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Detection of receptor ligands by monitoring selective stabilization of a *Renilla* luciferase-tagged, constitutively active mutant, G-protein-coupled receptor

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1 The wild-type β_2-adrenoceptor and a constitutively active mutant of this receptor were C-terminally tagged with luciferase from the sea pansy *Renilla reniformis*.

2 C-terminal addition of *Renilla* luciferase did not substantially alter the levels of expression of either form of the receptor, the elevated constitutive activity of the mutant β_2-adrenoceptor nor the capacity of isoprenaline to elevate cyclic AMP levels in intact cells expressing these constructs.

3 Treatment of cells expressing constitutively active mutant β_2-adrenoceptor-*Renilla* luciferase with antagonist/inverse agonist ligands resulted in upregulation of levels of this polypeptide which could be monitored by the elevated luciferase activity.

4 The pEC_{50} for ligand-induced luciferase upregulation and ligand affinity to bind the receptor were highly correlated.

5 Similar upregulation could be observed following sustained treatment with agonist ligands.

6 These effects were only observed at a constitutively active mutant of the β_2-adrenoceptor. Co-expression of the wild-type β_2-adrenoceptor C-terminally tagged with the luciferase from *Photinus pyralis* did not result in ligand-induced upregulation of the levels of activity of this luciferase.

7 Co-expression of the constitutively active mutant β_2-adrenoceptor-*Renilla* luciferase and an equivalent mutant of the α_{1b}-adrenoceptor C-terminally tagged with green fluorescent protein allowed pharmacological selectivity of adrenoceptor antagonists to be demonstrated.

8 This approach offers a sensitive and convenient means, which is amenable to high throughput analysis, to monitor ligand binding to a constitutively active mutant receptor.

9 As no prior knowledge of receptor ligands is required this approach may be suitable to identify ligands at orphan G protein-coupled receptors.

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Abbreviations: CAM, constitutively active mutant; DHA, dihydroalprenolol; DMEM, Dulbecco's Minimum Essential Medium; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; PBS, phosphate buffered saline

Introduction

Mutations in G protein-coupled receptors (GPCRs) which impart higher levels of agonist-independent, constitutive regulation of second messenger levels than are produced by the wild-type receptors have been studied extensively in recent years (Lefkowitz *et al*., 1993; Scheer & Cotecchia, 1997; Leurs *et al*., 1998; Pauwels & Wurch, 1998). This has been, in part, to explore whether such mutants can provide insights into the conformational changes which must occur in a GPCR upon agonist binding to allow activation of G proteins. Many wild-type receptors also display agonist-independent function which can often be detected as increases in a functional endpoint measure in parallel with higher levels of expression of the receptor. Both of these approaches have been useful in demonstrations that many ligands, previously described as antagonists, have a capacity to dampen such constitutive activity (Lefkowitz *et al*., 1993; Milligan *et al*., 1995). As such ligands are thought to favour the presence of inactive or ground states of GPCRs they are generally now referred to as 'inverse' agonists because they mirror the ability of agonist ligands to favour the enrichment of GPCRs in active conformational states (Milligan *et al*., 1995; Chen *et al*., 2000).

Recently, the constitutive activity of a GPCR has been suggested to provide a useful strategy in drug discovery which might be amenable for both GPCRs with known natural ligands and for so-called 'orphan' GPCRs for which the natural ligands remain unidentified (Chen *et al*., 2000). One of the most studied constitutively active mutant (CAM) GPCRs is a form of the human β_2-adrenoceptor in which a small segment of the distal region of the third intracellular loop was replaced by the equivalent sequence from the α_{1b}-adrenoceptor (Samama *et al*., 1993; 1994; Pei *et al*., 1994; MacEwan & Milligan, 1996; Gether *et al*., 1997a; Javitch *et al*., 1997; McLean *et al*., 1999). As well as producing

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significantly greater levels of cyclic AMP in an agonist-independent manner than the wild-type β_2-adrenoceptor, this mutant displays a substantially higher affinity to bind agonist but not antagonist/inverse agonist ligands. Furthermore, a Cys residue in transmembrane helix VI which can provide a monitor of agonist binding (Gether *et al*., 1997b) is closer to the ligand binding pocket in the CAM β_2-adrenoceptor than in the wild-type receptor (Javitch *et al*., 1997). This CAM receptor has also been reported to be more structurally unstable and to denature more easily than the wild-type receptor (Gether *et al*., 1997a). These effects can be reversed by ligand binding to the CAM receptor (Gether *et al*., 1997a). Herein, we take advantage of this feature to demonstrate ligand-induced upregulation of a form of the CAM β_2-adrenoceptor which has luciferase from the sea pansy *Renilla reniformis* linked in-frame to its C-terminal tail. Ligand-induced upregulation of the CAM receptor thus results in elevated cellular levels of luciferase activity which can be measured easily in formats appropriate to high throughput ligand screening programmes. The pEC_{50} for antagonist/inverse agonist-induced upregulation of the CAM β_2-adrenoceptor-*Renilla* luciferase construct was well correlated with the pK_i of the ligands, suggesting ligand occupancy of the GPCR binding site was the basis for the effect.

Methods

[^{3}H]-dihydroalprenolol, ([^{3}H]-DHA, 64 Ci mmol^{-1}), [^{3}H]-adenine and [^{3}H]-cyclic AMP were purchased from Amersham Pharmacia Biotech. All reagents for cell culture were from Life Technologies (Paisley, Strathclyde, U.K.). Receptor ligands were from RBI (Gillingham, Dorset, U.K.). All other reagents were from Sigma or Fisons and were of the highest purity available.

