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Agonist Actions of "β-Blockers" Provide Evidence for Two Agonist Activation Sites or Conformations of the Human β_1-Adrenoceptor

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ABSTRACT

Previous work with 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one (CGP 12177) has led to the suggestion that there are two different agonist conformations of the human β_1-adrenoceptor: 1) where classic agonists (catecholamines) and β-antagonists act, and 2) where CGP 12177 is an agonist and relatively resistant to inhibition by β-adrenoceptor antagonists. In the present study, we have used studies of cAMP response element-regulated gene transcription to confirm the presence of these two β_1-adrenoceptor sites/conformations and to provide strong evidence that a range of clinically used β-adrenoceptor blockers (β-blockers) exhibit differential agonists and/or antagonist actions at the two sites. [2-(3-Carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulphonate (CGP 20712A) and atenolol act as classic antagonists at the catecholamine binding site but have much lower affinity for the secondary CGP 12177 site. CGP 12177 and carvedilol are potent antagonists at the catecholamine site but mediate substantial agonist actions on gene transcription via the secondary antagonist-resistant site at higher concentrations. Agonist effects of β-blockers are not, however, confined to this secondary site, and we show that some (particularly acebutolol and labetolol) act primarily via the catecholamine site, whereas others (pindolol and alprenolol) can stimulate both. The different responses to β-blockers seen in the clinic may therefore be caused in part by these β-blocker agonist responses and the differential activation of the two sites or conformations.

The human β_1-adrenoceptor is known to couple to Gαs and stimulate adenylyl cyclase to produce a rise in intracellular cAMP (Kobilka, 1992). CGP 12177 was originally developed as a β-adrenoceptor antagonist (Haddad et al., 1987); recently, however, it has been reported to stimulate cAMP accumulation in cellular studies (CHO-K1 cells transfected with the human β_1-adrenoceptor; Pak and Fishman, 1996; Konkar et al., 2000a) and to act as a cardiostimulant in whole-animal studies (Kaumann et al., 1998). Furthermore, this β_1-agonist effect of CGP 12177 was relatively resistant to antagonism by classic β-adrenoceptor antagonists. Concentrations of antagonists at least 10-fold higher than needed to antagonize the isoprenaline-mediated response were required to inhibit the CGP 12177 agonist responses (Konkar et al., 2000a; Lowe et al., 2002).

The novel pharmacology observed with CGP 12177 in the heart was initially thought to be caused by a new β_4-adrenoceptor (Kaumann et al., 1998; Cohen et al., 2000). Later studies, however, showed that the cardiostimulant effects of CGP 12177 were absent in β_1-adrenoceptor knockout mice (Kaumann, 2000; Konkar et al., 2000b; Kaumann et al., 2001). This led to the concept of two different active sites or conformations of the human β_1-adrenoceptor: 1) where classic agonists (catecholamines) and β-antagonists act, and 2) where CGP 12177 is an agonist and relatively resistant to inhibition by β-adrenoceptor antagonists (Konkar et al., 2000a). To date, no other ligands have been conclusively shown to stimulate functional responses via this secondary site on the human β_1-adrenoceptor.

β-Antagonists are used clinically to reduce heart rate and force of contraction in hypertension, angina, and acute myocardial infarction by directly blocking endogenous catecholamine activity (Heidenreich et al., 1999; Wright, 2000; Morgan et al., 2001). It is also now well established that some "β-blockers" are beneficial in the treatment of chronic heart failure (CIBIS-II, 1999; MERIT-HF, 1999; Packer et al.,

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ABBREVIATIONS: CGP 12177, 4-[3-[(1,1-dimethylethyl)amino]2-hydroxypropoxy]-1,3-dihydro-2H-benzimidazol-2-one; CHO, Chinese hamster ovary; CRE, cAMP response element; SPAP, secreted placental alkaline phosphatase; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; CGP 20712A, [2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulphonate; ICI 118551, *erythro*-(±)-3-isopropylamino-1-(7-methylindan-4-yloxy)butan-2-ol; HEK, human embryonic kidney.

2001; Tendera and Ochala, 2001). However, unlike β-blocker therapy for ischemic heart disease and hypertension, successful treatment in heart failure requires a longer-term, lower-dose approach (Tendera and Ochala, 2001; Wehling, 2002). Interestingly, however, this does not seem to be a simple class effect, because treatment with bucindolol was not found to be beneficial (BEST, 2001; Port and Bristow, 2001), and xamoterol increased mortality (Nicholas et al., 1990). However, it is not known whether the beneficial effects of β-antagonists can simply be attributed to long-term antagonism of endogenous catecholamine responses or some of these drugs have direct effects of their own. These could include partial agonist effects at either site of the β_1-adrenoceptor, allosteric modulation of the receptor, short-term secondary messenger changes, or long-term gene transcription changes within the cell.

We recently observed that CGP 12177, although stimulating only a small increase in cAMP accumulation, stimulated a large increase in CRE-mediated gene transcription at the human β_2-adrenoceptor (Baker et al., 2002) suggesting that weaker partial agonists are able in induce substantial gene transcription changes. The aim of this study was therefore to see whether CGP 12177 was able to stimulate gene transcription via the human β_1-adrenoceptor and whether the different pharmacological effects seen at the level of cAMP accumulation (Pak and Fishman, 1996; Konkar et al., 2000a) can be demonstrated at the level of gene transcription. Furthermore, we have investigated whether a range of clinically used β-blockers can stimulate gene transcription via the human β_1-adrenoceptor and whether they do this via the catecholamine or secondary CGP 12177 site of the β_1-adrenoceptor.

Materials and Methods

Materials

Cell culture reagents were from Sigma Chemical (Poole, Dorset, UK) except fetal calf serum, which was from PAA Laboratories (Teddington, Middlesex, UK). [^{3}H]Adenine, [^{3}H]CGP 12177, and [^{14}C]cAMP were obtained from Amersham Biosciences UK, Ltd. (Buckinghamshire, UK); CGP 12177 was from Tocris Cookson Inc. (Avonmouth, Bristol, UK); Microscint-20, the scintillation fluid Cocktail Plus, and the Luclite Plus Assay System kit were from PerkinElmer (Groningen, Netherlands). Bupranolol was a gift from Prof. S. Harding (Imperial College, London, UK). Sigma Chemical supplied all other reagents.

Cell Culture

CHO cells stably expressing the human β_1-adrenoceptor (a gift from S. Rees, GlaxoSmithKline, Stevenage, UK) were secondarily transfected with the reporter gene secreted placental alkaline phosphatase (SPAP), under the transcriptional control of a six-CRE promoter (McDonnell et al., 1998) using LipofectAMINE and OptiMEM according to the manufacturer's instructions. After selection using resistance to geneticin (1 mg/ml for the β_1-receptor) and hygromycin (200 μg/ml for SPAP reporter), a clone was isolated by dilution cloning (CHO-β_1-SPAP). A second line of CHO cells, stably transfected with the human β_1-receptor and a six-CRE promoter luciferase reporter gene, were also used (CHO-β_1-luciferase; a gift from S. Rees, GlaxoSmithKline). Untransfected cells (CHO-K1) and those transfected only with the CRE-SPAP reporter (CHO-SPAP) were used as controls where stated. All CHO cell lines were grown at 37°C in Dulbecco's modified Eagle's medium/Ham's F12 nutrient mix (DMEM/F12) containing 10% fetal calf serum and 2 mM L-glutamine in a humidified 5% CO_2/95% air atmosphere.

