

Up-regulation of the levels of expression and function of a constitutively active mutant of the hamster α_{1B} -adrenoceptor by ligands that act as inverse agonists

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The α_1 -adrenergic agonist phenylephrine stimulated phospholipase D (PLD) activity in Rat 1 fibroblasts transfected to express either the wild-type hamster α_{1B} -adrenoceptor or a constitutively active mutant (CAM) form of this receptor. The EC_{50} for agonist stimulation of PLD activity was substantially lower at the CAM receptor than at the wild-type receptor as previously noted for phenylephrine stimulation of phosphoinositidase C activity. Sustained treatment of cells expressing the CAM α_{1B} -adrenoceptor with phentolamine resulted in a marked up-regulation in levels of this receptor with half-maximal effects produced within 24 h and with an EC_{50} of approx. 40 nM. Such an up-regulation could be produced with a range of other ligands generally viewed as α_1 -adrenoceptor antagonists but equivalent

treatment of cells expressing the wild-type α_{1B} -adrenoceptor was unable to mimic these effects. After sustained treatment of the CAM α_{1B} -adrenoceptor expressing cells with phentolamine, basal PLD activity was increased and phenylephrine was now able to stimulate PLD activity to greater levels than in vehicle-treated CAM α_{1B} -adrenoceptor-expressing cells. The EC_{50} for phenylephrine stimulation of PLD activity was not altered, however, by phentolamine pretreatment and the associated up-regulation of the receptor. After phentolamine-induced up-regulation of basal PLD activity, a range of α_1 -antagonists were shown to possess the characteristics of inverse agonists of the CAM α_{1B} -adrenoceptor as they were able to substantially decrease the elevated basal PLD activity.

INTRODUCTION

Sustained agonist treatment of cells expressing a variety of G-protein-coupled receptors (GPCRs) regularly results in a decrease in levels of the receptor [1]. This process is known as down-regulation and often reflects a combination of enhanced degradation of the receptor polypeptide and alterations in its rate of synthesis [1,2]. Recent studies on the histamine H_2 receptor have shown that sustained treatment of Chinese hamster ovary cells transfected to express this receptor with ligands that function as inverse agonists, judged by their capacity to inhibit basal adenylate cyclase activity, results in up-regulation of the receptor [3]. Equivalent experiments performed with an antagonist ligand that had no ability to alter basal adenylate cyclase activity failed to alter levels of expression of the receptor [3]. An interpretation of these findings is that inverse agonists might have the capacity to cause up-regulation of receptors that display significant agonist-independent signal transduction activity, perhaps because they relax the structure of the receptor to one in which this activity is decreased or minimized.

Both naturally occurring and experimentally introduced mutations of a number of GPCRs are known to result in higher levels of agonist-independent effector regulation than is produced by the wild-type receptors [4–8]. Such mutations are called constitutively active mutant (CAM) variants of the receptors, although the degree of spontaneous activity produced can vary widely. In general they are still responsive to the addition of agonist ligands but are being widely examined as contributors to an understanding of the structural changes that must accompany the activation of a receptor [4,5]. We have recently shown that sustained treatment of neuroblastoma \times glioma hybrid NG108-

15 cells transfected to express stably a CAM variant of the human β_2 -adrenoceptor with the inverse agonists betaxolol and sotalol results in strong up-regulation of this receptor, whereas equivalent treatment of cells harbouring the wild-type human β_2 -adrenoceptor produces much less marked alterations in receptor expression [9]. However, as the levels of expression of adenylate cyclase in NG108-15 cells represent the limiting element of the signal transduction cascade [10], the higher levels of expression of the CAM β_2 -adrenoceptor after inverse agonist treatment did not result in greater agonist stimulation of adenylate cyclase activity after removal of the inverse agonist [9].

Unlike the adenylate cyclase system, lipid signalling pathways are not generally limited in intensity by levels of expression or activity of the catalytic effector enzyme and thus in the current study we examined the ability of phentolamine, a ligand usually viewed as an α_1 -adrenoceptor antagonist but which has previously been shown to act as an inverse agonist at a CAM variant of the hamster α_{1B} -adrenoceptor [11] to regulate levels of expression of this receptor and to alter the effectiveness of signal transmission via this receptor.

MATERIALS AND METHODS

Materials

All materials for tissue culture were supplied by Life Technologies (Paisley, Strathclyde, Scotland, U.K.). [3 H]Prazosin (24 Ci/mmol), *myo*-[2- 3 H]inositol (17.6 Ci/mmol) and [3 H]-palmitate were obtained from Amersham International (Little Chalfont, Bucks., U.K.). All other chemicals were from Sigma Chemical (Poole, Dorset, U.K.) and were of the highest purity available.

Abbreviations used: CAM, constitutively active mutant; DMEM, Dulbecco's modified Eagle's medium; GPCR, G-protein-coupled receptor; PLD, phospholipase D; YM-12617, 5-(2-([2-(ethoxyphenoxy)ethyl]amino)propyl)-2-methoxybenzenesulphonamide HCl.

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Cells

The Rat 1 fibroblast clones transfected to express stably either the wild-type hamster α_{1B} -adrenoceptor or the CAM mutant of this receptor have been described previously [12,13]. These were maintained in tissue culture in Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) newborn calf serum, 2 mM glutamine, 100 units penicillin and 100 μ g/ml streptomycin. For most experiments cells were grown until close to confluency and then either harvested or subcultured in a 1:10 ratio. In a number of experiments cells were treated for varying periods of time with concentrations (up to 1 μ M) of either phentolamine or other ligands with α_1 -adrenoceptor-binding affinity. Before experiments were performed on these cells the medium containing the ligand was removed and the cells were washed three times with fresh medium lacking the ligand.

