The three Gα subunits were independently depleted from rat pituitary GH4C1 cells by stable transfection of each Gα subunit antisense rat cDNA construct. Depletion of any Gα subunit eliminated receptor-induced inhibition of basal cAMP production, indicating that all Gα subunits are required for this response. By contrast, receptor-mediated inhibition of vasoactive intestinal peptide (VIP)-stimulated cAMP production was blocked by selective depletions for responses induced by the transfected serotonin 1A (5-HT1A) (Gαi3 or Gαbg) or endogenous muscarinic-M4 (Gαi1 or Gαbg) receptors. Strikingly, receptor activation in Gαi1-depleted clones (for the 5-HT1A receptor) or Gαi2-depleted clones (for the muscarinic receptor) induced a pertussis toxin-sensitive increase in basal cAMP production, whereas the inhibitory action on VIP-stimulated cAMP synthesis remained. Finally, in Gαi2-depleted clones, activation of 5-HT1A receptors increased VIP-stimulated cAMP synthesis. Thus, 5-HT1A and muscarinic-M4 receptor may couple dominantly to Gαi1 and Gαi3, respectively, to inhibit cAMP production. Upon removal of these Gα subunits to reduce inhibitory coupling, stimulatory receptor coupling is revealed that may involve Gβγ-induced activation of adenyl cyclase II, a Gαi-stimulated cyclase that is predominantly expressed in GH4C1 cells. Thus Gαi-coupled receptor activation involves integration of both inhibitory and stimulatory outputs that can be modulated by specific changes in Gαi subunit expression level.

Heterotrimeric G proteins transduce signals generated by hormone receptors with seven transmembrane domain α-helices to various effectors such as AC, phospholipase C, and ion channels (1–3). These G proteins are composed of a Gα subunit and a tight complex of Gβ and Gγ subunits. The binding of agonist to receptors allows them to interact with G proteins, which subsequently accelerates the rate of dissociation of GDP and the binding of GTP to the Gα subunits. Both GTP-ligated Gα subunits and Gβγ dimers then regulate downstream effector activities. The intrinsic GTPase activity of Gα subunits hydrolyzes GTP to GDP, which then associates with Gβγ dimers to inactivate the complex.

The cyclic AMP-forming enzyme, adenyl cyclase, is one of the ubiquitous effectors that is regulated by G protein-coupled receptors. The pathways of Gαi-coupled receptor-induced stimulation of AC have been well characterized (1, 4). However, the inhibitory regulation of AC by Gαi-coupled receptors is far less clear, and the underlying mechanisms seem to be rather complex. For example, in several cell systems, e.g. mouse Ltk-, Rat-1, and NIH-3T3 fibroblast cells, inhibition of cAMP synthesis by Gαi-coupled receptors was only observed when AC was activated by forskolin or Gαi-coupled receptors (5–7). On the other hand, in neuronal and endocrine cells, Gαi-coupled receptors inhibited both unstimulated and stimulated AC activity (2, 5, 8). In addition, some Gαi-coupled receptors have even been reported to stimulate AC, e.g. α2-adrenergic receptor in PC-12 pheochromocytoma cells (7, 9) or 5-HT1A receptors in hippocampus (10, 11).

The specificity of distinct G proteins in receptor-AC coupling remains incompletely understood. For example, using anti-Gαi1 subunit antibodies to block receptor coupling, it has been reported that inhibition of AC by αGαi1-adrenergic receptors in platelet membranes is mediated by Gαi3 (12) and that 5-HT1A receptor-inhibited induction of cAMP synthesis in HeLa cell membranes is preferentially mediated by Gαi3 (13). This approach, however, is limited to cell-free preparations and depends on the specificity of the antibodies used. To assess the roles in receptor coupling of particular G proteins in whole cells, transfection of PTX-insensitive Gαi mutant proteins has been used to rescue receptor-mediated signaling following PTX pretreatment. For example, the dopamine-D2S receptor appears to couple to Gαi2 and Gαi3 to mediate inhibition of forskolin and Gαi-stimulated AC, respectively (14, 15). This approach depends on the specificity and functionality of the Gαi mutants. We have used expression of antisense constructs to selectively deplete particular G proteins and assessed their contribution to receptor coupling (16–18).