CRE-Mediated Gene Transcription (SPAP)

Cells were grown to confluence in 24-well plates then serum-starved for 24 h before experimentation in DMEM/F12 containing 2 mM L-glutamine. On the day of experimentation, the medium was replaced with 1 ml of fresh serum-free medium. Where used, antagonists were added to this medium and incubated for 30 min at 37°C in a humidified atmosphere of 5% CO_2/95% air. Agonists (in 10 μl, each condition in triplicate) were then added and incubated for 5 h in the same atmosphere. Media and drugs were then removed and replaced with 300 μl of fresh serum-free media and incubated for a further hour; 20-μl samples of media from each well were then transferred to 96-well plates and heated to 65°C for 30 min to destroy any endogenous alkaline phosphatases. CRE-dependent SPAP reporter activity was quantified by following the color change caused by the hydrolysis of *p*-nitrophenol phosphate (Cullen and Malim, 1992); 200 μl of *p*-nitrophenol phosphate in diethanolamine buffer was added to each sample and incubated at 37°C in air for 1 h. The plates were then read at 405 nm using an MRX plate reader (Dynatech Laboratories, Chantilly, VA) and the data converted to SPAP concentration in milliunits per milliliter as described previously (McDonnell et al., 1998).

CRE-Mediated Luciferase Production

Cells were grown to confluence in white 96-well plates. On the day of experimentation, the medium was removed and replaced with 200 μl of fresh serum-free medium or a medium containing the final antagonist concentration and incubated for 30 min at 37°C. Agonists in 20 μl were then added to each well and incubated in the presence or absence of antagonist for a further 5 h at 37°C. The drugs and media were then removed and the cells washed twice with 200 μl of phosphate-buffered saline (PBS); 2 ml of PBS containing 1 mM Ca^{2+}/1 mM Mg^{2+} were mixed with 2 ml Luclite Plus, 40 μl of this was added to each well, and the plate counted on a Topcount liquid scintillation counter (PerkinElmer).

Cyclic AMP Accumulation

Cells were grown to confluence in 24-well plates then prelabeled with [^{3}H]adenine (4 μCi/ml) for 2 h at 37°C in 1 ml/well Hanks' balanced salt solution containing 20 mM HEPES, pH 7.4). The [^{3}H]adenine was removed, and each well was washed twice with 1 ml of Hanks' balanced salt solution/HEPES each time, then incubated for 30 min with 1 ml of medium containing 3-isobutyl-1-methylxanthine (100 μM). Agonists in 10 μl of Hanks' balanced salt solution/20 mM HEPES were then added and the cells incubated for a further hour before the reaction was terminated by the addition of 50 μl of concentrated HCl. [^{3}H]cAMP was separated from other [^{3}H]adenine nucleotides by sequential Dowex and alumina chromatography, and each column corrected for efficiency by comparison with [^{14}C]cAMP recovery as described previously (Donaldson et al., 1988).

[^{3}H]CGP 12177 Whole-Cell Binding

Cells were grown to confluence in white 96-well plates. The medium was removed and replaced with 200 μl of DMEM/F12 medium containing the relevant concentration of [^{3}H]CGP 12177 either with or without 100 nM CGP 20712A (to define nonspecific binding). The media and drugs were then removed and each well was washed twice with 200 μl of PBS; 200 μl of Microscint 20 were added to each well, and the plates were counted on a Topcount (PerkinElmer). Protein content was determined by the method of Lowry et al. (1951).

Data Analysis

One-Site Agonist Curves. A maximal isoprenaline concentration was included in each separate experiment for both [^{3}H]cAMP accumulation and SPAP gene transcription to allow agonist responses to be expressed as a percentage of the isoprenaline maximum. Agonist concentration-response curves were fitted to a four-parameter logistic equation through computer-assisted nonlinear

regression using the program Prism 2 as described previously (Hopkinson et al., 2000).

Antagonist K_D Value Calculations. All antagonist dissociation constants were assessed at fixed antagonist concentrations (assuming competitive antagonism) by observing the shift in the agonist concentration-response curve using the equation $DR = 1 + [A]/K_d$, where DR (dose-ratio) is the ratio of the concentrations of agonist required to produce an identical response in the presence and absence of antagonist, [A] is the concentration of antagonist, and K_D is the antagonist dissociation constant. Schild Plots were determined using the equation $\log(DR - 1) = \log[A] - \log[K_d]$.

Two-Site Agonist Curves. Concentration-response curves for pindolol and alprenolol were fitted to two sites with Prism 2 using the equation

$$\%\ \text{maximal stimulation} = \frac{[A]\cdot N}{([A] + EC1_{50})} + \frac{[A]\cdot(100 - N)}{([A] + EC2_{50})}$$

where N is the percentage of site 1, [A] is the concentration of agonist, and $EC1_{50}$ and $EC2_{50}$ are the respective EC_{50} values for the two agonist sites.

Two-site analysis was also applied to the concentration response curves obtained with CGP 12177 in the presence of a fixed concentration of isoprenaline (see Fig. 3A). The equation fitted by Prism 2 was:

$$\text{Response} = \text{Basal} + (\text{Iso}_R - \text{Basal})\left[1 - \frac{[C]}{([C] + IC_{50})}\right] + CGP_S\left[\frac{[C]}{([C] + EC_{50})}\right]$$

where Basal is the response produced in the absence of agonist, Iso_R is the measured response to the fixed concentration of isoprenaline ($[Iso]_1$), [C] is the concentration of CGP 12177, IC_{50} is the concentration of CGP 12177 required to inhibit isoprenaline-stimulated SPAP production by 50%, CGP_S is the maximal stimulation of SPAP production produced by CGP 12177, and EC_{50} is the concentration of CGP 12177 required to produce 50% of the maximal stimulation of SPAP secretion produced by CGP 12177. The antagonist dissociation constant for CGP 12177 was also calculated from the IC_{50} value obtained from this analysis by use of the null method described by Lazareno and Roberts (1987): $K_D = IC_{50}/([Iso]_1/[Iso]_2 - 1)$, where $[Iso]_1$ is the concentration of isoprenaline used in the competition experiment above and $[Iso]_2$ is the concentration of isoprenaline (deduced from a concentration-response curve to isoprenaline measured in the same experiment) that produced 50% of the response achieved by $[Iso]_1$.

[^{3}H]Ligand Binding. Curves of the specific binding (SB) of [^{3}H]CGP 12177 at different concentrations of the [^{3}H]ligand were fitted using the nonlinear regression program Prism 2 to the equation $SB = (A \times B_{max})/(A + K_D)$, where A is the concentration of [^{3}H]CGP 12177, B_{max} is the maximal specific binding, and K_D is the dissociation constant of [^{3}H]CGP 12177. All data are presented as mean ± S.E.M. The n in the text refers to the number of separate experiments.

