Factors Determining the Specificity of Signal Transduction by Guanine Nucleotide-binding Protein-coupled Receptors

I. COUPLING OF α₂-ADRENERGIC RECEPTOR SUBTYPES TO DISTINCT G-PROTEINS*

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α₂-Adrenergic receptor (α₂-AR) subtypes couple to pertussis toxin (PT)-sensitive G-proteins to elicit both stimulatory and inhibitory cell responses. Signal specificity may be generated by the ability of the receptor subtypes to "recognize" distinct G-proteins with different affinity.

To address this issue we stably expressed three α₂-AR subtypes, RNα₂ (α₂B-AR), RG10 (α₂c-AR), and RG20 (α₂D-AR), in NIH-3T3 fibroblasts, which express two PT-sensitive G-proteins (Gαi3, GαS), and analyzed receptor/G-protein interactions by determining: 1) functional coupling to adenylcyclase and 2) the ability of the receptors to exist in a high affinity state for agonist. In α₂D-AR transfectants expressing 200 or 2,200 fmol of receptor/mg of protein, epinephrine (10 μM) inhibited forskolin-induced elevation of cellular cAMP by 26 ± 4.8% and 72 ± 6.2%, respectively. Similar results were obtained in α₂B-AR transfectants. However, in α₂c-AR transfectants (200 fmol/mg) the forskolin-induced elevation of cellular cAMP was not altered by agonist treatment. In α₂c-AR transfectants expressing higher receptor densities (650–1,200 fmol/mg), epinephrine inhibited the effect of forskolin by 30 ± 3.2%. This difference in functional coupling among the α₂-AR subtypes is reflected at the receptor/G-protein interface. In membrane preparations of α₂B and α₂D-AR but not α₂c-AR transfectants, agonist competition curves were biphasic, indicating high and low affinity states of the receptor for agonist. The high affinity state was guanyl-5'-yl imidodiphosphate- and PT-sensitive, indicative of receptor coupling to a specific class of G-proteins (G1α, G2α, Gαi3-restricted to rod and cone cells of retina) (4, 24). α₂-AR are negatively coupled to adenylcyclase in several tissues, but many cell responses to α₂-AR activation are independent of cyclase inhibition, suggesting receptor recognition of additional PT-sensitive G-proteins and/or activation of different effector molecules. However, the precise coupling pathways utilized by each receptor subtype are not defined.

The roles of various factors (i.e. relative affinity or stoichiometry of receptor/G-protein effectors) in determining the selection of a particular transduction pathway by the receptor subtypes are unknown. The transduction pathway utilized by each receptor subtype is likely cell-specific depending on the particular G-proteins and effector molecules expressed in a given cell phenotype (25). To test this hypothesis we are stably transfecting different cell phenotypes with α₂-AR subtype genes following by analysis of functional coupling and receptor/G-protein interaction.

The present study indicates the utility of such an approach. Utilizing NIH-3T3 transfectants expressing different α₂-AR subtypes (α₂B, α₂c, and α₂D), we report that the α₂c-AR subtype differs from the α₂B and α₂D-AR subtypes in terms of both receptor/G-protein interaction and the efficiency with which it couples to adenylcyclase.

EXPERIMENTAL PROCEDURES

Materials—[3H]Rauwolscine (87 Ci/mmol), [3H]cAMP (30 Ci/ mmol), [α-32P]ATP (30 Ci/mmol), cAMP 125i radioimmunoassay kit, Gαi and Gαo antisera were purchased from Du Pont-New England Nuclear. Tissue culture supplies were obtained from JRH Bioscience (Lenexa, KS). Rauwolscine was obtained from Atomergic Chemicals (Farmingside, NY). Phentolamine was obtained from Ciba-Geigy, and prazosin was a gift from Dr. Hess (Pfizer, Groton, CT). Genetin

