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Contribution of Ligand Structure to Activation of α_2-Adrenergic Receptor Subtype Coupling to G_s

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SUMMARY

Recently, we have demonstrated that α_2-adrenergic receptors (α_2AR) functionally couple not only to G_i but also to G_s. This α_2AR-G_s coupling was subtype selective, in that the degree of α_2AR-G_s (but not -G_i) coupling was different between α_2AR subtypes. It is not known whether the determinants of this subtype selectivity are found within the ligand-binding region of the receptor or within the intracellular G protein-coupling domains of the individual subtypes. We therefore expressed the three cloned human α_2AR (α_2C10, α_2C4, and α_2C2) in Chinese hamster ovary cells and studied the contribution of the ligand-binding domain to functional G_i versus G_s coupling, by determining the ability of various agonists (catecholamines, imidazolines, and azepines) to elicit α_2AR-mediated inhibition and stimulation of adenylyl cyclase activity. Isolation of G_i and G_s responses was accomplished by incubating cells with cholera or pertussis toxin, respectively. Although each compound was found to be a full agonist for α_2AR-G_i coupling, the efficacy of these agonists to elicit α_2AR-G_s coupling was markedly different, not only among drugs but also among the three α_2AR subtypes. The most notable differences occurred with the imidazoline agonists. Specifically, oxymetazoline stimulated adenylyl cyclase activity 210 ± 17% for α_2C2 and 22 ± 2.6% for α_2C10 and displayed no stimulation for α_2C4. UK-14304 stimulated adenylyl cyclase activity 240 ± 16% for α_2C10, 160 ± 14% for α_2C4, and 86 ± 9% for α_2C2. Overall, the rank order of efficacy of these agonists to elicit stimulation of adenylyl cyclase activity by α_2C10 was epinephrine = norephinephrine = UK-14304 > BHT-933 > BHT-920 > oxymetazoline. For α_2C4 the rank was epinephrine = norepinephrine = UK-14304, with oxymetazoline, BHT-920, and BHT-933 not eliciting any stimulation. For α_2C2 the rank was epinephrine = norepinephrine > oxymetazoline > UK-14304 = BHT-920 > BHT-933. Thus, the coupling of α_2AR subtypes to G_s occurs with endogenous catecholamines as well as multiple synthetic agonists, and the degree of G_s coupling is highly dependent on the structure of the agonist. Also, compounds that act as full agonists for G_i coupling are not necessarily full agonists for G_s coupling.

AR are members of a large family of receptors that signal to the interior of the cell via G proteins. Although classically a given G protein-coupled receptor is thought to initiate a single second messenger pathway, activation of multiple biochemical signals within the cell is usually observed. This is primarily due to downstream effects of the second messenger, but evidence has accumulated over the past few years that multifunctional signaling can be accomplished in other ways. One mechanism for this is the coupling of a receptor to two different G proteins (reviewed in Ref. 1). Traditionally, α_2AR have been described as being coupled to G_i, with agonist occupancy resulting in a decrease in adenylyl cyclase activity and thus a decrease in intracellular cAMP (2, 3). However, the α_2AR are known to couple to multiple effector systems (2), and a few studies have demonstrated paradoxical α_2AR-mediated increases in cAMP (4–7). Until recently, the mechanism of this stimulatory response was not known. Studies from our laboratory (8), as well as that of Bourne and colleagues (9), have now demonstrated that α_2AR-mediated stimulation of adenylyl cyclase activity occurs via direct α_2AR coupling to G_s. The α_2AR are thus unique among the AR, in that they can couple to two G proteins with opposing effects on the same effector enzyme.

The ability to bind and initiate α_2AR coupling to G_i has been well established for a number of synthetic compounds, which are not limited to the catecholamine family. Although many of these compounds are considered full agonists (based on their ability to elicit α_2AR-mediated inhibition of adenylyl cyclase activity), the efficacy of these same compounds to fully activate α_2AR-G_s coupling has yet to be investigated. Previously, we showed that the three cloned human α_2AR subtypes, α_2C10

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ABBREVIATIONS: AR, adrenergic receptor(s); α_2C10, α_2C4, and α_2C2, human α_2-adrenergic receptor subtypes localized to chromosomes 10, 4, and 2, respectively; UK-14304, 5-bromo-6-(2-imidazolin-2-yl-amino)quinozaline; BHT-920, 6-allyl-2-amino-5,6,7,8-tetrahydro-4*H*-thiazolo[4,5-*d*]azepin dihydrochloride; BHT-933, 2-amino-6-ethyl-4,5,7,8-tetrahydro-6*H*-oxazolo[5,4-*d*]azepin dihydrochloride; 5-HT, 5-hydroxytryptamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHO, Chinese hamster ovary.