Results

Receptor Expression in CHO-β_1-SPAP and CHO-β_1-Luciferase Cells. The specific binding of [^{3}H]CGP 12177 to whole CHO-β_1-SPAP cells yielded a K_D value of 0.42 ± 0.01 nM (n = 3) for the radioligand, giving a B_{max} value of 1146.7 ± 132.1 fmol/mg of protein (n = 3). In CHO-β_1-luciferase cells, specific binding of [^{3}H]CGP 12177 revealed a K_D value of 0.15 ± 0.01, n = 7, and a B_{max} value of 79.0 ± 9.7 fmol/mg of protein [i.e., 14.5 times lower receptor expression than the CHO-β_1-SPAP cell line (n = 3)].

Luciferase Production in CHOβ_1-Luciferase Cells. Isoprenaline stimulated an increase in luciferase activity that was 5.3 ± 0.4-fold over basal (log EC_{50} value of −7.69 ± 0.14, n = 42, Fig. 1A). In these cells with lower receptor expression, CGP 12177 appeared as a partial agonist, stimulating an increase in luciferase production that was 52.3 ± 2.5% of the maximal isoprenaline response (log EC_{50} −7.74 ± 0.04, n = 36, Fig. 1B).

Atenolol inhibited the isoprenaline-induced response to yield a log K_D value of −6.57 ± 0.13 (n = 19, Fig. 1A); however, even 100 μM atenolol was unable to cause a substantial shift of the CGP 12177-induced response (n = 6), suggesting that the K_D value for atenolol is at least 2 orders of magnitude above that achieved for the isoprenaline-stimulated response (Fig. 1B). Carvedilol (Fig. 2), propranolol, and CGP 20712A also showed this differential antagonist ability for the isoprenaline and CGP 12177-stimulated responses (Table 1). Thus, the concentrations of β-antagonist required to antagonize the CGP 12177-induced responses were consistently at least an order of magnitude larger than those required to inhibit the isoprenaline-stimulated responses at this physiological level of expression (Table 1).

Potent antagonism by CGP 12177 of the catecholamine site

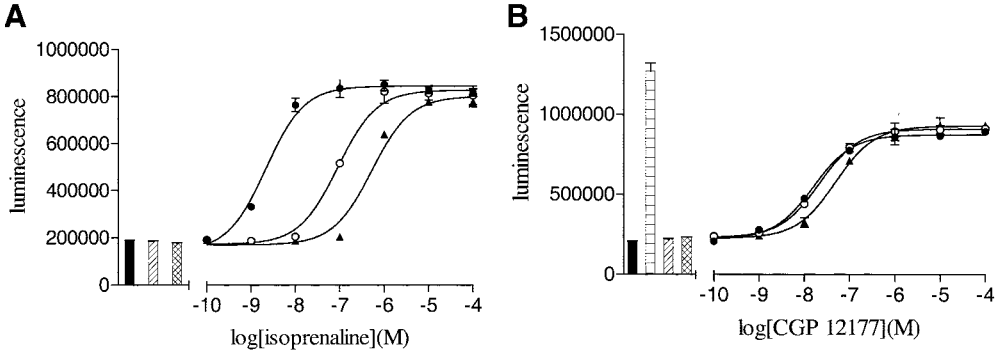

Fig. 1. A, CRE-luciferase activity in response to isoprenaline in the absence and presence of 3 or 30 μM atenolol. Luciferase activity is shown as the luminescence (relative light units) obtained as described under *Materials and Methods*. Bars show the basal luciferase activity (■) and that obtained in the presence of 3 μM atenolol alone (▨; i.e., in the absence of isoprenaline), and 30 μM atenolol alone (▩). ●, isoprenaline; ○, isoprenaline + 3 μM atenolol; ▲, isoprenaline + 30 μM atenolol. B, CRE-luciferase activity in response to CGP 12177 in the absence and presence of 10 μM or 100 μM atenolol. Bars show basal luciferase activity (■) and that obtained in the presence of 10 μM isoprenaline alone (▤), 10 μM atenolol alone (▨), and 100 μM atenolol alone (▩). Data points (mean ± S.E.M.) are from a single experiment. Similar data were obtained on three other occasions. ●, CGP 12177; ○, CGP 12177 + 10 μM atenolol; ▲, CGP 12177 + 100 μM atenolol.

is demonstrated in Fig. 3A, where the response to isoprenaline (at a fixed concentration of 100 nM) became inhibited by increasing concentrations of CGP 12177 (up to 10 nM CGP 12177). As the concentration of CGP 12177 increased further, however, the agonist effects of CGP 12177 were seen. Thus, in Fig. 3A, both higher-affinity antagonism of the catecholamine site and lower-affinity agonist action at the secondary CGP 12177 site have been clearly demonstrated with this compound. Estimation of the antagonist K_D value for the catecholamine site (from the IC_{50} as described under *Materials and Methods*) yielded a log K_D value of -9.96 ± 0.09, $n = 3$. This was very similar to the value obtained from radioligand binding studies for [^{3}H]CGP 12177 (above).

Further evidence of potent antagonism of the catecholamine site by CGP 12177 is seen in Fig. 3B, where 3 nM CGP 12177 was able to produce a parallel shift (10-fold) of the upper portion of the concentration response to isoprenaline, consistent with a partial agonist action (Fig. 3B). However, at the higher concentrations of CGP 12177, where significant responses are observed, there was no further shift of the isoprenaline curve beyond that achieved with 3 nM CGP 12177 (Fig. 3B, $n = 8$). This result is intriguing and would be consistent with an additional agonist action of isoprenaline at the secondary site.

To determine whether isoprenaline was indeed acting at the secondary site and thus limiting the extent of the shift in Fig. 3B, we have determined whether the concentration-response curve for isoprenaline can be shifted beyond this point with other antagonists. The isoprenaline concentration response curve was shifted by increasing concentrations of CGP 20712A in a competitive manner (Schild slope = 0.99 ± 0.05, $n = 4$) to yield a K_D value for CGP 20712A of -9.59 ± 0.06, $n = 16$ (Fig. 4A). CGP 12177 was also antagonized in a competitive manner by CGP 20712A (Schild slope = 1.05 ± 0.06, $n = 4$) yielding a K_D value of $-7.15 + 0.05$, $n = 23$ (Fig. 4B). The isoprenaline response was shifted beyond the limited position observed in Fig. 3B (in the presence of CGP 12177), which suggests that isoprenaline does not have a marked agonist action at the second site.

SPAP Production in CHO-β_1-SPAP. Isoprenaline stimulated a maximal increase in SPAP secretion of 2.85 ± 0.09-fold over basal with a log EC_{50} value of -8.59 ± 0.12 ($n = 18$; Fig. 5A). In this higher-expressing cell system, CGP 12177 (log EC_{50} -9.13 ± 0.04, $n = 16$, Fig. 5B) was virtually a full agonist, stimulating a maximal SPAP response equivalent to $84.9 \pm 2.7\%$ of that produced by isoprenaline. As expected for a cell line with higher β_1-adrenoceptor expression, the log EC_{50} values for both isoprenaline and CGP 12177 were shifted left to lower agonist concentration with respect to the CHO-β_1-luciferase line. This is further evidence that both responses are indeed occurring via the transfected β_1-adrenoceptor.

