British Journal of Pharmacology (2002) 137, 400–408
www.nature.com/bjp

Pharmacological characterization of CGP 12177 at the human β_2-adrenoceptor

[1]Jillian G. Baker, [1]Ian P. Hall & *,[1]Stephen J. Hill

[1]Institute of Cell Signalling, University of Nottingham, Queen's Medical Centre, Nottingham NG7 2UH

1 It has recently been reported that CGP 12177 can act as an agonist at a novel secondary site within the human β_1-adrenoceptor. The aim of this study was to undertake a detailed pharmacological study of the effects of CGP 12177 on the human β_2-adrenoceptor.

2 CGP 12177 acted as a potent partial agonist of ^{3}H-cyclic AMP accumulation (log $EC_{50} -8.90 \pm 0.06$) and CRE-mediated reporter gene transcription (log $EC_{50} -9.66 \pm 0.04$) in CHO-K1 cells expressing the human β_2-adrenoceptor. These CGP-induced responses were antagonized by the β_2-selective antagonist ICI 118551 (apparent log K_D values of -8.84 ± 0.15 and -9.51 ± 0.02 for the cyclic AMP and reporter gene responses respectively).

3 CGP 12177 was also able to antagonize both cyclic AMP and reporter gene responses to more efficacious β_2-agonists with similar log K_D values (e.g. -9.57 ± 0.15 and -10.04 ± 0.096 respectively with salbutamol as agonist).

4 ^{3}H-CGP 12177 binding to β_2-adrenoceptors in intact CHO-β_2 cells yielded a log K_D value of -9.84 ± 0.06, but indicated that the ligand dissociates very slowly from the receptor ($t_{\frac{1}{2}}$ for dissociation = 65 min). However, studies with a Green Fluorescent Protein (GFP)-tagged β_2-adrenoceptor indicated that CGP 12177 does not stimulate β_2-adrenoceptor internalization.

5 This study demonstrates that CGP 12177 is a high affinity partial agonist of both cAMP accumulation and CRE-mediated gene transcription at the human β_2-adrenoceptor. It provides no evidence that CGP 12177 can discriminate a secondary site on the β_2-adrenoceptor analogous to that observed for the human β_1-adrenoceptor. However, despite its very weak actions on cAMP accumulation, the potent agonist effects of CGP 12177 on CRE-mediated gene transcription at the human β_2-adrenoceptor, coupled with its long duration of action, offers a potential lead for drug development for the treatment of chronic inflammatory airway diseases.
British Journal of Pharmacology (2002) **137,** 400–408. doi:10.1038/sj.bjp.0704855

Keywords: CGP 12177; β_2-adrenoceptor; partial agonist; gene transcription; cAMP

Abbreviations: B_{MAX}, maximal specific binding; cAMP, cyclic adenosine monophosphate; CHO, Chinese hamster ovary; CRE, cyclic AMP response element; DMEM/F12, Dulbecco's modified Eagle's medium/Nutrient mix F12; DR, dose ratio; GFP, green fluorescent protein; HBH, Hank's balanced salt solution containing HEPES; HBS, HEPES buffered saline; IBMX, 3-isobutyl-1-methylxanthine; LSM, laser scanning microscope; PNPP, p-nitrophenol phosphate; SPAP, secreted placental alkaline phosphatases

Introduction

CGP 12177 has generally been thought to be a high affinity antagonist at both β_1- and β_2-adrenoceptors (Lang & Lemmer, 1985; Thede-Reynolds *et al*., 1986; Haddad *et al*., 1987; Steinkraus *et al*., 1990; Law, 1993). More recently, agonist effects of CGP 12177 have been noted in transfected CHO–K1 cells expressing only the human β_1-adrenoceptor (Pak & Fishman, 1996; Konkar *et al*., 2000a). In these studies, CGP 12177 acted as an antagonist of isoprenaline-induced cAMP accumulation at low concentrations (CGP 12177 K_D 2 nM), while at higher concentrations it had a partial agonist effect (EC_{50} 12–15 nM). Furthermore, these CGP 12177-induced responses were much less sensitive to inhibition by classical β_1-antagonists than responses to isoprenaline (Kaumann, 1996; Konkar *et al*., 2000a; Lowe *et al*., 2002).

CGP 12177 has also been found to have a low affinity partial agonist action at β_3-adrenoceptors in brown adipose tissue (Mohell & Dicker, 1989; Muzzin *et al*., 1992; D'Allaire *et al*., 1995). In whole animal studies, CGP 12177 acted as a cardiostimulant in both wild-type and β_3-adrenoceptor knockout mice (Kaumann *et al*., 1998). It was therefore suggested that this agonist effect of CGP 12177 may be occurring *via* a novel β_4-adrenoceptor (Kaumann *et al*., 1998; Cohen *et al*., 2000). However, recent data obtained in β_1-adrenoceptor knockout mice provide strong evidence that the cardiostimulant effects of CGP 12177 require the presence of a β_1-adrenoceptor (Konkar *et al*., 2000b; Kaumann, 2000; Kaumann *et al*., 2001). This suggests that different conformations of the β_1-adrenoceptor, rather than a putative β_4-adrenoceptor, may be responsible for the different pharmacological actions seen with CGP 12177 in whole animals.

Both the cell system and knockout animal data therefore suggest that the β_1-adrenoceptor has more than one binding site, leading Konkar *et al*. (2000a) to propose that the β_1-

*Author for correspondence;
E-mail: stephen.hill@nottingham.ac.uk

adrenoceptor possesses: (1) a 'catecholamine' site or conformation at which catecholamines and β-antagonists act, and where CGP 12177 is a potent antagonist, and (2) a 'CGP 12177' site or conformation where CGP 12177 is an agonist. Other members of the aryloxypropanolamine family also share these properties of CGP 12177 (Kaumann, 2000; Konkar *et al*., 2000a) and recently the ethanolamine sotalol has also been shown to have detectable affinity for these two states (Lowe *et al*., 2002).

CGP 12177 was also noted to stimulate a small cyclic AMP response in recombinant CHO–K1 cells expressing the human β_2-adrenoceptor (Pak & Fishman, 1996), although the pharmacological characteristics remain to be established. The aim of the present study was therefore to undertake a detailed pharmacological analysis of the effects of CGP 12177 on the human β_2-adrenoceptor. A preliminary account of this work has been presented to the British Pharmacological Society (Baker *et al*., 2001; 2002).

Methods

Materials

Cell culture reagents were from Sigma Chemicals (Poole, Dorset, U.K.) except foetal calf serum which was from PAA Laboratories (Teddington, Middlesex, U.K.). ^{3}H-adenine, ^{3}H-CGP 12177 and ^{14}C-cAMP were obtained from Amersham International (Buckinghamshire, U.K.); ICI 118551 and CGP 12177 were from Tocris Cookson (Avonmouth, Bristol, U.K.). All other reagents were supplied by Sigma Chemicals.