1 The abbreviations used are: α₂-AR, α₂-adrenergic receptor(s); G-protein, guanine nucleotide-binding protein; PT, pertussis toxin; Gpp(NH)p, guanyl-5'-yl imidodiphosphate; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid.
(G418) was obtained from GIBCO-BRL. Aluminum oxide (90% activated) was obtained from EM Science (Cherry Hill, NJ). Dowex 50W-X4 (100–200 mesh, hydrogen form), acrylamide, bisacrylamide, and SDS were purchased from Bio-Rad. Nitrocellulose and Westman membrane were obtained from Schleicher & Schuell. RNazol was obtained from Tel-Test Laboratory (Maywood, IL). Protein-A-Sepharose (G-200) was obtained from Bethesda Research Laboratories. Propranolol, [3H]epinephrine, forskolin, prostaglandin E1, phenylmethylsulfonyl fluoride, pepstatin A, aprotinin, and pertussis toxin were obtained from Sigma. The antisera for G-11 and G-12 were provided by Drs. C. Bianchi and C. Homcy (Cardiac Unit, Massachusetts General Hospital, Boston, MA). DNase I was purchased from National Diagnostics (Manville, NJ). All other materials were obtained as described previously (6, 7, 25).

**Membrane Preparations and Radioligand Binding Studies**—NIH-3T3 fibroblasts were grown as monolayers at 37°C (5% CO2) in Dulbecco's modified Eagle's medium with high glucose (4.5 g/liter), supplemented with 10% bovine calf serum plus penicillin (100 units/ml), streptomycin (100 µg/ml), and fungizone (0.25 µg/ml). In some experiments, cells were pretreated with 100 ng/ml pertussis toxin for 18 h (37°C) before functional studies or membrane preparation.

For membrane preparations, cells were washed twice with washing solution (157 mM NaCl, 2.6 mM KCl, 10 mM NaHPO4, 1.8 mM KH2PO4) and harvested with a rubber policeman. Cells were pelleted at 4°C at 200 g in a Sorvall RT6000 centrifuge. The pellet was resuspended in 1 ml/dish of lysis buffer at 4°C (5 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin A, and 10 µg/ml aprotinin) and homogenized in a Dounce homogenizer. The cell lysate was then centrifuged at 17,000 × g (Sorvall RC5B, type SS34 rotor) for 15 min and the pellet resuspended in membrane buffer (50 mM Tris-HCl, pH 7.5, 0.6 mM EDTA, 5 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin A, and 10 µg/ml aprotinin). The protein concentration was determined by the method of Lowry et al. (26). Radioligand binding studies were performed as described previously (6, 7, 25). For saturation binding studies, membranes (25–50 µg) were incubated with increasing concentrations of [3H]rauwolscine or [3H]HUK 14,304 in a total volume of 1 ml for 60 min at 25°C. For competition binding studies, membranes (20–100 µg) of protein were incubated with [3H]rauwolscine (2 nM [3H]a2c, 0.8 nM [3H]a2A, 12 nM a2B) and increasing concentrations of competing ligands for 60 min at 25°C in a final volume of 100 µl. The reaction was terminated by adding 5 ml of ice-cold 100 mM Tris-HCl, pH 7.4, followed by rapid filtration over glass fiber filters. The filters were then washed with 2 × 5 ml of cold buffer and radioactivity quantified by scintillation counting at 50% efficiency. Non-specific binding was determined in the presence of 10 µM rauwolscine or 10 µM epinephrine. Binding data were analyzed as described above (6, 7, 25) utilizing the nonlinear least square curve fitting procedure, LIGAND (27).

