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β_1- and β_2-Adrenergic Receptors Display Subtype-Selective Coupling to G_s

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SUMMARY

β-Adrenergic receptor (βAR) subtypes differ in their affinities for some agonists and antagonists and thus may potentially impart different cellular effects based on this ligand-binding specificity. However, the possibility that there may be subtype-specific events subsequent to ligand binding has not been evaluated extensively. In particular, although βARs stimulate adenylyl cyclase by coupling to the guanine nucleotide-binding protein G_s, no studies have directly assessed the coupling efficiencies among isolated βAR subtypes. We, therefore, permanently transfected the mammalian fibroblast cell line CHW-1102 with β_1- or β_2AR cDNAs and studied the coupling characteristics of these two receptor subtypes, each expressed at ~335 fmol/mg of protein. Both receptors mediated equivalent maximal increases in adenylyl cyclase activities (6.63 ± 1.85-fold for β_1AR versus 6.10 ± 0.53-fold for β_2AR; p = not significant). However, the isoproterenol dose-response curves for the β_2AR were shifted to the left, compared with those for the β_1AR (EC_{50} of 52.3 ± 2.87 nM and 191 ± 10.5 nM, respectively; $p < 0.05$), resulting in an ~4-fold greater potency for the β_2AR versus the β_1AR. Thus, at the submaximal isoproterenol concentration of 30 nM, the β_2AR stimulated adenylyl cyclase ~50% more than did the β_1AR. This finding was not due to a difference in the affinities of isoproterenol for these receptors, which were found to be the same, as determined by competition binding studies with ^{125}I-cyanopindolol in the presence of GTP. The ability of β_1- and β_2ARs to form the high affinity ternary complex was assessed in agonist competition studies without guanine nucleotide. We found that, whereas the proportion of receptors in the high affinity state was equivalent between the two receptor subtypes, the affinity of this state for isoproterenol was ~5-fold greater for the β_2AR, compared with the β_1AR (K_H for β_2AR, 11.8 ± 3.1 nM; K_H for β_1AR, 61.7 ± 18.3 nM; $p < 0.05$). In addition, we examined physical and functional coupling of β_1- and β_2ARs to G_s using the agonist epinephrine, which also has equal binding affinity for both receptor subtypes. As with isoproterenol, epinephrine was more potent in stimulating adenylyl cyclase and promoted a higher affinity ternary complex for the β_2AR. Thus, a greater degree of both physical and functional agonist-promoted coupling occurs between G_s and β_2AR, compared with β_1AR. We conclude that coupling to G_s by β_1- and β_2ARs is subtype selective and is a potentially important distinguishing feature among these members of the βAR family.

βARs mediate catecholamine-induced increases in intracellular cAMP via the stimulatory G protein G_s. Studies of the mechanisms of βAR-G_s coupling have been performed using almost exclusively the β_2AR subtype (1–3). Although it is clear that the β_1AR also couples to G_s (4), the relative efficacy of such coupling, compared with that of the β_2AR, has not been assessed. Studies that have previously examined heterogeneity among βAR subtypes have focused on ligand binding interactions (4, 5); indeed, such agonist and antagonist binding patterns are used to define βAR subtypes. However, the assumption that events in the signal-transduction cascade subsequent to ligand-receptor binding are functionally homologous among βAR subtypes has not been tested. To separate effects due exclusively to differences in coupling, studies should ideally use tissues or cells expressing pure populations of receptor subtypes in identical mammalian cells, which also provide equivalent amounts of G proteins, adenylyl cyclase, and other components necessary for measuring the coupling process.

Expression of β_1- and β_2AR subtypes in *Escherichia coli* has been reported (5, 6); these receptors demonstrate comparable physical coupling with reconstituted G_s in solubilized membranes, but functional coupling to adenylyl cyclase could not be determined. For the purpose of studying the structural requirements for ligand binding, Frielle *et al.* (4) expressed β_1- and β_2ARs in *Xenopus laevis* oocytes, but G protein coupling studies were not undertaken. Tissues (such as heart or lung) or cells (such as C_6 glioma cells) that have mixed populations of

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ABBREVIATIONS: βAR, β-adrenergic receptor; ICYP, ^{125}I-cyanopindolol; K_i, equilibrium dissociation constant (one-site model); K_H and K_L, high and low affinity equilibrium dissociation constants for agonist (two-site model); R_H or R_L, percent of receptors in the high or low affinity agonist binding state; G protein, guanine nucleotide-binding regulatory protein; Gpp(NH)p, guanosine 5′-(β,γ-imido)triphosphate.

