Agonist-Directed Trafficking of Porcine $\alpha_{2A}$-Adrenergic Receptor Signaling in Chinese Hamster Ovary Cells: $I$-Isoproterenol Selectively Activates $G_s$

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ABSTRACT

In this study, we investigated the hypothesis of agonist-directed trafficking of receptor signaling for the $\alpha_{2A}$-adrenergic receptor ($\alpha_{2A}$-AR), $\alpha_{2A}$-ARs couple to both $G_s$ and $G_l$ to stimulate or inhibit adenyl cyclase activity. Chinese hamster ovary-K1 cell lines expressing the porcine $\alpha_{2A}$-AR at high ($\alpha_{2A}$-H) and low ($\alpha_{2A}$-L) levels were used to estimate the relative efficacies (R.e.s) of a series of agonists for the $G_s$ and $G_l$ pathways. $G_s$-mediated responses were measured after pertussis toxin treatment to inactivate $G_l$ in $\alpha_{2A}$-H, whereas $G_s$ responses were measured in $\alpha_{2A}$-L, where $G_s$ responses were absent. The full agonist UK-14,304 showed a large receptor reserve for $G_l$ responses in $\alpha_{2A}$-H but little receptor reserve for $G_s$ responses in $\alpha_{2A}$-L for $G_l$ responses in $\alpha_{2A}$-H or for $G_s$ responses in $\alpha_{2A}$-L. With the exception of $I$-isoproterenol (ISO), all agonists showed similar R.e.s at the $\alpha_{2A}$-AR for $G_s$ and $G_l$ responses, with rank orders of R.e.s as follows: $\beta$-epinephrine $= l$-norepinephrine $= UK-14,304 > p$-aminoclonidine $\geq BHT-920 \geq BHT-933 > clonidine = p$-iodoclonidine $\geq$ xylazine $\geq$ guanabenz. Interestingly, ISO had the highest efficacy at the $\alpha_{2A}$-AR for activating $G_s$ versus $G_l$ (9-fold higher); however, it had low potency for both. By several criteria, the ISO response was mediated by the $\alpha_{2A}$-AR, supporting the hypothesis of agonist-directed trafficking of receptor signaling or agonist-specific $G$ protein selectivity. In contrast, the apparent $G_l$ pathway selectivity of oxytetracycline appears to be mediated by an endogenous serotonergic receptor. It is intriguing that a classic $\beta$-AR agonist that activates $G_l$ through $\beta$-ARs also appears to produce a $G_s$-selective conformation of the $\beta$-coupled $\alpha_{2A}$-AR.

The hypothesis of agonist-directed trafficking of receptor signaling (ADTRS; Kenakin, 1995) predicts that when a receptor signals through two or more independent signal transduction pathways, the relative efficacies (R.e.s) of a series of agonists may differ for the pathways. This contrasts with the classic concept that intrinsic efficacy is a solely agonist-dependent pharmacodynamic parameter (Furchgott, 1966). This hypothesis builds on the ideas that a receptor can exist in distinct states (or conformations) and that the ability of those states to activate different $G$ protein types or subtypes may differ.

The idea of ADTRS led to the development of the “N-state receptor models,” in which the receptor is assumed to exist in N states that may be “inactive” (R) or “active” (R*, R**, etc.). As a special case of the N-state receptor models, Leff et al. (1997) developed a mathematical model for three receptor states that accommodates the concept of ADTRS. They were able to predict differential R.e.s from this model. In this regard, Berg et al. (1998) recently reported strong evidence for the existence of pathway-dependent R.e.s for a series of five agonists at 5-hydroxytryptamine (5-HT)2A and 5-HT2C receptors when looking at the phospholipase C and phospholipase A2 signal transduction pathways. They also pointed out that for ADTRS to be possible, it is critical that the independent pathways diverge at the receptor $G$ protein level (e.g., receptor activates two $G$ proteins independently, leading...
ing to the transduction of the stimulus through two separate effector pathways) and not downstream from a common G protein (e.g., receptor activates a G protein such as $G_i$ that subsequently activates phospholipase C, which in turn activates two independent effector pathways via release of inositol trisphosphate and diacylglycerol).

According to the three-state model by Leff et al. (1997), two cases can be distinguished. The first is when the two effector pathways result in two independently measurable responses, as in the work of Berg et al. (1998) mentioned earlier. Second, the two pathways can diverge and eventually recombine to modulate one measurable response (e.g., regulation of adenylyl cyclase by both $G_s$ and $G_i$ proteins). If one pathway is isolated by inactivating the other selectively, R.e.s can be determined for the active pathway. It should now be possible to obtain an estimate of the R.e.s for each pathway independently, and theoretically their R.e.s may be different.

$\alpha_2A$-Adrenergic receptors ($\alpha_2A$-ARs) have previously been shown to activate three G proteins: $G_s$, $G_i$, and $G_q$ (Eason et al., 1992; Chabre et al., 1994). The former study reported a pertussis toxin-insensitive stimulation of cAMP accumulation in Chinese hamster ovary (CHO) cells expressing high amounts of $\alpha_2A$-AR. In addition to functional studies, direct, agonist-dependent, physical coupling of the $\alpha_2A$-AR to $G_s$ was demonstrated (Eason et al., 1992). For the $G_q$ coupling, Chabre et al. (1994) used human embryonic kidney (HEK) 293 cells transiently transfected with the porcine $\alpha_2A$-AR and murine $G_q$ or rat $G_s$. They estimated that the efficiency of coupling of the $\alpha_2A$-AR to endogenous $G_i$ was approximately 1000 times higher than that to $G_s$ or $G_q$.

Although no conclusive evidence was provided to support the hypothesis that ADTRS may occur for $\alpha_2$-ARs, three previous reports suggested such a possibility. Using CHO cells expressing the human $\alpha_2A$-AR ($\alpha_2C10$), Eason et al. (1994) showed a rank order of intrinsic activities of agonists for the $G_s$ signal transduction pathway to be epinephrine = norepinephrine $>$ UK-14,304 $>$ BHT-933 $>$ BHT-920 $>$ oxymetazoline. For the $G_i$ signal transduction pathway, they found that the intrinsic activities of these agonists were remarkably similar. They did not, however, take into account receptor reserve, so the true relative efficacies of these drugs cannot be determined from their data. Kenakin (1995) found that oxymetazoline selectively activated $G_i$ (compared with $G_s$) in CHO cells stably transfected with $\alpha_2A$-AR. Yang and Lanier (1999) found differential regulation of $G_i$ and $G_s$ by clonidine and epinephrine. A potential mechanism for ADTRS has been suggested by reports that different amino acid residues of the $\alpha_2A$-AR are required for $G_i$ and $G_s$ activation (Eason and Liggett, 1996; Wade et al., 1999).