The aim of the present study was to evaluate the contribution of the three known Gαi subunits (19) in relaying inhibitory signals from 5-HT1A and muscarinic M4 receptors to AC in intact GH4C1 rat pituitary cells. We analyzed Gαi protein sub-
type specificity in receptor-effector coupling by stably introducing distinct full-length rat Go3 antisense constructs into GH4ZD10 cells (GH4C1 cells transfected with the rat 5-HT1A receptor (5)). This approach produced a specific block of the gene expression of these proteins (18). Characterization of these different Go3-deficient antisense clones indicates that Go3 proteins specifically link receptors to inhibition of cAMP synthesis but not to closure of calcium channels and that the combined presence of all three Go3 subunits is essential for receptor-mediated inhibition of unstimulated but not of VIP receptor-stimulated cAMP synthesis. Strikingly, upon depletion of distinct Go3 subunits, the Gi4 coupled-5-HT1A and muscarinic M4 receptors switched to stimulate AC activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—5-HT, baclofen, VIP, PTK, and isobutylmethylxanthine were purchased from Sigma. Hygromycin B was from Calbiochem (La Jolla, CA). BakY-8644 was from Research Biochemicals Inc. (Natick, MA). Fura 2-AM was from Molecular Probes (Eugene, OR). PTK was from List Biological Laboratories (Campbell, CA). Rat G protein Go3, Go3, and Go3 subunits and AC type II cDNAs were gifts of Dr. R. Reed. G protein Go3 subunit antibodies were kindly donated by Dr. D. Manning.

**Cell Culture**—All cells were grown as monolayer in Ham’s F-10 medium with 8% fetal bovine serum at 37 °C in a humidified atmosphere with 5% CO2. Media were changed 12–24 h prior to experimentation.

**Preparations of the Antisense Clones**—The 2.0-kb EcoRI-EcoRI Go3 cDNA fragment, the 1.8-kb EcoRI-EcoRI Go3 cDNA fragment, or the 3.1-kb EcoRI-EcoRI Go3 cDNA fragment (13), containing the full coding sequences and 0.5 to 0.8 kb of 5’ and 3’ noncoding regions were excised using EcoRI. The cDNA fragments were ligated into pcDNA (Invitrogen) in the reverse orientation with respect to the cytomembrane promoter, resulting in Go3, Go3, or Go3 antisense expression vectors. The constructs were confirmed by restriction enzyme analysis and by DNA sequencing. A modified transfection procedure was used; 300–500 µg of each Go3 antisense construct was cotransfected separately with 30 µg of pY-3 hygromycin B resistance plasmid into G418-resistant GH4ZD10 cells by standard calcium phosphate co-precipitation protocol (20). The selection was initiated after 24 h by adding 150 µg/ml hygromycin B into the culture medium to select the clones with expression of the antisense RNAs and to allow the clones to adapt the cytotoxicity of hygromycin B. After 2 weeks, the colonies formed on hygromycin B plates were picked, solubilized, and total RNA was subjected to Northern analysis, using a pair of oligonucleotides specific for each G protein subunit antibody.

**Preparation of the Anti-Ga Go3 cDNA Constructs**—To specifically block the protein expression of distinct Gαi subunits, the Gi4 coupled-5-HT1A and muscarinic M4 receptors switched to stimulate AC activity.

**Assay Conditions**—Measurement of cAMP was performed as described previously (5). In brief, the cells were plated in six-well 35-mm dishes. After removal of the medium, the cells were preincubated in 2 ml of HBSS for 5–10 min at 37 °C. The buffer was replaced by 1 ml of HBSS containing 100 µg isobutylmethylxanthine, a cAMP phosphodiesterase inhibitor, and the incubation was continued for another 5 min. Then the various test compounds were added to the cells, and the incubation was allowed at room temperature for 20 min. The buffer was collected for cAMP assay using a specific radioimmunoassay (ICN) as described before (5).