(10), α_2C4 (11), and α_2C2 (12), displayed G_s coupling in a subtype-selective manner, with a rank order for the stimulation of adenylyl cyclase activity of α_2C10 > α_2C4 > α_2C2 (8). These studies were performed with the specific α_2AR agonist UK-14304, which is considered to be a full agonist in its efficacy to elicit inhibition of adenylyl cyclase activity.

The underlying mechanism for these differences in G_s coupling found between the α_2AR subtypes might be due to structural determinants within the intracellular portions of these receptors that interact directly with G proteins (13). Another mechanism by which subtype-selective G protein coupling could occur would be through the specific interactions of agonists with regions within the ligand-binding portion of the receptor. Such interactions, if different between the subtypes, might cause different conformational changes in the third intracellular loop region, thereby resulting in different degrees of α_2AR-G_s coupling. In the present study, the contribution of ligand structure to α_2AR-G_s coupling specificity was investigated using the three cloned human α_2AR subtypes expressed in CHO cells and six different α_2AR agonists, two from each of three representative molecular classes of compounds, i.e., catecholamines (epinephrine and norepinephrine), imidazolines (UK-14304 and oxymetazoline), and azepines (BHT-920 and BHT-933).

Experimental Procedures

Cell transfection and culture. The constructs consisting of α_2C10, α_2C4, and α_2C2 cloned into the mammalian expression vector pBC12BI have been described previously (8). CHO cells (which normally express no AR) were co-transfected with 30–40 μg of the aforementioned vectors and 3 μg of psV$_2$neo (which provides for resistance to G418) using a calcium phosphate precipitation method, as described (8). Selection was performed with 1.0 mg/ml G418. Clonal cell lines were screened for α_2AR expression using a [^{3}H]yohimbine binding assay, as described below. Cells permanently expressing either α_2C10, α_2C4, or α_2C2 were grown in monolayers in Ham's F12 medium supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 units/ml penicillin, and, for the maintenance of selection pressure, 80 μg/ml G418. Transient transfections of COS cells were performed as described elsewhere (14).

Cholera and pertussis toxin treatment. To ablate α_2AR-G_i or α_2AR-G_s coupling, cultured CHO cells expressing the α_2AR subtypes were incubated with either pertussis or cholera toxin, respectively, as described (8). Briefly, CHO cells in monolayers were rinsed three times with phosphate-buffered saline and then incubated for 24 hr with either 500 ng/ml pertussis toxin or 20 μg/ml cholera toxin in fresh medium without serum. These concentrations of pertussis and cholera toxin have been previously shown to effectively ablate G_i and G_s coupling, respectively, without detrimental effects to the cells (8). Incubations were terminated by rinsing each flask five times with phosphate-buffered saline. Membranes were then prepared by hypotonic lysis in ice-cold 5 mM Tris, pH 7.4, 2 mM EDTA, and scraping with a rubber policeman. The particulate suspension was then pelleted by centrifugation at 40,000 × g for 10 min and the crude membrane pellet was resuspended in the appropriate assay buffer as described below.

Adenylyl cyclase assays. Membranes from CHO cells that separately expressed each of the α_2AR subtypes and that had been exposed to cholera toxin or pertussis toxin were prepared as described above and then resuspended in a buffer that provided 25 mM NaCl, 1.6 mM $MgCl_2$, 0.8 mM EDTA, and 40 mM HEPES, pH 7.4, in the assay. Adenylyl cyclase activities were determined by incubating membranes in the presence of water (basal), 1.0 μM forskolin, or 1.0 μM forskolin with various concentrations of agonist, in a reaction mixture of 2.7 mM phosphoenolpyruvate, 0.1 mM cAMP, 0.12 mM ATP, 50 μg/ml myokinase, 0.5 mM ascorbic acid, and 1.0 μCi of [α-^{32}P]ATP, for 45 min at 37°. The reaction mixture also contained 50 μM GTP, which we have found, along with high receptor expression levels, to be optimal for promoting α_2AR stimulation of adenylyl cyclase activity. In some studies, incubations were carried out in the presence of various antagonists (10 μM) as indicated. Reactions were terminated by the addition of 1.0 ml of an ice-cold solution consisting of excess ATP, cAMP, and [^{3}H]cAMP, which was used to quantitate individual column recovery. [^{32}P]cAMP was isolated by sequential chromatography over Dowex and alumina columns by the method of Salomon *et al.* (15), as modified (16). No modulation of adenylyl cyclase activity was observed in nontransfected cells with any of these α_2AR agonists, and in cells transfected with the separate α_2AR subtypes stimulatory and inhibitory responses to α_2AR agonists were blocked by α_2AR antagonists but not by antagonists for βAR, α_1AR, or 5-HT receptors (see Results). For some studies, accumulation of whole-cell cAMP was quantitated using a radioimmunoassay, as described previously (17).

