A constitutively active mutant of the α_{1B}-adrenergic receptor can cause greater agonist-dependent down-regulation of the G-proteins $G_q\alpha$ and $G_{11}\alpha$ than the wild-type receptor

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Rat 1 fibroblasts transfected to express either the wild-type hamster α_{1B}-adrenergic receptor or a constitutively active mutant (CAM) form of this receptor resulting from the alteration of amino acid residues 288–294 to encode the equivalent region of the human β_2-adrenergic receptor were examined. The basal level of inositol phosphate generation in cells expressing the CAMα_{1B}-adrenergic receptor was greater than for the wild-type receptor. The addition of maximally effective concentrations of phenylephrine or noradrenaline resulted in substantially greater levels of inositol phosphate generation by the CAMα_{1B}-adrenergic receptor, although this receptor was expressed at lower steady-state levels than the wild-type receptor. The potency of both phenylephrine and noradrenaline to stimulate inositol phosphate production was approx. 200-fold greater at the CAMα_{1B}-adrenergic receptor than at the wild-type receptor. In contrast, endothelin 1, acting at the endogenously expressed endothelin ET_A receptor, displayed similar potency and maximal effects in the two cell lines. The sustained presence of phenylephrine resulted in down-regulation of the α subunits of the phosphoinositidase C-linked, pertussis toxin-insensitive, G-proteins G_q and G_{11} in cells expressing either the wild-type or the CAMα_{1B}-adrenergic receptor. The degree of down-regulation achieved was substantially greater in cells expressing the CAMα_{1B}-adrenergic receptor at all concentrations of the agonist. However, in this assay phenylephrine displayed only a slightly greater potency at the CAMα_{1B}-adrenergic receptor than at the wild-type receptor. There were no detectable differences in the basal rate of $G_q\alpha/G_{11}\alpha$ degradation between cells expressing the wild-type or the CAMα_{1B}-adrenergic receptor. In both cell lines the addition of phenylephrine substantially increased the rate of degradation of these G-proteins, with a greater effect at the CAMα_{1B}-adrenergic receptor. The enhanced capacity of agonist both to stimulate second-messenger production at the CAMα_{1B}-adrenergic receptor and to regulate cellular levels of its associated G-proteins by stimulating their rate of degradation is indicative of an enhanced stoichiometry of coupling of this form of the receptor to G_q and G_{11}.

INTRODUCTION

Different receptors for adrenaline and noradrenaline couple preferentially to different subfamilies of guanine nucleotide-binding proteins (G-proteins) to allow the same hormones to regulate distinct effector pathways [1]. The α_1-adrenergic receptor subtypes represent the prototypical examples of G-protein-coupled receptors (GPCRs), which interact with pertussis toxin-insensitive G-proteins to cause stimulation of phosphoinositidase C activity [2]. In experiments designed to analyse which segments of the intracellular loops of receptors define the identity of the G-proteins regulated by different adrenergic receptors, replacement of a small region of the human β_2-adrenergic receptor at the extreme distal end of the third intracellular loop with the equivalent region from the hamster α_{1B}-adrenergic receptor resulted in the expressed construct displaying elevated basal activation of adenylate cyclase activity compared with the wild-type receptor in the absence of an agonist ligand [3,4]. This construct was designated the constitutively active mutant (CAM) β_2-adrenergic receptor. Generation of a reciprocal construct in which a small section of the α_{1B}-adrenergic receptor was replaced with the equivalent region of the β_2-adrenergic receptor resulted in this construct (CAMα_{1B}-adrenergic receptor) [5–7] displaying elevated basal activation of phosphoinositidase C.