Cell culture

Chinese Hamster Ovary cells stably expressing both the human β_2-adrenoceptor (CHO-β_2) and a reporter gene, Secreted Placental Alkaline Phosphatase (SPAP), under the transcriptional control of a six CRE promoter (McDonnell *et al*., 1998) were grown at 37°C in Dulbecco's modified Eagle's medium/Nutrient mix F12 (DMEM/F12) containing 10% foetal calf serum and 2 mM L-glutamine in a humidified 5% CO_2 : 95% air atmosphere. Other cell lines were established by transfection with either a C-terminal GFP tagged human β_2-adrenoceptor cDNA (CHO-β_2-GFP DNA; a gift from Prof. G. Milligan, University of Glasgow; McLean *et al*., 1999) or the CRE–SPAP reporter alone (CHO–SPAP) using Lipofectamine and Optimem according to the manufacturer's instructions. Transfected cells were selected using resistance to geneticin (1 mg ml^{-1}; for CHO-β_2-GFP) and hygromycin (200 μg ml^{-1}; for CHO–SPAP). A single clone was isolated for the CHO-β_2-GFP cell line by dilution cloning. CHO–K1 cells (not transfected), grown in identical conditions were used as controls as appropriate.

Cyclic AMP accumulation

Cells were grown to confluence in 24-well plates then pre-labelled with ^{3}H-adenine (2 μ Ci ml^{-1}) for 2 h at 37°C in 1 ml well^{-1} Hanks balanced salt solution containing HEPES 20 nM, pH 7.4, HBH). The ^{3}H-adenine was removed, each well washed with 1 ml HBH, then incubated for 30 min with 1 ml medium containing IBMX (100 μM) and where appropriate, antagonists. Agonists in 10 μl were then added and the cells incubated for a further 10 min before the reaction was terminated by the addition of 50 μl concentrated HCl. ^{3}H-cyclic AMP 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).

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 media was replaced with 1 ml of fresh serum-free media. Where used, antagonists were added to this media 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 fresh serum-free media and incubated for a further hour. Twenty 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 colour change caused by the hydrolysis of p-nitrophenol phosphate (PNPP) (Cullen & Malim, 1992). 200 μl of p-nitrophenol phosphate (PNPP) 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 a Dynatech Laboratories MRX plate reader and the data converted to SPAP concentration mU ml^{-1} as previously described (McDonnell *et al*., 1998).

^{3}H-CGP 12177 whole cell binding

Cells were grown to confluence in 24-well plates. The media was removed and replaced with 1 ml HBH containing 0.3 nM ^{3}H-CGP 12177. Competing drugs were added in 10 μl and the plates incubated at 37°C for 1 h. Non-specific binding was determined using 100 nM ICI 118551. The HBH and drugs were then removed and each cell washed with 500 μl HBH. Cells were then dissolved by incubation with 500 μl 0.5 M NaOH/well at 37°C for an hour. The entire well contents were then mixed with scintillation fluid (Cocktail Plus, Packard) and counted on a β-counter for 3 min. Protein content was determined by the method of Lowry *et al*. (1951).

Confocal microscopy

Confocal microscopy was performed using a Zeiss LSM 510 laser scanning microscope (Argon laser, 488 nm line; 505 nm long-pass filter) with a Zeiss ×40, 1.3 NA oil immersion lens. CHO-β_2-GFP cells were grown on glass coverslips in 6-well plates containing 3 ml DMEM–F12 media containing 10% foetal calf serum and 2 mM glutamine. The coverslips were transferred to a specially designed holder in a heated stage to form the base of a sealed chamber to which 1 ml HEPES Buffered Saline (HBS) was added. The microscope objective and stage were maintained at 37°C throughout the experiments. Agonists (in 10 μl HBS) were added for 30 min and

the cells imaged in the continued presence of agonist (1024×1024 pixels; averaging at four frames).

Data analysis

A maximal isoprenaline concentration was included in each separate experiment for both ^{3}H-cAMP accumulation and SPAP gene transcription to allow the CGP 12177 responses to be expressed as a percentage of the isoprenaline maximum. Agonist and antagonist concentration–response curves were fitted to a four-parameter logistic equation through computer-assisted non-linear regression using the programme Prism 2 as previously described (Hopkinson *et al*., 2000). 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.

Where appropriate, partial agonist dissociation constants were estimated according to the method of Stephenson (1956). Relative agonist efficacy was estimated as a ratio of the maximal response (cyclic AMP or SPAP) of the partial agonist compared with the maximal response to isoprenaline measured in the same experiment.

Curves of the specific binding (SB) of ^{3}H-CGP 12177 at different concentrations of the ^{3}H-ligand were fitted using the non-linear regression programme Prism 2 to the equation: $SB = (B_{MAX} \times A)/(A + K_D)$ 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. Curves of the inhibition of specific ^{3}H-CGP 12177 binding were also fitted by non-linear regression to the equation: % of inhibited $SB = 100 - ((100.D^n)/(D^n + IC_{50}^n))$ where D is the inhibitor concentration, n is the Hill coefficient and IC_{50} is the concentration of non-radioactive inhibitor producing 50% inhibition of specific ^{3}H-CGP 12177 binding. K_i values for inhibitors were determined from the IC_{50} values according to the expression: $K_i = IC_{50}/(1 + A/K_D)$, where A is the concentration of ^{3}H-CGP 12177 used in displacement experiments.

All data are presented as mean ± s.e.mean. The n in the text refers to the number of separate experiments. Statistical significance was determined by Student's unpaired t-test and analysis of variance (ANOVA; $P < 0.05$ was considered statistically significant).

Results

^{3}H-Cyclic AMP accumulation

In CHO-β_2 cells, isoprenaline produced a concentration dependent increase in ^{3}H-cAMP accumulation (log $EC_{50} - 8.88 \pm 0.08$; 6.79 ± 0.61 fold over basal; $n = 11$; Figure 1). ICI 118551, a selective β_2-adrenoceptor antagonist, antagonized this isoprenaline-induced response yielding a log K_D of -9.11 ± 0.10 nM ($n = 3$) consistent with the value

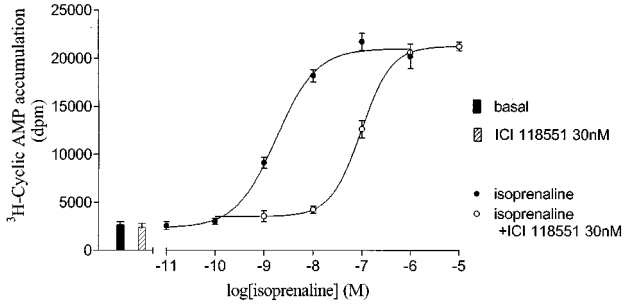

Figure 1 ^{3}H-cyclic AMP accumulation in CHO-β_2 cells in response to isoprenaline in the presence and absence of 30 nM ICI 118551. Bars show basal ^{3}H-cAMP accumulation and that in response to 30 nM ICI 118551. All data points are mean ± s.e.mean from quadruplicate determinations in a single experiment which is representative of three separate experiments.