Radioligand binding assays. For screening purposes, α_2AR expression levels were measured by incubation of membranes with a saturating concentration (25 nM) of [^{3}H]yohimbine in the absence (total binding) or the presence (nonspecific binding) of 100 μM phentolamine, in a buffer containing 75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, and 2 mM EDTA, for 30 min at 37°. Specific binding was defined as the difference between total and nonspecific binding and was normalized for protein concentration. Incubations were terminated by dilution with ice-cold 10 mM Tris buffer, pH 7.4, and rapid filtration over Whatman glass fiber filters. Protein concentration was measured using the copper-bicinchoninic acid method, with bovine serum albumin as standard. We have previously shown that higher expression levels facilitate the observation of α_2AR-G_s coupling (8). At levels of expression higher than 5 pmol/mg, no significant increase in maximal stimulation of adenylyl cyclase was found (data not shown). In these studies, clonal cell lines expressing the α_2AR subtypes at levels of 6–10 pmol/mg were used.

Data analysis. Adenylyl cyclase dose-response data and radioligand binding data were analyzed by iterative nonlinear least squares techniques, as described (8). For adenylyl cyclase studies, data are reported as mean ± standard error of the R_{max} (maximal response, either stimulation or inhibition) and the EC_{50} for a given response from four to eight individual experiments. Comparisons were made by two-tailed t tests, with significance imparted at $p < 0.05$.

Materials. [^{3}H]Yohimbine (80 Ci/mmol), [α-^{32}P]ATP (30 Ci/mmol), and [^{3}H]cAMP (31 Ci/mmol) were from DuPont-New England Nuclear. Pertussis toxin was from List Biologicals. Cholera toxin, (−)-epinephrine, (−)-norepinephrine, oxymetazoline, and forskolin were from Sigma. pBC12BI was a gift from Brian Cullen (Duke University), UK-14304 was a gift from Pfizer, phentolamine was a gift from Ciba-Geigy, and BHT-920 and BHT-933 were gifts from Boehringer Ingelheim. Geneticin (G418) was from GIBCO. Ham's F12 medium and fetal bovine serum were from JRH Biosciences. CHO-K1 and COS-7 cells were obtained from the American Type Culture Collection. All other reagents were obtained from standard commercial sources.

Results

We investigated the role of ligand binding in α_2AR-mediated stimulation of adenylyl cyclase using various agonists with transfected cells expressing the three human α_2AR subtypes. As shown in Fig. 1, this stimulatory effect, which occurred in transfected CHO and COS cells, was clearly mediated by the α_2AR, rather than an endogenous receptor on these cells. Nontransfected cells showed no modulation of adenylyl cyclase even at high concentrations of agonist. On the other hand, a marked increase (~400%) in adenylyl cyclase was observed under the same conditions after transient (COS) or permanent (CHO) transfection of α_2C10. (For CHO cells G_i coupling was blocked by pertussis toxin.) The lack of agonist-mediated changes in

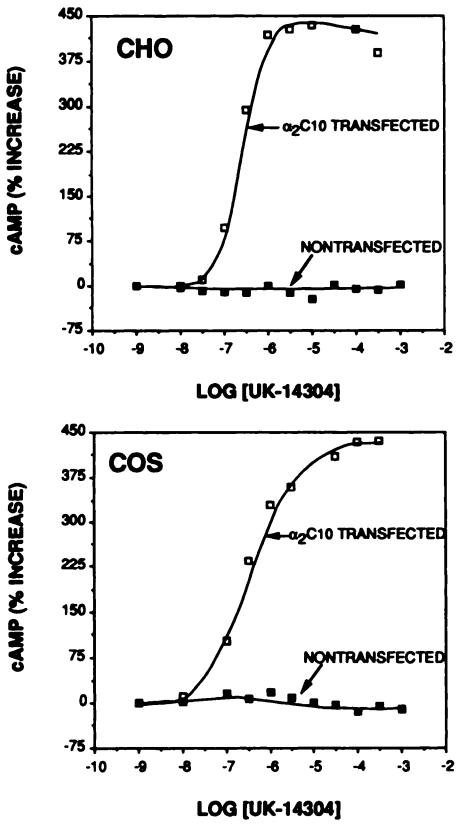

Fig. 1. α_2AR-mediated stimulation of adenylyl cyclase activity in CHO and COS cells. The effects of the α_2AR agonist UK-14304 on cAMP accumulation in nontransfected cells and cells expressing α_2C10 were studied using both CHO and COS-7 cells. In nontransfected cells, no modulation of cAMP levels by UK-14304 was observed. In contrast, transfected cells expressing α_2C10 exhibited ~400% stimulation of cAMP levels. CHO cells were pretreated with pertussis toxin to ablate G_i coupling. Shown is a single representative experiment.

