0026-895X/92/010006-04$03.00/0

MOLECULAR PHARMACOLOGY, 42:6-9

ACCELERATED COMMUNICATION

Selective Interaction of β_2- and α_2-Adrenergic Receptors with Stimulatory and Inhibitory Guanine Nucleotide-Binding Proteins

YOSHIO OGINO, CLAIRE M. FRASER, and TOMMASO COSTA

Laboratory of Theoretical and Physical Biology, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland 20892 (Y.O., T.C.), and Section on Molecular Neurobiology, Laboratory of Neurogenetics, National Institute on Alcohol Abuse and Alcoholism, Rockville, Maryland 20852 (C.M.F.)

Received February 27, 1992; Accepted April 27, 1992

SUMMARY

In Chinese hamster ovary cells expressing recombinant β_2-adrenergic receptors, isoproterenol enhanced cholera toxin-catalyzed ADP-ribosylation of the large form of $G_{s\alpha}$. The effect was stereoselectively blocked by the enantiomers of propranolol, indicating receptor mediation. The ADP-ribosylated form of G_s α-subunit was resolved into a triplet in gradient gels. β_2-Adrenergic receptors increased both the labeling and the apparent mass of the slower migrating forms of large $G_{s\alpha}$, as determined by autoradiography and immunoblotting, suggesting that $G_{s\alpha}$ can incorporate more than one ADP-ribose per molecule. In cells coexpressing similar amounts of β_2-adrenergic, α_2-adrenergic, and m1 muscarinic receptors, β_2 receptors stimulated the ADP-ribosylation of only large G_s and α_2 receptors that of only G_i; muscarinic receptors had no apparent effect. Thus, in native membranes there appears to be a selectivity for the interaction between adrenergic receptor subtypes and $G_{s\alpha}$ or $G_{i\alpha}$ subunits.

It is known that cholera toxin, the multimeric toxin of *Vibrio cholerae*, preferentially catalyzes the incorporation of ADP-ribose into G_s (1, 2), the G protein mediating stimulation of adenylyl cyclase (3). It is also known, however, that, under favorable experimental conditions, cholera toxin can ADP-ribosylate other G proteins, such as transducin (4) and G_i or G_o (5, 6), which are preferential substrates of pertussis toxin. Cholera toxin-mediated ADP-ribosylation of the G_i/G_o-type of G proteins is considerably potentiated by agonist occupation of G_i/G_o-coupled receptors (7–9). Thus, agonist-mediated enhancement of cholera toxin-catalyzed ADP-ribosylation of G protein represents a means for demonstrating receptor-G_i/G_o coupling in isolated membrane preparations.

A similar effect of receptor occupation on cholera toxin-mediated labeling of the α-subunit of G_s has not been described. On the contrary, agonist occupation of the β-adrenergic receptor in pigeon erythrocytes has been shown to reduce the labeling of G_s in the presence of GTPγS (10). Because erythrocytes contain only the faster migrating form of G_s, it is not clear whether the lack of observable receptor effect on labeling is related to this type of membrane and G_s subtype or reflects a mechanistic difference in the way that receptors interact with G_i- and G_s-type G proteins.

In this study, we have utilized transfected CHO cell lines, constitutively expressing the human β_2-adrenergic receptor, to show that receptor stimulation results in enhancement of cholera toxin-induced labeling of the endogenous G_s in isolated membranes. Furthermore, in cell lines expressing both α_2- and β_2-adrenergic receptors, we have demonstrated a selective interaction of these receptors with inhibitory and stimulatory G proteins, respectively.

Materials and Methods

Culture of transfected cells. Transfected CHO cell lines expressing β_2-adrenergic (β_2-CHO) (1.2 pmol of receptor/mg of membrane protein) (11), α_2-adrenergic (α_2-CHO) (1.2 pmol of receptor/mg) (12), and ml muscarinic receptors (ml-CHO) (1.5 pmol of receptor/mg) (13) were obtained as previously described (11–13). Another cell line, expressing all three receptors at ~1 pmol of receptor/mg of membrane protein (ABM-CHO), was obtained by transfection of CHO cells with a mixture of the pSVL expression vector containing the genes encoding the human β_2-adrenergic (11), human α_2-adrenergic (12), and rat ml muscarinic acetylcholine (13) receptors plus pMSV*neo* (14), at a ratio of 1:1:1:0.2, using the calcium phosphate precipitation technique (14). Stable transfectants were obtained as described (15).