In the current study, we asked whether convincing evidence for the ADTRS hypothesis could be found for the regulation of $G_s$ and $G_i$ by the $\alpha_2A$-AR. To account for the difference in spare receptors (for a full agonist) when measuring $G_s$ or $G_i$ responses, we used two CHO-K1 cell lines: one expressing the porcine $\alpha_2A$-AR at high levels and the other expressing the porcine $\alpha_2A$-AR at low levels. We estimated the R.e.s for a series of agonists for the $G_s$ and $G_i$ signal transduction pathways, measuring $[^3H]$cAMP accumulation as the response. Interestingly, $l$-isoproterenol (ISO), but not oxymetazoline, exhibits ADTRS.

### Experimental Procedures

#### Materials

**Radiochemicals.** $[2-^3H]$Adenine (21–25 Ci/mmol) was obtained from Amersham Life Science (Piscataway, NJ). $[^3H]$Yohimbine (74.5–78 Ci/mmol) was obtained from DuPont-New England Nuclear (Boston, MA).

**Other Chemicals.** CAMP, ATP, 3-isobutyl-1-methyl-xanthine (IBMX), $l$-epinephrine-$(-)$-bitartrate, ISO, and yohimbine HCl were purchased from Sigma (St. Louis, MO). UK-14,304, $p$-iodoclonidine HCl, $l$-norepinephrine bitartrate, BHT-933 dihydrochloride, xylazine HCl, and $S$(-)$-$cyanoopindolol hemifumarate were obtained from Research Biochemicals Inc. (Natick, MA). Clonidine HCl and $p$-aminochloridaine were purchased from Boehringer Ingelheim (Ingelheim, Germany). Guanabenz acetate was obtained from Wyeth Laboratories (Philadelphia, PA). BHT-920 Cl$_2$ was purchased from Dr. Karl Thomaes Inc. (Biberach, Germany). Oxymetazoline HCl was purchased from Schering Corporation (Bloomfield, NJ). Propranolol HCl was purchased from Ayerst Laboratories Inc. (New York, NY). Benextramine tetrahydrochloride monohydrate was obtained from Aldrich (Milwaukee, WI). Pertussis toxin (PTX) was obtained from List Biological Laboratories (Campbell, CA). Forskolin was obtained from Calbiochem-Novabiochem Corp. (San Diego, CA). LipofECTAMINE and genetecin (G418) were obtained from Life Technologies (Gaithersburg, MD). Fluorescein-conjugated 12CA5 anti-hemagglutinin monoclonal antibody was purchased from Boehringer Mannheim (Indianapolis, IN).

**$\alpha_2A$-AR Expressing Cell Lines.**

In this study, we used several cell lines. The first are CHO-K1 cell lines expressing high concentrations of either the wild type or one of two mutant porcine $\alpha_2A$-ARs (Wade et al., 1999). All receptor constructs contain an amino-terminal HA tag. The receptor concentrations were estimated as 19 ± 2 pmol/mg membrane protein for the wild-type $\alpha_2A$-AR (clone 1 designated $\alpha_2A$-H in this report), 10 ± 1 pmol/mg for the R3 mutant $\alpha_2A$-AR (mutating RW5GR to AWAGA at residues 361–365 of the receptor, designated $\alpha_2A$-R3 in this report), and 36 ± 3 pmol/mg for the B2 mutant (mutating basic residues 388–391 of the membrane-proximal 13c region to form BXXA, designated $\alpha_2A$-B2 in this report). The $\alpha_2A$-R3 and $\alpha_2A$-B2 mutations disrupt coupling to $G_s$ and $G_i$, respectively (Wade et al., 1999).

For the purpose of this study, we isolated two additional CHO-K1 cell lines expressing a low concentration of the wild-type porcine $\alpha_2A$-AR. This was done with another flow cytometry sorting selection from the original transformation of Wade et al. (1999) from which the WT (clone 1) had been isolated. The cell line expressing the lowest concentration of the $\alpha_2A$-AR, as determined by $[^3H]$Yohimbine binding ($B_{max}$ $-$ 1 pmol/mg, clone 101), was selected for this study (designated $\alpha_2A$-L). This line exhibited a similar EC$_{50}$ value for the dose-response curve of UK-14,304 through the $G_s$ pathway as did $\alpha_2A$-H through the $G_i$ pathway, simplifying comparison of the $G_s$ and $G_i$ responses. The Neo cells containing the selection plasmid but no $\alpha_2A$-AR vector were used as controls.

#### Measurement of Whole-Cell $[^3H]$cAMP Accumulation

CHO-K1 cells were maintained in Ham's F-12 medium with 10% fetal bovine serum, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin at 37°C in 5% CO$_2$. Selection for stable expression was maintained by the addition of 0.4 mg/ml G418 (active). $[^3H]$cAMP accumulation was determined in whole cells in 24-well plates as described by Wade et al. (1999), adding 1 $\mu$Ci/well $[^3H]$adenine and, when indicated, 100 ng/ml PTX for at least 18 h before the assay. Cells were then washed once with Dulbecco's modified Eagle's medium (DMEM), after which the assay was initiated by adding DMEM with 1 mM IBMX and 30 $\mu$M forskolin and the appropriate drug or drugs. After a 20-min incubation, the medium was aspirated, and the reaction was terminated with 1 ml of 5% trichloroacetic acid (TCA) containing 1 mM...
ATP and 1 mM CAMP. The acid-soluble nucleotides were separated on Dowex and alumina columns as described by Salomon et al. (1974). The cAMP accumulation was normalized by dividing the \(^{[3]H}\)cAMP counts by the total \(^{[3]H}\)nucleotide counts. The control percent conversion of ATP to cAMP was 10 to 14% for non-PTX-treated cells \((n = 81)\) and 3.7 to 7.4% for PTX-treated cells \((n = 66)\) and did not vary significantly for high, low, or no \(\alpha_{2A}\)-AR expression. This percent conversion value was then divided by the corresponding value obtained with only IBMX and forskolin and no drug (to calculate percent of control).