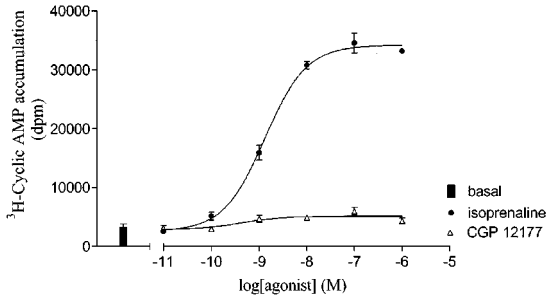

Figure 2 ^{3}H-cyclic AMP accumulation in response to isoprenaline and CGP 12177. The bar represents basal ^{3}H-cAMP accumulation. Data points are quadruplicate mean ± s.e.mean from a single experiment and this is representative of three separate experiments.

previously reported for the human β_2-adrenoceptor (Bylund *et al*., 1994; Hopkinson *et al*., 2000; Figure 1). CGP 12177 stimulated a small but statistically significant increase in ^{3}H-cAMP accumulation (6.50 ± 0.38% of the maximal isoprenaline; Figure 2; $P < 0.05$, two-way ANOVA). When used as an antagonist of the isoprenaline stimulated response, CGP 12177 (1, 10, 100 nM) produced a parallel shift of the isoprenaline concentration–response curve yielding a log K_D of -9.77 ± 0.07 ($n = 21$; Figure 3).

In order to see whether the small CGP 12177-induced cAMP response was also sensitive to the selective β_2-inverse agonist ICI 118551 (which has a long dissociation time; Hopkinson *et al*., 2000), the method was optimized for detecting small changes in ^{3}H-cAMP measurement by doubling the ^{3}H-adenine concentration and extending the CGP 12177 incubation time to 60 min. Under these conditions CGP 12177 produced a consistent accumulation of ^{3}H-cAMP (log $EC_{50} = -8.90 \pm 0.06$; 2.35 ± 0.18 fold over basal; $n = 11$; Figure 4). The log EC_{50} value for isoprenaline after 60 min incubation however remained similar (log $EC_{50} - 8.47 \pm 0.08$; $n = 5$) to that obtained at 10 min. This CGP 12177-induced response was then clearly antagonized by ICI 118551 to yield an apparent log K_D of -8.84 ± 0.15 (Figure 4). The reduction in maximal response in the presence of ICI 118551 is most likely due to the hemi-equilibrium produced as a consequence of its slow dissociation from the receptor (see below).

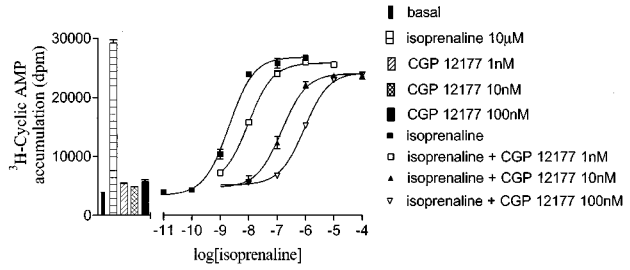

Figure 3 ^{3}H-cyclic AMP accumulation in response to isoprenaline in the presence and absence of 1, 10 and 100 nM CGP 12177. Bars show basal ^{3}H-cAMP accumulation and that in response to CGP 12177. All data points are mean ± s.e.mean from quadruplicate determinations in a single experiment which is representative of five separate experiments. The Schild slope in this experiment was 1.048.

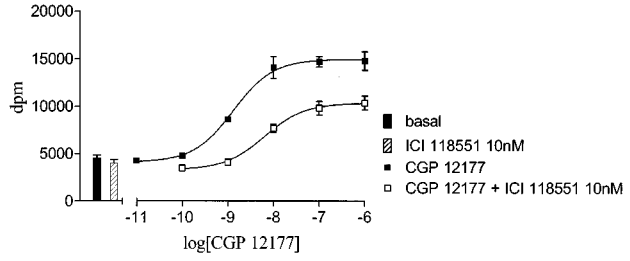

Figure 4 ^{3}H-cyclic AMP accumulation in response to CGP 12177 (and CGP 12177 in the presence of 10 nM ICI 118551) where the ^{3}H-adenine concentration has been doubled and the CGP 12177 incubation time extended to 60 min to maximise the response seen (see text). Bars represent basal ^{3}H-cAMP accumulation and that in response to 10 nM ICI 118551 under the same conditions. Data points are quadruplicate mean ± s.e.mean from a single experiment and this is representative of three separate experiments.

CRE-mediated gene transcription (SPAP)

Isoprenaline stimulated CRE-mediated gene transcription to give a concentration dependent increase in SPAP secretion (log EC_{50} −8.29 ± 0.08; 8.46 ± 0.54 fold over basal; $n=30$; Figure 5). This response was antagonized by increasing concentrations of ICI 1185551 to yield a series of parallel shifts consistent with competitive competition at the β_2-adrenoceptor (log K_D −9.06 ± 0.17; $n=8$; Schild slope = 0.93 ± 0.07 $n=3$; Figure 5; Arunlakshana & Schild, 1959).

CGP 12177 was also able to induce a concentration dependent increase in SPAP production (log EC_{50} −9.66 ± 0.04; $n=28$) that represented 59.8 ± 2.8% of the maximal response to isoprenaline (Figure 6a). ICI 118551 also antagonized this CGP 12177 induced response in a competitive manner giving a log K_D for ICI 118551 of −9.51 ± 0.02 ($n=6$; Figure 6b).

When examined as an antagonist, CGP 12177 was able to antagonize the isoprenaline-stimulated response in a manner consistent with its partial agonist actions. When the log K_D was calculated by the partial agonist method of Stephenson (1956) a value of −8.87 ± 0.09; $n=17$ was obtained (Figure 7). This was ten fold higher than its log EC_{50} for stimulation of SPAP secretion and the log K_D obtained from antagonism of the isoprenaline-stimulated cAMP accumulation ($P<0.01$, unpaired t-test).