Preparation of membranes

Membrane fractions were prepared from cell pastes that had been stored at -80°C after harvesting. Cell pellets were resuspended in 5 ml of 10 mM Tris/HCl (pH 7.5)/0.1 mM EDTA (buffer A) and rupture of the cells was achieved with 25 strokes of a hand-held Teflon-glass homogenizer. Unbroken cells and nuclei were removed from the resulting homogenate by centrifugation at 500 *g* for 10 min in a Beckman L5-50B centrifuge with a Ti-50 rotor. The supernatant fraction was then centrifuged at 48 000 *g* for 10 min and the pellet was washed and resuspended in 10 ml of buffer A. Membrane fractions were recovered after a second centrifugation at 48 000 *g* for 10 min and pellets were resuspended in buffer A to a final protein concentration of 1–3 mg/ml and stored at -80°C until required.

[^3H]Prazosin-binding experiments

Binding assays were initiated by the addition of 5–15 μ g of protein to an assay buffer [50 mM Tris/HCl (pH 7.4)/0.5 mM EDTA] containing [^3H]prazosin (0.005–1 nM in saturation assays and between 0.1 and 1 nM for competition assays) in the absence or presence of increasing concentrations of the test drugs (500 μ l final volume). Non-specific binding was determined in the presence of 10 μ M phentolamine. Reactions were incubated for 30 min at 25°C and bound ligand was separated from free ligand by vacuum filtration through GF/B filters. The filters were washed twice with 5 ml of assay buffer and bound ligand was estimated by liquid-scintillation spectrometry.

α_1 -Adrenoceptor regulation of inositol phosphate production

Cells were seeded in 24-well plates and labelled close to isotopic equilibrium by incubation with 1 μ Ci/ml *myo*-[2- ^3H]inositol in 0.5 ml inositol-free DMEM containing 1% (v/v) dialysed newborn calf serum for 36 h. On the day of experiments, the labelling medium was removed and cells were washed twice with 0.5 ml of Hanks buffered saline, pH 7.4, containing 1% (w/v) BSA and 10 mM glucose (HBG). Cells were then washed twice for 10 min with HBG supplemented with 10 mM LiCl (HBG/LiCl) and subsequently stimulated with agonist in HBG/LiCl for 20 min. All incubations were performed at 37°C . Reactions were terminated by the addition of 0.5 ml of ice-cold methanol. Cells were then scraped and transferred to vials, after which chloroform was added to a ratio of 1:2 (CHCl₃/MeOH). Total inositol phosphates were extracted for 30 min before the addition of chloroform and water to a final ratio of 1:1:0.9 (CHCl₃/MeOH/H₂O). The upper phase was taken and total inositol phosphates were analysed by batch chromatography on Dowex-1 formate as previously described [13,14].

Regulation of Phospholipase D (PLD) activity

This activity was examined essentially as described by MacNulty et al. (14). Cells were seeded in 24-well plates and labelled close to isotopic equilibrium by incubation with 4 μ Ci/ml of [9,10- ^3H]palmitic acid (51 mCi/ml) in DMEM containing 5% (v/v) newborn calf serum for 24–36 h. On the day of the experiment, the medium was replaced with 500 μ l of HBG as described above and incubated at 37°C for 20 min. Cells were then incubated with 500 μ l of HBG containing 0.3% (v/v) butan-1-ol (HBG/butanol) at 37°C for 10 min and subsequently treated with test drugs in HBG/butanol for 20 min. Reactions were terminated by removal of the drug-containing solution and the immediate addition of 500 μ l of ice-cold analytical grade methanol. Cells were scraped and extracted with 700 μ l of analytical grade chloroform at room temperature for 15 min and 585 μ l of deionized water was then added to give a final ratio of chloroform/methanol/water (1:1:0.8, by vol.) before centrifugation at 1200 rev./min in a table-top centrifuge for 5 min. A 450 μ l sample of the lower chloroform phase was taken and dried down by vacuum centrifugation in a Jouan RC centrifugal evaporator for 30 min. Dried samples were resuspended twice in 25 μ l of solvent II [chloroform/methanol (19:1, v/v)] and loaded on TLC plates (Whatman LK5-DF) together with phosphatidylbutanol standard on each lane. The plates were run to 1–2 cm from the top in solvent I [2,2,4-trimethylpentane (iso-octane)/ethyl acetate/acetic acid/deionized water (final ratio 5:11:2:10, by vol.)] and then stained with iodine for 5 min. The stained area was then scraped and counted by liquid-scintillation spectrometry.