Construction of wild-type and CAM β_2-adrenoceptor/luciferase fusion proteins

Both human wild-type and CAM β_2-adrenoceptor-*Renilla* luciferase fusion proteins were generated. A β_2-adrenoceptor fragment was generated *via* PCR amplification of an existing β_2-adrenoceptor DNA in pcDNA3. Generation of the CAM β_2-adrenoceptor-fragment was also *via* the same PCR amplification of the CAM β_2-adrenoceptor in pcDNA3. The mutations which comprise the CAM consist of four amino acid substitutions in intracellular loop 3 of the receptor. The primers used were as follows: 5′ forward primer 5′-AAA AAG CTT GCC ACC ATG GGG CAA CCC GGG AA-3′ which incorporates both a 5′ *Hind*III cloning site and Kozak sequence. The 3′ reverse primer had sequence 5′-CCT CTC GAG CAG TGA GTC ATT-3′ which incorporates an *Xho*I cloning site to allow linkage to the *Renilla* luciferase gene. Introducing the *Xho*I site results in an insertion of a glutamate residue between the β_2-adrenoceptor DNA and the *Renilla* luciferase. It also resulted in alteration of the last nucleotide in the amino acid coding sequence of β_2-adrenoceptor (G→C). This did not alter the amino acid sequence as the new codon CTC still encodes leucine. *Renilla* luciferase was similarly generated *via* PCR amplification of a *Renilla* luciferase DNA cloned into plasmid pRLCMV (Promega). The primers used for amplification were as follows: 5′ forward primer 5′-TCG CTC GAG ACT TCG AAA GTT TAT G-3′ which incorporates an *Xho*I site at the 5′ end of the gene to allow linkage to either the β_2-adrenoceptor or the CAM β_2-adrenoceptor fragment. The reverse primer had the sequence 5′-GCG TCT AGA TTA TTG TTC ATT TT-3′ which incorporates an *Xba*I site into the 3′ end of the gene immediately downstream of the stop codon.

Following PCR reactions the resultant fragments were digested with the appropriate enzymes and subsequently gel purified. pcDNA3 was digested with *Hind*III and *Xba*I to provide a recipient vector for the ligated fragments. Ligations were performed using Fast-link DNA ligation kit (CAMBIO) in which digested pcDNA3, β_2-adrenoceptor (or CAM β_2-adrenoceptor fragment) and *Renilla* luciferase fragments were mixed and ligated together.

Construction of a GFP-tagged form of the 3CAMα_{1b}-adrenoceptor

Production and subcloning of the 3CAM hamster α_{1b}-adrenoceptor-GFP fusion protein (Stevens *et al*., 2000) was performed in two separate stages. In the first step the coding sequence of a modified form of GFP (Zernicka-Goetz *et al*., 1997) was modified by polymerase chain reaction (PCR) amplification. Using the amino-terminal primer 5′-GGAAGGTACCAGTAAAGGAGAAGAACTT-3′ the initiating Met of GFP was removed and both a *Kpn*I restriction site (underlined) and a 2-amino acid spacer (Gly-Asn) were introduced. Using the carboxy-terminal primer 5′-TGCTCTAGATTATTTGTATAGTTCATCCATGCATG-3′ an *Xba*I restriction site (underlined) was introduced downstream of the stop codon of GFP. The amplified fragment of GFP digested with *Kpn*I and *Xba*I was subcloned into similarly digested pcDNA3 expression vector (Invitrogen). To obtain the 3CAM α_{1b}-adrenoceptor-GFP fusion protein, the coding sequence of the 3CAM α_{1b}-adrenoceptor was amplified by PCR. Using the amino-terminal primer 5′-GACGGTACCTCTAAAATGAATCCCGAT-3′, a *Kpn*I restriction site (underlined) was introduced upstream of the initiator Met. Using the carboxy-terminal primer 5′-GTCCCTGGTACCAAAGTGCCCGGGTG-3′, a *Kpn*I restriction site (underlined) was introduced immediately upstream of the stop codon. Finally, the GFP construct in pcDNA3 was digested with *Kpn*I and ligated together with the PCR product of the α_{1b}-adrenoceptor amplification also digested with *Kpn*I.

Transient transfection of HEK293 cells

HEK293 cells were maintained in Minimum Essential Medium supplemented with 0.292 g L^{-1} L-glutamine, and 10% newborn calf serum at 37°C. Cells were grown to 60–80% confluency prior to transient transfection. Transfection was performed using LipofectAMINE reagent (Life Technology, Inc.) according to manufacturer's instructions. To generate cell lines stably expressing the various constructs, cells were seeded/diluted 2 days after transfection and maintained in DMEM supplemented with 1 mg ml^{-1} Geneticin sulphate (Life Technologies, Inc.). The medium was replaced every 3 days with DMEM containing 1 mg ml^{-1} Geneticin sulphate.

[^{3}H]-ligand binding studies

Cells were grown in 6 cm dishes and treated with or without isoprenaline for various times. After treatment the cells were washed three times with ice-cold phosphate-buffered saline (PBS (mM): KCl 2.7, NaCl 137, KH_2PO_4 1.5, Na_2HPO_4 8, pH 7.4). Cells were then detached from plates with PBS/0.5 mM EDTA, pelleted and resuspended in ice cold TE buffer (10 mM Tris HCl, 0.1 mM EDTA pH 7.5) and lysed with 2×10 s bursts of a polytron. The homogenate was centrifuged at $500 \times g$ to remove unbroken cells and nuclei. The supernatant fraction was then centrifuged at $48{,}000 \times g$ for 30 min and the pellet resuspended in TE buffer and stored at -80°C until use.

