Coupling of the \( \alpha_{2A} \)-Adrenergic Receptor to Multiple G-proteins

A SIMPLE APPROACH FOR ESTIMATING RECEPTOR-G-PROTEIN COUPLING EFFICIENCY IN A TRANSIENT EXPRESSION SYSTEM*

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It is now widely appreciated that G-protein-coupled cell-surface receptors can modulate distinct signal transduction pathways via coupling to different GTP-binding proteins. In the present study, we have used a transient co-expression approach to study the coupling of a single \( \alpha_{2A} \)-adrenergic receptor (\( \alpha_{2A} \)AR) population to three different G protein subtypes (G\(_1\), G\(_2\), and G\(_3\)) acting on two different cellular effectors in HEK 293 cells. In all cases, the affinity of the receptor for the \( \alpha_{2A} \)-adrenergic agonist, UK14304, is unchanged (\( K_D \approx 670 \) nM). However, there is a dramatic difference in the EC\(_{50}\) of UK14304 in eliciting inhibition of endogenous adenyl cyclase via endogenous G\(_1\) (0.09 \( \mu \)M) versus activation of phospholipase C via co-transfected G\(_3\) (50 \( \mu \)M) or stimulation of endogenous adenyl cyclase via co-transfected G\(_2\) (70 \( \mu \)M) in HEK 293 cells. These findings are consistent with the interpretations that the \( \alpha_{2A} \)AR preferentially interacts with G\(_3\) rather than G\(_1\) or G\(_2\). When the \( \alpha_{2A} \)AR was mutated at Asp\(^{79}\), a residue highly conserved among G-protein-coupled receptors, the mutant D79N \( \alpha_{2A} \)AR lost the ability to couple to G\(_3\) and G\(_2\), and, although it was able to couple to inhibition of cyclase via pertussis toxin-sensitive pathways (G\(_3\)), it did so with a lower potency than observed for the wild-type \( \alpha_{2A} \)AR (EC\(_{50}\) = 7.2 ns). The most straightforward interpretation of these data is that the D79N mutation in the \( \alpha_{2A} \)AR reduces the efficiency of coupling of the \( \alpha_{2A} \)AR to all G-proteins, thus eliminating signal transduction through those pathways less efficiently coupled to the \( \alpha_{2A} \)AR. Since the transient expression assays described permit manipulation of the structure of both the receptor and the G-protein, the present strategies could be exploited to delineate the complementary domains specifying the affinity and/or efficacy of receptor coupling to distinct GTP-binding proteins.

A large family of cell surface receptors conforming to the predicted topography of seven transmembrane spanning proteins elicit their physiological effect by first coupling to and activating a population of heterotrimeric GTP-binding proteins that then mediate the responses of a plethora of cellular effectors, including enzymes and ion channels. Recently, it has been appreciated that cell surface receptors often are coupled to a variety of effectors, and this is likely due to the interaction of the receptor with multiple distinct GTP-binding proteins (1–8).

A number of experimental strategies have revealed that a single receptor population is capable of interacting with multiple GTP-binding proteins. For example, reconstitution of purified receptors with differing GTP-binding proteins into lipid vesicles or a mixed detergent/lipid milieu restores receptor-G-protein coupling disrupted by biological detergents, as assessed using agonist-dependent GTPase or GTP\(_\gamma\)S\(^{32}\) binding activities. Such strategies have revealed, for example, that the \( \beta_{2}\)-adrenergic receptor can couple not only to G\(_1\), but also to G\(_3\) (9) and that the muscarinic receptor can couple both to G\(_3\) and to G\(_1\) (10). The ability of receptors to couple to multiple families of GTP-binding proteins can also be revealed by overexpression of a given receptor or G-protein in heterologous cells. Such studies, for example, have demonstrated at the \( \alpha_{2A} \)AR can couple not only to pertussis toxin substrates (G\(_1\) and G\(_2\)), as it does in physiological settings, but also to activation of phospholipase C (3), presumably through interaction with G\(_3\)/(G\(_{11}\)), and to stimulation of adenyl cyclase through G\(_1\) (11, 12). Similarly, co-expression of \( \alpha_{2A} \)AR and G\(_1\) leads to the pertussis toxin-insensitive inhibition of adenyl cyclase (13).

