0026-895X/91/050707-05$03.00/0

MOLECULAR PHARMACOLOGY, 40:707-711

Multiple G_i Protein Subtypes Regulate a Single Effector Mechanism

MARK A. GERHARDT and RICHARD R. NEUBIG

Departments of Pharmacology (M.A.G., R.R.N.) and Internal Medicine (Hypertension Division) (R.R.N.), The University of Michigan Medical School, Ann Arbor, Michigan 48109-0626

Received April 23, 1991; Accepted July 29, 1991

SUMMARY

α_2-Adrenergic receptor (α_2-AR) responses are mediated by the pertussis toxin-sensitive guanine nucleotide-binding protein (G protein) G_i. Because all three known G_i subtypes are inactivated by pertussis toxin, it has been difficult to determine which of the subtypes are involved in α_2-AR responses. In order to investigate α_2-AR/G_i coupling, we performed binding and adenylyl cyclase experiments in membranes from CHO-K1 cells transfected with the human α_{2A}-AR. Antisera directed against the carboxyl-terminal region of the G_{i1}/G_{i2} or the G_{i3} proteins were used to determine which subtypes were important for high affinity agonist binding and inhibition of adenylyl cyclase. The CHO-K1 cell membranes exhibited immunoreactivity at an apparent molecular mass of 40–41 kDa for both G_{i1}/G_{i2} and G_{i3} antisera. Western blot analysis, using purified bovine brain G proteins for comparison, demonstrated that the transfected CHO-K1 cells possess G_{i2} and G_{i3}. High affinity guanosine 5′-(β,γ-imido) triphosphate-sensitive binding of the α_2-AR agonists [^{3}H]bromoxidine and *p*-[^{125}I]iodoclonidine ([^{125}I]PIC) was reduced by 30–50% by either the G_{i1}/G_{i2} or G_{i3} antiserum. Bromoxidine (1 μM) and PIC (1 μM) inhibited membrane adenylyl cyclase by 34 and 27%, respectively. G_{i3} antiserum reduced the inhibition by 26% and 67% for bromoxidine and PIC, respectively. The G_{i1}/G_{i2} antiserum reduced the inhibition by 56% and 63% for bromoxidine and PIC, respectively. Furthermore, when both antisera were used together, there was a complete reversal of α_2-AR-mediated inhibition. These observations provide evidence of α_{2A}-AR coupling to at least two subtypes of G_i proteins and the first evidence of functional involvement of G_{i3} in the inhibition of adenylyl cyclase.

G proteins are heterotrimeric proteins, with $\alpha\beta\gamma$ subunits, that transduce signals from receptors to effector proteins (1). At the present time, >80 receptors have been reported to couple to G proteins (1). The initial classification of G proteins was based on their ability to stimulate or inhibit adenylyl cyclase. G proteins that stimulate adenylyl cyclase were termed G_s, whereas those that inhibited cAMP formation were termed G_i. Advances in cloning techniques have led to the discovery of a large family of G proteins. There are three known subtypes of G_i (based on sequence differences of the α subunit), termed G_{i1}, G_{i2}, and G_{i3}, all of which serve as substrates for ADP-ribosylation by PTX. PTX also ribosylates a major brain G protein (G_o). The functional significance of the G_i subtypes is not fully understood, but G_{i2} has been shown to be required for inhibition of adenylyl cyclase in human platelet membranes (2), δ-opioid receptor stimulation of GTPase in NG108 cells (3), and agonist binding to the α_{2B}-AR in NG108 cells (4). All three subtypes of recombinant G_i can activate K^+ channels (5) in reconstitution assays, but it is not known whether multiple G_i proteins couple to adenylyl cyclase or other signaling pathways.

The α_2-AR has been used as a model to study G_i-receptor interactions and generation of second messengers (6, 7). Inhibition of adenylyl cyclase is the most well known α_2-AR mechanism, but the α_2-AR has been reported to activate other signal transduction systems (8). Three subtypes of the α_2-AR have been described pharmacologically and structurally (9), but the distribution and function of α_2-AR subtypes are not completely understood.

