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Generation and Analysis of Constitutively Active and Physically Destabilized Mutants of the Human β_1-Adrenoceptor

ALISON J. MCLEAN, FU-YUE ZENG, DOMINIC BEHAN, DEREK CHALMERS, and GRAEME MILLIGAN

Molecular Pharmacology Group (A.J.M., G.M.), Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow, Scotland, United Kingdom; and Arena Pharmaceuticals (F.-Y.Z., D.B., D.C.), San Diego, California

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ABSTRACT

Constitutive activity of wild-type and mutant forms of human β_1- and β_2-adrenoceptors was measured by guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) binding assays using fusion proteins between these receptors and $G_s\alpha$. Constitutive activity of the β_1-adrenoceptor is enhanced by mutation of Leu^{322}. The ability of ligands to suppress receptor instability and produce up-regulation is often associated with constitutively active mutants. Leu^{322}Lysβ_1-adrenoceptor, but not wild type, was up-regulated by exposure to the β_1-adrenoceptor selective blocker betaxolol. More extensive sequence alterations of the β_1-adrenoceptor were generated to mimic the initially described constitutively active mutant (CAM) of the β_2-adrenoceptor that is up-regulated strongly by betaxolol. Substitution of amino acids 316 to 324 of the β_1-adrenoceptor with the equivalent α_{1b}-adrenoceptor sequence did not result in up-regulation by betaxolol. However, these forms of both β_1- and β_2-adrenoceptors displayed substantial and equivalent constitutive activity. The addition of the Leu^{322}Lys mutation into the α_{1b}-adrenoceptor substituted β_1-adrenoceptor to produce the CAMKβ_1-adrenoceptor allowed substantially greater levels of up-regulation by betaxolol without enhancement of constitutive [^{35}S]GTPγS binding. Arg^{156}Alaβ_1-adrenoceptor was up-regulated strongly by betaxolol but displayed lower constitutive activity than did other mutants. Binding of [^{35}S]GTPγS binding to all the fusion proteins was increased substantially by isoprenaline. Despite the ability of betaxolol to cause up-regulation of many mutants, only for the CAMβ_2-adrenoceptor-$G_s\alpha$ and CAMKβ_1-adrenoceptor-$G_s\alpha$ fusion proteins was the basal binding of [^{35}S]GTPγS decreased by betaxolol. Clear resolution between receptor constitutive activity and ligand suppression of receptor instability can be obtained for mutant β-adrenoceptors, and potential inverse agonists do not function equally at phenotypically apparently equivalent CAM receptors.

Although the β_1- and β_2-adrenoceptors respond to the same natural ligands, are highly homologous, are often coexpressed, and can both elevate intracellular levels of cAMP, they have a number of distinct properties. These include their different distributions in cells in which they are coexpressed (Rybin et al., 2000; Steinberg and Brunton, 2001) and potentially in their effectiveness of coupling to the G protein $G_s\alpha$ (Levy et al., 1993), although this has not been observed in all studies (Guerrero and Minneman, 1999). Another point of difference is in their degree of agonist-independent or constitutive activity. In general, higher levels of agonist-independent signal transduction are noted for the β_2-adrenoceptor than for the β_1-adrenoceptor (Zhang et al., 2000; Zhou et al., 2000; Engelhardt et al., 2001). The degree of constitutive activity of many receptors can be enhanced by mutation in a number of regions of the sequence (Leurs et al., 1998; Pauwels and Wurch, 1998; Gether, 2000). In the case of the β_2-adrenoceptor, such studies have concentrated on alterations at the interface of the third intracellular loop and the sixth transmembrane domain. Although less studied, mutation of a single amino acid in this region (Leu^{322}) of the β_1-adrenoceptor can also result in enhanced constitutive activity (Lattion et al., 1999). The most studied constitutively active mutant (CAM) of the β_2-adrenoceptor was produced by the replacement of a short segment of the distal region of the third intracellular loop with the homologous region from the α_{1b}-adrenoceptor (Samama et al., 1993). An interesting feature of this receptor is that it is physically destabilized compared with the wild-type β_2-adrenoceptor (Gether et al., 1997), and this can be suppressed by the binding of ligands. Thus in the face of ongoing synthesis, the addition of β_2-adrenoceptor ligands to cells expressing the CAMβ_2-adrenoceptor results in up-regulation of the polypeptide (Pei et al., 1994; MacEwan and Milligan, 1996; McLean et al., 1999; Ramsay et al., 2001). This has been demonstrated to provide a useful

ABBREVIATIONS: [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; CAM, constitutively active mutant; GPCR, G protein–coupled receptor; VSV, vesicular stomatitis virus; PCR, polymerase chain reaction; WT, wild-type; HEK, human embryonic kidney; PBS, phosphate-buffered saline; MOPS, 4-morpholinepropanesulfonic acid; CS, C terminus of $G_s\alpha$; ICI118551, (±)-1-(2,3-[dihydro-7-methyl-1H-inden-4-yl]oxy)-3-([1-methylethyl]-amino)-2-butanol.

means to identify, without any *a prioiri* knowledge, ligands that interact with the receptor (Milligan et al., 2002).

In this study, we generated and analyzed markedly destabilized mutants of the human β_1-adrenoceptor and developed a novel [^{35}S]GTPγS binding assay to measure the constitutive activity of such mutants that is based on the immunoprecipitation of receptor-$G_s\alpha$ fusion proteins (Stevens et al., 2001). Parallel use of these two strategies overcomes the historical limitations in attempts to measure receptor-dependent guanine nucleotide exchange on $G_s\alpha$ (Wieland and Jakobs, 1994) and allows the determination of the relative constitutive activity of different receptor mutants.

Materials and Methods

Materials. All materials for tissue culture were supplied by Invitrogen (Carlsbad, CA). [^{3}H]Dihydroalprenolol (40 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were from PerkinElmer Life Sciences (Boston, MA). Oligonucleotides were purchased from Interactiva (Ulm, Germany). Sources of all other reagents have been described previously (Stevens et al., 2000, 2001).

Construction of Mutants and Fusion Proteins. CAM mutations within the human WT β_1-adrenoceptor were generated using a three-reaction PCR approach. Boldface type represents the nucleotides designed to introduce the desired mutations. First, PCR [95°C for 5 min (95°C for 1 min, 51°C for 1 min, and 73°C for 2 min) for 20 cycles; 73°C for 10 min) on WT β_1-adrenoceptor cDNA with a forward mutagenic primer (Leu^{322}Lysβ_1-adrenoceptor, CTC GTG GCC CTA CGA CGC GAG CAG AAG GCG **AAA** AAG ACG; CAMβ_1-adrenoceptor, CTC GTG GCC **TCA** CGC GAG **AAG** AAG GCG **GCC**AAG ACG; or CAMKβ_1-adrenoceptor, CTC GTG GCC **TAC** CGC GAG **AAG** AAG GCG **AAA** AAG ACG) and a reverse primer tagged with a VSV sequence (Feng et al., 2002), also containing a unique restriction site, *Xho*I, and a silent mutation creating a new unique restriction site, *Pvu*II (GAT ACT GGG CTA TCC GCT CGA GTC GCT GTC CGC AGC TGC CCC), generated a super primer used in a subsequent PCR extension reaction. This reaction (95°C for 6 min, 51°C for 1 min, and 73°C for 12 min) generated an amplicon or template for the third and last PCR reaction [95°C for 5 min (95°C for 1 min, 42°C for 1 min, and 73°C for 1 min) for 20 cycles; 73°C for 10 min). This generated a final product of 600 bases, using a forward primer with a unique restriction site, *Not*I (CCA GCG CGG CCG CCC), and a VSV reverse primer (GAT ACT GGG CTA TCC). This product was digested with *Not*I and *Xho*I and subsequently ligated into human β_1-adrenoceptor cDNA, which was also digested with *Not*I and *Xho*I.