The biochemical reconstitution or heterologous expression strategies of recombinant proteins outlined above have been useful in identifying which receptor-G-protein interactions can occur, and have demonstrated that receptors are capable of interacting with a wide array of GTP-binding proteins when high concentrations of receptor and G-protein are provided in biochemical or recombinant "reconstitution" paradigms. In contrast, a greater fidelity of receptor-G-protein interactions apparently occurs in the physiological setting. For example, virtually all \( \alpha_{2A} \)AR receptor-mediated physiological effects are sensitive to pertussis toxin, implying a role for the G\(_1\) and G\(_2\) GTP-binding protein family in mediating the physiological consequences of \( \alpha_{2A} \)AR activation in vivo. Similar conclusions about fidelity in receptor-effector interactions in vivo derive from recent studies using antisense strategies which reveal that receptor-G-protein interactions giving rise to a particular cellular response are very stringent in terms of the \( \alpha \) (14) as well as the \( \beta \) (15) and \( \gamma \) (16) subunits involved.

The present study was undertaken to measure receptor-GTP-binding interactions that occur in a given physiological setting, but rather to provide a straightforward analysis of the...
efficacy of coupling of a particular receptor, the α2AAR, to various G-proteins expressed in intact cells and thereby allow a prediction of the selectivity of receptor interaction with given sub-populations of G-proteins in the intact cell. For these studies, the α2AAR was introduced into HEK-293 cells, and its coupling to various GTP-binding proteins and subsequent effectors was assessed following transient expression of the receptor and particular G-protein α subunits. The studies revealed that the α2AAR preferentially interacts with the G_{i} protein family and to a lesser extent with G_{o} and G_{z}. Interestingly, the D79N mutation of the α2AAR decreases receptor coupling to the G_{i} protein as well as to G_{o} and G_{z} such that, following this mutation, only receptor-mediated attenuation of adenylyl cyclase through the G_{i} pathway can be detected, and this inhibition occurs at higher receptor occupancy than required for inhibition of adenylyl cyclase by the wild-type α2AAR. This transient expression approach, then, provides a reasonably rapid and straightforward analysis of the efficacy of receptor coupling to a particular GTP-binding protein family. Such an analysis could be of value in predicting the preferential signal transduction pathway utilized by a given receptor system or, following intentional structural alterations via mutagenesis, the domains within receptors and/or G-protein α subunits involved in specifying the affinity or efficacy of a given receptor-G-protein interaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—The wild-type porcine α2AAR (17) and the α2AAR D79N mutant in which Aasp was mutated to C (2× C-dubbed D79N in the present study (18)) were inserted in the pCMV4 vector. Other cDNAs were generous gifts: murine G_{o} (Melvin I. Simon, California Institute of Technology, Pasadena CA) (19) in pCDNA (Invitrogen), human LH receptor in pCIS vector (Deborah Segaloff, University of Iowa, Iowa City, IA) (20), and rat G_{i} (21) in pCDNA. The α2AAR agonist, bromoxidine (UK14304), was obtained from Research Biochemical Inc., as part of the National Pituitary Agency, NIDDK, Baltimore, MD; AG1-8X Dowex columns (Bio-Rad); plasmid purification columns (Qiagen, Chatsworth, CA); [3H]yohimbine (Du Pont NEN). All other reagents were obtained from Sigma.

**Plasmid Transfections**—HEK-293 cells were propagated in minimum essential medium (Earle's) containing 10% fetal calf serum in 5% CO_{2} in 100 mm dishes or as monolayers (250 µl/well) in the presence of DEAR-dextran (250 µg/ml) and cholera toxin (100 µM) for 90 min. Cells were washed with phosphate-buffered saline containing 10% dimethyl sulfoxide for 2 min, then washed once with phosphate-buffered saline and allowed to grow overnight. The next day cells were detached in 24-well plates (1-2×10⁶ cells/well) for analysis of receptor binding or for detection cAMP and inositol phosphate (IP) production.

**cAMP and IP Assays**—cAMP and IP production in HEK-293 cells were measured as described (22, 23). For cAMP, data are presented as 1000 times the ratio of [3H]cAMP/[3H]ATP + [3H]cAMP and [3H]H_{100} to IP as 1000 times the ratio of [3H]H_{100}/[3H]inositol + [3H]IP. Data were analyzed using GraphPad/InPlot Software.