[^{3}H]-DHA binding assays were performed at 30°C for 45 min in TEM buffer (mM): Tris HCl 75, EDTA 1, $MgCl_2$ 12.5, pH 7.4. Parallel studies with 10 μM propranolol allowed assessment of non-specific binding. All experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes with ice-cold TE.

Intact cell adenylyl cyclase activity measurements

Were performed essentially as described in (Wong, 1994; Merkouris *et al*., 1997). Cells were split into wells of a 12-well plate and the cells were allowed to reattach. Cells were then incubated in medium containing [^{3}H]-adenine (1.5 μCi well^{-1}) for 16–24 h. The generation of [^{3}H]-cyclic AMP in response to treatment of the cells with various ligands and other reagents was then assessed.

Luciferase activity assay

A stably transfected cell line of HEK293 cells expressing the CAM β_2-adrenoceptor C-terminally tagged with *Renilla* luciferase was used to seed 96 well microtitre plates in 90 μl of medium. The 96 well plate was then incubated overnight and on the following day the cells were about 80% confluent. Drug dilutions were then prepared in phenol red-free medium and 10 μl from each of 10 concentration stock solutions were then added to wells of the 96 well plate. These plates were then incubated for 24 h and the medium pipetted off from each well.

Fifty μl of phenol red-free medium was then added to each well plus 50 μl of LucLite luciferase assay solution (Packard Biosciences). Fifty μl of 15 μM coelenterazine in phenol red-free medium was added (to produce a final concentration of 5 μM). The plates were then assayed immediately on a top count luminometer to determine the light intensity in relative light units.

Results

cDNAs encoding both the wild-type and a previously well characterized, constitutively active mutant (CAM), form of the human β_2-adrenoceptor were modified to remove the stop codon and allow in-frame ligation of the luciferases from either the sea pansy *Renilla reniformis* or the firefly *Photinus pyralis*. As demonstrated previously (MacEwan & Milligan, 1996), transient expression of the unmodified, wild-type, β_2-adrenoceptor resulted in higher levels of expression of this protein than was achieved for the C-terminally unmodified CAM β_2-adrenoceptor when this was monitored by the specific binding of the β-adrenoceptor antagonist [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) (Figure 1). The *Renilla* luciferase-tagged forms of both the wild-type and CAM β_2-adrenoceptor were expressed at similar levels as the unmodified forms (Figure 1) but for both forms of the β_2-adrenoceptor, C-terminal addition of *Photinus* luciferase significantly reduced levels of expression as monitored by specific [^{3}H]-DHA binding (data not shown) and thus these constructs were only used in a limited series of further experiments (see later). Saturation [^{3}H]-DHA binding studies demonstrated also that the K_d for this ligand (0.3 ± 0.1 nM) was little affected by addition of *Renilla* luciferase to the C-terminal tail of the CAM β_2-adrenoceptor (data not shown).

Although levels of expression are low (Figure 1), HEK293 cells express the β_2-adrenoceptor endogenously. Thus, in intact cell adenylyl cyclase assays, addition of the β-adrenoceptor agonist isoprenaline (10 μM) to mock transfected cells resulted in a significant stimulation of cyclic AMP generation (Figure 2). However, in cells transfected with either the unmodified wild-type β_2-adrenoceptor or the *Renilla* luciferase-tagged form of this receptor both basal cyclic AMP levels and the stimulation produced by isoprenaline was substantially greater (Figure 2). Following expression of either the C-terminally unmodified CAM β_2-adrenoceptor or the equivalent *Renilla* luciferase-tagged form, basal cyclic AMP levels were greater than observed with expression of the equivalent wild-type receptor forms. This is the basic characteristic of CAM forms of GPCRs and confirmed that C-terminal addition of *Renilla* luciferase did not suppress the CAM nature of this construct nor indeed the spontaneous, agonist-independent, signalling capacity of the wild-type β_2-adrenoceptor. Addition of isoprenaline now

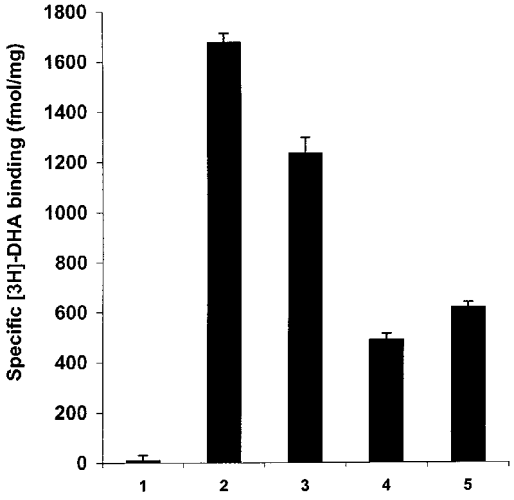

Figure 1 Expression levels of forms of the CAM β_2-adrenoceptor are lower than equivalent forms of the wild-type β_2-adrenoceptor. HEK293 cells were mock transfected (1) or transfected transiently with cDNAs encoding wild-type β_2-adrenoceptor (2), β_2-adrenoceptor-*Renilla* luciferase (3), CAM β_2-adrenoceptor (4) or CAM β_2-adrenoceptor-*Renilla* luciferase (5). Forty-eight hours later membranes were prepared and the specific binding of [^{3}H]-DHA (2 nM) measured. Data are means $\pm$ s.e.mean values of triplicate assays from a representative transfection. Two further experiments produced similar results.