The Arg^{156}Ala β_1-adrenoceptor mutant was generated using the same principle. The mutagenic primer used was CAT TGC CCT GGA C***GC C***TA CCT CGC CAT, and the reverse primer was tagged with a VSV sequence and contained a unique restriction site, *Not*I, and a silent mutation creating a new unique restriction site, *Apa*I (GAT ACT GGG CTA TCC GCG GCC GCG CGG GCC CGC CGA). The forward primer used in the third PCR reaction contained a FLAG sequence and a unique *Hind* III site (AAA AAA AAG CTT GCC ACC ATG GAC TAC AAG GAC GAC GAT GAT AAG GGC GCG GGG GTG CTG).

A CAMβ_2-adrenoceptor-$G_s\alpha$ (long isoform) fusion protein was generated using the $G_s\alpha$ portion of a WT β_2-adrenoceptor-$G_s\alpha$ construct in a PCR reaction [95°C for 5 min (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min) for 20 cycles; 72°C for 10 min) with forward *Xho*I primer (AAA AAC TCG AGG GCT GCC TCG GCA ACA GTA AG) and a reverse *Xba*I primer (AAA AAT CTA GAT TAG AGC AGC TCG TAT TG). Consequently, a CAMβ_2-adrenoceptor–*Renilla reniformis* luciferase construct (Ramsay et al., 2001) was digested with *Xho*I and *Xba*I to eliminate *R. reniformis* luciferase to ligate the digested $G_s\alpha$ fragment from the PCR in-frame with the CAMβ_2-adrenoceptor.

WT β_1-adrenoceptor-$G_s\alpha$ and Arg^{156}Alaβ_1-adrenoceptor-$G_s\alpha$ were generated by PCR [95°C for 5 min (95°C for 1 min, 60°C for 1 min, and 72°C for 2 min) for 20 cycles; 72°C for 10 min] of WT β_1-adrenoceptor and Arg^{156}Alaβ_1-adrenoceptor, respectively, with forward *Nhe*I primer (AAA AAG CTA GCG CCA CCA TGG ATA CTG GGC TAT CCG GCG CGG GGG TGC TC) and reverse *Kpn*I primer (AAA AAA GGT ACC CAC CTT GGA TTC CGA GGC) followed by the digestion of product with these two enzymes. The $G_s\alpha$ portion of a WT β_1-adrenoceptor-$G_s\alpha$ fusion construct was used in a PCR reaction [95°C for 5 min (95°C for 1 min, 55°C for 1 min, and 72°C for 1 min) for 20 cycles; 72°C for 10 min[with forward *Kpn*I primer (AAA AAA GGT ACC GGC TGC CTC GGC AAC AGT AAG) and a reverse *Xba*I primer (AAA AAT CTA GAT TAG AGC AGC TCG TAT TG). The $G_s\alpha$ 1-Kb product was digested with *Kpn*I and *Xba*I and ligated along with WT β_1-adrenoceptor into pcDNA3.1(+) digested with *Nhe*I and *Xba*I. The new WT β_1-adrenoceptor-$G_s\alpha$ construct was now digested with *Not*I and *Xho*I to insert the mutant CAMβ_1-adrenoceptor fragments generated previously.

Transient Transfection of HEK293 Cells. HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 0.292 g/l L-glutamine and 10% (v/v) newborn calf serum at 37°C in a 5% CO_2 humidified atmosphere. Cells were grown to 60 to 80% confluence before transient transfection in 100-mm dishes. Transfection was performed with the use of 5 μg of cDNA construct using LipofectAMINE reagent (Invitrogen) according to the manufacturer's instructions.

Up-Regulation Studies and Sample Preparation. Transiently transfected cells were treated with or without ligand in cell culture medium (concentrations are indicated in figure legends) for 0 to 72 h. Cell monolayers were washed three times in PBS (2.7 mM KCl, 137 mM NaCl, and 1.5 mM KH_2PO_4, pH 7.4) and then scraped into tubes and centrifuged for 10 min at 4000 rpm. Cell pellets were ruptured in Tris/EDTA buffer (10 mM Tris, and 0.1 mM EDTA, pH 7.4) by 50 passes of a glass-on-Teflon homogenizer. The resulting suspension was centrifuged at 1200 rpm for 10 min at 4°C. The supernatant was subsequently centrifuged at 50,000 rpm for 30 min at 4°C. The resulting pellet was resuspended through a syringe and attached to a 25-gauge needle. Membranes were quantified, placed into aliquots accordingly, and stored at −80°C.

Immunoblotting. Membrane sample (40 μg) prepared in Laemmli buffer was loaded onto a 1-mm thick 4 to 12% Bis-Tris precast gel (Invitrogen) and electrophoresed in MOPS buffer for approximately 1 h at 200 V. Protein was transferred from the gel to nitrocellulose membrane for 1 h at 30 V. The membrane was blocked overnight in 5% (w/v) nonfat milk at 4°C. Once washed briefly in PBS 0.1% and Tween 20, the membrane was exposed to anti-β_1-adrenoceptor antiserum (Santa Cruz Biochemicals, Santa Cruz, CA) at a dilution of 1:1000 in 1% (w/v) nonfat milk for 2 h at room temperature. After washing the membrane 3 times in PBS and 0.1% Tween 20, it was then exposed to secondary anti-rabbit antiserum (Amersham Biosciences Inc., Piscataway, NJ) at a dilution of 1:10000 for 1 h. Again, the membrane was washed thoroughly. To develop the blot, enhanced chemiluminescence reagent was added to the membrane for 5 min. The membrane was then exposed to film, and the film was developed.

[^{3}H]Dihydroalprenolol Binding Studies. Saturation binding studies were performed using Tris/EDTA/MgCl buffer (75 mM Tris, 1 mM EDTA, and 12.5 mM $MgCl_2$, pH 7.4) in 96-well blocks using 5 to 20 μg of membrane preparation with 0.1 to 10 nM [^{3}H]dihydroalprenolol and 10 μM propranolol or betaxolol for nonspecific binding at the β_2- and β_1-adrenoceptor constructs, respectively. For displacement binding studies, 0.5 or 1.0 nM [^{3}H]dihydroalprenolol was used for the β_2- and β_1-adrenoceptor constructs, respectively, along with a range of concentrations of isoprenaline (10^{-10}-10^{-3} M). After incubation at 30°C for 45 min, samples were harvested onto 96-well filters with ice-cold Tris/EDTA buffer (75 mM Tris, and 1 mM EDTA, pH 7.4). Once soaked in scintillation fluid for 1 h or more, the filters were counted in a Packard Top Count scintillation counter (Hewlett Packard, Palo Alto, CA). Because all studies were performed on crude membrane preparations, the data represent the full cellular

receptor complement and not only receptors present at the cell surface.