**Ligand Binding Assays**

The \(K_i\) values of the \(\alpha_{2A}\)-AR agonists were determined from competition binding curves in whole cells against 5 nM \(^{[3]H}\)yohimbine \((K_D = 3 \text{ nM})\). The buffer used in these binding studies was comparable with that used for measuring cAMP accumulation. Cells were plated and incubated as before but without \(^{[3]H}\)adenine and PTX. Cells were then washed once with OptiMEM, after which the assay was initiated by adding OptiMEM with 5 nM \(^{[3]H}\)yohimbine and different concentrations of the testing drug. After a 30-min incubation, the medium was aspirated, the cells were washed twice, and the reaction was terminated with 1 ml of 5% TCA and allowed to stand for at least 30 min to allow the cells to lyse. The TCA from each well was then transferred directly into scintillation vials using transfer pipettes, and the \(^{[3]H}\)yohimbine was counted.

**Pharmacological Receptor Inactivation**

Benexatrine was used as an irreversible competitive antagonist of \(\alpha_{2A}\)-ARs. The cells were incubated overnight in 24-well plates as described earlier. The cells were then washed once with DMEM and incubated with 0, 1, 10, or 100 \(\mu\)M benexatrine in PBS (containing 0.8% NaCl, 0.02% KCl, 0.09% \(\text{Na}_2\text{HPO}_4\), and 0.02% \(\text{KH}_2\text{PO}_4\)) for 20 min at room temperature. This was followed by two washes with DMEM. With the second wash, the cells were let to stand in the DMEM for at least 5 min to ensure that all unbound benexatrine was removed. The measurement of \(^{[3]H}\)cAMP accumulation then proceeded as described earlier.

**Data Analysis**

Functional data, except where indicated otherwise, were obtained from three or more independent and comparable experiments, each in triplicate, and expressed as mean ± S.E.

Analyses of dose-response curves were made with the nonlinear least-squares method of the computer program Prism (GraphPad Software, San Diego, CA), setting the Hill slope factor at 1. Results are expressed as the mean ± S.E. To verify statistical significance of differences between mean values, the nonparametric Student’s \(t\) test was used. After the Bonferroni correction for multiple comparison, a value of \(P < .05\) was taken as statistically significant.

The relative intrinsic activity (RIA) was determined from functional data and expressed as the maximal response (\(E_{\text{max}}\)) of an agonist relative to that of the full agonist UK-14,304. The apparent dissociation constant of an agonist-receptor complex \((K_\alpha_{\text{app}})\), and the fraction of receptors still functional after partial receptor alkylation (\(q\)) was estimated from Furchgott analysis of dose-response curves of UK-14,304 before and after partial receptor alkylation (Furchgott, 1966).

Because there appeared to be a small receptor reserve remaining for the full agonists (i.e., \(\alpha\)-epinephrine, \(\alpha\)-norepinephrine, UK-14,304) in \(\alpha_{2A}\)-L for \(G_i\) and \(\alpha_{2A}\)-H for \(G_s\) responses, we estimated the R.e.s of full agonists as follows:

\[
\text{R.e.} = \frac{E_{50}\text{(drug)}}{E_{50}\text{(UK-14,304)}}
\]

where \(E_{50}\) is the response at a drug concentration equal to its \(K_i\) value. Relative efficacies of partial agonists were determined as:

\[
\text{R.e.} = \frac{E_{\text{max}}\text{(drug)}}{S_{\text{max}}\text{(UK-14,304)}}
\]

where \(S_{\text{max}}\) and \(E_{\text{max}}\) are as defined later.

This analysis for full agonists is similar to that used by Van Rossum (1966) and Van den Brink (1977) to define a “corrected” intrinsic activity constant \(\alpha^3\) or by Venter (1997) to define the efficacy-related parameter \(e^E\). It assumes that 50% of the functionally coupled receptors are occupied at a concentration of agonist equal to the \(K_i\) value and that the relationship between receptor occupation and stimulus is linear over the range studied. Because the maximal stimulus should be proportional to twice the stimulus at the \(K_i\), we also define \(S_{\text{max}}\) to equal two times the response (linearly related to stimulus) obtained at the \(E_{50}\). Supporting this assumption of linearity, the \(S_{\text{max}}/E_{\text{max}}\) value for UK-14,304 determined in this manner is also similar to the \(S_{\text{max}}/E_{\text{max}}\) value from the \(q\) value estimated from Furchgott analysis before and after partial receptor alkylation (see above), where \(E_{\text{max}}\) was obtained before partial receptor alkylation. In this case, \(S_{\text{max}}\) was estimated from \(E_{\text{max}}/q\), where \(E_{\text{max}}\) represents the reduced \(E_{\text{max}}\) after partial receptor alkylation.

**Results**

\(\alpha_{2A}\)-ARs Expressed in \(\alpha_{2A}\)-H and \(\alpha_{2A}\)-L Are Comparable Pharmacologically. From saturation binding with \(^{[3]H}\)yohimbine, the \(B_{\text{max}}\) values in \(\alpha_{2A}\)-H and \(\alpha_{2A}\)-L cells were 2.0 ± 0.1 \(\times\) 10\(^6\) and 0.27 ± 0.09 \(\times\) 10\(^6\) \(\alpha_{2A}\)-ARs per cell, respectively. Thus, \(\alpha_{2A}\)-H cells express ~10 times more of the porcine \(\alpha_{2A}\)-AR than \(\alpha_{2A}\)-L cells. The \(pK_i\) values for yohimbine in \(\alpha_{2A}\)-H and \(\alpha_{2A}\)-L were similar, being 8.57 ± 0.06 and 8.57 ± 0.24, respectively.

