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Molecular Interaction of the Human α_2-C10-Adrenergic Receptor, When Expressed in Rat-1 Fibroblasts, with Multiple Pertussis Toxin-Sensitive Guanine Nucleotide-Binding Proteins: Studies with Site-Directed Antisera

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SUMMARY

DNA encoding the human α_2-C-10-adrenergic receptor was transfected into Rat-1 fibroblasts by $CaPO_4$ precipitation, and clones expressing the receptor were isolated and expanded. One clone (1C) expressing high levels of the receptor was studied in order to determine the contacts between this receptor and guanine nucleotide-binding proteins (G proteins) mediating second messenger signaling. The α_2-adrenergic agonist UK14304 stimulated high affinity GTPase activity in membranes from these cells. Incubation of these membranes with Protein A-purified fractions from an antiserum able to identify the carboxyl-terminal decapeptide common to $G_i1\alpha$ and $G_i2\alpha$ was partially able to prevent agonist stimulation of high affinity GTPase activity. Similar results were produced with an antiserum that identifies the carboxyl-terminal decapeptide of $G_i3\alpha$. In contrast, equivalent fractions of antisera that identify the carboxyl-terminal decapeptides of $G_o\alpha$ and $G_s\alpha$ did not inhibit receptor stimulation of high affinity GTPase activity. Coincubation of the membranes from the cells with Protein A-purified fractions from the anti-$G_i1\alpha$+$G_i2\alpha$ antiserum and the anti-$G_i3\alpha$ antiserum produced greater inhibition of UK14304-stimulated GTPase activity than did either of the two antisera in isolation. These data show direct interaction of the human α_2-C10-adrenergic receptor, when expressed in this clone of Rat-1 fibroblasts, with multiple pertussis toxin-sensitive G proteins and demonstrate that a single receptor has the physical capacity to interact functionally with more than a single pertussis toxin-sensitive G protein in a native membrane. Furthermore, because the two antisera were able to inhibit receptor stimulation of high affinity GTPase activity to similar degrees, the G protein pools identified by these antisera must contribute similar amounts of the total receptor activation of pertussis toxin-sensitive G proteins in these cells.

α_2-Adrenergic receptors are one of the most extensively studied members of the family of G protein-coupled receptors (1). Although all α_2-adrenergic receptors can act to mediate inhibition of adenylyl cyclase, it is now clear that a variety of pharmacologically and genetically distinct forms of this receptor exist (1, 2). Indeed, at least four distinct cDNA clones have been isolated that correspond to subtypes of α_2-adrenergic receptors (2–6), and at least three forms of the α_2-adrenergic receptor have been well characterized on a pharmacological basis. Detailed characterization of the individual gene products can be achieved by the stable expression of these proteins in cell lines that do not normally produce α_2-adrenergic responses. We have recently expressed the α_2-C10-adrenergic receptor in Rat-1 fibroblasts (7) and demonstrated that activation of the receptor in one of the clones isolated produced not only inhibition of adenylyl cyclase but also an agonist-activated stimulation of phospholipase D-mediated hydrolysis of phosphatidylcholine (8). Both of these receptor responses were abolished by pretreatment of the cells with pertussis toxin (8). This observation indicates that the two effector systems are regulated by one or more pertussis toxin-sensitive G proteins. In this report, we analyze the functional interactions of this defined receptor with pertussis toxin-sensitive G proteins, in this transfected clone of the Rat-1 fibroblast cell line, by using anti-G protein antisera of defined specificity to interfere with the transmission of information between receptor and G protein (9–12). These antisera are directed against the extreme carboxyl terminus of the α subunit of the various G proteins. This region of the G protein is known to be important for the coupling of receptors and G proteins (13–15). We demonstrate that in this cell line the expressed α_2-C10 receptor is able to interact with, and to activate, multiple pertussis toxin-sensitive G proteins.

Experimental Procedures

Materials

Reagents were obtained from the following sources. [^{3}H]Yohimbine (80 Ci/mmol) and [γ-^{32}P]GTP (>10 Ci/mmol) were obtained from

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Amersham International and [^{32}P]NAD$^+$ (800 Ci/mmol) from Dupont/New England Nuclear. Pertussis toxin was from Porton Products (Porton Down, Wiltshire, UK). All materials for tissue culture were from GIBCO/BRL.