Reverse transcriptase-PCR

The reverse transcriptase-PCR procedure was essentially as described previously [13]. Total RNA was extracted with RNAzol B (Biogenesis). Purity and quantification of RNA were assessed by spectrophotometric A_{260}/A_{280} ratios. Samples of 10–20 μ g of RNA (20 μ l) were denatured by incubation at 65°C for 10 min followed by chilling on ice and reverse transcribed in 33 μ l of reaction mixture with a first-strand cDNA synthesis kit (Pharmacia LKB Biotechnology) as detailed by the manufacturer. Incubation was performed at 37°C for 1 h. The reactions were terminated by heating samples at 95°C for 5 min followed by transfer to ice. PCR reactions were performed with the following primers: α_{1B} -sense, 5'-GACGACAAGGAATGCGGAGTC-3'; α_{1B} -anti-sense, 5'-GTCCACGGCCGATAGGTGTAA-3'. Amplifications were performed in 50 μ l of buffer containing 25 pmol of primers and 2.5 units of *Taq* polymerase (Promega) using a HYBAID Omnigene temperature cycler and were performed as follows: 94°C for 1 min, 60°C for 2 min, 72°C for 3 min (33 cycles), 94°C for 1 min, 60°C for 2 min, 72°C for 5 min (1 cycle). In a number of cases samples were then digested with the restriction enzyme *Sma*I. Reaction products were then separated on a 1.8% (w/v) agarose gel. In each case the size of the generated product was that expected from the selected primers.

Data analysis

Analysis was performed with the Kaleidagraph (version 2.1) curve-fitting package with an Apple Macintosh computer.

RESULTS

We have previously reported that both the wild-type hamster α_{1B} -adrenoceptor and a CAM variant of this receptor respond to

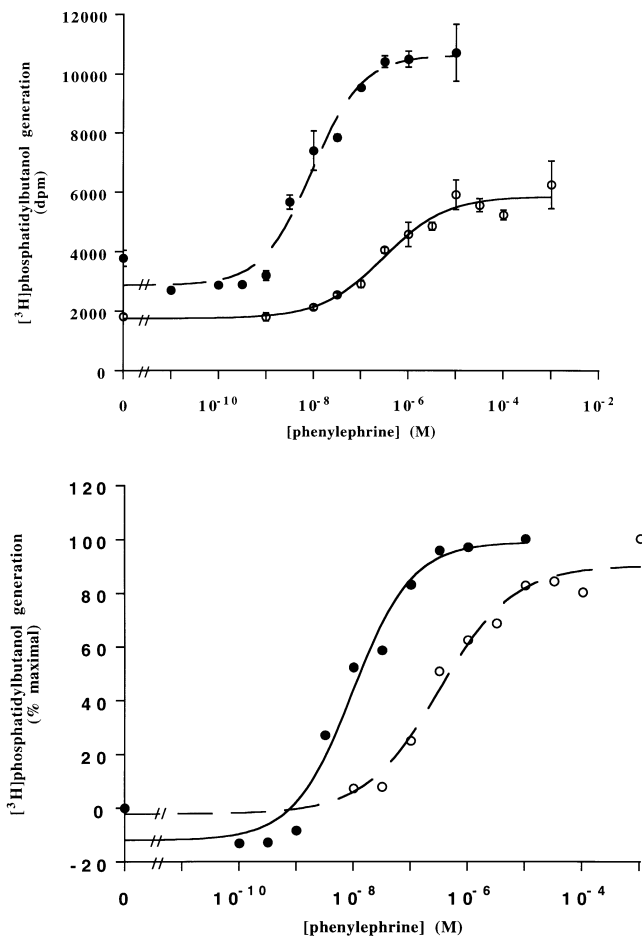


Figure 1 Increased basal PLD activity and maximal response to phenylephrine in Rat 1 fibroblasts expressing a CAM form of the α_{1B} -adrenoceptor compared with those expressing the wild-type receptor

Basal PLD activity and its stimulation by varying concentrations of phenylephrine were measured in Rat 1 fibroblasts expressing either the wild-type (○) or a CAM variant (●) of the hamster α_{1B} -adrenoceptor (upper panel). Data represent means \pm S.D. for quadruplicate assays from a representative experiment of four (CAM) or two (wild-type) performed (see the Results section for details). Lower panel: the data from the upper panel are presented as a percentage of the maximal effect of phenylephrine.

the agonist phenylephrine with a large stimulation of inositol phosphate generation when stably expressed in Rat 1 fibroblasts [11,13]. Equivalent experiments on these cells, after their labelling with [³H]palmitate and short-term treatment with butan-1-ol to allow production of [³H]phosphatidylbutanol and hence measure receptor regulation of the activity of phospholipase D [14], demonstrated that basal PLD activity was significantly higher (3.0 ± 1.0 -fold, mean \pm S.E.M., $n = 3$, $P < 0.01$) in the cells expressing the CAM α_{1B} -adrenoceptor (Figure 1, upper panel). Both the wild-type α_{1B} -adrenoceptor-expressing cells and those expressing the CAM α_{1B} -adrenoceptor displayed concentration-dependent increases in PLD activity in response to phenylephrine (Figure 1). The absolute level of [³H]phosphatidylbutanol produced by maximally effective concentrations of phenylephrine was substantially greater in the CAM α_{1B} -adrenoceptor-expressing cells than in those expressing the wild-type α_{1B} -adrenoceptor (2.9 ± 0.4 -fold, mean \pm S.E.M., $n = 5$, $P < 0.01$) even though levels of expression of the wild-type receptor were higher (results not shown, but see [13]). As observed when measuring [³H]inositol