[^{35}S]GTPγS Binding. [^{35}S]GTPγS binding experiments were initiated by the addition of membranes containing 10 fmol of the β-adrenoceptor-$G_s\alpha$ fusion constructs to an assay buffer (20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, 100 mM NaCl, 1 μM GDP, 0.2 mM ascorbic acid, and 50 nCi [^{35}S]GTPγS) containing the indicated concentrations of receptor ligands. Nonspecific binding was determined in the same conditions but in the presence of 100 μM GTPγS. Reactions were incubated for 10 min at 30°C and were terminated by the addition of 0.5 ml of ice-cold buffer containing 20 mM HEPES, pH 7.4, 3 mM $MgCl_2$, and 100 mM NaCl. The samples were centrifuged at 16,000*g* for 15 min at 4°C, and the resulting pellets were resuspended in solubilization buffer (100 mM Tris, 200 mM NaCl, 1 mM EDTA, and 1.25% Nonidet P-40) plus 0.2% SDS. Samples were precleared with normal rabbit serum followed by immunoprecipitation with C terminus of $G_s\alpha$ (CS) antiserum (Milligan and Unson, 1989). Finally, the immunocomplexes were washed twice with solubilization buffer, and bound [^{35}S]GTPγS concentration was estimated by liquid-scintillation spectrometry.

Results

The mutation of Leu^{322} of the β_1-adrenoceptor can enhance the capacity of this receptor to elevate cAMP levels in the absence of a ligand (Lattion et al., 1999). This elevated constitutive activity is dependent on the identity of the replacement amino acid, but Lys seems particularly effective (Lattion et al., 1999). Sustained treatment of HEK293 cells transiently expressing the wild-type β_1-adrenoceptor with the β_1-adrenoceptor selective blocker betaxolol had no significant effect on the levels of this receptor, as monitored subsequently by the specific binding of [^{3}H]dihydroalprenolol to membranes prepared from these cells (Fig. 1). In contrast, equivalent treatment of cells expressing the Leu^{322}Lys β_1-adrenoceptor resulted, over time, in a significant up-regulation, whether levels of the mutated receptor were assessed in [^{3}H]dihydroalprenolol binding studies (Fig. 1A) or by immunoblotting cell membrane fractions with a selective anti-β_1-adrenoceptor antiserum (Fig. 1B). In such immunoblots, both the Leu^{322}Lys β_1-adrenoceptor and the various other forms of the β_1-adrenoceptor used in these studies (see below) migrated as a distinct doublet with apparent molecular masses of 45 and 60 kDa. It is likely that these represent differentially glycosylated forms of the receptor.

The originally described CAM of the β_2-adrenoceptor was produced by the substitution of a short segment of the distal section of the third intracellular loop of this receptor with the equivalent section from the hamster α_{1b}-adrenoceptor (Samama et al., 1993). Because the β_1-and β_2-adrenoceptors are highly homologous in this region with only two variations in sequence (Fig. 2), we generated a form (CAMβ_1-adrenoceptor) of the β_1-adrenoceptor in which the α_{1b}-adrenoceptor sequence replaced the wild-type β_1-adrenoceptor sequence in this region (Fig. 2). However, although the CAMβ_2-adrenoceptor is strongly up-regulated by exposure to betaxolol (Fig. 3), the effects of this ligand on the CAMβ_1-adrenoceptor were very modest (Fig. 3) and, indeed, insignificant. The CAMβ_1-adrenoceptor sequence has Ala rather than Lys at position 322 (Fig. 2). Although the Leu^{322}Ala β_1-adrenoceptor has been reported to display enhanced constitutive activity compared with wild-type, this is not as great as for the Leu^{322}Lys β_1-adrenoceptor (Lattion et al., 1999). We thus introduced Lys into this position in the CAMβ_1-adrenoceptor to generate the CAMKβ_1-adrenoceptor (Fig. 2). The CAMKβ_1-adrenoceptor was up-regulated to a significantly greater degree by sustained exposure to betaxolol than either the Leu^{322}Lys β_1-adrenoceptor or the CAMβ_1-adrenoceptor (Fig. 3). However, this effect was still less impressive than for the CAMβ_2-adrenoceptor (Fig. 3). None of the mutants of the β_1-adrenoceptor bound [^{3}H]dihydroalprenolol with affinity that was substantially different from that of the wild-type receptor (Table 1), but each of the Leu^{322}Lys β_1-adrenoceptor, the CAMβ_1-adrenoceptor, and particularly the CAMKβ_1-adrenoceptor bound the agonist isoprenaline with significantly higher affinity than did the wild-type β_1-adrenoceptor (Table 2).

Although enhanced agonist affinity and up-regulation of protein levels in response to ligand challenge are properties often associated with receptors that display elevated constitutive activity, we wished to examine this directly. Constitutive activity of $G_s\alpha$-coupled receptors is most often measured at the level of cAMP generation. However, a direct and potentially more quantitative approach is to measure guanine nucleotide exchange on $G_s\alpha$ induced by the receptor. Previously, this has been very difficult to monitor because of the low rates of basal guanine nucleotide exchange of G_s compared with that of G_i-family G proteins (Wieland and Jakobs, 1994). To address this, we generated fusion proteins between each of the wild-type, the Leu^{322}Lys, the CAM, and the CAMK forms of β_1-adrenoceptor and the long isoform of $G_s\alpha$. When these were expressed in HEK293 cells, prior satura-

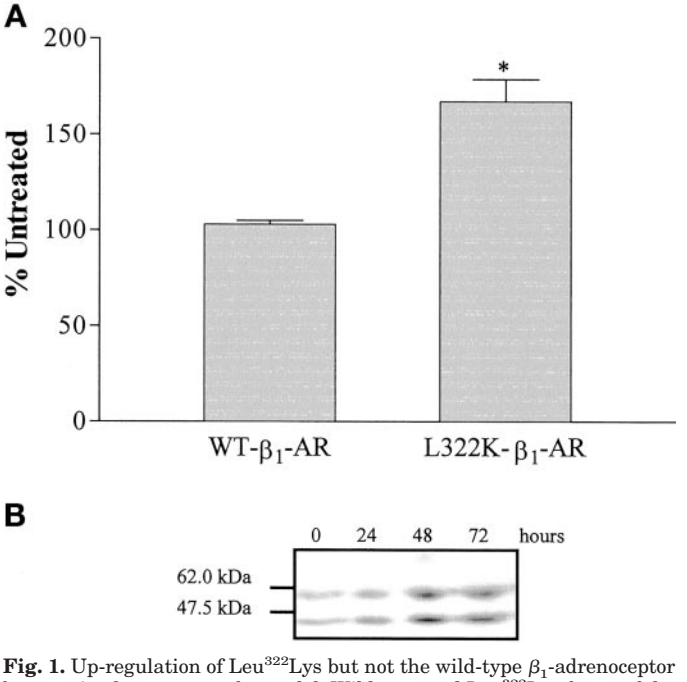

Fig. 1. Up-regulation of Leu^{322}Lys but not the wild-type β_1-adrenoceptor by sustained exposure to betaxolol. Wild-type and Leu^{322}Lys forms of the β_1-adrenoceptor were expressed transiently in HEK293 cells. These were untreated or exposed to betaxolol (0.1 μM) for varying times. A, membranes prepared from cells treated with and without betaxolol for 72 h were used to measure the specific binding of [^{3}H]dihydroalprenolol. *Enhanced by betaxolol treatment, $p < 0.05$. 100% corresponds to 0.82 ± 0.15 pmol/mg membrane protein for the wild-type β_1-adrenoceptor and 1.60 ± 0.22 pmol/mg membrane protein for the Leu^{322}Lysβ_1-adrenoceptor. B, membranes expressing the Leu^{322}Lysβ_1-adrenoceptor from cells treated with betaxolol for periods between 0 and 72 h were resolved by SDS-PAGE and immunoblotted with an antiserum against the β_1-adrenoceptor.

