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A comparison of the antagonist affinities for the Gi and Gs-coupled states of the human adenosine A1 receptor.

Jillian G Baker, Stephen J Hill

Institute of Cell Signalling (JGB, SJH)

Medical School

University of Nottingham

Queen's Medical Centre

Nottingham

NG7 2UH

UK

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Correspondence to:

Dr Jillian G Baker

Institute of Cell Signalling,

Queen's Medical Centre,

Nottingham,

NG7 2UH, UK

Tel: +44-115-8230085

Fax: +44-115-8230081

E-Mail: jillian.baker@nottingham.ac.uk

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Abbreviations:

AAC, adenosine amine congener

cAMP, adenosine-3',5'-cyclic monophosphate

CHO, Chinese hamster ovary

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CRE, cyclic AMP response element;

2-CA, 2-chloroadenosine

2C-CPA, 2-chloro- N^6-cyclopentyladenosine

CGS 15943, 5-amino-9-chloro-2-(2-furyl)1,2,4-trizolo[1,5-*c*]quinazoline

CHA, N^6-cyclohexyladenosine

CPA, N^6-cyclopentyladenosine

DMEM/F12, Dulbecco's modified Eagles medium/nutrient mix F12;

DPCPX, 8-cyclopentyl-1,3-dipropylxanthine

DPPX, 1,3-dipropyl-8-phenylxanthine

GR 79236, N-[(1S,2S)-2-hydroxycyclopentyl]adenosine

GPCR, G-protein coupled receptor

NECA, 5'(N-ethylcarboxamideo)adenosine

pNPP 4-nitrophenyl phosphate

PIA, $N^6$2-phenylisopropyladenosine

PTX, pertussis toxin

SPAP, secreted placental alkaline phosphatase

XAC, xanthene amine congener

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Abstract

The antagonist affinity for a given receptor is traditionally considered to be constant reflecting the chemical nature of the specific ligand-receptor interaction. However, recent observations with all three β-adrenoceptors have cast doubt on this basic pharmacological principle. The extent to which this finding applies to other GPCRs and their interaction with different G-proteins is unknown. We therefore studied the influence of different agonists on antagonist affinity measurements for Gi and Gs-coupled conformations of the adenosine A1-receptor in CHO cells stably expressing the human adenosine A1-receptor and a CRE-SPAP reporter gene. Gi-coupled inhibition of ^{3}H-cAMP accumulation via the A1-receptor was observed at low concentrations of agonist, however a small increase in ^{3}H-cAMP accumulation was also seen at higher agonist concentrations. This biphasic response was more evident for A1-stimulated CRE-gene transcription. The inhibitory component was abolished by pre-treatment with pertussis toxin whilst the stimulatory component was augmented suggesting that the responses were due to an A1-Gi-coupled inhibition followed by an A1-Gs-coupled stimulation. The antagonist affinity values measured at the Gi-coupled and Gs-coupled conformations of the receptor were, however, the same in both functional responses and whole cell binding. Thus, in marked contrast to the β-adrenoceptors, the A1-receptor conforms to the long-held principle of pharmacology that antagonist affinity measurements are constant regardless of the response being measured and the competing agonist used to stimulate that response. This was true even when the receptor was shown, in the same assay, to exist in two different conformational states coupled to two different G-proteins.

Introduction

The ability of an antagonist to bind to a given receptor is traditionally considered to be constant reflecting the nature of the specific chemical interaction between a ligand and its receptor (Kenakin et al., 1995). Consequently, antagonist affinity measurements at a given species homologue of a particular receptor should remain constant regardless of the method used to measure it, provided that the chemical composition of the receptor has not changed. Neither should it depend on the efficacy of the competing agonist used nor the downstream level at which the final response is measured. This premise has been the cornerstone of classical receptor pharmacology and has underpinned major advances in drug discovery and the definition of novel receptor subtypes (Arunlakshana and Schild, 1959; Black et al., 1965, 1972). However, recent observations with all three members of the β-adrenoceptor family of G-protein-coupled receptors (GPCRs) have begun to cast doubt on this basic pharmacological principle (Konkar et al., 2000; Lowe et al., 2002; Baker et al., 2003a,b; Baker 2005a,b).

Changes in antagonist affinity measurements have been noted at all human β-adrenoceptors. At both the human β1 and β3-adrenoceptor, responses to some agonists are readily inhibited by antagonists (giving high antagonist affinity values) whilst others are relatively resistant to antagonism (giving low antagonist affinity values for the same antagonist at the same receptor; Pak and Fishman, 1996; Konkar et al., 2000; Lowe et al., 2002; Baker et al., 2003b, Baker 2005a,b). Furthermore, at both receptors certain agonist ligands demonstrate two components in their concentration-response curves, one component of which is more readily antagonised than the other (Baker et al., 2003b; Baker 2005b). These observations have led to the proposal that the β1 and β3-adrenoceptors can

exist in at least two different states or conformations with different sensitivities to antagonists (Granneman 2001; Molenaar, 2003; Arch, 2004). At the human β2-adrenoceptor, antagonist affinities also differ 10-fold depending on the agonist used and the level response measured (Baker et al., 2003a). This appears to depend upon the length of agonist incubation, the efficacy of the competing agonist and the response being measured i.e. cAMP versus CRE-gene transcription measurements.

The observations with the β-adrenoceptors suggest that the underlying principle on which original receptor classification was based is in need of re-examination. Antagonist affinity estimates at many GPCRs may indeed depend upon the nature of the agonist used. For example, it has been proposed that agonists can differentiate between different G-protein-coupled conformations of a GPCR thus leading to agonist-trafficking (Kenakin, 1995; Berg et al., 1998). In this case, antagonist affinities may well vary for the different G-protein-coupled conformations of the same receptor. Kenakin (2002) has also proposed that receptors can adopt a variety of different conformations and that different ligands may stabilise a specific set of conformations leading to different responses. Thus again, antagonist affinity may vary in the presence of different agonists. Alternatively, the observations made with the β2-adrenoceptor may simply reflect receptor-specific alterations in the chemical structure of the receptor over time and with different agonists which may be a consequence of receptor phosphorylation or dephosphorlyation (Iyer et al., 2006; Vaughan et al., 2006). In addition, the observations made concerning the β1 and β3-adrenoceptor may reflect physical differences in the conformation of the receptor recognised by different agonists as a consequence of the association with other signalling or scaffolding proteins (including receptor dimerization; Hall and Lefkowitz, 2002; Milligan,

2004; Bulenger et al., 2005). In short, it is not currently known how widespread the patterns of changing antagonist affinity measurements are, to what extent this applies to other families of GPCRs or the effects of interactions of the same receptor with different G-proteins.

The adenosine A1-receptor is a GPCR that primarily couples to Gi-proteins and thus produces an inhibition in the production of intracellular cAMP (Libert et al., 1992; Olah and Stiles, 1995). However, the human A1-receptor has also been shown to couple to Gs-proteins in CHO-K1 cells (Cordeaux et al., 2000, 2004). The A1-receptor therefore provides a model system in which to evaluate whether antagonist affinity values vary for a Gi-coupled receptor, and whether the values obtained vary with the response measured. Furthermore, as the receptor is known to exist in different G-protein-coupled states, antagonist affinities can be examined in two different states of the receptor (Gi and Gs-coupled), within the same assay. We have therefore studied the influence of different agonists on antagonist affinity measurements for Gi and Gs-coupled pathways at the level of ligand binding, cAMP accumulation and CRE-gene transcription.

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Methods

Materials

Fetal calf serum was from PAA laboratories (Teddington, Middlesex, UK). ^{3}H-DPCPX, ^{3}H-adenine and ^{14}C-cAMP were obtained from Amersham International (Buckinghamshire, UK). CGS 15943, DPCPX, DPPX, GR 79236, NECA and CPA were from Tocris Cookson (Avonmounth, Bristol, UK). AAC, 2-chloroadenosine, 2C-CPA, CHA, PIA, and XAC were from Sigma Chemicals (Poole, Dorset, UK) who also supplied all other reagents.

Cell Culture

CHO cells stably expressing the human A1-adenosine receptor (Hill et al., 2003) were secondarily transfected with CRE-SPAP reporter gene using Lipofectamine and OPTIMEM as per manufacturer's instructions. Transfected cells were selected for 3 weeks using resistance to neomycin (1mg/ml; for A1 receptor) and hygromycin (200μg/ml; for CRE-SPAP reporter gene). A single clone was then isolated by dilution cloning. These cells, CHO-A1-CRE-SPAP cells (or CHO-A1 cells) were used throughout this study. A control CHO cell line stably expressing the CRE-SPAP reporter gene but not the A1-receptor was also used. Cells were grown in Dulbecco's modified Eagles medium/Nutrient mix F12 (DMEM/F12) containing 10% fetal calf serum and 2mM L-glutamine in a humidified 5% CO_2 : 95% air atmosphere at 37°C.