**Results**

**Independent Depletion of Distinct Go3 Subunits from GH4ZD10 Cells**—Reverse transcription-PCR and Western blot analysis indicated the presence of all three known Go3 subunits in GH4C1 cells (Fig. 1), although Ga11 may be the least abundant based on the weakness of the signal. The three Ga3 subunits are highly homologous in their coding regions but exhibit a clear variability in their 5’- and 3’-noncoding sequences (19). To specifically block the protein expression of distinct Go3 subunits, the antisense constructs were chosen to include the full coding sequences and, in addition, 500–800 base pairs of the 5’- and 3’-untranslated sequences (18). Selection for stably transfected clones exhibiting the highest levels of antisense RNA expression was accomplished by raising temporarily the concentration of hygromycin B to 400 µg/ml. The expression of antisense RNA was detected by reverse transcription-PCR analysis, using a pair of oligonucleotides specific for each Go3 subunit (not shown). The extent of depletion of distinct Go3 subunits was verified by Western blot analysis, using antibodies specific for each Go3 subunit (22, 23). It was found that Go3 and Go3 subunits were virtually eliminated in clones Gi3ZD-3 and Gi3ZD-5 (Fig. 1A) and Gi3ZD-4 and Gi3ZD-5 (Fig. 1B), respectively, whereas Go3 subunits were largely depleted in clones Gi3ZD-3 and Gi3ZD-4 (Fig. 1C). To examine the extent of cross-hybridization of different Go3 antisense RNAs with...
Effects of elimination of different $G_i$ subunits on receptor-mediated inhibition of calcium entry.

Data are presented as the means ± S.D. of three independent experiments in which the actions of 5-HT (1 μM) and carbachol (10 μM) on the BayK 8644 (1 μM)-induced increase in [Ca$^{2+}$]i were measured in GH4ZD10 and various $G_i$ antisense clones as described under "Experimental Procedures." Values are expressed as fold basal level of [Ca$^{2+}$]i.

| Treatment | GH4ZD10 | G12ZD-3 | G12ZD-5 | G32ZD-5 |
|-----------|---------|---------|---------|---------|
| Basal     | 2.15 ± 0.06 | 2.15 ± 0.06 | 2.15 ± 0.06 | 2.15 ± 0.06 |
| 5-HT      | 3.12 ± 0.06 | 3.12 ± 0.06 | 3.12 ± 0.06 | 3.12 ± 0.06 |
| Carbachol | 3.12 ± 0.06 | 3.12 ± 0.06 | 3.12 ± 0.06 | 3.12 ± 0.06 |

* Significant difference ($p < 0.05$) compared with BayK 8644 alone.

### Table 1

| Treatment | GH4ZD10 | G12ZD-3 | G12ZD-5 | G32ZD-5 |
|-----------|---------|---------|---------|---------|
| Basal     | 2.15 ± 0.06 | 2.15 ± 0.06 | 2.15 ± 0.06 | 2.15 ± 0.06 |
| 5-HT      | 3.12 ± 0.06 | 3.12 ± 0.06 | 3.12 ± 0.06 | 3.12 ± 0.06 |
| Carbachol | 3.12 ± 0.06 | 3.12 ± 0.06 | 3.12 ± 0.06 | 3.12 ± 0.06 |

* Significant difference ($p < 0.05$) compared with BayK 8644 alone.
that of VIP. The 5-HT-induced stimulation of basal cAMP accumulation in these cells, an action almost as efficacious as
Surprisingly, 5-HT induced a 5-fold increase in basal cAMP and carbachol failed to inhibit basal cAMP production (Fig. 3)
Measurements of carbachol-induced inhibition of cAMP accumulation. This novel stimulatory action of 5-HT on basal cAMP formation was also observed in another Go_{i1} depleted clone, Gi1ZD-5 (Table II). In the same Go_{i1}-depleted cells, however, 5-HT inhibited VIP-stimulated cAMP accumulation by some 45%, similar to the results obtained in GH4ZD10 cells. These data indicate that the presence of Go_{i3} subunits is not essential for the 5-HT1A receptor-mediated inhibition of G_{i}-stimulated cAMP accumulation. In contrast to 5-HT1A receptor action, in both Gi1ZD-3 and Gi1ZD-5 clones, carbachol-induced inhibition of VIP-stimulated cAMP accumulation was blocked (Fig. 3A).

As observed in Go_{i1}-depleted clones, the ability of 5-HT1A and muscarinic M4 receptors to inhibit basal cAMP synthesis was also ablated in Go_{i3}-depleted clones, Gi2ZD-4 (Fig. 3B). In addition, both receptors failed to inhibit VIP-stimulated cAMP synthesis. Activation of 5-HT1A receptors potentiated slightly VIP-stimulated cAMP accumulation by 35%, an action sensitive to PTX treatment.