**[3H]Yohimbine Binding Assay**—HEK-293 cells were transfected with the porcine α2AAR (3 µg of DNA/100-mm dish), and membranes were prepared and assayed as described elsewhere (15). Binding was analyzed in the presence of 100 mM NaCl, to maximally impose regulatory effects of monovalent cations (24), and 100 µM Gpp(NH)p, to eliminate effects of GTP-binding protein-receptor interactions on α2AAR affinity for agonists (25). Although agonist potency at the D79N mutant α2AAR is not modulated by monovalent cations (26), in contrast to wild-type α2AAR, Na+ was present in the binding assay for evaluation of receptor affinity for agonist at both wild-type and D79N α2AAR.

**RESULTS AND DISCUSSION**

To study the EC_{50} for a given α2-adrenergic agonist, UK14304 (bromoxidine), on second messenger production via a variety of G-proteins, we expressed the α2AAR in HEK-293 cells, which lack endogenous α2AAR binding activity, in the absence or presence of DNA coding for G_{i} and G_{o}. Regardless of whether the α2AAR was transfected in the absence or presence of G-proteins, the K_{p} for UK14304 at the receptor was 0.67 µM (data not shown).

Fig. 1 demonstrates that introduction of α2AAR into HEK 293 cells permits detection of inhibition of endogenous adenylyl cyclase by the α2AAR due to interaction with endogenous G-proteins. To facilitate detection of inhibition of the adenylyl cyclase enzyme by transfected α2AAR, DNA coding for the α2AAR was co-transfected with that coding for the hCG/LH receptor, which is not expressed in HEK-293 cells. It has been determined empirically that the same fraction of cells that take up the DNA coding for α2AAR also take up that coding for the LH receptor, and thus UK14304-elicited inhibition of cAMP production elevated by hCG provides a sensitive tool for monitoring exogenous α2AAR-mediated inhibition of adenylyl cyclase via a pertussis toxin-sensitive, G_{i}-coupled pathway (22).

As shown in Fig. 1, the EC_{50} for inhibition of cAMP accumulation by UK14304 was 0.07 ± 0.05 (n = 4). The UK14304-elicited inhibition of cyclase was sensitive to pertussis toxin, indicating that G_{i} mediates this effect. In the absence of introduction of the α2AAR, no inhibition of cAMP accumulation by UK14304 could be detected.

Fig. 2 demonstrates that co-expression of the α2AAR with G_{i} results in the activation of phospholipase C as measured by production of [3H]IP. Although HEK-293 cells do have endogenous G_{i} or members of the G_{i} family, activation of phospholipase C by the α2AAR required co-expression of G_{i}. Further evidence that α2AAR activation of phospholipase C was occurring via a G_{i}-dependent pathway was the insensitivity of inositol phosphate production to pertussis toxin. The EC_{50} of UK14304 for activation of the phospholipase C pathway was 60 ± 22 nM (n = 3).

In contrast to inhibition of cyclase, stimulation of endogenous adenylyl cyclase via α2AAR in HEK 383 cells could be detected only following co-expression of α2AAR with G_{o} (Fig. 3). As also shown in Fig. 3, UK14304-elicited stimulation of adenylyl cyclase via the α2AAR could be increased by prior pertussis toxin treatment, presumably due to the abolition of receptor-mediated inhibition of the cyclase. The EC_{50} of UK14304 for stimulation of cyclase via G_{o} was 70 ± 3 nM.