tion [^{3}H]dihydroalprenolol binding studies monitored expression levels in membrane preparations and allowed the addition of equal amounts (10 fmol) of each construct to [^{35}S]GTPγS binding assays, even though the various constructs were expressed at markedly different levels (Table 1). At the termination of the experiment, the samples were immunoprecipitated with an antiserum (CS), that identifies the extreme C terminus of $G_s\alpha$ (Milligan and Unson, 1989) before

WT-β_2-AR	*L*	*K*	E	*H*	K	A	*L*	K	T
CAM- β_2-AR	*S*	*R*	E	*K*	K	A	*A*	K	T
WT-β_1-AR	*L*	*R*	E	*Q*	K	A	*L*	K	T
L322A	*L*	*R*	E	*Q*	K	A	*A*	K	T
L322K	*L*	*R*	E	*Q*	K	A	*K*	K	T
CAM	*S*	*R*	E	*K*	K	A	*A*	K	T
CAMK	*S*	*R*	E	*K*	K	A	*K*	K	T
WT-α_{1B}-AR	*S*	*R*	E	*K*	K	A	*A*	K	T
CAM- α_{1B}-AR	*S*	*K*	E	*H*	K	A	*L*	K	T

Fig. 2. Construction of constitutively active mutants of the β_1-adrenoceptor. The sequence 316 to 324 of the human β_1-adrenoceptor is shown and compared with equivalent regions of the wild-type β_2-adrenoceptor and α_{1b}-adrenoceptor. The best studied CAMβ_2-adrenoceptor (Samama et al., 1993) resulted from the replacement of the sequence in this region with the equivalent section from the α_{1b}-adrenoceptor. The initially studied CAM α_{1b}-adrenoceptor (Allen et al., 1991) was produced by reciprocal replacement with the β_2-adrenoceptor sequence. The sequences of the Leu^{322}Lys-, CAM-, and CAMKβ_1-adrenoceptors used in this study are also shown. Residues underlined are different from the sequence of the equivalent wild-type receptor.

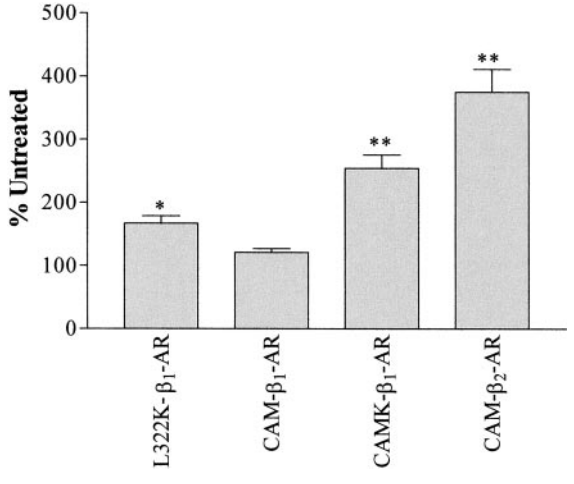

Fig. 3. Comparisons of the effects of betaxolol treatment on levels of mutant β_1- and β_2-adrenoceptors. Leu^{322}Lys-, CAMβ_1-, CAMKβ_1-, and CAMβ_2-adrenoceptors were expressed transiently in HEK293 cells and treated for 72 h with or without betaxolol (0.1 μM for β_1-adrenoceptors, 10 μM for CAMβ_2-adrenoceptor). Membranes were prepared, and the specific binding of [^{3}H]dihydroalprenolol was assessed. Data are presented as the percentage of the receptor levels in membranes of untreated cells. Significant up-regulation by betaxolol *, $p < 0.05$; **, $p < 0.01$.

scintillation counting. In the absence of ligand, the wild-type β_1-adrenoceptor-$G_s\alpha$ construct bound very low levels of nucleotide. However, in individual studies, this amount was increased between 20- and 30-fold when the experiments were performed in the presence of 10 μM isoprenaline (Fig. 4A). The binding of [^{35}S]GTPγS to the Leu^{322}Lys-, CAM-, and CAMKβ_1-adrenoceptor-$G_s\alpha$ fusion proteins in the absence of agonist was substantially higher than that for the wild-type β_1-adrenoceptor-$G_s\alpha$ fusion protein (Fig. 4), and binding of the nucleotide to each fusion protein increased in a linear fashion with time over at least a 20-min period (Fig. 4B). These results demonstrate that each of these mutated forms of the β_1-adrenoceptor is indeed substantially more constitutively active in its capacity to exchange the guanine nucleotide on $G_s\alpha$ and hence activate the G protein than the wild-type receptor. Moreover, the extent of constitutive activity was not different between these three mutants (Fig. 4). Many CAM receptors remain responsive to agonist ligands, indicating that the conformational changes associated with the mutations are not equivalent to those produced by agonist binding. The addition of isoprenaline to [^{35}S]GTPγS binding assays using each of the Leu^{322}Lys-, CAM-, and CAMKβ_1-adrenoceptor-$G_s\alpha$ fusion proteins also resulted in a large increase in nucleotide binding (Fig. 4), although when calculated as a fold increase over basal, this increase was much less than that for the construct containing the wild-type receptor because of the very low levels of nucleotide incorporation into the fusion protein containing the wild-type receptor in the absence of agonist. The basal incorporation of [^{35}S]GTPγS into the wild-type β_1-adrenoceptor-$G_s\alpha$ fusion protein was sufficiently low that it was not feasible to examine possible inverse agonism of ligands. However, the extra basal incorporation into the mutated β_1-adrenoceptor-$G_s\alpha$ fusion proteins allowed such an examination. For the CAMKβ_1-adrenoceptor, this was reduced significantly by the presence of 10 μM betaxolol (Fig. 4), indicating this ligand to be an inverse agonist at this construct. However, betaxolol did not produce a significant reduction in basal binding of [^{35}S]GTPγS to either the CAMβ_1-adrenoceptor-$G_s\alpha$ fusion protein or the Leu^{322}Lys β_1-adrenoceptor–containing construct (Fig. 4).