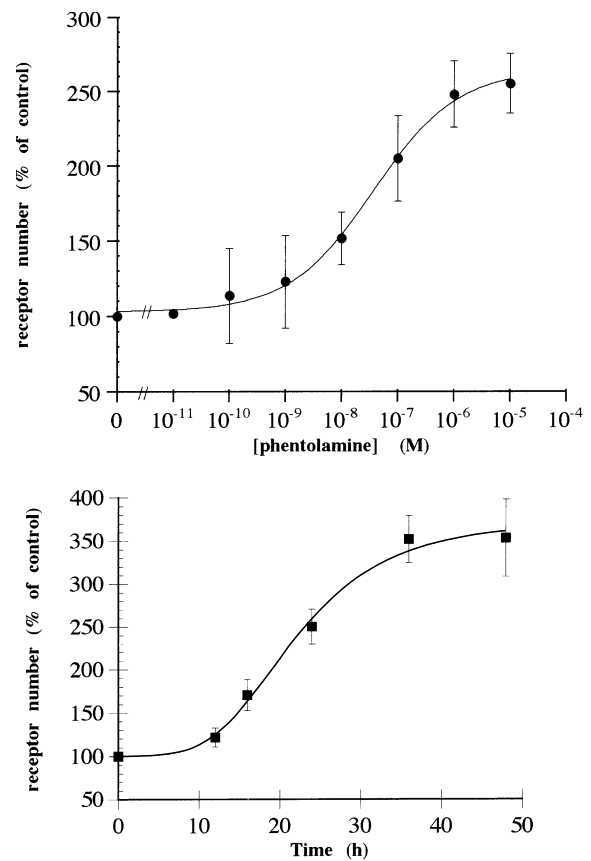


Figure 2 Sustained treatment of CAM α_{1B} -adrenoceptor-expressing Rat 1 fibroblasts with phentolamine increases levels of specific [³H]prazosin binding

Upper panel: concentration studies. Rat 1 fibroblasts expressing the CAM α_{1B} -adrenoceptor were treated for 24 h with various concentrations of phentolamine. After removal of phentolamine and membrane preparation, the specific binding of [³H]prazosin (1 nM) was measured. Data represent means \pm S.D. for triplicate measurements from a representative experiment of three performed (see the Results section for details). In these experiments basal levels of the CAM α_{1B} -adrenoceptor were 1.2 ± 0.1 pmol/mg of protein. Lower panel: time dependence. Rat 1 fibroblasts expressing the CAM α_{1B} -adrenoceptor were treated for various times with 1 μ M phentolamine. After removal of phentolamine and membrane preparation, the specific binding of [³H]prazosin (1 nM) was measured. Data represent means \pm S.E.M. for three independent experiments. [³H]Prazosin binding at zero time was 1.4 ± 0.1 pmol/mg of protein.

phosphate generation [13], the EC_{50} for phenylephrine stimulation of PLD activity was substantially less in the CAM α_{1B} -adrenoceptor-expressing cells (14 ± 4 nM, mean \pm S.E.M., $n = 4$) than in those expressing the wild-type α_{1B} -adrenoceptor (330 ± 10 nM, mean \pm range, $n = 2$) (Figure 1, lower panel). It must be noted, however, that the difference in EC_{50} for phenylephrine stimulation of PLD activity at the CAM α_{1B} -adrenoceptor compared with the wild-type α_{1B} -adrenoceptor was not as pronounced as we have previously recorded for [³H]inositol phosphate generation (greater than 100-fold) [13]. We have no obvious explanation for these differences.

Sustained treatment of the CAM α_{1B} -adrenoceptor-expressing cells with phentolamine resulted in a marked concentration-dependent (Figure 2, upper panel) and time-dependent (Figure 2, lower panel) increase in levels of specific [³H]prazosin (1 nM) binding to membrane preparations of the cells. The EC_{50} for increases in specific [³H]prazosin binding induced by 24 h of treatment with phentolamine was 37 ± 9 nM (mean \pm S.E.M.,

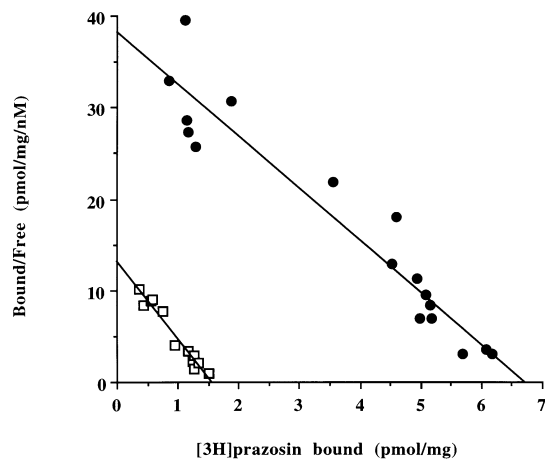


Figure 3 Phentolamine-induced increase in [^3H]prazosin binding in CAM $\alpha_{1\text{B}}$ -adrenoceptor-expressing Rat 1 fibroblasts represents an up-regulation in receptor number

Rat 1 fibroblasts expressing the CAM $\alpha_{1\text{B}}$ -adrenoceptor were treated for 36 h with or without $1 \mu\text{M}$ phentolamine. After removal of phentolamine and membrane preparation, saturation binding studies were performed with [^3H]prazosin. Data are presented as a Scatchard plot. In the experiment displayed the maximal specific binding of [^3H]prazosin was 1.7 pmol/mg of membrane protein and the estimated K_{d} for this interaction was 120 pM without phentolamine pretreatment (\square) and 6.6 pmol/mg membrane protein and 180 pM after pretreatment (\bullet).

