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Efficacy of inverse agonists in cells overexpressing a constitutively active β_2-adrenoceptor and type II adenylyl cyclase

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1 Maximal stimulant output from the adenylyl cyclase cascade in neuroblastoma × glioma hybrid, NG108-15, cells is limited by the levels of expression of isoforms of adenylyl cyclase. Stable expression in these cells of a constitutively active mutant (CAM) version of the human β_2-adrenoceptor resulted in higher basal adenylyl cyclase activity than following expression of the human wild type β_2-adrenoceptor. Isoprenaline acted as a full agonist in membranes from both wild type and CAM β_2-adrenoceptor expressing clones.

2 Expression of type II adenylyl cyclase resulted in a substantially elevated capacity of isoprenaline to stimulate [^{3}H]-forskolin binding, whereas in CAM β_2-adrenoceptor expressing cells the basal high affinity [^{3}H]-forskolin binding represented a markedly greater % of the maximal effect which could be produced by addition of isoprenaline, and the EC_{50} for isoprenaline was some 10 fold lower than in cells expressing the wild type β_2-adrenoceptor.

3 Further transfection of the CAM β_2-adrenoceptor expressing cells with type II adenylyl cyclase greatly increased both absolute basal and agonist-stimulated levels of adenylyl cyclase activity.

4 Betaxolol, ICI 118,551, sotalol and timolol acted as inverse agonists with varying degrees of efficacy, whereas propranolol functioned as a neutral antagonist and alprenolol as a partial agonist.

5 Pretreatment of the CAM β_2-adrenoceptor and type II adenylyl cyclase expressing clones with the irreversible alkylating agent BAAM (1 μM) did not reduce the efficacy of isoprenaline but eliminated efficacy from all the inverse agonist ligands. This effect was dependent upon the concentration of BAAM employed, with half-maximal effects being produced between 10 nM and 100 nM of the alkylating agent, which is similar to the concentrations required to prevent subsequent ligand access to some 50% of the CAM β_2-adrenoceptor population.

6 These data demonstrate that inverse agonist efficacy can be modulated by receptor availability and also indicate why in physiological systems, inverse agonism can be difficult to detect.

Keywords: Inverse agonist; β_2-adrenoceptor; constitutively active mutant receptor; type II adenylyl cyclase; NG108-15 cells

Introduction

Efficacy (Stephenson, 1956; Kenakin, 1989) of agonist ligands at guanine nucleotide-binding protein (G protein)-coupled receptors (GPCRs) can be influenced by the levels of expression of the individual elements of the signal transduction cascade (GPCR, G protein and effector enzyme) (Whaley *et al*., 1994; MacEwan *et al*., 1995) and, dependent upon the limiting component in the cascade, upon the assay point (GPCR-G protein interaction, effector enzyme function) used to measure efficacy. In the neuroblastoma × glioma hybrid cell line, NG108-15, we have previously demonstrated that the cellular level of adenylyl cyclase is quantitatively the limiting element in the stimulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production (Kim *et al*., 1994; MacEwan *et al*., 1996). Following expression of the human β_2-adrenoceptor in these cells, a combination of studies with clones expressing varying levels of the receptor and an irreversible alkylating agent, to limit ligand access to varying proportions of the receptor, have demonstrated that agonist efficacy is dependent upon levels of the receptor and that for ligands usually considered as 'full agonists', efficacy can only be restricted to 50% by reducing receptor availability until the ratio of receptor to adenylyl cyclase is less than 1:5 (MacEwan *et al*., 1995).

Studies on inverse agonist ligands and how their efficacy might be modified are generally restricted by the requirement that their function be measured by their capacity to inhibit 'basal' levels of second messenger generation. One strategy to improve the basal activity of a GPCR-initiated cascade is to express mutationally modified forms of the GPCR which display agonist-independent regulation of second messenger production and are thus generically described as constitutively active mutant (CAM) forms (Samama *et al*., 1993; Lefkowitz *et al*., 1993; Barker *et al*., 1994; Hogger *et al*., 1995; Scheer *et al*., 1996; Perez *et al*., 1996). Because of the limiting levels of adenylyl cyclase activity in membranes of NG108-15 cells, overexpression of adenylyl cyclase would also be expected to increase basal adenylyl cyclase activity (MacEwan *et al*., 1996). Providing this was controlled to reasonable levels, this would be anticipated to increase agonist-mediated output without resulting in a major alteration in ligand EC_{50} values (MacEwan *et al*., 1996).

In the present studies we have used a combination of both of these approaches, by generating stable cell lines derived from parental NG108-15 cells which express both a CAM form of the human β_2-adrenoceptor (Samama *et al*., 1993; Lefkowitz *et al*., 1993) and type II adenylyl cyclase (Pieroni *et al*., 1995; Sunahara *et al*., 1996). Type II adenylyl cyclase was selected for these studies both because it is not expressed endogenously by NG108-15 cells (MacEwan *et al*., 1996), which allowed easy identification of positive clones, and because it is known to have a high basal activity compared to a number of other adenylyl cyclase isoforms (Pieroni *et al*., 1995) resulting in an effective assay system for inverse agonist efficacy.

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Methods

Generation and isolation of cell lines

Plasmid pJM16 (Gunning *et al*., 1987) which harbours a copy of the neomycin resistance gene was cut with the restriction enzymes BamH1 and Xho1 to allow a cDNA encoding the wild-type human β_2-adrenoceptor to be ligated downstream of the β-actin promoter of this plasmid. 'Genecleaned' (Life Technologies) human wild-type β_2-adrenoceptor cDNA, with 5′ BamH1 and 3′ Xho1 sites, was ligated into the digested pJM16. For the CAM β_2-adrenoceptor, the cDNA in vector pRK5 was co-transfected with the neomycin-resistance plasmid, pSV-neo. Ten micrograms of these DNAs were transfected into NG108-15 cells using Lipofectin reagent (Life Technologies) according to the manufacturer's instructions. Clones that were resistant to geneticin sulphate (800 μg ml^{-1}) were selective and expanded. Expression of the forms of β_2-adrenoceptor in membranes from these clones was assessed by the specific binding of the β-adrenoceptor antagonist [^{3}H]-dihydroalprenolol (see below). Clone βN22 used within the present studies has previously been characterized in detail (Adie & Milligan, 1994a).