This study tests the hypothesis that multiple G_i proteins are coupled to the α_{2A}-AR. We used G_i subtype-specific antisera in binding and adenylyl cyclase assays to examine coupling to the receptor. By performing the experiments in transfected cells that do not express endogenous α_2-AR, we can unambiguously assign coupling to the α_{2A}-AR subtype. We report here that the α_{2A}-AR does couple to more than one G_i protein, and we provide the first evidence that the G_{i3} protein participates in the attenuation of adenylyl cyclase.

This work was supported by National Institutes of Health Grant GM39561, funds from the Office of the Vice President for Research at the University of Michigan, and a Rackham Dissertation/Thesis Grant. M.A.G. is supported by Grant GM97863 and a Hartford Foundation Geriatric Fellowship. R.R.N. is an American Heart Association/Genentech Inc. Established Investigator.

ABBREVIATIONS: G protein, guanine nucleotide-binding protein; AR, adrenergic receptor(s); bromoxidine, UK14,304 [5-bromo-6-*N*-(2,4,5-dihydroimidazoyl)quinoxaline]; G_i, "inhibitory" guanine nucleotide-binding protein; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate; PIC, *p*-iodoclonidine; PTX, pertussis toxin; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

Materials and Methods

[^{125}I]PIC, [^{3}H]bromoxidine, [^{3}H]cAMP, and the specific antisera against G_{i1}/G_{i2} (AS/7) and G_{i3} (EC/2) were obtained from Dupont/NEN. PIC was purchased from Research Biochemicals, Inc. Bromoxidine was a gift from Pfizer. [α-^{32}P]ATP and ^{125}I-labeled donkey anti-rabbit antibody were purchased from Amersham. All other reagents and materials were obtained from standard suppliers.

Cell culture. The MAG-2 cell line was derived from CHO-K1 cells by transfection with PSVα2-neo (10) and selection of stable clones with G418. MAG-2 cells possess about 1–2 pmol of α_2 receptor/mg of membrane protein (11).[1] When confluent, cell membranes were prepared as described by Huang *et al.* (10), except that membranes were pelleted at 100,000 × *g* and resuspended in either TME (50 mM Tris, 10 mM $MgCl_2$, 1 mM EGTA) or TE (50 mM Tris, 10 mM $MgCl_2$) buffer at pH 7.5.

Western blots. MAG-2 membrane proteins were separated on a 10% (Fig. 1A) or 11% (Fig. 1B) sodiumdodecylsulfate-polyacrylamide gel. Proteins were transferred to Immobilon, and a Western blot was performed with G protein antisera at 1/1000 dilution. In Fig. 1A, incubation with biotin-IgG complex and ExtrAvidin preceded visualization by dye precipitation (nitro-blue tetrazolium and 5-bromo-4-chloro-3-indoyl phosphate). In Fig. 1B, the Western blots were incubated with 1 μCi of ^{125}I anti-rabbit antibody. The autoradiograms were exposed at −70°.

Binding and functional assays. Binding and adenylyl cyclase assays were performed as described (12), except that membranes were incubated at room temperature for 45 min with buffer alone, with G_i antisera, or with nonimmune rabbit serum, before the assay was started. Incubations with serum were at 1/100 dilution unless otherwise specified. The protein concentration during incubation with antisera ranged from 0.04 to 0.16 mg/ml.

Statistical analysis. *p* values were calculated from paired, one-tailed, *t* tests of the results with specific serum compared with a nonimmune serum control. Data are expressed as mean ± standard error unless otherwise indicated.