adenylyl cyclase in nontransfected cells was observed for all of the other agonists that were used in this study (data not shown). As additional evidence that this G_s coupling is an α_2AR-mediated event in CHO cells, the stimulation of adenylyl cyclase by all six agonists was evaluated in the presence of 10 μM concentrations of the following receptor-specific antagonists: propranolol (βAR), prazosin (α_1AR), ketanserin (5-HT receptor), and yohimbine (α_2AR). Shown in Fig. 2 are representative results from such experiments with α_2C10. As can be seen, only yohimbine blocked the stimulation of adenylyl cyclase by each of the agonists. Studies with cells expressing α_2C4 and α_2C2 gave the same results (data not shown). The clonal cell lines expressing the three α_2AR subtypes chosen for these studies were also assessed for the equivalancy of their signal transduction properties. For cells expressing α_2C10, α_2C4, and α_2C2, basal (4.7 ± 0.5, 4.8 ± 0.4, and 4.3 ± 0.4 pmol/min/mg, respectively), forskolin-stimulated (14.7 ± 1.0, 13.7 ± 1.7, and 14.3 ± 1.7 pmol/min/mg, respectively), and NaF-stimulated (48 ± 1.4, 43.1 ± 3.1, and 46.1 ± 2.6 pmol/min/mg, respectively) adenylyl cyclase activities and epinephrine-mediated inhibition (39 ± 5%, 31 ± 3%, and 33 ± 4%, respectively) of forskolin-stimulated adenylyl cyclase activities were the same among all cell lines (four experiments, p is not significant).

In previous work (8) we found that responses obtained from CHO cells expressing α_2AR at this level are of a complex biphasic nature, exhibiting both inhibitory and stimulatory

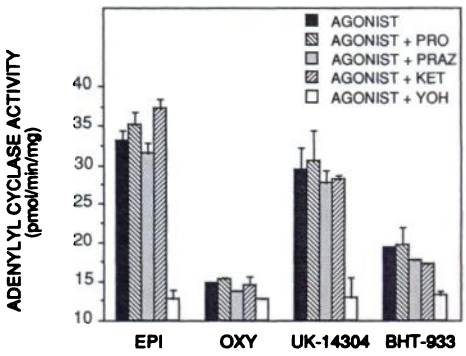

Fig. 2. Specificity of α_2AR-mediated stimulation of adenylyl cyclase activity. Adenylyl cyclase activities were determined in membranes from CHO cells that had been incubated with pertussis toxin to isolate the α_2AR-G_s response. Activities were determined with the indicated agonists (0.3–1.0 mM) in the presence of 10 μM concentrations of various antagonists. The stimulation of adenylyl cyclase was blocked only by the α_2AR antagonist yohimbine. Values shown are mean ± standard deviation of a single representative experiment. The same results were also obtained with α_2C4- and α_2C2-expressing cells. *PRO*, propranolol; *PRAZ*, prazosin; *KET*, ketanserin; *YOH*, yohimbine; *EPI*, epinephrine; *OXY*, oxymetazoline. Results from experiments with norepinephrine and BHT-920 were the same as those with epinephrine and BHT-933, respectively.

components. Preincubation of cells with cholera toxin or pertussis toxin resulted in monophasic inhibitory or stimulatory curves. Thus, in the present study, the ability of each ligand to elicit α_2AR-mediated inhibition and stimulation of adenylyl cyclase activity was evaluated for the three α_2AR subtypes after such toxin treatment.

As shown in Fig. 3 and Table 1, all six drugs elicited maximal G_i coupling for α_2C10, α_2C4, and α_2C2 that produced ~35% inhibition of forskolin-stimulated adenylyl cyclase activity. In marked contrast, the ability of these drugs to elicit α_2AR-G_s coupling differed depending on the compound and on the receptor subtype. For α_2C10, epinephrine, norepinephrine, and UK-14304 all elicited stimulation of adenylyl cyclase activity to an equal extent (291 ± 23%, 329 ± 50%, and 240 ± 16%, respectively) (Fig. 3; Table 1), whereas oxymetazoline, BHT-920, and BHT-933 were much less efficacious, eliciting α_2C10-mediated stimulation of adenylyl cyclase activity of 22 ± 2.6%, 34 ± 1.8%, and 49 ± 8.4%, respectively (Fig. 3; Table 1). The efficacy of these agonists for α_2C4-G_s coupling had some similarities to the profile found for α_2C10-G_s coupling; however, the maximal extent of coupling was less than that obtained for α_2C10. Again, epinephrine, norepinephrine, and UK-14304 elicited α_2C4-G_s coupling to an equal extent (190 ± 35%, 170 ± 20%, and 160 ± 14%, respectively) (Fig. 3; Table 1). In contrast to what was found with α_2C10, BHT-920, BHT-933, and oxymetazoline elicited no detectable stimulation of adenylyl cyclase activity (Table 1). For α_2C2, the efficacy of these agonists for G_s coupling was markedly different from that for the other two α_2AR subtypes. The catecholamines elicited similar levels of α_2C2-mediated stimulation of adenylyl cyclase activity (epinephrine, 340 ± 26%; norepinephrine, 330 ± 39%) (Fig. 3; Table 1). However, α_2C2-mediated stimulation of adenylyl cyclase activity in the presence of UK-14304 was much smaller (86 ± 9.3%) than that with the catecholamines and was similar to that with BHT-920 (92 ± 10%). Interestingly, whereas oxymetazoline elicited very minimal α_2C10-G_s coupling and no α_2C4-G_s coupling, marked α_2C2-G_s coupling in the presence of this compound was found, with a stimulation of adenylyl cyclase activity of 210 ± 17% (Fig. 3; Table 1). α_2C2-mediated