^{3}H-CGP 12177 whole cell binding

The specific binding of ^{3}H-CGP 12177 to whole CHO-β_2 cells yielded a log K_D of −9.84 ± 0.06 $n=4$ and B_{MAX} of 466.0 ± 36.2 fmol.mg protein^{-1} (Figure 8). ^{3}H-CGP 12177 binding to the transfected cells was inhibited by ICI 118551 to give a log K_i for ICI 118551 of −9.25 ± 0.004 ($n=4$; Figure 9a), consistent with the values obtained in both functional studies above. CGP 12177 was able to inhibit the binding of ^{3}H-CGP 12177 to yield a log K_i −9.40 ± 0.02 ($n=3$; Figure 9b). ^{3}H-CGP 12177 (1 nM) binding to intact cells yielded values of $9.78 \pm 0.70 \times 10^7$ M^{-1} min^{-1} ($t_{\frac{1}{2}} = 6.3 \pm 0.7$ min, $n=4$; Figure 10a) for k_{on} and

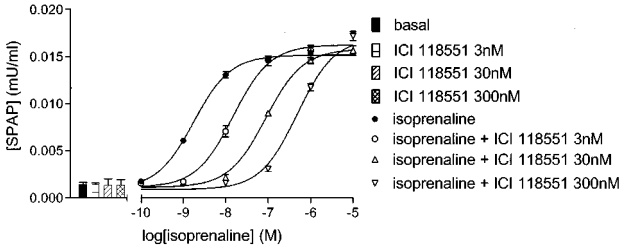

Figure 5 CRE-mediated SPAP production in CHO-β_2 cells in response to isoprenaline in the absence and presence of 3, 30 and 300 nM ICI 118551. Bar represents basal SPAP production and that in response to 3, 30 and 300 nM ICI 118551. The data points are mean ± s.e.mean (triplicate determinations) from a single experiment and are representative of four separate experiments.

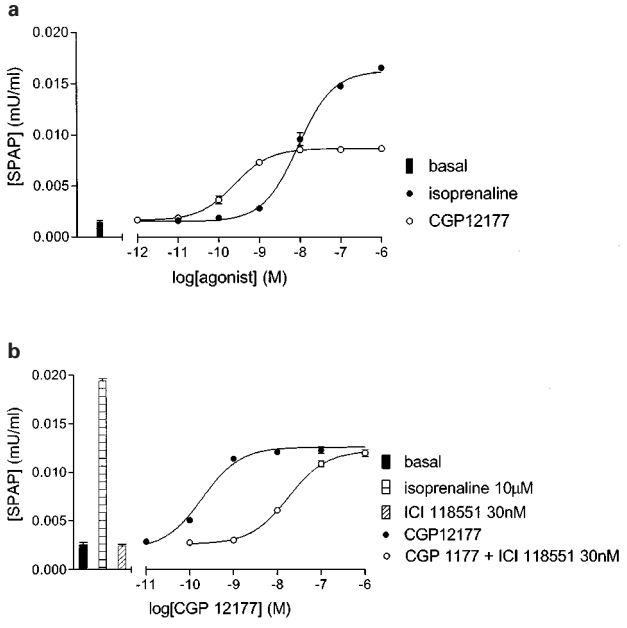

Figure 6 (a) CRE-mediated SPAP production in response to isoprenaline and CGP 12177. Data points are mean ± s.e.mean (triplicate determinations) from a single experiment and are representative of at least 12 separate experiments. The bar represents the SPAP production from unstimulated cells. (b) SPAP production induced by CGP 12177 in the presence and absence of 30 nM ICI 118551. Points represent mean ± s.e.mean of triplicate determinations. The bar shows basal SPAP production, that in response to maximal isoprenaline stimulation and that in response to 30 nM ICI 118551. This single experiment is representative of six separate experiments.

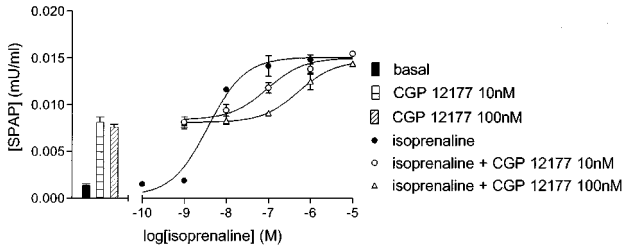

Figure 7 SPAP production induced by isoprenaline in the presence and absence of 10 nM and 100 nM CGP 12177. Bars represent basal response and SPAP production in the presence of 10 nM and 100 nM CGP 12177. Data points are mean ± s.e.mean (triplicate determinations) and this single experiment is representative of 14 separate experiments.

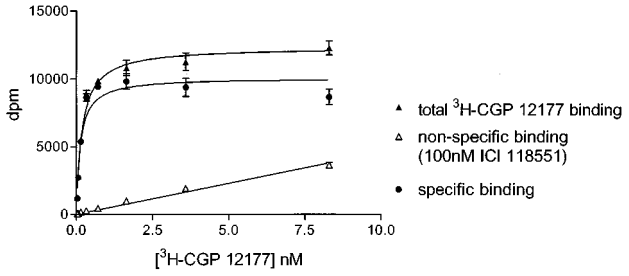

Figure 8 ^{3}H-CGP 12177 binding to whole CHO-β_2 cells showing total ^{3}H-CGP 12177 binding, non-specific binding as defined in the presence of 100 nM ICI 118551 and specific binding. Data points are mean ± s.e.mean of triplicate determination and this single experiment is representative of four separate experiments.

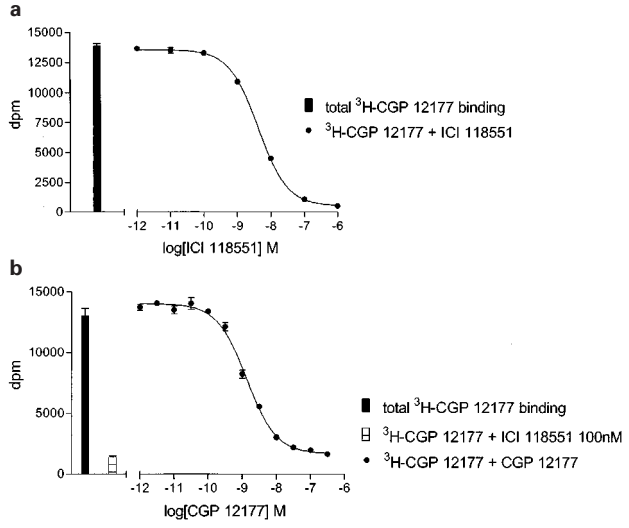

Figure 9 Inhibition of ^{3}H-CGP 12177 binding to whole cells by (a) ICI 118551 and (b) CGP 12177. Bars represent total ^{3}H-CGP 12177 binding and non-specific ^{3}H-CGP 12177 binding in the presence of 100 nM ICI 118551. Data points are mean ± s.e.mean (triplicate determinations) from single experiments which are representative of at least three separate experiments in each case. Where not seen, the error bars are within the symbol.