Results

In order to determine which G_i subtypes were present in MAG-2 cells, we performed a Western blot with the G_i-specific antisera (Fig. 1A). The α subunits of G_{i1} and G_{i3} are 41-kDa proteins, whereas that of G_{i2} is a 40-kDa protein (1). Specific immunoreactivity was defined as bands that were detected with the G_i antisera but not with the nonimmune rabbit serum. Specific immunoreactivity was detected at the 40–41-kDa level with both G_{i3} and G_{i1}/G_{i2} antisera. Nonimmune rabbit serum did not reveal any immunoreactivity at 40–41 kDa (data not shown). A comparison of the immunoreactivity in MAG-2 cells with that of purified bovine brain G_o/G_i permitted a more definitive identification of the G_i proteins. Bovine brain G_o/G_i contains primarily G_{i1} (41 kDa) and G_o (39 kDa). The immunoreactivity of MAG-2 membrane proteins, when probed with the G_{i1}/G_{i2} antiserum, appeared as a single band at the 40-kDa level (Fig. 1B, *top*). The immunoreactivity of bovine brain G_o/G_i had a higher molecular weight, which would correspond to G_{i1}. When probed with the G_{i3} antiserum, MAG-2 membranes exhibited a single band of immunoreactivity at 41 kDa, but there was no staining at the 39-kDa level (Fig. 1B, *bottom*). The G_{i3} antiserum did weakly react with the 39-kDa G_o protein of bovine brain (Fig. 1B, *bottom*). Thus, the transfected CHO-K1 cells (MAG-2 cells) contain primarily G_{i2} and G_{i3}.

In order to discern which G_i subtypes couple to the α_{2A}-AR,

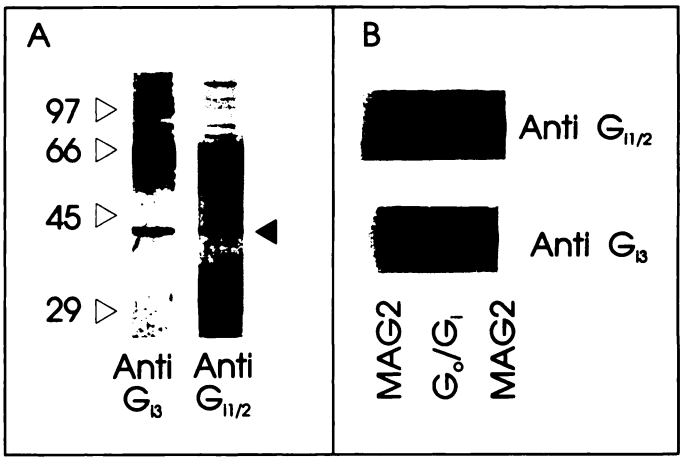

Fig. 1. Western blot of G proteins in MAG-2 membranes. A, MAG-2 membrane proteins (75 μg/lane) were separated on a 10% sodium dodecyl sulfate-polyacrylamide gel. After transfer to Immobilon-P, the blot was probed with antisera (1/1000 dilution) directed against the carboxyl-terminal region of G_{i3} α subunit or the G_{i1}/G_{i2} α subunits. *Open arrows*, molecular mass markers, in kDa. *Solid arrow*, G_i α subunits (40–41 kDa). Nonimmune serum did not react with the 40–41-kDa band but did show immunoreactivity to the other bands seen (not shown). B, MAG-2 membrane proteins (35 μg/lane) or purified bovine brain G_o/G_i proteins (0.1 μg/lane) were separated on an 11% sodium dodecyl sulfate-polyacrylamide gel and probed as described for A.

we tested the effects of antisera on binding of the α_2-AR full agonist [^{3}H]bromoxidine and the α_2-AR partial agonist [^{125}I]PIC (Fig. 2). When compared with the nonimmune control, specific [^{3}H]bromoxidine binding was decreased 25% by G_{i1}/G_{i2} antiserum, G_{i3} antiserum, or both antisera together. Gpp(NH)p (10 μM) reduced binding by 49%. The effect of G_{i3} antiserum, G_{i1}/G_{i2} antiserum, or both antisera used together was statistically significant, compared with the nonimmune control ($p = 0.002$, 0.03, and 0.01, respectively). Binding experiments performed with [^{125}I]PIC produced similar results (Fig. 2). When compared with nonimmune control, specific [^{125}I]PIC binding was decreased 16–27% by G_{i1}/G_{i2} antiserum, G_{i3} antiserum, or the combination of antisera. Again, Gpp(NH)p (10 μM) reduced binding by 62%. Statistically, the results were similar to those described above. G_{i3} antiserum treatment was different from the nonimmune control ($p < 0.0001$), as was treatment with both antisera ($p = 0.03$). G_{i1}/G_{i2} antiserum treatment did not achieve a statistical difference. Agonist binding in MAG-2 cells is only partially inhibited by Gpp(NH)p (65% versus 90% in platelet membranes) (12). Because we would not expect antisera to affect the Gpp(NH)p-insensitive component of agonist binding, it is appropriate to express the effects of antisera as a percentage of the Gpp(NH)p-sensitive binding. When compared with nonimmune control, G_{i3} antiserum abolished 50% and 31% of Gpp(NH)p-sensitive binding for [^{3}H]bromoxidine and [^{125}I]PIC, respectively. For G_{i1}/G_{i2} antiserum, the respective values were 53% and 49% and, for both antisera together, 52% and 51%.