**Cell Transfection**—The a2c-AR (RG10) (~1,700 base pairs) or a2a-AR (RG20) (~2,100 base pairs) receptor gene fragment was inserted into the pMSV expression vector downstream of the murine sarcoma virus long terminal repeat (7). The RG10 and RG20 constructs contain ~400 bp of sequence 3' to the stop codon. The RG20 receptor cDNA in the expression vector pRlkb2 (51) was kindly provided by Dr. Kevin Lynch (10). Constitutive gene expression was achieved by cotransfecting the a2c-AR and a2a-AR constructs and pNEO (resistance plasmid) into NIH-3T3 cells by calcium precipitate containing a mixture of 16 µg of expression vector plus 4 µg of a plasmid encoding neomycin resistance. Transfected cells were selected for their resistance to the antibiotic G418 (0.5 mg/ml). Selection was begun 3 days after transfection and continued for 10 days. G418-resistant colonies were screened for the expression of the receptor subtypes by RNA blot analysis and by their ability to bind the a2c-selective antagonist [3H]rauwolscine. Transfectants expressing appropriate sizes of receptor mRNA and low or high receptor densities were selected for functional studies. The expected size of receptor mRNA and low or high receptor densities were calculated from RNA blotting with 10× SSC. The size of mRNA was estimated by comparison with the migration of the 0.24–0.95-kilobase RNA ladder run in parallel with RNA samples. After transfer, RNA blot hybridizations were performed in an aqueous phosphate buffer system as described previously (29). Briefly, nitrocellulose membranes were first prehybridized in phosphate buffer (0.6 M NaHPO4, pH 7.2, 1 mM EDTA, pH 8.0, 7% SDS, 1% bovine serum albumin) at 65°C for 60 min. Incubation was continued overnight in the same solution containing 32P-labeled probe (~2 × 106 cpm/ml) generated with the multiprime DNA labeling system (Amersham Corp.). The hybridized blots were then washed (60°C) twice, 30 min each, in 1× SSC, 1 mM EDTA, pH 8.0, 0.5× SSC, and once in buffer containing 40 mM NaHPO4, pH 7.5, 1% SDS, and 1× EDTA, pH 8.0 for 60 min. The blots were then dried and exposed to Kodak XAR-5 film at −70°C with Du Pont Cronex Quanta III intensifying screens for 3–15 h.

**Photoaffinity Labeling**—The expressed rat a2-AR subtypes were photoaffinity labeled as described previously utilizing [125I]Rau-AzPC, a photolabile probe that selectively recognizes a2-AR (6, 30). Optimal conditions for photolabeling of the receptor subtypes in terms of [125I-Rau-AzPC and protein concentration were determined in a series of preliminary experiments. Aliquots of cell membranes were incubated with [125I-Rau-AzPC (250, 500, 1000, 2500, or 5000 cpm) and glycylglycine, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml pepstatin A, 10 µg/ml aprotinin) with [125I]Rau-AzPC for 30 min at 25°C under reduced light and then cooled on ice for 5 min. Samples were then placed in a Ray-O-Vac photolysis chamber (300 W, 300 nm) and 1 ml of labeling buffer containing 2 µM dithiothreitol was added prior to photolysis (5 min, 4°C). Because of its higher affinity for rauwolscine, it was possible to photoaffinity label the a2c-AR in the intact cell. Confluent cultures of fibroblasts were harvested as described above and resuspended in washing solution. Aliquots of transfected cells (50,000–500,000) were then incubated with 0.8 nM [32P]labeled rauwolscine or 10 nM epinephrine. Binding data were analyzed as described above (6, 7, 25). The photolyzed samples were centrifuged at 14,000 × g (Beckman Microfuge E) for 10 min at 4°C and the pellet solubilized in 0.04 ml of sample buffer and subjected to electrophoresis in 10% SDS-polyacrylamide gels according to the Laemmli protocol (31). Gels were dried and autoradiograms were obtained by exposing the dried gels to Kodak XAR-5 film at −70°C in a cassette containing Du Pont Cronex Quanta III intensifying screens.

**Measurement of Cellular cAMP**—NIH-3T3 cells were plated at a density of ~5 × 104 cells/well in six-well plates. After 24 h the growth medium was replaced with 4 ml of serum-free Dulbecco's modified Eagle's medium containing 20 mM HEPES, pH 7.5 and 10 µM propranolol for 30 min at 37°C. This medium was aspirated, and cells were then incubated for 10 min with various epinephrine concentrations in the absence and presence of 10 µM forskolin in the same medium containing 250 µM isobutyl-1-methylxanthine. The reaction was terminated by aspiration of the medium and addition of 1 ml of ice-cold 10% trichloroacetic acid. The precipitate was then washed using RIANEN radioimmunoassay for nonacetylated CAMP (31-CAMP). Data for CAMP assays are expressed as pmol/mg of protein. Protein concentration was determined in cell precipitates solubilized in 0.2 n NaOH by the method of Lowry et al. (28).