^{3}H-DPCPX whole cell binding.

CHO-A1 cells were grown to confluence in white-sided 96-well view plates. On the day of experimentation, the media was removed and replaced with 100μl serum-free media (i.e.

DMEM/F12 containing 2mM L-glutamine only) containing the competing ligand and immediately followed by the addition of 100µl serum-free media containing ^{3}H-DPCPX (to give a final concentration of ^{3}H-DPCPX of 2.67-3.60nM). Total and non-specific binding (as defined by 10µM XAC) were measured in every experiment. In saturation studies, 100µl serum free media or serum free media containing 20µM XAC was added to each well, immediately followed by 100µl serum free media containing ^{3}H-DPCPX to give final well concentrations of 10µM XAC (to define non-specific binding) and ^{3}H-DPCPX in the range 0.005 – 43.89nM. The cells were incubated for 90 minutes at 37°C in a humidified 5% CO_2 : 95% air atmosphere. After 90 minutes, the media and drugs were removed and the cells washed twice by the addition and removal of 2 x 200µl PBS/well. A white base was then added to the plate, followed by 100µl Microscint 20 per well and a sealant film placed over the wells. The plates were then counted the following day on a Topcount (Packard) 2 minutes per well. The protein content was determined by the method of Lowry et al., (1951).

Binding studies were also carried out after pre-treatment with pertussis toxin (PTX). Here, after the cells had grown to confluence, the media was removed and replaced with either 100µl serum free media (to ensure that the serum starving step did not cause any changes in the ligand affinities) or 100µl serum free media containing 100ng PTX/ml. The cells were incubated for a further 24 hours before whole cell binding was undertaken as described above.

^{3}H-cAMP accumulation

Cells were grown to confluence in 24-well plates. 24 hours before experimentation, the media was removed and replaced with serum-free media (with or without PTX at 100ng/ml). On the day of experimentation, the media was removed and the cells pre-labelled with ^{3}H-adenine by incubation with 2μCi/ml ^{3}H-adenine in serum-free media (0.5ml per well) for 3 hours at 37°C (5% CO_2). The ^{3}H-adenine was removed and each well washed by the addition and removal of 1ml serum-free media. 1ml serum-free media containing 10μM rolipram with or without the final required concentration of DPCPX, was added to each well and the cells incubated for 30 minutes at 37°C (5% CO_2). Agonist in 10μl was added to each well and the plates incubated for 10 minutes at 37°C. Forskolin was then added to each well (except basal) and the plates incubated for a further 10 minutes at 37°C before the reaction was terminated by the addition of 50μl concentrated HCl per well. The plates were then frozen, thawed and ^{3}H-cAMP separated from other ^{3}H-nucleotides by Dowex and alumina column chromatography, with each column being corrected for efficiency by comparison with ^{14}C-cAMP recovery as previously described (Donaldson et al., 1988).

In experiments where the response to DPCPX, XAC, CGS 15943 and DPPX were examined alone (e.g. Figure 3), following the forskolin addition, the ligands were incubated for 1 hour (37°C, 5% CO_2) in order to maximise any observed responses. For the same reason, all ^{3}H-cAMP experiments involving the control CHO-CRE-SPAP cells (i.e. those without the transfected A1 receptor) were also incubated for 1 hour at 37°C.

CRE-SPAP gene transcription

Cells were grown to confluence in 96-well plates in 100µl DMEM/F12 containing 10% fetal calf serum and 2mM L-glutamine. Once confluent, the media was removed and replaced with 100µl serum free media (DMEM/F12 containing 2mM L-glutamine) and the cells incubated for a further 24 hours. Where used, pertussis toxin (PTX) was added to the serum free media (100ng/ml) thus the cells received 24 hours pre-treatment with PTX. On the day of experimentation, the serum-free media was removed and replaced with 100µl serum-free media or 100µl serum-free media containing an antagonist at the final required concentration and the cells incubated for 1 hour at 37°C (5% CO_2). Agonist in 10µl (diluted in serum free media) was then added to each well and the plate incubated at 37°C for 10 minutes. Forskolin in 10µl was then added to all but the basal wells and the cells incubated for 5 hours at 37°C (5% CO_2). After 5 hours, the media and drugs were removed, 40µl serum-free media was added to each well and the cells incubated for a further 1 hour at 37°C. The plates were then incubated at 65°C for 30 minutes to destroy any endogenous phophatases. The plates were then cooled to 37°C. 100µl 5mM pNPP in diethanolamine buffer was added to each well and the plates incubated at 37°C until the yellow colour developed. The plates were then read on a Dynatech MRX plate reader at 405nm.

Data Analysis

^{3}H-DPCPX whole cell binding

Saturation curves for specific ^{3}H-DPCPX binding were fitted to the following equation using GraphPad Prism 2:

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Equation 1: $$\text{Specific binding} = \frac{B_{max} \times K_D}{([L] + K_D)}$$

where B_{MAX} is the maximum specific binding, K_D is the dissociation constant of ^{3}H-DPCPX and [L] is the concentration of ^{3}H-DPCPX.

Curves for inhibition of specific binding of ^{3}H-DPCPX by a range of A1-antagonists and agonists were fitted to the following equation:

Equation 2: $$\text{\% of specific binding} = 100 - \frac{100 \times IC_{50}}{[A] + IC_{50}}$$

where [A] is the concentration of competing antagonist and IC_{50} is the concentration which inhibits specific binding by 50%. Antagonist dissociation constants (K_B) were then determined from the following expression:

Equation 3: $$K_B = \frac{IC_{50} \times K_D}{(K_D + [L])}$$

where [L] and K_D are the concentration and dissociation constant of ^{3}H-DPCPX respectively.

Functional experiments

One-site concentration responses curves

Sigmoidal agonist concentration-response curves were fitted to the data using the following equation through computer-assisted non-linear regression using the programme Graphpad Prism 2:

Equation 4: $$\text{Response} = \frac{Emax \times [A]}{EC_{50} + [A]}$$

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where Emax is the maximal response, [A] is the agonist concentration and EC_{50} is the concentration of agonist that produces 50% of the maximal response.

Antagonist K_D values were then calculated from the shift of the agonist concentration responses in the presence of a fixed concentration of antagonist using the following equation:

Equation 5: $$DR = 1 + \frac{[B]}{K_D}$$

where DR (dose ratio) is the ratio of the agonist concentration required to stimulate an identical response in the presence and absence of a fixed concentration of antagonist [B].

In experiments where three different fixed concentrations of the same antagonist were used, Schild plots were constructed using the following equation:

Equation 6: $$\text{Log } (DR-1) = \log [B] - \log (K_D)$$

These points were then fitted to a straight line. A slope of 1 then indicates competitive antagonism (Arunlakshana and Schild, 1959).

Two site agonist curves

As many concentration response curves clearly contained two components, two-site analysis was required for most ligands. This was performed using the following equation:

Equation 7: $$\text{Response} = \text{Basal} + (FK - \text{Basal})\left[1 - \frac{[A]}{([A] + IC_{50})}\right] + S_{MAX}\left[\frac{[A]}{([A] + EC_{50})}\right]$$

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where basal is the response in the absence of agonist, FK is the response to a fixed concentration of forskolin, [A] is the concentration of A1-agonist, IC_{50} is the concentration of A_1-agonist that inhibits 50% of the response to forskolin, S_{MAX} is the maximum stimulation of the Gs-component of the response by the A1-agonist and EC_{50} is the concentration of A1-agonist that stimulated a half maximal Gs-response.

For analysis of the cyclic AMP responses to DPCPX and CGS15943 in which a secondary inhibitory effect was observed at high concentrations the equation fitted was:

Equation 8: $$\text{Response} = \text{Basal} + S_{MAX}\left[\frac{[A]}{([A]+EC_{50})}\right] - I_{MAX}\left[\frac{[A]}{([A]+IC_{50})}\right]$$

Where basal is the response in the absence of ligand, [A] is the concentration of DPCPX or CGS 15943, S_{MAX} is the maximum stimulation produced by the inverse agonist, EC_{50} is the concentration of inverse agonist that produced 50% of its maximal response, I_{MAX} is the maximal extent of inhibition observed at high concentrations and IC_{50} is the concentration of DPCPX or CGS 15943 required to produce 50% of this maximal inhibitory effect.

