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Up-regulation of a constitutively active form of the β_2-adrenoceptor by sustained treatment with inverse agonists but not antagonists

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Abstract In neuroblastoma×glioma hybrid, NG108-15, cells transfected to stably express a constitutively active mutant (CAM) form of the human β_2-adrenoceptor, the β-adrenoceptor ligands sotalol and betaxolol functioned as inverse agonists as they reduced basal adenylyl cyclase activity whereas the antagonists dihydroalprenolol and propranolol did not. Maintained presence of the CAM β_2-adrenoceptor inverse agonists but not the antagonists in the culture medium of the cells resulted in a substantial, concentration-dependent, up-regulation of the CAMβ_2-adrenoceptor. Up-regulation of the CAMβ_2-adrenoceptor by the inverse agonists was prevented by co-incubation of the cells with either propranolol or dihydroalprenolol. Neither maintained elevation of cAMP levels nor the inhibition of adenylyl cyclase activity altered the ability of the inverse agonist ligands to cause receptor up-regulation.

Key words: Beta-adrenoceptor; Constitutively active mutant; Inverse agonist

1. Introduction

Inverse agonists are ligands which, by binding to a receptor, have the capacity to modulate the basal activity of a cell signalling cascade in the opposite direction to that produced by an agonist [1–3]. Although it was applied originally to agents functioning, probably as negative allosteric modulators, at the $GABA_A$ receptor [4], the term has recently gained acceptance in the field of G protein-mediated signalling. Conceptually, agonist ligands are envisaged to stimulate signalling cascades by binding with higher affinity, and thus preferentially stabilising, an active conformation of a G protein-coupled receptor (GPCR) resulting in more effective activation of the relevant G protein [1–3]. As a corollary, inverse agonists should interact preferentially with an inactive conformation of the GPCR and thus limit G protein activation [1–3]. Such modulation implies that active and inactive conformations of a GPCR must have the thermodynamic capacity to interconvert spontaneously, a situation which has required extension and re-analysis of the ternary complex model [5,6]. As such, basal activity of a GPCR-regulated signalling cascade is defined by the position of the equilibrium between these conformations [1].

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Abbreviations: Sotalol, (*N*-[4-[1-hydroxy-2-[(methylethyl)amino]ethyl]phenyl]methanesulphonamide; Betaxolol, 1-[4-[2-(cyclopropylmethoxy)ethyl]phenoxy]-3-isopropylamino-2-propanol); SQ 22,536, 9-(tetrahydro-2-furanyl)-9*H*-purin-6-amine; CAM, constitutively active mutant; cAMP, cyclic 3′,5′-adenosine monophosphate; GPCR, G protein-coupled receptor

Certain mutations in GPCRs, both found naturally in the human population and produced artificially by site-directed mutagenesis, act in a manner consistent with them functioning as stabilised, partially active, conformations in the absence of agonist ligands [7]. These are termed constitutively active (CAM) mutations [7]. As they produce greater regulation of the signalling pathway in the absence of ligands than the wild-type forms of the same receptor then, theoretically, they may be very useful in the development of systems suitable for the easy detection and analysis of inverse agonist ligands. In the current study we use a CAM mutant of the human β_2-adrenoceptor [5] to show: (i) that sustained treatment of cells expressing this receptor with the inverse agonists sotalol and betaxolol results in up-regulation of the receptor; (ii) that neutral antagonists do not produce a similar effect; (iii) that the effect of the inverse agonists is prevented by co-incubation with the antagonist ligands; and (iv) that this effect is neither prevented by sustained elevation of cellular cAMP levels nor mimicked by inhibition of cAMP production.

2. Materials and methods

2.1. Materials

All reagents for tissue culture were purchased from Life Technologies, Paisley, Strathclyde, UK. [^{3}H]Dihydroalprenolol (56 Ci/mmole), α[^{32}P]ATP and [^{3}H]cAMP were obtained from Amersham International. 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, UK) or from Research Biochemicals International (Natick, MA, USA). Iloprost was a kind gift of Schering Health Care, Burgess Hill, Sussex, UK. 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 CAMβ_2-adrenoceptors [5] were a gift from Dr. R.J. Lefkowitz (HHMI, Duke University, NC, USA).