The isoprenaline-induced SPAP response was antagonized by the selective β_1-antagonist atenolol to yield a log K_D value of -6.88 ± 0.06 ($n = 12$; Fig. 5A). Atenolol also inhibited the CGP 12177-stimulated response to yield a log K_D value of -5.21 ± 0.18 ($n = 6$, Fig. 5B). CGP 20712A and propranolol showed similar differences in the K_D values obtained with the two different agonists (Table 1). Thus, in a similar manner to the lower-expressing system, the concentrations of β-antagonist required to antagonize the CGP 12177-induced responses were consistently at least an order of magnitude larger than those required to inhibit the isoprenaline-stimulated responses. This suggests that the discrepancies seen

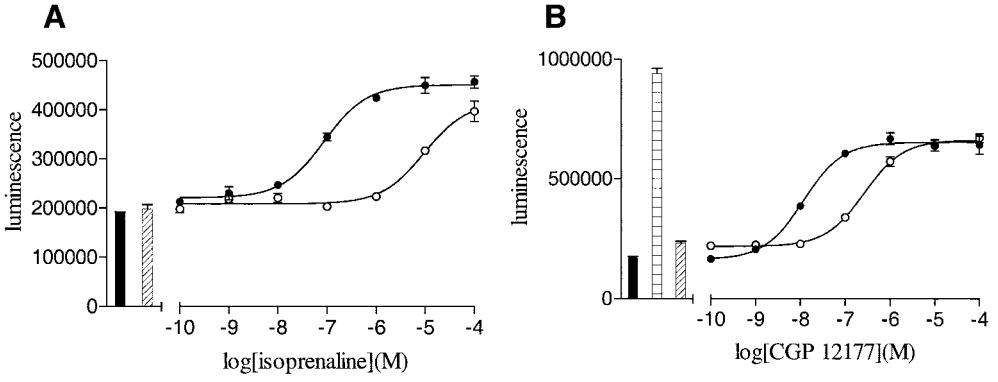

Fig. 2. A, luciferase activity in response to isoprenaline in the absence and presence of 100 nM carvedilol. Bars show basal luciferase activity (■) and that in the response to 100 nM carvedilol alone (▨). ●, isoprenaline; ○, isoprenaline + 100 nM carvedilol. B, luciferase activity in response to CGP 12177 in the absence and presence of 1 μM carvedilol. Bars show basal luciferase activity (■) and that in response to 10 μM isoprenaline alone (▤) and 1 μM carvedilol alone (▨). Data points are mean ± S.E.M. of triplicate determinations from a single experiment.●, CGP 12177; ○, CGP 12177+ 1 μM carvedilol. Similar data were obtained in three (A) and six (B) other separate experiments.

TABLE 1

log K_D values for antagonism of isoprenaline- or CGP 12177-stimulated responses by a range of antagonists

Values are mean ± S.E.M. of n determinations. log K_D values were determined from parallel shifts of agonist concentration-response curves. Measurements were made with either isoprenaline or CGP 12177 as agonists.

β-Blocker	Isoprenaline log K_D	n	CGP 12177 log K_D	n
CHO-β1-luciferase cells (low receptor expression)				
Atenolol	-6.57 ± 0.13	19	>-4	6
Propranolol	-7.45 ± 0.07	8	-6.09 ± 0.21	6
CGP 20712A	-9.59 ± 0.06	16	-7.15 ± 0.05	23
Carvedilol	-9.32 ± 0.14	4	-7.44 ± 0.11	7
CHO-β1-SPAP cells (high receptor expression)				
Atenolol	-6.88 ± 0.06	12	-5.21 ± 0.18	6
Propranolol	-8.32 ± 0.11	8	-6.91 ± 0.14	5
CGP 20712A	-8.91 ± 0.08	9	-7.93 ± 0.08	10

were not a result of either receptor expression or interference from the reporter product itself.

Close inspection of the basal response to carvedilol (1 μM, Fig. 2B) in the lower-expressing cell line suggests that there may by a direct effect of carvedilol on β_1-adrenoceptor-mediated gene transcription. We have therefore examined the

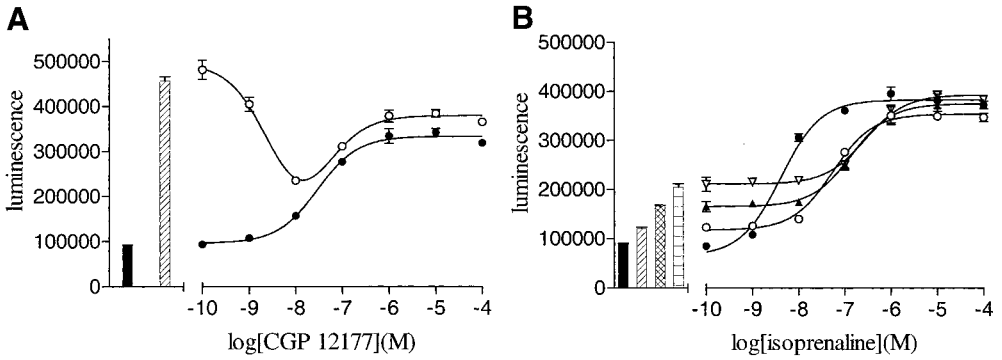

Fig. 3. A, luciferase activity in response to increasing concentrations of CGP 12177 in the presence and absence of 100 nM isoprenaline. Here, the isoprenaline and CGP 12177 were added simultaneously. Bars show basal luciferase activity (■) and that in response to 100 nM isoprenaline alone (▨). Data points are mean ± S.E.M. of triplicate determinations, and this single experiment is representative of three separate experiments. Curves through the data points represent single (CGP 12177 alone) or two-site (CGP 12177 + 100 nM isoprenaline) nonlinear regression analyses as described under *Materials and Methods*. ●, CGP 12177; ○, CGP 12177 + 100 nM isoprenaline. B, luciferase activity in response to isoprenaline in the presence and absence of 3, 10, or 30 nM CGP 12177. Bars represent basal luciferase activity (■) and that in response to 3 nM CGP 12177 alone (▨), 10 nM CGP 12177 alone (▩), or 30 nM CGP 12177 alone (▤). CGP 12177 was added 30 min before isoprenaline as described under *Materials and Methods*. ●, isoprenaline; ○, isoprenaline + 3 nM CGP 12177; ▲, isoprenaline + 10 nM CGP 12177; ▽, isoprenaline + 30 nM CGP 12177. Data points are mean ± S.E.M. of triplicate determinations. Similar data were obtained on seven other separate occasions.