$7.03 \pm 0.48 \times 10^{-3}$ min^{-1} ($t_{\frac{1}{2}} = 65.4 \pm 9.4$ min, $n = 4$, Figure 10b) for k_{off} of this ligand. The equilibrium dissociation constant K_D, obtained from the ratio of K_{off}/K_{on} was 72 pM (log $K_D = -10.13 \pm 0.03$). The agonist isoprenaline also

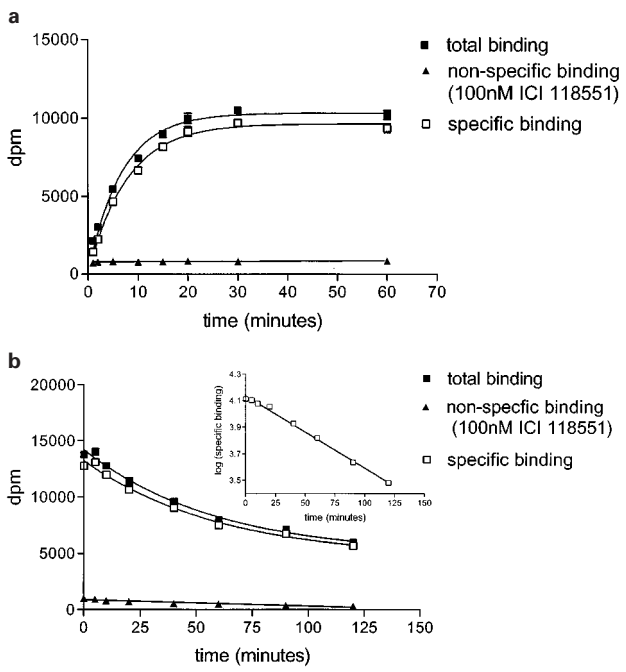

Figure 10 (a) Association of ^{3}H-CGP 12177 (1 nM) binding to β_2-adrenoceptors in intact CHO-β2 cells. Data points are mean ± s.e.mean (triplicate determinations) from single experiments which are representative of four separate experiments. (b) Dissociation of ^{3}H-CGP 12177 from β_2-adrenoceptor in CHO-β_2 cells. Cells were incubated with ^{3}H-CGP 12177 1 nM for 1 h at 37°C, the cells were then washed (time = 0) with 1 ml HBH. All cells were washed with 1 ml HBH at each remaining time point and the total and non-specific binding measured at the times indicated. Data points are mean ± s.e.mean (triplicate determinations) from single experiments which are representative of four separate experiments. The inset shows a log plot of the specific binding dissociation data.

inhibited ^{3}H-CGP 12177 binding with a log K_D of -7.05 ± 0.09 $n = 3$.

Salbutamol stimulated gene transcription and ^{3}H-cAMP accumulation

In order to investigate the difference in the log K_D of CGP 12177 between the two functional assays, and the apparent 'stickiness' of this ligand, we investigated the ability of CGP 12177 to inhibit the SPAP responses to another β_2-adrenergic agonist, salbutamol. Salbutamol was a full agonist on CRE-mediated gene transcription in this system (log $EC_{50} - 8.62 \pm 0.09$, $n = 11$; Figure 11) causing a maximal response which represented $101.6 \pm 2.3\%$ ($n = 11$) of that obtained with isoprenaline. When CGP 12177 was used as an antagonist of this salbutamol-induced gene transcription response, the log K_D value obtained was -10.04 ± 0.10 ($n = 9$, Figure 11). This was significantly different from the value obtained from antagonism of the isoprenaline-induced response ($P < 0.001$, unpaired t-test). Salbutamol produced a maximal rise in ^{3}H-cAMP accumulation which represented $86.8 \pm 1.43\%$ of the maximum response to isoprenaline (log $EC_{50} - 7.31 \pm 0.05$ $n = 5$; Figure 12). Increasing concentration of CGP 12177 appeared to produce a non-competitive antagonism of this response to salbutamol ($n = 5$; Figure 12). However, an estimate of the antagonist log K_D value for

CGP 12177 was determined from the lowest concentration of the antagonist used (1 nM) where the maximal response to salbutamol was better maintained (log K_D CGP 12177 of -9.57 ± 0.15; $n=4$; Figure 12).

Lack of β-agonist mediated ^{3}H-cAMP accumulation and gene transcription responses in native CHO-K1 cells

There was no increase in the ^{3}H-cAMP accumulation in response to isoprenaline, CGP 12177 or salbutamol in untransfected CHO–K1 cells ($n=3$) or in SPAP production in CHO–SPAP cells transfected with the CRE–SPAP reporter but not the β_2-adrenoceptor ($n=3$). 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 ($n=4$; data not shown). These data confirm that the cAMP and SPAP responses to β_2-agonists described above are dependent upon the presence of the human β_2-adrenoceptor.

Confocal microscopy with β_2-GFP-adrenoceptor

In order to examine internalization of the β_2-adrenoceptor in response to the different β_2-agonists, we utilized a CHO–K1 cell line stably expressing a β_2-adrenoceptor–GFP fusion protein. Under basal conditions the fluorescently-tagged β_2-adrenoceptor in CHO-β_2-GFP cells was localized to the plasma membrane (Figure 13a, approximately 250 cells, 50 wells imaged). Following a 30 min incubation with isoprenaline (1 μM and above), the GFP-tagged receptor moved from the membrane into punctate lesions within the cytoplasm of the cells where it remained for at least 1 h (Figure 13a,b), 32 cells, 18 wells). CGP 12177 (up to 10 μM) did not cause this internalization of the β_2-GFP-adrenoceptor and the β_2-GFP-adrenoceptor remained on the cell surface (Figure 13e,f), 17 cells, 6 wells) whilst salbutamol only caused slight internalization (Figure 13c,d, 30 cells, 12 wells).

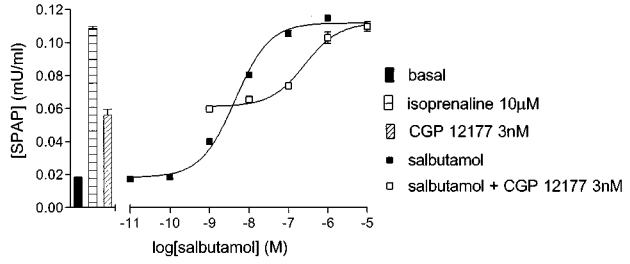

Figure 11 SPAP production induced by salbutamol in the presence and absence of 3 nM CGP 12177. Bars represent basal response and SPAP production in the presence of 10 μM isoprenaline and 3 nM CGP 12177. Data points are mean ± s.e.mean (triplicate determinations) and this single experiment is representative of seven separate experiments.