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βAR subtypes have also been studied (7–9) but have yielded little in the way of detailed analysis of the functional coupling of these subtypes to G_s. Receptor-G protein coupling also appears to be markedly cell type specific. Recently, the neurotumor cell line SK-N-MC has been reported to express the β_1AR exclusively (10), but a rigorous comparison of the coupling characteristics of this receptor in these cells with those of the β_2AR expressed in different cells cannot be made.

To address the G_s coupling between these two βAR subtypes, we transfected a Chinese hamster fibroblast cell line (CHW-1102), which is normally devoid of adrenergic receptors, with the human β_1- or β_2AR cDNA. The resulting permanent cell lines provided two clonal lines expressing the same densities of β_1- or β_2AR, which were chosen for further characterization. Our results reveal a selectivity of coupling of βAR subtypes to G_s.

Materials and Methods

Cell culturing. CHW-1102 cells were grown as monolayers in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin, in an atmosphere of 95% air/5% CO_2, at 37°. In order to maintain selective pressure, transfected cells were grown in the presence of 50 μg/ml G418. Studies were performed when the cells were at 90% confluency.

Constructs and transfections. Permanent expression of β_1- and β_2AR in CHW cells was carried out by methods similar to those previously described (11). The human β_1AR (12) and β_2AR (13) cDNA were inserted into the mammalian expression vector pBC12BI (14) downstream of the RSV long terminal repeat transcription-control region. CHW cells (2×10^7 cells) were co-transfected by calcium phosphate precipitation with the aforementioned β_1- or β_2AR-containing vectors (30 μg) and pSV_2neo (3.0 μg) (15), which provides G418 resistance. The same parental CHW cells were utilized for each transfection. Selection was performed in 300 μg/ml G418. Expression of β_1- and β_2AR was determined using an ICYP binding assay, as described below. Subtype-specific pharmacological properties of the expressed βAR were verified as described in Results. Clonal lines expressing the β_1AR or β_2AR at identical expression levels were used for this comparative study.

Radioligand binding. Cell membranes were prepared essentially as described previously (16, 17). Briefly, attached cells were washed three times in phosphate-buffered saline, scraped in 5 mM Tris, 2 mM EDTA, pH 7.4, buffer, centrifuged at 38,000 × *g* for 10 min, homogenized, and centrifuged again. Radioligand binding studies were carried out in 75 mM Tris, 12 mM $MgCl_2$, 2 mM EDTA buffer, at 37° for 1.5 hr, and the reactions were stopped by dilution with cold buffer and rapid filtration over glass fiber filters. For saturation isotherms, membranes were incubated with varying concentrations of ICYP (5–300 pM), in the absence (total binding) or presence (nonspecific binding) of 1 μM propranolol. Competition studies were carried out as described (17), using 30 pM (β_1AR) or 20 pM (β_2AR) ICYP (concentrations equal to the K_d for ICYP, as determined from the binding isotherms) and various concentrations of agonists or antagonists, as shown. Some assays contained 100 μM GTP, as indicated. For these radioligand binding studies, the amount of bound ICYP was typically <10% of that which was added.

Adenylyl cyclase assays. Assays were performed by the method of Salomon *et al.* (18), as modified (11, 17). Membranes were incubated in 30 mM Tris, 5 mM $MgCl_2$, 0.8 mM EDTA, 0.12 mM ATP, 0.06 mM GTP, 2.8 mM phosphoenolpyruvate, 2.2 μg of myokinase, 0.1 mM cAMP, 1.0 μCi [α-^{32}P]ATP, in a final volume of 50 μl, for 45 min at 37°. [^{32}P]cAMP was separated using sequential chromatography over Dowex and alumina columns (18). Activities were determined in the presence of water (basal) and various concentrations of agonist as indicated.