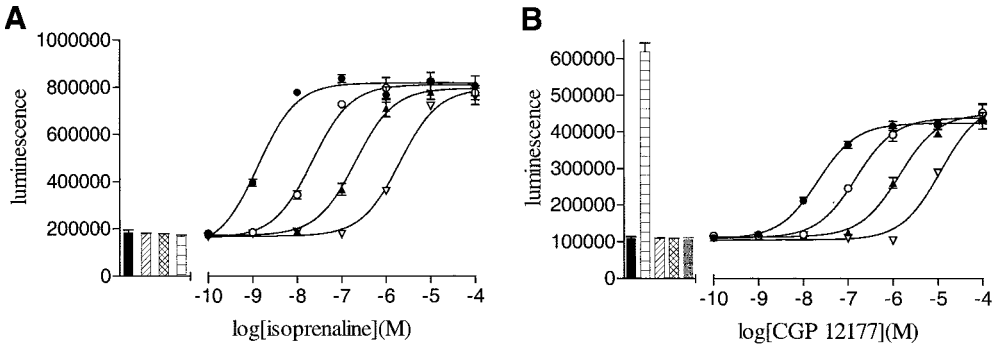

Fig. 4. A, luciferase activity in response to isoprenaline in the absence and presence of increasing concentrations (3, 30, and 300 nM) of CGP 20712A. Bars represent basal luciferase activity (■) and that in the presence of 3 nM CGP 20712A alone (▨), 30 nM CGP 20712A alone (▩), and 300 nM CGP 20712A alone (▤). Data points are mean ± S.E.M. of triplicate determinations from a single experiment. Similar data were obtained in three other separate experiments. ●, isoprenaline; ○, isoprenaline + 3 nM CGP 20712A; ▲, isoprenaline + 30 nM CGP 20712A; ▽, isoprenaline + 300 nM CGP 20712A. B, luciferase activity in response to CGP 12177 in the absence and presence of increasing concentrations (300 nM, 3 μM, and 30 μM) of CGP 20712A. Bars represent basal luciferase activity (■), that in response to 10 μM isoprenaline alone (▤), in the presence of 300 nM CGP 20712A alone (▨), 3 μM CGP 20712A alone (▩), and 30 μM CGP 20712A alone (▤). ●, CGP 12177; ○, CGP 12177 + 300 nM CGP 20712A; ▲, CGP 12177 + 3 μM CGP 20712A; ▽, CGP 12177 + 30 μM CGP 20712A. Data points are mean ± S.E.M. of triplicate determinations from a single experiment. Similar data were obtained in seven other separate experiments.

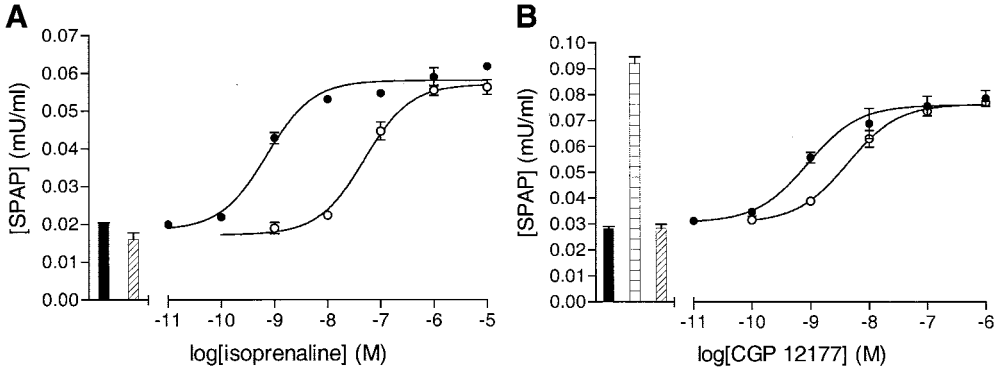

Fig. 5. A, SPAP production induced by isoprenaline in the absence and presence of 10 μM atenolol. Points represent mean ± S.E.M. of triplicate determinations. The bars show basal SPAP production (■) and that in the presence of 10 μM atenolol alone (▨) in a single experiment. Similar results were obtained in five other experiments. ●, isoprenaline; ○, isoprenaline + 10 μM atenolol. B, SPAP production induced by CGP 12177 in the presence and absence of 100 μM atenolol. Points represent mean ± S.E.M. of triplicate determinations. The bars show basal SPAP production (■), that in response to 10 μM isoprenaline alone (▤), or 100 μM atenolol alone (▨); this single experiment is representative of six separate experiments. ●, CGP 12177; ○, CGP 12177 + 100 μM atenolol.

effects of other β-blockers as stimulants of β_1-adrenoceptor mediated gene expression. In the following experiments, the higher-expressing CHO-β_1-SPAP line was used because the increased receptor numbers allowed easier detection of smaller agonist responses. CGP 20712A was used as the antagonist because it did not cause any stimulatory or inverse agonist effects of its own.

Activation of CRE-SPAP Reporter Gene Expression by Other β-Blockers. In CHO-β_1-SPAP cells, acebutolol (Fig. 6A), labetolol (Fig. 6B), and carvedilol (Fig. 6C) all stimulated an increase in SPAP reporter in CHO-β_1-SPAP cells. These acebutolol, labetolol, and carvedilol responses were all antagonized by CGP 20712A, yielding log K_D values of -9.94 ± 0.09 ($n = 5$), -9.12 ± 0.09 ($n = 5$), and -8.27 ± 0.13 ($n = 9$), respectively (Fig. 6, a–c). Propranolol also stimulated a dose-dependant increase in CRE-SPAP production of $13.7 \pm 3.5\%$ of the maximum isoprenaline response and although consistent, this was too small to evaluate antagonism by CGP 20712A (Fig. 6D). Atenolol, bisoprolol, CGP 20712A, ICI 118551, metoprolol, and sotalol had no effect on gene transcription at concentrations up to 100 μM ($n = 3$–4; see Table 2).

Concentration response curves to alprenolol ($47.9 \pm 4.0\%$ of isoprenaline maximum) and pindolol ($66.4 \pm 4.2\%$), however, were fitted best by a two-component analysis (Fig. 7). For alprenolol, site 1 accounted for $52.9 \pm 4.0\%$ of the total response (site 1 log EC_{50}, -8.66 ± 0.14; site 2 log EC_{50}, -6.13 ± 0.19; $n = 9$, Fig. 7A), whereas for pindolol (site 1 log EC_{50}, -8.51 ± 0.20; site 2 log EC_{50}, -5.30 ± 0.20, $n = 8$, Fig. 7B) site 1 accounted for $43.6 \pm 2.8\%$ of the maximal response. In the presence of 100 nM CGP 20712A, however, the responses to these two ligands seemed to contain only a single component with log EC_{50} values of -6.03 ± 0.03 ($n = 5$, Fig. 7A) and -6.20 ± 0.12 ($n = 5$, Fig. 7B) for alprenolol and pindolol, respectively.

[^{3}H]cAMP Accumulation. To determine that these agonist responses to β-blockers observed at CRE-mediated gene transcription were not an unusual effect of the reporter assay, cAMP accumulation was measured for all of the ligands mentioned above. All those ligands that caused an increase in CRE-SPAP production in CHO-β_1-SPAP cells also caused an increase in cAMP accumulation (see Table 2, Fig. 8). In addition, isoprenaline stimulated an increase in cAMP accumulation that was 37.8 ± 3.4-fold over basal (log EC_{50} -8.81 ± 0.12, $n = 3$). Two-component responses were also seen for alprenolol and pindolol (alprenolol total response $12.1 \pm 1.3\%$ isoprenaline maximum; site 1 log EC_{50}, -7.91 ± 0.17, site 2 log EC_{50}, -5.86 ± 0.15, site 1 accounted for $45.9 \pm 3.8\%$ of the total response, $n = 6$, Fig. 8C; pindolol total response $23.0 \pm 1.5\%$ of isoprenaline, site 1 log EC_{50}, -8.47 ± 0.14, site 2 log EC_{50}, -5.88 ± 0.15, site 1 accounted for $33.4 \pm 1.4\%$ of the maximal response, $n = 4$, Fig. 8D). Atenolol, bisoprolol, CGP 20712A, ICI 118551, metoprolol, and sotalol did not cause any demonstrable change in cAMP accumulation (see Table 2).