Generation of adenylyl cyclase type II expressing clones

Cells of clones βN22 and CAM22 were co-transfected with a 10:1 ratio of a cDNA encoding adenylyl cyclase type II (a kind gift from Dr H.R. Bourne, UCSF, CA) in plasmid pcDNA1 and plasmid pBABEhygro, which is able to direct expression of the hygromycin B resistance marker, by using DOTAP transfection reagent (Boehringer Mannheim) according to the manufacturer's instructions. After transfection the cells were maintained in medium containing hygromycin B (200 μg ml^{-1}). Individual colonies were selected and expanded and subsequently examined for the maintained expression of forms of the β_2-adrenoceptor and novel expression of adenylyl cyclase type II (see Results). Clone AC 2.4 which forms part of the present studies has previously been characterized in detail (MacEwan *et al*., 1996).

Membrane preparation

Membrane fractions were prepared from cell pastes which had been stored at −80°C following harvest, essentially as described by Milligan (1987). Frozen cell pellets were suspended in 5 ml 10 mM Tris/HCl, 0.1 mM EDTA, pH 7.5 (TE buffer) and rupture of the cells achieved with 25 strokes of a hand-held Teflon on glass homogenizer. The resulting homogenate was centrifuged at $500 \times g$ for 10 min in a Beckman L5-50B centrifuge with a Ti50 rotor to remove unbroken cells and nuclei. The supernatant fraction from this treatment was then centrifuged at $48{,}000 \times g$ for 10 min and the pellet from this treatment washed and resuspended in 10 ml TE buffer. Following a second centrifugation at $48{,}000 \times g$ for 10 min the membrane pellet was resuspended in TE buffer to a final protein concentration between 1–3 mg ml^{-1} and stored at −80°C until required.

Adenylyl cyclase activity assays

These assays were performed as described by Milligan *et al*. (1987). Each assay contained 100 mM Tris HCl, pH 7.5, 20 mM creatine phosphate, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM cyclic AMP (adenosine 3′:5′-cyclic monophosphate), 1 μM GTP, 10 u creatine phosphokinase and 0.2 mM ATP containing 1 μCi α[^{32}P]ATP. Separation of radiolabelled cyclic AMP and ATP was achieved by use of the double column method described by Johnson and Salomon (1991).

Reverse transcriptase-polymerase chain reaction

RNA extractions Total RNA was extracted according to the acid phenol/guanidinium thiocyanate method of Chomczynski and Sacchi (1987) with RNAzol B (Biogenesis). Purity and quantification of RNA were assessed by A_{260}/A_{280} nm ratios.

Reverse transcription The reverse transcriptase/polymerase chain reaction (RT-PCR) procedure was carried out as follows. Samples of 5 μg RNA (8 μl) were denatured by incubation at 65°C for 10 min followed by chilling on ice and reverse transcribed in 33 μl of reaction mixture by use of the first strand cDNA synthesis kit (Pharmacia LKB Biotechnology), as detailed by the manufacturer. Incubation was carried out at 37°C for 1 h after which time the reaction was stopped by heating at 95°C for 5 min followed by chilling on ice.

PCR PCR reactions on the reverse transcribed samples or on 200 ng of type II adenylyl cyclase cDNA were carried out with the following primers: adenylyl cyclase type II sense 5′ GCTGTGCAAGGCTGTGCTCT 3′; adenylyl cyclase type II antisense 5′ CCAGACGATGACGAAGATGTG 3′.

Amplifications were performed in 100 μl of buffer containing 20–40 pmol of primers, 2.5 units of Taq polymerase (Promega) in a HYBAID Omnigene temperature cycler. Cycles were as follows: 95°C for 5 min; 55°C for 1 min, 72°C for 1 min (1 cycle): 95°C for 30 s, 55°C for 1 min, 72°C for 1 min (30 cycles); 95°C for 30 s, 55°C for 1 min, 72°C for 5 min (1 cycle). Reaction products were separated by 1.5–1.75% agarose gel electrophoresis.

Cell preparation for [^{3}H]-forskolin binding experiments

The specific high affinity binding of [^{3}H]-forskolin to the adenylyl cyclase-stimulant G-protein α subunit ($G_s\alpha$)-adenylyl cyclase complex (GSAC) in whole cells was performed essentially as described by Kim *et al*. (1995) and Alousi *et al*. (1991). Cells were harvested into HEPES-buffered Dulbecco's modified Eagle's medium (DMEM), counted with a haemocytometer and then added to tubes containing [^{3}H]-forskolin (approximately 20 nM) in either the absence or presence of 10 μM cold forskolin to define non-specific binding. The tubes also contained varying concentrations of the individual receptor ligands. Samples were incubated at 4°C for 60 min and then filtered through Whatman GF/B filters followed by liquid scintillation counting.

Binding experiments with [^{3}H]-dihydroalprenolol

In experiments designed to assess the maximal binding capacity of membranes for [^{3}H]-dihydroalprenolol ([^{3}H]-DHA), concentrations of this ligand were varied between 0.1 and 5 nM in the absence and presence of 10 μM propranolol to define maximal and non-specific binding, respectively. Assays were performed at 30°C for 30 min in 10 mM Tris/HCl (pH 7.5), 50 mM sucrose, 20 mM $MgCl_2$ (buffer C). Specific binding, defined as above, represented greater than 90% of the total binding of [^{3}H]-DHA. All binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes (5 ml) with ice-cold buffer C. In experiments designed to assess the effectiveness of a range of concentrations of bromoacetyl alprenolol menthane (BAAM)

in producing irreversible blockade of the β_2-adrenoceptor population, cells were treated with the appropriate concentration of this agent for 4 h. After harvest, the cells were washed extensively with phosphate-buffered saline by a series of centrifugation and resuspension steps. Membranes prepared from BAAM-treated cells were incubated as above with varying concentrations (0.1 nM – 5 nM) of [^{3}H]-DHA in the absence and presence of 10 μM propranolol to define maximal and non-specific binding, respectively.

Chemicals

All reagents for tissue culture were purchased from Life Technologies (Paisley, Strathclyde, U.K.). [^{3}H]-dihydroalprenolol (56 Ci mmol^{-1}), α[^{32}P]-ATP and [^{3}H]-cyclic AMP were obtained from Amersham International. [^{3}H]-forskolin (36 Ci mmol^{-1}) was obtained from Dupont/New England Nuclear. Betaxolol was a kind gift from Dr V. Rovei (Synthelabo Recherche, Bagneux, France). Other β-adrenoceptor active compounds were purchased from the Sigma Chemical Company (Poole, U.K.) or from Research Biochemicals International (Natick, MA, U.S.A.). Iloprost was a kind gift of Schering Health Care (Burgess Hill, Sussex, U.K.). All other chemicals were bought from Sigma or British Drug Houses (BDH) and were of the highest purity available. The cDNAs for the human wild type and CAM β_2-adrenoceptors were a gift from Dr R.J. Lefkowitz (HHMI, Duke University, NC, U.S.A.). ICI 118,551 (erythro-1-(7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol).