In the absence of PTX, \(G_i\)-mediated inhibition of adenylyl cyclase by the full agonist UK-14,304 predominates over the \(G_s\) response (Fig. 1, bottom). The \(E_{50}\) value for UK-14,304 in the \(\alpha_{2A}\)-H cells (~PTX) is ~1000-fold lower than the \(K_i\) value determined from \(^{[3]H}\)yohimbine competition (Table 1), suggesting that there is a large receptor reserve. The \(E_{50}\) value of the dose-response curve for \(\alpha_{2A}\)-L (~PTX) is 2 logs higher than that in \(\alpha_{2A}\)-H, which is expected with the lower receptor bin.
TABLE 1

The $K_i$ values and $q$ values for UK-14,304 as calculated from Furchgott analysis of dose-response curves of UK-14,304 in $\alpha_{2A}$-H (+PTX) and $\alpha_{2A}$-L (−PTX) before and after partial receptor alkylation by the irreversible inhibitor benextramine.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$G_s$ Pathway: $\alpha_{2A}$-H (+PTX)</th>
<th>$G_i$ Pathway: $\alpha_{2A}$-L (−PTX)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$pK_i$(app.)</td>
<td>$K_i$(app./$K_i$)</td>
</tr>
<tr>
<td>Benextramine = 1 µM</td>
<td>6.39</td>
<td>0.85</td>
</tr>
<tr>
<td>Benextramine = 10 µM</td>
<td>6.31</td>
<td>1.02</td>
</tr>
<tr>
<td>Benextramine = 100 µM</td>
<td>5.83</td>
<td>3.08</td>
</tr>
<tr>
<td>[UK] = 1× $K_i$</td>
<td>1.7</td>
<td></td>
</tr>
</tbody>
</table>

expression and therefore smaller receptor reserve for the $\alpha_{2A}$-L.

In $\alpha_{2A}$-H (−PTX), at concentrations of UK-14,304 of greater than $10^{-8}$ M, a modest stimulation of adenyl cyclase is seen, confirming the $G_s$ protein activation previously reported (Eason et al., 1992; Wade et al., 1999). In $\alpha_{2A}$-L (−PTX), the stimulation of adenyl cyclase is not observed. After PTX treatment, which inhibits receptor activation of $G_i$, only the $G_i$-mediated stimulation of adenyl cyclase can be seen in $\alpha_{2A}$-H (Fig. 1, top). However, even in the presence of PTX, the stimulation of adenyl cyclase is not evident in $\alpha_{2A}$-L (−PTX). Thus, the inhibition of adenyl cyclase in $\alpha_{2A}$-L (−PTX) will not be functionally antagonized by $G_s$-mediated stimulation, as may be occurring in $\alpha_{2A}$-H (−PTX). The 120-fold difference in the $E_{C50}$ values for the dose-response curves of UK-14,304 for the $G_s$ versus the $G_i$ pathways [i.e., in $\alpha_{2A}$-H (−PTX) versus $\alpha_{2A}$-L (−PTX), respectively] suggests a much more efficient coupling of the $\alpha_{2A}$-AR to $G_s$ than to $G_i$. It should also be noted that stimulation of adenyl cyclase in $\alpha_{2A}$-H (−PTX) occurs at nearly the same concentration of UK-14,304 as does inhibition in $\alpha_{2A}$-L (−PTX). Although not a prerequisite for comparison, this renders these two cell lines ideal for comparison of $G_s$ and $G_i$-mediated responses.

We investigated the receptor reserve for UK-14,304 in $\alpha_{2A}$-H (±PTX) and $\alpha_{2A}$-L (−PTX) by irreversible inhibition of $\alpha_{2A}$-ARs by benextramine. The $E_{C50}$ value for the dose-response curves in $\alpha_{2A}$-H (−PTX) before benextramine treatment is more than 100-fold lower than the $K_i$ value for UK-14,304 binding in cells, and the curves are shifted progressively to the right in a parallel fashion by increasing concentrations of benextramine (Fig. 2B). Even 100 µM benextramine for 20 min is not sufficient to eliminate receptor reserve as the $E_{C50}$ is maintained and the $E_{max}$ is still ~10-fold lower than the $K_i$ value. The $E_{C50}$ values for the dose-response curves in $\alpha_{2A}$-H (−PTX) and $\alpha_{2A}$-L (−PTX) before benextramine treatment are ~6- and ~4-fold lower, respectively, than the $K_i$ value (Fig. 2, A and C). With 1 µM benextramine treatment, these curves are shifted to the right with $E_{C50}$ values comparable with the $K_i$ value. The $E_{max}$ is also decreased, indicating that the receptor concentration is decreased sufficiently to eliminate receptor reserve with even the smallest concentration of benextramine. Results from Furchgott analysis of the data in $\alpha_{2A}$-H (−PTX) and $\alpha_{2A}$-L (−PTX) are presented in Table 1.

