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Inverse Agonist-Induced Up-Regulation of the Human β_2-Adrenoceptor in Transfected Neuroblastoma X Glioma Hybrid Cells

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SUMMARY

Neuroblastoma X glioma hybrid NG108-15 cells were transfected to express stably either the wild-type human β_2-adrenoceptor or a constitutively active mutant (CAM) version of this receptor. Basal adenylyl cyclase activity in cells expressing the CAM β_2-adrenoceptor correlated well with the level of expression of the receptor and was substantially greater than that in cells expressing the wild-type β_2-adrenoceptor. The CAM β_2-adrenoceptor displayed higher affinity for the agonist isoprenaline than the wild-type receptor but not for the antagonist alprenolol or the inverse agonist betaxolol. Pretreatment of cells harboring the CAM β_2-adrenoceptor with betaxolol resulted in a large (4–7-fold within 24 hr) up-regulation in levels of this receptor. This was not observed after exposure of the CAM β_2-adrenoceptor-expressing cells to alprenolol, and a much smaller effect of betaxolol was produced in cells expressing the wild-type receptor. Betaxolol-mediated up-regulation of the CAM β_2-adrenoceptor was both time and concentration dependent. However, this up-regulation did not result in a substantial alteration in the cellular distribution profile of the receptor. Half-maximal up-regulation of the CAM β_2-adrenoceptor required concentrations of betaxolol similar to those needed to cause half-maximal inhibition of basal adenylyl cyclase activity, indicating the receptor up-regulation is associated with the inverse agonist properties of this compound. Despite the large up-regulation of CAM β_2-adrenoceptor levels, treatment with betaxolol did not significantly alter levels of the G protein that couples to this receptor ($G_{s\alpha}$). After sustained treatment with betaxolol, Northern analyses did not demonstrate up-regulation of either CAM β_2-adrenoceptor or $G_{s\alpha}$ mRNA, and up-regulation of the receptor was prevented by cotreatment of the cells with cycloheximide. These data indicate that the up-regulation of the receptor by betaxolol is likely to reflect an increase in translational efficiency of existing mRNA and/or stabilization of the receptor polypeptide from proteolytic degradation and indicate that such effects can be produced by inverse agonists but not by neutral antagonists.

During studies to determine which elements of the intracellular loops of adrenergic receptors were responsible for the selective coupling of the receptors to different G protein families, Samama *et al.* (1) produced a constitutively active chimeric receptor in which a small section of the distal end of the third intracellular loop of the human β_2-adrenoceptor was replaced with the equivalent section in the hamster α_{1B}-adrenoceptor. This construct was able to stimulate adenylyl cyclase activity in an agonist-independent manner to a greater extent than the wild-type β_2-adrenoceptor when cells expressing similar levels of these receptors were examined (1). Given the agonist-independent activation of adenylyl cyclase produced by this construct, it was described as a CAM receptor (1, 2).

In a recent reappraisal of receptor theory, it was concluded that even wild-type receptors are not entirely quiescent in the absence of agonist ligands (1–5) and that the position of the equilibrium between an inactive conformation of the receptor (R) and a conformation (R*) capable of activating a cognate G protein may vary among receptors and can be modulated by mutation (1–6). The current view is that agonist ligands function because they display preferential affinity for R*, act to selectively stabilize this form of the receptor, and thus move the equilibrium between R⇆R* to the right (1–4, 6). Ligands that display preferential affinity for R will move the equilibrium in the other direction and will reduce agonist-independent activity, and on this basis, they have been termed "inverse agonists" (1–11). If CAM variants provide useful models for an R* state of a receptor, it must be predicted that as long as such mutations have the inherent capacity to relax to the R conformation, they will potentially provide a means by which to explore the mechanisms of action of inverse agonists. In the current study, we compare clones of NG108-15 cells transfected to express either the

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ABBREVIATIONS: CAM, constitutively active mutant; DHA, dihydroalprenolol; TE, Tris/EDTA; TME, Tris/magnesium chloride/EDTA.

human wild-type or CAM β_2-adrenoceptor to explore differences in their regulation by the β-adrenoceptor inverse agonist betaxolol.

Experimental Procedures

Materials. All reagents for tissue culture were purchased from Life Technologies (Paisley, Strathclyde, UK). [^{3}H]DHA (56 Ci/mmol), [α-^{32}P]ATP, and [^{3}H]cAMP were obtained from Amersham International (Buckinghamshire, UK). Betaxolol was a kind gift from Dr. V. Rovei (Synthelabo Recherche, Bagneux, France). Other β-adrenoceptor active compounds were purchased from Sigma Chemical (Poole, Dorset, UK) or Research Biochemicals (Natick, MA). Iloprost was a kind gift of Schering Health Care (Burgess Hill, Sussex, UK). All other chemicals were purchased from Sigma (Poole, Dorset, UK) or British Drug Houses (Lutterworth, Leicestershire, UK) 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 (Howard Hughes Medical Institute, Duke University, NC).

Generation and isolation of cell lines. Plasmid pJM16 (12), which harbors 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, 13). Wild-type human β_2-adrenoceptor cDNA (with 5′ *Bam*HI and 3′ *Xho*I sites) that had been treated with GeneClean (Life Technologies) was ligated into the digested pJM16. For the CAM β_2-adrenoceptor, the cDNA in vector pRK5 was cotransfected with the neomycin-resistance plasmid, pSV-neo. Ten micrograms 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 sulfate (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]DHA (see below).

Membrane preparation. Membrane fractions were prepared from cell pastes that had been stored at −80° after harvest as previously described (14). Frozen cell pellets were suspended in 5 ml of TE buffer (10 mM Tris·HCl, 0.1 mM EDTA, pH 7.5), and rupture of the cells was achieved with 25 strokes of a hand-held Teflon-on-glass homogenizer. The resulting homogenate was centrifuged at 500 × g for 10 min in a Beckman Instruments (Palo Alto, CA) 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 was washed and resuspended in 10 ml of TE buffer. After 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° until use.