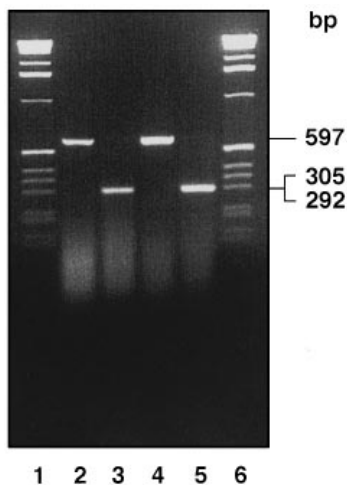


Figure 4 All the $\alpha_{1\text{B}}$ -adrenoceptor mRNA in CAM $\alpha_{1\text{B}}$ -adrenoceptor-expressing Rat 1 fibroblasts encodes the mutant protein both with and without pretreatment with phentolamine

CAM $\alpha_{1\text{B}}$ -adrenoceptor-expressing Rat 1 fibroblasts were either untreated (lanes 2 and 3) or treated with phentolamine ($1 \mu\text{M}$, 24 h) (lanes 4 and 5). RNA was subsequently isolated, reverse transcribed and PCR was performed to amplify a 597 bp fragment of the CAM $\alpha_{1\text{B}}$ -adrenoceptor (lanes 2 and 4). The CAM mutant contains a *Sst*I restriction site not present in the wild-type $\alpha_{1\text{B}}$ -adrenoceptor DNA. Digestion with this enzyme restricted all the generated PCR product in both treatments (lanes 3 and 5). Size markers are shown in lanes 1 and 6.

$n = 3$) and maximal up-regulation of the CAM $\alpha_{1\text{B}}$ -adrenoceptor was achieved within 36 h when using $1 \mu\text{M}$ phentolamine. Saturation binding analyses of membranes of vehicle-treated (K_{d} for [^3H]prazosin $110 \pm 10 \text{ pM}$) and phentolamine-treated (K_{d} for [^3H]prazosin $170 \pm 20 \text{ pM}$) CAM $\alpha_{1\text{B}}$ -adrenoceptor-expressing cells (Figure 3) confirmed that the measured increase in

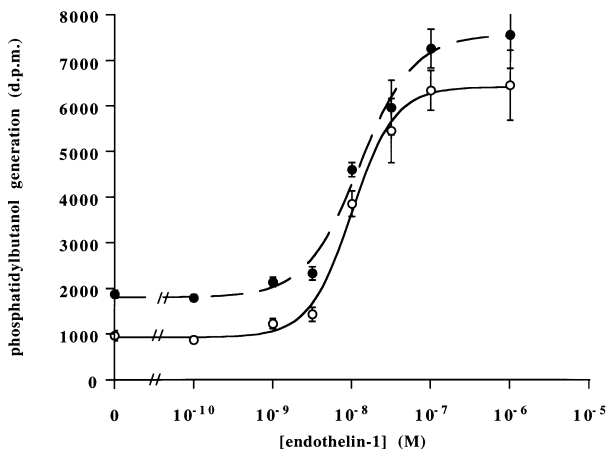
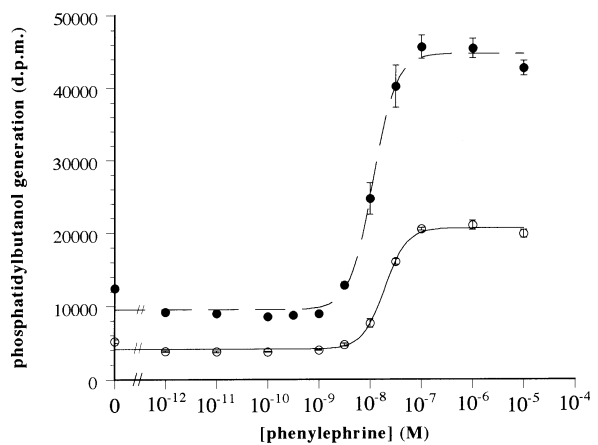
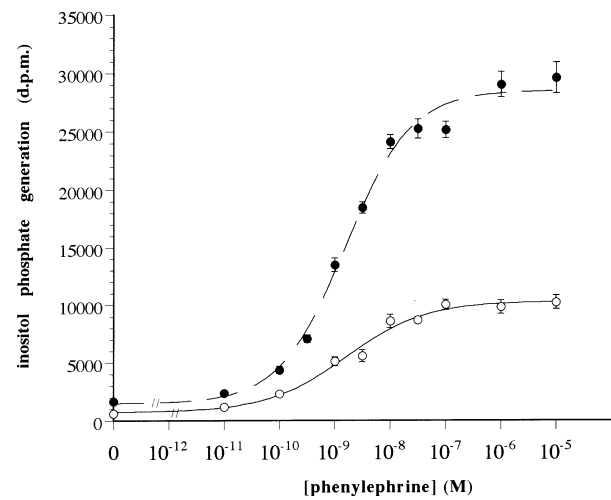


Figure 5 Phentolamine pretreatment of CAM $\alpha_{1\text{B}}$ -adrenoceptor-expressing Rat 1 fibroblasts results in greater maximal signalling capacity in response to phenylephrine

CAM $\alpha_{1\text{B}}$ -adrenoceptor-expressing Rat 1 fibroblasts were treated with either vehicle (\circ) or phentolamine ($1 \mu\text{M}$) (\bullet) for 24 h. Subsequently the phentolamine was removed and basal and phenylephrine (top and middle panels) or endothelin-1 (bottom panel) regulation of phosphoinositidase C (top panel) or PLD (middle and bottom panels) measured. Representative experiments of three performed for each panel are displayed (see the Results section for details).

[^3H]prazosin binding observed in Figure 2 was truly a reflection of an increase in the number of receptor-binding sites with only a small alteration ($P < 0.05$) in the measured K_{d} for [^3H]prazosin.

