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Resolution of Inverse Agonist-Induced Up-Regulation from Constitutive Activity of Mutants of the α_{1b}-Adrenoceptor

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ABSTRACT

Constitutively active forms of the hamster α_{1b}-adrenoceptor can be produced from the point mutations $Asp^{142}Ala$ or $Ala^{293}Glu$ or exchange of a small segment of the third intracellular loop with the equivalent region of the β_2-adrenoceptor. Green fluorescent protein (GFP)-tagged forms of each of these mutants and of the wild type α_{1b}-adrenoceptor were expressed stably in HEK293 cells. The wild type α_{1b}-adrenoceptor-GFP was expressed both at the plasma membrane and with a distinctly perinuclear punctate pattern. Sustained treatment with a range of antagonist/inverse agonist ligands failed to modulate the cellular distribution or levels of expression of this construct. The form of the α_{1b}-adrenoceptor containing the β_2-adrenoceptor sequence substitution was predominantly located in punctate intracellular vesicles and sustained challenge with the same series of antagonists/inverse agonists produced a 5-fold up-regulation of protein levels with elevation of both plasma membrane and intracellular receptor. Quantification of these effects could be produced by spectrofluorometric analysis of cells grown in a 96-well microtiter plate. In contrast, both the $Asp^{142}Ala$ and $Ala^{293}Glu$ forms of the α_{1b}-adrenoceptor-GFP were located predominantly at the plasma membrane. Levels of these two point mutants were not increased by any of the antagonist/inverse agonist ligands tested, although the sequence substitution mutation encompasses codon 293. Resolution of constitutive activity and ligand-induced up-regulation was further exemplified by a mutant lacking eight serine residues in the C-terminal tail that displayed little constitutive activity but was up-regulated by sustained ligand challenge. These results demonstrate the nonequivalence of mutations in their regulation by antagonist/inverse agonist ligands.

Mutationally induced agonist-independent activity of G protein-coupled receptors (GPCRs) has been widely studied to provide insights into possible conformational changes that must occur on ligand binding to result in guanine nucleotide exchange on, and subsequent activation of, heterotrimeric G proteins (Scheer and Cotecchia, 1997; Leurs et al., 1998). Such constitutive activity can be imparted to class I GPCRs by mutations at a considerable range of locations in the primary sequence. The most commonly studied mutations, however, have tended to cluster either at the interface of transmembrane region VI and the end of the third intracellular loop or at the interface of transmembrane region III and the beginning of the second intracellular loop. This reflects, at least in part, that the former location was the first identified region in the rhodopsin-like GPCRs where mutation produced such a phenotype (Lefkowitz et al., 1993) and that the latter region contains the Asp-Arg-Tyr (DRY) motif, which is the most highly conserved sequence element in this family of proteins. Mutation of either Ala^{293} or Asp^{142} of the hamster α_{1b}-adrenoceptor to any other amino acid has been reported to increase the capacity of the expressed GPCR to stimulate phosphoinositidase C activity and the extent of production of inositol phosphates (Scheer and Cotecchia, 1997; Scheer et al., 1997, 2000). Not all constitutively active mutations of this GPCR can be considered to be equivalent, however. For example, the specific amino acid selected to replace either Ala^{293} or Asp^{142} determines the degree of constitutive activity revealed (Scheer et al., 1996, 1997, 2000). Furthermore, a $Cys^{128}Phe$ mutant displays constitutive activity when phosphoinositidase C activation is monitored but not when phospholipase A2 activity is the measured endpoint, whereas an $Ala^{293}Glu$ mutant has constitutive activity in both pathways (Perez et al., 1996). It has also been noted that the regulatory features of an $Ala^{293}Glu$ and an $Asp^{142}Ala$ mutant are distinct in terms of their interactions with β-arrestin-2 (Mhaouty-Kodja et al., 1999).

Previous studies (Lee et al., 1997) on a constitutively active

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ABBREVIATIONS: GPCR, G protein-coupled receptor; GFP, green fluorescent protein; PCR, polymerase chain reaction; DMEM, Dulbecco's modified Eagle's medium; CAM, constitutively active mutant.

mutant of the hamster α_{1b}-adrenoceptor resulting from replacement of a short segment of the third intracellular loop with the equivalent segment from the β_2-adrenoceptor have shown that it can be stabilized by binding ligands with antagonist/inverse agonist pharmacology. It has been impossible to resolve the relevance of inverse agonism to this feature, however, because all ligands that are classical blockers at the α_{1b}-adrenoceptor display inverse agonism at both this (Lee et al., 1997), the Ala^{293}Glu mutant, and the wild type α_{1b}-adrenoceptor (Rossier et al., 1999).

The addition of green fluorescent protein (GFP) to the C-terminal tail of a considerable range of GPCRs has been extremely useful in monitoring their cellular distribution and regulation (for review, see Milligan, 1999). As the attached GFP is thus in a 1:1 M ratio with the GPCR it also provides an ideal means to monitor, at least qualitatively, alterations in expression levels of such a construct (McLean et al., 1999). In this study, we use cell lines stably expressing GFP-tagged forms of each wild type and the three most widely studied constitutively active mutants of the hamster α_{1b}-adrenoceptor (Fig. 1) to explore differences in their cellular location and regulation by receptor ligands.

Experimental Procedures

Materials. All reagents for tissue culture were purchased from Life Technologies, Inc. (Paisley, Strathclyde, UK). Oligonucleotides were purchased from Cruachem Ltd. (Glasgow, UK). [^{3}H]Prazosin was purchased from NEN Life Science Products (Boston, MA), and *myo*-[^{3}H]inositol was obtained from Amersham Pharmacia Biotech (Amersham, UK). Receptor ligands were purchased from RBI (Gillingham, Dorset, UK). All other reagents were obtained from Sigma (Poole, UK) and were of the highest grade available.

Construction of GFP-Tagged Forms of the α_{1b}-Adrenoceptor. Production and subcloning of wild type, 3CAM (constitutively active mutant), Ala^{293}Glu, Asp^{142}Ala, and M8 hamster α_{1b}-adrenoceptor-GFP fusion proteins were performed in two separate stages. In the first step, the coding sequence of a modified form of GFP (Zernicka-Goetz et al., 1997) was modified by polymerase chain reaction (PCR) amplification. Using the amino-terminal primer 5′-GGAAGGTACCAGTAAAGGAGAAGAACTT-3, the initiating Met of GFP was removed, and both a *Kpn*I restriction site (underlined) and a two-amino acid spacer (Gly-Asn) were introduced. Using the carboxyl-terminal primer 5-TGCTCTAGATTATTTGTATAGTTCATCCATGCCATG-3′, an *Xba*I restriction site (underlined) was introduced downstream of the stop codon of GFP. The amplified fragment of GFP digested with *Kpn*I and *Xba*I was subcloned into similarly digested pcDNA3 expression vector (Invitrogen). To obtain the various α_{1b}-adrenoceptor-GFP fusion proteins, the coding sequence of each form of the α_{1b}-adrenoceptor was amplified by PCR. Using the amino-terminal primer 5′-GACGGTACCTCTAAAATGAATCCCGAT-3′, a *Kpn*I restriction site (underlined) was introduced upstream of the initiator Met. Using the carboxyl-terminal primer 5′-GTCCCTGGTACCAAAGTGCCCGGGTG-3′, a *Kpn*I restriction site (underlined) was introduced immediately upstream of the stop codon. Finally, the GFP construct in pcDNA3 was digested with *Kpn*I and ligated together with the PCR product of the α_{1b}-adrenoceptor amplification, also digested with *Kpn*I. The open reading frames thus produced represent the coding sequence of either the wild type, 3CAM, Ala^{293}Glu, Asp^{142}Ala, or M8 α_{1b}-adrenoceptor-GFPs. Each was fully sequenced before its expression and analysis.