Adenylyl cyclase activity assays. Adenylyl cyclase activity assays were performed according to Milligan *et al.* (15). 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 units of creatine phosphokinase, and 0.2 mM ATP containing 1 μCi of [α-^{32}P]ATP. Separation of radiolabeled cAMP and ATP was achieved using the double-column method described by Johnson *et al.* (16, 17).

[^{3}H]DHA binding assays. In routine experiments, a single concentration (2 nM) of [^{3}H]DHA in the absence and presence of 10 μM propranolol was used to define total and nonspecific binding, respectively. In experiments designed to assess the maximal binding capacity of membranes, the binding of a single concentration of [^{3}H]DHA was competed for by increasing concentrations of nonradioactive DHA, and the measured specific binding was subsequently recalculated to generate a saturation curve based on the dilution of the specific activity of [^{3}H]DHA (18). Assays were performed at 37° for 30 min in TSM buffer (20 mM Tris·HCl, pH 7.5, 50 mM sucrose, 20 mM $MgCl_2$). All binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes (5 ml) with ice-cold TSM buffer.

Northern analysis. Total RNA was extracted according to the acid guanidinium thiocyanate-phenol method of Chomczynski and Sacchi (19) using RNAzol B (Biogenesis, Poole, Dorset, UK). Purity and quantification of RNA were assessed by A_{260nm}/A_{280nm} ratios. Electrophoresis of RNA through agarose, transfer to Nytran+ (Amersham), and radiolabeled-cDNA probe Northern analyses were performed as previously described (20). Full-length β_2-adrenoceptor and $G_{s\alpha}$ cDNAs or a specific polymerase chain reaction-amplified fragment of the β-actin cDNA was [^{32}P]dCTP random-primed for use as probes in the Northern analyses.

Western analysis. Membrane proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [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 (21–23) were raised in New Zealand White rabbits after immunization with a glutaraldehyde conjugate of keyhole limpet hemocyanin and the synthetic peptides RMHLRQYELL and QLNLKEYNLV, which correspond to the carboxyl-terminal decapeptide regions of the $G_{s\alpha}$ isoforms and $G_{q\alpha}/G_{11\alpha}$, respectively.

Subcellular fractionation of clone 22 cells on sucrose density gradients. CAM β_2-adrenoceptor-expressing clone 22 cells either untreated or pretreated with betaxolol (10 μM for 24 hr; 3× 75-cm^2 flasks/sample) were harvested by centrifugation for 10 min at 900 × g; washed in 140 mM NaCl, 20 mM Tris·HCl, 3 mM $MgCl_2$, and 1 mM EDTA, pH 7.4; resuspended in TME homogenization buffer (20 mM Tris, 3 mM $MgCl_2$, 1 mM EDTA, pH 7.4); and frozen at −80° for ≥1 hr. After thawing, the samples were homogenized in a volume of 2 ml of TME buffer with the use of a Potter-Elvehjem Teflon-and-glass homogenizer (Philip Harris Scientific, Clydebank, Strathclyde, UK). The homogenate was layered on the top of a discontinuous sucrose density gradient consisting of (from top to bottom) 19% (5 ml), 23% (5 ml), 27% (5 ml), 31% (5 ml), 35% (5 ml), and 43% (10 ml) sucrose (all w/w); 20 mM Tris·HCl; 3 mM $MgCl_2$; and 1 mM EDTA, pH 8.0, according to Svoboda *et al.* (24). The gradient was centrifuged for 30 min at 27,000 rpm in a Beckman SW 28 rotor and fractionated manually from the meniscus (fractions 1–7, 5 ml each). The first 5 ml (fraction 1) represented an interphase between the overlaid homogenate and 19% (w/w) sucrose. The TME buffer was also used to resuspend the gradient pellet (fraction 8). The gradient fractions were frozen at −80° until use. Details of marker enzyme assays that were used to detect the presence of individual membrane fractions have been described in detail (24).

Data analysis. All binding data were analyzed using the Kaleidagraph curve-fitting program (version 2.1; Synergy Software, Reading, PA) with an Apple Macintosh computer.

Results

Neuroblastoma X glioma hybrid NG108-15 cells were transfected with a cDNA encoding the CAM β_2-adrenoceptor. A selection of clones were isolated and expanded. Northern blot analysis of RNA isolated from these clones and from parental NG108-15 cells to detect mRNA corresponding to the β_2-adrenoceptor showed the presence of varying levels of this message in clones 8, 21, 22, 24, and 34 but not in the parental cells (data not shown). Preparation of membranes from each of these clones allowed demonstration of varying levels of specific binding of the β-adrenoceptor antagonist [^{3}H]DHA (Fig. 1), with clone 22 displaying the highest levels. Parallel assessments of adenylyl cyclase activity, measured in the absence of agonist ligands, also showed variability among these clones, with those expressing higher steady state levels of the CAM β_2-adrenoceptor displaying higher

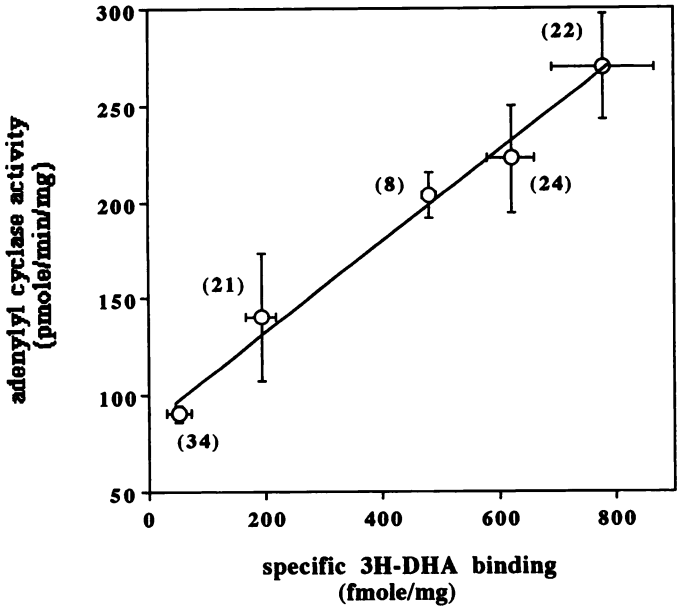

Fig. 1. Correlation between levels of expression of the CAM β_2-adrenoceptor and basal adenylyl cyclase activity. Membranes produced from various CAM β_2-adrenoceptor mRNA positive clones were used either in ligand binding assays to estimate the specific binding of a single concentration (2 nM) of [^{3}H]DHA or to measure basal adenylyl cyclase activity. Data are from a typical experiment in which both assays were performed on the same membrane preparations and represent mean ± standard deviation values of quadruplicate determinations. *Numbers in parentheses*, specific clones analyzed (see text). Two additional independent experiments produced similar results.