Parallel experiments were performed using fusion proteins between both the wild-type and CAMβ_2-adrenoceptors and $G_s\alpha$ (Fig. 5A). Basal incorporation of [^{35}S]GTPγS into the wild-type β_2-adrenoceptor-$G_s\alpha$ construct was again low (although significantly higher than for the β_1-adrenoceptor-$G_s\alpha$ fusion protein) and increased greatly in response to isoprenaline (Fig. 5A). The same amount of CAMβ_2-adrenoceptor-$G_s\alpha$ fusion protein, again monitored by the specific binding of [^{3}H]dihydroalprenolol, produced markedly elevated levels of nucleotide binding in the absence of ligand (Fig. 5A). This was also increased substantially by isoprenaline and inhibited significantly by betaxolol (Fig. 5A). Increased binding of [^{35}S]GTPγS in immunoprecipitates of endogenously expressed $G_s\alpha$ could also be produced by the addition of isoprenaline to membranes of cells transfected to express the isolated β_2-adrenoceptor (Fig. 5B). However, the agonist-induced signal was substantially weaker than when studies were performed using equal amounts of the GPCR-G protein fusion (Fig. 5B). Furthermore, no significant constitutive activity of the isolated receptor to activate $G_s\alpha$ could be observed when compared with mock-transfected cells (Fig. 5B).

The addition of $G_s\alpha$ to the C terminus of constitutively active forms of either the β_1- or β_2-adrenoceptor did not alter the capacity of betaxolol to cause their up-regulation as monitored in subsequent [^{3}H]dihydroalprenolol binding studies (Fig. 6). The extent of up-regulation of the CAMKβ_1-adrenoceptor-$G_s\alpha$ fusion protein was the same as for the isolated CAMKβ_1-adrenoceptor, and the same was true for comparisons between CAMβ_2-adrenoceptor-$G_s\alpha$ and the CAMβ_2-adrenoceptor (Fig. 6). Equally, fusion of $G_s\alpha$ to the C terminus of the wild-type forms of the β_1-adrenoceptor or the β_2-adrenoceptor did not allow significant up-regulation by betaxolol (Fig. 6).

In certain receptors, such as the histamine H_2 receptor (Alewijnse et al., 2000), mutations within the highly conserved DRY domain at the interface of transmembrane helix III and the second intracellular loop produce significant receptor instability and the capacity of receptor blockers to produce very high degrees of up-regulation of these mutants. We thus also produced an Arg^{156}Alaβ_1-adrenoceptor and generated an Arg^{156}Alaβ_1-adrenoceptor-$G_s\alpha$ fusion protein. The addition of membranes containing equal amounts of the wild-type β_1-adrenoceptor-$G_s\alpha$ and Arg^{156}Alaβ_1-adrenoceptor-$G_s\alpha$ fusion proteins to [^{35}S]GTPγS binding assays followed by immunoprecipitation with the anti-$G_s\alpha$ antiserum demonstrated that the Arg^{156}Alaβ_1-adrenoceptor was capable of producing enhanced guanine nucleotide exchange on $G_s\alpha$ in the absence of ligand (Fig. 7A). However, the extent of constitutive activity in this mutant was significantly lower than for the series of mutants at the intracellular loop III/transmembrane helix VI interface examined earlier. Although the Arg^{156}Alaβ_1-adrenoceptor constructs bound [^{3}H]dihydroalprenolol with equivalent affinity to the other β_1-adrenoceptor mutants used in this study (Table 1), this form of the receptor did not display an affinity that was higher than that of the wild-type receptor to bind isoprenaline (Table 2). This form of the receptor, both with and without C-terminal attachment of $G_s\alpha$, was up-regulated, however, by betaxolol treatment at least as effectively as the Leu^{322}Lysβ_1-adrenoceptor (Fig. 7B). Furthermore, as we noted previously for the CAMβ_2-adrenoceptor (Ramsay et al., 2001), ligand-induced up-regulation was not restricted to antagonist/inverse agonist ligands. Up-regulation of both the CAMKβ_1-adrenoceptor-$G_s\alpha$ and the Arg^{156}Alaβ_1-adrenoceptor-$G_s\alpha$ constructs was produced to similar extents by sustained exposure to isoprenaline (Fig. 7C). There was, however, ligand specificity for up-regulation because neither of these constructs was up-regulated by exposure to the markedly β_2-adrenoceptor-selective blocker ICI118551 (10μM) (Fig. 7C), which does cause up-regulation of the CAMβ_2-adrenoceptor (McLean et al., 1999; Ramsay et al., 2001).

Discussion

A number of studies have noted that the β_1-adrenoceptor displays relatively low levels of constitutive activity compared with the β_2-adrenoceptor (Lattion et al., 1999; Zhou et al., 2000). However, enhanced signal transduction in the absence of agonist that correlates with expression levels of this receptor has been noted (Engelhardt et al., 2001). The basis for the differences between these two β-adrenoceptors is unclear but may relate to their differential targeting in cells because it seems that the β_2-adrenoceptor is more selectively targeted to detergent-insensitive membrane domains that also allow marked concentration of heterotrimeric G proteins (Rybin et al., 2000). Although constitutively active mutants of the β_2-adrenoceptor that have a markedly higher capacity for signaling in the absence of agonist have been widely studied (Samama et al., 1993; MacEwan and Milligan, 1996; Gether et al., 1997; Ramsay et al., 2001), much less information is available on the β_1-adrenoceptor. In many regards, this is surprising given the quantitative importance of this receptor in cardiac function. Moreover, the single available study on constitutively active mutants of the β_1-adrenoceptor concentrated on a mutation of a single (Leu^{322}) amino acid (Lattion et al., 1999).

Many constitutively active mutants of G protein–coupled receptors are significantly destabilized compared with the wild type (Samama et al., 1993; MacEwan and Milligan, 1996; Gether et al., 1997; Li et al., 2001a,b), and this can frequently be overcome by the binding of ligands to the mutated receptor. Such ligand-induced stabilization can be visualized directly if the mutant receptor is tagged with a fluorescent protein (McLean et al., 1999) or converted to a useful ligand screen if tagged with an enzyme whose activity is easy to measure (Ramsay et al., 2001). Leu^{322}Lys was the most constitutively active point mutant of the β_1-adrenoceptor reported by Lattion et al. (1999). Sustained treatment of cells expressing this mutant with the β_1-adrenoceptor selective blocker betaxolol resulted in significant up-regulation of the number of [^{3}H]dihydroalprenolol binding sites and immunodetected amounts of the polypeptide, whereas equivalent treatment of cells expressing the wild-type β_1-adrenoceptor was without effect. The originally defined CAMβ_2-adrenoceptor is known to be up-regulated strongly by sustained treatment with betaxolol (Pei et al., 1994; MacEwan and Milligan, 1996). This was confirmed in the present

TABLE 1

Binding affinity of β_1-adrenoceptor mutants for [^{3}H]dihydroalprenolol

The binding affinity for [^{3}H]dihydroalprenolol and steady-state levels of expression of the various forms of the β1-adrenoceptor were assessed in saturation ligand binding studies. Such studies allowed membrane amounts corresponding to 10 fmol of each construct to be added to [^{35}S]GTPγ S binding assays.