Table 1 Sustained treatment with a range of α_1 -adrenoceptor 'antagonists' results in up-regulation on the CAM α_{1B} -adrenoceptor but not the wild-type receptor

Rat 1 fibroblasts expressing either the CAM α_{1B} -adrenoceptor or the wild-type receptor were treated for 24 h with vehicle or one of the range of α_1 -adrenoceptor ligands examined in Figure 6 (1 μ M of each). After removal of the ligands and membrane preparation, the specific binding of [3 H]prazosin (1 nM) was measured and compared with that in membranes of untreated cells. Results are presented as means \pm S.E.M for three independent sets of experiments. All of the ligands produced a significant ($P < 0.05$) increase in levels of the CAM α_{1B} -adrenoceptor but none of them altered levels of the wild-type receptor. Abbreviations: HV-723, α -ethyl-3,4,5-trimethoxy- α -(3-{[2-(2-methoxyphenoxy)ethyl]amino}propyl)benzeneacetonitrile fumarate; WB4101, 2-(2,6-dimethoxyphenoxyethyl)aminomethyl 1,4-benzodioxane.

Ligand	CAM α_{1B} -adrenoceptor (pmol/mg of membrane protein)	Wild-type α_{1B} -adrenoceptor (pmol/mg of membrane protein)
None	2.15 \pm 0.23	2.64 \pm 0.13
Phentolamine	6.11 \pm 0.56	2.59 \pm 0.28
YM-12617	4.76 \pm 0.61	2.82 \pm 0.23
HV-723	6.48 \pm 1.02	2.93 \pm 0.29
Corynanthine	5.02 \pm 0.66	2.62 \pm 0.08
5-Methyl urapidil	4.62 \pm 0.63	2.75 \pm 0.14
WB4101	6.25 \pm 0.82	2.80 \pm 0.20

The CAM α_{1B} -adrenoceptor cDNA construct contains a restriction site for the enzyme *Stu*I that is not present in the wild-type receptor sequence [13]. Reverse transcription/PCR performed on RNA isolated from vehicle-treated and phentolamine-treated CAM α_{1B} -adrenoceptor-expressing cells led to the identification of a single PCR product, the entire population of which was cleaved by treatment with *Stu*I (Figure 4), demonstrating that both before and after phentolamine treatment all of the α_{1B} -adrenoceptor-expressing mRNA in these cells encoded the CAM α_{1B} -adrenoceptor and not wild-type protein.

After treatment of the CAM α_{1B} -adrenoceptor-expressing cells with or without phentolamine (1 μ M) for 24 h and washing out of this ligand, both basal phosphoinositidase C activity and the capacity of phenylephrine to stimulate this activity in *myo*-[3 H]inositol-labelled cells was assessed. Basal phosphoinositidase C activity was increased by phentolamine treatment (2.2 \pm 0.4-fold, mean \pm S.E.M., $n = 4$). Substantially greater (2.3 \pm 0.2-fold, mean \pm S.E.M., $n = 5$) maximal levels of [3 H]inositol phosphates were generated in the phentolamine-pretreated cells but the EC₅₀ for phenylephrine was not altered [2.5 \pm 0.9 nM without phentolamine treatment and 2.9 \pm 0.9 nM (means \pm S.E.M., $n = 3$ in each case) after such treatment] (Figure 5, top panel). A similar pattern was observed when phenylephrine activation of PLD activity was assessed by measuring the generation of [3 H]phosphatidylbutanol (Figure 5, middle panel). Both basal activity (2.4 \pm 0.02-fold) and maximal phenylephrine stimulation (1.8 \pm 0.3-fold) were increased without a substantial alteration in EC₅₀ (21 \pm 2 nM without phentolamine treatment and 13 \pm 2 nM after such treatment; means \pm S.E.M., $n = 3$ in each case). In contrast, the ability of endothelin 1 to stimulate PLD activity after occupancy of the endogenously expressed endothelin ET_A receptor was not increased by phentolamine pretreatment, nor was the measured EC₅₀ for endothelin different (19 \pm 5 nM without phentolamine treatment and 22 \pm 7 nM after such treatment; means \pm S.E.M., $n = 3$ in each case) (Figure 5, bottom panel).

Sustained treatment of the CAM α_{1B} -adrenoceptor-expressing cells with a variety of ligands normally considered to be α_1 -adrenoceptor antagonists (at 1 μ M) for 24 h resulted in a

Table 2 A range of α_1 -adrenoceptor ligands function as inverse agonists at the CAM α_{1B} -adrenoceptor

After treatment with phentolamine (1 μ M, 24 h) and removal of this ligand by washing, basal PLD activity and its acute regulation by a series of α_1 -adrenoceptor ligands (1 μ M) were measured in Rat 1 fibroblasts expressing the CAM α_{1B} -adrenoceptor as described in the Materials and methods section. Data are presented as means \pm S.E.M. for three independent experiments. Basal PLD activity after phentolamine treatment (100%) ranged between 10620 and 18940 d.p.m. in the three experiments. Abbreviations: HV-723, α -ethyl-3,4,5-trimethoxy- α -(3-{[2-(2-methoxyphenoxy)ethyl]amino}propyl)benzeneacetonitrile fumarate; WB4101, 2-(2,6-dimethoxyphenoxyethyl)aminomethyl 1,4-benzodioxane.