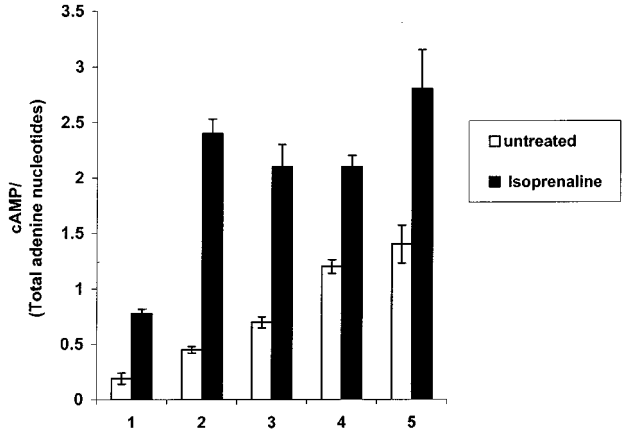

Figure 2 C-terminal addition of *Renilla* luciferase neither inhibits constitutive activity of the CAM β_2-adrenoceptor nor the capacity of agonist to stimulate cyclic AMP production. HEK293 cells were mock transfected (1) or transfected transiently with cDNAs encoding wild-type β_2-adrenoceptor (2), β_2-adrenoceptor-*Renilla* luciferase (3), CAM β_2-adrenoceptor (4) or CAM β_2-adrenoceptor-*Renilla* luciferase (5). Twenty-four hours later cells were labelled with ^{3}H-adenine and after a further 24 h cyclic AMP generation measured as in Methods in the absence or presence of the β-adrenoceptor agonist isoprenaline (10 μM). Results are taken from a representative experiment of three performed.

further increased cyclic AMP generation to levels similar to those obtained for the wild-type receptors (Figure 2).

Sustained (24 h) treatment of HEK293 cells transiently expressing the untagged or *Renilla* luciferase-tagged CAM β_2-adrenoceptor with the β-adrenoceptor antagonist/inverse agonist betaxolol (10 μM) resulted in the presence of substantially increased levels of [^{3}H]-DHA binding sites in membranes prepared from these cells (Figure 3A). Saturation [^{3}H]-DHA binding studies confirmed this to represent a true upregulation of the *Renilla* luciferase-tagged CAM β_2-adrenoceptor (Figure 3B). Parallel measures of the upregulation of [^{3}H]-DHA binding sites and *Renilla* luciferase luminescence produced by treatment with varying concentrations of betaxolol showed these parameters to overlap (Figure 3C). This suggested a novel strategy to identify antagonist/inverse agonist ligands at this receptor which would potentially be amenable to high throughput analysis. HEK293 clones stably expressing the *Renilla* luciferase-tagged CAM β_2-adrenoceptor were thus isolated and characterized for expression of the construct based on a combination of [^{3}H]-DHA binding and the presence of *Renilla* luciferase activity. A single clone was selected for detailed study. Following plating on wells of a 96 well microtitre plate, the cells were incubated with a range of concentrations of betaxolol at 37°C for 24 h. The luciferase activity of plate wells was subsequently monitored using 5 μM colenterazine. Betaxolol produced a concentration-dependent upregulation of *Renilla* luciferase activity with $EC_{50} = 1.2 \pm 0.26 \times 10^{-7}$ M (Figure 4A). The maximal effect of betaxolol was between 2–3 fold in individual experiments. Equivalent experiments with propranolol produced equivalent results except with $EC_{50} = 3.9 \pm 0.2 \times 10^{-9}$ M (Figure 4A). Parallel binding experiments confirmed upregulation of [^{3}H]-DHA binding sites (data not shown) which would be expected to be in a 1 : 1 ratio with the increase in *Renilla* luciferase activity based on the 1 : 1 stoichiometry of the receptor and luciferase in the fusion construct. Betaxolol was able to compete with [^{3}H]-DHA for binding to the CAM β_2-adrenoceptor-*Renilla* luciferase with a $K_i = 6.9 \times 10^{-8}$ M (Figure 5b). Equivalent experiments with propranolol (Figure 4b) resulted in a $K_i = 6.8 \times 10^{-10}$ M. A range of other β-adrenoceptor antagonists/inverse agonists, including ICI118551, CGP12177A and sotolol produced similar concentration-dependent upregulation of *Renilla* luciferase activity. These compounds had varying affinity to compete with [^{3}H]-DHA in binding experiments. However, a strong correlation was observed between the pK_i values of these ligands to bind to the *Renilla* luciferase-tagged CAM β_2-adrenoceptor and their pEC_{50} values for upregulation of this construct (Figure 5). Interestingly, equivalent long-term treatment with the agonists isoprenaline, salbutamol and salmeterol also produced significant, concentration-dependent, upregulation of the *Renilla* luciferase activity of cells expressing the tagged CAM β_2-adrenoceptor (Figure 6).

To ascertain the importance of the CAM nature of the β_2-adrenoceptor to observe ligand-mediated upregulation of luciferase activity we transiently co-expressed CAM β_2-adrenoceptor-*Renilla* luciferase and wild-type β_2-adrenoceptor-*Photinus* luciferase in HEK293 cells. Following sustained treatment of the cells with or without betaxolol we sequentially monitored *Photinus* luciferase and *Renilla* luciferase activity using a dual luciferase assay system. *Photinus* luciferase activity, as a monitor of levels of the wild-type β_2-adrenoceptor, was barely increased by betaxolol treatment whereas *Renilla* luciferase activity was increased substantially (Figure 7). Betaxolol substantially increased the *Renilla*/*Photinus* activity ratio and therefore the ratio of expression of CAM to wild-type β_2-adrenoceptors.