Data analysis

All binding data were analysed using the Kaleidograph curve fitting programme (Version 2.1) driven by an Apple MacIntosh computer.

Results

Following stable expression of a constitutively active mutant (CAM) version of the human β_2-adrenoceptor in neuroblastoma × glioma hybrid, NG108-15, cells (clone CAM22) basal adenylyl cyclase activity was increased in these membranes compared to those from cells (clone βN22) which had been transfected to stably express the wild type form of this receptor (Figure 1). This was despite higher steady state levels of expression of the wild type receptor (data not shown but see MacEwan & Milligan, 1996a).

Despite the higher basal adenylyl cyclase activity in membranes of the CAM β_2-adrenoceptor expressing cells and previous evidence that such mutants can respond more effectively to agonist than the wild type receptor (Samama *et al*., 1993), addition of a maximally effective concentration of isoprenaline (10 μM) resulted in adenylyl cyclase activity being stimulated to reach the same levels in the two cell systems (Figure 1). Addition of iloprost (10 μM), an agonist at the IP prostanoid receptor which is expressed endogenously by NG108-15 cells and clones derived from them, to membranes of both clone βN22 and CAM22 β_2-adrenoceptor expressing cells also resulted in a stimulation of adenylyl cyclase activity, which reached the same activity as that produced by isoprenaline (Figure 1). By contrast, subsequent re-transfection of clone βN22 also to express stably type II adenylyl cyclase (clone AC 2.4) (MacEwan *et al*., 1996) resulted in a higher basal adenylyl cyclase activity than that produced by agonist stimulation of either the clone βN22 or the CAM β_2-adrenoceptor expressing cells (Figure 1). Addition of either isoprenaline or iloprost to membranes of clone AC 2.4 now resulted in equivalent levels of stimulation of adenylyl cyclase, which were substantially greater than could be achieved in the clones which had not been transfected with type II adenylyl cyclase (Figure 1). Such data define that the cellular level of adenylyl cyclase is the limiting element for cyclic AMP generation in NG108-15 cells (MacEwan *et al*., 1996).

High affinity specific binding of [^{3}H]-forskolin can be used to detect the complex formed between activated $G_s\alpha$ and adenylyl cyclase (GSAC) (Alousi *et al*., 1991; Kim *et al*., 1995). In intact cells specific binding of [^{3}H]-forskolin can be promoted by addition of agonists which activate adenylyl cyclase (Alousi *et al*., 1991; Kim *et al*., 1995). In cells of clone βN22 specific binding of [^{3}H]-forskolin was induced in a concentration dependent manner by isoprenaline with an $EC_{50} = 3 \pm 2$ nM (Figure 2a). In clone AC2.4 basal specific [^{3}H]-forskolin binding was higher than in clone βN22 and the capacity of isoprenaline to induce this binding was much greater. Despite this, the EC_{50} for isoprenaline (6 ± 2 nM) was not substantially different (Figure 2a). By contrast, although the maximal levels of isoprenaline-stimulated [^{3}H]-forskolin binding were similar in cells of clones βN22 and CAM22, the basal levels of [^{3}H]-forskolin binding were greater in clone CAM22 (65 ± 14 and 136 ± 12, respectively ($n = 4$; $P = 0.004$)) and the EC_{50} for isoprenaline (0.4 ± 0.1 nM) was lower (($n = 4$; $P = 0.02$) Figure 2b). To demonstrate that these differences were not simply a reflection of clonal variation, the capacity of iloprost to stimulate [^{3}H]-forskolin binding was examined in these three clones. As noted for isoprenaline, the presence of adenylyl cyclase type II in clone AC2.4 resulted in a greater maximal level of stimulation by iloprost than in clone βN22 without an alteration in these EC_{50} (βN22 20 ± 10 nM, AC2.4 = 40 ± 20 nM) (data not shown). However, by contrast with isoprenaline, the EC_{50} for iloprost was not different in clone CAM22 (data not shown).

To provide a suitable cell system to examine how β-adrenoceptor ligand efficacy and potency might alter with regulation of both CAM β_2-adrenoceptor and adenylyl cyclase levels, clone CAM22 cells were re-transfected to express stably

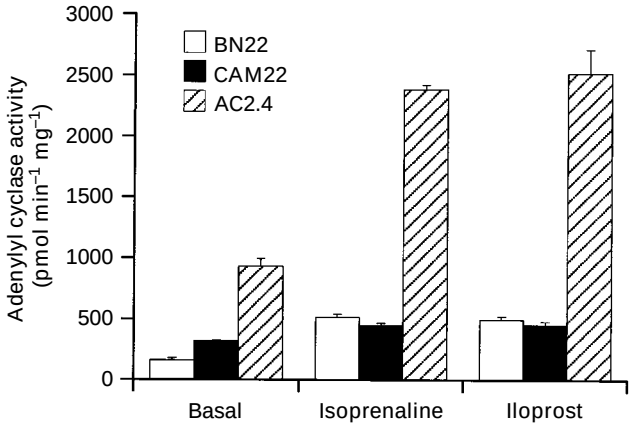

Figure 1 Regulation of adenylyl cyclase in clones of NG108-15 cells expressing forms of the human β_2-adrenoceptor or type II adenylyl cyclase. Basal adenylyl cyclase and its stimulation by either isoprenaline (10 μM) or iloprost (10 μM) was measured in membranes of clone βN22 (wild type β_2-adrenoceptor), CAM22 (CAM β_2-adrenoceptor) or AC2.4 (wild type β_2-adrenoceptor and type II adenylyl cyclase). Results represent mean ± s.d. of triplicate determinations. The experiment shown is typical of three independent experiments.

