Fig. 7), or challenged with NaF, a nonselective G protein activator (lower panels; A, B, and C) and cAMP accumulation (D and E, and F). Results are expressed as percentages of the respective basal value without agonists for IP production or as cellular content (nmol/mg of protein) for cAMP accumulation. Normalized basal values (cpm) for IP production in each transfected cell were 596 ± 21, 818 ± 55, and 566 ± 21 for control, β1;2-expressing, and G2α(Q205L)-expressing cells, respectively. The data are means ± S.E. of four or five separate experiments.

FIG. 5. Effect of PIA on the dose-dependent TSH-induced actions in the cells overexpressing G2α(Q205L) or β subunits. COS-7 cells were transfected with expression plasmids encoding Gβ1 (40 μg) and Gγ2 (40 μg) (B and E), G2α(Q205L) (50 μg) (C and F), or pCMV5 vector (80 μg) (A and D) together with TSHR (20 μg) and A1R (20 μg). The cells were then cultured in medium containing [3H]inositol for 2 days. The cells were incubated with the indicated doses of TSH in the presence (●) or absence (○) of 100 nM PIA for measurement of IP production (upper panels; A, B, and C) and cAMP accumulation (lower panels; D, E, and F).

DISCUSSION

In the present study, we constructed two Ptx-insensitive mutant DNAs of G2α and G3α, both with a mutation in the sequence encoding the Ptx-sensitive domain of the G proteins. By using the cells expressing each one of these mutant Gα subunits, we were able to analyze the functions of a single subtype species of Gi in the cells where all of the native wild type Gα/Gγ proteins are inactivated by the Ptx treatment. We demonstrated that a single type of G protein, either G2 or G3, mediates both the inhibition of TSH-induced AC activation and the enhancement of TSH-induced PLC activation. A similar strategy had been chosen by other groups to identify a particular Ptx-sensitive G protein subtype to inhibit AC through dopamine D2 receptor isoforms (45) and to regulate multiple effectors including AC, PLC, and phospholipase A2 through the m2 muscarinic acetylcholine receptor (46).

On the other hand, one might consider that the molecular mutation affects its activity and hence results in a misleading conclusion, because, in this mutant α subunit, a glycine residue substitutes for a cysteine residue near the C terminus, where inhibitory action of an α subunit on activated AC has been reported by several groups (40, 42, 43), and this mechanism may account for the AαR-mediated inhibition of AC in the present COS-7 cell system. As with the analogy of the α subunit, it might be possible that the βγ subunit complex directly interacts with the activated PLC and further stimulates the activated enzyme. If this is the case, PLC activated by any means should be further stimulated by the overexpressed βγ subunit complex even in the absence of TSH. Since the TSH-induced activation of PLC is mediated by the TSHR activation of Gq/G11 (10), we planned to provide activated Gq/G11 to TSH-induced activation of PLC is mediated by the TSHR activator (10), we planned to provide activated Gq/G11 or NaF, the level of inositol phosphate production (Fig. 6, A and B), and these activities were further stimulated by TSH (Fig. 6B). This eliminates the possibility that failure of the βγ subunits to enhance the G1α(Q209L)-induced PLC activation is due to the saturation of the enzyme activity. Likewise, the NaF-induced PLC activation was not enhanced but rather was inhibited by the overexpression of the βγ subunits (Fig. 7, A and B, compare first columns). These results support the idea that the target of the βγ subunits is not PLC itself.

As mentioned above, in this experiment, we noticed that the NaF-induced action was slightly but significantly (p < 0.05) inhibited by the overexpressed βγ subunit complexes. This was somewhat unexpected because we expected the positive role for the βγ subunit complexes in the PLC activation. In relation to this, we found that this NaF-induced PLC activation is inhibited by PIA (Fig. 7A). This inhibitory effect of PIA on the NaF-induced PLC activation was significantly reduced in the βγ overexpressed cell, in parallel with the reduction in the stimulatory effect of PIA on TSH action (Fig. 7B). This suggests that the PIA inhibition of the NaF-induced action is also mediated by the βγ subunits. One might wonder why PIA can inhibit NaF-induced PLC activation, because it might be expected that PTX-sensitive G proteins would also be stimulated by a nonselective G protein activator, NaF. This may be simply explained if, in our COS-7 cell system, NaF is an activator for Gi2α/G11 but not for G3α NaF hardly influenced the TSH-induced cAMP accumulation but PIA inhibited it even in the presence of NaF to the same extent as in its absence (data not shown).
the PTX-treated cells was comparable with that in the control cells. This suggests that the change in the expression level of the mutant Gi2 for A1R, even if occurring, is negligible. During the coupling of the D2 dopamine short form receptor to Gi2, no detectable difference in affinity has been observed between the native and the PTX-insensitive mutant Gi2 (45). Thus, the application of this technique is reasonable for the evaluation of the role of a particular G protein in living cells.