To demonstrate pharmacological specificity of ligand-induced upregulation we established a HEK293 clone stably expressing a C-terminally GFP-tagged form of a previously characterized CAM form of the α_{1b}-adrenoceptor which we designate 3CAM (Stevens *et al*., 2000). These cells were then transiently transfected with CAM β_2-adrenoceptor-*Renilla* luciferase and the cells subsequently treated with either betaxolol or the α_1-adrenoceptor antagonist/inverse agonist phentolamine (each at 10 μM, 24 h). These cells were then monitored for either *Renilla* luciferase activity or GFP fluorescence. Betaxolol increased levels of luciferase activity without a significant effect on levels of GFP whereas phentolamine had the opposite effect, increasing GFP fluorescence but not luciferase activity (Figure 8).

Discussion

A number of CAM forms of GPCRs appear to be physically destabilized compared to the wild-type receptors (MacEwan & Milligan, 1996; Lee *et al*., 1997; Gether *et al*., 1997b). Following either transient or stable transfection antagonist/inverse agonist binding to such CAM GPCRs can result in their upregulation without alteration in mRNA levels (MacEwan & Milligan, 1996; Lee *et al*., 1997; McLean *et al*., 1999; see Milligan & Bond, 1997 for review). Such results have added to the concept that such CAM GPCRs may provide a useful model of conformationally active, R*, states

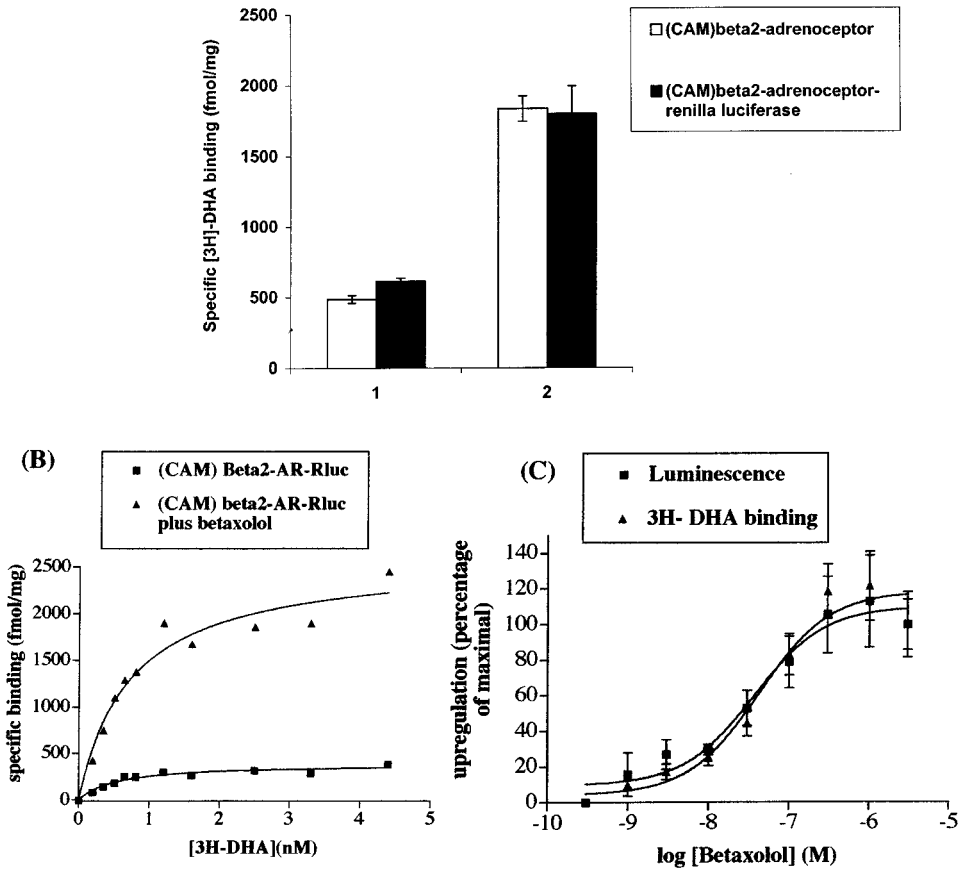

Figure 3 Sustained treatment with betaxolol upregulates levels of the untagged and *Renilla* luciferase-tagged forms of the CAM β_2-adrenoceptor. (**A**) HEK293 cells were transfected transiently with cDNAs encoding CAM β_2-adrenoceptor or CAM β_2-adrenoceptor-*Renilla* luciferase. Twenty-four hours later the cells were exposed to betaxolol (10 μM) (2) or vehicle (1). Twenty-four hours later membranes were prepared and the specific binding of [^{3}H]-DHA (2 nM), measured. (**B**) HEK293 cells were transfected transiently to express the CAM β_2-adrenoceptor-*Renilla* luciferase construct. Twenty-four hours later the cells were exposed to betaxolol (10 μM) or vehicle. Twenty-four hours later membranes were prepared and the specific binding of a wide range of [^{3}H]-DHA concentrations measured. Results are representative of three independent experiments. (**C**) HEK293 cells were transfected transiently to express the CAM β_2-adrenoceptor-*Renilla* luciferase construct. Twenty-four hours later the cells were exposed to varying concentrations of betaxolol. The specific binding of [^{3}H]-DHA (2 nM) and the luminescence from *Renilla* luciferase were measured in parallel 24 h later. Data are presented as per cent of maximal effect with results derived from three independent experiments.

of the GPCR with antagonist/inverse agonist binding resulting in a relaxation of the CAM receptor to ground state, inactive, R, forms. Such effects of antagonist/inverse agonist ligands on CAM GPCRs have been examined using approaches as diverse as alterations in fluorescence of a conformation-sensitive reporter in the purified receptor (Gether *et al*., 1997b) and analysis of increased levels of receptor binding sites present in cells following treatment with appropriate ligands (MacEwan & Milligan, 1996; Lee *et al*., 1997).