Transient and Stable Transfection of HEK293 Cells. HEK293 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.292 g/liter 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% confluency before transient transfection. Transfection was performed using LipofectAMINE reagent (Life Technologies, Inc.) according to the manufacturer's instructions. To generate cell lines stably expressing the various constructs, cells were seeded and diluted 2 days after transfection and maintained in DMEM supplemented with 1 mg/ml Geneticin sulfate (Life Technologies, Inc.). The medium was replaced every 3 days with DMEM containing 1 mg/ml Geneticin sulfate. Receptor-expressing clones were identified initially by fluorescence microscopy, and the clones chosen for further study were selected and expanded.

Inositol Phosphate Assays. Measurement of inositol phosphate accumulation was performed essentially as described previously (Drmota et al., 1998). HEK293 cells stably expressing the various α_{1b}-adrenoceptor-GFP fusion proteins were seeded into 12-well plates and allowed to reattach. Cells were then labeled with [^{3}H]inositol (1 μCi/ml) in inositol-free DMEM supplemented with 2% (v/v) newborn calf serum and 1% L-glutamine for 24 h. The accumulation of inositol phosphates in response to increasing concentrations of phenylephrine during a 15-min incubation period was then assessed in the presence of LiCl (15 mM). [^{3}H]Inositol and [^{3}H]inositol phosphates were batch separated by Dowex-formate chromatography as detailed previously (Drmota and Milligan, 2000). Data are

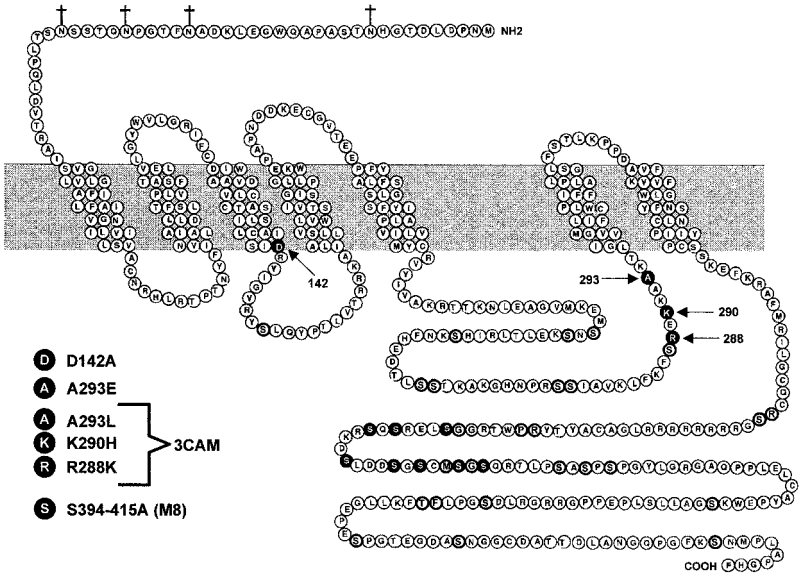


Fig. 1. Location of mutations that imbue constitutive activation of phosphoinositidase C activity or ligand-induced up-regulation to the α_{1b}-adrenoceptor. A ribbon diagram of the primary sequence of the hamster α_{1b}-adrenoceptor indicates the mutants used herein. Dark circles indicate amino acids altered in the individual mutants.

presented as the quotient of [^{3}H]inositol phosphates divided by inositol phosphates plus [^{3}H]inositol.

Preparation of Membranes. HEK293 cells stably expressing each of the α_{1b}-adrenoceptor-GFP fusion proteins were grown to confluence on 6-cm dishes. Before harvesting, cells were washed twice with 4 ml of ice-cold TE buffer (10 mM Tris, 0.1 mM EDTA, pH 7.5) and then scraped into 1 ml of the same buffer. Rupture of the cells was achieved with 25 strokes of a hand-held glass Dounce homogenizer on ice. The suspension was centrifuged at 16,000*g* for 15 min, and the resulting pellets resuspended in ice-cold TE buffer to final protein concentrations of 0.035 to 0.16 mg/ml.

[^{3}H]Prazosin Binding Experiments. Binding experiments were initiated by the addition of 0.7 to 3.2 μg of protein to an assay buffer [75 mM Tris/HCl (pH 7.5), 5 mM EDTA, 12.5 mM $MgCl_2$ (buffer A)] containing [^{3}H]prazosin (0.01–2.0 nM in saturation assays and between 0.08 and 0.9 nM for competition assays) in the absence or presence of increasing concentrations of the test drugs (500 μl, final volume). Nonspecific binding was determined in the presence of 10 μM phentolamine. Reactions were incubated for 30 min at 30°C, and bound ligand was separated from free ligand by vacuum filtration through GF/B filters. The filters were washed twice with buffer A, and bound ligand was estimated by liquid-scintillation spectrometry.

Confocal Laser Scanning Microscopy. Cells were observed with a laser scanning confocal microscope (Zeiss Axiovert 100) using a Zeiss Plan-Apo 63 × 1.40 NA oil immersion objective, pinhole of 35, and electronic zoom 1 or 3. The GFP was exited using a 488-nm argon/krypton laser and detected with a 515- to 540-nm band pass filter. The images were manipulated with Zeiss LSM or MetaMorph software. Cells on glass coverslips were washed with PBS and fixed for 20 min at room temperature using 4% paraformaldehyde in PBS, 5% sucrose, pH 7.2. After one wash with PBS, coverslips were mounted on microscope slides with 40% glycerol in PBS.

Studies in Microtiter Plates. Cells were seeded into black Costar view plates the day before the experiment. On the day of the experiment, the medium was removed from the cells, and drug was added to the well in a final volume of 100 μl. The experiments were performed in phenol red-free F12 medium containing 10% fetal calf serum. A Spectrafluor Plus fluorimeter was used to read the plates, reading from the bottom at a gain of 100. A blank plate was initially read on the fluorimeter, and then the plates of cells were read at time 0 and after 22 h of incubation at 37°C with drug. Results were calculated by subtracting the blank plate from the fluorescence values obtained to control for plate autofluorescence.

Results

Compared with the wild type hamster α_{1b}-adrenoceptor point, mutations at either amino acid Asp^{142} or Ala^{293} have been demonstrated to result in significantly enhanced basal (also called constitutive) stimulation of phosphoinositidase C activity in the absence of an agonist ligand. This is also true of a form of the receptor in which a small segment of the distal region of the third intracellular loop, which encompasses Ala^{293}, was replaced with the equivalent segment of the β_2-adrenoceptor. Herein this form of the receptor is designated 3CAM. C-terminally GFP-tagged forms of each of wild type, Asp^{142}Ala, Ala^{293}Glu, and the 3CAM α_{1b}-adrenoceptor were generated and expressed stably in HEK293 cells. Clones were initially screened on the basis of their GFP autofluorescence and subsequently by their capacity to specifically bind the selective α_1-adrenoceptor antagonist/inverse agonist [^{3}H]prazosin. Over a large number of individual clones screened in this preliminary manner, distinct patterns of expression were observed. The wild type α_{1b}-adrenoceptor-GFP demonstrated clear plasma membrane-associated fluorescence. However, a significant fraction of the GFP signal was observed to occupy an intracellular, punctate, perinuclear location (Fig. 2A). Both the Asp^{142}Ala and Ala^{293}Glu forms of the α_{1b}-adrenoceptor-GFP were heavily concentrated at the plasma membrane with little evidence for a significant perinuclear or other intracellular component (Fig. 2, B and C). In contrast, although plasma membrane located 3CAM α_{1b}-adrenoceptor-GFP could be observed, much of the fluorescence in these clones was widely distributed in punctate, intracellular vesicles (Fig. 2D). It was also obvious, over a wide range of clones, that those expressing the 3CAM α_{1b}-adrenoceptor-GFP were substantially less fluorescent and thus appeared to express the construct at lower levels, whereas the two point mutants were present in even