In the present study, we mainly presented the results obtained from the cells expressing the Gi2 mutant because we observed no appreciable difference between the cells expressing the mutant Gi2 and those expressing the mutant Gi3. It has been previously shown, however, that in a liposome system constituted with purified bovine brain A1R and one of recombinant subunits of various PTX-sensitive G proteins, the affinity of Gi3 for A1R was higher than that of other G protein subtypes tested, including Gα1, Gα2, Gα3, and Gαi4 (47–49). This discrepancy may be explained by the saturation of transfected Gα1 cDNAs in our case where the cells were transfected with cDNAs at doses needed for maximal responses. Even in the liposome experiments mentioned, the maximal response to Gα1 was the same as that to Gα3.

The present results showed that the expression of a constitutively active mutant of the Gi2αa subunit caused an appreciable inhibition of the TSH-induced AC activation. This is consistent with the current idea of the Gi action mechanism, such that the α subunit of the PTX-sensitive G protein liberated by receptor activation directly inhibits AC, but the β subunits act as either an inhibitor or activator of the enzyme depending on the type of enzyme isoform (42). In HEK293 cells transfected by cDNAs encoding the GTPase-deficient derivatives of Gα1, Gα2, or Gα3, AC is inhibited, which is consistent with our results with COS-7 cells (40). However, a previous paper has reported that the transfection of COS-7 cells with the same mutant Gα2 was sufficient to inhibit AC (34). This discrepancy between the two COS-7 cell experiments might be due to the presence or absence of staurosporin, a potent protein C inhibitor, in the assay medium. Actually, under our experimental conditions, AC in COS-7 cells was not inhibited even by PIA, a typical inhibitory receptor agonist, unless staurosporin was added (21). Although the mechanism by which the suppression of protein kinase C discloses the Giα-induced inhibition of AC has not yet been elucidated, this phenomenon might be related to the ability of the enzyme to phosphorylate the Giα (50).

The PTX-insensitive nature of the TSH-induced PLC activation suggests that the activation occurs on the β1 or β3 isofoms of PLC through a PTX-insensitive G protein, Gi or G simmered (38, 51, 52). The present study demonstrates the participation of βγ subunits in the A1R-linked enhancement of the TSH-induced PLC activation. Recently, βγ subunits of Gβγ have also been shown to activate PLCβ2 and β3 isomons in a PTX-sensitive manner, using cell-free reconstituted systems and cells transfected with cDNAs (53, 54). Therefore, one may presume that in our experimental system, the βγ subunit of the Gαiβγ complex dissociated from the α subunit upon A1R receptor stimulation directly activates the β2 or β3 isoform of PLC in COS-7 cells where at least the PLCβ3 expression has been reported (52). This mechanism, however, would not explain the permissive or synergistic nature of the AC-inhibiting receptor agonist-enhanced enhancement of PLC activation observed in the many cell systems including the present COS-7 cell system, where PIA alone induced only a small, if any, activation of PLC, but it markedly enhanced the enzyme in concert with Ca2+-mobiliz-
FIG. 8. A possible mechanism by which the increase in βγ subunits either enhances or inhibits the TSH- or AlF₄⁻-induced PLC activation. The production of βγ subunits could theoretically either enhance (a) or inhibit (b) the TSH-induced PLC activation, depending on the stoichiometry with the α subunits of the Gq/G11 proteins (A). The production of βγ subunits could theoretically inhibit the AlF₄⁻-induced PLC activation regardless of the stoichiometry with the α subunits of the Gq/G11 proteins (B). See "Discussion" for more details.

The present study also excluded the possibility that PLC prestimulated by activated Gq/G11 interacts with the βγ subunits, because PLC prestimulated by the expression of a constitutively active mutant of Gq/11α was not further stimulated by the overexpression of the β1 and γ2 subunits. Furthermore, when PLC was prestimulated by NaF through the AlF₄⁻-activated G protein, the enzyme was even inhibited by the A1R agonist or the βγ overexpression.