**Adenylylcyclase Assay**—The conversion of [a-32P]ATP to [32P]cAMP was determined essentially as described by Salomon et al. (32). The membranes were prepared as described before and resuspended in 50 mM Na-HEPES, pH 8.0, 1 mM EDTA at 4°C. The assay buffer contained 50 mM Na-HEPES, pH 8.0, 1 mM EDTA, 4 mM MgCl2, 100 mM NaCl, 0.24 mM ATP, 0.1 M GTP, 0.2 mM cAMP, 0.2% propranolol, 0.1% crystalline albumin, 100 µM GDP, and ATP-regenerating system consisting of 90 mM creatine phosphate and 10 units/ml creatine kinase in a final volume of 100 µl. Reactions were initiated by the addition of membranes (50 µg of protein) which had been preincubated with 10 µM propranolol at 4°C for 30 min. The reaction was conducted in duplicate for 30 min at 37°C in the presence of 0.5 mM [32P]ATP (2.5 × 104 cpm/tube), and a ATP-regenerating system consisting of 90 mM creatine phosphate and 10 units/ml creatine kinase in a final volume of 100 µl. The reaction was terminated by addition of 0.5 ml of ice-cold 10% trichloroacetic acid. The precipitate was then washed, and the radioactivity was measured in a liquid scintillation counter.

**Immunoblotting**—To achieve maximal resolution by SDS-polyacrylamide gel electrophoresis, NIH-3T3 cell membranes were treated as described by Stierweil and Robinshaw (33). Solubilized membranes (20–50 µg) were loaded into a 10% SDS-polyacrylamide gel. Proteins...
RESULTS

Stable Expression of Rat \(\alpha_2\)-Adrenergic Receptor Subtypes in NIH-3T3 Fibroblasts—NIH-3T3 cells were transfected separately with the cDNA encoding \(\alpha_2\)-AR or the genes coding for \(\alpha_2\)-AR and \(\alpha_2\)-AR subtypes. Thirty to fifty G418-resistant clonal cell lines were screened for receptor expression. The transfectants selected for functional studies expressed a range of receptor densities (50-5,500 fmol/mg of protein) and were evaluated by determining (1) the size of the receptor’s mRNA; (2) the receptor’s ligand recognition properties; and (3) the apparent molecular weight of the receptor protein. RNA blot analysis of total RNA prepared from the transfected cells indicated the size of the \(\alpha_2\)-AR, \(\alpha_2\)-AR, and \(\alpha_2\)-AR mRNA species as ~3,100, ~3,200, and ~4,000, respectively (Fig. 1). Only transfectants expressing the appropriate size of receptor mRNA were selected for further characterization. The \(\alpha_2\)-AR transfectant B(2) expressed ~1,000 fmol of receptor/mg of protein, the \(\alpha_2\)-AR transfectants (10/17, 10/6, and 10/1) expressed ~200, ~650, and ~1,200 fmol of receptor/mg of protein, whereas the \(\alpha_2\)-AR transfectants (20/21, 20/44, 20/38, and 20/20) expressed ~200, ~2,200, ~3,400, and ~5,500 fmol of receptor/mg of protein.