Although it has been well established that sustained exposure of many GPCRs to an agonist can result in a decrease in cellular levels of the receptor (a process known as down-regulation) [8] it is only in the recent past that it has also become clear that agonist-occupation of GPCRs can result in a decrease in cellular levels of the G-protein(s) activated by the receptor (reviewed in [9]). The mechanism responsible for agonist-mediated G-protein regulation seems primarily to be enhanced proteolysis of the activated G-protein α subunit, as we have recently demonstrated in clones of Rat 1 fibroblasts transfected to express individual α_1-adrenergic receptor subtypes [10]. In the present study we have compared the ability of the α_1-adrenergic receptor agonist phenylephrine to regulate cellular levels of $G_q\alpha$ and $G_{11}\alpha$ in Rat 1 fibroblasts transfected to express stably either the wild-type or the CAMα_{1B}-adrenergic receptor.

MATERIALS AND METHODS

Materials

All materials for tissue culture were supplied by Life Technologies (Paisley, Strathclyde, Scotland). [^{3}H]Prazosin (24 Ci/mmol) and *myo*-[2-^{3}H]inositol (17.6 Ci/mmol) were obtained from Amersham International. Tran^{35}S-label (1180 Ci/mmol) was purchased from ICN Biomedicals. All other chemicals were from Sigma or Fisons 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; RT–PCR, reverse transcriptase–PCR.

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Cells

The Rat 1 fibroblast clones transfected to express stably either the wild-type hamster α_{1B}-adrenergic receptor or the CAM of this receptor have been described previously [6,7].These were maintained in tissue culture in Dulbecco's modified Eagle's medium (DMEM) containing 5% (v/v) newborn calf serum, glutamine, penicillin and streptomycin. For most experiments cells were grown until close to confluency and then either harvested or subcultured in a 1:10 ratio. In preparation for labelling with Tran^{35}S-label for the pulse–chase experiments, cells were trypsin-treated and seeded in 6-well culture plates. At approx. 70% confluency, two-thirds of the growth medium was replaced with DMEM lacking methionine and cysteine, supplemented with glutamine, antibiotics and 50 μCi/ml Tran^{35}S-label (final concentration in well). After the labelling period (16 h) the radioactive medium was removed and the now nearly confluent cell layer was washed once with 1 ml of normal DMEM culture medium. They were subsequently incubated in normal DMEM culture medium (1.5 ml per well) in the presence or absence of phenylephrine (100 μM). At appropriate times the medium was removed and cells were dissolved in 1% (w/v) SDS (200 μl per well). The cell suspension was heated in a screw-capped test tube to 100 °C for 20 min to denature proteins and nucleic acids; samples were then either stored at −20 °C or processed directly for immunoprecipitation.

Preparation of membranes

Membrane fractions were prepared from cell pastes that had been stored at −80 °C after being harvested. 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-on-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 48000 ***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 48000 ***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.

[^{3}H]Prazosin-binding experiments

Binding assays were initiated by the addition of 5–15 μg of protein to an assay buffer [50 mM Tris/HCl (pH 7.4)/0.5 mM EDTA] containing [^{3}H]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 μl final volume). Non-specific binding was determined in the presence of 10 μM 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 μCi/ml *myo*-[2-^{3}H]inositol in 0.5 ml of inositol-free DMEM containing 1% (v/v) dialysed newborn calf serum for 36 h. On the day of the experiments the labelling medium was removed and cells were washed twice with HBG buffer [0.5 ml Hanks buffered saline, pH 7.4, containing 1% (w/v) BSA and 10 mM glucose]. Cells were then washed twice for 10 min with HBG/LiCl buffer (HBG supplemented with 10 mM LiCl) and subsequently stimulated with agonist in HBG/LiCl for 20 min. All incubations were conducted at 37 °C. Reactions were terminated by the addition of 0.5 ml of ice-cold methanol. Cells were then scraped, transferred to vials and chloroform was added to a $CHCl_3$/MeOH ratio of 1:2 (v/v). Total inositol phosphates were extracted for 30 min before the addition of chloroform and water to a final ratio of 1:1:0.9 ($CHCl_3$/MeOH/H_2O, by vol). The upper phase was taken and total inositol phosphates were analysed by batch chromatography on Dowex-1 formate as previously described [11,12].