A remaining possibility for the βγ subunits to participate in the synergistic or permissive stimulation of PLC is their interaction with Gq/G11. The βγ subunits released by the action of A1R stimulation or overexpression by transfection of their cDNAs could theoretically either enhance or inhibit the activity of PLC depending on their stoichiometry with the α subunits of the Gq/G11 proteins (Fig. 8A). A similar model has already been presented for the explanation of the inhibitory or stimulatory effect of free βγ subunits on the receptor-mediated PLC activation (55, 56). If there is an excess of Gq/G11α(GDP) over the βγ subunits, the increase in this subunit complex could lead to the formation of a heterotrimeric form of Gq/G11 protein (Gq/G11γ(GDP) βγ) that can couple to TSHR, thereby potentiating the TSH-induced action (through mechanism a in Fig. 8A). On the other hand, if there is no excess of Gq/G11α(GDP) over the βγ subunits or if the formation of the heterotrimeric form of the Gq/G11 protein is not rate-limiting, the production of free βγ subunits could have an inhibitory effect by binding to the active form of Gq/G11 (Gq/G11α(GDP-AlF₄⁻)) through mechanism b in Fig. 8B.

AlF₄⁻, which is produced from the added F⁻ ions and traces of Al³⁺ ion, converts the Gq/G11 protein to the active form (Gq/G11α(GDP-AlF₄⁻)) in this case) by interacting with the inactive GDP forms of the monomeric as well as the heterotrimeric G proteins (57) (Fig. 8B). In this case, the formation of the active form of the Gq/G11 protein is rate-limiting regardless of the excess of the inactive form of Gq/G11α(GDP) over the βγ subunits. Thus, according to the model shown in Fig. 8B, the production of βγ subunits would result in the inhibition of NaF-induced PLC activation by inhibiting the formation of the active form of the Gq/G11 protein. Actually, A1R stimulation and also overexpression of the βγ subunits inhibited the NaF-induced PLC activation (Fig. 7). This inhibition also supports this model.

In addition to transfected COS-7 cells used in the present study, the permissive or synergistic stimulation by Gq/G11 proteins of the receptor-mediated PLC activation has been observed in a variety of cells including FRTL-5 thyroid cells, NG108−15 cells, and smooth muscle cells (see Introduction). In these systems as well, a similar stimulatory mechanism through the βγ subunits (Fig. 8A) might account for the positive cross-talk as in the case of the present COS-7 cellular reconstituted system. A similar βγ subunit action has also been observed by Harden and colleagues (55) in a cell-free reconstituted system using turkey erythrocyte membranes; the reconstituted βγ subunits of the G proteins alone hardly affected the basal activity of PLC but enhanced the P2 receptor-mediated activation of PLC. In this cell-free system, a large amount of βγ subunits significantly inhibited the AlF₄⁻-induced activation of the enzyme as in the case of the present cellular system. In some cases (e.g. in GH3 cells), however, AC-inhibiting receptor agonists inhibit, through the Gq/G11 proteins, the receptor-induced PLC activation (58). In this case, the inhibitory mechanism as described in Fig. 8A might operate.

In conclusion, using COS-7 cells transfected with cDNAs encoding a receptor and one of the G protein subunits, we have shown that a single type of PTX-sensitive G protein, G2 or G3, upon stimulation of A1R, can couple to PLC in a stimulatory fashion and to AC in an inhibitory fashion in collaboration with an activated TSHR. Within the subunits of the PTX-sensitive G proteins, the α subunit seems to be involved in the inhibition of AC activity, while the βγ subunits are involved in the enhancement of PLC. The cross-talk between the βγ subunits derived from PTX-sensitive G proteins and the α subunit of G proteins, which couples to TSHR (probably one of the Gq/G11 family members) might account for the permissive or synergistic activation of PLC by simultaneous stimulation of A1R and TSHR.

Acknowledgments—We thank Dr. Y. Kaziro for valuable discussions and Drs. G. Vassart, M. I. Simon, T. Nakuda, Y. Takebe, Y. Kanaho, and T. Katada for the supply of DNAs and antibodies used in the present study.