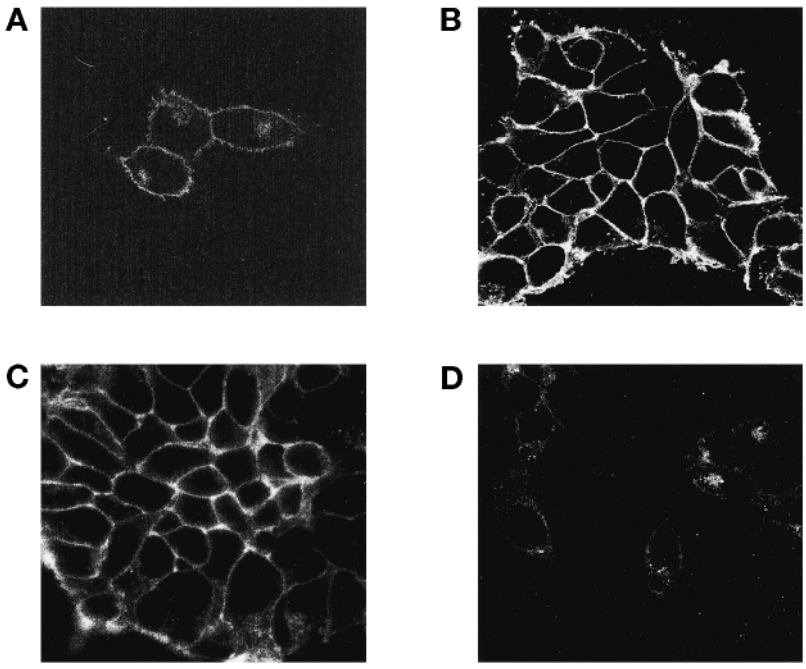


Fig. 2. Cellular distribution patterns and qualitative comparison of expression levels of GFP-tagged wild type and constitutively active mutants of the hamster α_{1b}-adrenoceptor. C-terminally GFP-tagged forms of wild type (A), Asp^{142}Ala (B), Ala^{293}Glu (C), and 3CAM (D) mutants of the hamster α_{1b}-adrenoceptor were expressed stably in HEK293 cells. Cells of representative clones were then examined confocally.

higher amounts than the wild type receptor-GFP (Fig. 2). A further mutant (designated M8) of the α_{1b}-adrenoceptor, which has eight serine residues between amino acids 394 and 415 in the C-terminal tail mutated to alanines (Diviani et al., 1997) (Fig. 1), was also C-terminally tagged with GFP and expressed stably in HEK293 cells. Clones expressing this mutant displayed a very similar pattern of distribution of the receptor-GFP construct to those expressing the wild type α_{1b}-adrenoceptor-GFP (see Fig. 7).

Representative individual clones were selected for detailed analysis. After preparation of a crude particulate fraction from these cells, saturation-specific [^{3}H]prazosin binding experiments were performed (Table 1). These confirmed the 3CAM α_{1b}-adrenoceptor-GFP to be expressed at considerably lower levels than the other four constructs in the clones selected. However, the estimated K_d for [^{3}H]prazosin was similar for each construct (Table 1) and similar to values that Lee et al. (1997) previously reported for nonGFP-tagged forms of both the wild type and the 3CAM α_{1b}-adrenoceptor. To confirm that addition of GFP to the C-terminal tail of these forms of the α_{1b}-adrenoceptor did not either compromise coupling to phosphoinositidase C or obscure the features of constitutive activity previously associated with these mutations, cells expressing each construct were labeled with *myo*-[^{3}H]inositol, and the generation of [^{3}H]inositol phosphates was measured in the absence of ligand and in the presence of varying concentrations of the agonist phenylephrine. When corrected for expression levels of the receptor constructs, each of the previously characterized mutants caused substantially greater accumulation of [^{3}H]inositol phosphates in the absence of ligand than did the wild type α_{1b}-adrenoceptor-GFP (Fig. 3A). However, it was clear that the 3CAM form of α_{1b}-adrenoceptor-GFP was substantially more constitutively active than the other mutants (Fig. 3A). Even the M8 mutant produced slightly greater basal stimulation of [^{3}H]inositol phosphate production than the wild type receptor (Fig. 3A), but this was lower than any of the other mutants studied. Constitutively active mutants still generally produce a further stimulation in response to agonist. This was the case for each of the mutants, including the M8 mutant, on addition of phenylephrine (10 μM), although again 3CAM α_{1b}-adrenoceptor-GFP produced the greatest response (Fig. 3A). When compared with the wild type α_{1b}-adrenoceptor-GFP, agonist potency to stimulate [^{3}H]inositol phosphate production was greater for the Ala^{293}Glu, Asp^{142}Ala, and 3CAM mutants, but not for the M8 mutant, with the 3CAM α_{1b}-adrenoceptor-GFP displaying the most exaggerated shift in potency (Fig. 3B and Table 2). Agonist affinity, measured by the capacity of phenylephrine to compete with [^{3}H]prazosin for receptor binding, was also substantially higher for the 3CAM α_{1b}-adrenoceptor-GFP (Table

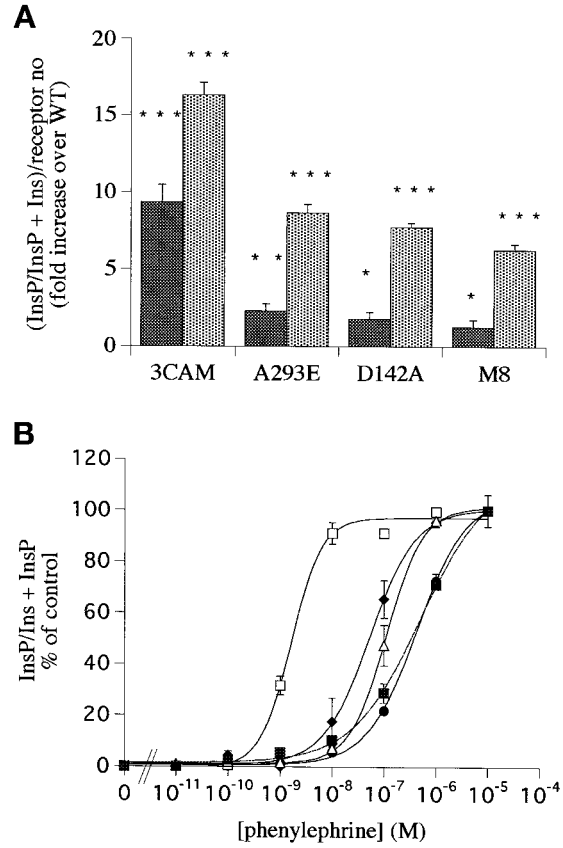


Fig. 3. Constitutive activity and agonist potency at forms of the hamster α_{1b}-adrenoceptor. A, cells expressing C-terminally GFP-tagged forms of wild type, the 3CAM, Ala^{293}Glu, Asp^{142}Ala, or M8 mutants of the hamster α_{1b}-adrenoceptor, were labeled with *myo*-[^{3}H]inositol. The generation of [^{3}H]inositol phosphates was then monitored in the presence of LiCl in the absence (basal or constitutive activity) (▦) or presence (▨) of phenylephrine (10 μM). Data are presented as -fold increase over wild type after standardization for receptor expression levels (see Table 1). *$P < .05$, **$P < .001$, ***$P < .0001$ when compared with wild type α_{1b}-adrenoceptor-GFP. B, cells expressing C-terminally GFP-tagged forms of wild type (●), Asp^{142}Ala (△), Ala^{293}Glu (◆), M8 (■), or 3CAM (□) mutants of the hamster α_{1b}-adrenoceptor were labeled with *myo*-[^{3}H]inositol. The generation of [^{3}H]inositol phosphates was then monitored in the presence of LiCl (15 mM) after addition of varying concentrations of phenylephrine. The effects of phenylephrine above the constitutive activity measured as in Fig. 3A are shown as percent maximal effect and represent mean ± S.E. from three independent experiments.