In all cases, the pathways activated by UK14304 were strictly dependent on the expression of transfected α2AAR. In addition, α2AAR receptor density achieved in each transfection, as assessed using Scatchard transformation of [3H]yohimbine.
saturation binding data, was not significantly different:

whether the α2AAR was transfected alone or with Gα or Gq and ranged from 2.8 to 3.6 pmol [3H]yohimbine binding per mg of protein. Thus, the dramatic difference in EC50 of UK14304 for inhibition of adenyl cyclase (0.07 nM) via activation of phospholipase C via Gq (60 nM) or stimulation of a adenyl cyclase versus Gα (70 nM) when compared to the Kp of the α2AAR for UK14304 expressed in HEK 293 cells, 670 nM, suggests that inhibition of adenyl cyclase is achieved by occupancy of only a small fraction of the α2aAR that are expressed in the HEK 293 cells when compared to effects of the α2aAR on Gqi or Gq-mediated signal transduction pathways. The fact that activation of adenyl cyclase and of phospholipase C in HEK 293 cells requires both increased fractional occupancy of the α2aAR (EC50 = 60–70 nM, i.e. 1000-fold greater agonist concentrations required for inhibition of adenyl cyclase via Gq and co-expression of heterologous Gαs or Gqα subunits indicates that encounters among α2AR and these signal-transducing molecules are of lesser affinity (lower frequency) and of lower efficacy than among α2aAR, Gq, and cyclase. Said another way, the present findings suggest that α2aAR preferentially associates with GTP-binding proteins of the Gq family in contrast to the Gα or Gq family.

Implicit in the above interpretations is the premise that agonist potency in activating various signal transduction pathways via a single receptor population reflects not only the intrinsic receptor affinity for the agonist but also the efficacy of receptor coupling to a given G-protein population and the degree of amplification in downstream reactions (27). We therefore decided to compare the potency of UK14304 in inhibition of adenyl cyclase via Gqi, activation of adenyl cyclase via Gq, and stimulation of phospholipase C via Gq when HEK 293 cells were transfected with the D79N mutant of the α2aAR instead of the wild-type α2aAR. Such studies should allow a direct comparison of receptor-G-protein coupling efficiency of the wild-type versus D79N α2aAR, as receptor affinity for agonist is not markedly different at the two receptors (Kp = 70 nM at the wild-type α2aAR and 300 nM at the D79N α2aAR) and the downstream consequences of G-protein activation should be the same for both preparations. Previous studies with other G-protein-coupled receptors mutated at a topographically homologous position indicate that receptor interaction with G-proteins is perturbed by this mutation (28–30), and similar findings have been reported for the α2aAR, at least as assessed in broken cell assays of receptor-G-protein coupling (18, 31).

In contrast, introduction of the D79N α2aAR mutant into AtT20 cells results in detectable α2a-mediated inhibition of cAMP accumulation in intact cells and suppression of voltage-gated Ca2+ currents measured in the cell-attached patch configuration, despite a loss of D79N α2aAR-elicited activation of receptor-operated K+ currents in the AtT20 cells (18). These findings could arise either by selective loss of the ability of the D79N receptor to couple to the G-protein population that mediates K+ channel activation or, alternatively, by perturbation of D79N α2aAR coupling to all GTP-binding proteins, with sufficient coupling remaining to permit inhibition of cAMP accumulation and of voltage-gated Ca2+ currents via the D79N α2aAR but not sufficient to activate receptor operated K+ channels via this mutant receptor.

To evaluate whether the D79N mutation of the α2aAR perturbs receptor-G-protein coupling, as manifest in the transient transfection assays described above, HEK 293 cells were transfected with DNA coding for the D79N α2aAR mutant in the absence or presence of DNA coding for Gα or Gqα. The receptor density achieved in these transfections (2.5 pmol of [3H]yohimbine binding per mg of protein) was comparable from that for the wild-type receptor (2.8–4.6 pmol of [3H]yohimbine binding per mg of protein). Nonetheless, as shown in Fig. 4A, UK14304 is considerably less potent in inhibiting adenyl cyclase through the D79N receptor (EC50 = 7.2 nM) than through the wild-type α2aAR (EC50 = 0.09 nM), implying that the mutant D79N α2aAR is less efficacious than the wild-type α2aAR in interacting with the Gα proteins that mediate inhibition of adenyl cyclase. This difference in EC50 values for wild-type versus D79N α2aAR in inhibiting adenyl cyclase is not unique to the UK14304 agonist, but also is detected for the agonist

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**FIG. 2.** Dose-response of the α2aAR agonist UK14304 for stimulation of phospholipase C through transfected Gα. HEK-293 cells were transfected with 3 μg of α2aAR cDNA and 9 μg of Gαs cDNA or 9 μg of vector per 2 × 10⁶ cells. IP production was measured under basal conditions and under stimulation with the indicated concentrations of UK14304, without and with pertussis toxin (PT). Data are calculated as 1000 times the ratio of [3H]cAMP ([H] cAMP) over [3H]lyohimbine ([H] lyohimbine). The data shown are the mean ± S.D. of triplicate measurements in the same experiment. Two other dose-response experiments yielded similar results. In the absence of added Gα, the IP production detected in the presence of 10–519 K14304 was unperturbed by overnight treatment with 2400 ng/ml pertussis toxin.