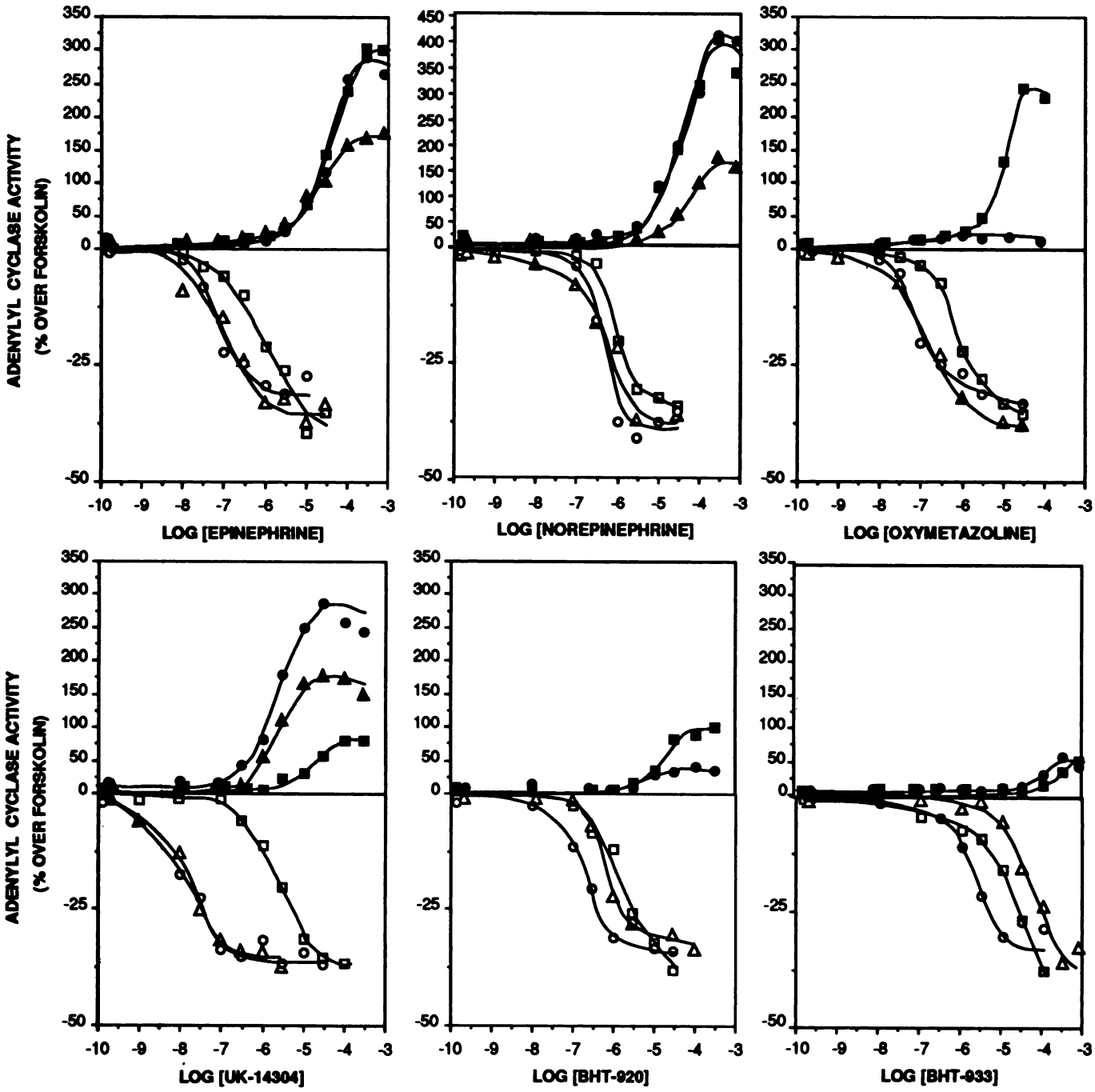

Fig. 3. Dependency on agonist structure for functional G_s and G_i coupling of α_2C10, α_2C4, and α_2C2. CHO cells separately expressing the three α_2AR subtypes were incubated with either cholera or pertussis toxin to isolate G_i or G_s coupling, respectively, membranes were prepared, and adenylyl cyclase assays were performed as described in Experimental Procedures. Activities were determined in the presence of 1.0 μM forskolin or 1.0 μM forskolin and various concentrations of agonists as indicated. *Circles*, α_2C10; *triangles*, α_2C4; *squares*, α_2C2. *Closed symbols*, results from experiments in which cells were pretreated with pertussis toxin to isolate α_2AR-G_s coupling; *open symbols*, results from experiments in which cells were pretreated with cholera toxin to isolate α_2AR-G_i coupling. With all six agonists, α_2AR-G_i coupling occurred in a similar manner for each subtype, with ~35% inhibition of forskolin-stimulated activities. In contrast, α_2AR-G_s coupling exhibited marked differences in the presence of the different agonists and between the α_2AR subtypes, as summarized in Tables 1 and 2. Shown are representative results from four to eight experiments performed.

stimulation of adenylyl cyclase activity in the presence of BHT-933 was 59 ± 6.5%, clearly less than that with the other five compounds (Fig. 3; Table 1).

Thus, for each of the α_2AR subtypes there is a subtype-specific ranking of the six agonists, based on their ability to elicit α_2AR-mediated stimulation of adenylyl cyclase activity. For α_2C10 the order is epinephrine = norepinephrine = UK-14304 > BHT-933 > BHT-920 > oxymetazoline and for α_2C4 it is epinephrine = norepinephrine = UK-14304; in the presence of oxymetazoline, BHT-920, and BHT-933 there was no α_2C4-mediated stimulation of adenylyl cyclase activity. For α_2C2 the order is epinephrine = norepinephrine > oxymetazoline > UK-14304 = BHT-920 > BHT-933. These data can also be expressed based on a given compound, with the α_2AR-G_s coupling