A 3μM (maximal) forskolin concentration was included in each CRE-gene transcription plate and a 10μM (maximal) forskolin included in each cAMP plate for each separate experiment. All data are presented as mean ± s.e.m. of triplicate or quadruplicate determinations and n in the text refers to the number of separate experiments.

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Results

^{3}H-DPCPX Whole cell binding

As an important aim of this study was to compare the antagonist affinity measurements obtained in different functional assays, the affinity of each antagonist was firstly determined directly by competition binding studies. The K_D value of ^{3}H-DPCPX determined from saturation binding experiments was 3.61 ± 0.28nM (n=6) and the expression of the human A1 adenosine receptor in this stable cell line 3767 ± 310 fmol/mg protein (n=6; Figure 1a). The affinity of the A1 antagonists was then determined in the presence and absence of pertussis toxin (PTX) from competition binding studies and the log K_D values are shown in Table 1 (Figure 1b and c). PTX ADP ribosylates a cysteine residue in the C-terminal region of the Gαi/o subunit that is responsible for receptor selectivity and coupling (Jajoo et al., 2006). Pre-treatment with PTX therefore prevents the A1 receptor coupling to Gαi/o proteins and as shown in the functional responses below can therefore be used to isolate the A1-Gs coupled responses. The affinity of the agonists used was also determined in order to give an indication of relative efficacy of the agonists when comparing the EC_{50} values of functional studies later (Table 1).

^{3}H-cAMP accumulation

2-chloro-cyclopentyladenosine (2C-CPA) inhibited forskolin-stimulated ^{3}H-cAMP accumulation in CHO-A1 cells to yield a log IC_{50} value of -9.06 ± 0.05 (n=10) in keeping with its agonist activation of the Gi-coupled A1-receptor (Figure 2a). This response was inhibited by DPCPX to yield a log K_D value for DPCPX of -8.62 ± 0.10 (n=9, Figure 2a). However, close examination reveals a small increase in cAMP at the higher concentrations

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of 2C-CPA. When the cells were pre-incubated with PTX (100ng/ml) for 24 hours, the inhibitory response to 2C-CPA was abolished confirming that this is due to Gi-coupling. However, the stimulatory response remained. It is therefore likely that this PTX-insensitive stimulatory response seen at higher concentrations of 2C-CPA is due to the A1-receptor coupling to Gs proteins. This stimulatory response was also inhibited by DPCPX to yield a log K_D value of -8.54 ± 0.11 (n=6) very similar to that seen for antagonism of the inhibitory response.

Similar responses were seen with a range of adenosine A1-receptor agonists (Figure 2, Table 2) and all of these responses were inhibited by DPCPX to yield comparable log K_D values (Figure 2, Table 2). However, although the PTX insensitive stimulatory response seen with adenosine amine congener (AAC, Figure 2d) was antagonised by DPCPX, a maximum response was not reached in the presence of DPCPX thus making a log K_D value for DPCPX was therefore unobtainable.

DPCPX alone, at concentrations of 10nM and 30nM, appeared to stimulate a response (Figure 2). The response to DPCPX and the other antagonists used in this study were therefore examined more closely. DPCPX, XAC, CGS 15943 and DPPX all caused stimulatory responses in CHO-A1 cells (log EC_{50} -8.43 ± 0.07, 1.61 ± 0.07 fold over basal; -7.80 ± 0.15, 1.51 ± 0.07 fold over basal; -8.61 ± 0.07, 1.45 ± 0.06 fold over basal and -7.79 ± 0.09, 1.37 ± 0.05 fold over basal, respectively all n=4; Figure 3). Pre-treatment with PTX however abolished the stimulatory effect of these compounds (Figure 3). However, higher concentrations of DPCPX and CGS 15943 caused a decrease in ^{3}H-cAMP accumulation regardless of whether the cells had been pre-incubated with PTX (Figure 3). This was also

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seen in CHO cells not expressing the A1-receptor (CHO CRE-SPAP cells; Figure 3) and is therefore likely to be a non-specific effect of the compounds at these high concentrations.

A1-CRE-gene transcription

2-chloro-cyclopentyladenosine (2C-CPA) inhibited forskolin-stimulated CRE-gene transcription (SPAP) in CHO-A1 cells to yield a log IC_{50} value of -8.42 ± 0.08 (n=7) in keeping with its agonist activation of the Gi-coupled A1-adenosine receptor (Figure 4a). However at higher concentrations of 2C-CPA, a substantial stimulatory response was also seen with a log EC_{50} -6.66 ± 0.03 (n=7; Figure 4a). DPCPX antagonised both the inhibitory and stimulatory responses to 2C-CPA with similar affinities (log K_D -8.76 ± 0.05, n=15 and -8.81 ± 0.05, n=11 respectively). Furthermore in experiments where several different concentrations of DPCPX were used, a Schild plot of the inhibitory component was constructed and the slope was 1.04 ± 0.07 (n=3) consistent with competitive antagonism. A Schild plot was not possible for the stimulatory component as inhibition of the response by only two concentrations of DPCPX were possible within the concentration window available for the solubility of the compounds (Figure 4a). When a similar experiment was performed after 24 hours pre-incubation with PTX, the inhibitory response to 2C-CPA was abolished confirming that this is due to Gi-coupling. However, once again the stimulatory response remained suggesting coupling to Gs proteins. This stimulatory response was also inhibited by DPCPX to again yield a similar log K_D value for DPCPX of -8.84 ± 0.09 (n=8).

Once again, similar responses were seen to the other adenosine agonists (Figure 4 and 5, Table 4). All of the inhibitory and stimulatory response yielded similar log K_D values for

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DPCPX and all inhibitory responses were found to be competitive (Figures 4 and 5, Table 5). All log K_D values determined in the presence of PTX were again found to be similar, with the exception of AAC, where once more a log K_D value was not obtainable due to the decreasing maximums seen in the presence of DPCPX (Figure 5d).

When this study was extended to different antagonists (XAC, CGS 15943 and DPPX) similar antagonist affinity measurements were obtained for each antagonist at the inhibitory response and the stimulatory responses in both the absence and presence of PTX (Table 6).

Lack of responses in CHO CRE-SPAP cells

Full 6 or 7 point concentration response curves were performed in CHO CRE-SPAP cells (i.e. CHO cells stably expressing the CRE-SPAP reporter but not the human A1-adenosine receptor) both in ^{3}H-cAMP accumulation and CRE-SPAP gene transcription assays in a manner identical to that performed in the CHO-A1 cells for every ligand used in this study (n=3 for each ligand in each assay). There was no response to any compound except for the decrease in response to DPCPX and CGS 15943 mentioned above (Figure 3) and a small decrease in response at the highest concentration (100μM) of PIA and 2-chloroadenosine. This suggests that all the stimulatory Gs and inhibitory Gi responses discussed above are occurring via the transfected human A1-adenosine receptor and not a native receptor present in the parent cell line.

Discussion

Antagonist affinity measurements have traditionally been considered to be constant for a given receptor-ligand interaction and this property has been used to define which receptors are present within a given tissue (Kenakin et al., 1995; Arunlakshana and Schild, 1959; Black et al., 1965, 1972; Hill, 2006). However, recent evidence obtained for the three Gs-coupled human β-adrenoceptors indicate that this is not always the case (Konkar et al., 2000; Lowe at al, 2002; Baker et al., 2003a,b; Baker 2005a,b) . Changes in antagonist affinity at the human β-adrenoceptors have been attributed to two agonist conformations or states of the receptor (β1 and β3; Pak and Fishman, 1996; Konkar et al., 2000; Lowe et al., 2002; Baker et al., 2003b, Baker 2005a,b) or have been shown to vary depending upon the assay used, the time of incubation employed and the nature of the competing agonist (β2; Baker et al., 2003a) . A similar difference has been noted for the histamine H_1-receptor where antagonist affinity estimates varied according to the response measured (inositol phosphate accumulation or NFκB gene transcription; Bakker et al., 2001). Here, we have examined a Gi-coupled receptor (the human adenosine A1-receptor; Libert et al., 1992; Olah & Stiles, 1995; Cordeaux et al., 2000) and evaluated, using a range of different agonists, the extent to which antagonist affinity estimates depend on the level of the signalling cascade at which responses are measured, the length of incubation time and the nature of the stimulating agonist.