Membranes prepared from transfected cells displayed saturable binding of the radioligand [\(^3\)H]rauwolscine. Scatchard analysis of saturation binding studies indicated that [\(^3\)H]rauwolscine exhibited 6-fold higher affinity for the \(\alpha_2\)-AR (\(K_d = 2 \pm 0.15 \text{ nM}\)) and 15-fold higher affinity for the \(\alpha_2\)-AR (\(K_d = 0.8 \pm 0.07 \text{ nM}\)) than for the \(\alpha_2\)-AR (\(K_d = 12 \pm 1.76 \text{ nM}\)). The differences among the three receptor subtypes are further illustrated by the results of competition binding studies with various ligands (Fig. 2). The \(\alpha_2\)-AR as compared with the \(\alpha_2\)-AR, exhibited 6-fold higher affinity for phentol-
with the photoaffinity adduct in the absence to the transfectants obtained from two different clonal cell lines. The results of photolabeling experiments at these probe concentrations were 3.2%, 6.2% (IC50 = 20 nM), and 8.26 ± 0.07 pmol/mg protein, respectively. Forskolin increased the respective levels of cAMP in the a2c- and CYD-AR transfectants to 7.87 ± 0.86 and 8.14 ± 1.12 pmol/mg protein. Data are expressed as percent inhibition of forskolin-induced elevation of cAMP and represent the mean ± S.E. of four separate experiments performed in duplicate.

FIG. 4. Effect of epinephrine on forskolin-induced elevation of cAMP in a2c- and a2D-AR transfectants expressing low receptor densities. Transfectants expressing a2c (1,000 fmol/mg protein), a2c (1,200 fmol/mg protein), and a2D (2,200 fmol/mg protein) receptor subtypes were incubated with 10 μM forskolin plus increasing concentrations of epinephrine for 10 min at 37°C, and cAMP was extracted and assayed as described under “Experimental Procedures.” The basal levels of cAMP in the a2c- and a2D-AR transfectants were 0.82 ± 0.07 and 0.79 ± 0.09 pmol/mg protein, respectively. Forskolin increased the respective levels of cAMP in the a2c- and a2D-AR transfectants to 7.87 ± 0.86 and 8.14 ± 1.12 pmol/mg protein. Data are expressed as percent inhibition of forskolin-induced elevation of cAMP and represent the mean ± S.E. of four separate experiments performed in duplicate.

FIG. 5. Effect of epinephrine on forskolin-induced elevation of cAMP in receptor transfectants expressing high receptor densities. Transfectants expressing a2B (1,000 fmol/mg protein), a2c (1,200 fmol/mg protein), and a2D (2,000 fmol/mg protein) receptor subtypes were incubated with 10 μM forskolin plus increasing concentrations of epinephrine for 10 min at 37°C, and cAMP was extracted and assayed as described under “Experimental Procedures.” The basal levels of cAMP in the a2B-, a2c-, and a2D-AR transfectants were 0.81 ± 0.07, 0.84 ± 0.09, 0.82 ± 0.08, and 8.26 ± 0.97, 8.15 ± 1.04, 8.45 ± 1.13, respectively. Data are expressed as percent inhibition of forskolin-induced elevation of cAMP and represent the mean ± S.E. of four separate experiments performed in duplicate.

terminate if the differences in the ability of a2-AR subtypes to mediate reduction of cellular cAMP are reflected at the receptor/G-protein interface we performed agonist competition binding studies. In a2B- and a2D-AR transfectants, epinephrine displacement curves were biphasic and could be resolved into high and low affinity sites for the agonist (Fig. 6 and Table I). The high affinity state represented 34 ± 3.4% (a2B-AR) and 37 ± 2.9% (a2D-AR) of the total receptor population. The high affinity state of the receptor for agonist was not observed in the presence of 100 μM Gpp(NH)P, a nonhydrolyzable analogue of GTP, or after cell treatment with pertussis toxin. Similar results were obtained in three other transfectants expressing a third receptor subtype, the a2B-AR. In a2B- and a2D-AR transfectants, epinephrine inhibited the effect of forskolin by 30% (a2B-AR) and 72% (a2D-AR) of the total receptor population.
possibility we attempted to identify directly such a receptor expressed in NIH-3T3 fibroblasts. Curves were generated following data with pertussis toxin. Each incubation tube contained protein, ~zc-AR expressing receptor densities of ~650--1200 fmol/mg of protein. Agonist competition curves in a2c-AR transfectants were best fit to a two-site model in control and to a one-site model in the presence or absence of Gpp(NH)p or in membranes derived from cells treated with pertussis toxin (Fig. 6 and Table I). The complete absence of high affinity agonist binding was surprising in that the high affinity state is the functionally coupled receptor population (40) and that this receptor, at least in the intact cell, did mediate a small reduction in the intact cell, did mediate a small reduction in the intracellular levels of cAMP in response to epinephrine. Our inability to detect high affinity agonist binding may be a result of the sensitivity of agonist competition studies in terms of resolving and quantifying low affinity receptor/G-protein interactions or small populations of receptors which may exist in the high affinity state for agonist. To address this possibility we attempted to identify directly such a receptor population using [3H]UK 14,304, an a2-AR selective agonist. As shown in Fig. 7, Gpp(NH)p-sensitive binding of [3H]UK 14,304 was observed over a range of radioligand concentrations. The detection of the receptor-G-protein complex by agonist competition binding studies in azc-AR and a2c-AR but not a2c-AR transfectants suggests a difference in the affinity of the three receptor subtypes for PT-sensitive G-proteins expressed in NIH-3T3 fibroblasts and is consistent with the reduced coupling efficiency exhibited by the a2c-AR transfectants.