TABLE 1

Characteristics of the specific binding of [^{3}H]prazosin to GFP-tagged forms of the hamster α_{1b}-adrenoceptor

The specific binding of various concentrations of [^{3}H]prazosin to membranes of stable clones expressing each of the α_{1b}-adrenoceptor-GFP constructs was measured, and B_{max} and K_d values were estimated. Data are presented as mean ± S.E. from three independent experiments.

α_{1b}-Adrenoceptor-GFP Construct	[^{3}H]Prazosin	B_{max}
	K_d, *nM*	*pmol/mg*
Wild type	0.09 ± 0.02	7.0 ± 1.5
3CAM	0.12 ± 0.02	4.0 ± 0.8
A293E	0.10 ± 0.02	22 ± 4
D142A	0.10 ± 0.02	23 ± 3
M8	0.07 ± 0.02	11 ± 1

TABLE 2

Affinity for the α_{1b}-adrenoceptor-GFP constructs and potency of phenylephrine to stimulate inositol phosphate generation

The affinity of phenylephrine to bind to the various forms of the α_{1b}-adrenoceptor-GFP was estimated from competition binding experiments with [^{3}H]prazosin and corrected for receptor occupancy by [^{3}H]prazosin. EC_{50} values derive from stimulation of [^{3}H]inositol phosphate production above basal levels. Data are presented as mean ± S.E. from three independent experiments.

α_{1b}-Adrenoceptor-GFP Construct	K_i	EC_{50}
	μM	*nM*
Wild Type	30 ± 11	438 ± 9
3CAM	0.4 ± 0.05[a]	2.0 ± 0.4[a]
A293E	46 ± 12	80 ± 18[a]
D142A	61 ± 24	180 ± 60[a]
M8	34 ± 14	356 ± 54

[a] Significantly different from wild type, $P < .01$.

2). However, this was not apparent for the other mutant α_{1b}-adrenoceptor-GFP forms.

We have previously noted that long-term treatment of cells stably expressing an untagged form of the 3CAM α_{1b}-adrenoceptor with a range of α_{1b}-adrenoceptor antagonists/inverse agonists results in substantial up-regulation of the protein (Lee et al., 1997). After sustained treatment (24 h) of cells expressing the 3CAM α_{1b}-adrenoceptor-GFP with the antagonists/inverse agonists phentolamine, WB4101, and HV723 (each at 10 μM), the cells became markedly more fluorescent (Fig. 4A). This was not observed after equivalent treatment of cells expressing the wild type α_{1b}-adrenoceptor-GFP (Fig. 4B). Visual inspection of the cells indicated that the up-regulation produced by the antagonist/inverse agonist ligands did not alter the overall cellular distribution pattern of the 3CAM α_{1b}-adrenoceptor-GFP (Fig. 4A). Parallel [^{3}H]prazosin binding studies performed on untreated and antagonist/inverse agonist-treated cells, which were then well washed before membrane preparation, confirmed up-regulation of the 3CAM α_{1b}-adrenoceptor-GFP but not the wild type (Table 3).

The affinity of phentolamine, WB4101, and HV723 for wild type and 3CAM forms of α_{1b}-adrenoceptor-GFP was subsequently monitored based on their capacity to compete with [^{3}H]prazosin for the receptor ligand binding site (Table 3). Only in the case of phentolamine was the affinity of [^{3}H]prazosin higher at the 3CAM α_{1b}-adrenoceptor-GFP compared with the wild type.

Although clear-cut and containing higher information content, visual inspection of cells by confocal microscopy did not

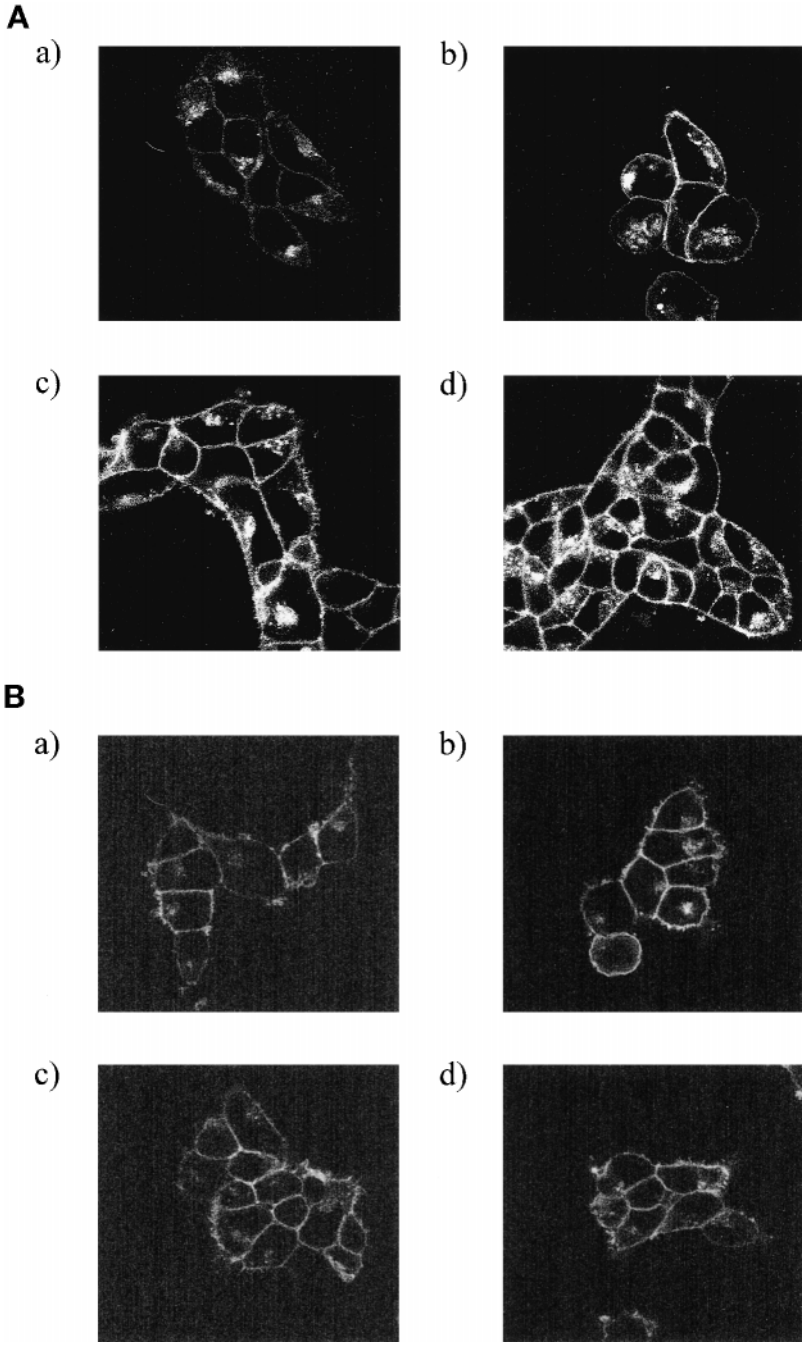


Fig. 4. Up-regulation of the 3CAM but not wild type hamster α_{1b}-adrenoceptor by sustained antagonist/inverse agonist challenge. Cells expressing either the 3CAM (panel A) or wild type (panel B) hamster α_{1b}-adrenoceptor grown on glass coverslips were treated with vehicle (a), phentolamine (b), WB4101 (c), or HV723 (d) (all at 10 μM) for 24 h. The cells were then visualized on a confocal microscope.

represent a convenient means to monitor ligand potency for receptor up-regulation. Cells expressing either the wild type or 3CAM α_{1b}-adrenoceptor-GFP were therefore plated into 96-well microtiter plates and individual wells treated with a range of concentrations of phentolamine, WB4101, or HV723. Fluorescence corresponding to the GFP was then monitored 22 h later. Each of the ligands produced a strong, concentration-dependent up-regulation of GFP fluorescence in cells expressing the 3CAM α_{1b}-adrenoceptor-GFP, with no detectable effect on GFP fluorescence in cells expressing the wild type α_{1b}-adrenoceptor-GFP (Fig. 5A). The time course of this up-regulation of 3CAM α_{1b}-adrenoceptor-GFP was examined with exposure to phentolamine, WB4101, or HV723 (all at 10 μM) for times up to 28 h (Fig. 5B). Half-maximal up-regulation was observed within 15 h.