As expected, stimulation of the A1-receptor by agonists caused a decrease in forskolin-stimulated ^{3}H-cAMP accumulation in keeping with a Gi-coupled GPCR. In addition, a small augmentation of forskolin-stimulated ^{3}H-cAMP accumulation was observed at higher agonist concentrations. Previous studies of ^{35}S-GTPγS binding to Gs-alpha proteins have

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shown that the A1-receptor expressed in CHO-K1 cells can also couple directly to Gs-proteins (Cordeaux et al., 2000, 2004). Experiments performed in the presence of PTX removed the inhibitory coupling to adenylyl cyclase via Gi-proteins and enhanced the observed stimulation of ^{3}H-cAMP accumulation. This confirmed that the stimulatory component was due to an A1-receptor-mediated stimulation of adenylyl cyclase via the Gs-protein. However, since much higher concentrations of agonist were required to achieve this Gs-coupling, the agonist efficacy (in terms of the ratio of agonist dissociation constant K_D determined from binding compared to its IC_{50} and EC_{50} values seen in the functional assays; Clark et al., 1999) was, by definition, lower at the A1-Gs-coupled conformation of the receptor than at the equivalent A1-Gi-coupled conformation. For example, in the case of 2-CCPA the K_D/IC_{50} ratio for the Gi response was 437 whereas that for the Gs response (K_D/EC_{50}) was 2. The other agonists gave very similar estimates of efficacy for the two responses (Table 7).

Close examination of the effect of DPCPX alone showed an increase in forskolin-stimulated ^{3}H-cAMP accumulation. This could have been secondary to DPCPX acting as either an A1-Gi-inverse agonist or a weak A1-Gs-agonist. An alternative explanation could be antagonism of secreted endogenous adenosine from the cells themselves. Concentration response curves were therefore constructed to the antagonists alone. An augmentation of forskolin-stimulated ^{3}H-cAMP accumulation was observed in response to all four antagonists. Furthermore, the log EC_{50} values of the antagonists for this response were very similar to the log K_D values obtained from whole cell binding. Closer examination of the responses to DPCPX and CGS 15943 indicated that the responses curves were biphasic. When the responses were examined in the parent CHO CRE-SPAP cells (i.e. those with the

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CRE-SPAP reporter but without the A1-receptor), a similar decrease in ^{3}H-cAMP was seen at matching higher concentrations. This suggests that the decrease in ^{3}H-cAMP is a non-A1 receptor mediated event and DPCPX and CGS 15943 may well have cytotoxic properties at these higher concentrations, a property not shared by XAC and DPPX. When the responses in CHO-A1 cells were examined in the presence of PTX, the stimulatory component to all antagonists was abolished. Therefore, unlike the Gi-coupled conformation, there was no suggestion of agonist effects on Gs-constitutive activity for the A1-receptor. This suggests that all four ligands were acting as Gi-coupled inverse agonists, although blockade of endogenous adenosine cannot be ruled out. Inverse agonist effects of DPCPX, XAC and CGS 15943 have also been observed on A_1-receptor-mediated GTPγS binding in CHO cells (Shryock et al., 1998).

When the ability of DPCPX to inhibit the 10 minute ^{3}H-cAMP agonist responses was examined, very similar log K_D values were obtained for DPCPX regardless of the competing agonist used to stimulate the A1-receptor. These were similar to the log K_D value determined for DPCPX from whole cell binding with ^{3}H-DPCPX. However, the agonist-induced Gs-mediated ^{3}H-cAMP response were too small to obtain a reliable measurement of DPCPX affinity. In order to isolate this Gs response and obtain antagonist affinity measurements at this conformation of the A1 receptor, the cells were pre-incubated with PTX for 24 hours prior to experimentation. With the Gi "break" removed, the ^{3}H-cAMP response to A1-agonists was far greater. However, it was striking that the antagonist affinity measurements for DPCPX obtained from antagonism of the Gs-coupled A1-response were very similar to those obtained from the Gi-mediated inhibition of cAMP accumulation.

It has previously been noted that tiny (3-5% of maximum) β-adrenoceptor Gs-coupled partial agonist cAMP responses can be markedly amplified (to 50% of maximum) when longer-term (5hour) Gs-coupled CRE-gene transcription is studied (Baker et al., 2003b, 2004, Baker 2005b). A1-CRE-gene transcription responses were therefore examined, and in keeping with this, both the Gi and Gs-mediated responses to A1-agonists were clearly seen without the requirement for PTX pre-incubation to isolate the Gs component. It is also interesting to note, however, that there was no obvious amplification (in terms of EC_{50} values) in either the Gi or Gs responses to any of the A1-agonists when comparing cyclic AMP accumulation with CRE-mediated gene transcription.

The affinity of DPCPX for both of these A1-receptor conformations was the same irrespective of the agonist used. The bigger window for Gi-coupled response allowed Schild analysis to be undertaken and confirmed that the interaction of DPCPX with the receptor was competitive. Although this was not possible for the less-well-coupled Gs-conformation, it also appeared competitive (see Figure 4 and 5). Pre-incubation with PTX also isolated the Gs component of the CRE-gene transcription response and the agonist EC_{50} values obtained were very similar to those determined from the two-component fits of the responses obtained in the absence of PTX. Furthermore, the affinity of DPCPX was also the same. When this analysis was extended to the other A1-antagonists, a similar pattern was observed.

Interestingly, antagonist affinities were similar for both Gi and Gs-mediated responses and these values did not change when longer term assays of CRE-mediated gene transcription

were undertaken in the same cell background. In the case of the β2-adrenoceptor longer term gene transcription assays reveal differences in antagonist affinities which depend on agonist efficacy but which are not evident in shorter term assays of ^{3}H-cAMP accumulation. It appears that certain agonists cause a chemical modification of the β2-adrenoceptor over time that alters antagonist affinity (Baker et al., 2003a). The fact that no similar effect is seen with the adenosine A1-receptor for both a well-coupled (Gi) and a poorly-coupled (Gs) response suggests that no chemical modification has been induced in the A1-receptor. Indeed, unlike the β_2-adrenoceptor, the adenosine A1-receptor is resistant to agonist-induced receptor phosphorylation in CHO-K1 cells (Kahout and Lefkowitz, 2003; Palmer et al., 1996). Both the cAMP and gene transcription A1-receptor responses were measured in the presence of forskolin which will have elevated cAMP levels within the cell. However, the affinity constants for A1-antagonists obtained from each functional studies were similar to those obtained from inhibition of ^{3}H-DPCPX binding (in the absence of forskolin). These data suggest that this has no major influence on the outcome of this study.

The data obtained with the A1-receptor confirm one of the longest-held principles of pharmacology (i.e. that provided the chemical nature of the receptor is not changed the affinity of an antagonist for a particular receptor should be constant regardless of the response being measured and the agonist used to stimulate that response). The fact that this has been demonstrated for two different G-protein bound conformations of the A1-receptor and that the observation appears to be independent of the time of incubation with agonists and antagonists, the efficacy of the agonist or the conformational state of the A1 receptor, provides strong evidence that the ligand binding site/conformation of the human A1-

receptor is not chemically altered as a consequence of G-protein-coupling or prolonged incubation with agonists of different efficacy. The conformational of this basic pharmacological principle for the A1-receptor strengthens the data obtained for other GPCRs where there are variations in antagonist affinity. For example the β-adrenoceptors, studied in a similar CHO-CRE-SPAP cell background, have variations in antagonist affinity which must therefore be due to a change in the chemical or conformational nature of the ligand binding pocket (e.g. agonist-induced conformations or sites of action, second-messenger-induced changes, agonist-induced auxiliary protein coupling or heterodimerisation).

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Footnotes

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Legends for Figures

Figure 1

a) ^{3}H-DPCPX binding to whole CHO A1-CRE-SPAP cells showing total binding and non-specific binding determined in the presence of 10μM XAC. Data points are mean ± s.e.m. of quadruplicate determinations. This single experiment is representative of 6 separate experiments.

b) and c) inhibition of ^{3}H-DPCPX binding to whole cells by DPCPX, DPPX, CGS 15943, and XAC in CHO A1-CRE-SPAP cells. In b), the cells have been serum starved for 24 hours before experimentation. In c) the cells have been incubated with PTX (100ng/ml) in serum free media for 24 hours before experimentation. Bars represent total ^{3}H-DPCPX binding and non-specific binding as determined by the presence of 10μM XAC and data points are mean ± s.e.m. of triplicate determinations. The concentration of ^{3}H-DPCPX was 3.60nM and these single experiments are representative of b) 4 and c) 4 separate experiments.

Figure 2

^{3}H-cAMP accumulation in response to 2-chloro-cyclopentyladenosine (2C-CPA) a) and b) and AAC c) and d) in the absence and presence of DPCPX in CHO A1-CRE-SPAP cells. b) and d) are following 24hours pre-treatment with PTX (100ng/ml). Bars show basal ^{3}H-cAMP accumulation, that in response to 10μM forskolin, and that in response to DPCPX in the presence of 10μM forskolin. Data points are mean ± s.e.m. of triplicate values from a single experiment and are representative of a) 9, b) 6, c) 4 and d) 4 separate experiments.