The ability of G_i to couple to the α_{2A}-AR in a binding assay does not necessarily mean that those G_i proteins are utilized in a functional response. Reduction of the α_{2A}-AR-mediated inhibition of adenylyl cyclase by the G_{i1}/G_{i2} antiserum and the G_{i3} antiserum is shown in Fig. 3. Forskolin (10 μM) routinely stimulated cAMP formation 10-fold over basal, and the antisera had little if any effect on forskolin-stimulated adenylyl cyclase (data not shown).

[1] The details of the preparation and characterization of MAG-2 cells will be published elsewhere.

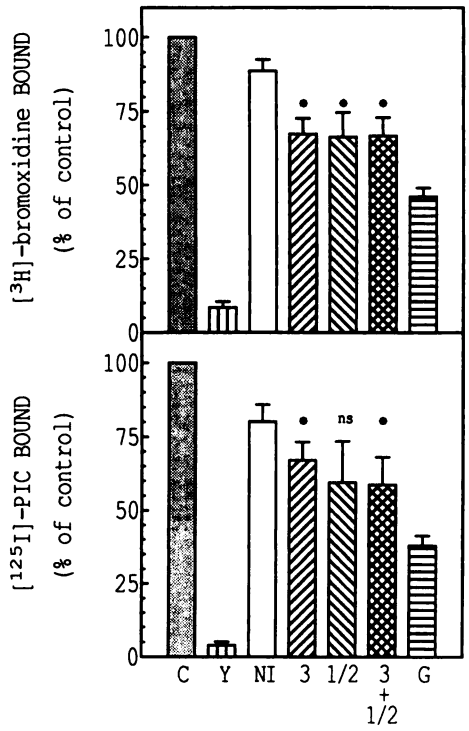

Fig. 2. Effects of $G_i\alpha$-specific antisera on α_2-AR agonist binding. MAG-2 membranes were incubated for 45 min at room temperature with buffer (*C*), 10 μM yohimbine (*Y*), 1/100 nonimmune serum (*NI*), 1/100 G_{i3} antiserum (*3*), 1/100 G_{i1}/G_{i2} antiserum (*½*), 1/100 G_{i1}/G_{i2} plus 1/100 G_{i3} antisera (*3 + ½*), or 10 μM Gpp(NH)p (*G*). The binding assay was initiated by the addition of 1 nM [^{3}H]bromoxidine (*top*) or 1 nM [^{125}I]PIC (*bottom*). Data for [^{3}H]bromoxidine represent six experiments, except G_{i1}/G_{i2} plus G_{i3}, which is from three experiments. Data for [^{125}I]PIC represent four experiments, except G_{i1}/G_{i2} plus G_{i3}, which is from three experiments. Data are presented as the mean ± standard error of experiments performed in triplicate. *, Statistically significant difference from the nonimmune control ($p < 0.05$); *ns*, no statistically significant difference ($p > 0.05$).

The full agonist bromoxidine inhibited forskolin-stimulated cAMP formation slightly but consistently more than did the partial agonist PIC (34 ± 3% versus 27 ± 3%), under these assay conditions. Both G_{i1}/G_{i2} and G_{i3} antisera blocked α_{2A}-AR-mediated inhibition. For bromoxidine (Fig. 3, *left*), the percentage of inhibition was reduced from 34 ± 3% in control and 31 ± 3% with nonimmune serum to 25 ± 5% with G_{i3} antiserum, 15 ± 3% with G_{i1}/G_{i2} antiserum, 9 ± 4% with twice as much G_{i1}/G_{i2} antiserum (1/50 dilution), and 0 ± 3% with both antisera. Statistically, both G_{i3} antiserum ($p = 0.04$) and G_{i1}/G_{i2} antiserum ($p = 0.0006$) treatments were different from the nonimmune control. The effect of G_{i3} antiserum was additive to that of G_{i1}/G_{i2}, in that addition of both antisera showed significantly greater inhibition than G_{i1}/G_{i2} antiserum alone ($p = 0.002$).