Unlike the situation with the 3CAM α_{1b}-adrenoceptor-GFP, treatment of cells expressing either the $Asp^{142}Ala$ α_{1b}-adrenoceptor-GFP or the $Ala^{293}Glu$ α_{1b}-adrenoceptor-GFP with concentrations of phentolamine, WB4101, or HV723, which were maximally effective in the up-regulation of the 3CAM α_{1b}-adrenoceptor-GFP, failed to modulate cellular levels of these constitutively active mutants (Figs. 6, A and B). Equivalent results on the capacity of phentolamine and WB4101 to differentially affect levels of the 3CAM α_{1b}-adrenoceptor-GFP and the $Ala^{293}Glu$ α_{1b}-adrenoceptor-GFP were obtained after transient expression of these two constructs in HEK293 cells and overnight treatment with the two ligands (data not shown).

Such results indicated nonequivalence of the different constitutively active mutants of the GFP-tagged α_{1b}-adrenoceptor. However, the level of constitutive activity for generation of inositol phosphates was clearly lower for the $Asp^{142}Ala$ α_{1b}-adrenoceptor-GFP and the $Ala^{293}Glu$ α_{1b}-adrenoceptor-GFP, compared with the 3CAM α_{1b}-adrenoceptor-GFP (Fig. 3A). We thus wished to garner further evidence to dissociate antagonist/inverse agonist-induced up-regulation and constitutive second messenger generation. Although the M8 α_{1b}-adrenoceptor-GFP displayed lower constitutive inositol phosphate generation than either the $Ala^{293}Glu$ or $Asp^{142}Ala$ forms of α_{1b}-adrenoceptor-GFP (Fig. 3A), this form of the receptor was up-regulated by sustained treatment with such ligands (Fig. 7).

To explore the separation of ligand regulation and constitutive activity further, equivalent experiments were performed using the agonist phenylephrine. Twenty-four-hour treatment with this agonist resulted in an up-regulation of the wild type α_{1b}-adrenoceptor-GFP with increased levels being observed at both the plasma membrane and perinuclear locations (Fig. 8A). Increased levels were also observed for all three of the constitutively active mutant α_{1b}-adrenoceptor-GFP constructs after treatment with phenylephrine (Fig. 8, B–D) and for the M8 mutant (Fig. 8E). However, from parallel [^{3}H]prazosin ligand binding studies the only mutant that was up-regulated to a greater extent than the wild type α_{1b}-adrenoceptor-GFP was the 3CAM α_{1b}-adrenoceptor-GFP construct (Fig. 8B). These studies also indicated that the degree of up-regulation observed was substantially lower than that achieved with the antagonist/inverse agonist ligands. For all of the constructs a greater proportion of the cellular receptor appeared to be intracellular after sustained challenge with this agonist.

TABLE 3

The affinity of antagonists/inverse agonists and their capacity to up-regulate the 3CAM α_{1b}-adrenoceptor

The capacity of varying concentrations of phentolamine, WB4101, and HV723 to compete with [^{3}H]prazosin for binding to wild type or 3CAM forms of the hamster α_{1b}-adrenoceptor-GFP was assessed, and K_i values were calculated by correction for receptor occupancy by [^{3}H]prazosin. Up-regulation of levels of 3CAM α_{1b}-adrenoceptor-GFP was also measured from the binding capacity of [^{3}H]prazosin. Data are presented as mean ± S.E. from three independent experiments.

Ligand	Wild Type-GFP	3CAM-GFP	3CAM-GFP Up-Regulation
	K_i, nM	*K_i, nM*	*-fold*
Phentolamine	75 ± 8	23 ± 10*	4.6 ± 0.21
WB4101	7 ± 1	5 ± 1	4.0 ± 0.14
HV723	10 ± 4	3 ± 2	5.3 ± 0.16

* $P < .01$ compared to wild type.

Discussion

Extensive study of the constitutive activity of GPCRs in recent years has provided a range of novel insights, including multiple receptor states must exist (Lefkowitz et al., 1993), a requirement for an extension of the ternary complex model (Samama et al., 1993), and an expansion of the basic lexicon of pharmacology to incorporate the term "inverse agonist" to describe a ligand that preferentially binds to and stabilizes the inactive, ground state conformation of a GPCR (Milligan et al., 1995).

Despite evidence that the detailed alterations resulting from specific point mutations are not identical in terms of the degree of constitutive activity produced (Scheer et al., 1996, 1997, 2000; Scheer and Cotecchia, 1997) and, indeed, that constitutive activity imbued by mutational alteration at different sites can differentially alter separate signaling cascades (Perez et al., 1996), there has been a tendency to treat constitutively active mutants and, thus, the conformational alterations they produce as equivalent. However, this is unlikely to be the case.

One of the interesting features of a constitutively active mutant of the human β_2-adrenoceptor has been its capacity to be stabilized from either denaturation or proteolytic degradation by the binding of ligands. When purified protein is examined, both agonist and antagonist/inverse agonist ligands have this capacity (Gether et al., 1997). However, in the intact cell situation, although the effects of antagonists/inverse agonists can clearly be observed as an up-regulation of GPCR protein levels over time (MacEwan and Milligan, 1996; for reviews see Milligan and Bond, 1997 and Leurs et al., 1998), the effect of agonist ligands is confounded because the mutant GPCR is further activated, resulting in its internalization. This effect has recently been visualized after C-terminal GFP-tagging of this constitutively active mutant β_2-adrenoceptor (McLean et al., 1999). After stable expression in HEK293 cells, this construct was markedly up-regulated by sustained treatment with the inverse agonist betaxolol but was then rapidly internalized on addition of isoprenaline. Because up-regulation by betaxolol of the constitutively active mutant β_2-adrenoceptor-GFP construct could be monitored in a 96-well microtiter plate format and occurred in a concentration-dependent manner (McLean et al., 1999), then equivalence of other constitutively active mutant GPCRs in this regard could provide a simple antagonist/inverse agonist identification strategy because mutations imparting constitutive activity have now been de-

scribed for a very wide range of GPCRs (Leurs et al., 1998; Pauwels and Wurch, 1998).