Identification of the Pertussis Toxin-sensitive G-protein Recognized by the a2c-AR Subtypes in NIH-3T3 Fibroblasts—Seven known G-proteins (G1,2,3, Gq1,2) are ADP-ribosylated by pertussis toxin. Tr and Te are found exclusively in rod and cone cells of the retina (4). To determine which of these G-proteins are expressed in NIH-3T3 fibroblasts we performed immunoblot analysis utilizing antisera that recognize the a-subunits. Such an analysis indicated the presence in membrane preparations of G11 and G12 but not the a-subunits of G11 or G12 (Fig. 8). Thus, either NIH-3T3 cells do not express G12 or G12 or else they are expressed at very low amounts.

**Table I**

| Transfectant | K<sub>H</sub> (nM) | nH<sub>H</sub> (%) | High affinity sites |
|--------------|------------------|------------------|-------------------|
| azc-AR       |                  |                  |                   |
| Control (4)  | 642 ± 80         | 0.65 ± 0.07      |                   |
| Two sites    | 5 ± 0.4          | 34.36 ± 3.6      |                   |
| Pertussis toxin pre,treatment (3) | 924 ± 102 | 0.82 ± 0.03 |                   |
| a2c-AR       |                  |                  |                   |
| Control (8)  | 460 ± 13         | 0.90 ± 0.01      |                   |
| Gpp(NH)p (8) | 469 ± 11         | 0.91 ± 0.04      |                   |
| a2c-AR       |                  |                  |                   |
| Control (8)  | 893 ± 137        | 0.65 ± 0.07      |                   |
| Two sites    | 7 ± 1            | 37.5 ± 2.9       |                   |
| Pertussis toxin pre,treatment (3) | 1405 ± 138 | 0.86 ± 0.03 |                   |

*K<sub>H</sub> values for high and low affinity states of the receptor for agonist.

1 Hill coefficient.

In azc- and a2c-AR transfectants, agonist competition curves were best fit to a two-site model in control and to a one-site model in the presence of Gpp(NH)p or after pertussis toxin treatment.
G-protein Recognition by α2-Adrenergic Receptor Subtypes

Effect of Gαi2, Gαi3, and Gαs antisera on epinephrine inhibition of forskolin-stimulated adenylyl cyclase activity in α2-AR subtype transfectants

The increase in adenylyl cyclase activity produced by forskolin (1 μM) was determined in the presence or absence of epinephrine (Epi) (10 μM) as described under “Experimental Procedures.” Experiments were conducted in membrane preparations preincubated with rabbit preimmune serum, Gαi2, Gαi3, or Gαs antisera for 60 min at 25°C. In each experiment the serum was diluted 1:50. Assays contained 50 μg of membrane protein in a 100-μl final reaction volume. Data represent the mean ± S.E. of four separate experiments with triplicate determinations.