For PIC (Fig. 3, *right*), the percentage of inhibition was reduced from 27 ± 3% (control) and 28 ± 7% with nonimmune serum to 9 ± 7% with G_{i3} antiserum, 10 ± 6% with G_{i1}/G_{i2} antiserum, and −8 ± 8% with both antisera. Statistically, α_2-AR-mediated inhibition of adenylyl cyclase in the presence of either G_{i1}/G_{i2} or G_{i3} antisera was significantly less than for the nonimmune control ($p = 0.03$). The effect of addition of G_{i3} antiserum with G_{i1}/G_{i2} antiserum was significantly greater ($p = 0.03$) than that of G_{i1}/G_{i2} antiserum alone.

Discussion

This study tests the hypothesis that multiple G_i proteins are coupled to the α_{2A}-AR. Antisera directed against specific G_i protein subtypes were evaluated in both binding and functional assays. The data demonstrate that at least two G_i subtypes, G_{i3} and G_{i2}, are involved in high affinity agonist binding and inhibition of adenylyl cyclase. The ability of PTX to disrupt the binding and functional activities of the α_2-AR is well known (13). However, PTX has at least four substrates (G_{i1}, G_{i2}, G_{i3}, and G_o), and it is not clear which of these proteins are coupled to the α_2-AR. This study demonstrates that the α_{2A}-AR interacts with at least two of the PTX substrates, G_{i2} and G_{i3}. Simonds *et al.* (2) proposed a role for G_{i2} in the α_2-AR-mediated inhibition of adenylyl cyclase in platelets, and our results show evidence for a similar role for G_{i2} in transfected Chinese hamster ovary cells. Birnbaumer and co-workers (5, 14) have reported that all three subtypes of G_i stimulate K^+ channels. This study is the first to describe a role for G_{i3} in inhibition of adenylyl cyclase in any system.

Western blot analysis of the MAG-2 cell membranes demonstrates the presence of G_{i3} and G_{i2}. Only a single band of immunoreactivity was detected on Western blot, at the 41-kDa level, with the G_{i3} antiserum.[2] The distinct molecular weight of the G_{i1} from bovine brain G_o/G_i demonstrates that G_{i1} is not detected in MAG-2 cells by the G_{i1}/G_{i2} (AS/7) antiserum. It is not surprising that G_{i1} was not detected in MAG-2 membranes. G_{i1} has a very limited distribution, primarily in neural tissue,

[2] The immunoreactive bands detected by the G_{i1}/G_{i2} and the G_{i3} antisera could not be resolved into a clear doublet in our hands, but the staining by G_{i3} antiserum consistently ran higher than the staining by the anti-G_{i1}/G_{i2} antiserum (data not shown).