Cells were grown in a mixture of Ham's F-12 and Dulbecco's modified Eagle's medium (1:1), containing 10% fetal calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 100 μg/ml Geneticin, in a humidified atmosphere of 5% CO_2 in air. Cells were harvested after incubation of the monolayers in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, containing 1 mM EDTA, and were stored frozen at −70° before preparation of membranes.

Toxin-mediated ADP ribosylation. Membranes from CHO cell lines were prepared as described previously (12). The reaction for cholera toxin-mediated ADP ribosylation was carried out in a buffer containing 100 mM potassium phosphate, pH 7.4, 10 mM thymidine, 10 mM arginine, 2.5 mM $MgCl_2$, 1 mM ATP, 1 mM dithiothreitol, 100 μg/ml bovine serum albumin, and 2–5 μCi of [α-^{32}P]NAD (0.8–1 Ci/μmol); NEN, DuPont). Each reaction contained 100 μg/ml cholera

ABBREVIATIONS: G_s, stimulatory guanine nucleotide-binding protein; G_i, inhibitory guanine nucleotide-binding protein; G_o, guanine nucleotide binding protein other than G_s or G_i; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; CHO, Chinese hamster ovary; ARF, ADP-ribosylation factor; G protein, guanine nucleotide-binding protein.

toxin A promoter (List Biologicals, Campbell, CA), 50–100 μg of membrane protein, and receptor ligands, as shown, in a total volume of 50 μl. Samples were incubated for 20 min at 37°, and reactions were stopped by the addition 0.5 ml of ice-cold 50 mM Tris·HCl, pH 7.8. Tubes were centrifuged (45,000 × *g*, 10 min at 4°), and the membrane pellets were resuspended in sample buffer, heated at 95° for 5 min, and applied to polyacrylamide gels. Proteins were separated on slab gels (14 × 16 cm; 1.5-mm thickness) with a stacking gel of 3 cm and a resolving gel containing a linear gradient from 8 to 12% acrylamide. The sodium dodecyl sulfate-containing buffer system of Laemmli (16) was used for electrophoresis. Separations were conducted at 4°, at constant current (60 mA), for 4–5 hr. After electrophoresis, the gels were dried and exposed to Hyperfilm-βmax (Amersham).

Immunoblot analysis of G_s. Proteins were electrotransferred to Immobilon membranes (Millipore) that were processed for Western blotting as described previously (9). The characteristics of the carboxyl terminus-directed anti-G_s antibody (RM) have been reported previously (17, 18). The antibody preparation used in this study was an affinity-purified immunoglobulin (17, 18) donated by Dr. W. F. Simonds (NIDDK, NIH) and was used in Western blotting at a concentration of 1 μg/ml.

Results

Incubation of membranes from β_2-CHO cells with cholera toxin A_1 fragment and [^{32}P]NAD resulted in the labeling of two major forms of G_s. The faster migrating band (42 kDa) displayed markedly lower levels of labeling, whereas a slower migrating form, usually resolved into two or three bands (45–48 kDa), was more prominently labeled (Fig. 1). Addition of isoproterenol to the reaction (10 μM) resulted in a marked increase in the labeling of the high molecular mass species of G_s (Fig. 1). The effect of isoproterenol was blocked by *l*-propranolol but not *d*-propranolol (both at 10 μM), indicating that the effect is stereoselective and mediated via activation of β-adrenergic receptors (Fig. 1). In some experiments, isoproterenol also elicited a slight increase in the labeling of the 42-kDa form of G_s. In contrast, addition of GTP alone stimulated the labeling of both the 42-kDa band and the 45–48-kDa triplets. Under these conditions, the addition of isoproterenol produced little or no further enhancement of the amount of [^{32}P]ADP-ribose incorporated into the 45–48-kDa species of G_s, indicating lack of additivity between agonist and guanine nucleotide. Similar effects were also observed upon addition of GDP (data not shown).