Ligand	Phospholipase D activity (% of basal)
None	100
Phentolamine	16.9 \pm 4.4
HV-723	17.8 \pm 5.4
YM-12627	51.6 \pm 5.3
Corynanthine	17.5 \pm 3.5
5-Methyl urapidil	37.5 \pm 2.9
WB4101	13.2 \pm 3.6

substantial up-regulation of receptor levels as measured after removal of the ligands and the preparation of cell membranes by the specific binding of [3 H]prazosin (Table 1). In contrast, equivalent treatment of cells expressing the wild-type α_{1B} -adrenoceptor failed to produce any significant alteration in levels of this receptor (Table 1). Each of these ligands was shown to have the ability to function as an inverse agonist at the CAM α_{1B} -adrenoceptor. The enhanced basal PLD activity produced after sustained treatment of the CAM α_{1B} -adrenoceptor-expressing cells with phentolamine (1 μ M for 24 h) was substantially decreased by acute incubation with each ligand (Table 2). Although all of the ligands tested inhibited basal PLD activity, 5-(2-{[2-(ethoxyphenoxy)ethyl]amino}propyl)-2-methoxybenzenesulphonamide HCl (YM-12617) was less effective than the others (Table 2).

To assess whether this might reflect a low affinity of this ligand for the CAM α_{1B} -adrenoceptor, competition for the specific binding of [3 H]prazosin to membranes of the CAM α_{1B} -adrenoceptor-expressing cells was performed with the same ligands as used in Table 2. These displayed varying potency (Figure 6) (estimated K_i values were: YM-12617, 0.66 \pm 0.1 nM; WB4101 [2-(2,6-dimethoxyphenoxyethyl)aminomethyl 1,4-benzodioxane], 1.9 \pm 0.1 nM; HV-723 [α -ethyl-3,4,5-trimethoxy- α -(3 - {[2 - (2-methoxyphenoxy)ethyl]amino}propyl)-benzeneacetonitrile fumarate], 2.2 \pm 0.2 nM; phentolamine, 8.1 \pm 0.3 nM; corynanthine, 87 \pm 7 nM; 5-methyl urapidil, 210 \pm 20 nM [means \pm range, $n = 2$ independent experiments]), with YM-12617 displaying the highest affinity.

DISCUSSION

A number of mutations within the sequence of the α_{1B} -adrenoceptor have been reported to impart agonist-independent 'constitutive' activity to this receptor as measured by enhanced basal activation of phosphoinositidase C and elevated generation of inositol phosphates in cells expressing these mutants compared with those expressing the wild-type receptor [11,12,15,16]. In the present study we have made use of the first described CAM of the hamster α_{1B} -adrenoceptor [12] to examine whether a number of ligands generally viewed as α_1 -adrenoceptor antagonists might

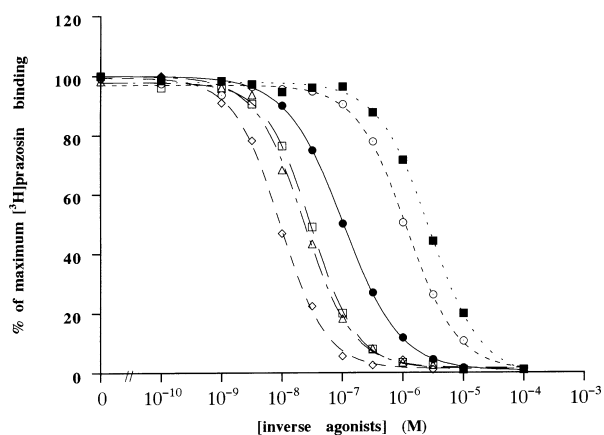


Figure 6 Comparison of the affinity of α_1 -adrenoceptor selective ligands in competition with [3 H]prazosin for binding to the CAM α_{1B} -adrenoceptor

The specific binding of [3 H]prazosin (1 nM) to membranes of the CAM α_{1B} -adrenoceptor-expressing Rat 1 fibroblasts was competed for by various concentrations of YM-12617 (\diamond), WB4101 [2-(2,6-dimethoxyphenoxyethyl)aminomethyl 1,4-benzodioxane] (Δ), HV-723 [α -ethyl-3,4,5-trimethoxy- α -(3-{[2-(2-methoxyphenoxy)ethyl]amino}propyl)benzeneacetonitrile fumarate] (\square), phentolamine (\bullet), corynanthine (\circ) and 5-methyl urapidil (\blacksquare). Data represent the mean values from two experiments performed.

function as 'inverse agonists' at this mutated receptor and, if so, whether they might have the capacity to regulate expression and the functional capacity of the receptor.

Current views imply that GPCRs must exist in at least two (and potentially a continuum of) conformational states [1,2,17]. These must include both inactive or ground states and active conformations adopted on the binding of agonist ligands to promote signal transduction. It is likely that the balance between these states defines the basal signalling capacity of a receptor. Because they display greater agonist-independent activity than the wild-type receptor, CAM receptors are expected to favour a conformation or conformations more akin to that of the wild-type receptor in the presence of agonist. Inverse agonists are ligands that favour the stabilization of a ground state and thus decrease basal signal generation in a receptor-dependent manner [17]. In contrast, antagonists might be defined as ligands that do not selectively stabilize or recognize different receptor conformations and thus do not alter basal signal transduction [17].