2.2. Methods

2.2.1. Generation and isolation of cell lines. Plasmid pJM16 [8], which harbours a copy of the neomycin resistance gene, was cut with the restriction enzymes *Bam*HI and *Xho*I to allow a cDNA encoding the wild-type human β_2-adrenoceptor to be ligated downstream of the β-actin promoter of this plasmid [9,10]. 'Genecleaned' (Life Technologies) wild-type human β_2-adrenoceptor cDNA, with 5′ *Bam*HI and 3′ *Xho*I sites, was ligated into the digested pJM16. For the CAMβ_2-adrenoceptor, the cDNA in vector pRK5 was co-transfected with the neomycin-resist nce plasmid, pSV-neo. 10 μg of these DNAs was 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) were selected and expanded. Expression of the β_2-adrenoceptor in membranes from these clones was assessed by the specific binding of the β-adrenoceptor antagonist [^{3}H]dihydroalprenolol (see below).

2.2.2. Membrane preparation. Membrane fractions were prepared from cell pastes which had been stored at −80°C following harvest essentially as described [11]. 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

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homogeniser. The resulting homogenate was centrifuged at 500×*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×*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×*g* for 10 min the membrane pellet was resuspended in TE buffer to a final protein concentration of 1–3 mg/ml and stored at −80°C until required.

2.2.3. Adenylyl cyclase activity assays. These were performed as described by Milligan et al. [12]. Each assay contained 100 mM Tris-HCl, pH 7.5, 20 mM creatine phosphate, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM cAMP, 1 μM GTP, 10 U creatine phosphokinase and 0.2 mM ATP containing 1 μCi α[^{32}P]ATP. Separation of radiolabelled cAMP and ATP was achieved using the double column method described by Johnson and Salomon [13].

2.2.4. [^{3}H]Dihydroalprenolol binding assays. In routine experiments a single concentration (2 nM) of [^{3}H]dihydroalprenolol ([^{3}H]DHA) in the absence and presence of 10 μM propranolol was used to define total and non-specific binding respectively. Assays were performed at 37°C for 30 min in 20 mM Tris-HCl (pH 7.5), 50 mM sucrose, 20 mM $MgCl_2$ (TSM buffer). All binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes (5 ml) with ice-cold TSM buffer.

2.2.5. Western analysis. Membrane proteins were resolved by SDS-PAGE (10% (w/v) acrylamide) and subsequently transferred to nitrocellulose for immunoblotting. For detection of $G_s\alpha$, antiserum CS was used. For detection of $G_q\alpha/G_{11}\alpha$, antiserum CQ was used. These antisera [14,15] were raised in New Zealand White rabbits after immunization with a glutaraldehyde conjugate of keyhole limpet haemocyanin and the synthetic peptides, RMHLRQYELL and QLNLKEYNLV, which correspond to the C-terminal decapeptide regions of the $G_s\alpha$ isoforms and $G_q\alpha/G_{11}\alpha$ respectively.

3. Results

The specific binding of [^{3}H]dihydroalprenolol ([^{3}H]DHA) (2×10^{-9} M) to membranes of clone 22 which stably expresses the CAMβ_2-adrenoceptor was competed for by varying concentrations of the β-adrenoceptor ligands sotalol, betaxolol, timolol, dihydroalprenolol and propranolol. Betaxolol and sotalol were substantially less potent than the other compounds tested (Fig. 1). [^{3}H]DHA had a measured K_d of $8.8\pm3.9\times10^{-10}$ M at this receptor (data not shown) allowing the following estimates for K_i: propranolol $3.0\pm0.6\times10^{-9}$ M, dihydroalprenolol $1.6\pm0.4\times10^{-9}$ M, timolol $2.5\pm0.8\times10^{-9}$ M, betaxolol $7.6\pm2.4\times10^{-7}$ M and sotalol $3.5\pm0.9\times10^{-6}$ M (all mean ± S.E.M., $n=3$).