In order to determine whether the three bands in the 45–48-kDa range that were ADP-ribosylated by cholera toxin in the presence of isoproterenol were distinct forms of G_s, membranes from β_2-CHO cells were ADP-ribosylated in the presence or absence of isoproterenol, using increasing concentrations of NAD (obtained by increasing the proportion of unlabeled NAD in the reaction). The labeled proteins were separated by electrophoresis and allowed to react with an anti-G_s specific antibody (17, 18), and the blots were both developed with an alkaline phosphatase-labeled second antibody, to reveal the immunoreactive bands, and subjected to autoradiography, to reveal the radioactive proteins. At very low concentrations of NAD, a prominent 45-kDa immunoreactive species was the main band present in the 45–48-kDa region of the immunoblots, whereas a minor 46-kDa band was barely detectable (Fig. 2, *bottom*). As the concentration of NAD increased, both the 46-kDa species and an additional 48-kDa band were progressively stained by the antibody, so that at 1 mM NAD the 45–48-kDa triplet, commonly seen in the autoradiograms, also became immunoreactive. Because the anti-G_s antibody is carboxyl terminus-directed, its interaction with $G_{s\alpha}$ is presumably not affected by ADP-ribosylation, which occurs at Arg^{201} (19). Thus, the NAD dependence of the appearance of the 45–48-kDa triplets in immunoblots suggests that the various forms of large G_s result from an altered electrophoretic mobility, due to the incorporation of multiple ADP-ribose molecules into the α-subunit, rather than the ADP-ribosylation of multiple forms of G_s. As indicated by the immunoblots, the threshold concentration of NAD at which the immunoreactive triplet becomes detectable was clearly reduced in the presence of agonist. At a saturating concentration of NAD (1 mM), the difference in ADP-ribosylation of $G_{s\alpha}$ in the absence or presence of isoproterenol was no longer perceptible (Fig. 2, *bottom*), whereas this difference was most evident at a tracer concentration of NAD (Fig. 2, *top*). These findings suggest that isoproterenol enhances ADP-ribosylation of $G_{s\alpha}$ by decreasing the K_m for NAD.

To examine the specificity of receptor-induced enhancement of ADP-ribosylation of G_s, we studied CHO cells expressing different types of receptors. In a cell line expressing α_2-adre-

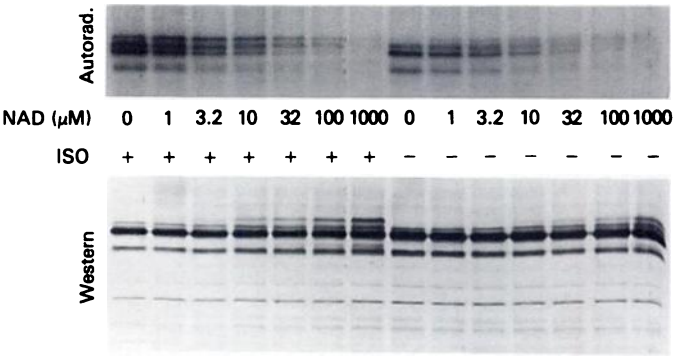

Fig. 2. Comparison between radioactivity and anti-G_s immunoreactivity of protein bands ADP-ribosylated by cholera toxin. Membranes from β_2-CHO cells were ribosylated using 2 μCi of [^{32}P]NAD and increasing concentrations of NAD as shown, either in the presence or in the absence of 10 μM *l*-isoproterenol (*ISO*). After sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the labeled proteins were transferred onto an Immobilon membrane and stained for anti-G_s immunoreactivity by using an alkaline phosphatase colorimetric procedure (see Materials and Methods). The Immobilon membrane was then dried and exposed to β-max films for autoradiography. *Top*, autoradiogram. *Bottom*, immunoblot. Note that in the autoradiogram the 45–48-kDa triplet is best seen at the lowest concentration of NAD, where the specific radioactivity is highest. In contrast, in the Western blot it is most evident at high concentrations of NAD, because sufficient amounts of protein have to be ADP-ribosylated in order to be detectable with the antibody. The appearance of the highest molecular weight band of the triplet occurs at noticeably lower concentrations of NAD in the presence of isoproterenol than in its absence.