In Go_{i2}-depleted clone Gi3ZD-3, activation of 5-HT1A or muscarinic M4 receptors did not result in inhibition of basal cAMP accumulation (Fig. 3C). Curiously, carbachol induced a 3-fold increase in cAMP production in these antisense clones. This stimulation was prevented by PTX pretreatment of the cells. In the same Go_{i2}-depleted clones, however, the inhibitory action of muscarinic M4 receptors on VIP-stimulated cAMP synthesis remained largely unaltered. In Gi3ZD-4 cells, another Go_{i3} depleted clone, carbachol-induced inhibition of VIP-stimulated cAMP accumulation was essentially unaltered (Table II).

Subtypes of AC Expressed in GH4C1 Cells—The possible mechanism of G_{i}-mediated stimulation of cAMP production upon depletion of specific Go subunits was addressed. Certain AC subtypes (types II, IV, and VII) have been demonstrated biochemically to be conditionally stimulated by Gi/Go subunits.
In addition ACII is known to mediate Gi-induced stimulation of cAMP levels when co-transfected with specific Gi-coupled receptors, such as the α2-adrenergic receptor (26). We examined the RNA expression of the most extensively characterized subtypes, AC types I–VI (4), using reverse transcription-PCR analysis at different concentrations of cDNA (Table III). In rat brain each subtype was present, although type I was weakly expressed (25). The rank order of expression of adenylyl cyclases in GH4C1 cells was II = VI > III >> (I, IV, and V). Of particular interest was the predominant expression in GH4C1 cells of AC type II, which was detected as a major species of 6.7 kb by Northern blot analysis of poly(A)+ RNA from GH4C1 cells (Fig. 4). By contrast, AC type II RNA was undetectable in various fibroblast cell lines (Ltk− and Balb/c-3T3), adrenocortical Y1, DDT1-MF2 smooth muscle, or PC-12 pheochromocytoma cells (data not shown and Refs. 15 and 25). Thus, AC II is abundantly expressed in pituitary GH4C1 cells and brain tissue, permitting the possibility for receptor coupling to Gα-dependent stimulation of cAMP accumulation in these tissues.

**DISCUSSION**

Using stable transfection of distinct Gαi full-length antisense constructs, we were able to specifically deplete the protein expression of individual Gai subunits from GH4C1 pituitary cells. It was found that knocking out of any of the three Gai subunits specifically altered 5-HT1A and muscarinic M4 receptor-mediated inhibition of AC but not the receptor-induced inhibition of calcium entry. The latter was achieved by specific ablation of αi subunits from GH4C1 cells (17). These data confirm that GAi subunits mediate inhibition of AC but not closure of calcium channels. Similar specificities have been reported for the coupling of different receptors to voltage-dependent calcium channels by αiA or αiB proteins but not Gαi proteins in GH3 cells (27–29).

One of the important findings of the present study is that although the three different GAi subunits all participate in receptor-mediated inhibition of AC, each GAi subunit apparently plays a distinct role in this signal transduction process. First, the contemporaneous expression of all three GAi subunits appeared to be essential for both 5-HT1A and muscarinic M4 receptor-mediated inhibition of unstimulated cAMP synthesis (Table III). Depletion of any of the three GAi subunits led to the inability of both receptors to inhibit basal cAMP synthesis in GH4C1 cells. This is consistent with our previous observations in GH4C1 cells that inhibition of basal cAMP level by dopamine-D2S, dopamine-D2L, and somatostatin receptors was blocked upon depletion of GAi3 (17). In cell types in which two or fewer types of GAi subunits are expressed, such as fibroblast Rat-1, Chinese hamster ovary, or JEG-3 chorionicarcinoma cells, Gi-coupled receptors inhibit stimulated cAMP production but do not inhibit basal cAMP accumulation (5–7, 30, 31). This supports the hypothesis that Gi-coupled receptors require all three Gai subunits for inhibition of basal cAMP accumulation. Second, different receptors link to different Gai subunits to inhibit Gαi-stimulated CAMP synthesis. For example, 5-HT1A receptors couple to Gai2 and Gai3 subunits to suppress cAMP accumulation stimulated by the Gi-coupled VIP receptor, because depletion of either subunit blocks this response. By contrast, muscarinic M4 receptors apparently couple to Gai1 and Gai2 subunits for inhibition of VIP-stimulated cAMP formation. Interestingly, each receptor interacts with and activates more than one type of Gαi proteins to inhibit Gαi-stimulated CAMP synthesis. Using immunoprecipitation with specific G protein antibodies and cholera toxin-catalyzed labeling of Gi proteins, it has been shown that Gi-coupled receptors may simultaneously activate more than one Gi protein and that different receptors apparently exhibit different preferences to different G proteins (6, 7, 31, 32).