Data analysis. Radioligand competition binding assays were fit to one- or two-site models, as appropriate, using iterative nonlinear least squares techniques (19). Competition curves for antagonists and for agonists with GTP had Hill coefficients of ~1.0 and fit to a single site, whereas agonist curves performed in the absence of GTP were best fit to two-site models. Comparisons were by *t* tests, with significance imparted at $p < 0.05$. Data are presented as means ± standard errors.

Materials. ICYP, [α-^{32}P]ATP, and [^{3}H]cAMP were from DuPont New England Nuclear. Dulbecco's modified Eagle's medium and fetal calf serum were from JRH Biosciences, and G418 was from GIBCO. ICI 118.551 was a gift from Imperial Chemical Industries, and betaxolol was a gift from Searle. pBC12BI was a gift from Brian Cullen, Duke University. All other reagents were from Sigma. Statistical analysis and computer modeling were performed using software from GRAPHPAD (San Diego, CA).

Results and Discussion

We expressed human β_1- and β_2ARs independently in the same cell line, in order to assess the agonist-promoted coupling of these receptors to G_s. This approach provides several advantages. First, it allows for assessment of either subtype without the potential difficulties encountered when tissue with a mixed population of β_1- and β_2AR subtypes is used. Most tissues that express predominantly one βAR subtype, for example β_1AR in heart or β_2AR in lung, have some amount of the other βAR subtype present. Thus, with these tissues, the relative functional coupling of these receptors to stimulation of adenylyl cyclase can potentially be examined only by the use of "selective" antagonists or agonists. Unfortunately, truly selective activation or blockade of one subtype, without any effect on the other, is difficult to attain. Also, it cannot be assumed that, in fact, β_1- or β_2ARs are expressed on the same cell type in a membrane preparation from heterogeneous tissue, making direct comparisons tenuous. For example, autoradiographic studies have shown that, in canine heart, β_2AR accounts for ~90% of the βARs in arterioles, compared with 15% in myocytes (20). In addition, evidence suggests that the β_1- and β_2ARs may be regulated differently *in vivo* (9); thus, βAR subtypes in tissue samples from animals may not be in their native state. Nor can the study of receptor coupling in different parental cell lines expressing βAR subtypes adequately address these questions, because G protein coupling appears to be somewhat cell type specific. By expressing these two recombinant subtypes in the same parental cell line, we have eliminated the aforementioned factors and thus are able for the first time to compare directly functional G_s coupling and agonist-promoted formation of the high affinity ternary complex of the human β_1- and β_2ARs.

The pharmacological properties of the β_1- and β_2ARs expressed in CHW cells are presented in Fig. 1 and Table 1. Both receptors demonstrated typical subtype-specific rank orders of potencies of agonists and antagonists for competition with ICYP, thus confirming the expressed receptors as β_1AR or β_2AR. Note in particular that the binding affinities of isoproterenol and epinephrine, as depicted by the K_i in competition assays performed in the presence of GTP, were the same for both β_1- and β_2ARs. The higher affinity of ICYP for the β_2AR is consistent with previous reports (21). In order to determine functional properties of these receptor subtypes, agonist-stimulated adenylyl cyclase activities were determined in membranes prepared from CHW cells expressing the two subtypes