TABLE 1

Functional α_2AR-G_i versus -G_s coupling for α_2C10, α_2C4, and α_2C2

Results are from experiments described in Fig. 3. For each agonist, the EC_{50} and R_{max} (maximum response) values for both G_i and G_s coupling are reported for each α_2AR subtype. EC_{50} and R_{max} values were determined using iterative nonlinear least squares analysis. 0, no functional G_s coupling was detected for a given agonist. –, EC_{50} could not be calculated due to little or no stimulation. Results shown are mean ± standard error from four to eight experiments performed.

	G_i coupling						G_s coupling					
	R_{max}			EC_{50}			R_{max}			EC_{50}		
	α_2C10	α_2C4	α_2C2	α_2C10	α_2C4	α_2C2	α_2C10	α_2C4	α_2C2	α_2C10	α_2C4	α_2C2
	% inhibition			nM			% stimulation			μM		
Epinephrine	39 ± 5	31 ± 3	33 ± 4	123 ± 20	150 ± 30	900 ± 280	291 ± 23	190 ± 35	340 ± 26	22.4 ± 2.6	27.1 ± 5.4	33.0 ± 2.4
Norepinephrine	32 ± 4	36 ± 3	39 ± 4	361 ± 14	400 ± 100	320 ± 220	329 ± 50	170 ± 20	330 ± 39	40.3 ± 2.2	6.5 ± 1.3	20.9 ± 2.3
Oxymetazoline	32 ± 3	27 ± 3	38 ± 6	22 ± 1	22 ± 10	500 ± 170	22 ± 3	0	210 ± 17	–	–	10.3 ± 1.3
UK-14304	36 ± 5	27 ± 2	33 ± 7	22 ± 3	15 ± 2	1500 ± 470	240 ± 16	160 ± 14	86 ± 9	2.8 ± 0.5	1.9 ± 0.3	11.5 ± 3.0
BHT-920	27 ± 2	32 ± 4	42 ± 9	301 ± 90	860 ± 290	1700 ± 640	34 ± 2	0	92 ± 10	3.6 ± 0.9	–	11.6 ± 1.7
BHT-933	29 ± 3	32 ± 2	45 ± 7	2880 ± 1280	8914 ± 206	7600 ± 2500	49 ± 8	0	59 ± 6	42.5 ± 7.6	–	146.5 ± 37.9

being ranked for each subtype. For example, for oxymetazoline the subtype rank for G_s coupling is α_2C2 > α_2C10 and coupling is absent for α_2C4. Table 2 provides in summary form these α_2AR-G_s coupling results for all six drugs and all three α_2AR subtypes.