Lack of Responses or [^{3}H]CGP 12177 Binding in Native CHO-K1 Cells. Although increases in the [^{3}H]cAMP accumulation and SPAP production were clearly demonstrated in response to the direct adenylyl cyclase activator forskolin, there was no response to any of the above agonists in either assay in CHO-K1 cells or cells transfected only with the CRE-SPAP reporter gene (CHO-SPAP cells); n = at least 3 for each drug in each assay up to concentrations of 100 μM. There was also no specific binding of [^{3}H]CGP 12177 to untransfected CHO-K1 cells, again confirming the absence of any other β-adrenergic receptors in these cells ($n = 4$). These

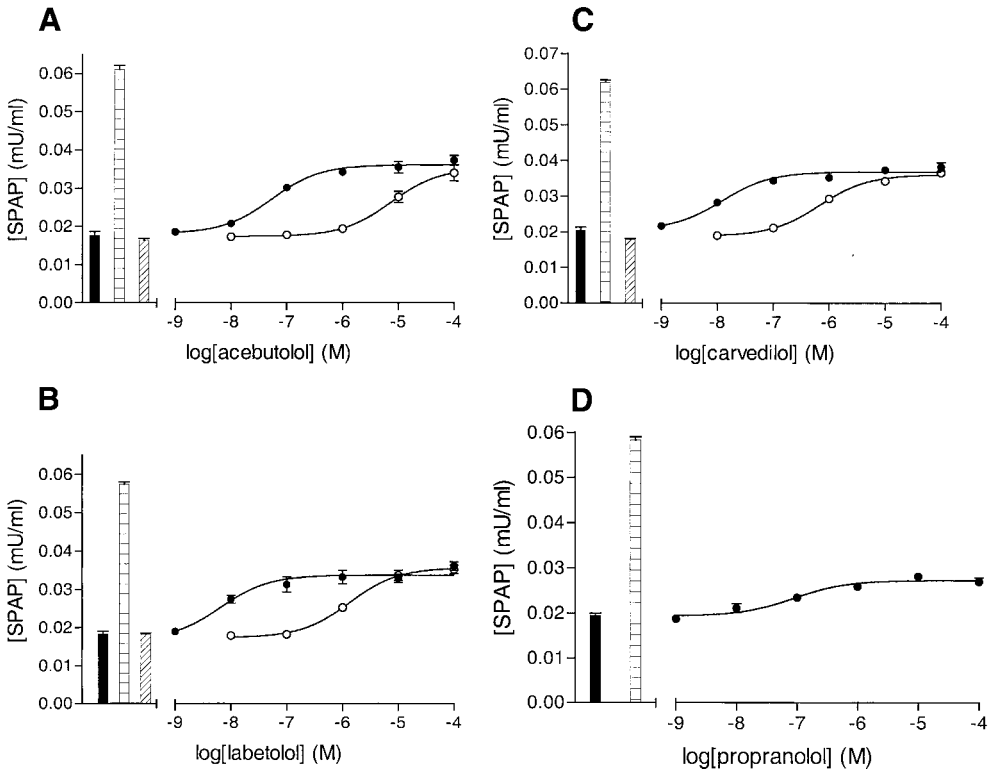

Fig. 6. A, SPAP production induced by acebutolol in the absence and presence of 10 nM CGP 20712A. Points represent mean ± S.E.M. of triplicate determinations in a single experiment. The bars show basal SPAP production (■), that in response to 10 μM isoprenaline alone (▤), and that obtained in the presence of 10 nM CGP 20712A alone (▨). Similar data were obtained in four further separate experiments. ●, acebutolol; ○, acebutolol + 10 nM CGP 20712A. B and C, SPAP production induced by labetolol (B) and carvedilol (C) in the absence and presence of 100 nM CGP 20712A. Points represent mean ± S.E.M. of triplicate determinations. The bars show basal SPAP production (■), that in response to 10 μM isoprenaline alone (▤), and that obtained in the presence of 100 nM CGP 20712A alone (▨). These single experiments are each representative of four (B) and five (C) further experiments. B, ●, labetolol; ○, labetolol + 100 nM CGP 20712A. C, ●, carvedilol; ○, carvedilol + 100 nM CGP 20712A. D, SPAP production after incubation with propranolol (●). Bars represent basal SPAP production (■) and that achieved in the presence of 10 μM isoprenaline alone (▤). Data points are mean ± S.E.M., and this single experiment is similar to six other separate experiments.

TABLE 2

Concentration-response parameters for β-blocker-stimulated cyclic AMP and SPAP responses in CHO-β1-SPAP cells

Data represent mean ± S.E.M. of n separate experiments. E_{max} represents the maximal response to each β-blocker expressed as a percentage of the response to 10 μM isoprenaline measured in the same experiment. Where the response was large enough, log K_D values were obtained for CGP 20712A from antagonism of the β-blocker–induced SPAP response.

β-Blocker	cAMP			SPAP				
	log EC_{50}	E_{max}	n	log EC_{50}	E_{max}	n	CGP 20712A log K_D	n
		%			%			
Acebutolol	−6.83 ± 0.09	8.4 ± 0.4	3	−7.16 ± 0.07	43.1 ± 5.4	8	−9.94 ± 0.09	5
Labetolol	−6.39 ± 0.13	7.2 ± 0.6	3	−7.60 ± 0.12	43.3 ± 3.1	8	−9.12 ± 0.09	5
Carvedilol	−7.64 ± 0.11	10.2 ± 1.8	8	−8.51 ± 0.13	39.7 ± 3.0	9	−8.27 ± 0.13	9
CGP 12177	−7.67 ± 0.15	63.0 ± 2.6	3	−9.13 ± 0.04	84.9 ± 2.7	16	−7.93 ± 0.08	10
Propranolol	−7.08 ± 0.35	3.2 ± 0.1	3	−7.19 ± 0.35	13.7 ± 3.5	7		
Bupranolol	−8.81 ± 0.39	1.13 ± 0.14	4	No response		3		
Atenolol	−6.74 ± 0.10	0.6 ± 0.2	3	No response		4		

data confirm that the cAMP and SPAP responses to β-agonists described above are dependent upon the presence of the human β_1-adrenoceptor.

Discussion

The β_1-adrenoceptor has recently been proposed to have two activation sites at which different agonists can elicit functional responses (Konkar et al., 2000a). The existence of multiple ligand binding sites on amine G-protein-coupled receptors is well known. A number of allosteric modulators have been shown to modulate the effects of classic agonists acting at different G-protein-coupled receptors [e.g., muscarinic receptors (Lazareno et al., 2000), the adenosine A_1 receptor (Mudumbri et al., 1993; Kollias-Baker et al., 1997), and the α_2-adrenoceptor (Leppik and Birdsall, 2000)]. An ectopic agonist site has also been identified on the muscarinic M1 receptor that is distinct from that used by traditional agonists (Spalding et al., 2002). The evidence for two agonist sites on the β_1-adrenoceptor so far is based on the observation that CGP 12177 induces stimulatory responses at the level of cAMP accumulation via the human β_1-adrenoceptor that are relatively resistant to antagonism by classic β-antagonists (Konkar et al., 2000a; Lowe et al., 2002). Here we have shown, using studies of gene transcription, that other β-blockers as well as CGP 12177 can stimulate responses via this second site or conformation of the human β_1-adrenoceptor.