A third major conclusion to be drawn from the data presented is that a receptor may dominantly link to different Gi subunits depending on whether Gs is engaged in stimulation of AC. For example, depletion of Gai1 and Gai2 subunits led to 5-HT1A and muscarinic M4 receptors, respectively, to stimulate basal CAMP synthesis. These depletions had no effect on inhibition of VIP-stimulated AC activity by the same receptors.
These results suggest that different G proteins regulate inhibition of basal (primarily G\(_i\)) and G\(_\text{S}\)-stimulated (primarily G\(_\text{S}\)) cAMP synthesis by the 5-HT1A receptor in GH4C1 cells. Results from transfections of PTX-insensitive G proteins in Ltk\(^{-}\) cells indicate that the D2S receptor couples to G\(_i\) to inhibit forskolin-induced cAMP accumulation but uses G\(_\text{S}\) to inhibit G\(_i\)-stimulated action (15). Taken together, these results are consistent with a state-dependent inhibition of adenylyl cyclase by specific G\(_i\) subunits.

A surprising finding of the present study was the efficient stimulation of basal cAMP synthesis in G\(_i\)- and G\(_\text{S}\)-depleted antisense clones by agonist-activated 5-HT1A and muscarinic M4 receptors, respectively. The weaker stimulation by muscarinic compared with 5-HT1A receptors (3-fold versus 5-fold basal cAMP) may reflect the less complete depletion of G\(_\text{S}\) compared with G\(_i\), or weaker efficacy of the muscarinic receptor for this signaling pathway. This stimulation of cAMP synthesis by 5-HT or carbococh was sensitive to PTX treatment, suggesting the involvement of the remaining G\(_i\)/G\(_\text{S}\) proteins to induce direct stimulation of AC. Functional analysis of AC enzymes I–VI indicates that all of them are stimulated by G\(_i\), and some of them (types I, V, and VI) have been shown to be inhibited by G\(_\text{S}\) proteins (4, 33–36). Interestingly, specific G\(_i\)/G\(_\text{S}\) dimer combinations potentiate G\(_i\) stimulation of AC types II, IV, and VII while either inhibiting (type I) or having no effect on the other isoenzymes (36–38). In cotransfection experiments, G\(_i\)-coupled receptors have been shown to potentiate G\(_i\) or protein kinase C-stimulated cAMP synthesis by AC type II, apparently by release of G\(_i\)/G\(_\text{S}\) dimers (26, 39). Our observations indicate that in the presence of multiple G\(_i\)/G\(_\text{S}\) subunits and AC subtypes, receptor-mediated inhibition is the dominant pathway. However, upon depletion of particular G\(_i\)/G\(_\text{S}\) subunits, the remaining PTX-sensitive G proteins stimulate cAMP levels. Because GH4C1 cells appear to express AC type II, the enhancement of cAMP by these receptors could be mediated by conditional activation of AC type II. We have identified G\(_i\) as the major G protein that mediates coupling of the 5-HT1A receptor to AC type II upon cotransfection in HEK-293 cells (40). These results suggest that upon depletion of G\(_i\) in GH4C1 cells, G\(_i\)/G\(_\text{S}\) subunits associated with G\(_i\) mediate positive coupling to AC type II resulting in enhanced cAMP production. Distinct G protein specificities of 5-HT1A or muscarinic M4 receptors to enhance cAMP levels may reflect the association of specific G protein complexes with each receptor. For example coupling of somatostatin and muscarinic M4 receptors to 

### Stimulation of Adenylyl Cyclase by G\(_i\)-coupled Receptors

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