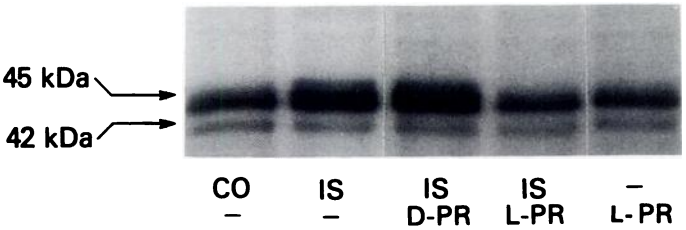

Fig. 1. Effect of β_2-adrenergic receptors on cholera toxin-catalyzed ADP-ribosylation of G_s. Membranes were ADP-ribosylated in the absence or presence of β_2-adrenergic receptor ligands as indicated. *CO*, control; *IS*, isoproterenol, 10 μM; *D-PR*, *d*-propranolol, 10 μM; *L-PR*, *l*-propranolol, 10 μM. The molecular size of the labeled bands is indicated.

nergic receptors (α_2-CHO cells), a similar pattern of cholera toxin labeling was observed in the absence of agonist as was observed with cells expressing β_2 receptors. However, addition of epinephrine did not alter the labeling of G_s but selectively enhanced the incorporation of radioactivity into a 40-kDa band, which corresponds to the mobility of a pertussis toxin-sensitive substrate. In CHO cells expressing m1 muscarinic receptors, the addition of carbachol had no effect on the pattern of ADP-ribosylation induced by cholera toxin (data not shown). These results were not unexpected, because the muscarinic receptor couples to phosphoinositide hydrolysis in these cells, and other transfected CHO cell lines (20), via a pertussis toxin-insensitive G protein.

We also examined a cell line in which ml muscarinic and β_2- and α_2-adrenergic receptors were constitutively coexpressed at comparable densities (ABM-CHO). Addition of epinephrine resulted in the increased labeling not only of the 45–48-kDa triplet, as observed for β_2-CHO cells, but also of the 40-kDa G_i band, as detected in α_2-CHO cells (Fig. 3). In the presence of RX 821002, a selective imidazoline antagonist of α_2-adrenergic receptors, the epinephrine-induced enhancement of labeling of the 40-kDa band was suppressed, but that of the 45–48-kDa triplet was not; instead, this was selectively blocked by propranolol. In the presence of both antagonists, neither the labeling of the 40-kDa band nor that of the 45–48-kDa triplet was enhanced by epinephrine (Fig. 3). RX 821002 and propranolol had no effect on incorporation of ADP-ribose in the absence of agonist. Thus, these data suggest a selectivity for the interaction between different types of adrenergic receptors and G proteins coexisting in the same membrane; β receptors stimulate only the labeling of G_s and α_2 receptors only that of G_i. Also in ABM-CHO cells, the muscarinic agonist carbachol had no effect on cholera toxin-catalyzed ADP ribosylation (Fig. 3), suggesting that the G protein mediating phosphoinositide turnover in CHO cells is a poor substrate for cholera toxin.

Discussion

In cells expressing β_2-adrenergic receptors, we have shown that cholera toxin-catalyzed ADP-ribosylation of $G_{s\alpha}$ is potentiated in the presence of agonists. Thus, the ability to change the sensitivity of the α-subunits to the enzymatic effect of this toxin is not a characteristic limited to receptors that interact with pertussis toxin substrates. Agonist-mediated enhancement of G_s labeling was clearly mediated by occupation of β_2-adrenergic receptors; (a) it was blocked only by the active enantiomer of the antagonist, propranolol, which, by itself, did not alter the reaction catalyzed by cholera toxin; (b) it did not occur in cells transfected with ml muscarinic receptors; and (c) it was selectively blocked by propranolol in cells in which the nonselective agonist epinephrine enhanced ribosylation of both G_i and G_s via α_2- and β_2-adrenergic receptors, respectively.

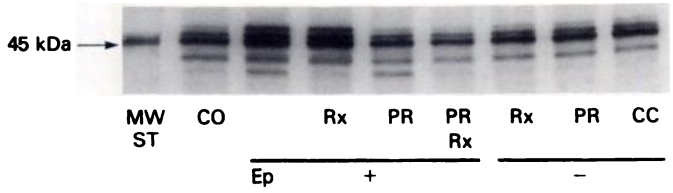

Fig. 3. Specificity of β_2-adrenergic receptors in enhacing cholera toxin effect on G_s. Membranes from a CHO cell line expressing β_2-adrenergic, α_2-adrenergic, and m1 muscarinic receptors (ABM-CHO) were ADP-ribosylated in the presence of the indicated agonists and antagonists. *CO*, control; *Ep*, epinephrine, 10 μM; *Rx*, RX 821002, 10 μM; PR, *l*-propranolol, 10 μM; *CC*, carbachol, 1 mM. The 45,000 molecular weight standard (MW ST) is indicated.