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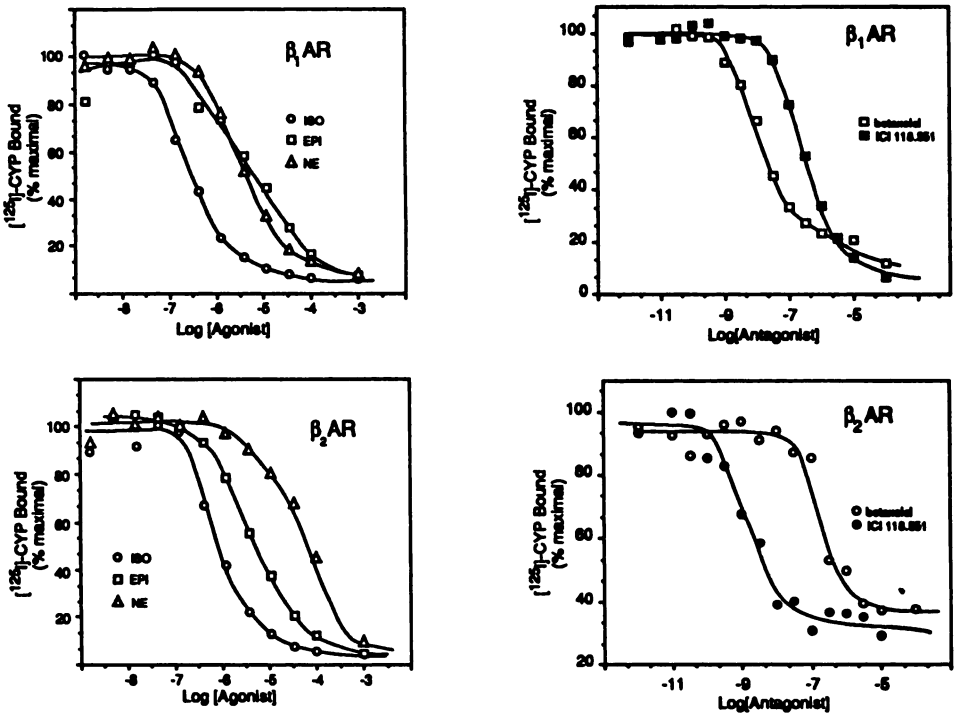

Fig. 1. ICYP competition curves with agonists or antagonists, in membranes prepared from CHW cells transfected with the β_1- or β_2AR constructs, as described in Materials and Methods. Experiments with agonists (*left*) were performed in the presence of 100 μM GTP. The rank order of potency of agonists for the β_1AR (isoproterenol > norepinephrine ≈ epinephrine) and β_2AR (isoproterenol > epinephrine ≫ norepinephrine) is characteristic, as are the potencies of the relatively selective antagonists. Data are representative of three to five experiments. *ISO*, isoproterenol; *EPI*, epinephrine; *NE*, norepinephrine.

TABLE 1

Pharmacological characteristics of ICYP binding to β_1AR and β_2AR expressed in CHW cells

The receptor density (B_{max}) and K_d were from saturation isotherms (three experiments), whereas the K_i values for various agonists and antagonists were derived from competition binding studies in the presence of GTP (four experiments), as described in Materials and Methods. The receptor densities were not statistically different between β_1- and β_2ARs, nor were the K_i values for isoproterenol and epinephrine. The other parameters are consistent with the expected rank order of potency of agonists and antagonists for β_1- and β_2AR subtypes.

	β_1AR	β_2AR
B_{max} (fmol/mg)	309 ± 6.3	362 ± 18.7
K_d ICYP (pM)	30.4 ± 5.5	21.7 ± 4.2
K_i (nM)		
Agonists		
Isoproterenol	366 ± 107	506 ± 20
Epinephrine	7640 ± 2180	3180 ± 530
Norepinephrine	3350 ± 624	44570 ± 1340
Antagonists		
ICI 118.551	102 ± 11	0.62 ± 0.121
Betaxolol	6.15 ± 2.40	128 ± 48