X Labels	K_d			B_{max}		
	Mean	S.E.M.	n	Mean	S.E.M.	n
	nM			*fmol/mg*		
WT-β1-AR	0.77	0.18	3	822	152	3
L322K-β1-AR	1.34	0.04	3	1603	215	3
CAM-β1-AR	1.46	0.21	3	2997	445	3
CAMK-β1-AR	1.36	0.24	3	385	41	3
R156A-β1-AR	1.28	0.23	4	4330	311	4

studies, but an equivalent mutant of the β_1-adrenoceptor in which the same segment of the α_{1b}-adrenoceptor was substituted was little affected by treatment with betaxolol (Fig. 3). An examination of the sequences of the adrenoceptors in this region (Fig. 2) showed that Leu^{322} of the β_1-adrenoceptor was altered to Ala by substitution of the segment from the α_{1b}-adrenoceptor. Although the $Leu^{322}Ala$ β_1-adrenoceptor has been reported to display a significant level of constitutive activity, it was not as marked as the $Leu^{322}Lys$ β_1-adrenoceptor (Lattion et al., 1999). We thus further modified the CAM β_1-adrenoceptor to encode Lys at residue 322. This construct was up-regulated significantly more strongly by treatment with betaxolol.

Most studies that examine the constitutive activity of receptors monitor agonist-independent regulation of either second-messenger levels or activity of a reporter gene construct. However, direct analysis of the activation of the relevant G protein provides inherently the most direct and quantitative measure. The most popular assay is to measure elevation of the binding of [^{35}S]GTPγS. Historically, however, this has been extremely difficult for G proteins other than those in the G_i-family because of combinations of their low basal guanine nucleotide exchange and relatively low expression levels (Wieland and Jakobs, 1994). We recently overcame these problems for the G_q/G_{11} family of G proteins by combining receptor G-protein fusion proteins (Milligan, 2002) with their effective immunoprecipitation after a [^{35}S]GTPγS binding assay (Stevens et al., 2001). We thus used the same strategy for the β_1-adrenoceptor and $G_s\alpha$. Fusion proteins were constructed between the wild-type and various mutants of the β_1- and β_2-adrenoceptor and the long isoform of $G_s\alpha$. Initially, we confirmed that the C-terminal addition of $G_s\alpha$ did not alter the effects of betaxolol on the extent of up-regulation of the β-adrenoceptors. Satisfied that this was the case (Fig. 6), we used membranes expressing these constructs to explore the loading of [^{35}S]GTPγS. Immunoprecipitation of the constructs with an antiserum directed to the extreme C terminus of $G_s\alpha$ resulted in very low levels of [^{35}S]GTPγS to the β_1-adrenoceptor-$G_s\alpha$ fusion protein in the absence of agonist. This could have been interpreted either as reflecting low levels of agonist-independent activation of the construct or that the basic assay concept was flawed. However, [^{35}S]GTPγS binding was increased some 20- to 30-fold by the addition of a maximally effective concentration of isoprenaline. The very low level of [^{35}S]GTPγS binding in the absence of ligand is consistent with previous indications that the wild-type β_1-adrenoceptor has low basal constitutive activity (Lattion et al., 1999). However, when the CAMK mutant was fused to $G_s\alpha$ and the same amount of this construct was assayed, the level of [^{35}S]GTPγS binding in the absence of agonist was markedly greater than that produced for the wild-type construct. Furthermore, betaxolol was shown to act as an inverse agonist for the CAMKβ_1-adrenoceptor because this ligand reduced the basal [^{35}S]GTPγS binding. As validation for this assay, equivalent studies were also performed with $G_s\alpha$ fusions incorporating the wild-type and CAM forms of the β_2-adrenoceptor. As anticipated from previous studies, the CAMβ_2-adrenoceptor produced marked levels of [^{35}S]GTPγS binding in the absence of ligand, and this was

TABLE 2

Mutants of the β_1-adrenoceptor: binding affinity for isoprenaline

The ability of varying concentrations of isoprenaline to compete for the binding of [^{3}H]dihydroalprenolol to various forms of the β_1-adrenoceptor were assessed. K_i values for isoprenaline for each construct were then determined using the information in Table 1.

Construct	K_i		
	Mean	S.E.M.	n
		nM	
WT-β1-AR	79.7*	7.90	3
L322K-β1-AR	42.3*	3.80	3
CAM-β1-AR	33.0*	9.00	2
R156A-β1-AR	107.3	7.60	4
CAMK-β1-AR	11.7**	2.30	3

*$p < 0.05$; **$p < 0.01$, significantly different from wild type.

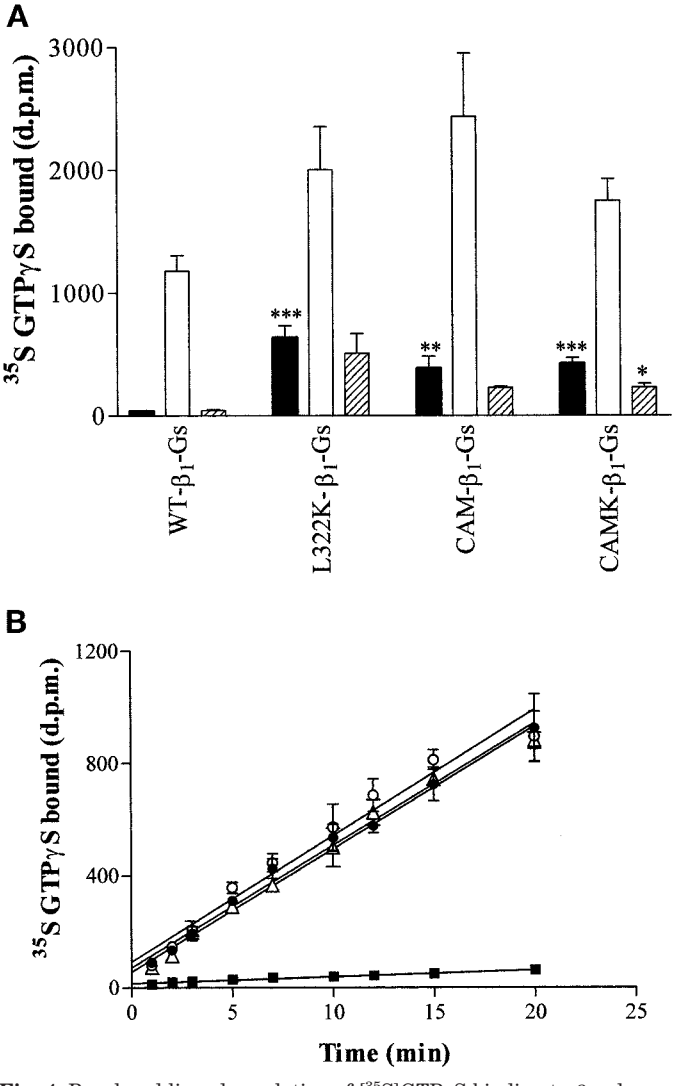

Fig. 4. Basal and ligand regulation of [^{35}S]GTPγS binding to β_1-adrenoceptor-$G_s\alpha$ fusion proteins. Fusion proteins between the wild-type, $Leu^{322}Lys$-, CAM-, or CAMKβ_1-adrenoceptors and the long isoform of $G_s\alpha$ were expressed transiently in HEK293 cells. A, membrane preparations were used to measure levels of [^{3}H]dihydroalprenolol binding sites in saturation binding assays, and 10 fmol of each construct was then used in [^{35}S]GTPγS binding assays in the absence of ligand (■) or the presence of isoprenaline (□) or betaxolol (▨) (both at 10 μM). At assay termination, samples were immunoprecipitated with antiserum CS before scintillation counting. **, $p < 0.01$; ***, $p < 0.001$, significantly different from basal of the wild-type construct. *, $p < 0.05$, significantly different from basal of the CAMKβ_1-adrenoceptor construct. B, the rate of binding of [^{35}S]GTPγS to fusion proteins containing the wild-type (■), $Leu^{322}Lys$ (□), CAM (△), or CAMK (○) forms of the β_1-adrenoceptor was assessed for periods of up to 20 min.