| Treatment          | α2B | α2C | α2D |
|--------------------|-----|-----|-----|
| Vehicle            | 21 ± 1.18 | 24 ± 1.87 | 19 ± 0.97 |
| Forskolin          | 126 ± 3.45 | 137 ± 4.58 | 133 ± 2.36 |
| Forskolin + Epi    | 86 ± 2.18 | 131 ± 6.27 | 86 ± 2.11 |
| Preimmune serum    | 24 ± 2.15 | 23 ± 2.58 | 16 ± 1.38 |
| Forskolin          | 120 ± 3.17 | 128 ± 6.23 | 109 ± 7.12 |
| Forskolin + Epi    | 84 ± 2.25 | 143 ± 8.82 | 74 ± 6.02 |
| Gαi2 antisera      | 28 ± 2.45 | 37 ± 2.57 | 21 ± 1.24 |
| Forskolin          | 135 ± 4.19 | 207 ± 18.23 | 141 ± 5.54 |
| Forskolin + Epi    | 147 ± 6.18 | 215 ± 25.45 | 137 ± 4.21 |
| Gαi3 antisera      | 20 ± 3.17 | 21 ± 1.78 | 21 ± 1.67 |
| Forskolin          | 118 ± 6.22 | 122 ± 3.64 | 133 ± 4.56 |
| Forskolin + Epi    | 90 ± 4.15 | 120 ± 2.47 | 90 ± 6.18 |
| Gαo antisera       | 22 ± 2.88 | 23 ± 3.17 | 18 ± 0.54 |
| Forskolin          | 123 ± 5.54 | 132 ± 6.54 | 127 ± 2.15 |
| Forskolin + Epi    | 80 ± 3.18 | 137 ± 7.88 | 80 ± 2.18 |

DISCUSSION

α2-AR can modulate a variety of transduction pathways including adenylyl cyclase, phospholipases, and various ion transport mechanisms (20–23). Coupling to various transduction pathways is tissue-dependent and is likely related to the specific function of the cell. The different cellular responses may be mediated by distinct receptor subtypes identified recently by molecular cloning (5–19). Alternatively, the various effector cell responses may be mediated by the same receptor subtype interacting with different G-proteins and/or effector molecules expressed in a particular cell phenotype. The transduction pathway selected by a receptor subtype may also depend on the relative amounts of various G-proteins and/or effector molecules or their colocalization in cellular microdomains. Each of these issues can now be addressed in a defined mammalian cellular environment utilizing the genomic and cDNA clones encoding the receptor subtypes. One basic question arises: Is there a prefered G-protein for each receptor subtype?

We have attempted to address this issue by analyzing receptor/G-protein interaction after stable expression of three rat α2-AR subtypes (RNGα2-C, RG10-α2C, and RG20-α2D) in NIH-3T3 fibroblasts. NIH-3T3 cells do not normally express α2-AR but do express many of the components of the transduction pathways utilized by α2-AR including Gαi and adenylyl cyclase. The subclassification of the cloned receptor subtypes is a matter of some debate (5, 7, 16–18). RG10 (α2C) and RG20 (α2D) are rat genomic clones encoding α2-AR subtypes that are 52% homologous (7). RG10 (α2C) exhibits binding properties similar to the human C-4 receptor (9), and its mRNA is primarily found in brain tissue. RG20 (α2D) exhibits ligand recognition properties distinct from other

FIG. 8. Identification of pertussis toxin-sensitive G-proteins present in NIH-3T3 membrane preparations. The presence of Gαi2 and Gαs in NIH-3T3 cell membranes was determined by immunoblot analysis with antisera that selectively recognize the Gα proteins as described under “Experimental Procedures.” Membrane preparations from whole brain and human platelets were included as internal controls for Gα protein identification. Each lane was loaded with 50 μg of membrane protein. The mark to the left of each autoradiograph indicates the relative migration of the 39,000 nonglycosylated molecular weight standard. In the lower two blots, the levels of Gαi2 (30-s exposure) or Gαs (120-s exposure) were determined in membranes derived from α2C-AR (RG10) and α2D-AR (RG20) transfectants. The two lower blots indicate that the two transfectants express similar levels of Gαi2 and similar levels of Gαs but do not provide information on the relative levels of these two G-proteins. The doublet observed in brain membranes in the Gαi2 blot is due to cross-reactivity of the antiserum with Gαz. The doublet observed in brain membranes in the lower left panel is due to recognition of the common carboxyl terminus of Gαs by the α2-AR antiserum.