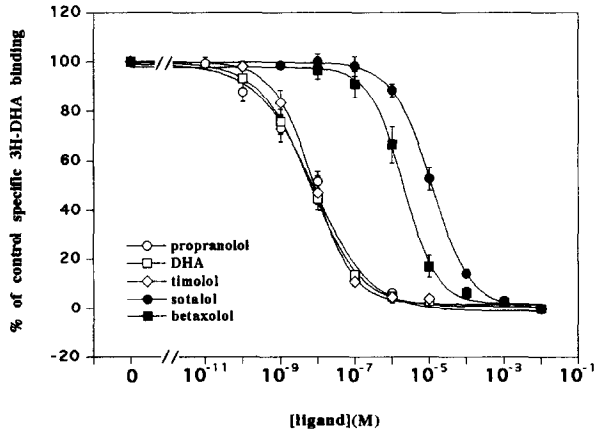

Fig. 1. Competition between [^{3}H]dihydroalprenolol and various β-receptor ligands for the CAMβ_2-adrenoceptor. Membranes prepared from clone 22 cells were incubated with [^{3}H]DHA (2×10^{-9} M) and varying concentrations of propranolol, dihydroalprenolol, timolol, sotalol or betaxolol and the specific binding of the radioligand assessed as described in Section 2.2. The results represent the means ± S.E.M., from three independent experiments.

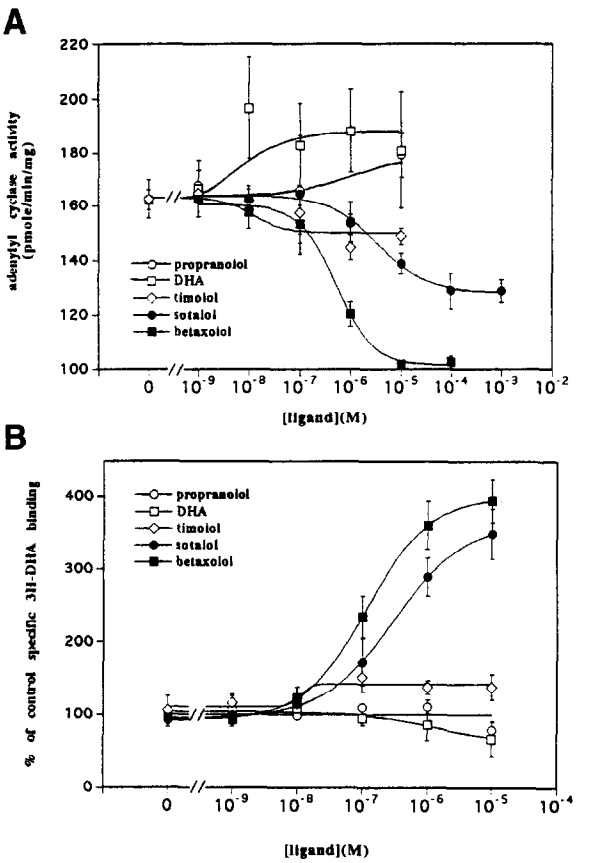

Fig. 2. Sotalol and betaxolol act as inverse agonists at the CAMβ_2-adrenoceptor and cause up-regulation of this receptor. A: Basal adenylyl cyclase activity and its regulation by varying concentrations of β-adrenoceptor ligands was measured in membranes of CAMβ_2-adrenoceptor expressing clone 22 cells. The data represent the means ± S.E.M., $n=3$. B: Clone 22 cells in culture were treated with or without a range of concentrations of a number of β-adrenoceptor ligands for 24 h. Membranes were subsequently prepared and the specific binding of [^{3}H]DHA measured. 100% specifc [^{3}H]DHA binding was equivalent to 790 ± 100 fmole/mg membrane protein. The data represent the means ± S.E.M., $n=3$.

Membranes prepared from cells of clone 22 displayed high adenylyl cyclase activity in the absence of ligands in comparison to untransfected NG108-15 cells or NG108-15 cells transfected to express varying levels of the wild-type β_2-adrenoceptor (see Fig. 2A and [10,16] for examples). This basal adenylyl cyclase activity was inhibited in a concentration-dependent manner by both betaxolol ($EC_{50}=5.2\times10^{-7}$ M) and sotalol ($EC_{50}=3.5\times10^{-6}$ M) (Fig. 2A), a response anticipated for inverse agonist ligands, whereas propranolol, timolol and dihydroalprenolol had no significant effects (Fig. 2A).