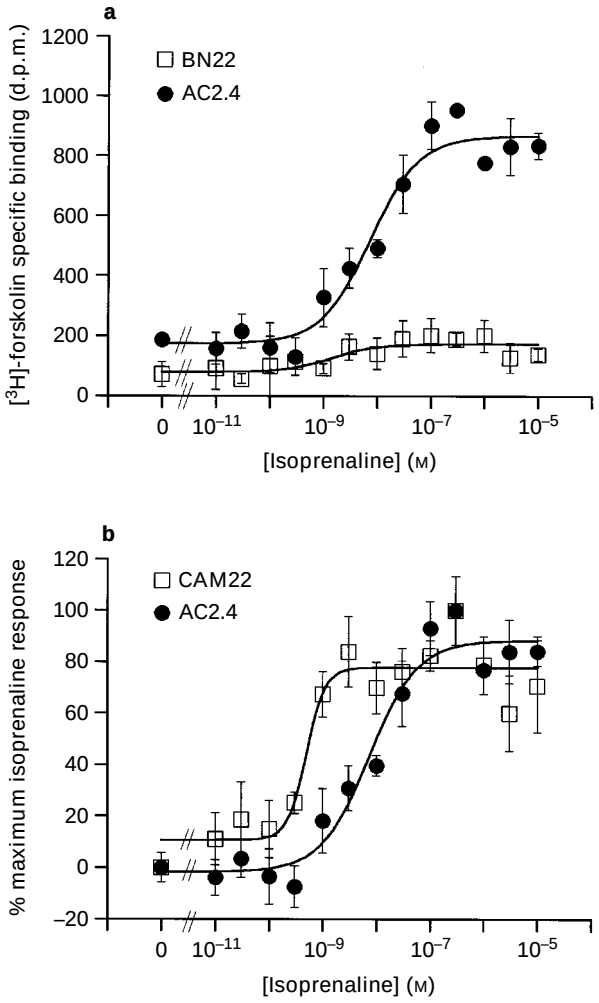

Figure 2 Regulation of high affinity [^{3}H]-forskolin binding by isoprenaline in clones of NG108-15 cells expressing forms of the human β_2-adrenoceptor or type II adenylyl cyclase. The specific binding of [^{3}H]-forskolin (20 nM) and its regulation by varying concentrations of isoprenaline was assessed in intact cells of clones βN22 (wild type β_2-adrenoceptor), CAM22 (CAM β_2-adrenoceptor) or AC2.4 (wild type β_2-adrenoceptor and type II adenylyl cyclase). In (a) data are expressed directly as d.p.m. of specific binding whereas in (b) data are expressed relative to the maximum achieved by addition of isoprenaline. Results represent mean$\pm$s.d. (vertical lines) of triplicate determinations. The experiment shown is representative of five independent experiments.

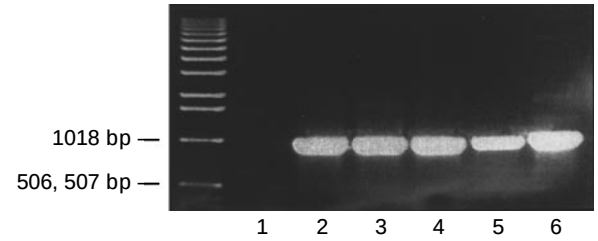

Figure 3 Stable expression of type II adenylyl cyclase in clones derived from clone CAM22. Total RNA was isolated from cells of clone CAM22 and clones 3, 5, 6 and 38 derived from stable transfection of clone CAM22 with a cDNA encoding type II adenylyl cyclase and subjected to RT-PCR by use of a primer pair (Methods) designed to amplify a 980 bp fragment from type II adenylyl cyclase (lanes 1, 2, 3, 4 and 5, respectively). PCR of the type II adenylyl cyclase in pcDNA 3 is displayed as a positive control in lane 6. The experiment shown is typical of two other independent experiments.

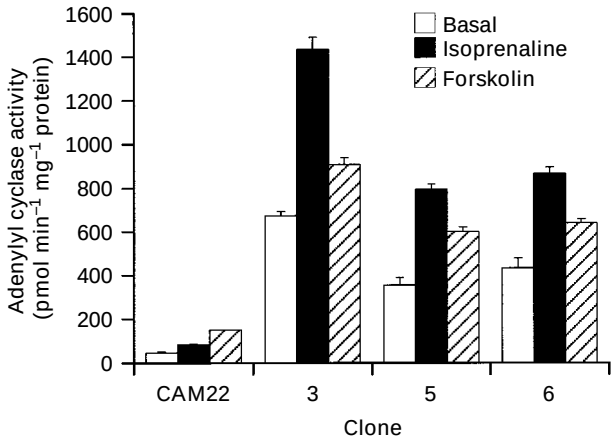

Figure 4 Basal adenylyl cyclase activity and its regulation in clones co-expressing the CAM β_2-adrenoceptor and type II adenylyl cyclase. Membranes from clone CAM22 and clones 3, 5 and 6 derived by stable transfection of clone CAM22 with a type II adenylyl cyclase cDNA were assessed for basal adenylyl cyclase activity and its regulation by either isoprenaline (1 μM) or forskolin (10 μM). Results represent mean$\pm$s.d. of triplicate determinations. The experiment shown is typical of at least four other experiments.

type II adenylyl cyclase. Three clones (3, 5, 6) expressing good levels of mRNA encoding type II adenylyl cyclase were identified (Figure 3) and examined in comparison to clone CAM22. Each of clones 3, 5 and 6 displayed substantially elevated basal adenylyl cyclase activity compared to their parental cells (Figure 4) and an increased response to both isoprenaline and forskolin (Figure 4). Despite the increased basal and isoprenaline-stimulated adenylyl cyclase activity in clones 3, 5 and 6, the EC_{50} for isoprenaline was not different in these clones compared to clone CAM22 (Figure 5). One clear advantage of the higher levels of basal adenylyl cyclase activity in the clones co-expressing the CAM β_2-adrenoceptor and adenylyl cyclase type II was the ease by which the capacity of inverse agonist ligands to inhibit basal adenylyl cyclase activity could be measured (Figure 6a). Clear inhibition of basal adenylyl cyclase activity could be recorded to varying concentrations of betaxolol in membranes of each of clones 3, 5 and 6. This effect of betaxolol was produced with a similar EC_{50} in each of these clones and in clone CAM22 (Figure 6).

ICI 118,551 also acted as an inverse agonist on basal adenylyl cyclase activity in CAM β_2-adrenoceptor expressing clones, the EC_{50} for which was not altered by the increased adenylyl cyclase levels (data not shown).