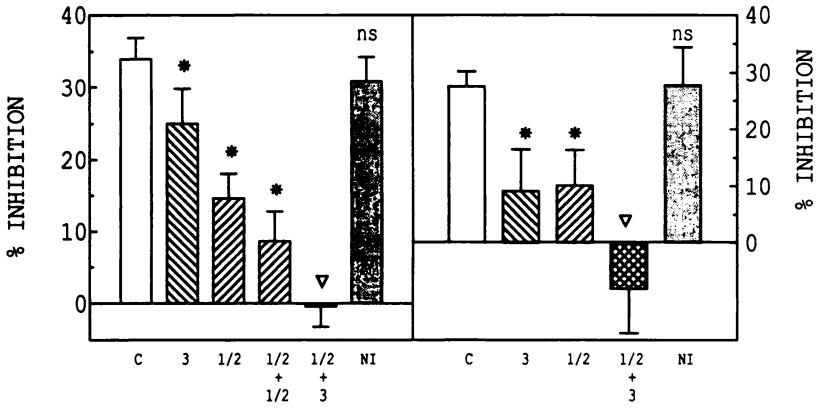

Fig. 3. Effects of G_i antisera on inhibition of adenylyl cyclase. MAG-2 membranes were incubated for 45 min with vehicle (*C*), 1/100 G_{i3} antiserum (*3*), 1/100 G_{i1}/G_{i2} antiserum (*½*), 1/50 G_{i1}/G_{i2} antiserum (*½ + ½*), 1/100 G_{i1}/G_{i2} plus 1/100 G_{i3} antisera (*½ + 3*), or 1/100 nonimmune serum (*NI*). The assay was initiated by the addition of reaction cocktail, forskolin, and 1 μM bromoxidine (*left*) or 1 μM PIC (*right*). Data are presented as mean ± standard error. *Left*, data represent three to five experiments; *right*, data represent three experiments. Individual experiments were performed in duplicate or triplicate. *, Statistically significant difference from nonimmune control ($p < 0.05$); *ns*, no statistically significant difference ($p > 0.05$). Nonimmune treatment was compared with control (*C*). ▽, Significant difference, compared with the result with G_{i1}/G_{i2} alone.

whereas G_{i2} is nearly ubiquitously distributed in tissues (1). Therefore, the G_{i1}/G_{i2} antiserum effects on high affinity binding and adenylyl cyclase can be attributed to G_{i2}.

The role of G_i subtypes has been examined previously by the use of specific carboxyl-terminal anti-G_i antisera. Spiegel, Milligan, and co-workers (2–4) reported that antiserum to G_{i2} blocks 1) inhibition of adenylyl cyclase in human platelet membranes (2), 2) stimulation of high affinity GTPase by the δ-opioid receptor in NG108 cells (3), and 3) high affinity norepinephrine binding to the α_{2B}-AR in NG108 cells (4). In the platelet studies (2), the anti-G_{i2} antiserum blocked 51% of the α_2-AR-mediated adenylyl cyclase inhibition, but the G_{i3} antiserum did not have any effect. The authors did not test the G_{i3} and G_{i1}/G_{i2} antisera in combination. A combination may have resulted in complete inhibition of the α_2 response, as observed here (Fig. 3). McClue and Milligan (4) used a different antibody (13B) against G_{i3} in studies of norepinephrine binding to the α_{2B} subtype of the α_2-AR in NG 108-15 cells. Also, they used an indirect method (agonist competition for [^{3}H]yohimbine binding), which is not as sensitive as direct agonist binding (15). The differences in cell and antibody type and assay methods may account for the differences between the results of McClue and Milligan and those reported here.

It is extremely unlikely that cross-reactivity of the G_{i3} antibody with G_{i2} accounts for our results. Although cross-reactivity between the anti-G_{i3} (EC) antiserum and G_{i2} has been reported (2), our functional data are inconsistent with an effect of the anti-G_{i3} antiserum solely on G_{i2}. A striking and consistent observation has been the complete block of adenylyl cyclase inhibition by the combination of anti-G_{i1}/G_{i2} and anti-G_{i3} antisera. The published effect of the G_{i1}/G_{i2} antisera on platelet adenylyl cyclase inhibition was never complete (51% block; range, 29–69%) (2). However, in seven different experiments, the combination of anti-G_{i1}/G_{i2} and anti-G_{i3} antisera resulted in complete block of adenylyl cyclase inhibition. Because the effects of 1/100 G_{i1}/G_{i2} plus 1/100 G_{i3} antisera on adenylyl cyclase inhibition are additive (Fig. 3, *left*) and are greater than the effects of simply doubling the G_{i1}/G_{i2} antiserum concentration (to 1/50), it can be concluded that cross-reactivity could not account for our observations. Thus, G_{i3}, as well as G_{i2}, couples to the α_{2A}-AR and participates in inhibition of adenylyl cyclase.