Binding Data for Selected $\alpha_{2A}$-AR Agonists. According to classic theory, ~95% receptor occupation, and therefore $E_{max}$, is expected at a concentration of 20× $K_i$ of an agonist. Thus, RIAs can be estimated from these single maximal concentrations of a series of agonists. Therefore, we calculated the $K_i$ values for a series of $\alpha_{2A}$-AR agonists from competition binding against 5 nM [3H]yohimbine (Table 2). All competition binding curves, except for l-epinephrine, l-norepinephrine, and clonidine, were monophasic and could be explained by a single binding site. The competition binding curves for l-epinephrine, l-norepinephrine, and clonidine were biphasic with high-affinity $pK_i$ values being 6.19, 5.25, and 9.18, respectively (and 57, 41, and 23% of the [3H]yohimbine displaced, respectively) and low-affinity $pK_i$ values being 4.07, 3.58, and 7.07, respectively. For l-epinephrine and l-norepinephrine, the biphasic competition binding curves can be explained by their hydrophilic properties, presumably hindering access to nonsurface $\alpha_{2A}$-ARs. The high-affinity $pK_i$ would therefore be expected to describe binding to the surface receptors. After 1 or 10 µM benextramine treatment, the $pE_{C50}$ values of the dose-response curves of l-epinephrine (data not shown) corresponded with the high-affinity $pK_i$ value as expected. Also, the high-affinity $pK_i$ values for l-epinephrine and l-norepinephrine were closest to the $pE_{C50}$ values measured from functional dose-response curves in $\alpha_{2A}$-H (−PTX) and $\alpha_{2A}$-L (−PTX) (see later). However, for the partial agonist clonidine, the $pE_{C50}$ values from functional dose-response curves (data not shown) corresponded best with the low-affinity $pK_i$. We cannot explain the small fraction of high-affinity clonidine binding sites observed here.
seen in Neo cells. The Gs response to ISO is small but also Gs selective response via cells (Fig. 4A). It is clear from Fig. 4C that ISO is inducing its cells to examine the Gi pathway and in the higher receptor represents maximal response relative to UK-14,304 rather than a true a response is mediated by a receptor type other than the seen in Fig. 4B that the inhibition of adenylyl cyclase by dose-response curves for oxymetazoline and ISO. It can be the control Neo cells without the porcine expressing a pathway. Very interestingly, ISO showed clear selectivity for inducing a PTX-sensitive G protein (e.g., Gi). The Gs-mediated after PTX treatment of Neo cells, this receptor is signaling through the Gs pathway. Neo cells without the porcine expressing a series of full and partial agonists for the Gs and Gi-mediated modulation of adenylyl cyclase activity. Because the a2a-AR couples more effectively to Gs than to Gi, [3H]cAMP production was measured in the lower receptor expressing a2a-L cells to examine the Gi pathway and in the higher receptor expressing a2a-H cells treated with PTX (+PTX) for the Gs pathway. Neo cells without the porcine a2a-AR were used as controls.

Dose-response curves in a2a-H (+PTX) and a2a-L (-PTX) are presented in Fig. 3. Of the agonists studied, l-epinephrine, l-norepinephrine, and UK-14,304 behaved as full agonists and BHT-920 behaved as a partial agonist for both the Gi and Gs-mediated responses. These agonists showed no significant differences in the relative maximum responses between the Gi and Gs pathways. However, oxymetazoline showed selectivity for inducing signaling through the Gi pathway compared with the other partial agonist BHT-920. Very interestingly, ISO showed clear selectivity for inducing signaling through the Gi pathway.

Having the two agonists, oxymetazoline and ISO, show opposite selectivity for the Gi and Gs pathways, it was important to investigate whether these responses were mediated via interaction with a2a-ARs. For this purpose, we used the control Neo cells without the porcine a2a-AR to study dose-response curves for oxymetazoline and ISO. It can be seen in Fig. 4B that the inhibition of adenylyl cyclase by oxymetazoline also occurs in Neo cells, suggesting that this response is mediated by a receptor type other than the a2a-AR. Because the inhibition of adenylyl cyclase is not seen after PTX treatment of Neo cells, this receptor is signaling through a PTX-sensitive G protein (e.g., Gs). The Gi-mediated stimulation of adenylyl cyclase by oxymetazoline, however, is mediated by a2a-ARs, because no response is seen in Neo cells (Fig. 4A). It is clear from Fig. 4C that ISO is inducing its Gi selective response via a2a-ARs because no response is seen in Neo cells. The Gi response to ISO is small but also appears to be mediated by the a2a-AR.

The list of agonists to be evaluated for Gi- and Gs-mediated responses in a2a-H (+PTX) and a2a-L (-PTX) was also extended. By assuming that an Emax is obtained at a concentration of agonist 20 times the Ki value as also used by Berg et al. (1998), RIA of the agonists were determined for the Gi and Gs pathways and are reported in Table 2. The RIA of the full agonists were similar, and the rank order of RIA for the

<table>
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<th>Drug</th>
<th>pKi</th>
<th>Gs Pathway</th>
<th>Gi Pathway</th>
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<tbody>
<tr>
<td>l-Epinephrine</td>
<td>6.19</td>
<td>Gs H (+PTX)</td>
<td>Gi L (-PTX)</td>
</tr>
<tr>
<td>l-Norepinephrine</td>
<td>5.25</td>
<td>Gs 6.00</td>
<td>Gi 6.64</td>
</tr>
<tr>
<td>l-Isoproterenol</td>
<td>2.91</td>
<td>Gs 3.79</td>
<td>Gi 5.13</td>
</tr>
<tr>
<td>p-Aminoclonidine</td>
<td>6.54</td>
<td>Gs 7.35</td>
<td>Gi 7.16</td>
</tr>
<tr>
<td>BHT-920</td>
<td>6.00</td>
<td>Gs 5.98</td>
<td>Gi 6.99</td>
</tr>
<tr>
<td>Tranylcypromine</td>
<td>6.51</td>
<td>Gs 7.63</td>
<td>Gi 7.88</td>
</tr>
</tbody>
</table>

Table 2

The Ki values, pEC50 values, R.I.A., and R.e. of selected agonists on a2a-ARs for the Gi, and Gs pathways

The Ki values were determined from competition binding against 5 nM [3H]yohimbine in a2a-H whole cells, and the pEC50, and R.I.A. values were determined from functional studies measuring [3H]cAMP accumulation. R.I.A.s were obtained at 20 × Ki concentrations of the agonists and are expressed relative to the maximal response of the full agonist UK-14,304, which showed no response in control Neo cells (+PTX). R.e. were estimated as described in the text (Data Analysis). Values are mean ± S.E.
Gα pathway can be given as l-epinephrine = l-norepinephrine = ISO = UK-14,304 > p-amino-clonidine > BHT-920 = BHT-933 > clonidine > xylazine = guanabenz > oxymetazoline. The small differences in the E_max values for the full agonists were all statistically insignificant after the Bonferroni correction for multiple comparison. However, for the G_i pathway, the difference between the E_max values for l-epinephrine and UK-14,304, but not for l-norepinephrine and UK-14,304, are statistically significant (P < 0.01 after the Bonferroni correction for multiple comparison). Some of these differences may be due to a small stimulation of adenyl cyclase in the Neo cells (Table 3). L-Epinephrine, l-norepinephrine, p-iodo-clonidine, and oxymetazoline exhibited responses that were significantly greater than zero (P < 0.05 after Bonferroni’s correction for multiple comparison) in Neo (+PTX). Because the endogenous full agonists l-epinephrine and l-norepinephrine showed modest stimulation of adenyl cyclase in both PTX-treated and untreated Neo cells, the RIAS of all agonists were calculated relative to UK-14,304.