C-terminally GFP-tagged forms of either the wild type or 3CAM α_{1b}-adrenoceptor were thus stably expressed in HEK293 cells. Initial characterization confirmed that the binding affinity of [^{3}H]prazosin to the two constructs was similar and was also unaffected by the addition of GFP (Table 1). It was immediately clear that although a significant amount of the wild type α_{1b}-adrenoceptor-GFP was located at the plasma membrane, there was a distinct fraction located with an intracellular, perinuclear distribution. Awaji et al. (1998) and Tsujimoto et al. (1998) have also noted a degree of intracellular localization of a wild type α_{1b}-adrenoceptor-GFP but also recorded that this was substantially more pronounced for an equivalent construct of the wild type α_{1a}-adrenoceptor. Interestingly, intracellular α_{1a}-adrenoceptors in LLCPK cells have been shown to be rapidly recruited to the cell surface by treatment with either a high concentration of phenylephrine or combinations of subthreshold levels of both phenylephrine and neuropeptide Y (Holtback et al., 1999). Significant levels of intracellular α_{1d}-adrenoceptors have also been recorded by monitoring their binding by a fluorescent quinazoline derivative (Daly et al., 1998). In contrast, a substantially greater fraction of the 3CAM α_{1b}-adrenoceptor-GFP was intracellular, but the pattern was not the same for the wild type receptor. Indeed, rather than being perinuclear, the bulk of the 3CAM α_{1b}-adrenoceptor-GFP was present in small punctate vesicles distributed throughout much of the cytoplasm (Fig. 2). Perhaps surprisingly then, after stable expression both the Asp^{142}Ala and Ala^{293}Glu α_{1b}-adrenoceptor-GFP constructs were heavily

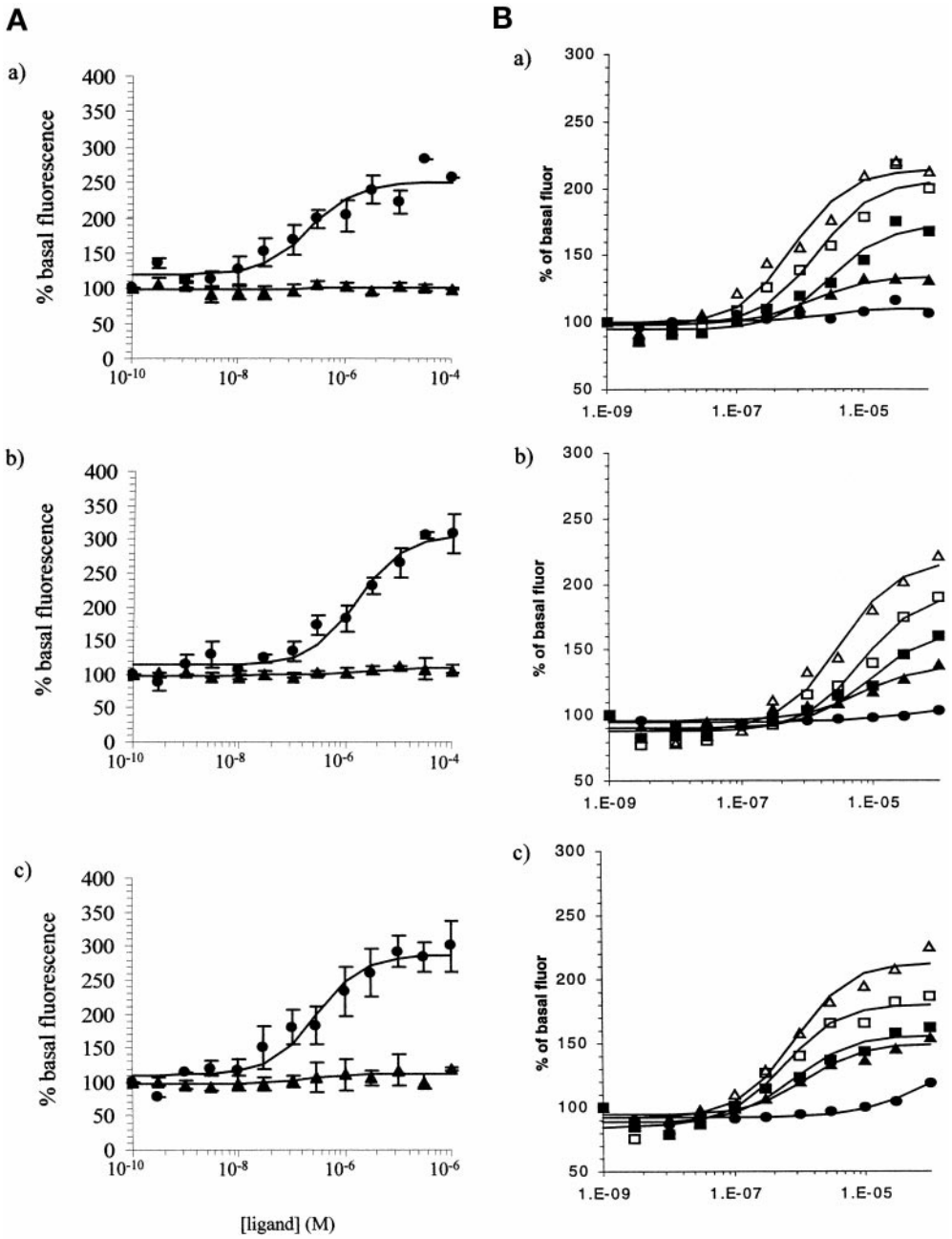


Fig. 5. Concentration dependence and time course of the up-regulation of 3CAM hamster α_{1b}-adrenoceptor-GFP by antagonist/inverse agonist ligands. Panel A, cells expressing either the 3CAM (circles) or wild type (diamonds) hamster α_{1b}-adrenoceptor-GFP were grown in wells of a 96-well microtiter plate. The cells were then exposed to varying concentrations of phentolamine (a), WB4101 (b), or HV723 (c), and fluorescence was measured on a Spectrofluor Plus fluorimeter after 22 h. Values are the mean percentages of basal fluorescence from six experiments performed in duplicate ± S.E.M. Panel B, cells expressing the 3CAM hamster α_{1b}-adrenoceptor-GFP were grown in wells of a 96-well microtiter plate and exposed to phentolamine (a), WB4101 (b), or HV723 (c) (all at 10 μM) for 140 min (●), 480 min (▲), 880 min (■), 1365 min (□), or 1690 min (△). Fluorescence was then measured as described in panel A with data derived from representative experiments.

concentrated at the plasma membrane with little evidence of significant intracellular levels (Fig. 2).

Sustained treatment of the 3CAM α_{1b}-adrenoceptor-GFP expressing cells with a wide range of antagonists/inverse agonists, including phentolamine, WB4101, HV723 (Fig. 4A), carvedilol, and prazosin (not shown), caused up-regulation of the construct. This increase in fluorescence could be monitored for cell populations in a microtiter plate format allowing EC_{50} values for ligand effects to be measured (Fig. 5A). However, the overall cellular distribution pattern remained unchanged with both increased plasma membrane and intracellular levels. [^{3}H]Prazosin binding studies on membranes of 3CAM α_{1b}-adrenoceptor-GFP cells indicated that the antagonists/inverse agonists produced up to a 5-fold increase in receptor levels (Table 3). As might be anticipated if enhanced constitutive activity was required to produce the up-regulation, these ligands had no significant effect on levels or the distribution pattern of the wild type α_{1b}-adrenoceptor-GFP, which could be monitored confocally (Fig. 4B), fluorometrically (Fig. 5A), or in [^{3}H]prazosin binding studies (not shown). However, although both the Asp^{142}Ala α_{1b}-adrenoceptor-GFP and Ala^{293}Glu α_{1b}-adrenoceptor-GFP constructs displayed clear constitutive stimulation of phosphoinositidase C activity, this was substantially less than that produced by equivalent expression of the 3CAM α_{1b}-adrenoceptor-GFP (Fig. 3A), and sustained treatment of these cells with the same set of antagonists/inverse agonists again failed to produce statistically significant up-regulation of these con-