Discussion

It has become apparent that α_2AR have the ability to both decrease and increase adenylyl cyclase activity. This dual modulation of adenylyl cyclase appears to be by direct α_2AR coupling to G_i as well as G_s (8, 9). In COS-7 cells transfected with the α_2C10 subtype, Bourne and colleagues (9) found that α_2AR agonists increase intracellular cAMP. However, in human embryonic kidney 293 cells, the transfected α_2AR displayed primarily G_i coupling, eliciting decreases in cAMP. Upon cotransfection with type II adenylyl cyclase, dual coupling to both G proteins became evident. This does not appear to be a property of all G protein-coupled receptors that are primarily coupled to G_i, however, because the somatostatin receptor did not display dual coupling under the same conditions as did the α_2AR (9). In CHO cells, dual modulation of adenylyl cyclase by α_2AR is manifested by biphasic curves, with an inhibitory component at lower agonist concentrations and a stimulatory component at higher agonist concentrations (4, 5, 8). In CHO cells we have shown by several approaches that this stimulatory component is due to direct G_s coupling to the α_2AR (8). First, stimulation of adenylyl cyclase by α_2AR agonists was observed in washed membrane preparations, which eliminates the possibility that some other downstream event causes an increase in cellular cAMP, as might be seen in whole-cell studies. Second, the biphasic curves were shown to become monophasic after ablation of the G_i- or G_s-coupled pathways with pertussis toxin or cholera toxin. We also showed that the α_2AR-mediated stimulation of adenylyl cyclase could be inhibited by anti-$G_{s\alpha}$ antibody in a cell-free system to the same extent as that found with β_2AR-G_s coupling. In addition, when α_2AR were exposed to agonist and then purified using an anti-α_2AR antibody, the immunoprecipitate revealed the presence of an α_2AR-G_s complex when probed with an anti-$G_{s\alpha}$ antibody in Western blots or assayed for cholera toxin-mediated ADP-ribosylation. Formation of the complex required agonist occupancy and was blocked by an α_2AR antagonist. In these same studies, dual coupling was not seen with the 5-HT_{1A} receptor expressed in CHO cells, again demonstrating that the phenomenon observed with the α_2AR was not common to other primarily G_i-coupled receptors. Finally, the extent of α_2AR-G_s coupling (using UK-

TABLE 2

Summary of G_s coupling between α_2C10, α_2C4, and α_2C2

As described in Results, the extent of functional α_2AR-G_s coupling was dependent on both the structure of the agonist and the α_2AR subtype. Summarized is the relative extent of G_s coupling for the six agonists studied with the three α_2AR subtypes. Comparisons within horizontal rows allow for assessment of the subtype dependence, whereas comparisons within vertical columns allow for assessment of compound dependence. Specific data are provided in Results and Table 1.

	α_2C10	α_2C4	α_2C2
Epinephrine	+++++	++++	+++++
Norepinephrine	+++++	++++	+++++
Oxymetazoline	+	–	++++
UK-14304	+++++	++++	+++
BHT-920	++	–	+++
BHT-933	++	–	++

14304) appeared to be different for the three human α_2AR subtypes.

The molecular determinants of α_2AR-G_s versus -G_i coupling are not known. In the present work we have explored whether agonists with divergent structures display different efficacies for G_i versus G_s coupling. We found that α_2AR-G_s coupling not only is receptor subtype selective but also is dependent on the particular agonist used. Six agonists, two from each of three molecular classes of compounds, were used to compare the efficacy of these agonists to elicit α_2AR-mediated inhibition and stimulation of adenylyl cyclase activity. We found that the efficacies of these agonists to couple to G_i were remarkably similar, both among various compounds and among the three α_2AR subtypes. In contrast, the various agonists differed markedly in their abilities to induce α_2AR-G_s coupling. It is important to note that, not only were there marked differences between the efficacies of various drugs to elicit G_s coupling for a given subtype, but between the three subtypes there were different levels of α_2AR-G_s coupling for a given drug. The efficacy of epinephrine to elicit α_2C4-G_s coupling was found to be far greater than that of oxymetazoline, which elicited no detectable α_2C4-G_s coupling. BHT-920 and BHT-933 also failed to induce α_2C4-G_s coupling, while inducing moderate G_s coupling for α_2C10 and α_2C2. The catecholamines epinephrine and norepinephrine were both capable of evoking pronounced stimulation of adenylyl cyclase, but the extent was less for α_2C4, compared with the other two subtypes. On the other hand, UK-14304, which induced G_s coupling to the same extent as did the catecholamines with α_2C10, showed a rank order of α_2C10 > α_2C4 > α_2C2. Oxymetazoline stimulation showed yet another pattern, being significant for α_2C2, minimal for α_2C10, and nonexistent for α_2C4. The divergent results among the α_2AR subtypes for G_s coupling prompted us to consider whether the introduction of the different α_2AR subtypes into CHO cells and subsequent selection of clonal cell lines could result in cell lines with differing levels of the various components of the signal transduction cascade. We investigated this with two approaches. First, more than one clonal cell line expressing each α_2AR subtype was studied and it was found that the rank orders for α_2AR-G_i and -G_s coupling, both among the α_2AR subtypes and among the various agonists, were comparable with different clonal cell lines. Second, we found identical basal and forskolin-, NaF-, and epinephrine-mediated inhibition of forskolin-stimulated adenylyl cyclase activities for all cell lines. Thus, in the present study, individual clonal cell lines did not vary in their functional capacity for signal transduction and, therefore, it seems that the subtype selectivity of α_2AR-G_s described here is not an artifact of transfection or clonal selection.