Addition and maintenance of varying concentrations of betaxolol or sotalol to cultures of clone 22 for 24 h prior to cell harvest resulted in a substantial, concentration-dependent, increase (some 4-fold in response to betaxolol (1×10^{-5} M) and greater than 3-fold in response to sotalol (1×10^{-5} M)) in membrane levels of the CAMβ_2-adrenoceptor (from a basal level of 790 ± 110 fmole/mg membrane protein) as measured by the specific binding of [^{3}H]DHA (Fig. 2B), correlating with the inverse agonism of these ligands noted above (Fig. 2A).

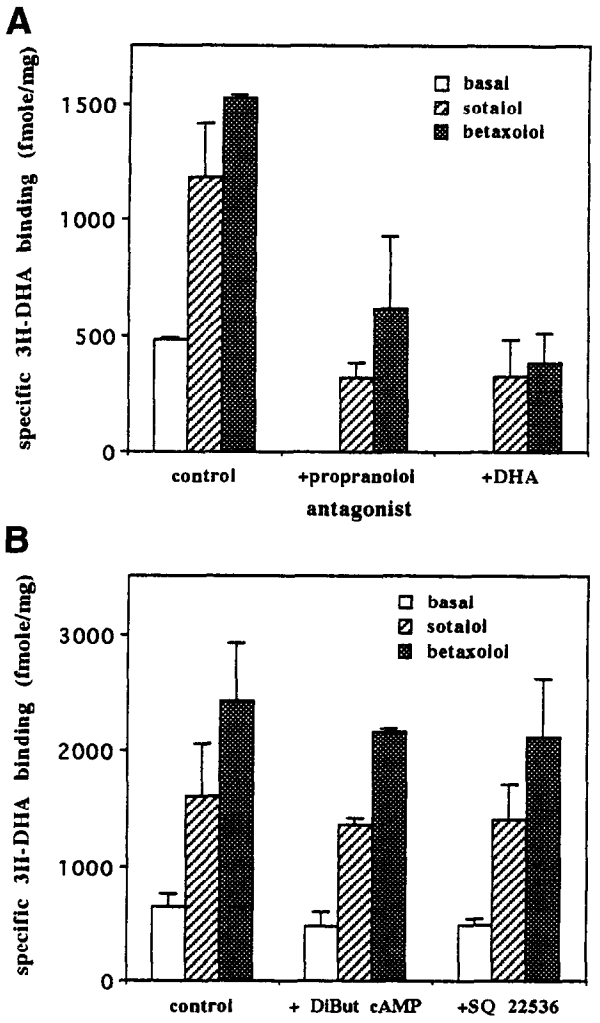

Fig. 3. Inverse agonist up-regulation of the CAMβ_2-adrenoceptor requires receptor occupancy but not alterations in cellular cAMP levels. A: Cells of clone 22 were treated for 24 h with either sotalol or betaxolol (1×10^{-5} M) or with either of these agents in combination with either propranolol or dihydroalprenolol (1×10^{-5} M) as indicated. Membranes were subsequently prepared and the specific binding of [^{3}H]DHA measured. The data represent the means ± S.D. of triplicate determinations. Similar data were produced in two independent experiments. B: Cells of clone 22 were treated for 24 h with either vehicle, sotalol or betaxolol (1×10^{-5} M) or with the agents dibutyryl cAMP (1×10^{-3} M) or SQ 22536 (1×10^{-4} M) added in parallel. The data represent the means ± S.D. of triplicate determinations. Similar data were produced in two independent experiments.

No significant alterations in levels of specific [^{3}H]DHA binding were observed following equivalent treatments with up to 1×10^{-5} M propranolol, timolol or dihydroalprenolol (Fig. 2B). Importantly for the contention that up-regulation of the CAMβ_2-adrenoceptor induced by betaxolol and sotalol was directly attributable to their interactions with the receptor and their function as inverse agonists, co-incubation of clone 22 cells with either betaxolol or sotalol (1×10^{-5} M) with either propranolol or dihydroalprenolol (1×10^{-5} M) for 24 h prevented the up-regulatory effects of both inverse agonist ligands (Fig. 3A).