In recent times, C-terminal addition of the 27 kDa green fluorescent protein to a wide range of GPCRs has been employed to monitor their cellular expression, distribution and trafficking in response to stimulation (see Milligan, 1999; Kallal & Benovic, 2000 for reviews). In general, such modification does not appear to prevent interaction of the GPCR with G proteins nor to substantially alter the ligand binding properties of the GPCR. Treatment of a cell line stably expressing a CAM β_2-adrenoceptor-GFP construct with a range of ligands with β_2-adrenoceptor antagonist/inverse agonist pharmacology resulted in upregulation of the fusion polypeptide and the cells becoming markedly more fluorescent over time (McLean *et al*., 1999). In essence such an approach should be more amenable to quantitative analysis if an easy to assay enzyme was linked in frame to the C-terminus of the GPCR providing that the modified GPCR functioned essentially as the unmodified. Herein, we have used forms of luciferase derived from either the firefly *Photinus pyralis* or the sea pansy *Renilla reniformis* and linked either of these to both the wild-type and a CAM β_2-adrenoceptor. Assays of *Photinus* luciferase activity are generally more widely employed than *Renilla* (Rees *et al*., 1999). However, presumably simply because *Photinus* luciferase is a relatively large (61 kDa) polypeptide, transient expression levels of the GPCRs tagged with this luciferase were relatively low. This restricted the use we made of these constructs. By contrast, C-terminal tagging with the 31 kDa *Renilla* luciferase had little effect on levels of transient

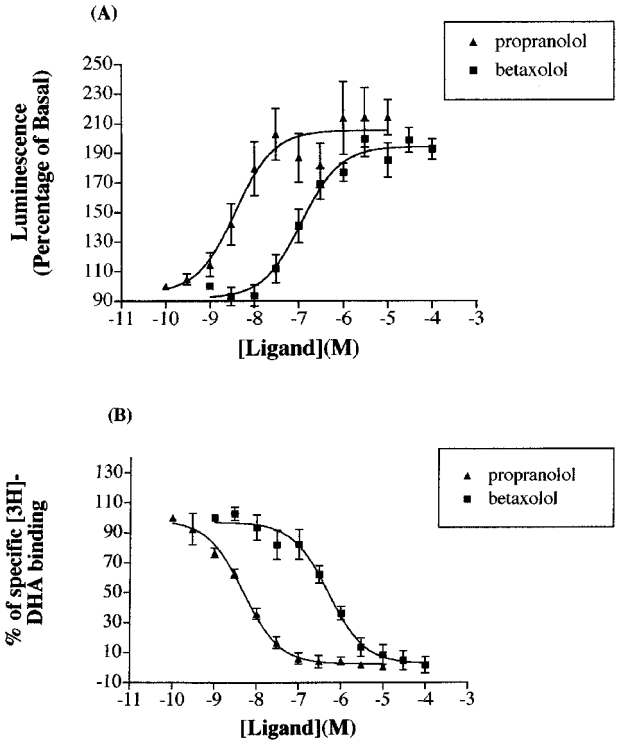

Figure 4 EC_{50} for betaxolol-induced upregulation of CAM β_2-adrenoceptor-*Renilla* luciferase is similar to ligand K_i. (**A**) Cells of a clone of HEK293 cells stably expressing CAM β_2-adrenoceptor-*Renilla* luciferase were grown in a 96 well microtitre plate and exposed to various concentrations of betaxolol or propranolol for 24 h. *Renilla* luciferase activity was then monitored as described in Methods. (**B**) The capacity of various concentrations of betaxolol or propranolol to compete with [^{3}H]-DHA (2 nM) to bind to CAM β_2-adrenoceptor-*Renilla* luciferase in membranes of the clone used in (**A**) was assessed. Data represent means ± s.e.mean from three experiments.

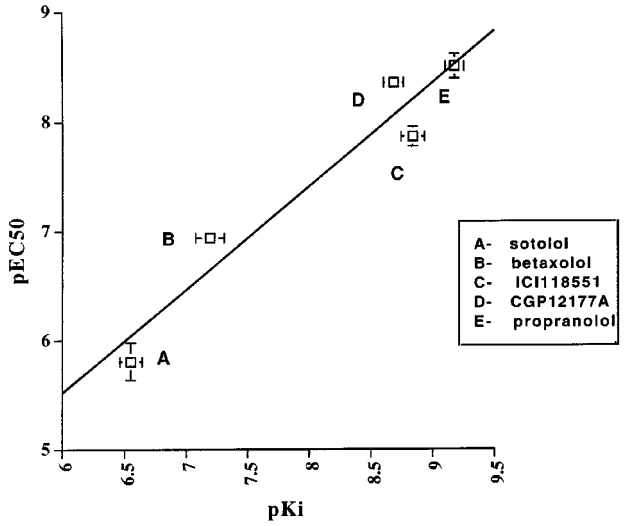

Figure 5 Correlation of pEC_{50} for ligand-induced upregulation and pK_i for CAM β_2-adrenoceptor-*Renilla* luciferase activity for a range of β_2-adrenoceptor antagonists/inverse agonists. Experiments similar to those described in Figure 4 were conducted using varying concentrations of sotolol (A), betaxolol (B), ICI118551 (C), CGP12177A (D) and propranolol (E). Measured EC_{50} values for upregulation and estimated K_i values from binding studies were then compared. Data represent means ± s.e.mean from three experiments.