Methods

Expression of recombinant DNA encoding the α_2-C10-adrenergic receptor. Stable expression of genomic DNA corresponding to the human platelet α_2-C10-adrenergic receptor (3) (clone, HPalpha2 GEN; American Type Culture Collection) was obtained using the mammalian expression vector pDOL (16), as described previously (7, 17). One specific clone was analyzed in detail in this study. Rat-1 α_{2A} clone 1C expressed high levels of the α_2-C10 receptor, as assessed by the specific binding of the α_2 receptor antagonist [^{3}H]yohimbine (see Results). Parental Rat-1 cells showed no specific [^{3}H]yohimbine binding (results not shown).

Cell culture. Cells of clone 1C were grown in Dulbecco's modified Eagles medium supplemented with 10% donor calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, in 5% CO_2 at 37°. Cells were grown in 75-cm^2 tissue culture flasks and were harvested just before confluency. In a number of cases, cells were treated with pertussis toxin (25 ng/ml) for 16 hr before cell harvest.

Membranes were prepared from the cells by homogenization with a Teflon-glass homogenizer and differential centrifugation, as described for a variety of other cells (18).

Pertussis toxin-catalyzed [^{32}P]ADP-ribosylation. [^{32}P]ADP-ribosylation of membranes of cells of clone 1C and of neuroblastoma × glioma hybrid NG108-15 was performed basically as in Ref. 19. Pertussis toxin was present in the assay at 10 μg/ml. Dried gels were autoradiographed for 48 hr, using Kodak X-O-Mat X-ray film.

Immunological studies. The generation and specificities of the various antisera used in this study are defined in Table 1. Each antiserum was produced in a New Zealand White rabbit, using a conjugate of a synthetic peptide and keyhole limpet hemocyanin (Calbiochem) as antigen. The details of this process have previously been recorded in detail (9). Immunoblotting of membrane samples was performed as in Ref. 10.

Generation of IgG fractions from anti-G protein antisera. Crude antisera were chromatographed on Protein A-Sepharose (Pharmacia). After elution of the IgG fractions at pH 4.0, the samples were restored to pH 7.0 and dialyzed overnight against 1000 volumes of 10 mM Tris·HCl, 0.1 mM EDTA, pH 7.5 (buffer A), lyophilized, and reconstituted with buffer A before use.

Binding experiments. Binding experiments were performed at 30° for 30 min in 10 mM Tris·HCl, 50 mM sucrose, 20 mM $MgCl_2$, pH 7.5 (buffer B). In saturation experiments using [^{3}H]yohimbine, the concentration of ligand was varied between 0.5 and 20 nM. Nonspecific binding was defined in all cases by parallel assays containing 100 μM idazoxan. Nonspecific binding increased with ^{3}H-ligand concentration in a linear manner. Binding experiments were terminated by rapid filtration through Whatman GF/C filters, followed by three washes of the filter with ice-cold buffer B (5 ml). Filters were left overnight in Hi-safe scintillant before liquid scintillation counting.

High affinity GTPase assays. GTPase assays were performed essentially as in described in Ref. 10, using [γ-^{32}P]GTP (0.5 μM, 60,000 cpm) and various concentrations of UK14304. Nonspecific GTPase was assessed by parallel assays containing 100 μM GTP.

TABLE 1

Generation and specificities of anti-G protein antisera

The antisera were generated in New Zealand White rabbits, using a conjugate of the synthetic peptide and keyhole limpet hemocyanin, as detailed in Experimental Procedures. $G_i1\alpha$ and $G_i2\alpha$ have identical carboxyl-terminal decapeptides and, thus, antiserum AS7 identifies each of these polypeptides equally. Transducin α is also identified by antiserum AS7 but, because this G protein is restricted in distribution ot photoreceptor-containing tissues, the antiserum can be used as a probe for G_i1 or G_i2 in all other locations.