The role of G protein subtypes in different cell systems is likely to depend on a variety of factors, including the relative amounts of the subtypes in that cell or tissue, the existence of receptor reserve, the efficiency of receptor/G protein coupling and the ability of that G protein subtype to activate the effector system. We do not understand why the α_{2A}-AR in human platelets does not appear to couple to G_{i3} in addition to G_{i2}, whereas the human α_{2A}-AR couples to both G_{i3} and G_{i2} in MAG-2 cells. One possibility is that spare receptors exist in the MAG-2 system. We have reported that few if any spare receptors are present for inhibition of adenylyl cyclase in human platelet membranes (6). The receptor content in MAG-2 cells (~1000 fmol/mg) is not markedly higher than that in platelets (~500 fmol/mg) (12, 16). However, it is possible that the receptor/G protein coupling is more efficient in the MAG-2 system, which allows detection of a role for G_{i3} in the binding and adenylyl cyclase assays. This is suggested by the lower IC_{50} for bromoxidine [about 20 nM for MAG-2 cells (11) versus 1.9 μM for platelets (12)]. These possibilities will be examined in a future study.

α_2-AR agonists bind preferentially to the high affinity state of the receptor, and our laboratory has reported that this is a functionally relevant conformation (6). Because only 65% of the agonist binding in MAG-2 cells is sensitive to Gpp(NH)p, the maximum effect of antiserum on binding might be expected to be 65%. When expressed as a fraction of the Gpp(NH)p-sensitive agonist binding, 30–50% of the binding is abolished in the presence of antisera.

It is intriguing that the antisera had greater effects on the adenylyl cyclase response than in the binding assay. Two mechanisms could account for this observation. First, the percentage of the G protein population that must be affected to influence binding may be greater than that required to perturb the inhibition of adenylyl cyclase. In functional responses, receptors activate multiple G proteins (17); thus, 5–10 G proteins may be required for each receptor to elicit a maximum response. Reducing the amount of functional G_i protein from a 10-fold to a 2-fold excess over receptor may have significant effects on responses. In contrast, the high affinity receptor state is a stoichiometric reaction of receptor and G protein, so the G protein complement must be reduced to a substoichiometric level to inhibit binding. Thus, inactivation of any given fraction of the G_i population might have a more pronounced effect in the functional assay than in binding. Second, it is possible that, when bound to the G protein, the antisera may sterically hinder G protein interaction with the effector protein, in addition to their effects on receptor/G_i coupling. However, it is unlikely that the antisera directly block the binding site.

For the α_2-AR, at least three proteins are involved in signal transduction, i.e., receptor, G protein, and effector. This study demonstrates that the α_{2A}-AR can couple to both the G_{i2} and G_{i3} proteins when inhibiting adenylyl cyclase.[3] It remains to be determined whether these proteins are utilized in other α_2-AR-mediated cellular responses or whether they couple to the α_{2B} and α_{2C} subtypes of the α_2-AR. We provide here the first evidence that G_{i3} can participate in the attenuation of adenylyl cyclase.

Acknowledgments

We would like to thank Drs. C. Strader and R. Dixon at Merck, Sharp, and Dohme for kindly providing the human α_{2A}-AR clone and Guim Kwon and Dr. Ann Remmers for the purified bovine brain G_o/G_i. We would also like to thank Dr. D. Ginsberg of the University of Michigan for his assistance in preparing Fig. 1 and Sue Lyons and Tim Angelotti for help in performing the transformations and transfections.

References

1. Birnbaumer, L. G proteins in signal transduction. *Annu. Rev. Pharmacol. Toxicol.* **30**:675–705 (1990).
2. Simonds, W. F., P. K. Goldsmith, J. Codina, C. G. Unson, and A. M. Spiegel. G_{i2} mediates α_2-adrenergic inhibition of adenylate cyclase in platelet membranes: *in situ* identification with G_α C-terminal antibodies. *Proc. Natl. Acad. Sci. USA* **86**:7809–7813 (1989).
3. McKenzie, F. R., E. C. H. Kelly, C. G. Unson, A. M. Spiegel, and G. Milligan. Antibodies which recognized the C-terminus of the inhibitory guanine nucleotide binding protein (G_i) demonstrate that opioid peptides and foetal-calf serum stimulate the high-affinity GTPase activity of two separate pertussis-toxin substrates. *Biochem. J.* **249**:653–659 (1988).
4. McClue, S. J., and G. Milligan. The α_{2B} adrenergic receptor of undifferentiated neuroblastoma × glioma hybrid NG108-15 cells interacts directly with guanine nucleotide binding protein, G_{i2}. *FEBS Lett.* **269**:430–434 (1990).
5. Yatani, A., R. Mattera, J. Codina, R. Graf, K. Okabe, E. Padrell, R. Iyengar, A. M. Brown, and L. Birnbaumer. The G protein-gated atrial K^+ channel is