In the CHO-β_1-luciferase cell line, with physiological levels of receptor expression, both isoprenaline and CGP 12177 induced agonist responses. The CGP 12177-induced responses were more resistant to antagonism by CGP 20712A, atenolol, propranolol, and carvedilol than responses induced by the classic agonist isoprenaline. This is consistent with the proposed two-site model suggested by Konkar et al. (2000a). Thus carvedilol, CGP 20712A, propranolol, and atenolol bind to the classic catecholamine site with much higher affinity than for the secondary site. This is also true for CGP 12177. Figure 3a clearly demonstrates potent antagonism (K_D value of 0.11 nM) of isoprenaline-stimulated gene transcription at concentrations of CGP 12177 much lower than those required to produce substantial agonist effects (EC_{50}, 18.2 nM in the same cells). Discrepancies in the K_D values for antagonists (CGP 20712A, propranolol, and atenolol) were also obtained in the higher-expressing CHO-β_1-SPAP cell line when CGP 12177 and isoprenaline were used an agonists. Because this unusual pharmacology is seen at both high and low levels of β_1-adrenoceptor expression with two different reporters, it is not likely to be a result of an overexpressed system or interference from the reporter product itself. The responses to both isoprenaline and CGP 12177 were competitively antagonized by increasing concentrations of CGP 20712A, although the concentrations required to shift the CGP 12177 response were much greater than those needed to shift the isoprenaline response. Because the isoprenaline response is shifted past the limited shift obtained

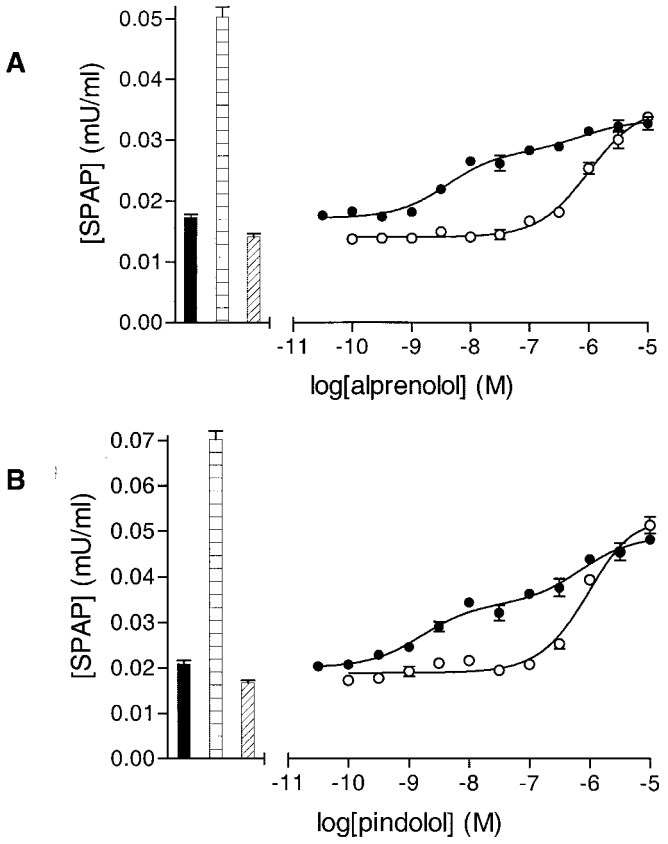

Fig. 7. SPAP production induced by alprenolol (A) and pindolol (B) in the absence and presence of 100 nM CGP 20712A. Points represent mean ± S.E.M. of triplicate determinations. The bars show basal SPAP production (■), that in response to 10 μM isoprenaline alone (▤), and that obtained in the presence of 100 nM CGP 20712A alone (▨). A, ●, alprenolol; ○, alprenolol + 100 nM CGP 20712A. B, ●, pindolol; ○, pindolol + 100 nM CGP 20712A. Concentration-response curves were fitted to a two-site model as described under *Materials and Methods*. These single experiments are representative of four further separate experiments.

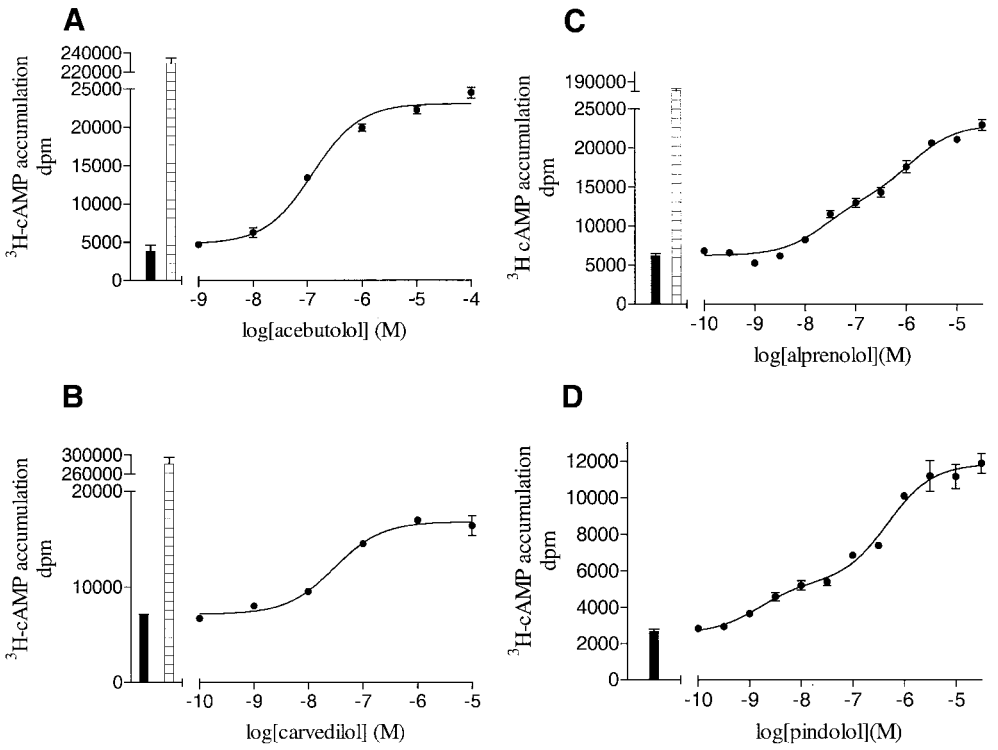

Fig. 8. [^{3}H]cyclic AMP accumulation in CHO-β_1-SPAP cells in response to acebutolol (A, ●), carvedilol (B, ●), alprenolol (C, ●), and pindolol (D, ●). C and D, the concentration response curves were fitted to a two-site model as described under *Materials and Methods*. Bars represent basal [^{3}H]cAMP accumulation (■) and that in response to 10 μM isoprenaline alone (▤). Data points are triplicate mean ± S.E.M. from a single experiment. Similar data were achieved in two (A), seven (B), five (C), and three (D) other separate experiments.

in the presence of CGP 12177 (Fig. 3B), there is no suggestion that isoprenaline is able to activate the second site. Schild analysis of the CGP 20712A antagonism of both isoprenaline and CGP 12177 responses provide strong evidence for competitive antagonism at both sites. The cause for the effect seen with CGP 12177 in Fig. 3B remains unknown.