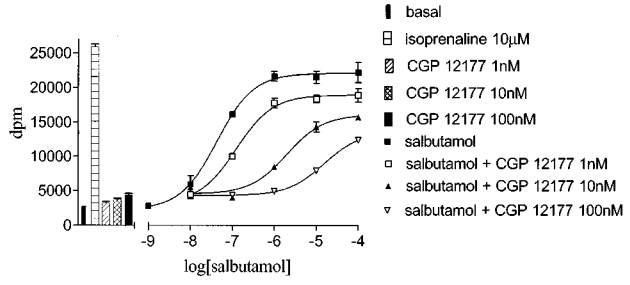

Figure 12 ^{3}H-cyclic AMP accumulation in CHO-β_2 cells in response to salbutamol in the presence and absence of 1, 10 and 100 nM CGP 12177. Bars show basal ^{3}H-cAMP accumulation and that in response to 10 μM isoprenaline and 1, 10, and 100 nM CGP 12177. All data points are mean ± s.e.mean from quadruplicate determinations in a single experiment which is representative of five separate experiments.

Discussion

CGP 12177 was generally thought to be a high affinity non-selective antagonist at β_1- and β_2-adrenoceptors coupled to stimulation of cAMP accumulation (Hertel *et al*., 1983; Lang & Lemmer, 1985; Thede-Reynolds *et al*., 1986; Haddad *et al*., 1987; Steinkraus *et al*., 1990; Law, 1993; Scarpace & Matheny, 1991) although Nanoff *et al*. (1987) have suggested that ^{3}H-CGP 12177 may have slight β_1-selectivity. Here, we have shown that CGP 12177 is a potential partial agonist at the human β_2-adrenoceptor with a log EC_{50} of -8.90 and -9.66 in two functional assays. The difference in size of the partial agonist response between cAMP accumulation and CRE-mediated gene transcription responses is most likely to be due to the magnification of response that occurs on progression along the cell signalling cascade from adenylyl cyclase to gene transcription, similar to that previously reported (McDonnell *et al*., 1998; Hill *et al*., 2001).

The log EC_{50} values obtained for CGP 12177 in both the ^{3}H-cAMP accumulation assay and the CRE-mediated gene transcription assay were similar to each other. Both of these log EC_{50} values were also similar to the log K_D values obtained for: (a) ^{3}H-CGP 12177 binding to whole cells; (b) inhibition of isoprenaline-stimulated cAMP accumulation and (c) inhibition of salbutamol-stimulated SPAP production. This is what would be predicted by classical receptor theory if these responses were mediated through the same receptor (Kenakin & Beek, 1984). In keeping with this, the CRE-

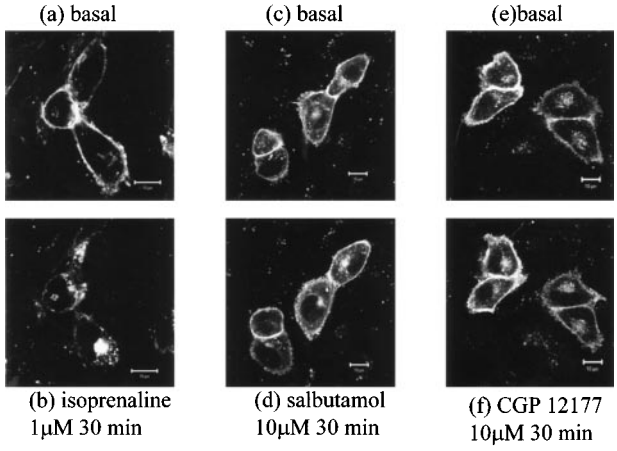

Figure 13 Representative examples of confocal images of CHO-β_2-GFP cells before (a), (c), (e) and after 30 min incubations (b) isoprenaline 1 μM, (d) salbutamol 10 μM and (f) CGP 12177 10 μM at 37°C.

mediated SPAP responses to CGP 12177 were antagonized by the selective β_2-adrenoceptor inverse agonist ICI 118551 with an affinity similar to that expected for classical β_2-adrenoceptor responses (log K_D −9.51; Bylund *et al*., 1994; Hopkinson *et al*., 2000).

The log K_D value obtained for CGP 12177 from inhibition of isoprenaline-stimulated CRE-mediated SPAP secretion however, is an order of magnitude higher than the K_D values measured in the other assays. It is possible that the differences in isoprenaline incubation period between the cAMP accumulation (10 min) and SPAP assays (5 h) could contribute to this difference, since isoprenaline would be expected to internalize the receptor during the time course of the 5 h incubation. Interestingly, a comparison of the ability of all three agonists to internalize the β_2-GFP-adrenoceptor indicated that only isoprenaline was able to consistently induce internalization of the β_2-adrenoceptor. These data were similar to the observations of McLean & Milligan (2000) who also noted that the internalization of β_2-GFP was slightly slower than the wild-type receptor. Other studies have also shown that low efficacy agonists such as salbutamol produce considerably reduced levels of desensitization and internalization compared to full agonists (January *et al*., 1998; Clark *et al*., 1999). However, the K_D values obtained for CGP 12177 as an inhibitor of SPAP responses to lower efficacy β_2-agonists that are less able to internalize the β_2-GFP-adrenoceptor (i.e. salbutamol) are identical to the other measures of this affinity. It is therefore quite likely that the isoprenaline induced SPAP responses are complicated by both time dependent increases in signal amplification and ongoing receptor internalization (leading to a decrease in response). In keeping with this hypothesis, it is striking that the log EC_{50} values for salbutamol (−7.31 for cAMP; −8.62 for SPAP production) show evidence of marked signal amplification while those for isoprenaline (−8.88 for cAMP and −8.29 for SPAP) show evidence for desensitization.

Salbutamol-induced cAMP responses were antagonized by low concentrations (1 nM) of CGP 12177 to yield a log K_D similar to that expected from the above studies. However, in contrast to isoprenaline, the higher concentrations of CGP 12177 appeared to produce a non-competitive antagonism of the salbutamol-induced response where a progressive decrease in maximal response to salbutamol was seen. Whole cell binding studies with ^{3}H-CGP 12177 indicated that rather surprisingly, CGP 12177, a small hydrophilic molecule (Staehelin *et al*., 1983; Lacasa *et al*., 1986), dissociated very slowly from the human β_2-adrenoceptor. Thus, one explanation for the decreasing maximal responses to salbutamol with increasing concentrations of CGP 12177 (over the 10 min of the cAMP assay) is that a hemi-equilibrium is reached where CGP 12177 effectively removes receptors from the free receptor pool. In this situation, a high efficacy agonist such as isoprenaline would still be able to occupy sufficient receptors to produce a maximal response. In the case of the less efficacious agonist, salbutamol, receptor number will have been effectively reduced by the slow dissociation from the receptor of the higher concentrations of CGP 12177, leading to a reduced maximal response. By the same mechanism, the CGP 12177-induced cAMP accumulation was also antagonized by ICI 118551, a 'sticky' ligand (Hopkinson *et al*., 2000) and again a reduced maximal response achieved.