levels of adenylyl cyclase activity (Fig. 1). A high correlation (r^2 = 0.987) of these two parameters was observed across the individual clones (Fig. 1).

Previous studies have indicated that the CAM β_2-adrenoceptor construct displays higher affinity to bind agonist ligands than the wild-type receptor (1). We compared the ability of varying concentrations of isoprenaline to compete for the binding of [^{3}H]DHA to membranes of clone 22 (transfected to express the CAM β_2-adrenoceptor) and a clone derived from transfection of NG108-15 cells (βN22; Ref. 18), which expresses the wild-type β_2-adrenoceptor. Isoprenaline was ~10-fold more effective at the CAM β_2-adrenoceptor (IC_{50} corrected for receptor occupancy = 16 ± 5 nM) than at the wild-type β_2-adrenoceptor (130 ± 60 nM) (mean ± standard error for three experiments in each case) (Fig. 2, *top*). In contrast, but anticipated on the basis of results of previous studies (1), the ability of the β-adrenoceptor antagonist alprenolol to compete with [^{3}H]DHA for binding to the wild-type (estimated K_i = 13 ± 2 nM) and CAM (estimated K_i = 12 ± 4 nM) β_2-adrenoceptors was not different (three experiments in each case) (Fig. 2, *middle*). Betaxolol also displayed similar affinity to compete for the binding of [^{3}H]DHA at both the wild-type (estimated K_i = 580 ± 50 nM) and CAM (estimated K_i = 520 ± 80 nM) β_2-adrenoceptors (three experiments in each case) (Fig. 2, *bottom*). Comparison of the ability of isoprenaline to stimulate adenylyl cyclase activity in clones βN22 and 22 demonstrated that half-maximal stimulation in clone 22 required a lower concentration of agonist than in clone βN22 (Fig. 3). However, the maximal level of adenylyl cyclase activity that could be achieved in response to isoprenaline was similar in the two clones (which is a

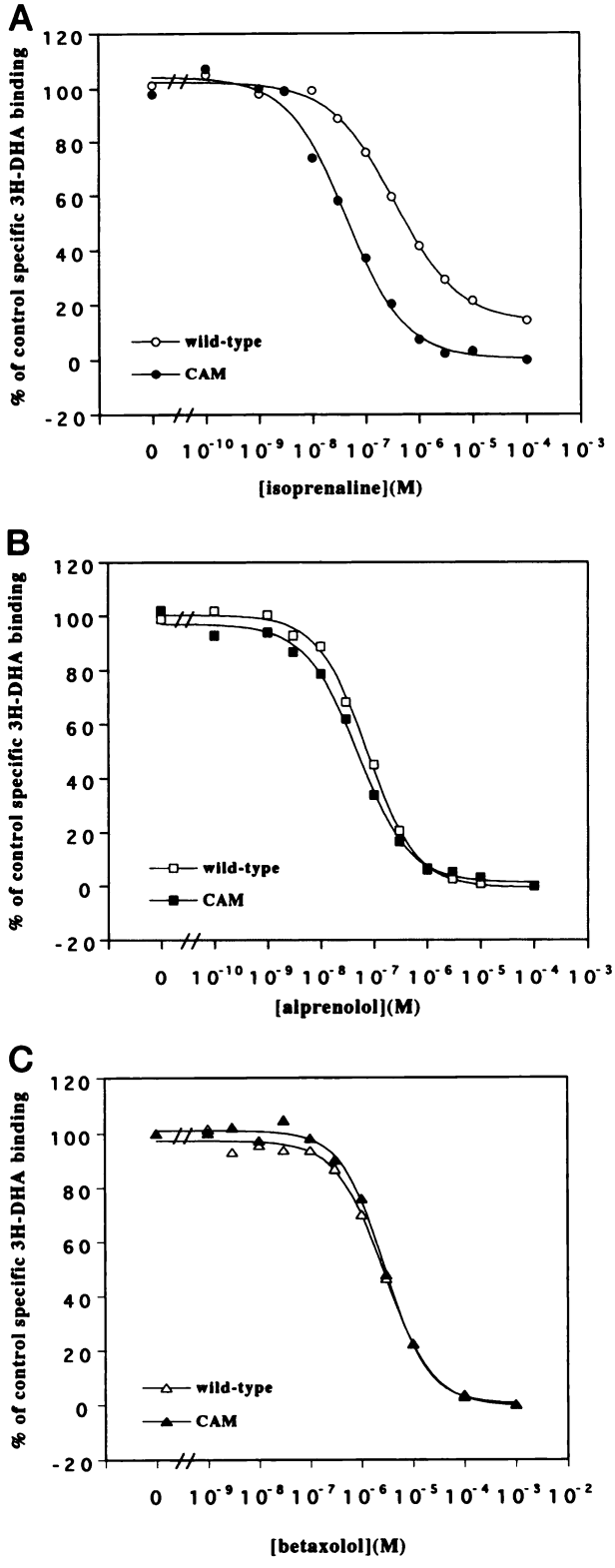

Fig. 2. Compared with the wild-type receptor, the CAM β_2-adrenoceptor has higher affinity for isoprenaline but not for alprenolol or betaxolol. The ability of varying concentrations of (*top*) isoprenaline, (*middle*) alprenolol, or (*bottom*) betaxolol to compete for the specific binding of 2 nM [^{3}H]DHA to membranes derived from either clone 22 (expressing the CAM β_2-adrenoceptor) or clone βN22 (expressing the wild-type β_2-adrenoceptor) was assessed as described in Experimental Procedures. Results represent mean values of duplicate determinations; the experiment shown is typical of three independent experiments that gave similar results (see text for quantitative details).

