British Journal of Pharmacology (2000) 131, 124–130

www.nature.com/bjp

Non-competitive antagonism of β_2-agonist-mediated cyclic AMP accumulation by ICI 118551 in BC3H1 cells endogenously expressing constitutively active β_2-adrenoceptors

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1 Constitutive activity of the β_2-adrenoceptor, which is sensitive to inhibition by an inverse agonist such as ICI 118551, has been readily demonstrated in recombinant systems expressing constitutively-active mutant receptors or over-expressing the wild-type β_2-adrenoceptor. Here we demonstrate the presence of constitutive β_2-adrenoceptor activity in BC3H1 cells which endogenously express this receptor.

2 In BC3H1 cells, only ICI 118551 behaved as an inverse agonist at β_2-adrenoceptors, while propranolol, ICI 118551, atenolol and, to a lesser extent, alprenolol exhibited inverse agonism in CHO-$\beta_2$4 cells transfected with cDNA for the human β_2-adrenoceptor (310 fmol.mg protein^{-1}). The level of expression of β_2-adrenoceptors in BC3H1 cells was not high (78 fmol.mg protein^{-1}) and the efficiency of receptor–effector coupling in this cell line was much lower than in the recombinant CHO-$\beta_2$4 cells (as judged by the partial agonist nature of both salbutamol and clenbuterol).

3 ICI 118551 (log $K_D - 9.73 \pm 0.07$) and propranolol (log $K_D - 9.25 \pm 0.12$) both behaved as conventional competitive antagonists of isoprenaline-stimulated cyclic AMP accumulation in high expressing CHO-$\beta_2$4 cells. In contrast, ICI 118551 appeared to act as a non-competitive antagonist in BC3H1 cells and in low expressing CHO-$\beta_2$6 cells (50 fmol.mg protein^{-1}).

4 This non-competitive effect of ICI 118551 in BC3H1 cells was also observed when either salbutamol was used as agonist, or the incubation period with isoprenaline was extended to 30 min.

5 The possibility that these effects of ICI 118551 are due to an interaction with different affinity states (R, R* and R′) of the receptor is discussed.

British Journal of Pharmacology (2000) **131,** 124–130

Keywords: BC3H1 cells; β_2-adrenoceptors; constitutive activity; ICI 118551; CHO-K1 cells; inverse agonism

Abbreviations: CAM, constitutively-active mutant; FCS, foetal calf serum; GPCR, G-protein-coupled receptor; ICI 118551; 1-[2,3-(dihydro-7-methyl-1H-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol); ICYP, iodocyanopindolol

Introduction

G-protein-coupled receptors (GPCRs) have classically been assumed to exist in a resting inactive state, but then undergo a conformational change when stimulated by an agonist leading to G-protein coupling and subsequent cellular responses (Stephenson, 1956). However, the recent observations that GPCRs can exist in a constitutively active state (i.e. in the absence of agonist) following site-directed mutagenesis of recombinant receptors (Cotecchia *et al*., 1990; Kjelsberg *et al*., 1992; Samama *et al*., 1993; Lefkowitz *et al*., 1993) or overexpression of wild-type receptors in transfected cell lines (Chidiac *et al*., 1994; Milligan & Bond, 1997) and transgenic mice (Milano *et al*., 1994; Bond *et al*., 1995), has led to the development of an extended ternary complex model for receptor activation (Samama *et al*., 1993; Lefkowitz *et al*., 1993). This model proposes that each member of the family of GPCRs can exist in two forms which are in equilibrium with each other i.e. R (with low affinity for G proteins) or R* (which has a high affinity for, and can bind G proteins in the absence of agonist) (Samama *et al*., 1993). High efficacy agonists are proposed to bind with higher affinity to the R* form and thus produce receptor activation by pulling the equilibrium between the two forms of the receptor in favour of R* (Samama *et al*., 1993; Lefkowitz *et al*., 1993). However, if sufficient receptors in a population exist in the R* form (in the absence of agonist), and the receptor signalling cascade is intact, the system will be constitutively active.

The β_2-adrenoceptor has been widely studied as a representative of the GPCR family in support of this theory (Samama *et al*., 1993; Milano *et al*., 1994; Chidiac *et al*., 1994; Adie & Milligan, 1994). Constitutively active mutant (CAM) β_2-adrenoceptors have been produced which are functionally active in the absence of agonist (Samama *et al*., 1993). Furthermore, β_3-adrenoceptor antagonists (e.g. ICI 118551) have been identified in these systems which can stabilize the R : R* equilibrium in the inactive conformation (R), leading to inverse agonist activity (Samama *et al*., 1993). ICI 118551 has also been shown to be an inverse agonist in tissues from transgenic mice (Bond *et al*., 1995) and in transfected cells overexpressing the wild-type receptor or CAM β_2-adrenoceptors (Samama *et al*., 1994; Stevens & Milligan, 1998). However, there are a number of inconsistencies in the reported properties of some β_2-antagonists in constitutively active systems. Thus, while ICI 118551 behaves consistently as an inverse agonist in constitutively active cells (containing either CAM-β_2 or overexpressed wild-type β_2-receptors; Samama *et al*., 1994; Stevens & Milligan, 1998), propranolol has been

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reported to be a neutral antagonist (equal affinity for both R & R*) in cells containing CAM-β_2s (Samama *et al*., 1994; Stevens & Milligan, 1998; MacEwan & Milligan, 1996), but an inverse agonist in sf9 insect cells (Chidiac *et al*., 1994) or NG108-15 cells (Adie & Milligan, 1994) overexpressing the wild-type receptor.