inhibited in the presence of betaxolol (Fig. 5A). Although the ability of ligands to modulate the binding of [^{35}S]GTPγS to fusion proteins containing the β_2-adrenoceptor and $G_s\alpha$ has been monitored previously (Seifert et al., 1999; Milligan, 2002), such studies have not incorporated an immunoprecipitation step. Thus, in general, such studies have been restricted to experiments performed in systems such as insect Sf9 cells that have low levels of expression of endogenous G_i family G proteins that elevate the background signal in mammalian cells and thus limit the signal to noise and sensitivity of assays. Prior ^{3}H-ligand binding studies also allowed for the addition of the same amount of the fusion constructs containing different mutations in the receptors to each [^{35}S]GTPγS binding assay, and thus they allowed direct comparisons of the level of constitutive activity imparted to the GPCR by each set of mutations. This was of particular relevance in these studies because mutations that imbue constitutive activity are known to alter levels of expression and stability of the receptor.

Mutations and alterations of the β_1-adrenoceptor that introduced similar levels of constitutive activity (Fig. 4) did not result in an equivalent capacity of an antagonist/inverse agonist to suppress this activity. Thus, although betaxolol has been described previously as an effective inverse agonist at the CAMβ_2-adrenoceptor (MacEwan and Milligan, 1996) and clearly functioned in this manner for the CAMKβ_1-adrenoceptor, this was not obviously the case for the Leu^{322}Lysβ_1-adrenoceptor (Fig. 4). This may indicate that not all constitutively active mutants of the same receptor should be considered to be equivalent and, individual ligands may suppress this activity to different degrees in what might have been considered to be phenotypically similar GPCR mutants. It is also obvious that none of the mutants used in these studies caused a level of binding of [^{35}S]GTPγS in the absence of ligands such that it was not increased markedly by the presence of the agonist isoprenaline. Thus although it has been suggested that such mutants may represent good models of the agonist-occupied or R* states of GPCRs (Samama et al., 1993; Scheer and Cotecchia, 1997), the current studies clearly indicate that they represent, at best, a rough approximation of an agonist-induced state. Other GPCRs may display significant agonist-independent G protein activation, and it is noteworthy that the wild-type melanocortin MC_4 receptor, at which the agouti-related peptide functions as an endogenous antagonist/inverse agonist (Adan and Vink, 2001), displays high levels of constitutive activity in this type of assay (G. Milligan, L. Ormiston, W. Nijenhuis, and R. Adan, unpublished observations). It is also of interest that mutant β_1-adrenoceptors displaying similar levels of constitutive activity to load [^{35}S]GTPγS onto $G_s\alpha$ were not up-regulated to the same extent by treatment with betaxolol.

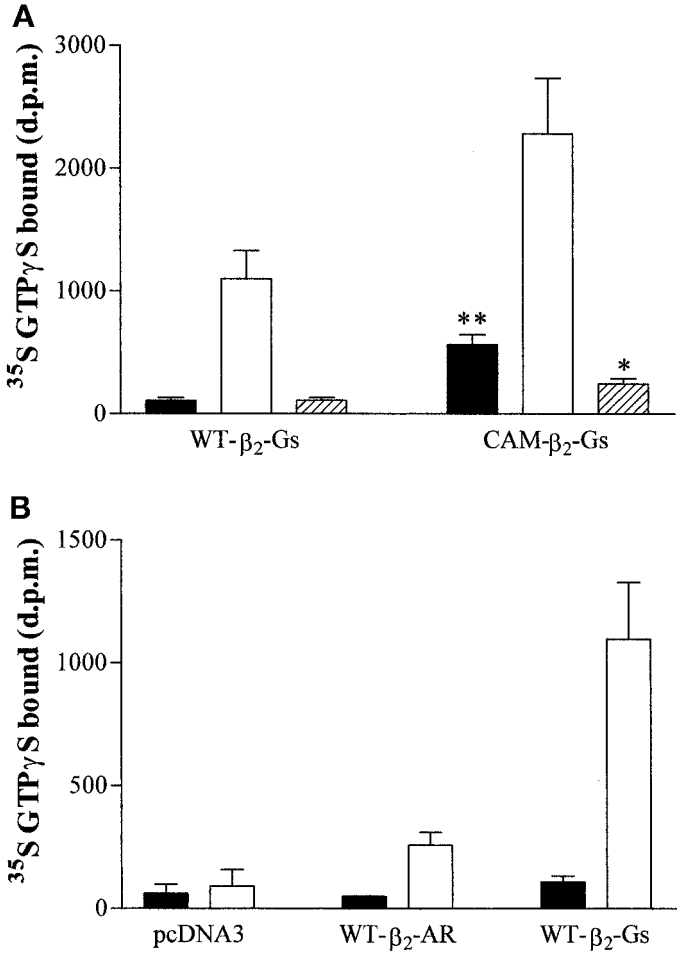

Fig. 5. Basal and ligand-regulation of [^{35}S]GTPγS binding to β_2-adrenoceptor-$G_s\alpha$ fusion proteins. A, fusion proteins between the wild-type or CAMβ_2-adrenoceptor and the long isoform of $G_s\alpha$ were expressed in HEK293 cells. Membrane preparations were used to measure levels of [^{3}H]dihydroalprenolol binding sites, and 10 fmol of each construct was used in [^{35}S]GTPγS binding assays in the absence of ligand (■) or the presence of isoprenaline (□) or betaxolol (▨) (both at 10 μM). At assay termination, samples were immunoprecipitated with antiserum CS before scintillation counting. **, significantly different from basal of the wild-type construct; *, significantly different from basal of the CAMβ_2-adrenoceptor construct. B, HEK293 cells were mock-transfected (pcDNA3) or transfected to express either the isolated wild-type β_2-adrenoceptor or a wild-type β_2-adrenoceptor-$G_s\alpha$ fusion protein. In [^{35}S]GTPγS binding assays, 10 fmol of each construct was used in the absence of ligand (■) or the presence of isoprenaline (□) before immunoprecipitation and counting as above.