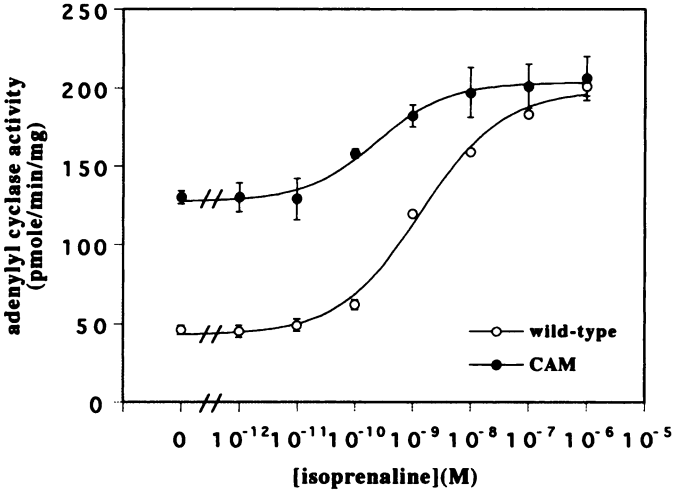

Fig. 3. Isoprenaline can produce similar maximal levels of adenylyl cyclase activity in membranes of wild-type- and CAM β_2-adrenoceptor-expressing NG108-15 cells. Basal adenylyl cyclase activity and its regulation by varying concentrations of isoprenaline were measured in membranes of clones 22 (●) and βN22 (○). Results represent mean ± standard deviation of triplicate determinations; the experiment shown is typical of at least three other independent experiments that gave similar results. The EC_{50} value was 1.4 ± 0.4 nM for isoprenaline stimulation of adenylyl cyclase in the wild-type receptor-expressing cells and 230 ± 80 pM for the CAM receptor-expressing cells.

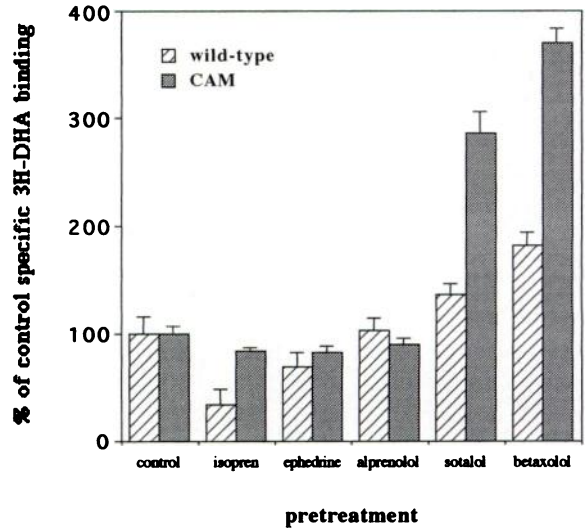

Fig. 4. Up-regulation of the CAM β_2-adrenoceptor after exposure to betaxolol. Cells of clones 22 (*CAM*) and βN22 (*wild-type*) were exposed to vehicle (control) or 10 μM isoprenaline, ephedrine, alprenolol, sotalol, or betaxolol for 24 hr. Membranes were prepared, and the specific binding of 2 nM [^{3}H]DHA was measured. Results are presented relative to the levels measured in the vehicle-treated cells and represent mean ± standard deviation of quadruplicate determinations. The experiment shown is typical of at least two other independent experiments. In the experiment shown, basal levels of specific [^{3}H]DHA binding were 1272 ± 196 fmol/mg of protein for wild-type receptor and 705 ± 48 fmol/mg of protein for CAM β_2-adrenoceptor (mean ± standard deviation of quadruplicate assays).

reflection of the fact that adenylyl cyclase is the limiting component for stimulatory regulation of this cascade in NG108-15 cells; Refs. 13 and 18); therefore, because the basal activity was substantially higher in membranes of clone 22, the actual increment of increase of adenylyl cyclase activity that could be achieved with isoprenaline was actually substantially lower in the CAM β_2-adrenoceptor-expressing membranes than in those expressing the wild-type β_2-adrenoceptor (Fig. 3).

Sustained (24 hr) treatment of the wild-type β_2-adrenoceptor-expressing cells with isoprenaline (10 μM) resulted in a substantial reduction in measurable membrane levels of the receptor (Fig. 4). The β-adrenoceptor partial agonist ephedrine (10 μM) was also able to cause down-regulation of the wild-type receptor but to a substantially lesser extent than isoprenaline (Fig. 4). Treatment with the β-adrenoceptor antagonist alprenolol had no significant effect on levels of this receptor, but equivalent treatment with betaxolol resulted in a significant up-regulation of levels of the wild-type receptor. Parallel treatment of clone 22 cells with betaxolol also resulted in up-regulation of the CAM β_2-adrenoceptor (Fig. 4), but the maximal effect of this ligand was now very much greater than that in cells expressing the wild-type receptor (Fig. 4). Betaxolol (24-hr treatment)-induced up-regulation of CAM β_2-adrenoceptor levels was also observed in the other CAM β_2-adrenoceptor-expressing clones (8, 21, 24, and 34) described (see Fig. 1), indicating that the CAM β_2-adrenoceptor up-regulation in response to betaxolol could be seen over a range of steady state receptor levels and in a number of individual clones (data not shown). Furthermore, this up-regulation of CAM β_2-adrenoceptor levels in clone 22 was not restricted to betaxolol. The β-blocker sotalol was also capable of markedly increasing levels of the CAM β_2-adrenoceptor expressed in clone 22 (Fig. 4).