The β_2-adrenoceptor couples to the G-protein G_s to mediate activation of adenylyl cyclase (Gilman, 1987). $G_{s\alpha}$ exists as two splice variants ($G_{s\alpha L}$ and $G_{s\alpha S}$) which differ by 15 aminoacids (inserted at position 72 of the polypeptide chain) and an exchange of glutamate for aspartate at position 71 (Robishaw *et al*., 1986; Seifert *et al*., 1998). Studies using β_2-adrenoceptor-$G_{s\alpha}$ fusion proteins have suggested that the $G_{s\alpha L}$ variant induces constitutive receptor activity because of its lower affinity for GDP and the consequent availability of more nucleotide-free $G_{s\alpha L}$ to stabilize the β_2-adrenoceptor in the active R* state (Seifert *et al*., 1998).

While constitutive receptor activity has been readily demonstrated in recombinant systems, there are few reports of this phenomenon in cells endogenously expressing native receptors. Spontaneous association between opioid receptors and G-proteins has been demonstrated using a GTPase assay in NG108-15 cell membranes (Costa *et al*., 1990). Thus, inverse agonism could be demonstrated with a peptidergic antagonist and this effect was enhanced by modifying the concentration of sodium ions in the incubation medium. Similarly, atenolol and propranolol have been shown to act as inverse agonists on β-adrenoceptors in membranes prepared from turkey erythrocytes (Gotze & Jakobs 1994). However, to date it has been difficult to demonstrate constitutive receptor activity of native receptors in intact cells (Costa *et al*., 1990).

In the present study we demonstrate constitutive activity in intact BC3H1 cells endogenously expressing native β_2-adrenoceptors. We also report differences in the inverse agonist properties of a range of β_2-antagonists between native β_2-adrenoceptors in these murine BC3H1 cells and CHO-K1 cells overexpressing the wild-type human β_2-adrenoceptor.

Methods

Cell culture

BC3H1 cells were cultured at 37°C under an atmosphere of 10% CO_2 in humidified air in 25 cm^2 or 75 cm^2 flasks. The growth medium was Dulbecco's modified Eagle's medium (DMEM) with 4 $g.l^{-1}$ glucose, supplemented with 2 mM L-glutamine and 20% (v v^{-1}) heat-inactivated foetal calf serum (FCS). Cells were passaged every fourth day using a split ratio of 1:6. CHO cells stably transfected with the human β_2-adrenoceptor (CHO-$\beta_2$4 or CHO-$\beta_2$6) were cultured at 37°C in 5% CO_2 in humidified air in 25 cm^2 or 75 cm^2 flasks. Growth medium was DMEM: Ham-F12 supplemented with 2 mM L-glutamine and 10% (v v^{-1}) heat inactivated FCS, and cells were passaged every third day using a split ratio of 1:20. HEK-293 cells were grown in Eagle's minimum essential medium (MEM) and Earle's balanced salt solution containing 1% MEM non-essential amino acids, 2 mM glutamine and 10% FCS at 37°C in humidified air: CO_2 (95:5).

[^{125}I]-iodocyanopindolol (ICYP) binding

Radioligand binding studies were based on a method by Neil *et al*. (1997) and were performed on membranes prepared from both cell types. Confluent cultures were scraped into phosphate buffered saline (PBS), centrifuged at 1000 r.p.m. for 10 min and resuspended in 50 mM Tris (pH 7.4) with 1 mM EDTA. This suspension was homogenized in a hand held homogenizer and centrifuged at $18{,}000 \times g$ for 10 min. Pellets were resuspended in Tris EDTA buffer and recentrifuged. The resulting pellet was resuspended in Tris-EDTA to a protein concentration of 1 $mg.ml^{-1}$. Membranes (10–20 μg protein) were incubated with 25–200 pM [^{125}I]-ICYP in a final volume of 250 μl for 1 h at 37°C. Reactions were stopped by dilution with Tris-ascorbate buffer (50 mM: 2 mM pH 8) and rapid filtration over Whatman GF-B filters. Bound [^{125}I]-ICYP was measured by gamma counting. Non-specific binding was determined in the presence of 1 μM propranolol. Protein content was determined by the method of Lowry *et al*. (1951).

[^{3}H]-cyclic AMP measurement

Cyclic AMP accumulation was assayed using a modification of the method described previously for brain slices (Donaldson *et al*., 1988). Monolayer cultures in 24-well plates were incubated with [^{3}H]-adenine (2 μCi per well) for 2 h at 37°C in 1 ml per well Hanks–HEPES buffer, pH 7.4. Prelabelled monolayers were then washed twice with Hanks–HEPES buffer and then incubated in the same buffer containing the phosphodiesterase (PDE) inhibitor rolipram (0.1 mM). β_2-adrenoceptor antagonists were equilibrated with the cells at the beginning of this incubation period, usually for 20 min. Agonists, or the appropriate vehicle controls, were added in 10 μl of buffer and the experiment was terminated 10 min later by the addition of 50 μl concentrated HCl. [^{3}H]-cyclic AMP together with a [^{14}C]-cyclic AMP tracer, was isolated by sequential Dowex-alumina chromatography and radioactivity determined by liquid scintillation counting. Accumulation of [^{3}H]-cyclic AMP was expressed as per cent conversion of the ^{3}H-adenine (taken up into the cells) to [^{3}H]-cyclic AMP, following correction for column recovery.