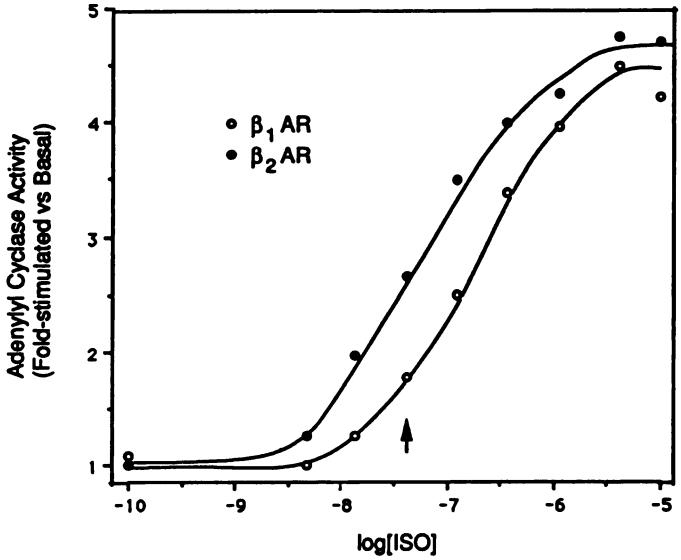

Fig. 2. Isoproterenol-stimulated adenylyl cyclase dose-response curves for CHW cells expressing the β_1AR (○) or the β_2AR (●), at identical receptor densities. The maximal isoproterenol-stimulated activities were not different between the two subtypes, but the EC_{50} for the β_2AR was ~4-fold less than that of the β_1AR. This leftward shift of the β_2AR curve results in an ~50% increase in adenylyl cyclase activity for the β_2AR versus β_1AR at the submaximal isoproterenol concentration of 30 nM (*arrow*). A representative experiment is shown. For five such experiments (β_1AR versus β_2AR), basal adenylyl cyclase activities were 2.36 ± 0.95 versus 1.34 ± 0.26 pmol/min/mg of protein (p = not significant), maximal stimulated activities were 6.63 ± 1.85-fold versus 6.10 ± 0.53-fold (p = not significant), and the EC_{50} values for isoproterenol stimulation were 191 ± 10.5 versus 52.3 ± 2.87 nM ($p < 0.05$).

(Fig. 2). Preliminary studies with these β_1- or β_2AR-expressing cells revealed that 100 μM forskolin-stimulated (21.9 ± 2.2- versus 26.4 ± 2.4-fold, respectively; n = 3; p = not significant) and 10 mM NaF-stimulated (7.35 ± 1.3- versus 10.0 ± 2.3-fold, respectively; n = 3; p = not significant) activities were not different. Maximal isoproterenol stimulation of adenylyl cyclase over basal levels was also identical between the subtypes (β_1AR, 6.63 ± 1.85-fold; β_2AR, 6.10 ± 0.53-fold; n = 5; p = not significant). However, the isoproterenol dose-response curve was shifted to the left for the β_2AR, compared with the β_1AR, resulting in a ~4-fold greater potency of isoproterenol for the β_2AR (EC_{50} = 52.3 ± 2.87 nM versus 191 ± 10.5 nM; n = 5; $p <$ 0.05). As revealed in Tables 1 and 2, this difference in the potency of isoproterenol for β_2AR versus β_1AR in stimulating adenylyl cyclase was not due to differences in the affinities of these receptors for binding the agonist. Studies performed with epinephrine gave similar results, with this agonist being more potent in stimulating β_2AR, despite having similar binding affinities for the two subtypes. For the β_2AR, the EC_{50} for stimulating adenylyl cyclase was 107 ± 26 nM, compared with 1439 ± 152 nM for the β_1AR (n = 4; $p < 0.03$).

To further assess this difference in functional activity of βAR subtypes, we performed agonist competition curves in the ab-

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TABLE 2

Agonist binding characteristics of β_1AR and β_2AR expressed in CHW cells

Membranes were prepared and isoproterenol/ICYP competition studies were carried out as described in Materials and Methods. The K_i was derived from three experiments performed in the presence of GTP, and the data were fit to a one-site model. The remainder of the presented data are from two-site fits from four experiments performed in the absence of guanine nucleotide. Low affinity binding (K_i, K_L, R_L) was not different between the β_1AR and the β_2AR (p = not significant). However, the high affinity binding state was clearly different between the two subtypes, with the K_H of the β_2AR being ~5-fold less than that of the β_1AR and the ratio of K_L/K_H being greater for the β_2AR, compared with the β_1AR.