**FIG. 3.** Dose-response of the α2aAR agonist UK14304 for stimulation of adenyl cyclase through transfected Gα. HEK-293 cells were transfected with 3 μg of α2aAR cDNA and 9 μg of Gαs cDNA or 9 μg of vector, cAMP production was measured under basal conditions and under stimulation with the indicated concentrations of UK14304, without or with pertussis toxin (PT). Data are presented as 1000 times [3H]cAMP ([H] cAMP) over [3H]ATP ([H] ATP) + [3H]cAMP. Data shown are the mean ± S.D. of triplicate measurements in the same experiment. Two other experiments yielded similar results.
clonidine (EC_{50} for inhibition of adenyl cyclase via wild-type α_{2A}AR at 0.07 nM; EC_{50} via D79N α_{2A}AR at 10 nM) data not shown. Furthermore, the mutant D79N α_{2A}AR receptor is not capable of activating phospholipase C via G_q (Fig. 4B) or adenyl cyclase via G_s (Fig. 4C). Although these results could be interpreted as a selective effect of this mutation on coupling of α_{2A}AR to G_q or G_s, the finding that agonist potency for inhibition of adenyl cyclase via G_s was markedly decreased for the D79N mutant when compared to the wild-type α_{2A}AR suggests that mutation of Asp^{79} to Asn^{79} in the α_{2A}AR creates a receptor that, upon agonist occupancy, is less effective in coupling to all G-proteins, with the result of eliminating signal transduction through these G-proteins less efficiently coupled to the α_{2A}AR. Consistent with this interpretation is our observation that transfection of 1/10 of the α_{2A}AR plasmid (using 0.3 μg instead of 3.0 μg of DNA coding for α_{2A}AR/100-mm dish) results in no reduction in the extent of attenuation of adenyl cyclase by the wild-type α_{2A}AR, but a loss in readily detectable attenuation by the D79N α_{2A}AR.

SUMMARY

A transient co-transfection approach has been used to study the coupling of a single α_{2A}AR population to multiple GTP-binding proteins. We observed that the EC_{50} of an agonist (UK14304) was dependent on which G-protein the wild-type α_{2A}AR was coupled to in order to elicit its effects on second messenger production. The EC_{50} of the agonist was not due to changes in intrinsic receptor affinity for UK14304, as receptor affinity for UK14304 was indistinguishable regardless of the G-protein composition of the transfected HEK293 cells evaluated. Instead, the EC_{50} reflects the efficiency of receptor-G-protein coupling as well as G-protein-effector interaction to elicit second messenger production. Furthermore, the comparison of agonist potency eliciting second messenger production via the wild-type versus the D79N mutant α_{2A}AR allowed a direct comparison of receptor-G-protein coupling efficiency for these two receptors, as the downstream consequences of G-protein activation elicited by wild-type versus D79N α_{2A}AR should not be different in these experiments, and the intrinsic affinity of the wild-type (K_D = 0.67 μM) and D79N (K_D = 0.3 μM) α_{2A}AR for UK14304 are essentially indistinguishable. The lower potency of the D79N α_{2A}AR in inhibiting adenyl cyclase via the endogenous G_q proteins and the inability of the mutant α_{2A}AR to activate phospholipase C via G_q or adenyl cyclase via G_s is consistent with the interpretation that the D79N mutation perturbs the efficiency of agonist-facilitated receptor-G-protein interaction.

It is clear that the experimental paradigm of transient expression of receptors and G-proteins described in the present report should be of considerable value in comparing the efficacy of receptor-G-protein coupling for wild-type and intentionally mutated receptors, and perhaps assist in revealing the preferred signal transduction pathway of newly discovered receptors identified by molecular cloning strategies.

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