Because betaxolol produced a large up-regulation of the CAM β_2-adrenoceptor, we examined this effect over a range of concentrations of this ligand. Over a 24-hr treatment period, half-maximal up-regulation was produced by 200 ± 80 nM betaxolol (mean ± standard deviation, five experiments; Fig. 5, *top*). Betaxolol was clearly capable of acting as an inverse agonist at this receptor as measured by its ability to inhibit basal adenylyl cyclase activity in membranes of clone 22 (Fig. 5, *bottom*), with the half-maximal effect of betaxolol in this assay at 250 ± 150 nM (three experiments). Because the estimated K_i value for betaxolol at the CAM β_2-adrenoceptor was ~520 nM (Fig. 2, *bottom*), both the induced up-regulation of CAM β_2-adrenoceptor levels and its inverse agonist properties were closely correlated with receptor occupancy. To confirm that the enhanced binding of a single concentration of [^{3}H]DHA in membranes of betaxolol-treated clone 22 cells (Figs. 4 and 5, *top*) truly represented an increase in receptor density, saturation binding analyses were conducted using a range of [^{3}H]DHA concentrations. As shown in Fig. 6, conversion of these saturation curves to Scatchard plots demonstrated the total number of receptor sites to be increased greatly after betaxolol treatment (untreated, 750 ± 110 fmol/mg of protein; betaxolol treated, 3970 ± 970 fmol/mg of protein; mean ± standard error for four experiments in each case), whereas the affinity for [^{3}H]DHA for the CAM β_2-adrenoceptor was similar after treatment with (K_d = 1.1 ± 0.3 nM) or without (K_d = 0.9 ± 0.4 nM) betaxolol (four experiments in each case). Time course studies indicated that the bulk of up-regulation of the CAM β_2-adrenoceptor in response to the presence of betaxolol was produced within 24 hr (Fig. 7). Furthermore, this betaxolol-stimulated up-regulation of the CAM β_2-adrenoceptor seemed to require *de novo* protein synthesis because concur-

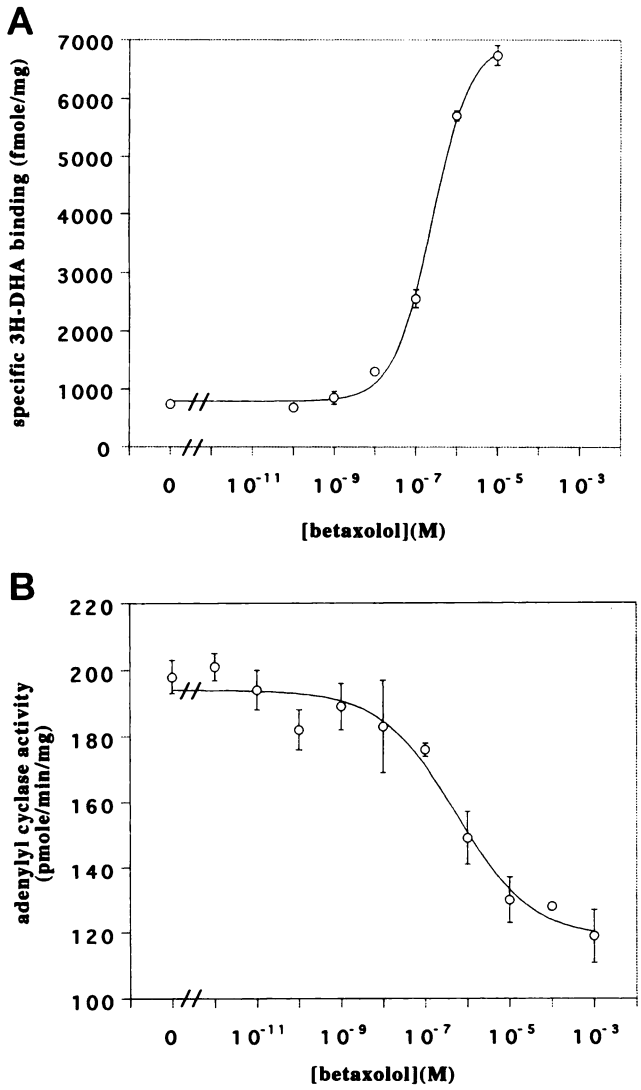

Fig. 5. Similarities in the concentration-effect curves for betaxolol-mediated inhibition of basal adenylyl cyclase activity and up-regulation of the CAM β_2-adrenoceptor. *Top*, the concentration-dependence (24-hr treatment) of betaxolol-mediated up-regulation of the CAM β_2-adrenoceptor in clone 22 was assessed by measuring the specific binding of [^{3}H]DHA. *Bottom*, basal adenylyl cyclase activity and its regulation by varying concentrations of betaxolol were measured in membranes prepared from clone 22 cells. Results represent mean ± standard deviation of triplicate determinations. The experiment shown is typical of at least three other independent experiments.

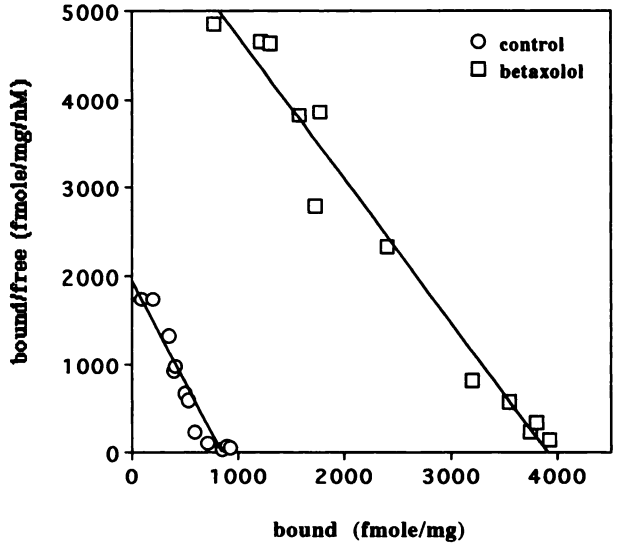

Fig. 6. Betaxolol treatment produces an up-regulation of the CAM β_2-adrenoceptor without altering the affinity of [^{3}H]DHA binding. Clone 22 cells were incubated for 24 hr with or without betaxolol (10 μM), and then membranes were prepared. Saturation binding studies were performed with [^{3}H]DHA. The specific binding data from a typical experiment are displayed as a Scatchard plot. Results represent mean of duplicate determinations. The experiment shown is typical of at least three other independent experiment. In the experiment shown, the estimated K_d value for [^{3}H]DHA was 0.43 nM in the untreated cells and 0.62 nM in the betaxolol-treated samples.