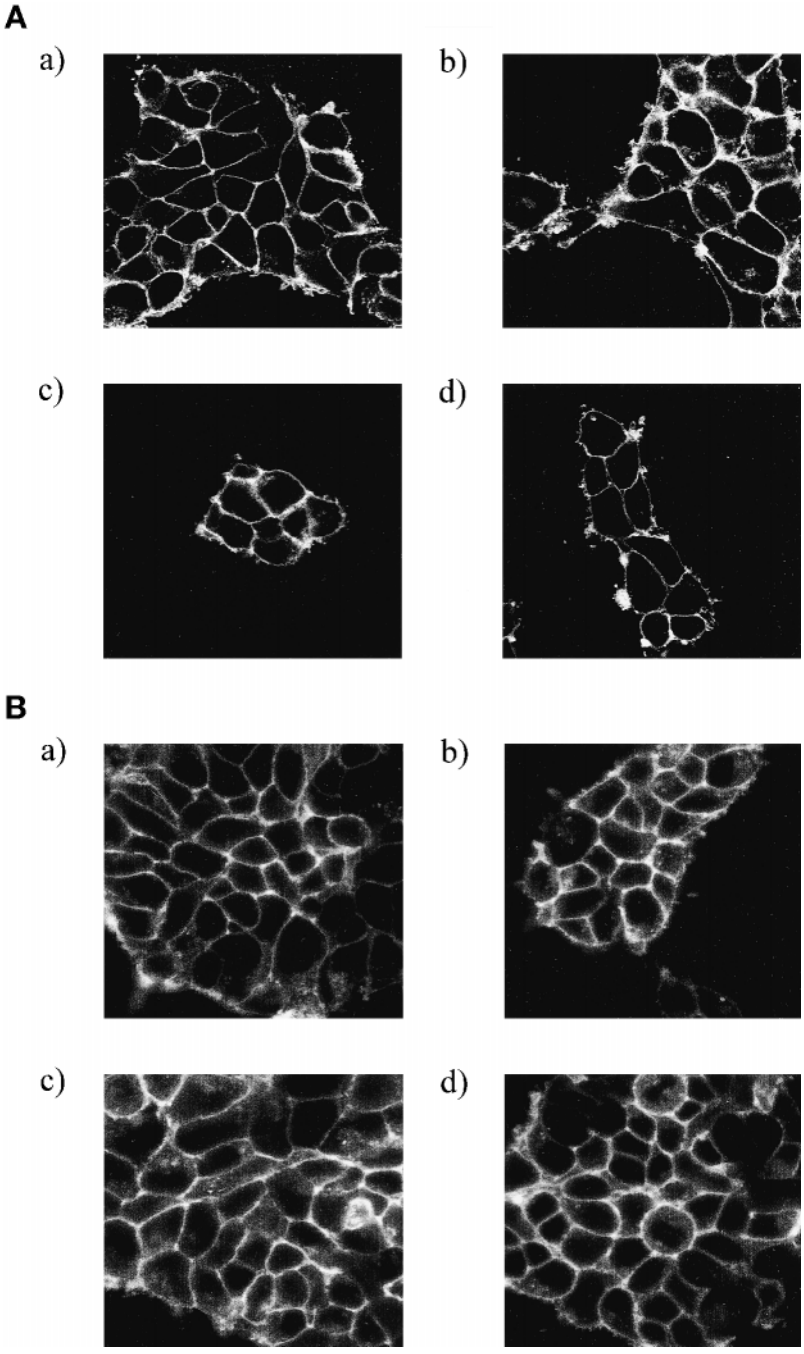


Fig. 6. Lack of up-regulation of Asp^{142}Ala and Ala^{293}Glu forms of the hamster α_{1b}-adrenoceptor by sustained antagonist/inverse agonist challenge. Cells expressing either the Asp^{142}Ala (panel A) or Ala^{293}Glu (panel B) hamster α_{1b}-adrenoceptor grown on glass coverslips were treated with vehicle (a), phentolamine (b), WB4101 (c), or HV723 (d) (all at 10 μM) for 24 h. The cells were then visualized with a confocal microscope.

structs (Fig. 6, A and B). These observations appear to provide clear evidence of the nonequivalence of the individual constitutively active mutant forms of the α_{1b}-adrenoceptor and indicate that suggestions that such ligand-induced up-regulation might provide a simple monitor of receptor constitutive activity (Milligan and Bond, 1997; Leurs et al., 1998) are too simplistic. However, it was noted that the 3CAM α_{1b}-adrenoceptor-GFP also had the most exaggerated shift in phenylephrine potency to stimulate second messenger generation (Fig. 3B, Table 2) and affinity for phenylephrine (Table 2) of the mutants tested, as well as the highest level of constitutive activity. Enhanced affinity and potency of agonist ligands is a feature often associated with the concept that constitutively active mutants may provide a useful model of the R* active conformation of wild type receptors (Lefkowitz et al., 1993). However, it is clear from these studies that the extent of this shift is variable between mutants. It has also been noted both that combinations of individual constitutively active point mutants can produce synergism in these effects (Hwa et al., 1997), and its manifestation can be dependent on the chemical structure of the agonist (Perez et al., 1996). To further resolve the features of constitutive activity and antagonist/inverse agonist-induced up-regulation of the α_{1b}-adrenoceptor, we searched for a mutant that would display low constitutive activity but would be up-regulated by antagonist/inverse agonist treatment. We identified a mutant (M8) with these characteristics that has the eight serines between amino acids 394 and 415 replaced with alanine (Diviani et al., 1997). After stable expression of a GFP-tagged form of this construct in HEK293 cells, the level of agonist-independent inositol phosphate generation was lower than for either the $Asp^{142}Ala$ α_{1b}-adrenoceptor-GFP or $Ala^{293}Glu$ α_{1b}-adrenoceptor-GFP constructs (Fig. 3A), and there was no shift in potency of phenylephrine to stimulate inositol phosphate production or in its affinity to bind to this variant receptor. However, this form of the receptor was up-regulated by antagonist/inverse agonist ligand treatment (Fig. 7). The serine residues removed in this mutant are key targets for both protein kinase C and G protein-coupled receptor kinase-mediated phosphorylation of the α_{1b}-adrenoceptor, and this form of the receptor is resistant to desensitization (Diviani et al., 1997), which may account for the higher inositol phosphate generation compared with the wild type α_{1b}-adrenoceptor-GFP in both basal conditions and on addition of phenylephrine (Fig. 3A).

Perhaps more surprisingly, sustained challenge with phenylephrine resulted in up-regulation of all the constitutively active mutants of the α_{1b}-adrenoceptor-GFP, although this was not as impressive an effect for the 3CAM α_{1b}-adrenoceptor-GFP as that produced by the antagonist-inverse agonist ligands. Although this is in accord with the view that binding of any ligand, no matter its functional characteristics, is associated with greater stability of the well studied constitutively active β_2-adrenoceptor (Gether et al., 1997), this up-regulation was also observed for both the wild type α_{1b}-adrenoceptor-GFP and the M8 form of the receptor (Fig. 8). Again, confocal images and [^{3}H]prazosin binding studies produced equivalent results with the greater information content present in the confocal images, indicating a more significant fraction of the constructs being intracellullar after agonist treatment (Fig. 8). Short-term agonist treatment of a wild type α_{1b}-adrenoceptor-GFP construct expressed in pituitary αT-3 cells has previously been shown to cause internalization of the construct (Awaji et al., 1998).

Direct measurements of the affinity of phentolamine, WB4101, and HV723 to bind to the 3CAM α_{1b}-adrenoceptor-GFP construct demonstrated that all these ligands to have low nanomolar affinity (Table 3). However, the EC_{50} for their capacity to cause up-regulation of this construct was substantially higher (Fig. 5A), being in the micromolar region. One scenario could envisage antagonist/inverse agonist binding causing stabilization of the construct and thus reducing its rate of degradation as has been proposed for the equivalent constitutively active mutant (CAM) version of the β_2-adrenoceptor (Gether et al., 1997). In the face of ongoing protein synthesis, this would result in time-dependent up-regulation of 3CAM α_{1b}-adrenoceptor-GFP as observed in Fig. 5B. However, in this most straightforward scenario, ligand binding and effect curves would be expected to be very similar, which is clearly not the case herein.