Data analysis

Cyclic AMP data were corrected for interwell variability in cell number and recovery of tracer [^{14}C]-cyclic AMP, from Dowex alumina chromatography. Agonist and antagonist concentration-response curves were fitted to a four-parameter logistic equation through computer-assisted curve fitting (Prism 2, GraphPad Software). The equation fitted was:

$$\text{Response} = E_{MIN} + \frac{(E_{MAX} - E_{MIN})}{(1 + 10^{(\log EC50 - X).n)}}$$

where E_{MIN} is the basal response, E_{MAX} is the maximal stimulation, X is the agonist concentration and n is the Hill coefficient.

Data represent mean ± standard error of triplicate or quadruplicate determinations in varying numbers of experiments (actual number given in the text). Statistical analysis was performed with the use of Student's *t*-tests and two-way analysis of variance.

Materials

All cell culture reagents were purchased from Sigma Chemicals (Poole, Dorset, U.K.). Radiolabelled compounds [^{3}H]-adenine, [^{14}C]-cyclic AMP, and [^{125}I]-cyanopindolol ([^{125}I]-CYP) were supplied by Amersham (Buckinghamshire, U.K.). Rolipram was a gift from Schering A.G. (Berlin, Germany); ICI 118551 was a gift from ICI Pharmaceuticals (Macclesfield, U.K.); other β-adrenoceptor active compounds and all other chemical reagents were purchased from Sigma. BC3H1 cells were

purchased from the European Collection of Animal Cell Cultures, Porton Down, Salisbury, U.K. CHO-K1 cells expressing the human β_2-adrenoceptor cDNA were a generous gift from GlaxoWellcome (Stevenage, U.K.). Chromatography columns and Dowex were purchased from Bio-Rad (Hemel Hempstead, Herts, U.K.).

Results

Pharmacological characterization of the β_2-adrenoceptor response in BC3H1 cells

Isoprenaline stimulated cyclic AMP accumulation in BC3H1 cells in a concentration-dependent fashion (log EC_{50} -6.93 ± 0.11, $n=16$) producing a maximum stimulation of between 5 and 6 fold over basal levels, at 10 μM isoprenaline. The β_2-adrenoceptor agonists salbutamol (log EC_{50} -6.12 ± 0.11, $n=3$) and clenbuterol (log EC_{50} -7.60 ± 0.10, $n=3$) behaved as partial agonists in this system yielding maximal responses which were $49.1\pm5.1\%$ (salbutamol) and $48.1\pm7.8\%$ (clenbuterol) of that produced by isoprenaline (10 μM; Figure 1). Propranolol (Figure 2a), atenolol (Figure 2b) and alprenolol (Table 1) produced parallel shifts in the concentration-response curves for isoprenaline, without any significant effect on basal levels of cyclic AMP. The β_2-selective antagonist ICI 118551, however, produced a non-parallel shift of the isoprenaline concentration-response curve at concentrations above 3 nM, with a corresponding reduction in the maximum response to isoprenaline (Figure 3). This effect of ICI 118551 was associated with a significant reduction in the basal cyclic AMP levels (Figures 3 and 4). Apparent dissociation constants (K_B) obtained for these β-antagonists are consistent with antagonism of β_2-adrenoceptors (Table 1).

Effects of β-adrenoceptor antagonists on basal cyclic AMP accumulation in BC3H1 cells

The basal accumulation of ^{3}H-cyclic AMP in these cells represented 15–20% of the maximum response to isoprenaline. In order to investigate whether a proportion of this basal production is due to constitutively β_2-adrenoceptor activity, the cells were exposed to antagonists in the absence of agonist. ICI 118551 produced a significant ($P<0.001$) and concentration-dependent (log IC_{50} -8.15 ± 0.12) reduction of basal ^{3}H-cyclic AMP accumulation in BC3H1 cells. In contrast, propranolol and atenolol produced no significant effect on basal cyclic AMP levels at concentrations up to 3 μM and 100 μM respectively (Figure 4a). In addition, alprenolol (100 nM) was able to attenuate the inhibitory effect of ICI 118551 on basal ^{3}H-cyclic AMP accumulation (Figure 4b). These data confirm that this inhibitory response to ICI 118551 is due to inverse agonism rather than antagonism of any response to endogenous catecholamines.

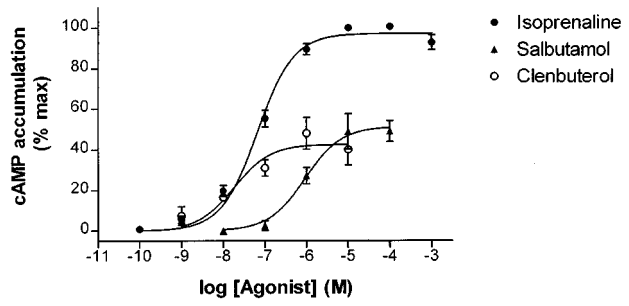


Figure 1 β_2-adrenoceptor agonist stimulated [^{3}H]-cyclic AMP accumulation in BC3H1 cell monolayers. Each point represents mean$\pm$s.e.mean of triplicate incubations from n repeat experiment (isoprenaline, $n=16$; clenbuterol, $n=3$; salbutamol, $n=3$). Results are expressed as a percentage of the maximal response to 10 μM isoprenaline which was measured in each experiment. All experiments were performed in the presence of 100 μM rolipram, and the agonist incubation time was 10 min in each case.