[3] Immediately before submission of this manuscript, Milligan *et al.* (18) published a paper providing evidence of the α_{2A}-AR coupling to G_{i2} and G_{i3} in Rat-1 fibroblasts. Those authors did not examine adenylyl cyclase responses.

stimulated by three distinct G_i α-subunits. *Nature (Lond.)* **336**:680–682 (1988).
6. Thomsen, W. J., J. A. Jacquez, and R. R. Neubig. Inhibition of adenylate cyclase is mediated by the high affinity conformation of the α_2-adrenergic receptor. *Mol. Pharmacol.* **34**:814–822 (1988).
7. Neubig, R. R., R. D. Gantzos, and W. J. Thomsen. Mechanism of agonist and antagonist binding to α_2 adrenergic receptors: evidence for a precoupled receptor-guanine nucleotide protein complex. *Biochemistry* **27**:2374–2384 (1988).
8. Limbird, L. E. Receptors linked to inhibition of adenylate cyclase: additional signaling mechanisms. *FASEB J.* **2**:2686–2695 (1988).
9. Bylund, D. B. Subtypes of α_2-adrenoceptors: pharmacological and molecular biological evidence converge. *Trends Pharmacol. Sci.* **9**:356–361 (1988).
10. Huang, R.-R. C., R. N. Dehaven, A. H. Cheung, R. E. Diehl, R. A. F. Dixon, and C. D. Strader. Identification of allosteric antagonists of receptor-guanine nucleotide-binding protein interactions. *Mol. Pharmacol.* **37**:304–310 (1990).
11. Gerhardt, M. A., and R. R. Neubig. α_{2A}-adrenergic receptors (α_2-AR) efficiently inhibit adenylate cyclase in transfected CHO-K1 cells. *FASEB J.* **5**:A1594 (1991).
12. Gerhardt, M. A., S. M. Wade, and R. R. Neubig. *p*-[^{125}I]Iodoclonidine is a partial agonist at the α_2-adrenergic receptor. *Mol. Pharmacol.* **38**:214–221 (1990).
13. Ui, M., and T. Katada. Bacterial toxins as probes for receptor-G_i coupling. *Adv. Second Messenger Phosphoprotein Res.* **24**:63–69 (1990).
14. Mattera, R., A. Yatani, G. E. Kirsch, R. Graf, K. Okabe, J. Olate, J. Codina, A. M. Brown, and L. Birnbaumer. Recombinant α_{i3} subunit of G-protein activates G_k-gated K^+ channels. *J. Biol. Chem.* **264**:465–471 (1989).
15. Graeser, C. G., and R. R. Neubig. Methods for the study of receptor/G-protein interactions, in *G Protein-Mediated Signal Transduction* (G. Milligan, ed.). IRL Press, Oxford, UK (in press).
16. Neubig, R. R., R. D. Gantzos, and R. S. Brasier. Agonist and antagonist binding to α_2-adrenergic receptors in purified membranes from human platelets: implications of receptor-inhibitory nucleotide binding protein stoichiometry. *Mol. Pharmacol.* **28**:475–486 (1985).
17. Pedersen, S. E., and E. M. Ross. Functional reconstitution of β-adrenergic receptors and the stimulatory GTP-binding protein of adenylate cyclase. *Proc. Natl. Acad. Sci. USA* **79**:7228–7232 (1982).
18. Milligan, G., C. Carr, G. W. Gould, I. Mullaney, and B. E. Lavan. Agonist-dependent, cholera toxin-catalyzed ADP-ribosylation of pertussis toxin-sensitive G-proteins following transfection of the human α_2-C10 adrenergic receptor in Rat 1 fibroblasts. *J. Biol. Chem.* **266**:6447–6455 (1991).

Send reprint requests to: Dr. Richard Neubig, M6322 Medical Science Building 1, Department of Pharmacology, University of Michigan, Ann Arbor, MI 48109-0626.