Receptor-mediated enhancement of cholera toxin-catalyzed modification of the α-subunit of the G protein can be used as a measure of the selectivity of coupling between distinct types of receptors and G proteins. In reconstituted systems, β-adrenergic receptors can activate not only G_s but also G_i subtypes and transducin, with differences in relative potency that are significant but not sufficient to indicate absolute specificity of interaction (21, 22). Here we find that the specificity of receptor-G protein interaction is much higher than expected from reconstitution experiments. In cells expressing β_2-adrenergic receptors, only the labeling of G_s was stimulated by agonists, whereas ADP-ribosylation of the 40-kDa G_i band was enhanced only in cells expressing α_2-adrenergic receptors. Labeling of both proteins was enhanced in cells where both receptors were present, whereas neither was stimulated in the same cell line when the m1 muscarinic receptor was selectively activated by carbachol.

This specificity of interaction in native membranes is relevant to the interpretation of the biphasic effect of recombinant α_2-adrenergic receptors on cAMP levels in transfected cells (12). In these cells, agonists at low concentrations produce a pertussis toxin-sensitive attenuation of forskolin-stimulated cAMP accumulation and at higher concentrations produce a pertussis toxin-insensitive potentiation of cAMP levels. One possible interpretation of these results is that α_2 receptors, when present at high density in the membrane, may cross-react with G_s and stimulate adenylyl cyclase. The data presented here, however, do not support the hypothesis of cross-reactivity and suggest that α_2-adrenergic receptors may increase cAMP levels by a mechanism involving an as yet unknown effector system.

Besides specificity for G_s, we also found a differential effect of β_2-adrenergic receptor stimulation on the ADP-ribosylation of the 'large' and 'small' forms of α_s-subunits; in all experiments, the slower migrating bands were those more clearly enhanced in the presence of adrenergic agonist. Yet, there are at least two reasons to doubt the conclusion that these data indicate preferential coupling of β_2-adrenoceptors to a particular subtype of G_s. First, as indicated by immunoblots, CHO cells express much more of the large than the small forms of G_s; therefore, the phenomenon may merely reflect this stoichiometric condition, rather than an intrinsic difference in affinity. Second, several studies based on the expression of recombinant long and short splice variants of $G_{s\alpha}$ did not show important differences in the way in which these two proteins interact with adenylyl cyclase and receptors (22–24). Nonetheless, the question of whether different subtypes of β-adrenergic receptors might have different affinities for distinct subtypes of G_s expressed in native membranes has not been addressed.

The large form of G_s, particularly when its ribosylation was enhanced by agonist binding to the β receptor, was resolved into a triplet by gradient gels. Similar anomalies in the migration of the high molecular mass form of G_s have been noted previously (10). Immunoblots of membranes ADP-ribosylated with increasing concentrations of NAD indicated that the appearance of the immunoreactive triplet was related to the concentration of NAD. Because antibody binding to G_s is not

affected by the presence or absence of ADP-ribose on the α-subunit, cholera toxin and NAD must be acting to increase the amount of G_s forms with slower mobility on sodium dodecyl sulfate-polyacrylamide gels. It is unlikely that the three bands represent pre-existing multiple forms of large G_s that are ribosylated with different efficiencies by cholera toxin, because all three forms should be immunoreactive, regardless of whether ADP-ribose has been covalently incorporated. These findings suggest, instead, that there may be multiple sites of ADP-ribosylation on G_s. Thus, upon stimulation with the agonist, a larger proportion of G_s carrying more than one ADP-ribose per molecule becomes detectable. In line with this idea is the observation of Landis *et al.* (25) that α_s mutated at Arg^{201} (the putative site of cholera toxin-catalyzed ADP-ribosylation) can still incorporate ADP-ribose, albeit less efficiently. Although the idea that α_s contains additional secondary acceptor sites for ADP-ribose is not unlikely, this hypothesis requires direct biochemical proof.