ISO Responses Are Blocked by α2A Antagonist Yohimbine but Not by β-Blocker Propranolol. To further verify that the G_i response seen with ISO in α2A-ARs, we obtained data for dose-response curves for ISO in the absence and presence of the β-AR antagonist propranolol (because ISO is a classic β-AR agonist) or the α2A-AR antagonist yohimbine. From Fig. 5A, it can be seen that 1 μM propranolol does not inhibit the response by ISO, whereas 1 μM yohimbine greatly inhibits this response. This would exclude the contribution of any endogenous β-ARs to the observed stimulation of adenyl cyclase by ISO. It also confirms that the G_i response is mediated by α2A-ARs, in agreement with data from the Neo cells. Results for the G_i response suggest the same with regard to the interaction of ISO with α2A-ARs (Fig. 5B).

Oxymetazoline but Not UK-14,304 Responses Are Blocked by 5-HT1 Antagonist (-)-Cyanopindolol. Figure 6 shows dose-response curves of UK-14,304 and oxymetazoline in the presence and absence of 100 nM (-)-cyanopindolol in α2A-L (–PTX). The G_i response of oxymetazoline, but not that of UK-14,304, was antagonized by 100 nM (-)-cyanopindolol. The estimated pA2 value is 8.44, indicating a K_i value of ~3.6 nM for (–)-cyanopindolol for the receptor involved. These data suggest that 5-HT1 receptors may mediate much of the G_i response of oxymetazoline (see Discussion). Also, 100 nM propranolol did not shift the response of oxymetazoline to the right, excluding any contribution of β-ARs to the response. Thus, the apparent ADTRs by oxymetazoline is an artifact of endogenous receptors, whereas that of ISO reflects a true property of the α2A-AR.

### Estimating Relative Efficacies of Agonists.

Because EC50/K_i ratios for l-epinephrine, l-norepinephrine, ISO, and UK-14,304 in α2A-H (+PTX) and for l-epinephrine, l-norepinephrine, and UK-14,304 in α2A-L (–PTX) show no response. However, enhanced oxymetazoline inhibition of adenyl cyclase is mediated by an endogenous G_i-coupled, non-α2-AR (see Neo cells). Data are averages of triplicate measurements from three experiments.

### Table 3

<table>
<thead>
<tr>
<th>Drug</th>
<th>R.E_max (R.E_max)</th>
<th>G_i</th>
<th>G_i-pathway</th>
<th>α2A-H</th>
<th>α2A-L</th>
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</thead>
<tbody>
<tr>
<td>l-Epinephrine</td>
<td>0.24 ± 0.02</td>
<td>0.69</td>
<td>0.40 ± 0.07</td>
<td>0.37 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>l-Norepinephrine</td>
<td>0.18 ± 0.04</td>
<td>0.69</td>
<td>0.26 ± 0.03</td>
<td>0.32 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>p-Iodosalolodine</td>
<td>0.16 ± 0.04</td>
<td>0.69</td>
<td>0.32 ± 0.03</td>
<td>0.30 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Oxymetazoline</td>
<td>0.05 ± 0.01</td>
<td>0.69</td>
<td>0.37 ± 0.06</td>
<td>0.38 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

[Fig. 5. Yohimbine, but not propranolol, blocks the response of l-isoproterenol. Whole-cell [3H]cAMP accumulation measurements with increasing concentrations of l-isoproterenol for the G_i (α2A-H: +PTX) (A) and G_i (α2A-L: –PTX) (B) pathways without (●) and with 1 μM β-AR antagonist propranolol (○) or 1 μM α2-AR antagonist yohimbine (◆). The data are averages of triplicate measurements from three experiments and indicate that the G_i-mediated response is mediated via α2A-ARs and not by β-ARs.]

[Fig. 6. Oxymetazoline but Not UK-14,304 Responses Are Blocked by 5-HT1 Antagonist (-)-Cyanopindolol. Figure 6 shows dose-response curves of UK-14,304 and oxymetazoline in the presence and absence of 100 nM (-)-cyanopindolol in α2A-L (–PTX). The G_i response of oxymetazoline, but not that of UK-14,304, was antagonized by 100 nM (-)-cyanopindolol. The estimated pA2 value is 8.44, indicating a K_i value of ~3.6 nM for (–)-cyanopindolol for the receptor involved. These data suggest that 5-HT1 receptors may mediate much of the G_i response of oxymetazoline (see Discussion). Also, 100 nM propranolol did not shift the response of oxymetazoline to the right, excluding any contribution of β-ARs to the response. Thus, the apparent ADTRs by oxymetazoline is an artifact of endogenous receptors, whereas that of ISO reflects a true property of the α2A-AR.]
At concentrations equal to their $K_i$ values, neither l-epinephrine nor l-norepinephrine showed any response in Neo (±PTX) (data not shown), making this concentration suitable for measuring $\alpha_{2A}$-AR-mediated responses. Therefore, an alternative for measuring R.e.s would be to use submaximal responses at concentrations of the agonists equal to their $K_i$ values (50% receptor occupation) as a measure of half-maximal stimulus and to calculate relative $S_{max}$ values from twice this response (see Experimental Procedures, Data Analysis).

One possible problem with this approach to keep in mind is the relationship between receptor occupation and response. This matter was clarified by estimating the $K_a$(app.) values (see Table 1) from Furchgott analysis of the dose-response curves of UK-14,304 before and after benextramine treatment, as in Fig. 2. At 1 and 10 $\mu$M concentrations of benextramine, the $K_a$(app.) values were almost identical with the $K_i$ values [see $K_a$(app.)/$K_i$ ratios in Table 1]. This would suggest a linear correlation between stimulus and response at concentrations of UK-14,304 yielding a submaximal response. Also, when the relative $S_{max}$ values were calculated from the fraction of receptors functional after alkalization (g) by 1 $\mu$M benextramine, it correlated very well with the relative $S_{max}$ values calculated from 1 $\times$ $K_i$ concentrations for both the $G_i$- and $G_s$-mediated responses. These results suggest that responses at 1 $\times$ $K_i$ concentrations can be used to estimate half-maximal stimulus (and therefore R.e.) in these cell lines. It should be noted that Furchgott analysis of dose-response curves of UK-14,304 before and after treatment with 100 $\mu$M benextramine gave $K_a$(app.) values higher than $K_i$ concentrations and the estimated relative $S_{max}$ values differed greatly from those calculated from responses at a 1 $\times$ $K_i$ concentration of UK-14,304. Increasing concentrations of benextramine treatment not only decreased the $E_{max}$, but also shifted the curves to the right beyond the $K_i$ value, suggesting that the mechanism of inhibition at higher concentrations of benextramine may not be purely due to irreversible competitive antagonism. More reliable $K_a$(app.) values are therefore calculated with the lowest concentration of benextramine (1 $\mu$M) where the least nonspecific effects are expected.