Figure 3

^{3}H-cAMP accumulation in response to DPCPX a) and b), XAC c) and d). a) and c) are in CHO A1-CRE-SPAP cells with and without a pre-incubation with 100ng/ml PTX. b) and d) are in CHO CRE-SPAP cells (i.e. the parent cells without the transfected receptor) in the absence of PTX. Bars show basal ^{3}H-cAMP accumulation, that in response to 10μM forskolin both with and without pre-incubation with PTX. Data points are mean ± s.e.m. of triplicate values from a single experiment and are representative of 4 separate experiments in each case. CGS 15943 stimulated responses in both CHO A1 SPAP cells (both with and without PTX) and CHO CRE-SPAP cells in an identical manner to those seen with DPCPX. DPPX stimulated responses identical to those seen with XAC.

Figure 4

CRE-SPAP gene transcription in response to 2-chloro-cyclopentyadenosine (2C-CPA) a) and b) and NECA c) and d) in the absence and presence of 3nM, 30nM and 300nM DPCPX in CHO A1-CRE-SPAP cells. b) and d) are following 24 hours pre-treatment with PTX (100ng/ml). Bars show basal CRE-SPAP gene transcription, that in response to 10μM forskolin, and that in response to 3nM or 30nM or 300nM DPCPX in the presence of 10μM forskolin. Data points are mean ± s.e.m. of triplicate values from a single experiment and are representative of 3 separate experiments in each case.

Figure 5

CRE-SPAP gene transcription in response to GR 79236 a) and b) and AAC c) and d) in the absence and presence of 3nM, 30nM and 300nM DPCPX in CHO A1-CRE-SPAP cells. b)

and d) are following 24hours pre-treatment with PTX (100ng/ml). Bars show basal CRE-SPAP gene transcription, that in response to 10μM forskolin, and that in response to 3nM or 30nM or 300nM DPCPX in the presence of 10μM forskolin. Data points are mean ± s.e.m. of triplicate values from a single experiment and are representative of a) 4, b) 3, c) 7 and d) 5 separate experiments.

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				After serum starving			After PTX pre-incubation	
Ligand	Log K_D	n		Log K_D	n		Log K_D	n
DPCPX	-8.43±0.03	6		-8.37±0.03	4		-8.52±0.07	4
XAC	-7.50±0.02	6		-7.25±0.02	4		-7.33±0.03	4
CGS 15943	-8.37±0.05	6		-8.35±0.05	4		-8.49±0.05	4
DPPX	-7.27±0.07	6		-6.96±0.11	4		-7.00±0.11	4
2C-CPA	-6.32±0.02	6		-6.42±0.04	4		-6.37±0.05	4
NECA	-5.75±0.05	6		-5.88±0.05	4		-5.74±0.03	4
GR 79236	-5.86±0.04	6		-6.03±0.07	4		-5.90±0.04	4
AAC	-4.89±0.04	6		-4.96±0.04	4		-4.97±0.05	4
PIA	-6.15±0.03	6		-6.24±0.02	4		-6.15±0.02	4
2-CA	-5.46±0.06	6		-5.66±0.05	4		-5.59±0.03	4
CHA	-5.92±0.02	6		-6.03±0.03	4		-6.04±0.07	4
CPA	-6.25±0.02	6		-6.40±0.03	4		-6.33±0.04	4

Table 1

Log K_D values of A1-antagonists and agonists obtained from inhibition of specific ^{3}H-DPCPX binding to the human A1 adenosine receptor in CHO A1-CRE-SPAP cells. The first column is from binding obtained 24 hours after plating cells into 96-well plates; the second column after serum starving for 24 hours before experimentation; and the third column following treatment with PTX in serum free media for 24 hours before experimentation. Values represent mean ± s.e.mean of triplicate deterinations of n different experiments in each case.

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				After PTX pre-incubation		
	Log IC_{50} (Gi)	n		Log EC_{50} (Gs)	Fold over basal	n
2C-CPA	-9.06±0.05	10		-6.77±0.05	2.36±0.09	7
NECA	-8.75±0.11	6		-6.22±0.02	3.01±0.33	4
GR 79236	-8.59±0.06	9		-6.21±0.04	2.86±0.52	6
AAC	-7.77±0.07	7		*	*	4
PIA	-8.67±0.12	5		-6.53±0.09	2.94±0.55	4
2-CA	-8.20±0.10	5		-5.88±0.06	2.60±0.21	5
CHA	-8.60±0.04	8		-6.39±0.08	2.00±0.07	5
CPA	-9.10±0.09	9		-6.84±0.03	3.02±0.41	9

Table 2

Log IC_{50} values (without PTX) and log EC_{50} values (following pre-incubation with PTX) for A_1-agonist-induced changes in forkolin-stimulated ^{3}H-cAMP accumulation in CHO A_1-CRE-SPAP cells. All agonists produced a maximal inhibition of forskolin-stimulated ^{3}H-cAMP accumulation and were therefore full agonists for the Gi-coupled response. Fold over basal values for the Gs stimulatory response are also shown. Values are mean ± s.e.mean of n separate experiments.

* In the case of AAC a maximal stimulatory response was not achieved at the highest agonist concentration used (10μM), however following PTX pre-incubation a stimulation in ^{3}H-cAMP accumulation of 1.66 ± 0.06 (n=4) fold over basal was achieved by 10μM AAC.

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				After PTX pre-incubation	
	Log K_D DPCPX (Gi)	n		Log K_D DPCPX (Gs)	n
2C-CPA	-8.62±0.10	9		-8.54±0.11	6
NECA	-8.71±0.09	6		-8.94±0.05	3
GR 79236	-9.08±0.11	9		-8.65±0.14	5
AAC	-8.64±0.07	4		*	4
PIA	-8.71±0.14	6		-8.59±0.08	4
2-CA	-8.77±0.03	4		-8.65±0.10	6
CHA	-8.67±0.12	7		-8.55±0.12	3
CPA	-8.86±0.11	6		-8.82±0.16	4

Table 3

Log K_D values for DPCPX from forskolin-stimulated ^{3}H-cAMP accumulation measured in the presence of the different agonists at the inhibitory Gi-coupled receptor conformation (without PTX pre-incubation) and at the stimulatory Gs-coupled receptor conformation. Values are mean ± s.e.mean of n separate experiments.

* it was not possible to determine the log K_D of DPCPX in the presence of AAC as maximal stimulation was not reached with AAC alone or in the presence of DPCPX.

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						With PTX pre-incubation		
	Log IC_{50} (Gi)	n	Log EC_{50} (Gs)	n		Log EC_{50} (Gs)	Fold over basal	n
2C-CPA	-8.42±0.08	7	-6.66±0.03	7		-6.98±0.07	1.91±0.24	9
NECA	-8.19±0.04	11	-6.35±0.05	11		-6.52±0.06	1.86±0.22	10
GR 79236	-8.06±0.04	9	-6.22±0.03	7		-6.36±0.07	2.07±0.20	12
AAC	-7.16±0.02	8	*	8		*		5
PIA	-8.15±0.09	8	-6.45±0.09	8		-6.87±0.06	1.69±0.15	10
2-CA	-7.67±0.05	7	-5.81±0.05	7		-6.19±0.06	1.73±0.15	10
CHA	-8.13±0.03	8	-6.33±0.05	8		-6.58±0.05	1.77±0.19	10
CPA	-8.51±0.05	19	-6.52±0.07	19		-6.81±0.06	2.15±0.20	16

Table 4

Log IC_{50} values and log EC_{50} values (determined from 2-site concentration-response analysis, without PTX) and log EC_{50} values following pre-incubation with PTX (which abolished the inhibitory response) of A1-agonist-induced changes in forskolin-stimulated CRE-SPAP production in CHO A1-CRE-SPAP cells. All agonists stimulated a maximal inhibition of forskolin-stimulated CRE-SPAP production and were therefore full agonists for the Gi-coupled response. Fold over basal values for the Gs stimulatory responses are also shown. Values are mean ± s.e.mean of n separate experiments.

* AAC did not stimulate a maximal Gs- response and therefore log EC_{50} values were not obtained however following pre-incubation with PTX, 10μM AAC stimulated a 1.43 ± 0.03 (n=5) fold over basal CRE-SPAP production.