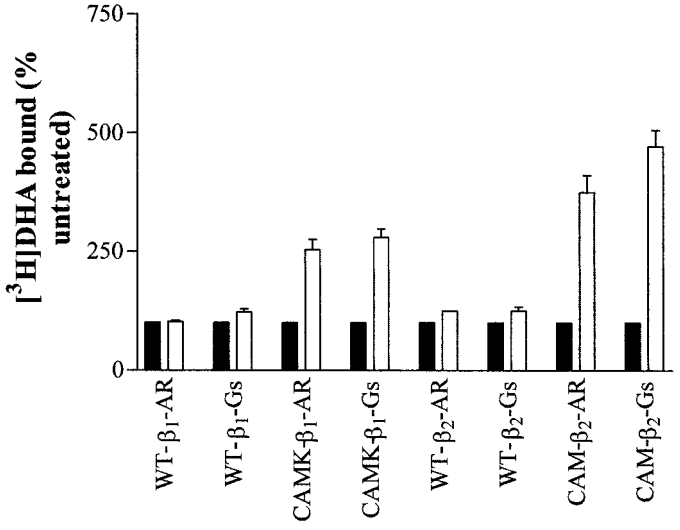

Fig. 6. C-terminal addition of $G_s\alpha$ does not alter betaxolol regulation of β-adrenoceptor mutants. $G_s\alpha$ fusions to each of the wild-type β_1- and β_2-adrenoceptors, and the CAMKβ_1 and CAM$\beta_2$adrenoceptors were expressed transiently in HEK293 cells, as were each of the equivalent receptor constructs without the C-terminal $G_s\alpha$ tag. These cells were treated with (□) or without (■) betaxolol (0.1 μM for β_1-adrenoceptors, 10 μM for β_2-adrenoceptors) for 72 h, the cells were harvested, and membrane preparations were used to measure the levels of [^{3}H]dihydroalprenolol binding sites.

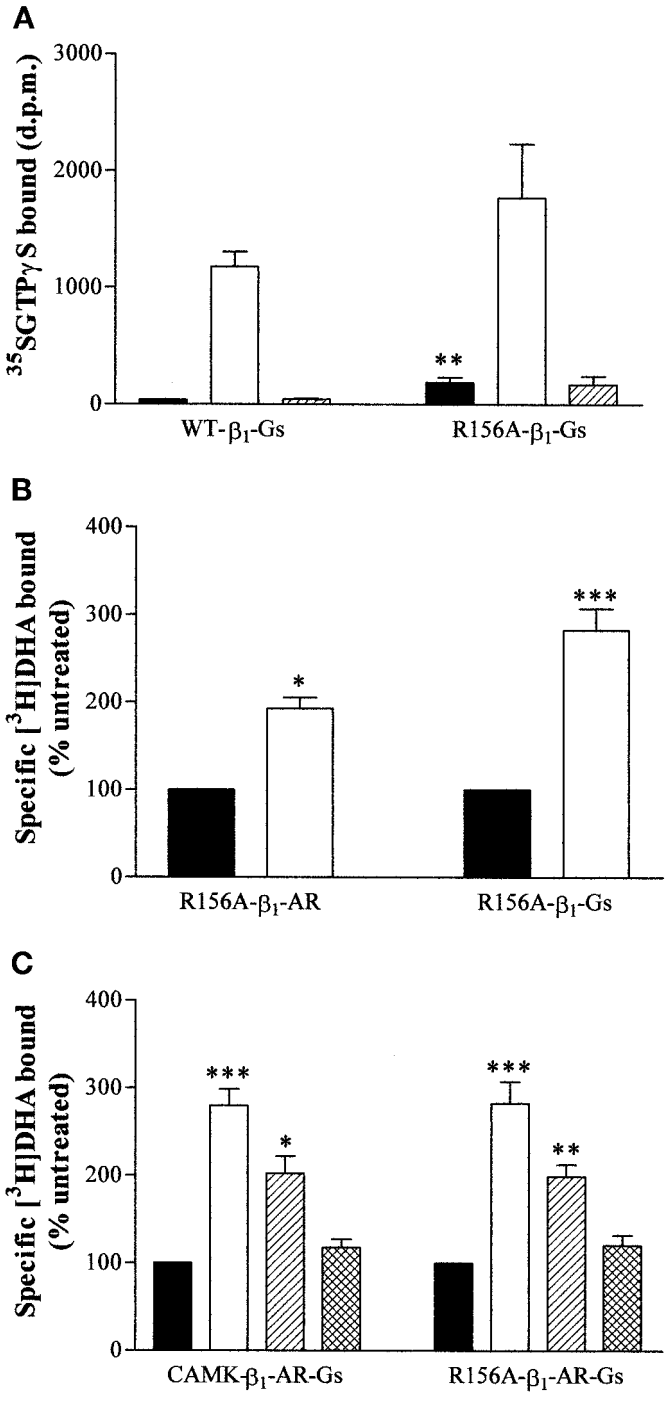

Fig. 7. Regulation and constitutive activity of a DRY domain mutant of the β_1-adrenoceptor. Wild-type, Arg^{156}Alaβ_1-adrenoceptor, or CAMKβ_1-adrenoceptor and fusion constructs of these with $G_s\alpha$ were expressed in HEK293 cells. A, membranes expressing 10 fmol of either the wild-type β_1-adrenoceptor-$G_s\alpha$ or Arg^{156}Alaβ_1-adrenoceptor-$G_s\alpha$ fusion proteins were used in [^{35}S]GTPγS binding assays as in Fig. 5 in the absence of ligand (■) or in the presence of isoprenaline (□) or betaxolol (▨) (both at 10 μM). **, $p < 0.005$, significantly different from basal of wild-type β_1-adrenoceptor-$G_s\alpha$. B, cells expressing the isolated Arg^{156}Ala β_1-adrenoceptor or the Arg^{156}Alaβ_1-adrenoceptor-$G_s\alpha$ fusion protein were exposed to vehicle (■) or betaxolol (0.1 μM) (□) for 72 h. Membranes were prepared, and the specific binding of [^{3}H]dihydroalprenolol was measured. Significant up-regulation by betaxolol was noted at *, $p < 0.05$; ***, $p < 0.001$. C, the CAMKβ_1-adrenoceptor-$G_s\alpha$ or the Arg^{156}Alaβ_1-adrenoceptor-$G_s\alpha$ fusion proteins were expressed in HEK293 cells and exposed to vehicle (■), betaxolol (0.1 μM) (□), isoprenaline (1 μM) (▨) or ICI118551 (10 μM) (▩) for 72 h. Membranes were prepared, and the specific binding of [^{3}H]dihydroalprenolol was measured. Significantly different from vehicle-treated was noted at *, $p < 0.05$, **, $p < 0.005$, and ***, $p < 0.001$.

It may be instructive to note in this regard that the largest degree of betaxolol-induced up-regulation was produced with the β_1-adrenoceptor mutant (CAMK), at which betaxolol clearly did function as an inverse agonist. It is also noteworthy that although there were marked differences in the steady-state levels of expression of the various forms of the β_1-adrenoceptor (Table 1), there was no obvious correlation between these and the extent of up-regulation produced by betaxolol treatment.

These studies confirm that mutations in hot spots at the end of transmembrane region III and the interface of transmembrane helix VI and the third intracellular loop can generate forms of the GPCR with elevated constitutive activity and provide a novel direct assay for the extent of activation of G proteins by such mutant GPCRs. However, at least for the β_1-adrenoceptor, there is no obvious direct correlation between the level of constitutive activity of such mutants and their apparent structural instability. An equivalent conclusion has been reached recently for distinct CAM forms of the α_{1b}-adrenoceptor (Stevens et al., 2000).

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Address correspondence to: Graeme Milligan, Davidson Building, University of Glasgow, Glasgow G12 8QQ, Scotland, United Kingdom. E-mail: g.milligan@bio.gla.ac.uk