Antiserum	Peptide used	Corresponding G protein sequence	Protein identified by antiserum
AS7	KENLKDCGLF	Transducin α 341–350	Transducin, G_i1, G_i2
OC1	ANNLRGCGLY	$G_o\alpha$ 345–354	G_o
I3B	KNNLKECGLY	$G_i3\alpha$ 345–354	G_i3
CS1	RMHLRQYELL	$G_s\alpha$ 385–394	G_s

Results

Transfection and stable expression of human α_2-C10 receptor DNA into the Rat-1 fibroblast cell line has been described (7). One clone, referred to here as 1C, was selected for this study.

Radioligand binding with the nonselective α_2-adrenergic ligand [^{3}H]yohimbine established that membranes from 1C cells possessed a high affinity yohimbine binding site. Saturation binding isotherms indicated a K_d of 1.0 ± 0.2 nM for the ^{3}H-ligand and a B_{max} of 3152 ± 310 fmol/mg of membrane protein (mean ± standard error; three experiments). Nonspecific binding was 6–15% of total binding. Parental Rat-1 cells do not bind [^{3}H]yohimbine in a specific manner (7). Characterization of the α_2-adrenergic receptor subtype was on the basis of differential affinity for the antagonist prazosin and the agonist oxymetazoline (20). Displacement of specific [^{3}H]yohimbine (5 nM) binding to membranes of 1C cells produced IC_{50} values of 8.1 ± 1.8 nM and 4.8 ± 1.5 μM for oxymetazoline and prazosin, respectively (mean ± standard error; three experiments for both drugs). Estimation of K_i by the method of Cheng and Prussoff (21) gave values of 1.4 nM and 0.9 μM, respectively. This high affinity for oxymetazoline and low affinity for prazosin is characteristic of a receptor of the α_{2A} subtype (20).

Clone 1C has previously been demonstrated to express the α subunits of G_i1, G_i2, G_i3, and both short and long forms of G_s (7). Because the α subunits of G_i1 and G_i2 share an identical carboxyl-terminal decapeptide, we examined the relative expression of these two G proteins in membranes of 1C cells by immunoblotting with an antiserum directed against this peptide sequence, under SDS-PAGE conditions (10) that conveniently separate these two G proteins. Results from these immunoblots indicated that G_i2 was much more highly expressed than G_i1 (Fig. 1a). Antisera raised against $G_o\alpha$ did not detect any protein on immunoblots of membranes of 1C cells but readily detected the presence of this polypeptide in membranes from rat brain (Fig. 1B) and from NG108-15 cells (Ref. 10 and results not shown). Pertussis toxin-catalyzed [^{32}P]NAD$^+$-dependent [^{32}P]ADP-ribosylation of membranes from 1C cells also failed to show any pertussis toxin substrate at 39 kDa, in contrast to membranes from neuroblastoma × glioma hybrid NG108-15 cells (Fig. 2). These experiments also indicated that the total level of pertussis toxin-sensitive G proteins was apparently considerably greater in membranes of 1C cells than in equivalent amounts of membranes of NG108-15 cells (Fig. 2).

The α_2-adrenergic agonist UK14304 stimulates a high affinity GTPase activity in membranes from the 1C cells (7), with an EC_{50} of 100 ± 10 nM in these experiments (mean ± standard error; three experiments). This GTPase response is entirely pertussis toxin sensitive (7). We have previously described a method of assessing G protein coupling to receptors that involves incubating membranes with specific antisera raised against the carboxyl-terminal decapeptides of a range of G protein α subunits (10). We have used this method here to determine which G protein(s) are coupled to the adrenergic receptor in rat 1C cells. Preincubation of membranes from 1C