REFERENCES
1. Kaziro, Y., Ishii, H., Kozasa, T., Nakafuku, M., and Satoh, T. (1991) Annu. Rev. Biochem. 60, 349–400
2. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
3. Birnbaumer, L. (1992) Cell 71, 1069–1072
4. Hille, B. (1992) Neuron 9, 187–195
5. Watson, A. J., Katz, A., and Simon, M. I. (1994) J. Biol. Chem. 269, 22150–22156
6. Watson, S., and Arkinstall, S. (1994) Trends Pharmacol. Sci., pp. 70–76
7. Clapham, D. E., and Neer, K. J. (1993) Nature 365, 403–406
8. Strathmann, M., Wilks, T. M., and Simon, M. I. (1989) Proc. Natl. Acad. Sci. U.S.A. 86, 7407–7409
9. Strathmann, M., and Simon, M. I. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 9113–9117
10. Allgeier, A., Offermanns, S., Van Sande, J., Spicher, K., Schultz, G., and Dumont, J. E. (1994) J. Biol. Chem. 269, 13753–13755
11. Shio, K., Okajima, F., Majid, M. A., and Kondo, Y. (1991) J. Biol. Chem. 266, 12150–12154
12. Okajima, F., Sato, K., Sho, K., and Kondo, Y. (1989) FEBS Lett. 245, 149–153
13. Okajima, F., Sato, K., Nazarea, M., Sho, K., and Kondo, Y. (1989) J. Biol. Chem. 264, 263,029–263,037
14. Nazarea, M., Okajima, F., and Kondo, Y. (1991) Eur. J. Pharmacol. 16081–16087
15. Ali, H., Cubha-Melo, J. R., Saul, W. F., and Beaven, M. A. (1990) J. Biol. Chem. 265, 745–755
16. Gerwins, P., and Fredholm, B. B. (1990) Proc. Natl. Acad. Sci. U.S.A. 89, 7330–7334
17. Gerwins, P., and Fredholm, B. B. (1992) J. Biol. Chem. 267, 16081–16087
18. Okajima, F., and Kondo, Y. (1992) *FEBS Lett.* **301**, 223–226
19. Tomura, H., Okajima, F., and Kondo, Y. (1992) *Neurosci. Lett.* **148**, 93–96
20. Okajima, F., Tomura, H., and Kondo, Y. (1993) *Biochem. J.* **290**, 241–247
21. Okajima, F., Tomura, H., She, K., Akbar, M., Majid, M. A., and Kondo, Y. (1995) *Biochem. J.* **306**, 709–715
22. Akbar, M., Okajima, F., Tomura, H., Shimegi, S., and Kondo, Y. (1994) *Mol. Pharmacol.* **45**, 1036–1042
23. Libert, F., Lefort, A., Gerard, C., Parmentier, M., Perret, J., Ludgate, M., Dumont, J. E., and Vassart, G. (1989) *Biochem. Biophys. Res. Commun.* **163**, 1220–1225
24. Libert, F., Schiffmann, S. N., Lefort, A., Parmentier, M., Gerard, C., Dumont, J. E., Vanderhaeghen, J. J., and Vassart, G. (1991) *EMBO J.* **10**, 1677–1682
25. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) *Mol. Cell. Biol.* **2**, 466–472
26. Okajima, F., and Kondo, Y. (1990) *J. Biol. Chem.* **265**, 21741–21748
27. Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katafu, T., Ui, M., Iwai, S., Ohtsuka, K., Kawasaki, H., Suzuki, K., and Kaziro, Y. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 3776–3780
28. Kozak, M. (1986) *Cell* **44**, 283–292
29. Itoh, H., Toyama, R., Kozasa, T., Tsukamoto, T., Matsuoka, M., and Kaziro, Y. (1988) *J. Biol. Chem.* **263**, 6656–6664
30. Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H., and Russell, D. W. (1989) *J. Biol. Chem.* **264**, 8222–8229
31. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
32. Yamauchi, J., Kaziro, Y., and Itoh, H. (1995) *Bioche. Biophys. Res. Commun.* **214**, 694–700
33. Okajima, F., Katada, T., and Ui, M. (1985) *Arch. Biochem. Biophys.* **281**, 298–304
34. Yasuda, K., Rens-Domiano, S., Breder, C. D., Law, S. F., Saper, C. B., Reisine, T., and Bell, G. I. (1992) *J. Biol. Chem.* **267**, 20422–20428