As expression of the CAMβ_2-adrenoceptor results in agonist-independent stimulation of adenylyl cyclase which is inhibited by the inverse agonists (see above) we examined whether the receptor up-regulation induced by betaxolol and sotalol would be prevented by maintained elevation of cAMP levels or mimicked by sustained inhibition of adenylyl cyclase. Addition of the cell-permeable second messenger analogue, dibutyryl cAMP (1×10^{-3} M) for 24 h neither altered basal steady-state levels of the CAMβ_2-adrenoceptor or prevented the ability of either betaxolol or sotalol to cause up-regulation of the receptor (Fig. 3B). Furthermore, co-incubation with the adenylyl cyclase P-site inhibitor [17,18], SQ 22536 (1×10^{-4} M), which acts to inhibit adenylyl cyclase activity (basal adenylyl cyclase activity in membranes of clone 22 cells in this set of experiments was 91.8 ± 6.4 pmole/min/mg protein and in the presence of SQ 22536 it was 27.1 ± 1.2 pmole/min/mg protein (mean ± S.D. of quadruplicate determinations)), was also without effect on either basal levels of the receptor or its up-regulation by betaxolol and sotalol (Fig. 3B).

Sustained (24 h) treatment of NG108-15 cells transfected to express the wild-type human β_2-adrenoceptor receptor (clone βN22) with isoprenaline resulted in a reduction in membrane-

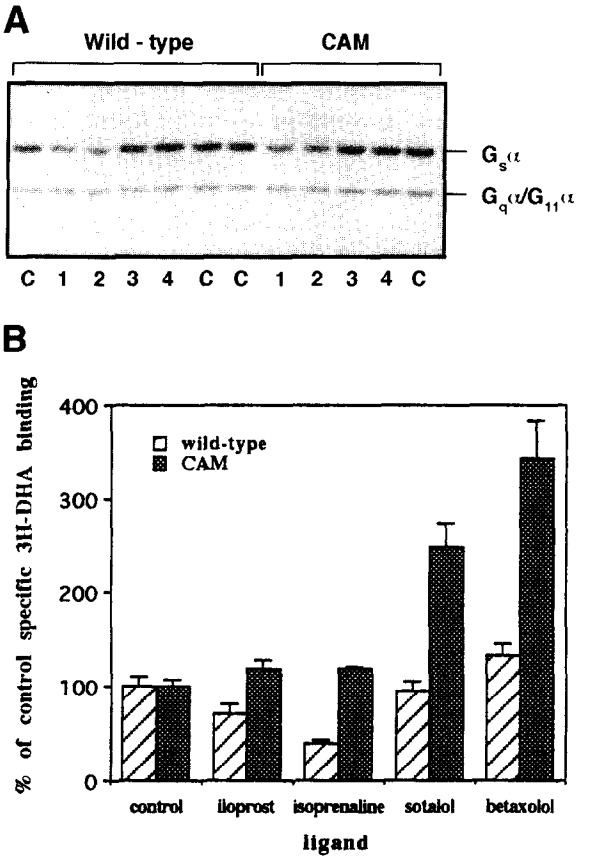

Fig. 4. Inverse agonists fail to regulate cellular G protein levels despite regulation of receptor levels. A: Cells of clone βN22 (expressing the wild-type β_2-adrenoceptor) or clone 22 (expressing the CAMβ_2-adrenoceptor) were maintained for 24 h in the presence of vehicle (C), or 1×10^{-5} M of the IP prostanoid receptor agonist iloprost (1), isoprenaline (2), sotalol (3) or betaxolol (4). Membranes were subsequently prepared from these cells and following resolution by SDS-PAGE the samples were immunoblotted with a combination of antiserum CS (to detect $G_s\alpha$) and CQ (to detect $G_q\alpha$ and $G_{11}\alpha$). Similar data were produced in two further independent experiments. B: The membranes produced in A had their specific [^{3}H]DHA binding capacity determined. The data represent the means ± S.D. of quadruplicate determinations. Similar data were produced in two further independent experiments.