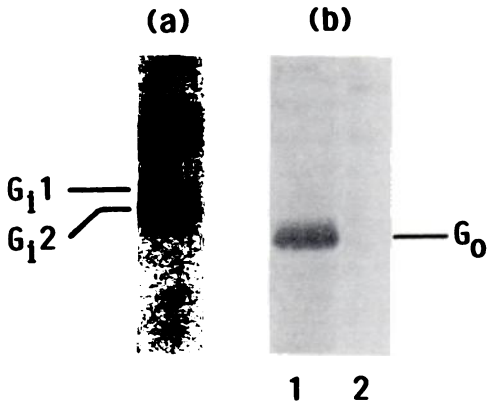

Fig. 1. a, $G_i2\alpha$ is more highly expressed than $G_i1\alpha$ in membranes of clone 1C cells. b, $G_o\alpha$ is expressed in brain but not by clone 1C cells. Membranes (50 μg/sample) were separated by SDS-PAGE [12.5% acrylamide, 0.06% (w/v) bisacrylamide] and the resolved proteins were transferred to nitrocellulose before blocking with 5% gelatin in Tris-buffered saline. Subsequent immunoblotting was performed as described in Experimental Procedures, with a 1/200 dilution of antiserum AS7 (9), which identifies the α subunits of G_i1 and G_i2 equally (a), or of antiserum IM1 (41, 42), which identifies the α subunit of G_o specifically (b). a, Membranes are from 1C cells; b, membranes are from rat cortex (*lane 1*) and 1C cells (*lane 2*).

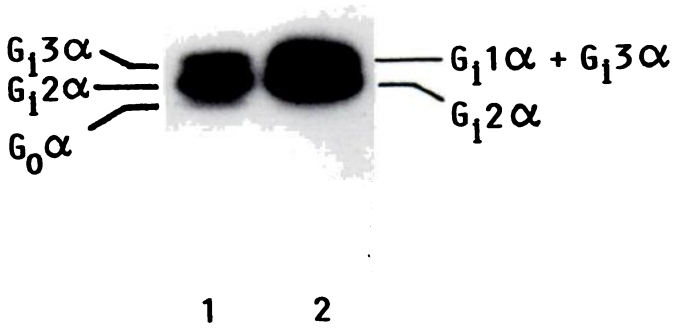

Fig. 2. Substrates for pertussis toxin-catalyzed [^{32}P]ADP-ribosylation in membranes from clone 1C cells. Membranes from NG108-15 (*lane 1*) or clone 1C (*lane 2*) cells (50 μg each) were [^{32}P]ADP-ribosylated with pertussis toxin (10 μg/ml), using [^{32}P]NAD$^+$ as substrate. Proteins were then separated on SDS-PAGE, as for Fig. 1. Gels were stained with Coomassie blue, dried, and autoradiographed. The three pertussis toxin-sensitive G proteins expressed in NG108-15 cells have been shown immunologically to correspond to G_o, G_i2, and G_i3 (10) and those in 1C cells to G_i1, G_i2, and G_i3 (7). It is not possible to resolve G_i1 and G_i3 using one-dimensional SDS-PAGE.

cells for 1 hr at 37°, in the absence or presence of nonimmune IgG, caused the loss of up to 47% of UK14304-stimulated GTPase activity (Table 2). However, we found that such preincubations were required to maximize the effects of added immune IgG fractions. Basal GTPase activity was also reduced by ~45% by this preincubation (results not shown).

Preincubation of membranes from clone 1C cells, for 1 hr at 37°, with Protein A-purified IgG fractions from antisera directed against the carboxyl-terminal decapeptides of the α subunits of G_o (antiserum OC1), G_i1 plus G_i2 (antiserum AS7), G_i3 (antiserum I3B), and G_s (antiserum CS1) resulted in a statistically significant reduction of UK14304-stimulated GTPase activity in the case of antisera AS7 and I3B ($p < 0.001$, Student's t test, compared with IgG fraction) but not in the cases of antisera OC1 or CS1 (Fig. 3). Incubation with a Protein

TABLE 2

Loss of GTPase activity upon preincubation of membranes from clone 1C cells

Membranes from clone 1C cells (10 μg/sample) were incubated for the times shown, before assay for UK14304 (10 μM)-stimulated GTPase activity. Results are mean ± standard error (three experiments), using different membrane preparations. Data are expressed as percentage of increase over basal GTPase (22.3 ± 2.2 pmol/min/mg).