	β_1AR	β_2AR
K_i (nM)	366 ± 107	506 ± 20
K_L (nM)	567 ± 137	1053 ± 266
K_H (nM)	61.7 ± 18.3	11.8 ± 3.1*
K_L/K_H	12.6 ± 2.2	143.0 ± 55.9*
R_L (%)	48.6 ± 9.5	66.0 ± 11.4
R_H (%)	51.4 ± 9.5	34.0 ± 11.4

*$p < 0.05$, β_1AR versus β_2AR.

sence of guanine nucleotides, thus promoting formation of the high affinity ternary complex. As shown in Fig. 3 and Table 2, a clear difference in K_H for isoproterenol was demonstrated between β_1AR and β_2AR. The K_H of the β_1AR was ~5-fold greater than that of the β_2AR (61.7 ± 18.3 nM versus 11.8 ± 3.1 nM; $p < 0.05$). The relative proportion of receptors in this high affinity state was not different between the two subtypes (Table 2). The K_L/K_H ratio has been considered a useful indicator of the relative tendency of a G protein-coupled receptor to form the high affinity ternary complex in the presence of a given agonist (22). We found that this ratio was ~10-fold greater for the β_2AR (Table 2), again revealing different degrees of G_s coupling between these two subtypes. Competition studies with epinephrine in the absence of GTP gave results similar to those with isoproterenol. The K_H of the β_1AR (89.4 ± 62 nM) was greater than that of the β_2AR (0.8 ± 0.3 nM; n = 4; $p <$ 0.05). The K_L, as determined from these two-site fits, was the same for both β_1- and β_2ARs (7.8 ± 0.9 μM versus 5.8 ± 1.4 μM; n = 4; p = not significant).

By expressing the β_1AR and the β_2AR independently in identical mammalian cells we have for the first time clearly demonstrated functional differences in receptor-G_s coupling between these βAR subtypes. Previous studies of the functional properties of β_1- and β_2ARs in cardiac tissues have revealed discrepant results. In the first study in cardiac membranes, Waelbroeck *et al.* (23) found virtually no stimulation of adenylyl cyclase by the β_1AR. Subsequent reports by Bristow *et al.* (7), using membranes from failing and nonfailing ventricular samples, suggested that, despite the smaller population of β_2ARs, this subtype was responsible for a significant proportion of isoproterenol-stimulated adenylyl cyclase activity. Noteworthy is the fact that, in the latter study, different assay conditions were required for assessment of agonist-stimulated adenylyl cyclase activities of the β_1AR and β_2AR, with the β_1AR conditions requiring Gpp(NH)p and forskolin for observation of weak agonist-mediated stimulation. Additionally, these studies used selective agonists and antagonists, making it difficult to assess with certainty any differences observed in G_s coupling between the two subtypes. Similar difficulties in interpretation were encountered by Dickinson and Nahorski (24), using radioligand binding in tissues expressing predominantly one or the other receptor subtype.