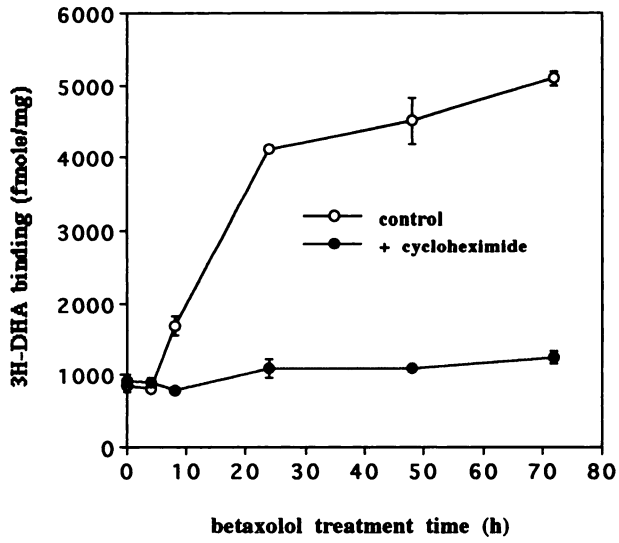

Fig. 7. Betaxolol-induced up-regulation of the CAM β_2-adrenoceptor is time dependent and requires new protein synthesis. Clone 22 cells were treated for varying times with either betaxolol (10 μM) alone or betaxolol plus cycloheximide (50 μg/ml). Membranes prepared from these cells were then assessed for levels of the CAM β_2-adrenoceptor as measured by the specific binding of [^{3}H]DHA. Results represent mean ± standard deviation of quadruplicate determinations. The experiment shown is typical of three independent experiments that gave similar results.

rent treatment with cycloheximide (50 μg/ml) almost completely prevented the up-regulation (Fig. 7). Not surprisingly, increases in basal adenylyl cyclase activity were also observed with betaxolol treatment of clone 22 cells that were statistically significant after treatment with 1 and 10 μM betaxolol (Fig. 8). Half-maximal increases were achieved with ~100 nM betaxolol. Despite the large increase in CAM β_2-adrenoceptor levels in these cells in response to betaxolol treatment, the maximal level of adenylyl cyclase activity that could be achieved through treatment of the membranes of these cells with a maximally effective concentration of isoprenaline did not increase (Fig. 8). The same was true when the endogenously expressed IP prostanoid receptor was occupied by the agonist iloprost (Fig. 8). Given that basal adenylyl activity in the untreated clone 22 cells was high due to the expression of the CAM β_2-adrenoceptor, that the presence of the increased levels of this receptor after betaxolol treatment failed to result in membrane preparations in which the adenylyl cyclase activity became completely adrenergic agonist independent was surprising (Fig. 8). In contrast to the up-regulation of the CAM β_2-adrenoceptor after betaxolol treatment, the total levels of adenylyl cyclase protein

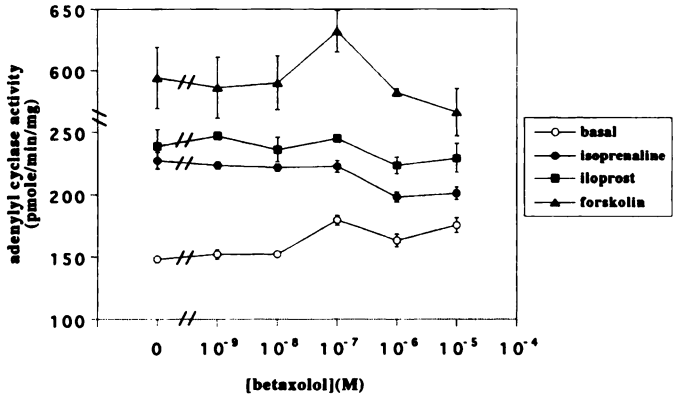

Fig. 8. Betaxolol treatment of CAM β_2-adrenoceptor-expressing cells increases basal but not agonist-regulated adenylyl cyclase activity. Clone 22 cells were exposed to varying concentrations of betaxolol for 24 hr. Membranes were subsequently prepared, and basal and 10 μM isoprenaline-, 10 μM iloprost-, and 100 μM forskolin-stimulated adenylyl cyclase activities were measured. Results represent mean ± standard deviation of triplicate determinations. The experiment shown is typical of three independent experiments. A statistically significant increase in the basal cyclase activity was observed in the 1 and 10 μM betaxolol-treated samples ($p < 0.05$) compared with the control values. No other samples displayed statistically significant differences compared with the controls.