Sustained treatment of cells or tissues expressing a particular GPCR with an agonist at that receptor frequently results in a decrease over time in the cellular levels of the receptor, a phenomenon described as down-regulation [1,2]. Although antagonist ligands have been shown to produce higher levels (up-regulation) of receptors in a number of physiological situations, this might reflect the fact that by competing to prevent the binding of endogenous agonists they are actually preventing a degree of receptor down-regulation rather than intrinsically themselves causing up-regulation on binding. However, it could be argued that an inverse agonist ligand might be expected to be capable of causing receptor up-regulation by favouring the ground state of the receptor if such ground states are more stable than activated conformations. If a CAM receptor adopts conformations more akin to the agonist-occupied wild-type receptor then potential inverse agonist up-regulation of a receptor might be more amenable to analysis with a CAM receptor if we accept the proviso that inverse agonists would have the capacity to 'relax' the CAM receptor to conformational states akin to the basal state of the wild-type receptor.

In the present study we demonstrate that a range of ligands long accepted as α_1 -adrenoceptor antagonists function as inverse agonists at the CAM α_{1B} -adrenoceptor. A clear indication of this is the capacity of these ligands to inhibit basal PLD activity (Table 2), a signalling function that is effectively stimulated by agonists at this receptor. A previous study has demonstrated that a number of these ligands also have the capacity to inhibit basal phosphoinositidase C activity in these CAM α_{1B} -adrenoceptor-expressing cells [11], a signalling function that seems to lie upstream of the activation of PLD activity [18,19]. It was thus of interest to note that sustained treatment of the cells with these ligands was able to cause a substantial up-regulation of cellular levels of the CAM α_{1B} -adrenoceptor but that equivalent treatments of cells expressing the wild-type α_{1B} -adrenoceptor had little effect on cellular levels of the receptor (Table 1). A similar pattern of receptor regulation has recently been noted for inverse agonists at a CAM mutant of the β_2 -adrenoceptor, in which betaxolol and sotalol caused strong increases in levels of this receptor after its stable expression in NG108-15 cells but considerably more modest increases in cellular levels of the wild-type β_2 -adrenoceptor [9]. Furthermore, Smit et al. [3] have recently reported a degree of up-regulation of the wild-type histamine H_2 receptor in response to long-term treatment with inverse agonists but not neutral antagonists. Although a lack of up-regulation of the CAM β_2 -adrenoceptor by long-term treatment with neutral antagonists was also observed in our studies on the CAM β_2 -adrenoceptor [9] we have not been able to assess this question for the CAM α_{1B} -adrenoceptor studied here, as all of the 'antagonist' ligands used in the study displayed marked inverse agonism. In previous studies of each of the histamine H_2 receptors [3], the δ opioid receptor [20,21] and the CAM β_2 -adrenoceptor [9,22], the availability of apparently neutral antagonists has been extremely useful. Such agents are expected to (and do) block the effects of the inverse agonist ligands in a concentration-dependent manner. This rather simple pharmacological feature has not been available in the current studies. YM-12617 did not cause the same degree of inhibition of basal PLD activity as the other ligands examined. As this compound had the highest affinity of all the ligands examined (Figure 6) this cannot reflect incomplete occupancy of the receptor at 1 μ M, as might have been suggested for 5-methyl urapidil, and thus it seems that YM-12617 acts as only a partial inverse agonist at the CAM α_{1B} -adrenoceptor.

Up-regulation of the CAM α_{1B} -adrenoceptor caused by sustained treatment with phentolamine was converted into greater maximal stimulation by phenylephrine of both phosphoinositidase C and PLD activities without significant alterations in the EC_{50} for the agonist (Figure 6). This was not true for stimulation of these enzymes by endothelin 1 acting at the endogenously expressed endothelin ET_A receptor (Figure 5, bottom panel), indicating that the observed effects were unlikely to be due to up-regulation of the G_q/G_{11} G-proteins or the phospholipases themselves.

Although it might seem intuitively obvious that up-regulation of a receptor should result in a greater maximal output of the relevant signalling cascades in response to agonists, this is only true if the effector enzymes are not the limiting element for signal transduction across the membrane. For example in NG108-15 cells, the model system we have used to study inverse agonist-induced up-regulation of the β_2 -adrenoceptor, increasing levels of expression of the β_2 -adrenoceptor results in the same maximal activation of adenylate cyclase activity but a shift to increased potency and efficacy of agonist ligands because the levels of adenylate cyclase place an upper limit on the activity that can be achieved [23]. On this basis, the levels of expression of the CAM α_{1B} -adrenoceptor achieved in these studies, even after treatment

with phentolamine, was clearly insufficient to outstrip the cellular G-protein and effector capacity.

One question that has been raised in relation to the potential use of inverse agonist ligands in a clinical setting is whether they might be expected to result in greater supersensitivity of response to the natural agonists on their withdrawal than the use of a neutral antagonist [17]. This issue remains unresolved because although up-regulation and enhanced signal transduction output were recorded by the maintained presence of the inverse agonists at the CAM α_{1B} -adrenoceptor, no up-regulation of the wild-type α_{1B} -adrenoceptor was produced by this treatment. This might reflect the fact that the wild-type α_{1B} -adrenoceptor is relatively silent in the absence of agonist ligands and thus the issue of inverse agonist-induced supersensitivity would not be a major concern at this receptor. However, other wild-type GPCRs can clearly show a substantial degree of spontaneous activity. This issue of potential supersensitivity after the withdrawal of inverse agonist treatment was not addressed in the study of Smits et al. [3] on the wild-type histamine H₂ receptor but is clearly deserving of detailed analysis at a range of receptor types.

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