Preincubation time	UK14,304-stimulated GTPase
min	%
0	100.0
15	93.9 ± 5.3
30	75.2 ± 4.1
45	60.4 ± 2.6
60	50.1 ± 4.3

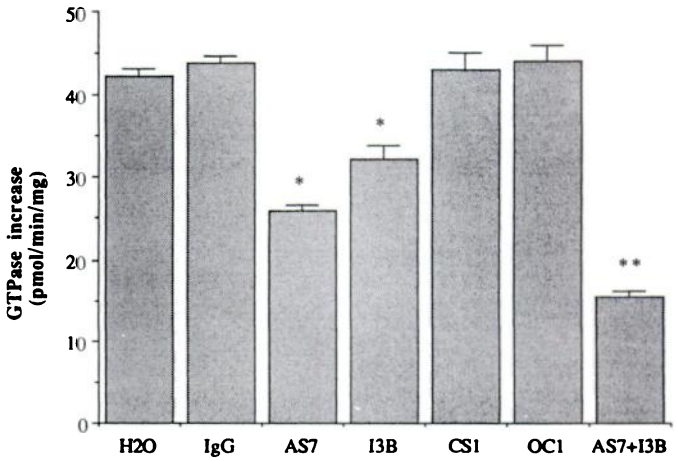

Fig. 3. Preincubation with Protein A-purified antisera causes specific reductions in UK14304-stimulated GTPase activity in membranes from clone 1C cells. Membranes (5 μg/sample) from clone 1C cells were incubated for 1 hr at 37° with Protein A-purified IgG (10 μg) from normal rabbit serum (*IgG*) or from anti-G protein antisera (10 μg) as shown, in the presence of GTPase assay mix without [γ-^{32}P]GTP. After preincubation, UK14304 (10 μM) and [γ-^{32}P]GTP were added, and incubation was continued for an additional 20 min. Points are means ± standard errors of four experiments performed using different membrane preparations. Antisera used were AS7 (versus G_i1 and G_i2), I3B (versus G_i3), OC1 (versus G_o), and CS1 (versus G_s). Results are expressed as increase over basal GTPase, which was 22.3 ± 1.4 pmol/min/mg of membrane protein (mean ± standard error; four experiments). *, Incubation with the fractions from antisera AS7 and I3B produced statistically significant decreases in the ability of UK14304 to stimulate high affinity GTPase activity ($p < 0.001$ for AS7 and I3B, compared with IgG fraction from normal rabbit serum; Student's t test). **, Incubation with AS7 plus I3B produced a statistically significant reduction, compared with either AS7 or I3B alone ($p < 0.001$ in each case).

A-purified, nonspecific IgG fraction of normal rabbit serum showed that antibody incubation *per se* did not affect basal or agonist-stimulated GTPase activity. Incubation with increasing concentrations of antisera AS7 and I3B demonstrated maximal reductions in UK14304-stimulated GTPase activity, with approximately 35–40% reduction in both cases, at a concentration of 10 μg/sample (Fig. 4). In some experiments, membranes were preincubated with Protein A-purified fractions from both AS7 and I3B. This resulted in a maximum reduction in UK14304-stimulated GTPase of approximately 70–80% (Fig. 3). Coincubation with IgG fractions from both antisera AS7 and I3B produced a statistically greater reduction in agonist-stimulated GTPase activity than either antiserum alone ($p < 0.001$, Student's t test, for AS7 plus I3B, compared with AS7 or I3B alone) and, within the errors of the experiment, the effects of the two antisera were additive. In no case, however, was com-