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Table 5 Log K_D DPCPX

								After PTX pre-incubation	
	Log K_D DPCPX (Gi)	n	slope	n	Log K_D DPCPX (Gs)	n		Log K_D DPCPX (Gs)	n
2C-CPA	-8.76±0.05	15	1.04±0.07	3	-8.81±0.05	11		-8.84±0.09	8
NECA	-8.92±0.04	15	1.10±0.02	3	-8.95±0.06	13		-8.93±0.08	8
GR 79236	-8.87±0.06	19	1.03±0.06	4	-8.93±0.06	14		-9.08±0.07	7
AAC	-8.80±0.04	19	1.02±0.02	5	*	8		*	5
PIA	-8.66±0.06	17	1.10±0.02	4	-8.98±0.04	19		-9.11±0.09	7
2-CA	-8.90±0.05	17	1.13±0.10	3	-8.73±0.07	16		-8.92±0.09	7
CHA	-8.76±0.04	23	1.01±0.04	7	-8.79±0.05	9		-8.60±0.12	8
CPA	-8.91±0.05	23	1.07±0.07	7	-8.88±0.05	19		-8.87±0.11	5

Table 5

Log K_D values for DPCPX obtained in CHO-A1-CRE-SPAP cells from antagonism of the CRE-SPAP responses to a range of different A1-agonists in the presence of 3μM forskolin. Measurements were made for both Gi-coupled and Gs-coupled (in the presence and absence of PTX pre-incubation) conformations of the human A1-receptor.

Values are mean ± s.e.mean of n separate experiments. Schild plots were performed for the Gi-inhibitory component of each agonist in the presence of 3nM, 30nM and 300nM DPCPX and the slopes for these plots shown.

* it was not possible to determine the log K_D value of DPCPX in the presence of AAC as a maximal stimulation was not reached with AAC alone.

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Table 6a Log K_D XAC

						After PTX pre-incubation	
	Log K_D XAC (Gi)	n	Log K_D XAC (Gi)	n		Log K_D XAC (Gi)	n
2C-CPA	-7.75±0.06	4	-7.92±0.04	3		-8.22±0.09	3
NECA	-7.77±0.05	11	-8.05±0.08	5		-8.01±0.07	6
GR 79236	-7.91±0.03	12	-8.05±0.10	6		-7.92±0.11	7
AAC	-7.78±0.06	8	*	8		*	5
PIA	-7.77±0.08	5	-7.91±0.13	5		-7.92±0.05	3
2-CA	-7.84±0.10	5	-7.80±0.09	5		-7.97±0.02	4
CHA	-7.86±0.03	8	-7.87±0.14	5		-7.69±0.12	7
CPA	-7.84±0.05	11	-7.92±0.15	5		-7.86±0.14	7

Table 6b Log K_D CGS 15943

						After PTX pre-incubation	
	Log K_D CGS (Gi)	n	Log K_D CGS (Gi)	n		Gs Log K_D CGS (Gi)	n
2C-CPA	-8.57±0.07	7	-8.67±0.09	6		-8.85±0.08	7
NECA	-8.73±0.10	8	-8.88±0.08	4		-8.83±0.16	6
GR 79236	-8.77±0.05	8	-8.93±0.10	4		-8.84±0.11	10
AAC	-8.65±0.09	8	*	8		*	5
PIA	-8.62±0.10	5	-8.84±0.05	5		-8.63±0.11	5
2-CA	-8.65±0.08	5	-8.69±0.05	5		-8.78±0.08	7
CHA	-8.71±0.07	8	-8.65±0.11	5		-8.82±0.16	6
CPA	-8.82±0.08	8	-8.91±0.06	3		-8.85±0.05	8

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Table 6c Log K_D DPPX

						After PTX pre-incubation	
	Log K_D DPPX (Gi)	n	Log K_D DPPX (Gi)	n		Log K_D DPPX (Gi)	n
2C-CPA	-7.44±0.10	7	-7.12±0.10	7		-7.21±0.10	9
NECA	-7.72±0.09	8	-7.32±0.10	3		-7.41±0.10	6
GR 79236	-7.70±0.05	11	-7.07±0.09	5		-7.20±0.08	9
AAC	-7.47±0.07	8	*			*	
PIA	-7.25±0.17	5	-7.32±0.10	8		-7.40±0.10	8
2-CA	-7.49±0.10	5	-7.17±0.05	3		-7.26±0.15	4
CHA	-7.51±0.10	8	-7.31±0.07	7		-7.27±0.04	5
CPA	-7.72±0.07	11	-7.21±0.14	3		-7.28±0.02	7

Table 6a-c

Log K_D values for (a) XAC, (b) CGS 15943 and (c) DPPX obtained from antagonism of the CRE-SPAP responses to a range of different A1-agonists in the presence of 3μM forskolin. Measurements were made for both Gi-coupled and Gs-coupled (in the presence and absence of PTX pre-incubation) conformations of the human A1-receptor. Values are mean ± s.e.mean of n separate experiments.

* it was not possible to determine the log K_D values of the antagonists in the presence of AAC as a maximal stimulation was not reached with AAC alone.

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	Log (K_D/IC_{50}) (Gi)			Log (K_D/EC_{50}) (Gs)		
Ligand	cAMP	CRE-SPAP		cAMP	CRE-SPAP	
2C-CPA	2.64	2.00		0.40	0.61	
NECA	2.87	2.31		0.47	0.78	
GR 79236	2.56	2.03		0.31	0.46	
AAC	2.81	2.20		*	*	
PIA	2.43	1.91		0.38	0.72	
2-CA	2.54	2.01		0.29	0.60	
CHA	2.57	2.10		0.35	0.54	
CPA	2.7	2.11		0.51	0.48	

Table 7

Agonist efficacy estimates for Gi- and Gs- mediated responses based on the extent of signal amplification achieved by each agonist for each response relative to the K_D value determined for each agonist from inhibition of ^{3}H-DPCPX ligand binding. For the Gi efficacy estimates, the K_D values were obtained from inhibition of ^{3}H-DPCPX binding (Table 1 column 2) and for Gs efficacy estimates the K_D values were obtained from ^{3}H-DPCPX binding in the presence of PTX (Table 1, column 3). Efficacy estimates are provided as log K_D – Log IC_{50} (or log EC_{50}). For example the value of 2.64 for 2C-CPA for inhibition of cyclic AMP accumulation indicates an amplification of 2.64 orders of magnitude (437 fold) over the ligand binding K_D value, whereas the value of 0.4 for the Gs effect indicates an amplification of 2-fold.

* it was not possible to determine the efficacy ratio for the Gs-coupled state of the receptor as a maximal stimulation was not reached with AAC alone.