Isoprenaline, which has previously been shown to function as a full agonist in NG108-15 cells expressing the wild type β_2-adrenoceptor, also functioned as a full agonist in each of CAM22 and clones 3 and 6 (Figure 7), in which basal adenylyl cyclase activity was close to 50% of the maximal level which could be achieved by addition of agonist. Interestingly, ephedrine, which is normally considered as a weak partial agonist, also functioned as a full agonist in each of these CAM β_2-adrenoceptor expressing clones (data not shown), a feature which we have previously not been able to observe in clones of NG108-15 cells expressing equivalent and indeed higher levels of the wild type β_2-adrenoceptors (MacEwan *et al.*, 1995). Alprenolol is routinely viewed as a β-adrenoceptor antagonist but in the CAM β_2-adrenoceptor expressing cells it clearly functioned as a partial agonist (Figure 7) and, although propranolol acted as a neutral antagonist in membranes from these cells, sotalol, timolol and ICI 118,551 each functioned as inverse agonists with differing degrees of efficacy (Figure 7).

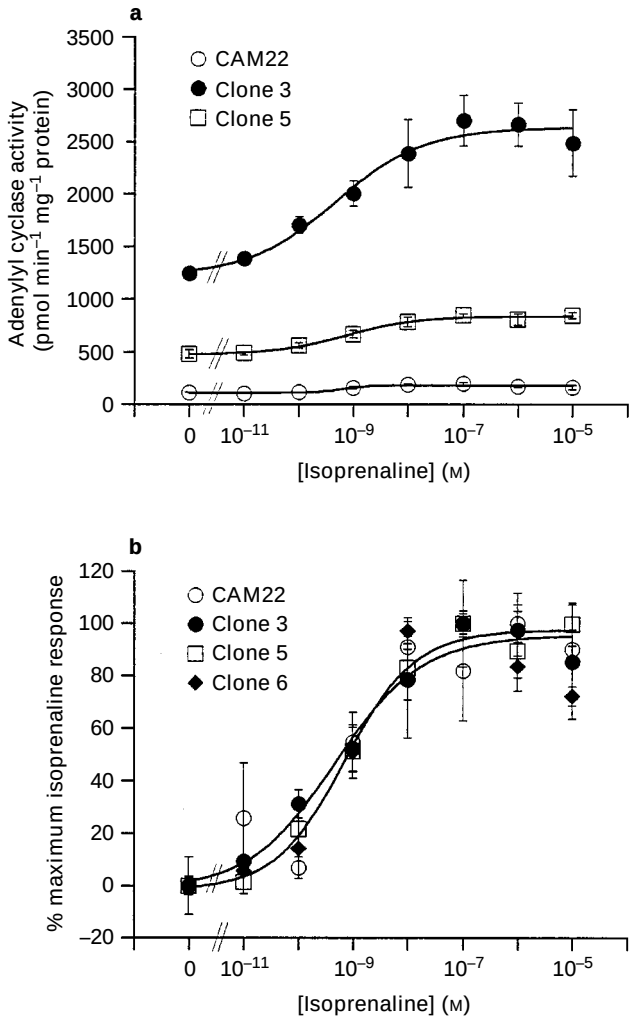

Figure 5 Concentration-dependence of isoprenaline regulation of adenylyl cyclase activity in clones expressing a combination of the CAM β_2-adrenoceptor and type II adenylyl cyclase. Membranes from a variety of the clones described in the legend to Figure 4 were used to measure basal adenylyl cyclase activity and its regulation by varying concentrations of isoprenaline. (a) Absolute activity measurements. (b) Normalization to maximal effect produced by isoprenaline. Results represent mean ± s.d. (vertical lines) of triplicate determinations. The experiment shown is typical of at least three other experiments.

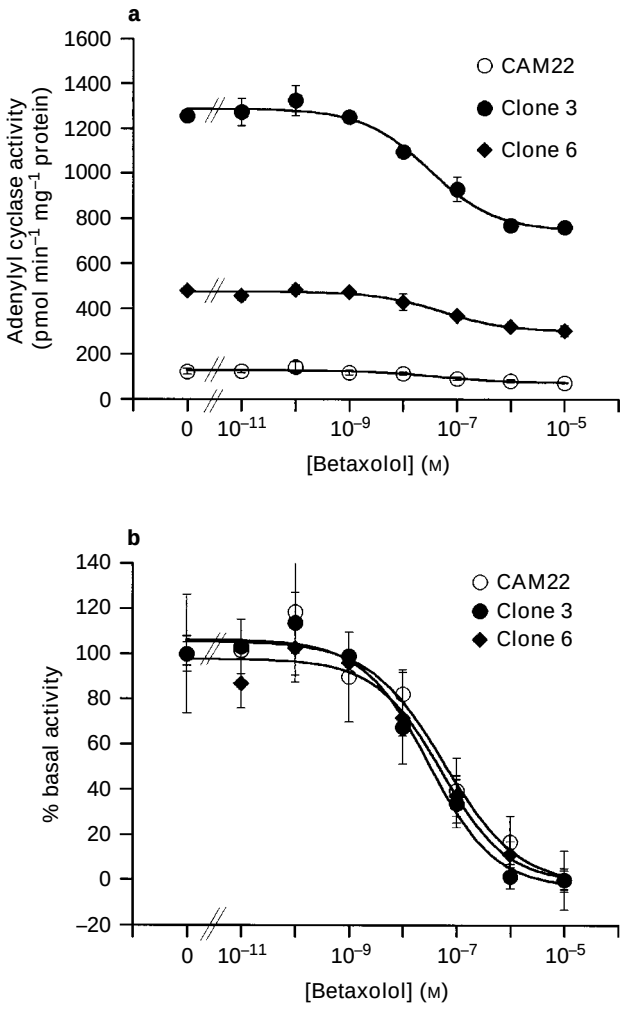

Figure 6 Detection of inverse agonism of betaxolol in clones expressing combinations of the CAM β_2-adrenoceptor and type II adenylyl cyclase. Membranes of a variety of the clones described in Figures 4 and 5 were exposed to a variety of concentrations of betaxolol. (a) Absolute activity measurements. (b) Data normalized with basal adenylyl cyclase activity presented as 100% and the maximal effect produced by betaxolol as 0%. Results represent mean ± s.d. (vertical lines) of triplicate determinations. The experiment shown is typical of three other independent experiments.

Analysis of the capacity of the irreversible β-adrenoceptor alkylating agent (bromoacetyl alprenolol methane (BAAM)) to restrict subsequent ligand access to the binding pocket of the CAM β_2-adrenoceptor was performed by saturation analysis of the binding of [^{3}H]-dihydroalprenolol (DHA) to membranes of clones CAM22 and 6 following exposure to varying concentrations of BAAM. At concentrations of BAAM up to 1 μM this resulted in a reduction in the total number of sites available to [^{3}H]-DHA without an alteration in the estimated K_d for the radioligand (Figure 8a). At concentrations of BAAM above 1 μM subsequent specific [^{3}H]-DHA binding was so low that analysis became problematic and reductions in apparent [^{3}H]-DHA binding affinity suggested that removal of BAAM by the wash procedure may have been incomplete (data not shown). Half of the initial CAM β_2-adrenoceptor binding sites were occluded by treatment with approximately 30 nM BAAM in both clones 6 and CAM22 (Figure 8b).