Calculation of the log K_D for ICI 118551 under these conditions may be further complicated by the inverse agonist nature of the inhibitor but the apparent log K_D (as estimated by comparing the agonist log EC_{50} values in the presence and absence of antagonist) yields a value in the same region (−8.84) consistent with β_2-adrenoceptor involvement.

The similarity between the log EC_{50} and log K_D values for CGP 12177 at the human β_2-adrenoceptor are in marked contrast to those seen at the human β_1-adrenoceptor where there is a substantial discrepancy between the log EC_{50} and log K_D values for CGP 12177 (Pak & Fishman, 1996; Kaumann, 2000; Konkar *et al*., 2000a). These authors proposed the existence of two active sites on the β_1-adrenoceptor: (1) a 'catecholamine' site where catecholamines and antagonists (including CGP 12177) act; and (2) a 'CGP 12177' site where CGP 12177 acts as an agonist but is insensitive to β_1-selective antagonists. However, unlike the situation with the β_1-adrenoceptor, the CGP 12177 response of the β_2-adrenoceptor delineated here was sensitive to selective β_2-antagonists. There is, therefore, no evidence that CGP 12177 interacts with an equivalent secondary site on the human β_2-adrenoceptor in this study. It therefore seems unlikely that the physico-chemical properties of CGP 12177 underlie its action at the β_1-adrenoceptor.

Conclusion

In summary, we therefore conclude that CGP 12177 is a high affinity partial agonist of both cAMP accumulation and CRE-mediated gene transcription at the human β_2-adrenoceptor. Unexpectedly, this small hydrophilic molecule dissociates slowly from the human β_2-adrenoceptor and this property is responsible for the ability of this ligand to expose less efficacious full agonists. This study also provides no evidence that CGP 12177 can discriminate a secondary site on the β_2-adrenoceptor analogous to that observed for the human β_1-adrenoceptor. However our data suggest that a comparative study of the β_1- and β_2-adrenoceptor sequences may be fruitful in identifying the CGP 12177 site on the β_1-adrenoceptor. Furthermore, despite its very weak actions on cAMP accumulation, the potent agonist effects of CGP 12177 on CRE-mediated gene transcription at the human β_2-adrenoceptor, coupled with its long duration of action, offers a potential lead for drug development for the treatment of chronic inflammatory airway diseases. This stimulant effect of a drug, generally thought to be a β_2-adrenoceptor antagonist, may yet provide a rationale for the potential use of 'β-blockers' in the chronic drug treatment of chronic inflammatory airways disease (Bond, 2001).

J.G. Baker holds a Wellcome Trust Clinical Training Fellowship.

References

ARUNLAKSHANA, O. & SCHILD, H.O. (1959). Some quantitative uses of drug antagonists. *Br. J. Pharmacol. Chemother.,* **14,** 48–58.

BAKER, J.G., HALL, I.P. & HILL, S.J. (2001). Agonist and antagonist effects of CGP 12177 on β_2-adrenoceptor-stimulated gene transcription in CHO cells transfected with the human β_2-adrenoceptor. *Br. J. Pharmacol.,* **134,** 112P.

BAKER, J.G., HALL, I.P. & HILL, S.J. (2002). Agonist specific temporal characteristics of β_2-adrenoceptor-stimulated gene transcription response in CHO-K1 cells transfected with the human β_2-adrenoceptor. *Br. J. Pharmacol.,* **135,** 182P.

BOND, R.A. (2001). Is paradoxical pharmacology a strategy worth pursuing? *Trends Pharmacol. Sci.,* **22,** 273–276.

BYLUND, D.B., EIKENBERG, D.C., HIEBLE, J.P., LANGER, S.Z., LEFKOWITZ, R.J., MINNEMAN, K.P., MOLINOFF, P.B., RUFFOLO, R.R. & TRENDELENBERG, U. (1994). IV International Union of Pharmacology nomenclature of adrenoceptors. *Pharmacol. Rev.,* **46,** 121–136.

CLARK, R.B., KNOLL, B.J. & BARBER, R. (1999). Partial agonist and G-protein-coupled receptor desensitization. *TIPS.,* **20,** 279–286.

COHEN, M.L., BLOOMQUIST, W., ITO, M. & LOWELL, B.B. (2000). Beta3 receptors mediate relaxation in stomach fundus whereas a fourth beta receptor mediates tachycardia in atria from transgenic beta3 receptor knockout mice. *Receptor Channels,* **7,** 17–23.

CULLEN, B.R. & MALIM, M.H. (1992). Secreted placental alkaline phosphatase as a eukaryotic reporter gene. *Methods Enzymol.,* **216,** 362–368.

D'ALLAIRE, F., ATGIE, C., MAURIEGE, P., SIMARD, P.M. & BUKOWIECKI, L.J. (1995). Characterization of beta 1- and beta 3-adrenoceptors in intact brown adipocytes of the rat. *Br. J. Pharmacol.,* **114,** 275–282.

DONALDSON, J., BROWN, A.M. & HILL, S.J. (1988). Influence of rolipram on the cyclic-3′,5′-adenosine monophosphate response to histamine and adenosine in slices of guinea-pig cerebral cortex. *Biochem. Pharmacol.,* **37,** 715–723.

HADDAD, C., WILKINSON, M., ROEDER, L.M., TILDON, J.T. & ARMOUR, J.A. (1987). Binding of the hydrophilic beta-adrenergic antagonist ^{3}H-CGP-12177 to cardiac tissue slices: characterization and ontogenetic studies in dogs. *Can. J. Physiol. Pharmacol.,* **65,** 1928–1933.

HERTEL, C., STAEHELIN, M. & PERKINS, J.P. (1983). Evidence for intravesicular beta-adrenergic receptors in membrane fractions from desensitised cells: binding of the hydrophilic ligand CGP-12177 only in the presence of alamethicin. *J. Cyclic Nucleotide Protein Phosphor. Res.,* **9,** 119–128.

HILL, S.J., BAKER, J.G. & REES, S. (2001). Reporter gene systems for the study of G-protein-coupled receptors. *Curr. Opin. Pharmacol.,* **1,** 526–532.

HOPKINSON, H.E., LATIF, M.L. & HILL, S.J. (2000). Non-competitive antagonism of β_2-agonist-mediated cyclic AMP accumulation by ICI 118551 in BC3H1 cells endogenously expressing constitutively active β_2-adrenoceptors. *Br. J. Pharmacol.,* **131,** 124–130.

JANUARY, B., SEIBOLD, A., ALLAL, C., WHALEY, B.S., KNOLL, B.J., MOORE, R.H., DICKERY, B.F., BARBER, R. & CLARK, R.B. (1998). Salmeterol-induced desensitisation, internalisation and phosphorylation of the human beta(2)-adrenoceptor. *Br. J. Pharmacol.,* **123,** 701–711.