Inverse agonism in CHO-K1 cells transfected with the human β_2-adrenoceptor cDNA

We have previously shown that the CHO-$\beta_2$4 cell line (which overexpresses the human β_2-adrenoceptor; 310 fmol.mg

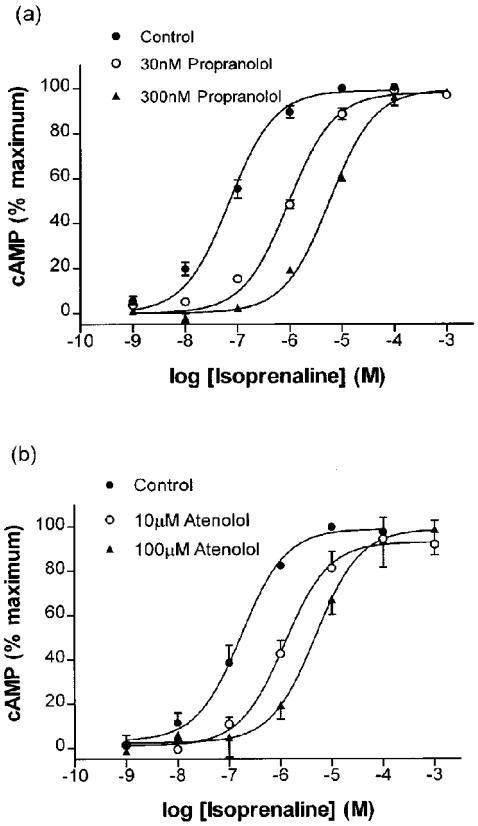


Figure 2 Antagonism of isoprenaline-stimulated [^{3}H]-cyclic AMP accumulation by (a) propranolol and (b) atenolol in BC3H1 cell monolayers. All experiments were performed in the presence of 100 μM rolipram; the antagonist was applied for 20 min prior to the agonist, and agonist incubation time was 10 min. Data are expressed as a percentage of the maximal response to 10 μM isoprenaline (in the absence of antagonist). Values show mean$\pm$s.e.mean from six (a) or three (b) separate experiments.

Table 1 Antagonist dissociation constants obtained for β-adrenoceptor antagonists from inhibition of cyclic AMP responses in BC3H1 cell monolayers

	Log antagonist K_D (M)	
	Isoprenaline	*Salbutamol*
ICI 118551	-8.98 ± 0.33 (4)	-9.30 ± 0.20 (4)
Propranolol	-8.69 ± 0.10 (6)	
Atenolol	-5.81 ± 0.15 (3)	-5.22 ± 0.11 (3)
Alprenolol	-8.62 ± 0.15 (3)	

Agonist concentration response curves were fitted using the program Prism2. Mean ($\pm$s.e.mean) log K_D values for each agonist-antagonist combination were derived from n separate experiments (n given in parentheses).

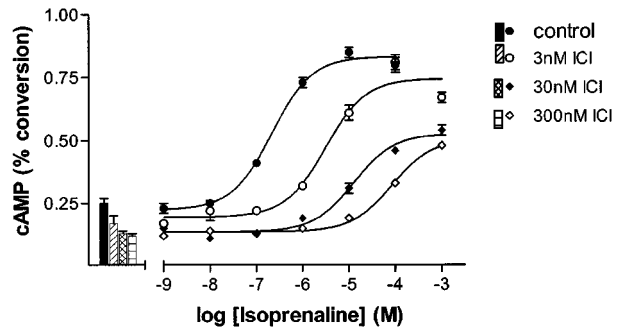


Figure 3 Antagonism of isoprenaline-stimulated cyclic AMP accumulation by ICI 118551 in BC3H1 cell monolayers. All experiments were performed in the presence of 100 μM rolipram; the antagonist was applied for 20 min prior to the agonist, and agonist incubation time was 10 min. Each point represents the mean$\pm$s.e.mean of triplicate determinations made in a single experiment.