Although the responses for all full agonists at concentrations equal to their $K_i$ values were submaximal, the responses were greater than 80% of the $E_{max}$ values, and theoretically one may expect the relationship between stimulus and response to become nonlinear when receptor reserve is present. This would mean that the relative $S_{max}$ values calculated from 1 $\times$ $K_i$ concentrations should be regarded as estimates.

The R.e.s of full agonists (relative to UK-14,304) were then calculated from $S_{max}$ values equal to twice the response obtained at 1 $\times$ $K_i$ concentrations of the agonists. The R.e.s of the partial agonists were calculated from the $E_{max}$ values obtained at 20 $\times$ $K_i$ concentrations of the agonists. The R.e.s are reported in Table 2. From the R.e.s it can be seen that ISO shows a ~9-fold higher efficacy for the $G_i$-mediated stimulation of adenylyl cyclase compared with the $G_i$-mediated inhibition of adenylyl cyclase.

**Fig. 6.** Adenylyl cyclase inhibition by oxymetazoline, but not that by UK-14,304, is antagonized by (−)-cyanoepinodilol. Whole-cell [3H]cAMP accumulation measurements with increasing concentrations of UK-14,304 (A) in the absence (●) or presence of 100 nM (−)-cyanoepinodilol (○) and oxymetazoline (B) in the absence (●) or presence of 100 nM (−)-cyanoepinodilol (○) or 100 nM propranolol (+). The data are averages of triplicate measurements from three experiments and indicate that a non-$\alpha_{2A}$-AR mediates the $G_i$-response of oxymetazoline.

**Fig. 7.** UK-14,304 and l-isoproterenol use similar effector regions on the $\alpha_{2A}$-AR for activation of $G_i$. Whole-cell [3H]cAMP accumulation measurements were made with increasing concentrations of UK-14,304 and l-isoproterenol. Shown are $G_s$ (A and C, +PTX) and $G_i$ (B and D, −PTX) responses with wild-type $\alpha_{2A}$-AR, the $\alpha_{2A}$-R3 mutant □, or the $\alpha_{2A}$-B2 mutant ○ (see text for full explanation). Note that l-isoproterenol acts as a full agonist in the high $\alpha_{2A}$-AR expressing $\alpha_{2A}$-H (−PTX) cell line compared with being a partial agonist in $\alpha_{2A}$-L (−PTX) in other figures. Data are averages of at least triplicate measurements from three experiments.
classic α2A-AR agonist UK-14,304. The effector region of the α2A-AR for Gi coupling also seems to be similar.

Discussion

The present study was undertaken to investigate agonist-directed trafficking of porcine α2A-AR signaling through the Gα and Gi-coupled signal transduction pathways.

**ISO Selectively Activates Gα Pathway by Interaction with α2A-ARs.** ISO was the only agonist tested to show clear selectivity for Gα or Gβγ at the α2A-AR. Interestingly, ISO had a 5-fold higher R. e. at the α2A-AR for Gα versus Gβγ, but it has low potency for both. It is clear from the data with both Neo control cells and pharmacological antagonists that this effect is mediated by the α2A-AR. These results support the hypothesis of ADTRS (Kenakin, 1995). We were not able to confirm the conclusion of Eason and Liggett (1996) that BHT-920 and BHT-933 lead to selective activation of Gα. The difference between these two conclusions is probably due to the fact that our analysis takes into account spare receptors, whereas theirs did not. A further difference is their use of human α2A-AR, whereas we have porcine receptors, although this does not seem to be a likely explanation. Other recently published reports of ADTRS include the study of Berg et al. (1998) in which 5-HT receptors couple to phospholipases C and A2 with efficacies dependent on the specific agonist; Bonhaus et al. (1998) found differential Gαi and Gαs coupling with cannabinoid (CB1) agonists, and Yang and Lanier (1999) found differential coupling of rat α2A-ARs to Gαi and Gαs in NIH 3T3 cells.

It is intriguing that a classic β2-AR agonist that activates Gαs through β2-ARs also appears to produce a Gα-selective conformation of the α2A-AR, which typically activates Gβγ. We could not, however, demonstrate significant Gαi or Gαs-mediated responses with other β2-AR agonists such as salbutamol or l-norephedrine because their affinities were too low (data not shown).

As a first step to understanding the possible conformational differences, we tested two recently described mutant α2A-ARs that selectively alter Gαi and Gαs coupling. However, the Gαs response in α2A-R3 and the Gαi response in α2A-B2 were inhibited similarly for UK-14,304 and ISO (see Fig. 7). We propose that the α2A-AR effector sites for Gαs and Gαi coupling when the α2A-AR is activated by ISO may be similar to those exposed when the α2A-AR binds other α2A-AR agonists.

**Oxymetazoline Activates Gβγ Pathway by Interaction with Non-α2A-ARs.** Enhanced inhibition of adenylyl cyclase by oxymetazoline in CHO-K1 cells as previously reported by Kenakin (1995), appears to be mediated via an endogenous 5-HT1 receptor (Figs. 4 and 6). Schoeffler and Hoyer (1991) estimated the affinities of oxymetazoline at 5-HT1 receptors as a Kd value of 26 nM. They also reported that stimulation of 5-HT1A, 5-HT1B, and 5-HT1D receptors inhibited adenylyl cyclase, whereas activation of 5-HT1C receptors stimulated adenylyl cyclase activity. Because CHO cells have an endogenous 5-HT1B receptor (Berg et al., 1994; Giles et al., 1996), that is the likely source of the excess Gαi response to oxymetazoline.