associated levels of the α subunit of the G protein G_s, which interacts with this receptor, as we have previously reported [9]. This was also true following sustained occupancy of the endogenously expressed (G_sα-linked) IP prostanoid receptor with the agonist iloprost (Fig. 4). In contrast to this, sustained treatment with either of the inverse agonists betaxolol or sotalol failed to cause any detectable alteration in levels of this G protein (Fig. 4). Similar steady-state levels of Gsα were present in membranes of the CAMβ2-expressing clone 22 cells as in those expressing the wild-type receptor. Equivalent down-regulation of G_sα was observed in response to isoprenaline and iloprost in the CAMβ_2-adrenoceptor expressing clone 22 (Fig. 4). Again, despite the strong up-regulation of the receptor in response to sotalol and betaxolol, no up-regulation of G_sα could be detected in parallel (Fig. 4). As a control in these experiments relative levels of the phosphoinositidase C-linked G proteins were also measured in the same assays. No alteration in levels of G_qα/G_{11}α G proteins were observed associated with any of the treatments employed (Fig. 4).

4. Discussion

Inverse agonists are expected to stabilise inactive conformations of GPCRs. Thus, they should reduce the basal input into signalling cascades produced by agonist-unoccupied GPCRs as this is thought to reflect the equilibrium position between inactive and active receptor conformations [1–3]. As such, mutations in GPCRs which show greater agonist-independent signalling than the wild-type receptors would be expected to provide easier model systems with which to examine the characteristics of inverse agonist ligands.

The current study demonstrates that sustained treatment of cells expressing a CAM human β_2-adrenoceptor with inverse agonist ligands can produce a marked upregulation of this receptor. A key feature of the action of an agonist ligand (whether positive or inverse) is that its effects should be prevented by the presence of an antagonist ligand. Although it is standard to demonstrate this for positive agonists it is a simple pharmacological test often overlooked in studies on potential inverse agonist ligands. As such, an important feature of these studies was the demonstration that betaxolol and sotalol-induced CAMβ_2-adrenoceptor up-regulation was blocked by co-incubation with either propranolol or dihydroalprenolol (Fig. 3A) even though these compounds alone had no significant effects on receptor levels (Fig. 2). The neutral antagonists used in this study display substantially greater affinity (at least 100-fold) to bind to the CAM β_2-adrenoceptor than the inverse agonists and thus it was a simple process to allow equimolar concentrations of the antagonists to compete with the inverse agonists for binding to the receptor. Such studies also help to define that the effects of betaxolol and sotalol require their interaction with the receptor and are not a reflection of some non-specific and trivial interaction with the cells. It is also of interest in this regard that the estimated K_i values for betaxolol and sotalol in this system were in close agreement with the measured EC_{50} values for inhibition of basal adenylyl cyclase activity but that the measured EC_{50} values for CAM β_2-adrenoceptor upregulation were some 3–5-fold lower (see Section 3). As noted above, both betaxolol and sotalol have relatively low affinity at the CAMβ_2-adrenoceptor whereas the high affinity ligands examined in this study were not inverse agonists. Further studies will be required to examine if this is significant, but one possibility derived from this observation is that the binding site for inverse agonists is not identical to that for antagonist ligands. This should not be considered too unlikely as in the catecholamine receptors sites of greater or lesser importance for the binding of agonist and antagonist ligands have been identified [19,20] and agonist ligands tend to have lower affinity at receptors than antagonists as their role involves selection between different receptor conformations and their stabilisation.

The demonstration that two β-blockers, sotalol and betaxolol, have the capacity to inhibit basal adenylyl cyclase activity in membranes of cells derived from neuroblastoma × glioma hybrid, NG108-15, cells following their transfection and stable expression of a CAM human β_2-adrenoceptor defines these compounds as inverse agonists at this receptor. It was thus of considerable interest to note that sustained (24 h) exposure of cells expressing the CAMβ_2-adrenoceptor to both betaxolol and sotalol resulted in a substantial up-regulation of levels of the receptor as subsequently measured by the binding of [^{3}H]DHA. The ability of a single concentration of betaxolol to cause up-regulation of the CAMβ_2-adrenoceptor has previously been noted in CHO cells [21]. However, as used in this report, the NG108-15 cell background provides more marked and reproducible inverse agonism responses than in other cell lines we have examined expressing the same CAMβ_2-adrenoceptor construct, including CHO cells (D.J. MacEwan and G. Milligan, unpublished observations).