The addition of guanine nucleotides to the reaction stimulated the labeling of both the 42-kDa band and the 45–48-kDa triplet, to an extent similar to that observed in the presence of isoproterenol, and obscured any further effect of the agonist. Because a guanine nucleotide is usually included in the reaction catalyzed by cholera toxin, this may explain why stimulation by G_s-coupled receptors has not been noted before. Both GDP and GTP stimulated the labeling of G_s, but this lack of difference between nucleotide di- and triphosphate may be due to reaction conditions, rather than being indicative of a specific mechanism. In fact, in the presence of Mg^{2+} and ATP, and at high concentrations of membranes, transphosphorylation reactions can easily generate GTP from GDP. Additional studies are needed to clarify this point. The site of action of guanine nucleotides on cholera toxin-induced ADP ribosylation is believed to be not the α-subunit of G_s but a 21-kDa protein (ARF) (26, 27) that interacts directly with the A_1 fragment of cholera toxin (28). If the effect of GTP reported here is mediated via ARF, the observation that agonist stimulation mimics, but is not synergistic with, that of guanine nucleotides suggests that ARF-like proteins may somehow be involved in the interaction between β-adrenergic receptors and G_s.

Acknowledgments

We thank Dr. W. F. Simonds and Dr. A. M. Spiegel for the generous gift of the RM antibody and Dr. David Rodbard for support and encouragement during this study.