KAUMANN, A.J. (1996). (-)-CGP 12177 induced increase of human atrial contraction through a putative third beta-adrenoceptor. *Br. J. Pharmacol.,* **117,** 93–98.

KAUMANN, A.J. (2000). Gs protein coupled receptors in human heart. *Handbook of Experimental Pharmacology,* Volume 148, pp 73–116, in 'The Pharmacology of functional, biochemical and recombinant receptor systems' eds T. Kenakin & J.A. Angus, Springer.

KAUMANN, A.J., ENGELHARDT, S., HEIN, L., MOLENAAR, P. & LOHSE, M. (2001). Abolition of (-)-CGP 12177-evoked cardiostimulation in double beta1/beta2-adrenoceptor knockout mice. Obligatory role of beta1-adrenoceptors for putative beta4-adrenoceptor pharmacology. *Naunyn Schmiedebergs Arch. Pharmacol.,* **363,** 87–93.

KAUMANN, A.J., PREITNER, F., SARSERO, D., MOLENAAR, P., REVELLI, J.P. & GIACOBINO, J.P. (1998). (-)-CGP 12177 causes cardiostimulation and binds to cardiac putative beta 4-adrenoceptors in both wild-type and beta 3-adrenoceptor knockout mice. *Mol. Pharmacol.,* **53,** 670–675.

KENAKIN, T.P. & BEEK, D. (1984). Relative efficacy of prenalterol and pirbuterol for beta-1 adrenoceptors: measurement of agonist affinity by alteration of receptor number. *J. Pharmacol. Exp. Ther.,* **229,** 340–345.

KONKAR, A.A., ZHAI, Y. & GRANNEMAN, J.G. (2000b). Beta1-adrenergic receptors mediate beta3-adrenergic-independent effects of CGP 12177 in brown adipose tissue. *Mol. Pharmacol.,* **57,** 252–258.

KONKAR, A.A., ZHENGXIAN, Z. & GRANNEMAN, J.G. (2000a). Arloxypropanolamine and catecholamine ligand interactions with the β_1-adrenergic receptor: evidence for interaction with distinct conformations of β_1-adrenergic receptors. *J. Pharmacol. Exp. Ther.,* **294,** 923–932.

LACASA, D., MAURIEGE, P., LAFONTAN, M., BERLAN, M. & GIUDICELLI, Y. (1986). A reliable assay for beta-adrenoceptors in intact isolated human fat cells with a hydrophilic radioligand ^{3}H-CGP 12177. *J. Lipid. Res.,* **27,** 368–376.

LANG, P.H. & LEMMER, B. (1985). Evidence for two specific affinity states of ^{3}H-antagonist binding to cardiac beta-adrenoceptors and influence of Gpp(NP)p. *J. Cyclic Nucleotide Protein Phosphor. Res.,* **10,** 341–360.

LAW, M.P. (1993). Demonstration of the suitability of CGP 12177 for in vivo studies of beta-adrenoceptors. *Br. J. Pharmacol.,* **109,** 1101–1109.

LOWE, M.D., LYNHAM, J.A., GRACE, A.A. & KAUMANN, A.J. (2002). Comparison of the affinity of β-blockers for the two states of the β_1-adrenoceptor in ferret ventricular myocardium. *Br. J. Pharmacol.,* **135,** 451–461.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.C. & RANDALL, R.J. (1951). Protein measurements with the folin phenol reagent. *J. Biol. Chem.,* **193,** 265–275.

MCDONNELL, J., LATIF, M.L., REES, E.S., BEVAN, N.J. & HILL, S.J. (1998). Influence of receptor number on the stimulation by salmeterol of gene transcription in CHO-K1 cells transfected with the human β_2-adrenoceptor. *Br. J. Pharmacol.* **125,** 717–726.

MCLEAN, A.J., BEVAN, N., REES, S. & MILLIGAN, G. (1999). Visualizing differences in ligand regulation of wild-type and constitutively active mutant β_2-adrenoceptor-green fluorescent protein fusion proteins. *Mol. Pharmacol.,* **56,** 1182–1191.

MCLEAN, A.J. & MILLIGAN, G. (2000). Ligand regulation of green fluorescent protein-tagged forms of the human β_1- and β_2-adrenoceptors; comparisons with the unmodified receptors. *Br. J. Pharmacol.,* **130,** 1825–1832.

MOHELL, S. & DICKER, A. (1989). The beta-adrenergic radioligand ^{3}H-CGP-12177, generally classified as an antagonist, is a thermogenic agonist in brown adipose tissue. *Biochem. J.,* **261,** 401–405.

MUZZIN, P., REVELLI, J.P., FRASER, C.M. & GIACOBINO, J.P. (1992). Radioligand binding studies of the atypical beta 3-adrenergic receptor in rat brown adipose tissue using ^{3}H-CGP-12177. *FEBS Lett.,* **298,** 162–164.

NANOFF, C., FREISSMUTH, M. & SCHUTZ, W. (1987). The role of a low beta 1-adrenoceptor selectivity of [3H]CGP-12177 for resolving subtype-selectivity of competitive ligands. *Naunyn Schmiedebergs Arch. Pharmacol.,* **336,** 519–525.

PAK, M.D. & FISHMAN, P.H. (1996). Anomalous behaviour of CGP 12177A on beta 1-adrenergic receptors. *J. Recept. Signal Transduct. Res.,* **16,** 1–23.

SCARPACE, P.J. & MATHENY, M. (1991). Adenylate cyclase agonist properties of CGP-12177A in brown fat: evidence for atypical beta-adrenergic receptors. *Am. J. Physiol.,* **260,** E226–231.

STAEHELIN, M., SIMONS, P., JAEGGI, K. & WIGGER, N. (1983). CGP-12177 A hydrophilic β-adrenergic receptor radioligand reveals high affinity binding of agonist to intact cells. *J. Biol. Chem.,* **258,** 3496–3502.

STEINKRAUS, V., NOSE, M., MENSING, H. & KORNER, C. (1990). Radioligand binding characteristics of beta 2-adrenoceptors of cultured melanoma cells. *Br. J. Dermatol.,* **123,** 163–170.

STEPHENSON, R.P. (1956). A modification of receptor theory. *Br. J. Pharmacol.,* **11,** 5109–5116.

THEDE-REYNOLDS, K.R., MOTULSKY, H.J. & FELDMAN, R.D. (1986). Temperature-dependent binding of hydrophilic beta-adrenergic receptor ligands to intact human lymphocytes. *Life Sci.,* **39,** 1325–1334.

(Received March 18, 2002
Revised May 27, 2002
Accepted June 24, 2002)