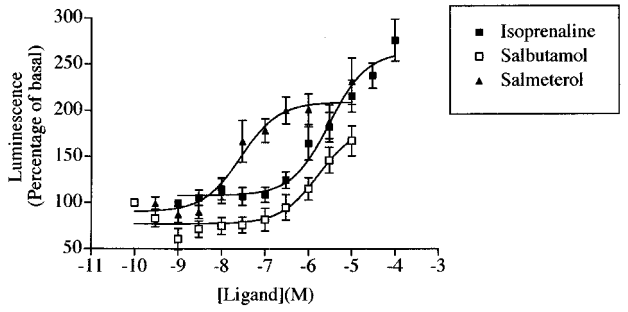

Figure 6 Sustained treatment with agonists produces concentration-dependent increases in CAM β_2-adrenoceptor-*Renilla* luciferase activity. Equivalent experiments to those of Figure 4A were performed using varying concentrations of isoprenaline, salbutamol and salmeterol. Data represent means ± s.e.mean from three experiments.

expression of either the wild-type or CAM β_2-adrenoceptor. Both the wild-type β_2-adrenoceptor-*Renilla* luciferase and the CAM β_2-adrenoceptor version of this construct were able to stimulate cyclic AMP levels upon addition of the agonist isoprenaline. As such, addition of the luciferase to the C-terminal tail of these GPCR forms does not prevent coupling of the receptors to G_S and thence to adenylyl cyclase. Equally, agonist stimulation of adenylyl cyclase activity by the β_2-adrenoceptor is not prevented by addition of GFP to the C-terminal tail (Barak *et al*., 1997; Kallal *et al*., 1998; McLean *et al*., 1999). Interestingly, both the wild-type β_2-adrenoceptor-*Renilla* luciferase and the CAM β_2-adrenoceptor version of this construct possessed the capacity to elevate cellular cyclic AMP levels in an agonist-independent manner (Figure 2). However, as expected from the untagged version of these GPCRs, the agonist-independent effect of the CAM β_2-adrenoceptor-*Renilla* luciferase was substantially more pronounced when expression levels were taken into consideration.

Upregulation of the CAM β_2-adrenoceptor-*Renilla* luciferase construct stably expressed in HEK293 cells was produced in a concentration-dependent manner by all the antagonist/inverse agonist ligands we tested. This was also the case in our previous studies using the CAM β_2-adrenoceptor-*Renilla* luciferase construct (McLean *et al*., 1999). However, as luciferase activity is both technically easy to measure and provides a robust and reproducible marker this system was ideally suited to the studies being performed in 96 well microtitre plates. The pEC_{50} for ligand upregulation of the CAM β_2-adrenoceptor-*Renilla* luciferase construct was well correlated with their pK_i to bind to the construct in intact cell binding assays (Figure 5). This suggests strongly that ligand occupancy is the key determinant which produces the upregulation. It has been shown previously that inverse agonist-induced upregulation of an unmodified CAM β_2-adrenoceptor occurs without alteration in levels of mRNA encoding the protein (MacEwan & Milligan, 1996). As such, although the activity of luciferases is most usually employed in transcriptionally-induced 'reporter gene' assays (Rees *et*

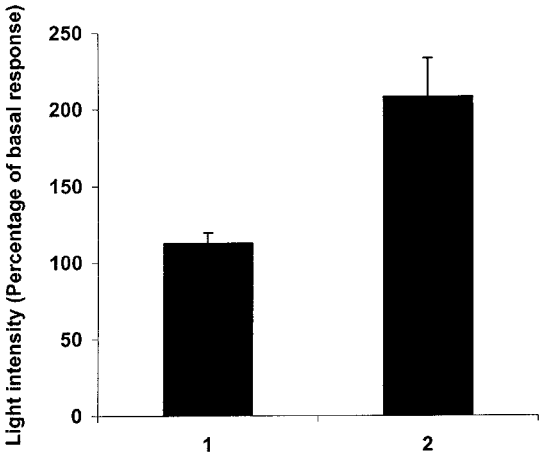

Figure 7 Ligand-induced upregulation of β_2-adrenoceptor-luciferase activity requires the CAM form of the receptor. HEK293 cells were transfected with a combination of CAM β_2-adrenoceptor-*Renilla* luciferase and wild-type β_2-adrenoceptor-*Photinus* luciferase constructs. Cells were then treated 24 h later without or with betaxolol (10 μM) for a further 24 h. *Photinus* (1) and *Renilla* (2) luciferase activities were then measured in parallel. Data represent means ± s.e.mean from three experiments.

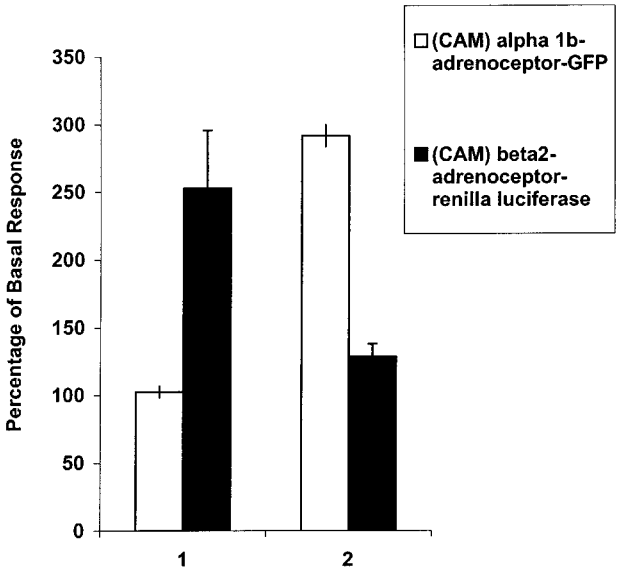

Figure 8 Pharmacological specificity of ligand-induced upregulation of CAM adrenoceptors. A clone of HEK293 cells stably expressing a CAM α_{1b}-adrenoceptor-GFP construct (Stevens *et al*., 2000) was transiently transfected for 24 h to express CAM β_2-adrenoceptor-*Renilla* luciferase. The cells were then exposed to either betaxolol (10 μM) (1) or to the α_1-adrenoceptor antagonist/inverse agonist phentolamine (10 μM) (2) for 24 h. *Renilla* luciferase activity (as bioluminescence) and GFP fluorescence were then monitored in parallel. Results are presented as per cent of the signals obtained by treatment of the cells with vehicle and represent means ± s.e.mean from three independent experiments.

al., 1999), the current studies do not require transcriptional modulation. Rather, they are based on ligand-induced protein stabilization of the construct. Coupled with unchanged rates of protein synthesis this results in time-dependent upregulation of these constructs.