To explore how efficacy would alter with changes in the access of β-adrenoceptor ligands to the CAM β_2-adrenoceptor, CAM22 and clone 6 cells were treated with either vehicle or with BAAM (1 μM) (Figure 9). Interestingly, treatment with BAAM routinely resulted in an increase in basal adenylyl cyclase activity in membranes of the CAM β_2-adrenoceptor and adenylyl cyclase II expressing clones. Such data indicate that BAAM does not function as a irreversible antagonist at the CAM β_2-adrenoceptor but, as noted above for its parent ligand alprenolol, as a weak partial agonist (Figure 9). BAAM (1 μM) treatment did not result in a reduction in efficacy of isoprenaline, but this treatment was sufficient essentially to prevent the inverse agonist effects of sotalol, betaxolol, timolol and ICI 118,551 (Figure 9). To explore this phenomenon further, CAM22 and clone 6 cells were treated with varying concentrations of BAAM before membrane preparation and analysis of basal adenylyl cyclase activity and its regulation by β-adrenoceptor ligands. In accord with the concept that BAAM functions as an agonist ligand at the CAM β_2-adrenoceptor, increasing concentrations of BAAM increased basal adenylyl cyclase activity until this reached the same levels as could be achieved by addition of isoprenaline to untreated membranes (Figure 10). Under these conditions adenylyl cyclase activity was essentially maximal and independent of

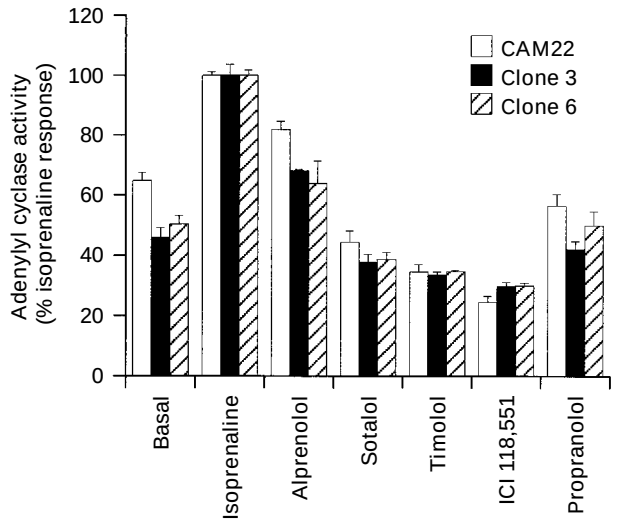

Figure 7 Efficacy of a range of β-adrenoceptor ligands was not affected by levels of adenylyl cyclase expression. Basal adenylyl cyclase activity and its regulation by a variety of β-adrenoceptor active ligands was assessed in membranes of clones expressing combinations of the CAM β_2-adrenoceptor and type II adenylyl cyclase. Data are presented as a % of the activity achieved by addition of isoprenaline (10 μM). Results represent mean $\pm$ s.d. (vertical lines) of triplicate determinations. The experiment shown is typical of three other independent experiments.

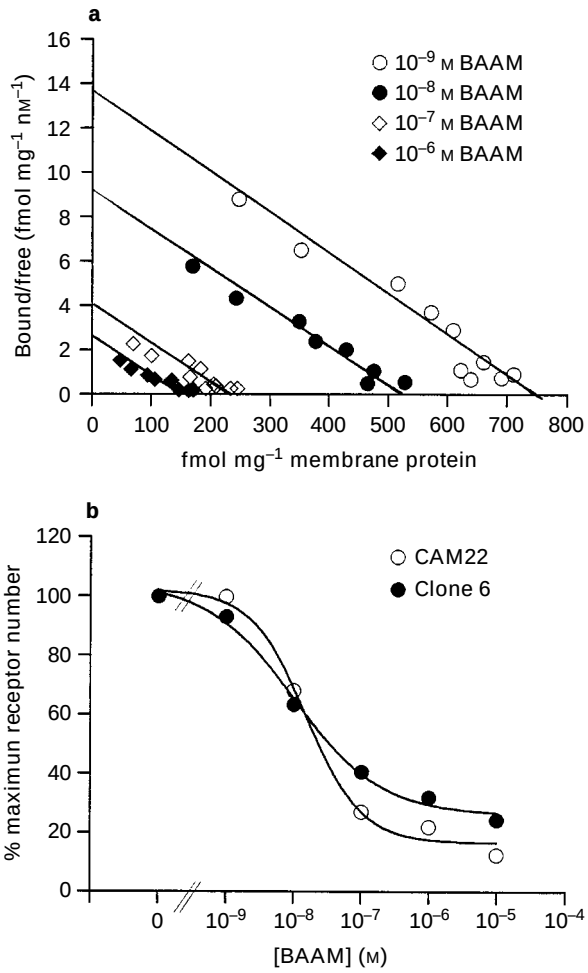

Figure 8 BAAM treatment reduced the number of available receptors without altering the affinity of [^{3}H]-DHA binding. Cells of clone CAM22 and clone 6 were treated with vehicle or varying concentrations of BAAM for 4 h before cell harvest. Binding of [^{3}H]-DHA (0.1 – 5 nM) in membranes from these cells was then assessed in the absence and presence of 10 μM propranolol to define maximal and non-specific binding respectively. (a) The specific binding data from a typical experiment are displayed as a Scatchard plot. (b) Results are expressed relative to the available receptor number in the absence of BAAM. Results represent mean of triplicate determinations. The experiment shown is typical of three other independent determinations.

further addition of agonist ligand. Most interestingly, the capacity of BAAM to prevent inhibition of basal adenylyl cyclase activity by the inverse agonist ligands was dose-dependent. Half-maximal reduction in the function of a range of inverse agonists (10 μM) was produced by treatment with between 10 nM and 100 nM BAAM (Figure 10). However, as anticipated from results presented above, propranolol had no significant effects on basal adenylyl cyclase activity under any condition and concentration of BAAM pretreatment (Figure 10).