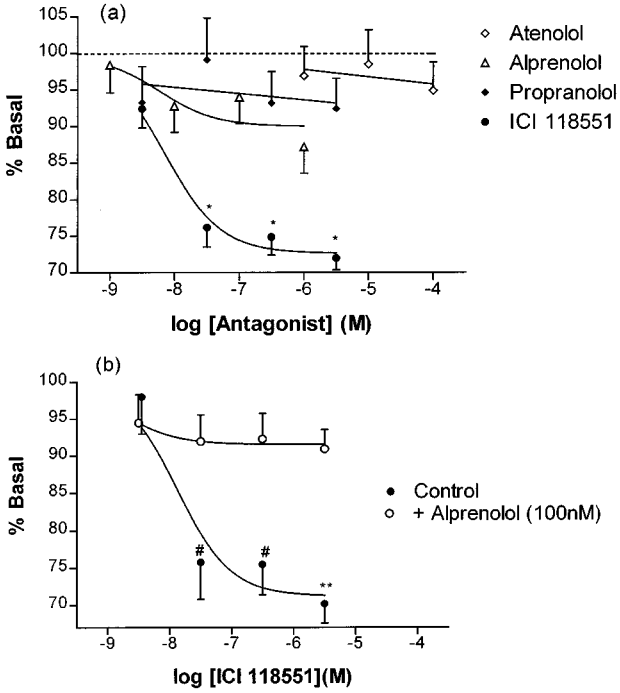


Figure 4 Effect of β-adrenoceptor antagonists on basal [^{3}H]-cyclic AMP accumulation in BC3H1 cells. Antagonists were incubated with cells for 30 min in the presence of 100 μM rolipram. The results are expressed as a percentage of the basal [^{3}H]-cyclic AMP accumulation in each experiment. Data points are mean$\pm$s.e.mean of quadruplicate incubations from n separate experiments. (a) propranolol, $n=9$; atenolol, $n=6$; alprenolol, $n=8$; ICI 118551 $n=9$. *$P<0.001$ with respect to basal (two-way ANOVA). (b) Effect of pre-incubation with 100 nM alprenolol for 10 min prior to the addition of ICI 118551 compared with the effect of ICI 118551 alone $n=3$; **$P<0.01$ or #$P<0.05$ with respect to basal (two-way ANOVA).

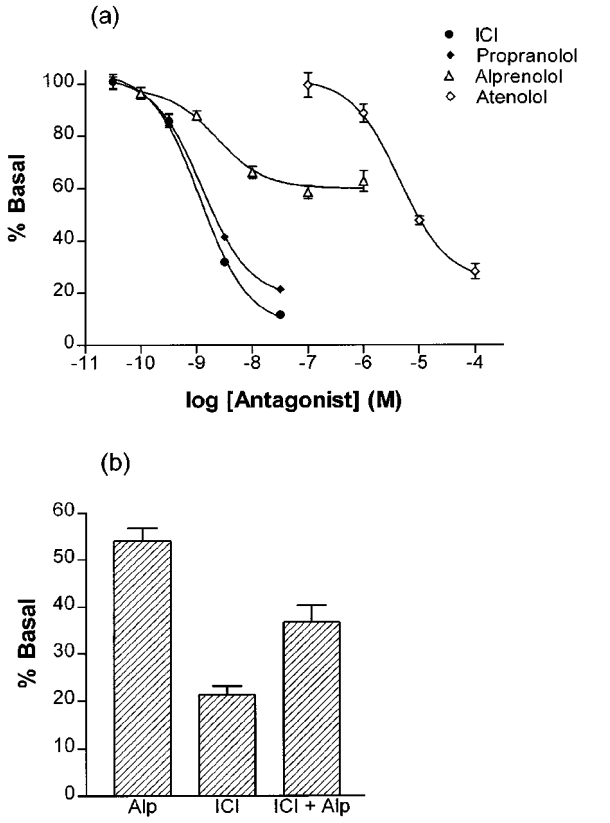


Figure 5 Effect of β-adrenoceptor antagonists on basal [^{3}H]-cyclic AMP accumulation in CHO-$\beta_2$4 cells. Antagonists were incubated with cells for 30 min in the presence of 100 μM rolipram. The results are expressed as a percentage of the basal [^{3}H]-cyclic AMP accumulation in each experiment. (a) Concentration-response curves for ICI 118551, propranolol, alprenolol and atenolol. Values are mean$\pm$s.e.mean from three separate experiments. Quadruplicate measurements were made in each experiment. (b) Effect of pre-incubation with 100 nM alprenolol (Alp) for 10 min prior to the addition of ICI 118551 (300 nM; ICI) on the inverse agonist activity of ICI 118551; $n=3$.

Table 2 Concentration-response parameters for inhibition of basal cyclic AMP production by β_2-adrenoceptor antagonists in CHO-$\beta_2$4 cells expressing constitutively active β_2-adrenoceptors

Antagonists	*Log IC_{50}*	*% reduction of basal cylic AMP accumulation*	n
Alprenolol	-8.60 ± 0.08	39.3 ± 5.8	3
Atenolol	-5.37 ± 0.06	73.5 ± 4.8	3
ICI 118551	-9.43 ± 0.05	93.0 ± 2.2	3
Propranolol	-9.42 ± 0.07	81.9 ± 2.6	3

Values represent mean$\pm$s.e.mean from n experiments.

protein^{-1}) demonstrates constitutive β_2-adrenoceptor activity (McDonnell *et al*., 1998). In order to evaluate whether there is a difference in the profile of β_2-antagonists as inverse agonists between BC3H1 cells and transfected CHO-K1 cells, we have also evaluated the extent of their inverse agonism in these latter cells. Basal cyclic AMP accumulation in the CHO-$\beta_2$4 cells was reduced by all four β_2-antagonists tested (Figure 5a, Table 2). Interestingly, the maximal reduction in basal cyclic AMP accumulation achieved by alprenolol was *ca* 50% of that obtained with the other antagonists (Figure 5a). Furthermore, 100 nM alprenolol was able to reverse the response produced by ICI 118551 (Figure 5b).