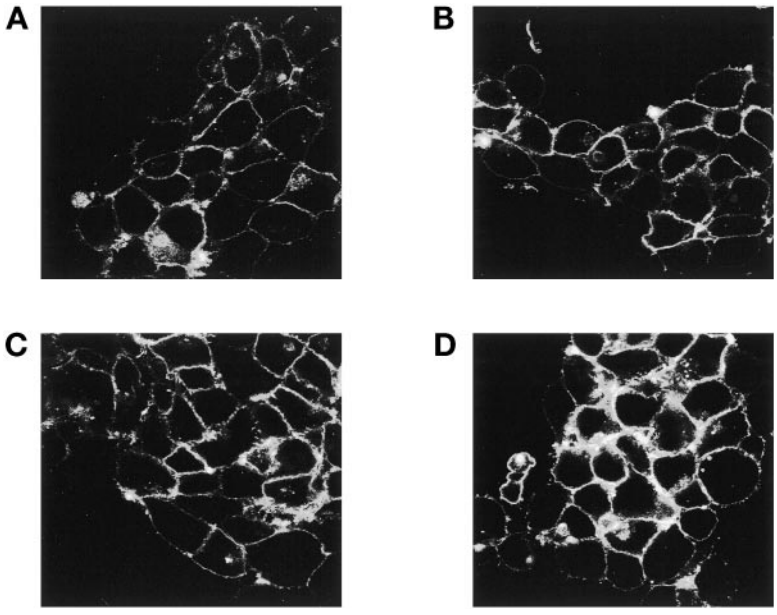
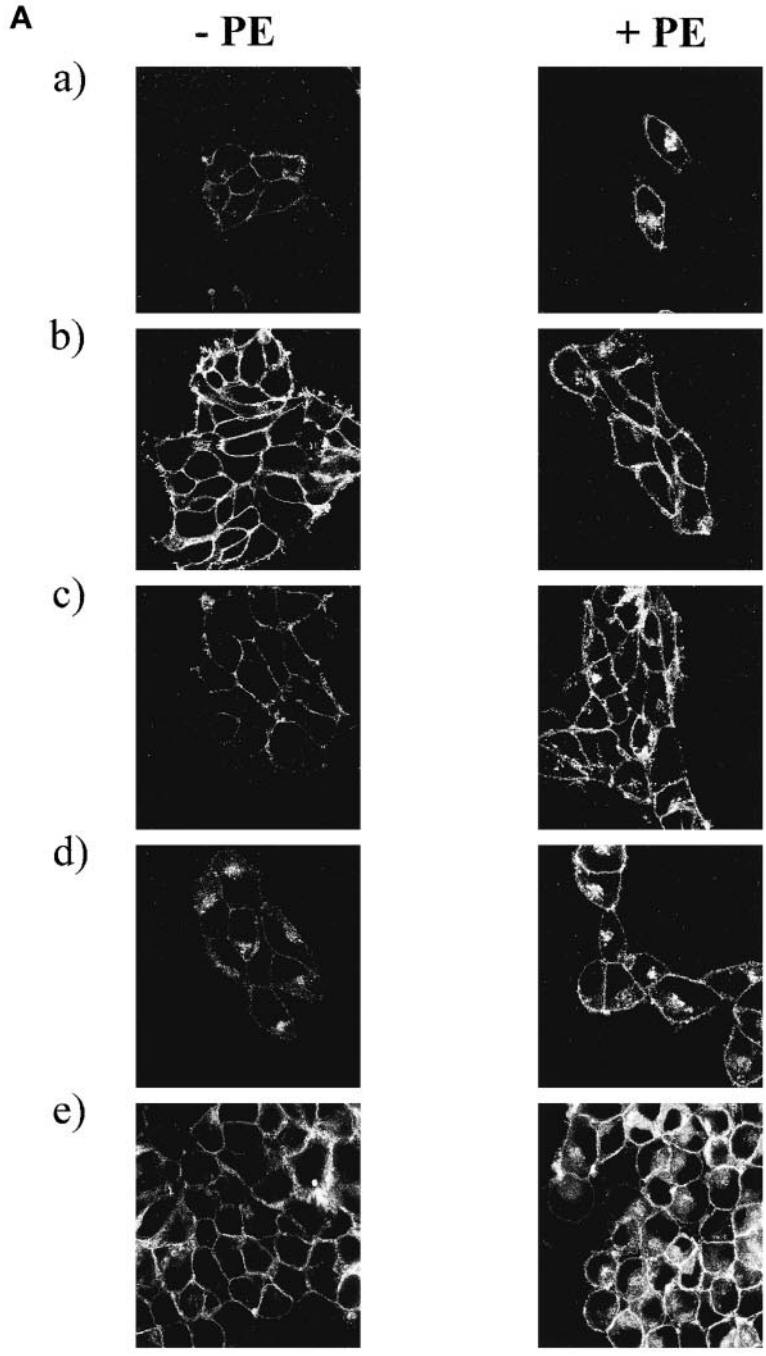


Fig. 7. The M8 form of the hamster α_{1b}-adrenoceptor is up-regulated by sustained antagonist/inverse agonist challenge. Cells stably expressing the M8 form of the hamster α_{1b}-adrenoceptor grown on glass coverslips were treated with vehicle (A), phentolamine (B), WB4101 (C), or HV723 (D) (all at 10 μM) for 24 h. The cells were then visualized with a confocal microscope.



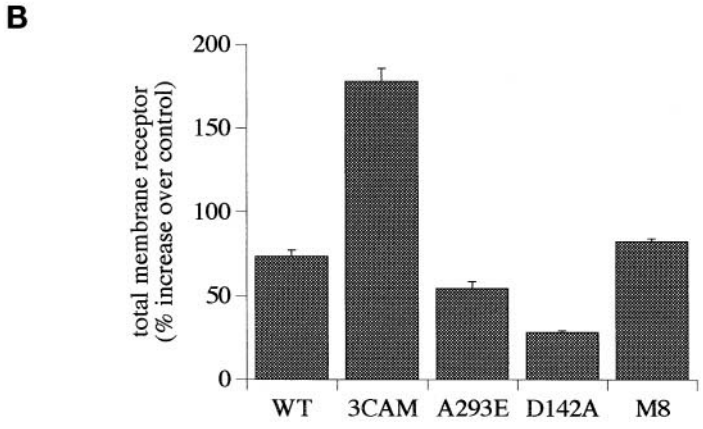


Fig. 8. Sustained challenge with phenylephrine causes upregulation of all forms of GFP-tagged hamster α_{1b}-adrenoceptor. Panel A, cells expressing GFP-tagged forms of either the wild type (a), $Asp^{142}Ala$ (b), $Ala^{293}Glu$ (c), 3CAM (d), or M8 (e) forms of the hamster α_{1b}-adrenoceptor were grown on glass coverslips and treated with either vehicle (−PE) or phenylephrine (+PE) (10 μM) for 24 h. The cells were then visualized. Panel B, equivalent cells grown in tissue culture dishes, treated with vehicle or phenylephrine (10 μM), were then washed and harvested. Membranes were prepared, and the specific binding capacity of a single concentration of [^{3}H]prazosin (2 nM) was then assessed. Results are presented as the percent increase in levels of the GFP fusion proteins produced by phenylephrine treatment.

These studies clearly demonstrate the nonequivalence of antagonist/inverse agonist regulation of individual constitutively active forms of the α_{1b}-adrenoceptor and also inherently dissociate constitutive activity of GPCR mutants from the destabilisation of G protein structure, which has been observed to be associated with certain constitutively active GPCR mutations. Further analysis will be required to fully understand the molecular and structural bases responsible for these two features.

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