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Fig 1a

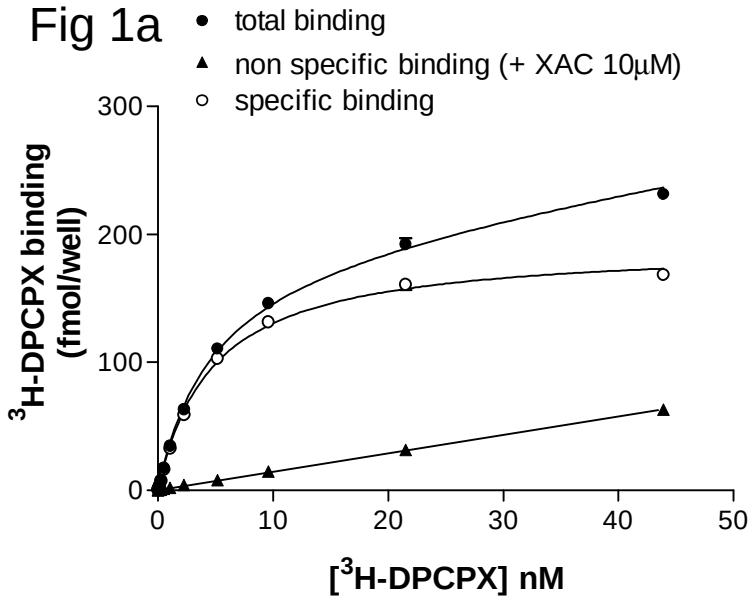

Fig 1b

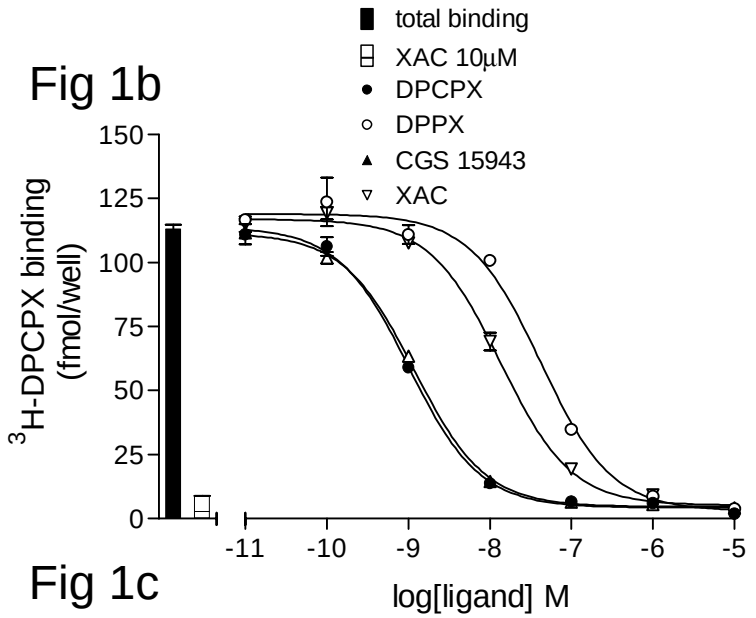

Fig 1c

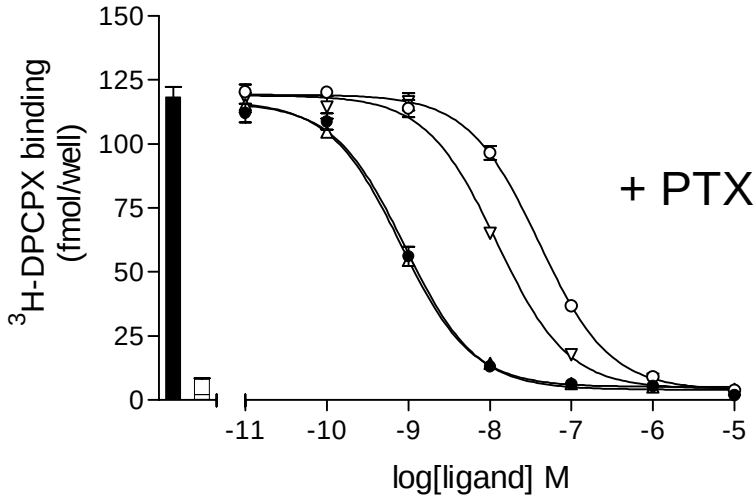

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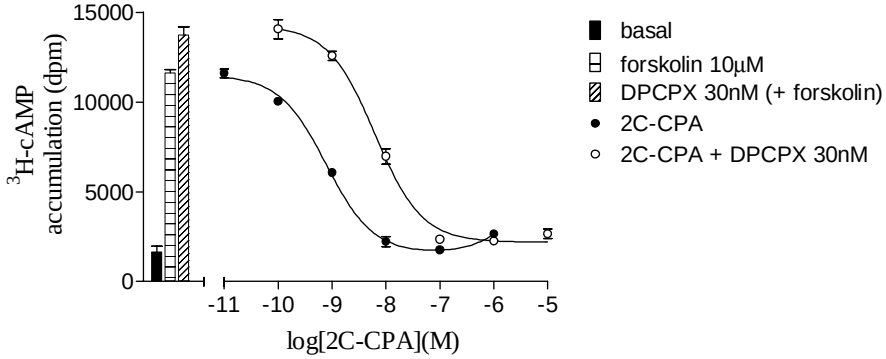
Fig 2a



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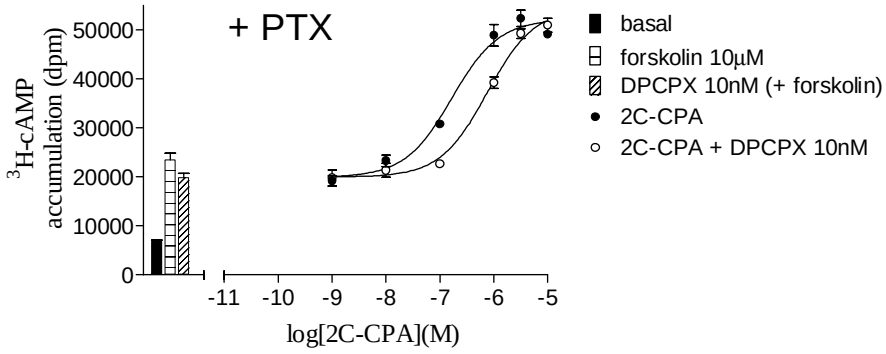

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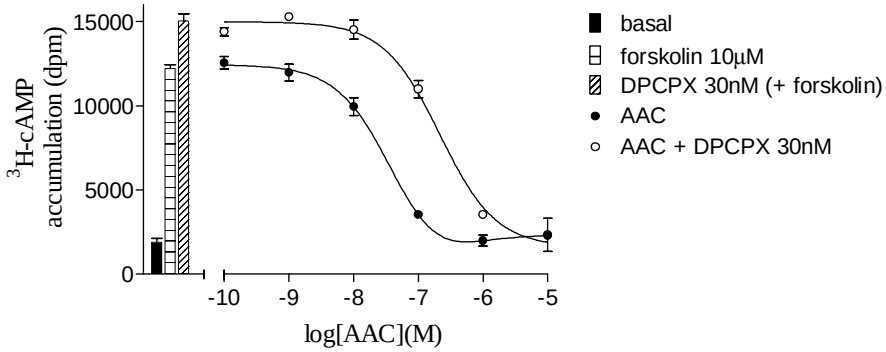
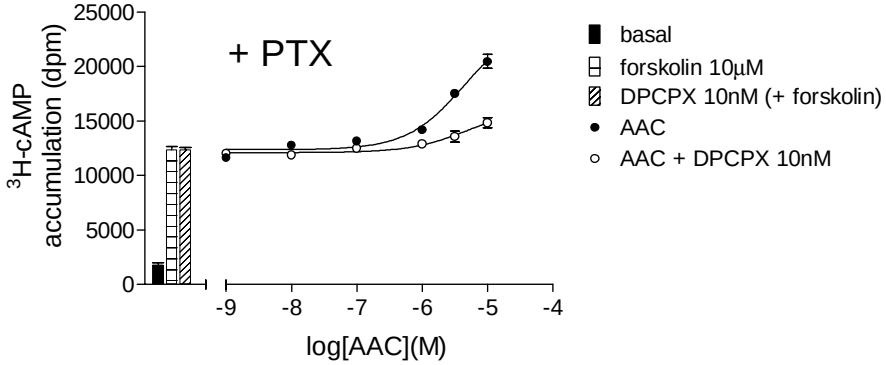


Fig 2d



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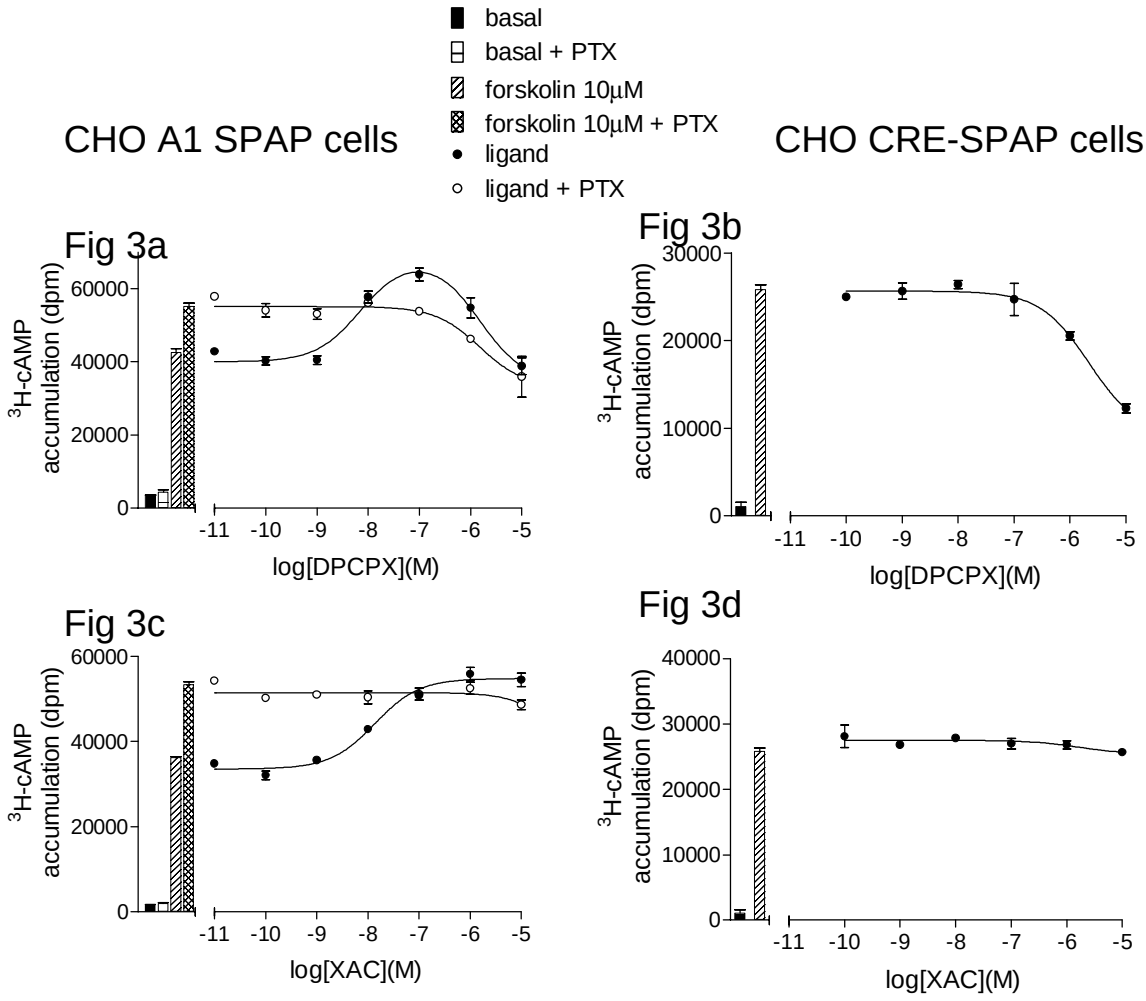