Differences in the nature of β_2-adrenoceptor antagonism by ICI 118551 in BC3H1 and CHO-$\beta_2$4 cells

ICI 118551 (log K_D -9.73 ± 0.07: $n=3$) and propranolol (log K_D -9.25 ± 0.12; $n=3$) both behaved as conventional competitive antagonists of isoprenaline without affecting the maximum response in CHO-$\beta_2$4 cells (Figure 6a). In contrast,

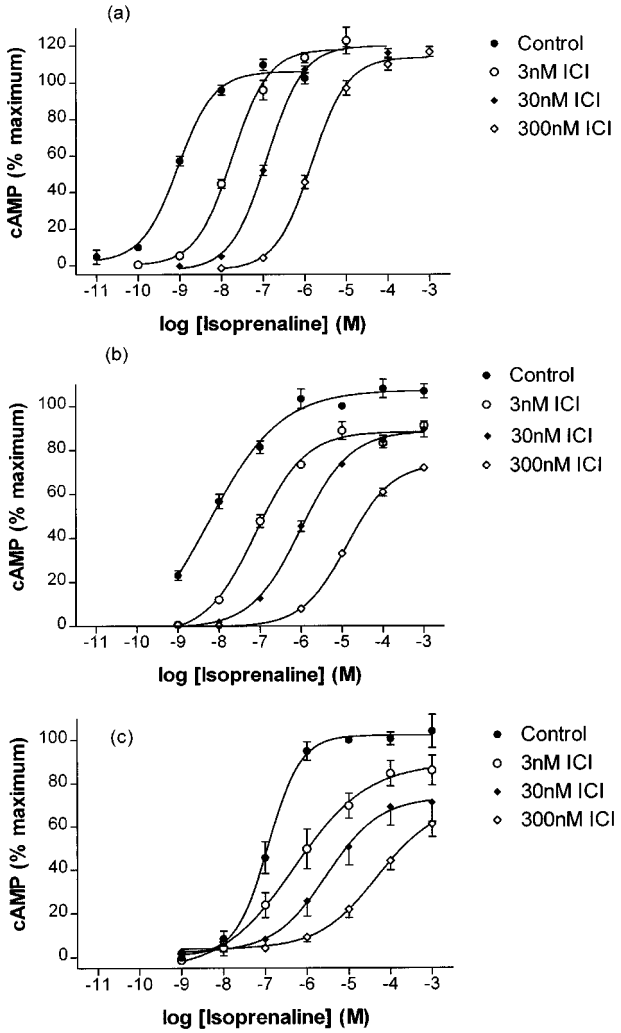


Figure 6 The influence of ICI 118551 on the concentration-response characteristics of isoprenaline-stimulated cyclic AMP accumulation in (a) CHO-$\beta_2$4 cells (310 fmol.mg protein^{-1}, human β_2-adrenoceptors), (b) CHO-$\beta_2$6 cells (50 fmol.mg proteins^{-1}, human β_2-adrenoceptor) and (c) BC3H1 cells 78 fmol.mg protein^{-1}, mouse β_2-adrenoceptor). Data have been normalized with respect to the control response to 10 μM isoprenaline in each cell line, after correction for the influence of antagonists on basal cyclic AMP accumulation. Values represent mean$\pm$s.e.mean of triplicate determinations in (a) three, (b) three or (c) four separate experiments.

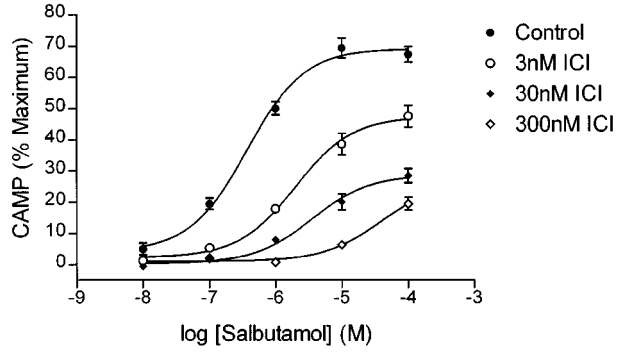


Figure 7 Antagonism by ICI 118551 of cyclic AMP responses to the β_2-selective agonist salbutamol in BC3H1 cells. Data have been normalized with respect to the control response to 10 μM isoprenaline in each experiment, after correction for the influence of ICI 118551 on basal cyclic AMP accumulation. Values represent mean$\pm$s.e.mean of triplicate determinations in four separate experiments.

ICI 118551 appeared to act as a non-competitive antagonist in BC3H1 cells (Figures 3 and 6c). To confirm that this was not a consequence of the involvement of β_1-adrenoceptors in the response to isoprenaline, experiments were also performed with the β_2-adrenoceptor agonist salbutamol (Figure 7 and Table 1). Consistent with an effect at the β_2-adrenoceptor, ICI 118551 produced a marked attenuation of the maximal response to salbutamol in these cells (Figure 7).

It is notable that there is a marked difference in the sensitivity to isoprenaline in the two cell lines (Figure 6a,c). This is most likely due to the lower receptor reserve in the BC3H1 cells. Binding experiments with [^{125}I]-iodocyanopindolol confirmed that BC3H1 cells have a lower expression of β_2-adrenoceptors (77.9$\pm$18.5 fmol.mg protein^{-1}; log K_D -11.1 ± 0.2; $n=4$) than CHO-$\beta_2$4 cells (310 fmol.mg protein^{-1}; McDonnell *et al.*, 1998). Interestingly, ICI 118551 produced a similar non-competitive antagonism of isoprenaline-stimulated cyclic AMP accumulation in CHO-$\beta_2$6 cells (Figure 6b) which express β_2-adrenoceptors at 50 fmol.mg protein^{-1} (McDonnell *et al.*, 1998). In these latter cells, ICI 118551 produced no significant reduction in basal ^{3}H-cyclic AMP accumulation.