Discussion

As noted in the Introduction, to establish a suitable system for analysis of inverse agonist efficacy and its regulation with receptor number required both selection of a GPCR with well established inverse agonist pharmacology and a system in which basal second messenger production is high. We selected the human β_2-adrenoceptor, as a variety of studies have indicated that a number of, but not all, β-blockers function as inverse agonists (Pei *et al*., 1994; Chidiac *et al*., 1994; Samama *et al*., 1994; Bond *et al*., 1995; MacEwan & Milligan, 1996b). Furthermore, as CAM mutants of this GPCR result in a boost in ligand-independent adenylyl cyclase activity (Samama *et al*., 1993), stable cell lines expressing this mutant were produced such that the capacity of the inverse ligand to relax the receptor structure towards the ground state would be anticipated to result in a depression of adenylyl cyclase activity (Pei *et al*., 1994; Chidiac *et al*., 1994; Samama *et al*., 1994; Bond *et al*., 1995; MacEwan & Milligan, 1996a,b). As the limiting element in stimulation of adenylyl cyclase in NG108-15 cells is the level of adenylyl cyclase (Kim *et al*., 1994; MacEwan *et al*., 1996) we also boosted this activity by stably co-expressing type II adenylyl cyclase. The independent benefits of each of these strategies are demonstrated in Figure 1, where separate expression of either the β_2-adrenoceptor or type II adenylyl cyclase increased basal adenylyl cyclase activity. Furthermore the expressed type II adenylyl cyclase was activated by ligands at $G_s\alpha$-coupled receptors.

An alternative method to examine the activity state of adenylyl cyclase in intact cells is to measure agonist regulation of the high affinity binding of [^{3}H]-forskolin (Alousi *et al*., 1991; Kim *et al*., 1995). As anticipated, expression of type II adenylyl cyclase resulted in a substantially elevated capacity of isoprenaline to stimulate [^{3}H]-forskolin binding, whereas in CAM β_2-adrenoceptor expressing cells the basal high affinity [^{3}H]-forskolin binding represented a markedly greater % of the maximal effect which could be produced by addition of isoprenaline and the EC_{50} for isoprenaline was some 10 fold lower than in cells expressing the wild type β_2-adrenoceptor. Importantly, to demonstrate that these features of the CAM β_2-adrenoceptor and type II adenylyl cyclase expressing clones were not simply due to specific features of the clones analysed, the capacity of an agonist at the IP prostanoid receptor, which is expressed endogenously in NG108-15 cells and clones derived from them (Kim *et al*., 1994), was assessed. Iloprost

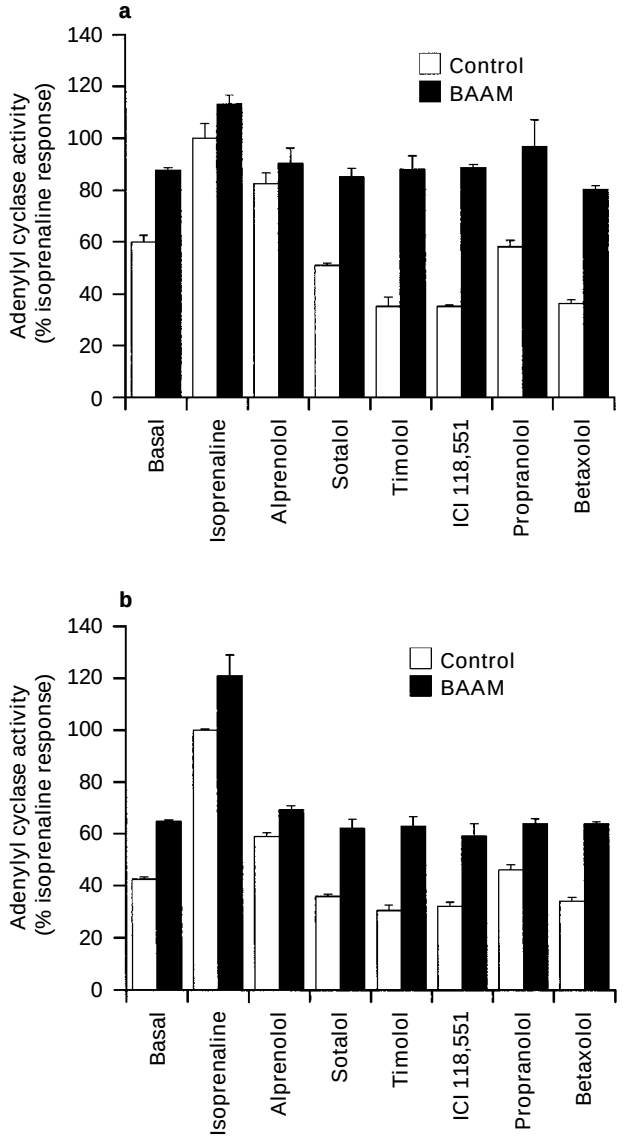

Figure 9 The effect of BAAM on basal adenylyl cyclase activity and the efficacy of β-adrenoceptor ligands. Clone CAM22 (a) and clone 6 (b) were treated with BAAM (1 μM) or vehicle (control) for 4 h. The unbound alkylating agent was then removed by washing and basal adenylyl cyclase activity and its regulation by a variety of β-adrenoceptor ligands (all at 10 μM) assessed in membranes derived from these cells. Results are presented relative to the adenylyl cyclase activity produced by isoprenaline in vehicle treated cells. Results represent mean $\pm$ s.d. of triplicate determinations. The experiment shown is representative of three other independent experiments.

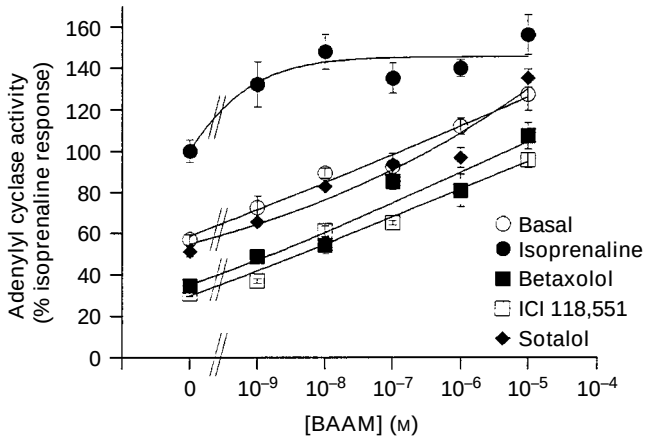

Figure 10 The capacity of BAAM to regulate the efficacy of inverse agonists in cell co-expressing the CAM β_2-adrenoceptor and type II adenylyl cyclase: concentration dependence. Cells of clone 6 were treated with vehicle or varying concentrations of BAAM for 4 h before cell harvest. Basal adenylyl cyclase activity and its regulation by a variety of β-adrenoceptor ligands (all at 1×10^{-5} M) was then assessed in membranes from these cells. Results are presented relative to the adenylyl cyclase activity produced by isoprenaline in vehicle-treated cells. Results represent mean $\pm$ s.d. (vertical lines) of triplicate determinations. The experiment shown is representative of two other independent determinations.