To demonstrate that the ligand effect required the structural variation present in the CAM receptor, we performed experiments in which the CAM β_2-adrenoceptor-*Renilla* luciferase was co-expressed with the *Photinus* luciferase tagged wild-type β_2-adrenoceptor. Initial transfection ratios were arranged such that dual luciferase assay produced similar levels of light output from the two forms of the enzyme. Now following sustained treatment with betaxolol the activity of *Photinus* luciferase was essentially unchanged although that of *Renilla* luciferase was substantially increased. We have recently demonstrated that a similar antagonist/inverse agonist-induced upregulation can be observed using a C-terminally GFP-tagged form of an equivalent CAM mutant of the α_{1b}-adrenoceptor (Stevens *et al*., 2000). We thus reasoned that a parallel monitor of ligand regulation of GFP fluorescence and *Renilla* luciferase luminescence could provide monitors of pharmacological selectivity of ligands following co-expression of the CAM α_{1b}-adrenoceptor-GFP and the CAM β_2-adrenoceptor-*Renilla* luciferase. As anticipated, the β-blocker betaxolol upregulated luciferase activity but not the GFP signal whereas the α_1-adrenoceptor antagonist/inverse agonist phentolamine produced the opposite profile (Figure 8). We were somewhat surprised to observe that a series of β-agonists were also able to cause upregulation of the CAM β_2-adrenoceptor-*Renilla* luciferase construct. When these experiments were performed, our only real expectation of agonists producing the upregulation we have observed was based on the results of Gether *et al*. (1997a). They have argued convincingly that the occupancy of the CAM β_2-adrenoceptor will produce protein stabilization, no matter the efficacy of the ligand. However, their experiments were performed with the purified CAM β_2-adrenoceptor. Thus, our initial hypothesis was that agonists would cause internalization of the CAM β_2-adrenoceptor-*Renilla* luciferase as we have previously noted for a GFP-tagged form of this receptor (McLean *et al*., 1999) and that with long-term agonist treatment downregulation of the construct would occur, as is often observed for the wild-type β_2-adrenoceptor (McLean & Milligan, 2000). This would be expected to result in lower levels of the constitutively active mutant β_2-adrenoceptor-*Renilla* luciferase protein and that consequent lower levels of luciferase activity would provide an assay for agonist. However, this is not what was observed. HEK293 cells find it significantly more difficult to destroy such tagged forms of receptors than they do the unmodified forms (McLean & Milligan, 2000). It is not important to the basis of the luciferase assay where the protein is located at the end of the treatment because addition of the luciferase assay reagents disrupts cell integrity. We thus envisage that the agonist-induced upregulation represents a balance between ligand-induced stabilization which favours upregulation of the construct and the poor capacity of the cells to destroy the tagged receptor. This, however, must remain speculation at this point. Recent studies on two mutated, constitutively active, forms of the rat histamine H_2 receptor have, however, also demonstrated substantial upregulation of these to be produced by sustained treatment with both an agonist and an inverse agonist ligand at the receptor (Alewijnse *et al*., 2000). By contrast, levels of wild-type rat histamine H_2 receptor were reduced by sustained exposure to the agonist and the upregulatory effect of the inverse agonist, although statistically significant, was much less pronounced

than for the CAM forms (Alewijnse *et al*., 2000). Interestingly, Zhu *et al*. (2000) have recently observed that sustained treatment with a variety of ligands which function as inverse agonist at a CAM form of the human α_{1a}-adrenoceptor produces substantial upregulation of the mutant but not the wild-type receptor. Moreover, they reported a lack of upregulation of this CAM form when using KMD-3213, which they indicated to be a neutral antagonist (Zhu *et al*., 2000), suggesting a possible means to discriminate between neutral and inverse agonist ligands. Although intriguing, the basis for these differences were not established and the efficacy of KMD-3213 was very similar to that of BMY7378, which did produce strong upregulation (Zhu *et al*., 2000).

It also appears that all CAM mutants of the same receptor are not equivalent. Stevens *et al*. (2000) demonstrated that only certain CAM forms of the GFP-tagged hamster α_{1b}-adrenoceptor are strongly upregulated by sustained exposure to antagonist/inverse agonist ligands and that this capacity was correlated with the level of constitutive activity imbued by distinct mutations.

The constitutive activity of GPCRs has been suggested to provide means of developing and enhancing drug discovery programmes (Chen *et al*., 2000). It has also been noted that mutations at different regions of a GPCR which result in constitutive activity can produce synergistic effects when combined (Hwa *et al*., 1997).

These studies provide a potential strategy for the identification of ligands which interact with a wide range of GPCRs, including those for which the natural ligands are currently unknown, if ligand-regulated protein stabilization either segregates with receptor constitutive activity or can be induced by limited mutation. These two issues will form the basis for future work, as will the concept that different cell types with varying overall rates of receptor internalization and recycling may be identified to optimise ligand regulation of the steady state levels of such CAM receptors.

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