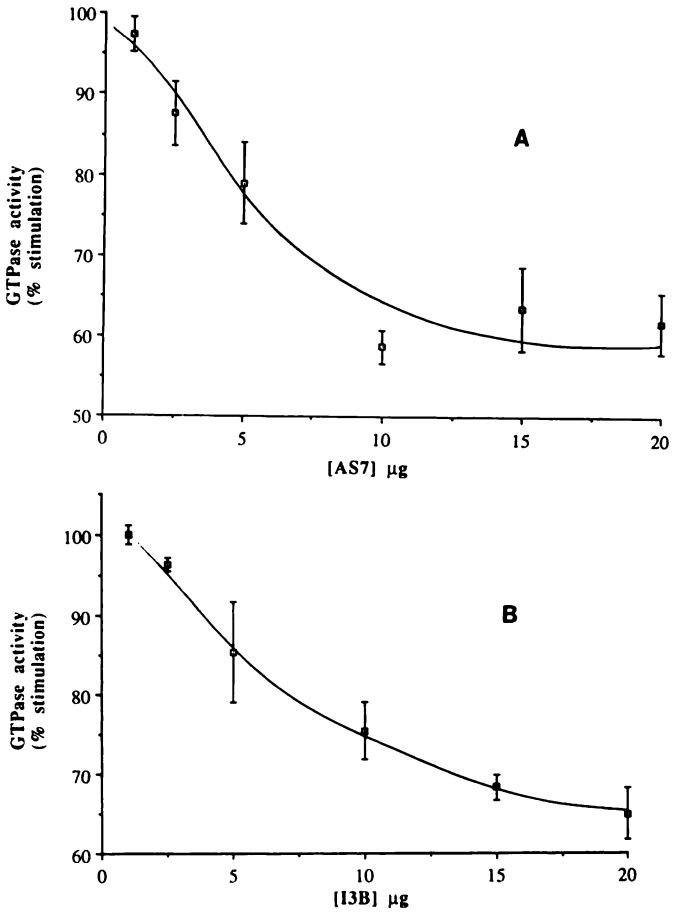

Fig. 4. Effect of increasing antiserum concentration on uncoupling of UK14304-stimulated GTPase activity in membranes from clone 1C. Experiments were performed as described in the legend to Fig. 3, but Protein A-purified antisera were present in the amounts indicated. Points are means ± standard errors of three experiments, using different membrane preparations. A, Effect of increasing AS7 concentration. *100%*, increase over basal high affinity GTPase activity of 41.2 ± 1.7 pmol/min/mg of membrane protein (mean ± standard error; three experiments). B, Effect of increasing I3B concentration. *100%*, increase over basal high affinity GTPase activity of 42.0 ± 2.0 pmol/min/mg of membrane protein (mean ± standard error; three experiments).

plete abolition of the UK14304-stimulated GTPase response observed (five experiments). Even at immune IgG concentrations up to 100 μg/sample, UK14304 stimulation of GTPase in the presence of AS7 plus I3B was still statistically significant ($p < 0.001$).

Discussion

Specificity of transmembrane signaling processes between G protein-linked cell surface receptors and effector systems, which are either ion channels or enzymes that regulate the rate of production of intracellular secondary messengers, is important to preserve the integrity of information passing across the cell membrane (22, 23). With information from both biochemical and molecular biological studies indicating that a considerable range of highly homologous G proteins can be expressed in a single cell (24), it then becomes important that a full understanding of the specificity of contacts between receptors and G proteins be achieved. Many early studies designed to assess this specificity utilized the reconstitution of purified or partially purified receptors with purified G proteins in artificial liposomes (25–27). Such studies frequently indicated that a single receptor was able to induce the activation of a number of different G proteins (25–27). More recent studies, however, have indicated a higher degree of specificity of these interactions (28–30). Reconstitution studies, therefore, although able to disclose the physical potential for interaction of receptor and G protein when they are placed in a single environment at relatively high concentrations, are naturally unable to assess directly whether such interactions take place within the membrane *in vivo*. Increasingly, the focus of research is to determine such *in vivo* interactions.

It has become clear that the carboxyl-terminal region of G proteins plays a key role in receptor G protein contacts. This is based on two pieces of information. Firstly, the observation has been made that pertussis toxin-catalyzed ADP-ribosylation of a cysteine residue located four amino acids from the carboxyl-terminal of a subset of G protein α subunits prevents functional contact between these G proteins and their receptors (15). Secondly, the analysis of cDNA clones corresponding to the α subunit of G_s in the *unc* mutation of S49 lymphoma cells, in which functional contact between G_s and its receptors does not occur, has indicated that the functional deficiency lies in a mutation of the sequence of the carboxyl-terminal region (13, 14).