References

1. Cassel, D., and Z. Selinger. Mechanism of adenylate cyclase activation by cholera toxin: inhibition of GTP hydrolysis at the regulatory site. *Proc. Natl. Acad. Sci. USA* **74**:3307–3311 (1977).
2. Gill, D. M., and R. Meren. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **75**:3050–3054 (1978).
3. Gilman, A. G. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* **56**:615–649 (1987).
4. Abood, M. E., J. B. Hurley, M. C. Pappone, H. R. Bourne, and L. Stryer. Functional homogeneity between signal coupling proteins: cholera toxin inactivates the GTPase activity of transducin. *J. Biol. Chem.* **257**:10540–10543 (1982).
5. Graves, C. B., N. B. Klaven, and J. M. MacDonald. Effects of guanine nucleotides on cholera toxin-catalyzed ADP-ribosylation in rat adipocyte plasma membranes. *Biochemistry* **22**:6291–6296 (1983).
6. Owens, J. R., L. T. Frame, M. Ui, and D. M. Cooper. Cholera toxin ADP-ribosylates the islet-activating protein substrate in adipocyte membranes and alters its function. *J. Biol. Chem.* **260**:15946–15952 (1985).
7. Gierschick, P., and K. H. Jakobs. Receptor-mediated ADP-ribosylation of a phospholipase C-stimulating G protein. *FEBS Lett.* **224**:219–223 (1987).
8. Milligan, G., and F. R. McKenzie. Opioid peptides promote cholera toxin-catalysed ADP-ribosylation of the inhibitory guanine nucleotide-binding protein in membranes of neuroblastoma × glioma hybrid cells. *Biochem. J.* **252**:369–373 (1988).
9. Klinz, F.-J., and T. Costa. Cholera toxin ADP-ribosylates the receptor-coupled form of pertussis toxin-sensitive G proteins. *Biochem. Biophys. Res. Commun.* **165**:554–560 (1989).
10. Gill, M. D., and M. J. Woolkalis. Cholera toxin-catalyzed [^{32}P]ADP-ribosylation of proteins. *Methods Enzymol.* **195**:267–280 (1991).
11. Owicki, J. C., J. W. Parce, K. M. Kersco, G. B. Sigal, V. C. Muir, J. C. Venter, C. M. Fraser, and H. M. McConnell. Continuous monitoring of receptor-mediated changes in the metabolic rates of living cells. *Proc. Natl. Acad. Sci. USA* **87**:4007–4011 (1990).
12. Fraser, C. M., S. Arakawa, W. R. McCombie, and J. C. Venter. Cloning, sequence analysis, and permanent expression of a human α_2-adrenergic receptor in Chinese hamster ovary cells. *J. Biol. Chem.* **264**:11754–11761 (1989).
13. Fraser, C. M., C.-D. Wang, D. A. Robinson, J. D. Gocayne, and J. C. Venter. Site-directed mutagenesis of m1 muscarinic receptors: conserved aspartic acids play important roles in receptor function. *Mol. Pharmacol.* **36**:840–847 (1989).
14. Graham, F., and A. van der Eb. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* **52**:456–467 (1973).
15. Muzzin, P., J.-P. Revelli, F. Kuhne, J. D. Gocayne, W. R. McCombie, J. C. Venter, J.-P. Giacobino, and C. M. Fraser. A novel adipocyte β-adrenergic receptor: molecular cloning and down-regulation in obesity. *J. Biol. Chem.* **266**:24053–24058 (1991).
16. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.)* **227**:680–685 (1970).
17. Simonds, W. F., P. K. Goldsmith, C. J. Woodard, C. G. Unson, and A. M. Spiegel. Receptor and effector interactions of G_s: functional studies with antibodies to the α_s carboxyl-terminal peptide. *FEBS Lett.* **249**:189–194 (1989).
18. Simonds, W. F., P. Goldsmith, C. Woodard, C. Unson, and A. Spiegel. G_{i2} mediates α_2-adrenergic inhibition of adenylyl cyclase in platelet membranes: *in situ* identification with G_α C-terminal antibodies. *Proc. Natl. Acad. Sci. USA* **86**:7809–7813 (1989).
19. Van Dop, C., M. Tsubokawa, H. R. Bourne, and J. Ramachandran. Amino acid sequences of retinal transducin at the site ADP-ribosylated by cholera toxin. *J. Biol. Chem.* **259**:696–698 (1984).
20. Buck, M. A., and C. M. Fraser. Muscarinic acetylcholine receptor subtypes which selectively couple to phospholipase C: pharmacological and biochemical properties. *Biochem. Biophys. Res. Commun.* **173**:666–672 (1990).
21. Cerione, R. A., C. Staniszewski, J. L. Benovic, R. J. Lefkowitz, M. G. Caron, P. Gierschik, R. Somers, A. M. Spiegel, J. Codina, and L. Birnbaumer. Specificity of the functional interactions of the β-adrenergic receptor and rhodopsin with guanine nucleotide regulatory proteins reconstituted in phospholipid vesicles. *J. Biol. Chem.* **260**:1493–1500 (1985).
22. Rubenstein, L. C., M. E. Linder, and E. M. Ross. Selectivity of the β-adrenergic receptor among G_s, G_i's, and G_o: assay using recombinant α-subunits in reconstituted phospholipid vesicles. *Biochemistry* **30**:10769–10777 (1991).
23. O'Donnell, J. K., R. W. Sweet, and J. M. Stadel. Expression and characterization of the long and short splice variant of $G_{s\alpha}$ in S49 *cyc*$^-$ cells. *Mol. Pharmacol.* **39**:702–710 (1991).
24. Freissmuth, M., E. Selzer, S. Marullo, W. Schütz, and A. D. Strosberg. Expression of two human β-adrenergic receptors in *Escherichia coli*: functional interaction with two forms of the stimulatory G protein. *Proc. Natl. Acad. Sci. USA* **88**:8548–8552 (1991).
25. Landis, C. A., S. B. Masters, A. Spada, A. M. Pace, H. R. Bourne, and L. Vallar. GTPase inhibiting mutations activate the α chain of G_s and stimulate adenylyl cyclase in human pituitary tumors. *Nature (Lond.)* **340**:692–696 (1989).
26. Enomoto, K., and D. M. Gill. Cholera toxin activation of adenylate cyclase. *J. Biol. Chem.* **255**:1252–1258 (1980).
27. Kahn, R. A., and A. G. Gilman. Purification of a protein cofactor required for the ADP-ribosylation of the stimulatory regulatory component of adenylate cyclase by cholera toxin. *J. Biol. Chem.* **259**:6228–6234 (1984).
28. Bobak, D. A., M. M. Bliziotes, M. Noda, S. C. Tsai, R. Adamik, and J. Moss. Mechanism of activation of cholera toxin by ADP-ribosylation factor (ARF): both low and high affinity interaction of ARF with guanine nucleotides promote toxin activation. *Biochemistry* **29**:855–861 (1990).

Send reprint requests to: Claire M. Fraser, Section on Molecular Neurobiology, Laboratory of Neurogenetics, NIAAA, 12501 Washington Ave., Rockville, MD 20852.