Our results with human β_1- and β_2ARs expressed in CHW

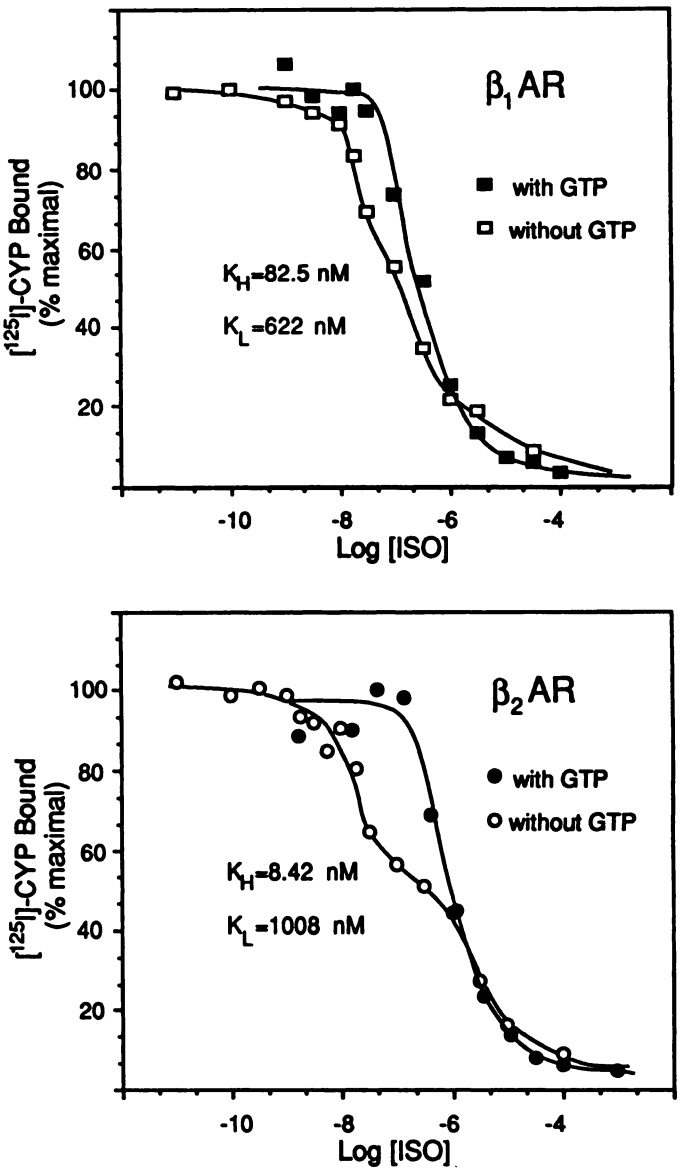

Fig. 3. ICYP/isoproterenol competition curves in membranes from CHW cells expressing the β_1AR or the β_2AR, performed in the presence (*closed symbols*) or absence (*open symbols*) of 100 μM GTP. Data were best fit to one-site (GTP present) or two-site (GTP absent) models by nonlinear iterative techniques, as described in Materials and Methods. A representative experiment is shown, similar to three to five performed (see Table 2).

cells show that agonist-mediated coupling to G_s is less efficient with the β_1AR than the β_2AR. This difference, which was consistent when examined with a variety of methods, does not, however, result in markedly depressed β_1AR function, as has been reported by some, using mixed tissues (7). At maximal concentrations of agonist, the stimulation of adenylyl cyclase over basal between the two subtypes is equivalent. At submaximal concentrations (such as at 30 nM, as shown in Fig. 2), an ~50% greater β_2AR-mediated stimulation of adenylyl cyclase is apparent, due to the ~4-fold greater potency of isoproterenol. The molecular determinates that account for these differences remain unknown at present. Studies with the β_2AR and other G protein-coupled receptors have suggested that regions in the second and third intracellular loops and the proximal cytoplasmic tails of these receptors are critical for G protein binding

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and activation (1–3). Although the β_1AR and β_2AR show an overall ~70% homology in primary amino acid structure in the transmembrane regions, this homology falls to as low as ~20% within some of the aforementioned intracellular regions. Further studies will be necessary to elucidate which regions of the β_1AR and β_2AR are responsible for the differences in coupling we have observed.

We and others have proposed that G protein coupling to receptors includes both a process of physical coupling (as assessed by high affinity agonist binding) and one of functional coupling (as assessed by activation of adenylyl cyclase) (17, 25–27). These processes appear to be distinct, with both having the potential to become impaired (25–28). Our current data suggest that a greater degree of both physical and functional agonist-mediated coupling occurs between G_s and the β_2AR, compared with the β_1AR. It is intriguing to speculate on the physiological relevance of these findings. Ultimately, the β_2AR appears to activate adenylyl cyclase to a greater extent at lower agonist concentrations, compared with the β_1AR, whereas at higher concentrations activation is similar (Fig. 2). In humans, the majority of β_1ARs are innervated and, as such, are potentially activated by high (micromolar) concentrations of agonist in the synaptic cleft. On the other hand, most β_2ARs are not innervated, thus potentially requiring enhanced activation during exposure to the lower (nanomolar) concentrations of catecholamines encountered in local tissues or the systemic circulation.

Our results reveal that a distinction, other than ligand binding specificity, can now be assigned to the β_1- and β_2ARs. We have demonstrated that G_s coupling of βARs displays subtype selectivity. Perhaps βAR subtypes, then, may have evolved to meet differing needs for agonist-mediated G_s coupling.

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