As cells expressing the CAMβ_2-adrenoceptor show elevated agonist-independent adenylyl cyclase activity, and thus generation of cAMP, we wished to ascertain whether inhibition of adenylyl cyclase and reduction in cAMP levels by a distinct mechanism would mimic the effect of the inverse agonists in inducing receptor up-regulation. To do so, we took advantage of the ability of a so-called adenylyl cyclase 'P-site' inhibitor [17,18] to block the generation of cAMP. Treatment of the CAMβ_2-adrenoceptor expressing cells with SQ 22536 had no significant effect on either steady-state levels of the receptor or on the ability of sotalol or betaxolol to increase these levels (Fig. 3B), although it reduced substantially the basal adenylyl cyclase activity in membranes of these cells. Furthermore, as treatment with the cell permeant and phosphodiesterase resistant analogue of cAMP, dibutyryl cAMP, was unable to prevent the effect of the inverse agonists we can eliminate a key role of the agonist-independent stimulation of adenylyl cyclase in these cells in the process of receptor up-regulation.

We have previously noted in a range of systems that sustained agonist occupancy of a GPCR can result in a selective down-regulation of the G protein α-subunit coupled to that receptor (as long as levels of receptor expression are sufficiently high such that a significant fraction of the relevant G protein α-subunit pool is activated) (see [22] for review). We confirmed that this phenomenon could be observed in the CAMβ_2-adrenoceptor expressing NG108-15 cells via both this receptor (in response to isoprenaline) and via an endogenously expressed IP prostanoid receptor (Fig. 4) and then further examined whether sustained occupancy of the CAMβ_2-adrenoceptor with inverse agonists might conversely result in an up-regulation of G protein levels. We were unable to record such an effect despite there being marked up-regulation of the CAMβ_2-adrenoceptor in the same preparations (Fig. 4). Thus, although down-regulation of both receptor and G-protein can

occur in response to positive agonists, the converse was not true of inverse agonists which resulted in up-regulation of receptor levels only. Although agonist-induced down-regulation of G proteins often occurs in the same temporal framework as down-regulation of the GPCR, these two processes were recently shown to be independent [23,24]. As such, it might be rather naive to anticipate that there would be coupled regulation of the levels of expression of different elements of a signalling cascade which would be dependent upon the conformational status of just the receptor.

The current studies provide evidence from a novel strategy, based on the up-regulation of ^3H ligand binding sites, that betaxalol and sotalol act as inverse agonists at the CAMβ_2-adrenoceptor and importantly demonstrate that this effect can be produced in a whole cell. A criticism of certain studies on inverse agonism has been that it is easier to observe responses consistent with such a pharmacology in assays which utilise broken cell preparations rather than whole cell systems.

A recent report has demonstrated a similar phenomenon to that reported herein for the histamine H_2 receptor [25]. Sustained treatment of CHO cells transfected to express this receptor with compounds such as cimetidine or ranitidine, which were shown to have inverse agonists properties, caused an upregulation of the receptor whereas equivalent treatment with burimamide, which acted as a neutral antagonist, was unable to replicate this effect [25]. Although the effects in the studies of Smits et al. [25] were relatively small compared to the degree of upregulation reported herein, they examined the wild-type rather than a mutationally modified form of histamine H_2 receptor. We have also noted that betaxolol treatment produces up-regulation of the wild-type human β_2-adrenoceptor [26] (Fig. 4B), although the effect is much smaller than when using the CAM version of the receptor. The mechanisms responsible for the upregulation does not seem to involve alteration in receptor mRNA but does require de novo protein synthesis [26] and it may be that the inverse agonists cause a stabilization of the receptor structure which would result in a decreased rate of turnover. This hypothesis, however, awaits direct examination.

It will be of interest to discover if these findings represent a general feature of inverse agonists compared to antagonists drugs at a wide range of GPCRs. A considerable number of activating mutations have now been recorded for a wide range of receptor types and as such these questions are amenable to direct examination [5–7,27,28].

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