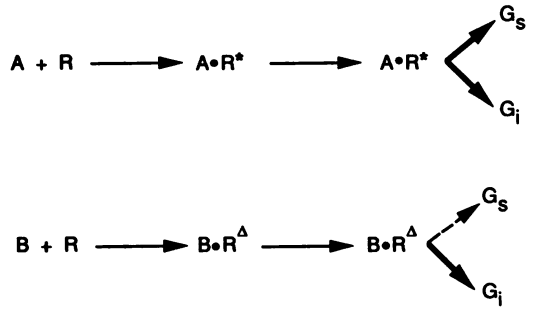

Fig. 4. Potential mechanism by which different α_2AR agonists promote different degrees of coupling to G_s. In this model agonist A binds to the α_2AR (R) and induces conformational changes in various regions of the receptor (A·R*). The conformational change in the third intracellular loop induces binding and functional coupling to G_i and G_s. Due to its different structure, agonist B induces a slightly different conformational change (B·R$^{\Delta}$), which is less favorable for G_s coupling but maintains full G_i coupling. An example from the current work would be norepinephine for agonist A and oxymetazoline for agonist B, with R being the α_2C10 subtype (see Table 1).

It is generally accepted that agonist binding to G protein-coupled receptors causes a conformational change of the receptor that results in binding and activation of the G protein. The critical receptor domains that must undergo these changes include the third intracellular loop, which has been implicated as the region involved in G protein coupling for most of these receptors. Our results suggest that these conformational changes may be different, depending on the structure of the agonist. This hypothesis is supported by a recent report by Fraser and colleagues (18). In that study, site-directed mutagenesis was used to delineate key amino acid residues critical for ligand binding and receptor activation of α_2C10 expressed in CHO cells. The important residues for these functions were found to be Asp-79, Asp-113, Asp-130, and Ser-204. The latter residue was critical for catecholamine, but not UK-14304, functional coupling. In addition, mutation of Asp-130 ablated agonist-dependent stimulation of adenylyl cyclase, but the inhibitory pathway remained intact. Taken together with the results of the current study, it is clear that certain requirements for agonist binding to the α_2AR that lead to G_i and G_s coupling are determined by different regions of the ligand-binding pocket. Thus, agonists with different structures bind to α_2AR within the pocket, causing different conformational changes and thus different degrees of G_i and G_s coupling (Fig. 4).

Given these results and those of other recent studies (4, 8, 9, 18), it is clear that the presence of functional α_2AR-G_s coupling in any given tissue is dependent on the level of α_2AR expression, the structure and concentration of the agonist, and the α_2AR subytpe. In addition, the proportion or availability of other components of the signal transduction system, particularly G_s, the various isoforms of G_i, and adenylyl cyclase, is also critical for the ultimate response of a given cell. This multifunctional signaling by G protein-coupled receptors appears to be another way in which cells that utilize the same receptor may nevertheless have differential responses. The role of dual coupling of the α_2AR to G_i and G_s in establishing biological responses to agonists is not clear at present. Some of the paradoxical effects of α_2AR are consistent with this proposed paradigm. These include α_2AR-mediated increases in cAMP found in at least four different cell lines under various conditions (8, 9, 19), in cortical brain slices (6), and in pancreatic islet cells (7) and a number of physiological responses in intact animals (reviewed in Ref. 2). Finally, the efficacy of an agonist to induce G_i coupling is not predictive of its efficacy to induce G_s coupling. This poses a new challenge for drug discovery and design, in addition to that of assigning the underlying α_2AR-mediated coupling to specific physiological processes. Indeed, the unique ability of α_2AR to couple to both G_i and G_s, eliciting opposing effects on the same signal transduction pathway, demonstrates the complexity of the system by which the final cellular response to endogenously released catecholamines or pharmacological agents is obtained.

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