It was, therefore, anticipated that site-directed antisera that identify the carboxyl-terminal region of a G protein could provide tools to interfere with receptor-G protein interaction and could be used potentially to define the specificity, or otherwise, of such interactions in membrane preparations or whole cells. These hopes have, indeed, been realized. δ-Opioid receptor-mediated inhibition of adenylyl cyclase in NG108-15 cells was initially demonstrated to be produced by 'G_i' (31) and, recently, the identity of this 'G_i' has been defined as G_i2 (10). α_{2B} Receptor-mediated inhibition of adenylyl cyclase in these NG108-15 cells (32) and α_{2A} receptor-mediated inhibition of adenylyl cyclase in human platelets (33) have also been shown to be transduced by G_i2. Antibodies to the carboxyl-terminal region of G_s have been demonstrated to prevent activation of adenylyl cyclase by β-adrenergic agonists in S49 lymphoma cells (34) and by both β-adrenergic agonists and epidermal growth factor in heart (35), and antibodies raised against the carboxyl terminus of G_o prevent α_{2B} regulation of voltage-operated Ca^{2+} channels in NG108-15 cells (36). We report here the use of such antisera to investigate the coupling of G proteins to the α_{2A} receptor in a transfected cell line.

The most appropriate and simple assay of functional contact between a receptor and a G protein is based on the stimulation of high affinity GTPase activity (15). This is produced as the consequence of guanine nucleotide exchange in the nucleotide binding site of the relevant G protein upon agonist occupation of the receptor to which it is coupled. Because nucleotide exchange is the rate-limiting step in the cycle of activation and deactivation of G proteins (22), stimulation of exchange of GTP for GDP will produce enhanced GTPase activity. Although, for a variety of technical reasons, agonist stimulation of high affinity GTPase cannot be measured in all situations (15), it has proved to be particularly suitable as an assay for receptor contacts with G proteins that are substrates for pertussis toxin-catalyzed ADP-ribosylation (15). Incubation of membranes of 1C cells with UK14304 caused a large increase in high affinity GTPase activity and, because this was not observed when the experiment was performed with membranes derived from cells that had been pretreated with pertussis toxin

(7), the G protein(s) responsible must be substrates for pertussis toxin-catalyzed ADP-ribosylation.

Preincubation of membranes of 1C cells with fractions of either the anti-G_i1+G_i2 antiserum (AS7) or the anti-G_i3 antiserum (I3B) partially prevented UK14304 stimulation of high affinity GTPase activity. In contrast, equivalent fractions of either the anti-G_o or anti-G_s antisera had no effect. None of the IgG fractions altered the basal high affinity GTPase activity and, therefore, the effects of the antibodies were only to prevent receptor activation of the G proteins. The lack of effect of the anti-G_o and anti-G_s antisera on UK14304 stimulation of high affinity GTPase activity in these cells was not surprising, because $G_o\alpha$ is not expressed at detectable levels (Fig. 2) and $G_s\alpha$ is not a substrate for pertussis toxin-catalyzed ADP-ribosylation. It should be noted, however, that both of these antisera have been shown previously to be capable of attenuating receptor regulation of appropriate effector systems (35, 36). Although antiserum AS7 has previously been reported to prevent G protein interaction with the α_{2A} receptor in human platelets (33) and with the α_{2B} receptor in NG108-15 cells (32), antibodies against the carboxyl-terminal region of $G_i3\alpha$ were ineffective in both of these studies. This would seem to imply a lack of interaction of naturally expressed α_2-adrenergic receptors with G_i3 in the cells and tissues studied to date. The α_{2A} receptor on the human platelet seems to interact only with G_i2 (34), yet in this transfected cell line it appears to interact also with G_i3, suggesting that there has been some loss of specificity of interaction with the α_{2A} receptor in the 1C clone. This has important implications for the use of transfected receptors in studies to determine G protein coupling.

We have recently provided an alternate demonstration of the interaction of the α_2-C10 receptor with multiple pertussis toxin-sensitive G proteins in clone 1C cells (7), by taking advantage of an assay in which cholera toxin can be induced to catalyze the [^{32}P]ADP-ribosylation of pertussis toxin-sensitive G proteins, in a manner that is dependent upon agonist activation of the receptors that interact with these G proteins (37–39). By thus incorporating a ^{32}P label into these G proteins, immunoprecipitation with selective antisera could be used to define which G proteins were involved in receptor coupling (7). However, although such experiments provided qualitative data similar to those found in this study, the use of the cholera toxin-catalyzed [^{32}P]ADP-ribosylation of "inappropriate" G proteins cannot offer any quantitative assessment of the degree of interaction of the receptor with the two G proteins and requires the assays to be performed in a distinctly artificial environment for the G proteins, i.e., in the absence of guanine nucleotides.