In order to determine whether the reduction in the maximum response to β_2-agonists produced by ICI 118551 in BC3H1 cells was due to a hemi-equilibrium caused by its slow dissociation from the receptor (effectively removing receptors from the pool used by agonist), the agonist incubation time was increased from 10 min to 30 min (Figure 8). This was to allow sufficient time for full equilibrium between isoprenaline, ICI 118551 and the β_2-adrenoceptor. In these experiments the reduced maximal response observed previously with ICI 118551 was still evident.

Discussion

The present study has shown that the BC3H1 cell line possesses a relatively low level of endogenous β_2-adrenoceptors (*ca* 80 fmol.mg protein^{-1}) coupled to adenylyl cyclase. This is consistent with the data obtained in functional studies with the low efficacy agonists salbutamol and clenbuterol, which show that they act as partial agonists in stimulating

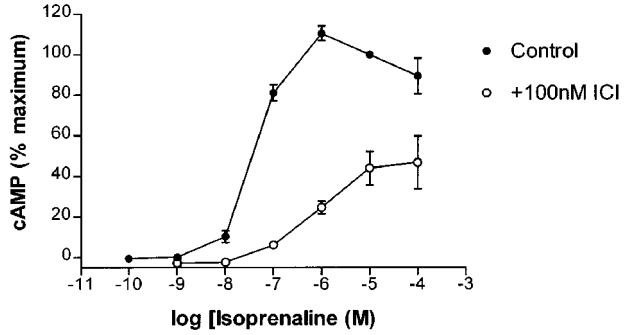


Figure 8 Influence of prolonged agonist-incubation on the antagonism by ICI 118551 (100 nM) of isoprenaline-stimulated cyclic AMP accumulation in BC3H1 cells. Values represent mean$\pm$s.e.mean of triplicate determinations in three separate experiments. Data have been normalized with respect to the control response to 10 μM isoprenaline in each experiment, after correction for the influence of ICI 118551 on basal cyclic AMP accumulation. Where appropriate cells were preincubated with ICI 118551 (100 nM) for 15 min prior to agonist addition. Incubations in the presence of agonist were then continued for a further 30 min.

cyclic AMP accumulation in this cell line. A comparison of the EC_{50} values obtained for isoprenaline-stimulated cyclic AMP accumulation in BC3H1 cells and CHO-$\beta_2$4 cells (which differ by two orders of magnitude; Figure 6) also suggests that the receptor reserve is much lower in the BC3H1 cell line. We have previously shown that the higher expression of β_2-adrenoceptors in CHO-$\beta_2$4 cells is associated with constitutive receptor activity (McDonnell *et al*., 1998). In the present study these latter cells exhibited an elevated basal cyclic AMP accumulation which could be reduced by ICI 118551, propranolol, atenolol and to a lesser extent alprenolol; consistent with inverse agonist (Figure 5). These data agree with previous work in cell systems overexpressing the wild-type receptor (Chidiac *et al*., 1994; Adie & Milligan, 1994). However, the data obtained with propranolol differ from those studies conducted with a constitutively-active mutant of the β_2-adrenoceptor (Samama *et al*., 1994; Stevens & Milligan, 1998; MacEwan & Milligan, 1996). This observation raises the possibility that the constitutively active conformation that results from mutations within the C terminus of the third intracellular loop of the β_2-adrenoceptor may differ from that produced when the wild-type receptor is overexpressed.

The demonstration of constitutive activity of the endogenous β_2-adrenoceptor in murine BC3H1 cells was unexpected in view of the low receptor expression and receptor reserve. Significant inhibition of basal cyclic AMP accumulation was observed with ICI 118551. Its potency (log IC_{50} −8.15) was similar to that obtained from antagonism of isoprenaline-stimulated cyclic AMP accumulation (log K_D −8.98) in the same cells, although lower than the log IC_{50} obtained for constitutive activity in CHO-$\beta_2$4 cells. The fact that the inhibition of basal cyclic AMP accumulation by ICI 118551 could be reversed by 100 nM alprenolol is consistent with ICI 118551 being an inverse agonist. However, it was notable that propranolol and atenolol, which both act as inverse agonists at the human receptor expressed in CHO-$\beta_2$4 cells, did not significantly inhibit basal cyclic AMP levels in BC3H1 cells. These data suggest that the constitutively active state in BC3H1 cells differs from that in CHO-$\beta_2$4 cells and perhaps resembles that obtained with constitutively active mutant forms of the β_2-adrenoceptor.