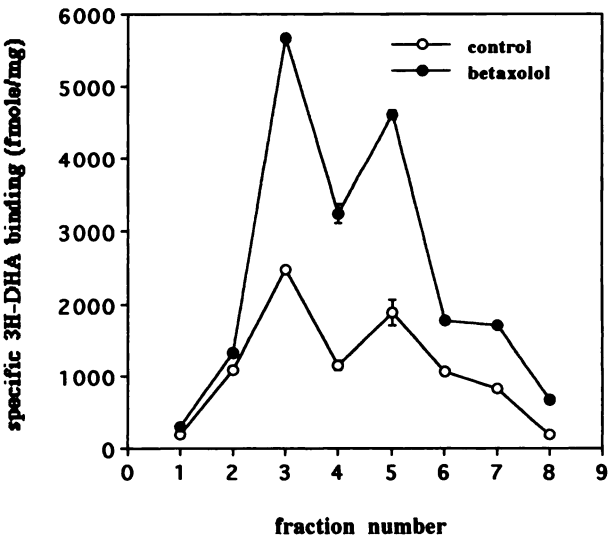

Fig. 9. Betaxolol treatment of CAM β_2-adrenoceptor-expressing cells up-regulates the receptor but does not alter its gross cellular distribution. Clone 22 cells were treated for 24 hr with or without 10 μM betaxolol and then fractionated on a sucrose density gradient as described in Experimental Procedures. Plasma membrane markers were enriched in fraction 3. Fraction 8 is the insoluble and nuclear pellet remaining after centrifugation. Data represent mean ± standard deviation of triplicate determinations. The experiment shown is representative of three independent experiments.

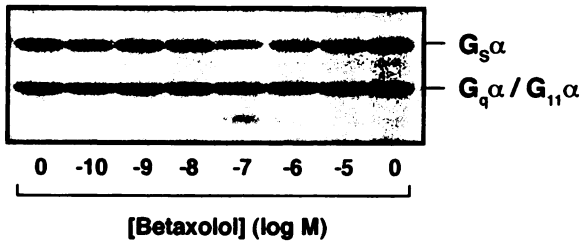

Fig. 10. Betaxolol treatment of CAM β_2-adrenoceptor-expressing cells does not alter membrane levels of $G_{s\alpha}$. Clone 22 cells were exposed to varying concentrations of betaxolol for 24 hr. Membranes (25 μg of protein/lane) prepared from these cells were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunoblotted to detect the presence of both $G_{s\alpha}$ and a combination of the phosphoinositidase C-linked G proteins $G_{q\alpha}$ and $G_{11\alpha}$. The experiment shown is typical of at least four other independent experiments that gave similar results.

in these cells (as determined through direct forskolin stimulation of adenylyl cyclase activity) were not increased by pretreatment with the inverse agonist (Fig. 8). In an attempt to explore whether this reflected a lack of appropriate localization/targeting of the up-regulated CAM β_2-adrenoceptor to the plasma membrane, whole-cell lysates of untreated and betaxolol-treated (10 μM) clone 22 cells were fractionated by sucrose density sedimentation. Two distinct peaks of [^{3}H]DHA binding were observed in the untreated cells, centered on fractions that we have previously shown to be enriched in plasma membrane and light vesicular membranes (24). Betaxolol treatment, while causing a large up-regulation of specific [^{3}H]DHA binding, did not seem to change the relative cellular distribution profile (Fig. 9).

Immunologically detectable membrane levels of $G_{s\alpha}$ were not significantly altered by betaxolol treatment in parallel with CAM β_2-adrenoceptor levels (Fig. 10), indicating that the increase in adenylyl cyclase activity was not due to increases in cellular G protein levels. Equally, no detectable changes in membrane levels of the phosphoinositidase C-linked G proteins $G_{q\alpha}/G_{11\alpha}$ were noted in response to betaxolol (Fig. 10). Northern blot analysis of RNA isolated from clone 22 cells that had been treated with varying concentrations of betaxolol for 7–14 hr showed no obvious steady state alteration in the mRNA encoding either the CAM β_2-adrenoceptor or $G_{s\alpha}$ (Fig. 11 and data not shown).

Discussion

The concept that CAM variants of G protein-coupled receptors represent at least a partial model of the activated (R*) state of a receptor (because they are able to produce greater activation of G proteins in a partly agonist-independent manner) suggests that they might provide the basis of useful systems with which to analyze the properties and mechanisms of action of inverse agonist ligands. In the current study, we assessed this for the β-adrenoceptor inverse agonist betaxolol using clones of NG108-15 cells transfected to express either the human wild-type or CAM β_2-adrenoceptor. Betaxolol was developed as a β_1-adrenoceptor selective antagonist, and it certainly displays good selectivity (10–100-fold) in affinity for this receptor compared with the β_2-adrenoceptor. However, as shown in Fig. 5, *bottom*, betaxolol acts as an effective inverse agonist at the CAM β_2-adrenoceptor and is able to cause a large, concentration-dependent inhibition of basal adenylyl cyclase activity.

Down-regulation of G protein-coupled receptors as a response to the maintained presence of agonist ligands is a well-established and -examined phenomenon. These effects seem to be produced by a combination of enhanced degradation of the receptor polypeptide and destabilization of receptor mRNA (25, 26). As such, it might be intuitively attractive to anticipate that inverse agonist ligands would cause an up-regulation of receptors. However, studies of the 5-hydroxytryptamine$_{2C}$ receptor have indicated that a num-

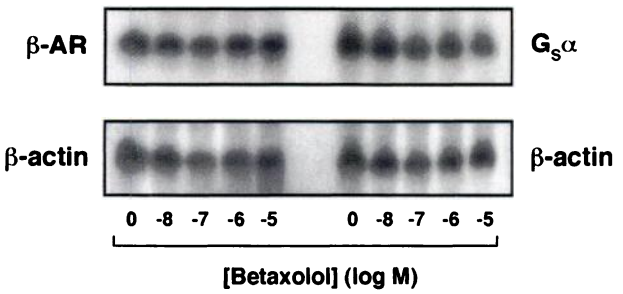

Fig. 11. Betaxolol treatment does not alter steady state CAM β_2-adrenoceptor mRNA levels. Clone 22 cells were exposed to varying concentrations of betaxolol for 14 hr. RNA was subsequently isolated from these cells, and after electrophoresis, relative mRNA levels were measured corresponding to the CAM β_2-adrenoceptor, $G_{s\alpha}$, and β actin. The experiment shown is typical of at least two other independent experiment that gave similar results. Shorter times of incubation (≥7-hr treatment) with betaxolol gave essentially the same results (data not shown).

ber of ligands that can be shown to have inverse agonist activity at this receptor actually cause down-regulation of the receptor on chronic exposure (27, 28), although these two properties may not be fundamentally linked (28).