35. Reins-Domiano, S., Law, S. F., Yamada, Y., Seino, S., Bell, G. I., and Reisine, T. (1992) *Mol. Pharmacol.* **42**, 28–34
36. Wu, D., LaRosa, G. J., and Simon, M. I. (1993) *Science* **261**, 101–103
37. West, R. E., Jr., Mass, J., Vaughan, M., Liu, T., and Liu, T.-Y. (1985) *J. Biol. Chem.* **260**, 14428–14430
38. Wong, Y. H., Federman, A., Pace, A. M., Zachary, I., Evans, T., Pouysségur, J., and Bourne, H. R. (1991) *Nature* **351**, 63–65
39. Simonds, W. F., Buttrynski, J. E., Gautam, N., Uncon, C. G., and Spiegel, A. M. (1991) *J. Biol. Chem.* **266**, 5363–5366
40. Tsanga, R., Iniguez Lluhi, J. A., and Gilman, A. G. (1993) *Science* **261**, 218–221
41. Chen, J., and Iyengar, R. (1993) *J. Biol. Chem.* **268**, 12253–12256
42. Qian, X. N., Winitz, S., and Johnson, G. L. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 4077–4081
43. Senogles, S. E. (1991) *Biochem. Biophys. Res. Commun.* **175**, 868–873
44. Martin, S. J., and Elledge, S. J. (1992) *Science* **260**, 665–668
45. Tsanga, R., Iniguez Lluhi, J. A., and Gilman, A. G. (1993) *Science* **261**, 218–221
46. Lam, A. Y., and Stiles, G. L. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 132–136
47. Keyser, R. M., and Snyder, S. H. (1991) *Science* **251**, 185–188
48. Stiles, G. L. (1992) *Science* **267**, 6451–6454
49. Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S., and Jakobs, K. H. (1985) *Biochem. Biophys. Res. Commun.* **126**, 213–218
50. Katada, T., Gilman, A. G., Watanabe, Y., Bauer, S., and Jakobs, K. H. (1985) *Biochem. Biophys. Res. Commun.* **126**, 213–218
51. Lefort, A., and Parmentier, M. (1989) *Science* **244**, 13483–13490
52. Sato, K., Okajima, F., Katada, T., and Kondo, Y. (1994) *Mol. Pharmacol.* **45**, 1036–1042
53. Libert, F., Lefort, A., Gerard, C., Parmentier, M., Perret, J., Ludgate, M., Dumont, J. E., and Vassart, G. (1989) *Biochem. Biophys. Res. Commun.* **163**, 1220–1225
54. Libert, F., Schiffmann, S. N., Lefort, A., Parmentier, M., Gerard, C., Dumont, J. E., Vanderhaeghen, J. J., and Vassart, G. (1991) *EMBO J.* **10**, 1677–1682
55. Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) *Mol. Cell. Biol.* **2**, 466–472
56. Okajima, F., and Kondo, Y. (1990) *J. Biol. Chem.* **265**, 21741–21748
57. Itoh, H., Kozasa, T., Nagata, S., Nakamura, S., Katafu, T., Ui, M., Iwai, S., Ohtsuka, K., Kawasaki, H., Suzuki, K., and Kaziro, Y. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83**, 3776–3780
58. Kozak, M. (1986) *Cell* **44**, 283–292
59. Itoh, H., Toyama, R., Kozasa, T., Tsukamoto, T., Matsuoka, M., and Kaziro, Y. (1988) *J. Biol. Chem.* **263**, 6656–6664
60. Andersson, S., Davis, D. L., Dahlback, H., Jornvall, H., and Russell, D. W. (1989) *J. Biol. Chem.* **264**, 8222–8229
61. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
62. Yamauchi, J., Kaziro, Y., and Itoh, H. (1995) *Bioche. Biophys. Res. Commun.* **214**, 694–700
63. Okajima, F., Katada, T., and Ui, M. (1985) *J. Biol. Chem.* **260**, 6761–6768
64. Okajima, F., Tokumitsu, Y., Kondo, Y., and Ui, M. (1987) *J. Biol. Chem.* **262**, 13483–13490
65. Sato, K., Okajima, F., Katada, T., and Kondo, Y. (1990) *Arch. Biochem. Biophys.* **281**, 298–304
66. Yasuda, K., Rens-Domiano, S., Breder, C. D., Law, S. F., Saper, C. B., Reisine, T., and Bell, G. I. (1992) *J. Biol. Chem.* **267**, 20422–20428