This was confirmed by the effects of cyanopindolol, a 5-HT1A/1B antagonist. (+)-Cyanopindolol binds the 5-HT1B receptor in rat brain membranes with a Kd value of 3.5 ± 0.4 nM (Ariani et al., 1989). The pA2 of 7.76 (Fig. 6) indicates a Kd value of ~18 nM for (+)-cyanopindolol for the receptor mediating this effect. This result strongly emphasizes the importance of nontransfected control cells when studying the pharmacological properties of cloned receptors.

**Rank Order of Relative Efficacies for Gαs and Gi Is Generally Similar.** With the exception of ISO, all agonists showed very similar R.e.s at the α2A-AR for the Gαs and Gi pathways. There may be modest differences in R.e.s, such as BHT-920 for the Gαs and Gi pathways (0.27 ± 0.02 and 0.19 ± 0.02, respectively), but this study did not have sufficient statistical power to define such a small difference. The rank order of R.e.s at the α2A-AR for activation of Gαs and Gi pathways are as follows.

For the Gαs pathway (rank order of R.e.s of oxymetazoline was not determined because of significant response in control Neo cells), the order is l-epinephrine = l-norepinephrine = ISO = UK-14,304 > p-aminoclonidine ≥ BHT-920 ≥ BHT-933 > xylazine ≥ p-idocloclidine ≥ guanabenz.

For the Gi pathway (rank order of R.e.s of p-iodocloclidine and oxymetazoline was not determined because of significant responses in control Neo cells), the order is l-epinephrine = l-norepinephrine = UK-14,304 > p-aminocloclidine ≥ BHT-920 > BHT-933 > xylazine ≥ clonidine ≥ ISO ≥ guanabenz.

The rank order of the R.e.s differs slightly from that found by Wise et al. (1997). They found that the R.e.s of l-epinephrine and l-norepinephrine were greater than that of UK-14,304 (i.e., epinephrine (adrenaline) = norepinephrine (noradrenaline) = α-methylnorepinephrine > UK-14,304 > BHT-933 ≥ xylazine = clonidine). However, their data were obtained in COS-7 cells transiently transfected to express the α2A-AR/Gs1 fusion protein, measuring GTPase activity as a readout of receptor activation. In CHO cells, Gα12 and Gα13 have been shown to mediate inhibition of adenylyl cyclase by the α2A-AR (Gerhardt and Neubig, 1991). It is possible that the difference in the Gαs (i.e., Gα1 versus Gα2 and Gα3) may contribute to the differences seen in the R.e. of UK-14,304 and the catecholamines.

**Defining α2A-H and α2A-L Pharmacologically.** The 120-fold difference in the EC50 value of the dose response curves for UK-14,304 for the Gαs pathway in α2A-H (−PTX) versus the Gi pathway in α2A-H (+PTX) suggests that the α2A-AR couples much more efficiently to the Gαs protein than to the Gαi protein. This preferential coupling of the α2A-AR to Gαi is in agreement with data from Eason et al. (1992, 1994) and Chabre et al. (1994). However, in HEK 293 cells transfected to transiently coexpress the porcine α2A-AR with either Gαs, Gαi, or Gαq, Chabre et al. (1994) found that the α2A-AR couples ~1000 times more efficiently to Gαq (endothelium to HEK 293) than to either Gαi (rat Gαi) or Gαq (murine Gαq). The reason for the difference in the Gα/Gi selectivity of the α2A-AR coupling as suggested by the data of Chabre et al. versus our data (1000 versus 120) is not known. It is possible that coupling to endogenous G proteins is more efficient than to transfected G proteins.

It is also important to note that UK-14,304 gave no significant Gαi response in α2A-L (+PTX), so the Gi pathway does not functionally antagonize the measured Gαs response in α2A-L (−PTX). Also, contrary to what is seen in α2A-H (−PTX) at supramaximal concentrations of UK-14,304, no Gαs-mediated stimulation of adenylyl cyclase is seen in α2A-L (−PTX). This is important because Gαs was not inactivated by...
cholera toxin when the $G_i$ responses were measured in $\alpha_{2A}-L$ (−PTX).

Because the large $K_{EC_{50}}$ ratio of UK-14,304 for the $G_i$ pathway in $\alpha_{2A}-H$ (−PTX) suggests a large receptor reserve, this was investigated by irreversible inhibition of the $\alpha_{2A}$-ARs by benextramine. Benextramine has been shown to irreversibly block $\alpha$-ARs (Melchiorre, 1981) and has been used as such in several studies (Timmermans et al., 1985; Brasili et al., 1986; Taouis et al., 1987; Galitzky et al., 1989; Karlsson et al., 1989). Because 100 $\mu$M benextramine (20 min) shifts the dose-response curve of UK-14,304 in $\alpha_{2A}-H$ (−PTX) by more than 2 logs to the right but is insufficient to reduce the $E_{max}$ it can be concluded that UK-14,304 has a large receptor reserve for the $G_i$ response in this cell line. In both $\alpha_{2A}-H$ (−PTX) and $\alpha_{2A}-L$ (−PTX), $1 \mu$M benextramine (20 min) is sufficient to decrease the $E_{max}$, suggesting that UK-14,304 has a much smaller receptor reserve for both the $G_a$ and $G_i$ responses, respectively, in these two cell lines.

Conclusions. We provide additional support for the ADTRS hypothesis in a pharmacologically well-defined system. High $\alpha_{2A}$-AR expression (higher than found in most mammalian tissue) is necessary for significant $G_i$ coupling, which may limit the relevance of these findings for normal physiological conditions. However, the implications of this important hypothesis for drug design are highly significant. To fully understand the molecular mechanisms of ADTRS, it will be important to extend these results to purified systems with direct measurements of $G$ protein activation.

The search for new drugs has classically been directed toward greater receptor subtype selectivity. However, ADTRS should now also be considered in the search for new drugs that we may look for agonists that selectively activate particular effector pathways. This should also prompt reevaluation of known agonists as a means to, we hope, decrease the unwanted side effects of some drug treatments.

Acknowledgments

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