Carvedilol clearly showed a small gene transcription response in the low-expressing luciferase cell line that, as expected, was much more noticeable in the higher-expressing CHO-β_1-SPAP cells. Furthermore, at the level of both cAMP accumulation and CRE-mediated gene transcription, several traditional β-antagonists (acebutolol, alprenolol, carvedilol, labetolol, pindolol, and propranolol) can stimulate agonist responses via the human β_1-adrenoceptor. Although the cAMP accumulation responses are small the gene transcription effects, seen after 5 h of addition of the ligands, are substantial. Given that all of these ligands are used clinically, these effects on gene transcription may well be important for their clinical use, for example in heart failure, because prolonged β-blocker treatment is required to demonstrate benefit. Small partial agonist responses of alprenolol, propranolol, carvedilol, and labetolol on cAMP accumulation have also been observed in HEK 293 cells transfected with wild-type and constitutively active mutants of human β_1-adrenoceptor (Lattion et al., 1999). Partial agonist responses to bucindolol (Maack et al., 2000) and pindolol (Lowe et al., 2002) have also been demonstrated in contractile studies in human and ferret myocardial preparations.

An important question raised by the demonstration of a substantial agonist effect of these β_1-adrenoceptor antagonists on gene transcription responses is whether the effects produced are mediated via the classic catecholamine site, the CGP 12177 site, or a combination of the two. To address this issue, we used CGP 20712A, which was devoid of any agonist or inverse agonist activity in both cell lines but was able to distinguish between the two sites based on its differential affinity as an antagonist. Acebutolol and labetolol agonist responses were potently antagonized by CGP 20712A in a manner that was consistent with an interaction predominately via the catecholamine site. In contrast, carvedilol agonist responses were relatively resistant to CGP 20712A antagonism in a manner more in keeping with the observed CGP 12177 responses (Fig. 9). These data therefore suggest that although both carvedilol and CGP 12177 are high-affinity antagonists at the catecholamine site, their agonist re-

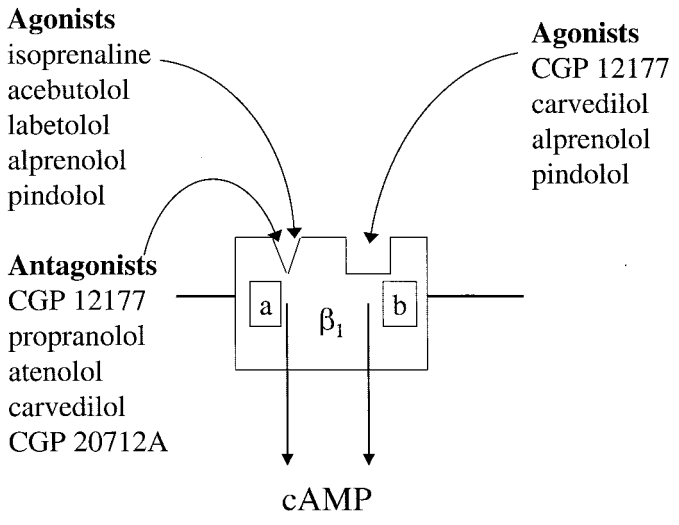

Fig. 9. Schematic diagram of the human β_1-adrenoceptor with agonists and antagonists acting through the 2 "sites", where a is the catecholamine site and b is the CGP 12177 site. These two "sites" may represent two separate binding sites on the β_1-adrenoceptor, a different conformational or active state (e.g., phosphorylation state or extent of association with particular scaffold proteins), or a difference in the receptors themselves (e.g., monomers or dimers).

sponses are mediated via the secondary CGP 12177 site, for which they both have lower affinity.

Perhaps the best evidence for the two-site hypothesis comes from the concentration response curves for alprenolol and pindolol. In both of these cases, not only does the β-blocker seem to act as an agonist, but each response also contains two components separated by a potency of 100-fold. In the presence of 100 nM CGP 20712A, both the alprenolol and pindolol responses are best described by only a single component. The simplest explanation for this is that CGP 20712A is able to produce a substantial shift of the concentration response curve for the high-potency component (i.e., log EC_{50} −8.66 for alprenolol) but has little effect on that for the lower potency component (log EC_{50} −6.13 for alprenolol). It is therefore likely that the high-potency component is occurring via activation of the classic catecholamine site, whereas the low-potency component is analogous to the antagonist-resistant site activated by high concentrations of CGP 12177.

Several β-blockers can therefore elicit agonist effects on gene transcription via the human β_1-adrenoceptor, and these actions can occur predominantly at the classic catecholamine site (acebutolol, labetolol), via the secondary site (CGP 12177, carvedilol), or via both sites (alprenolol, pindolol; Fig. 9). However, this "secondary site" could represent an independent site within the β_1-adrenoceptor monomer, a ligand-specific conformation (Seifert et al., 2001), or a conformation dependent upon the extent of receptor phosphorylation, dimerization (Salahpour et al., 2000), or association with particular scaffold proteins (e.g., AKAP79/150) (Fraser et al., 2000)

Close inspection of the K_D values obtained for antagonism by CGP 20712A of these responses, however, shows that there is actually a range of values (Table 2) rather than two clear groups. The position of any agonist concentration response curve will depend upon the relative affinity and efficacy of that agonist for each site and the degree to which the two sites contribute to the final response. Alprenolol and pindolol are clearly able to stimulate responses via both sites, but because the EC_{50} values for these two sites are 100-fold apart, the two components are easily distinguished. The intermediate K_D values obtained for CGP 20712A (i.e., those obtained between acebutolol or CGP 12177 as agonists) (Table 2) could have occurred because some of the other agents (e.g., labetolol) are not confined to one site, but their EC_{50} values are not sufficiently dissimilar to reveal the two components.

In summary, the data presented here provide strong evidence for the presence of two agonist activation sites within the human β_1-adrenoceptor. We show that a range of β-blockers are able to exhibit differential agonists and antagonist actions on these two sites. Thus, CGP 20712A and atenolol act as classic antagonists at the catecholamine binding site but have much lower affinity for the secondary CGP 12177 site. CGP 12177 and carvedilol are potent antagonists at the catecholamine site but mediate substantial agonist actions on gene transcription via the secondary antagonist-resistant site. The therapeutic plasma concentration of carvedilol in humans is approximately 100 ng/ml (300 nM; Swangkoon et al., 2000) and therefore would be sufficient to produce an agonist effect via the second site of the human β_1-adrenoceptor. Agonist effects of β-blockers are not, however, confined to this secondary site, and some (particularly acebutolol and labetolol) act primarily via the catecholamine site, whereas others (pindolol and alprenolol) can stimulate both (Fig. 9). Although the effects observed with these β-blockers at the level of cAMP accumulation are small, the resulting stimulation of gene transcription is substantial. It remains to be established whether the different responses to β-blockers seen in the clinic (MERIT-HF 1999; CIBIS-II 1999; BEST, 2001) are caused in part by these β-blocker agonist responses and the different activation of the two sites.

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