The expressed levels of Gαi2 were similar in transfectants expressing either α2C-AR and α2D-AR subtypes (lower left panel, Fig. 8), as was also the case for the levels of Gαs (lower right panel, Fig. 8).

These data suggest that either Gαz or Gαs is the G-protein that permits formation of the high affinity state of the receptor for agonist and thereby mediates the epinephrine effect on cellular cAMP. To determine which of these G-proteins is recognized by the α2-AR subtypes expressed in NIH-3T3 cells we examined the ability of a specific antisera to prevent receptor-mediated inhibition of adenylyl cyclase in membrane preparations. This approach has proven useful for identifying relevant functional interactions of G-proteins with receptors and effectors (42–44). The antisera utilized was obtained from rabbits immunized with COOH-terminal decapeptides of Gαi2, Gαs, or Gαz. The carboxyl-terminal region of these G-proteins is involved in receptor recognition and thus such antipeptide antibodies prevent receptor coupling to G-proteins (45).

In membrane preparations derived from α2C-AR, α2C-AR, and α2D-AR transfectants forskolin (1 μM) stimulated adenylyl cyclase activity 5–7-fold above basal values. In α2C-AR and α2D-AR transfectants epinephrine inhibited forskolin-stimulated adenylyl cyclase activity in a concentration-dependent manner with an IC50 value of 105 nM and 80 nM and a maximal effect of 36 ± 5.2% and 45 ± 4.7%, respectively (Table II). However, in α2C-AR transfectants the effect of forskolin was altered minimally by epinephrine. The effect of epinephrine in α2C-AR and α2D-AR transfectants was prevented by the α2C-AR antagonist rauwolscine and by membrane preincubation with Gαi2 antisera but not Gαs antisera (Table II). Similarly, the high affinity state of the receptor for agonists observed in α2D-AR transfectants was also lost after membrane preincubation with Gαs antisera.3

3. I. Coupy, E. Duzic, and S. Lanier, unpublished observations.
G-protein Recognition by α2-Adrenergic Receptor Subtypes

cloned receptor subtypes but similar to an α2-AR expressed by radioligand binding in submandibular gland, enterocytes, and adipoocytes of rat, and bovine pineal gland (18, 36–38). The RG20 gene encodes a receptor that is 85% homologous to the human C-10 (α2a), but it is termed the α2DP-AR based on its different binding properties (7, 8). RGA α (α2b) is a nonglycosylated α2-AR subtype that is expressed in neonatal rat lung and exhibits binding and structural properties similar to the human C-2 receptor (10–12, 16, 17). The receptor subtypes exhibit the highest homology in the postulated membrane-spanning regions, but sequence divergence is observed in certain domains suggested to interact with G-proteins.

Our results indicate that the α2C-AR differs from the α2B- and α2DP-AR receptor subtypes in its ability to couple to adenylcyclase. A 6-fold greater density of the α2DP-AR relative to the α2DP-AR subtype is required to elicit similar degrees of agonist-mediated effects on cellular levels of cAMP. This difference in coupling efficiency is not related to differences in the levels of Gia or Gao among the transfectants studied and is also observed when adenylcyclase was activated through the prostaglandin E, receptor. In addition, the degree of adenylcyclase stimulation by both forskolin and prostaglandin E, receptor, was identical in the α2B-, α2C-, and α2DP-AR transfectants, suggesting no differences in the levels of catalytic subunit in the selected cell lines.

Agnistion competition binding studies indicate that the difference in the ability of the receptor subtype to couple in an inhibitory fashion to adenylcyclase is reflected at the receptor/G-protein interface. For G-protein-coupled receptors such as the prostaglandin E, receptor. In addition, the degree of adenylcyclase stimulation by both forskolin and prostaglandin E, receptor, was identical in the α2B-, α2C-, and α2DP-AR transfectants, suggesting no differences in the levels of catalytic subunit in the selected cell lines.

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