This is, therefore, the first report to demonstrate the simultaneous interaction of a single defined α_2-adrenergic receptor subtype with multiple pertussis toxin-sensitive G proteins that also provides information indicating how quantitatively important these functional contacts might be. This assessment is based on the fact that neither the anti-G_i1+G_i2 antiserum nor the anti-G_i3 antiserum used in these studies was able, in isolation, to block receptor-stimulated GTPase activity completely. Indeed, the relative proportions of the receptor-activated GTPase that was blocked by each antiserum at maximally effective concentrations provide the first estimate of such interactions (Figs. 3 and 4). Our conclusion from these studies is that G_i1 plus G_i2 and G_i3 provide similar total proportions of the α_2-C10 adrenergic receptor-stimulated GTPase and, hence, by extension we conclude that the total pools of these two G protein populations activated by the receptor in these cells are similar in amount.

One slightly surprising aspect of the current studies is that, even when preincubation of the 1C cell membranes was performed with a mixture of maximally effective concentrations of the two functional antisera, there was still a measurable agonist-stimulated GTPase activity that remained. Two potential explanations for this observation can be considered. The first of these is that 1C cells express a pertussis toxin-sensitive G protein apart from G_i1, G_i2, and G_i3. It is, of course, possible that other pertussis toxin-sensitive G proteins, expressed by these cells but not recognized by our antisera, have yet to be identified. The second possibility to account for the remaining agonist-stimulated GTPase activity is that we have been unable to neutralize all of the available G protein within the membrane. We have been able in previous experiments to completely uncouple receptors from the G protein signaling cascade in membranes of NG108-15 cells, but it is certainly true that, based on the incorporation of [^{32}P]ADP-ribose into G protein substrates by pertussis toxin, there would appear to be considerably higher levels of these polypeptides in membranes of 1C cells than are present in equal amounts of membranes of NG108-15 cells (Fig. 2). However, addition of higher concentrations of either antiserum AS7 or I3B (up to 100 μg) was unable to produce further inhibition of GTPase stimulation (see Results). It is of vital importance to the conclusions generated by this study that the antisera used can be clearly demonstrated to have the designated specificities. We have recently shown that the $G_i3\alpha$ antiserum identifies a 41-kDa polypeptide in an *Escherichia coli* lysate expressing $G_i3\alpha$ but not in equivalent lysates expressing either $G_i1\alpha$ or $G_i2\alpha$, whereas the anti-G_i1+G_i2 antiserum identifies $G_i1\alpha$ and G_i2 but not $G_i3\alpha$ in these lysates.[1] Furthermore, immunoprecipitations with the respective antisera are also specific (7). We cannot address, within this study, whether the α_2-C10 receptor might be able to interact also with pertussis toxin-insensitive G proteins in membranes of these cells, because the receptor stimulation of high affinity GTPase activity that was used as the assay for functional interaction of the receptor and G protein was entirely attenuated by pretreatment of the cells with pertussis toxin. This fact establishes that it is only contacts between the receptor and G proteins of this subclass that we are measuring. There is evidence that muscarinic acetylcholine receptors can interact with both pertussis toxin-sensitive and -insensitive G proteins when they are expressed in Chinese hamster ovary cells (40). The functional consequences of this are that, within the same cell, a single receptor is able to regulate adenylyl cyclase via a pertussis toxin-sensitive G protein and also phosphoinositidase C-catalyzed generation of inositol phosphates via a pertussis toxin-insensitive mechanism.

We have recently demonstrated that clone 1C cells respond to α_2-adrenergic agonists both by producing inhibition of adenylyl cyclase and by stimulating the hydrolysis of phosphatidylcholine by a phospholipase D activity (8). Both of these effects are reversed by pertussis toxin pretreatment of the cells, but they appear to be independent, because maintenance of intracellular cyclic AMP concentrations does not lead to a reduction in the receptor activation of phospholipase D. Future experiments, which will require the ability to measure receptor

[1] A. Green, G. Milligan, and S. B. Belt. Submitted for publication.

stimulation of phospholipase D activity in a cell-free system, will be aimed at defining which of the activated G proteins mediate these two signaling processes in response to receptor activation.

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