also caused a substantially higher maximal increase in [^{3}H]-forskolin binding in the type II adenylyl cyclase expressing clone but the EC_{50} for this ligand was not different in the CAM β_2-adrenoceptor expressing clone (data not shown). The choice of type II adenylyl cyclase was at least partially dictated by the fact that NG108-15 cells do not express this isoform (MacEwan *et al*., 1996). This made identification of type II adenylyl cyclase positive clones easy by detection of expression of relevant mRNA by reverse transcriptase-PCR (Figure 3). Following re-transfection of CAM β_2-adrenoceptor expressing clone 22 with type II adenylyl cyclase we identified 4 clones with good levels of type II mRNA (Figure 3). All of these displayed markedly elevated basal adenylyl cyclase activity and the adenylyl cyclase activity of membranes of clones 3, 5 and 6 were further stimulated by addition of either isoprenaline or forskolin (Figure 4).

In clones 3, 5, and 6 (and indeed in their parental clone CAM22 which does not express type II adenylyl cyclase) basal adenylyl cyclase activity was close to 50% of the level which could be achieved by addition of maximally effective concentrations of isoprenaline or iloprost. Interestingly, although we have been unable to generate clones from NG108-15 cells by transfection of the wild type β_2-adrenoceptor in which ephedrine can function as a full agonist (MacEwan *et al*., 1995), this ligand functioned as a full agonist in the CAM β_2-adrenoceptor expressing clones (data not shown). In these clones ICI 118,551, betaxolol, timolol and sotalol functioned as inverse agonists, able to reduce basal adenylyl cyclase activity, whereas of the other β-blockers, propranolol functioned as a ligand without significant efficacy and alprenolol functioned as a partial agonist (Figure 7). Even in clones expressing high levels of wild-type β_2-adrenoceptor we have previously noted a capacity of alprenolol to function as a weak partial agonist (MacEwan *et al*., 1995). It was thus of interest to note that the irreversible alkylating 'β-blocker' BAAM, for which alprenolol is the parental structure, also displayed agonist activity. Pretreatment of the CAM β_2-adrenoceptor (and type II adenylyl cyclase) expressing clones with BAAM was used as a strategy to limit subsequent ligand access to the receptor in cell membrane preparations. However, treatment with varying concentrations of BAAM resulted in increasing basal adenylyl cyclase activity, such that following treatment with concentrations of BAAM above 1 μM, adenylyl cyclase activity was now close to maximal and became impervious to addition of isoprenaline. This was not a reflection that decreased receptor availability following BAAM treatment resulted in a decrease in agonist efficacy (as might be anticipated and indeed as we have previously shown for the wild type β_2-adrenoceptor (MacEwan *et al*., 1995)), but simply that the cellular adenylyl cyclase population was now fully active. A similar effect has been observed for isometric tension in the heart in mice which stably express the CAM β_2-adrenoceptor in a cardiac-specific manner (Bond *et al*., 1995).

As BAAM pretreatment resulted in essentially maximal 'basal' adenylyl cyclase activity in the absence of ligand this

might have been anticipated to provide the most robust conditions in which to observe inverse agonist function. However, following pretreatment with 1 μM BAAM, sotalol, betaxolol, ICI 118,551 and timolol displayed essentially no efficacy (Figure 9). Furthermore, the partial agonist effects of alprenolol were also removed, while propranolol remained without measurable efficacy (Figure 9). Pretreatment of clone 6 cells with varying concentrations of BAAM demonstrated that the degree of efficacy of sotalol, betaxolol and ICI 118,551 was dependent upon the availability of CAM β_2-adrenoceptor binding sites with half-maximal reduction in inverse efficacy of each of these ligands being produced at BAAM concentrations between 10 nM and 100 nM (Figure 10). We have previously demonstrated that sustained treatment of NG108-15 derived cells expressing the CAM β_2-adrenoceptor with inverse agonists, including betaxolol and sotalol, can result in a marked upregulation of this GPCR. This is likely to reflect the capacity of these ligands to stabilize the inherently unstable structure of the CAM β_2-adrenoceptor, resulting in a decreased rate of degradation of the protein in the face of unaltered rates of synthesis (MacEwan & Milligan, 1996a; Gether *et al*., 1997; Samama *et al*., 1997). The EC_{50} for betaxolol-induced upregulation of the CAM β_2-adrenoceptor is close to its estimated K_i at the receptor, as assessed from competition ^{3}H ligand binding studies (MacEwan & Milligan, 1996a), and its EC_{50} for inverse agonist function, as assessed by its capacity to inhibit basal adenylyl cyclase activity (MacEwan & Milligan, 1996a,b). Such results indicate that GPCR occupancy is a requirement for inverse agonist function and thus, that a receptor reserve would not be anticipated for inverse agonist function. This is entirely different for the situation for agonist ligands at either the wild type or CAM β_2-adrenoceptor following expression in the background of NG108-15 cells, where the limiting presence of adenylyl cyclase levels defines that the EC_{50} for agonist ligands should decline as levels of receptor expression increase, a situation which we have previously demonstrated directly (Adie & Milligan, 1994b). BAAM treatment of the CAM β_2-adrenoceptor cells indicated that a 50% reduction in available ligand binding sites was achieved with approximately 30 nM of the alkylating agent. This value is similar to the experimentally determined concentration of BAAM required to half-maximally reduce inverse agonist efficacy (Figure 8).

The data provided in this manuscript generate a clear understanding of why studies of inverse agonism are generally performed in cell systems expressing either high levels of expression of a wild type receptor or make use of CAM variants of these GPCRs (Tiberi & Caron, 1994; Labrecque *et al*., 1995; Mullaney *et al*., 1996). They furthermore display the benefit of enhancing second messenger generating capacity to allow ease of detection and analysis of inverse agonist function. Demonstration of the reduction in detectable inverse efficacy of ligands with decreasing receptor availability and the lack of receptor reserve also suggests why inverse agonism can often be difficult to detect in a range of physiological systems.

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