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Fig 4a

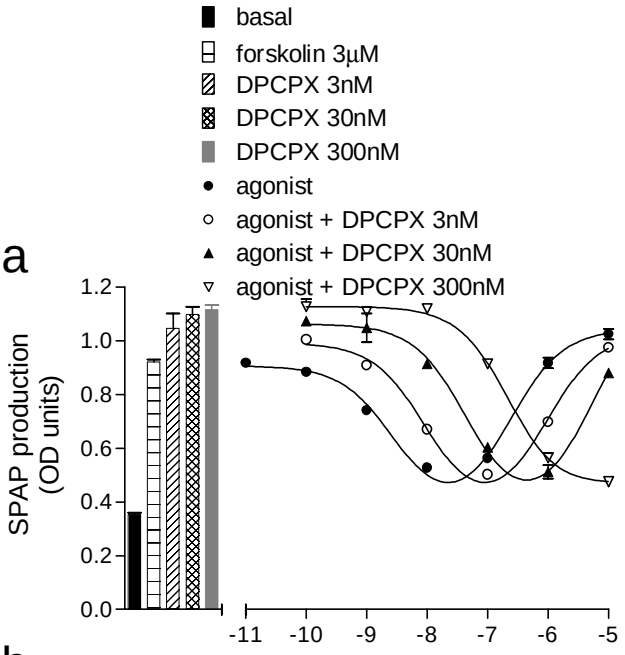

Fig 4b

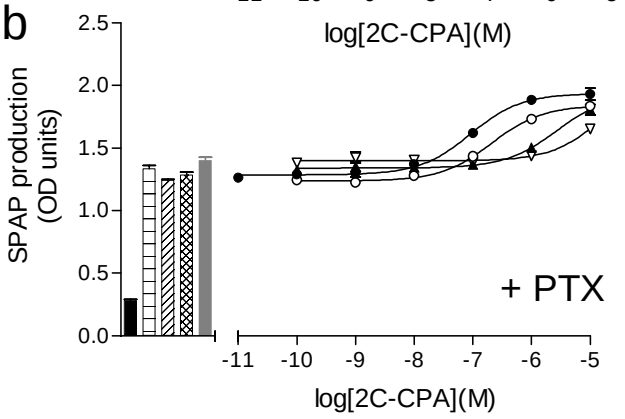

Fig 4c

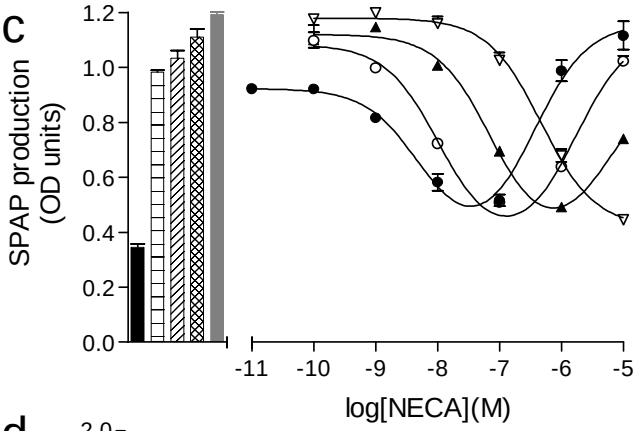

Fig 4d

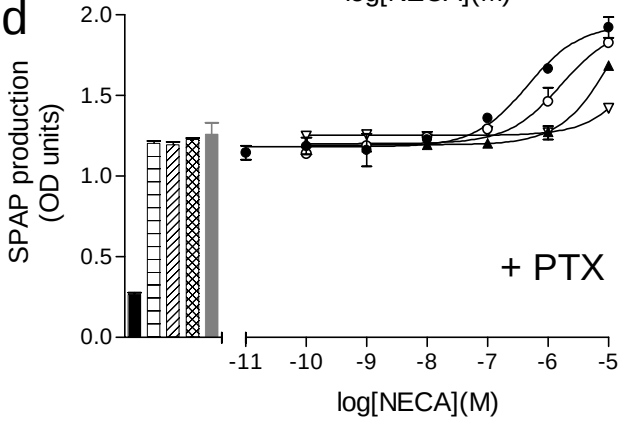

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Fig 5a

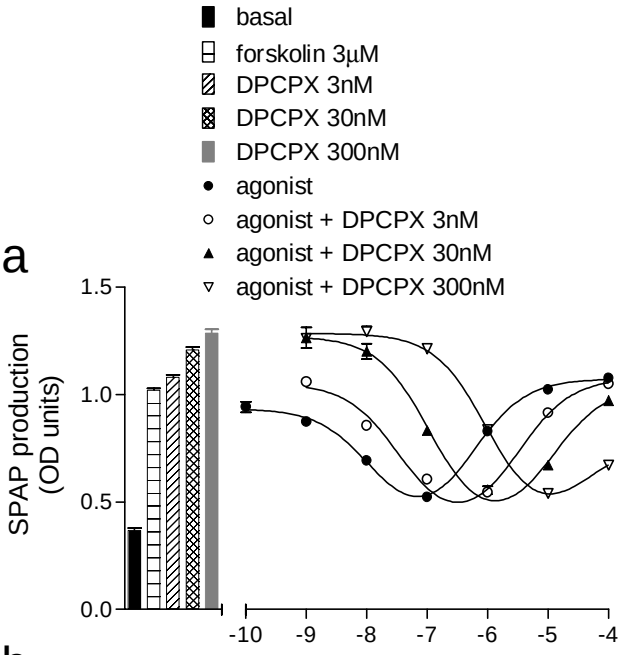

Fig 5b

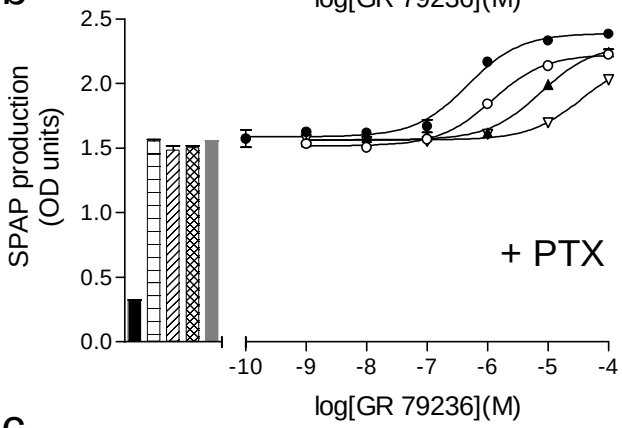

Fig 5c

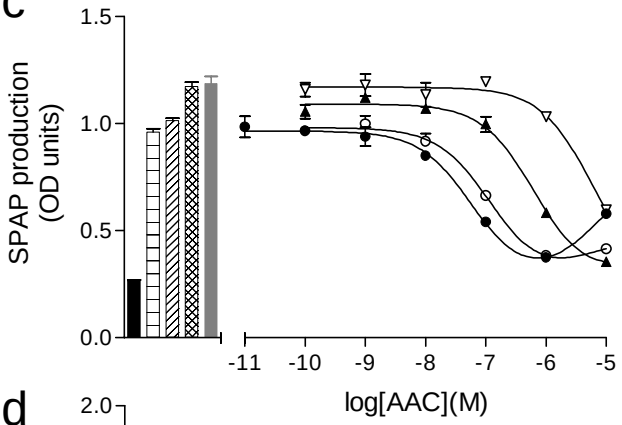

Fig 5d