The maintained presence of betaxolol in the medium surrounding the CAM β_2-adrenoceptor-expressing NG108-15 cells was able to cause a dramatic up-regulation of levels of this receptor as assessed in [^{3}H]DHA saturation binding analyses (Fig. 6), and a similar concentration of betaxolol was required to produce half-maximal increases in receptor levels as half-maximal inhibition of basal adenylyl cyclase activity (Fig. 5). An increase in levels of the wild-type β_2-adrenoceptor was also observed after sustained treatment of clone βN22 with betaxolol, but the effect of the drug was much less pronounced than that with the CAM β_2-adrenoceptor (Fig. 4). Pei *et al.* (29) noted an approximate doubling in the levels of the CAM β_2-adrenoceptor after treatment with 10 μM betaxolol of Chinese hamster ovary cells harboring this receptor. Interestingly, in their study, this was accompanied by a substantial increase in the maximal adenylyl cyclase response to isoprenaline, with perhaps a slight increase in potency of the agonist (29). In contrast, in the current study, although basal adenylyl cyclase activity increased in response to betaxolol in a concentration-dependent manner (Fig. 8), the maximal response to either isoprenaline or iloprost (acting at the endogenously expressed IP prostanoid receptor) was not increased (Fig. 8). Indeed, if presented as fold-stimulation of the basal adenylyl cyclase activity, the effects of the receptor ligands actually declined in membranes from the betaxolol-treated cells. The likely explanation for these results is that we have previously shown adenylyl cyclase to be quantitatively the limiting protein component of the stimulatory adenylyl cyclase cascade in NG108-15 cells (19, 30, 31). As shown in Fig. 3, in which we compared the ability of isoprenaline to regulate adenylyl cyclase activity in clones expressing either the wild-type or CAM β_2-adrenoceptor, very similar maximal adenylyl cyclase activity could be achieved but from a lower base-line activity in the membranes expressing the wild-type receptor. As such, a ~4-fold stimulation of adenylyl cyclase activity can be observed in response to isoprenaline in the wild-type receptor-expressing cells, whereas the maximal stimulation by isoprenaline is <2-fold in membranes of the CAM β_2-adrenoceptor-expressing cells. Therefore, it is not surprising that the apparent effect of isoprenaline becomes even more diminished as levels of the CAM β_2-adrenoceptor increase in response to betaxolol treatment. We are rather surprised, however, given the strong up-regulation in receptor levels achieved in response to betaxolol treatment, that the maximal adenylyl cyclase activity in membranes of these cells did not become entirely independent of agonist (Fig. 8). This was not a reflection of the fact that the elevated steady state amounts of the CAM β_2-adrenoceptor produced by betaxolol treatment (as measured in [^{3}H]DHA-binding studies) were not targeted to the plasma membrane. After resolution of whole-cell lysates of untreated and betaxolol-treated clone 22 cells by sucrose density sedimentation, the overall cellular distribution of the receptors was not appreciably altered (Fig. 9).

We previously noted the ability of isoprenaline to cause down-regulation of $G_{s\alpha}$ in clones of cells expressing the wild-type β_2-adrenoceptor (14, 32). Indeed, down-regulation of the G proteins activated by particular receptors is a commonly observed feature as long as levels of the receptor are such that a significant fraction of the G protein population of the cells is engaged and activated by the receptor (32). Based on these observations, we examined whether prolonged treatment with betaxolol would result in an equivalent up-regulation of G_s. No significant increase in $G_{s\alpha}$ (or, indeed, of the phospholipase Cβ-coupled G proteins, $G_{q\alpha}/G_{1\alpha}$, levels of which were measured as a control for these studies) was observed (Fig. 10). $G_{s\alpha}$ is expressed by NG108-15 at close to 1 million copies/cell (30). As such, observation of a significant increase in response to betaxolol would require that the effect be pronounced, and it may be that relative intensity immunoblotting is insufficiently accurate and quantitative to record a small change in cellular levels.

Betaxolol-induced increases in levels of the CAM β_2-adrenoceptor were almost completely prevented by coincubation with the protein synthesis inhibitor cycloheximide (Fig. 8), implying a requirement for *de novo* protein synthesis. However, the global inhibition of protein synthesis associated with cycloheximide treatment does not allow analysis of whether synthesis of the receptor itself or of proteins other than the receptor might be required to produce the observed effects of betaxolol on the cellular levels of the CAM β_2-adrenoceptor.

In an attempt to determine whether greater levels of CAM β_2-adrenoceptor might reflect an increase in transcription of the CAM β_2-adrenoceptor cDNA or stabilization of the mRNA, we performed Northern analysis on RNA isolated from cells exposed to varying concentrations of betaxolol for different periods of time. No obvious increases in levels of CAM β_2-adrenoceptor mRNA were observed, and the same was true for levels of $G_{s\alpha}$ mRNA (Fig. 11) (there seemed to be a small reduction in $G_{s\alpha}$ message, but as noted above, this was not reflected in alterations in protein levels). There have been a number of observations that indicated that agonist-induced down-regulation of the β_2-adrenoceptor can be mediated, at least in part, by destabilization of the receptor mRNA (33–35). This may be mediated by a 35-kDa polypeptide that is up-regulated on sustained challenge with β agonists and has been reported to bind selectively to β-adrenoceptor mRNA (36). As such, a possible explanation for betaxolol-induced up-regulation of the CAM β_2-adrenoceptor

would be stabilization of the mRNA (for reviews, see Refs. 37 and 38). In the absence of other regulatory features, such an effect would be likely to result in an increase in steady state β_2-adrenoceptor mRNA levels. However, as noted above, this was not observed (Fig. 11). The combination of the effect of cycloheximide and the lack of alteration in steady state levels of receptor mRNA suggests that the most likely locus for regulatory control of the amounts of the CAM β_2-adrenoceptor is at the level of translational control or that the inverse agonist causes stabilization of the receptor from proteolytic degradation. Future studies will address this issue.

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