A striking feature of the antagonism of isoprenaline-stimulated cyclic AMP accumulation in BC3H1 cells is the apparently non-competitive nature of the interaction with ICI 118551 (particularly at concentrations above 3 nM). Thus, while propranolol and atenolol produced parallel shifts in the agonist concentration-response curves, ICI 118551 reduces the maximum response to isoprenaline as well as the basal accumulation of cyclic AMP (Figure 3). This does not, however, occur with ICI 118551 in CHO-$\beta_2$4 cells (Figure 6). ICI 118551 is a lipophilic molecule and it is possible, particularly in a low receptor reserve system, that the reduced maximal response to agonist is due to the establishment of a hemi-equilibrium caused by the slow dissociation of antagonist from the receptor, which would have the effect of removing receptors from the agonist accessible receptor pool. In a higher β_2-receptor reserve system such as the CHO-$\beta_2$4 cell line, the removal of small numbers of receptors by this process would not impact on the maximum response achieved. In order to test this, we conducted experiments in which the agonist incubation period was increased from 10 to 30 min to allow full equilibrium of the receptor with both the agonist and antagonist. Under these conditions, the reduction in maximal response was, if anything, more marked (Figure 8).

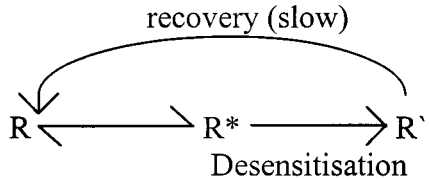
An alternative explanation is provided if one considers the possibility that there are other affinity states of the receptor, in addition to R and R*. The classic ternary complex model assumes that high efficacy agonists have higher affinity for R* (than R) and thus produce receptor activation by pulling the equilibrium between the two forms of the receptor in favour of R* (Samama *et al*., 1993; Lefkowitz *et al*., 1993). In the case of inverse agonists, these molecules are assumed to bind with higher affinity to R and therefore reduce basal responses, in conditions where there are significant levels of R* (i.e. constitutive activity). Analysis of this two-state model predicts that concentration-response curves to agonists (in the presence of inverse agonists) should not produce a reduced maximum response even in the presence of significant constitutive activity (Leff, 1995). This two-state model is difficult to reconcile, however, with the observations that both agonists and inverse agonists protect the β_2-adrenoceptor against thermal denaturation and proteolysis (Gether & Kobilka, 1998; Gether *et al*., 1997; Kobilka, 1990). These observations suggest that there are conformations of the β_2-adrenoceptor (inverse-agonist-bound and agonist-bound) which are more resistant to these processes than the unliganded form (Gether & Kobilka, 1998). Based on these latter observations, Gether & Kobilka (1998) have proposed that in addition to R, there are at least two other states of the receptor: (1) R* which binds to $G_{\alpha s}$ and is stabilized by agonists and (2) R′ which does not couple to $G_{\alpha s}$ and is stabilized by inverse agonists (e.g. ICI 118551) as depicted in (1) below:

1. $$R' \longleftrightarrow R \longleftrightarrow R^*$$

If the affinity of ICI 118551 is R′ > R > R* and the recovery from R′ to R is slow, then this will effectively result in the removal of receptors from the agonist accessible pool. In a low receptor reserve system such as the BC3H1 cell line this will lead to a reduction in the maximum response to isoprenaline. The shift in equilibrium to R′ will also produce inverse agonism. Under this model, the maximum response to a partial agonist (e.g. salbutamol) would be depressed more than that to a full agonist as observed in Figure 7. Similarly, in the lower expressing CHO-$\beta_2$6 cells. ICI 118551 would be expected to reduce the maximum response to isoprenaline, as was observed in Figure 6b. In higher receptor reserve systems (e.g. CHO-$\beta_2$4 cells), the decrease in available receptors (R and R*) by ICI 118551 will not decrease the maximal level of stimulation by agonist.

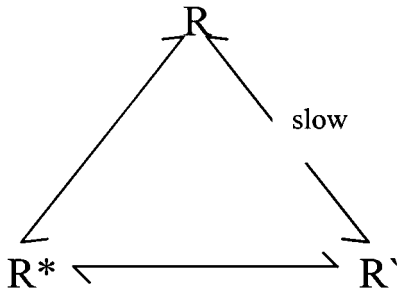
An alternative model is that the receptor state stabilized by ICI 118551 is a desensitized receptor conformation (R′):

2.



Where ICI 118551 has a higher affinity for R′ than either of the other two states (R and R*). This latter model might also explain the data of Chidiac *et al*. (1996) who found that agonist-promoted β_2-adrenoceptor desensitization could enhance the inhibitory effects of certain inverse agonists on constitutively active receptors in sf9 cells. This would also be consistent with the data we have obtained in Figure 8.

Models 1 and 2 are in fact essentially equivalent and can be depicted as follows:



If ICI 118551 and propranolol produce inverse agonism by interacting to differing extents with the various conformations of the β_2-adrenoceptor, then this may explain the observed differences in inverse agonist properties of these two ligands in BC3H1 cells, CHO-$\beta_2$4 cells and cells transfected with constitutively active mutants of the β_2-adrenoceptor.

In conclusion, we have demonstrated that constitutively active β_2-adrenoceptors are present in BC3H1 cells endogenously expressing the β_2-adrenoceptor. Furthermore, the observed differences in properties of different β_2-antagonists between the cell types studied here may also have important implications for the design of drugs capable of differentially modifying receptor function under different endogenous conditions.

Helen Hopkinson was an MRC Clinical Training Fellow. We thank the MRC and Wellcome Trust (ref 046755) for financial support, and Drs Paul Leff and Claire Scaramellini of Astra Zeneca for helpful discussions.

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(Received April 6, 2000
Revised June 14, 2000
Accepted June 14, 2000)