Immunological studies

The generation and specificity of antiserum CQ, which identifies the common C-terminal decapeptide of $G_q\alpha$ and $G_{11}\alpha$, has been fully described previously [13,14]. This antiserum was produced in a New Zealand White rabbit by using a conjugate of a synthetic peptide with keyhole-limpet haemocyanin (Calbiochem) as antigen. Membrane samples were resolved by SDS/PAGE [10% (w/v) acrylamide], in the presence or absence of 6 M urea, overnight at 60 V. Proteins were subsequently transferred to nitrocellulose (Schleicher and Schuell) and blocked for 3 h in 5% (w/v) gelatin in PBS, pH 7.5. The primary antiserum was added in 1% gelatin in PBS containing 0.2% (v/v) Nonidet P40 and incubated overnight. The primary antiserum was removed and the blot was washed extensively with PBS containing 0.2% Nonidet P40. Secondary antiserum (donkey anti-rabbit IgG coupled to horseradish peroxidase) in 1% gelatin/PBS/0.2% Nonidet P40 was added and left for 3 h. After removal of the secondary antiserum the blot was washed extensively as above and developed with *o*-dianisidine hydrochloride (Sigma) as substrate for horseradish peroxidase, as described [15].

Immunoprecipitation of [^{35}S]-labelled G-proteins

To 200 μl of SDS-denatured cell suspension was added 800 μl of solubilization buffer [1% (w/v) Triton X-100/10 mM EDTA/100 mM NaH_2PO_4/10 mM NaF/100 μM Na_3VO_4/50 mM Hepes (pH 7.2)] and 100 μl of Pansorbin (Calbiochem). Samples were incubated at 4 °C with continuous rotation for 1–2 h for non-specific preclearing. After centrifugation of the samples (16000 ***g*** for 2 min at 4 °C) the supernatant was collected and subjected to immunoprecipitation by the addition of 100 μl of Protein A–agarose along with 10 μl of the specific G-protein antiserum, and incubated at 4 °C for 4 h. Immune complexes were then recovered by centrifugation (16000 ***g*** for 30 s at 4 °C) and washed by resuspension and centrifugation three times each with 1 ml of wash buffer [1% (w/v) Triton X-100/100 mM NaCl/100 mM NaF/50 mM NaH_2PO_4/50 mM Hepes (pH 7.2)/0.5% (w/v) SDS]. The final Protein A–agarose pellet was resuspended in Laemmli sample buffer, heated at 100 °C for 5 min and subjected to SDS/PAGE [10% (w/v) acrylamide]. After resolution of the proteins, the gel was stained with Coomassie Blue and then dried. The dried gels were exposed either to photographic film or to phosphor-storage-screen autoradiography, in accordance with the manufacturer's instructions, with a Fujix Bio-imaging Analyser linked to an Apple Macintosh Quadra 650 personal computer. Autoradiographs were analysed such that the time-course data were expressed as a percentage of the control specific radiolabelling of $G_q\alpha/G_{11}\alpha$ protein found at zero time.

Reverse transcriptase–PCR (RT–PCR)

The RT–PCR procedure was essentially as described previously [10].

RNA extractions

Total RNA was extracted with RNAzol B (Biogenesis). Purity and quantification of RNA were assessed by spectrophotometric A_{260}/A_{280} ratios.

Reverse transcription

Samples of 10–20 μg of RNA (20 μl) were denatured by incubation at 65 °C for 10 min followed by chilling on ice and reverse-transcribed in 33 μl of reaction mixture by using a first-strand cDNA synthesis kit (Pharmacia LKB Biotechnology) as detailed by the manufacturer. Incubation was conducted 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

PCR reactions were performed with the following primers: α_{1B}sense, 5′-GACGACAAGGAATGCGGAGTC-3′; α_{1B} antisense, 5′-GTCCACGGCCGATAGGTGTAA-3′; $G_q\alpha$ sense, 5′-ATGACTTGGACCGTGTAGCCGACC-3′; $G_{11}\alpha$ sense, 5′-ACGTGGACCGCATCGCCACAGTAG-3′; $G_q\alpha/G_{11}\alpha$ antisense, 5′-CCATGCGGTTCTCATTGTCTGACT-3′. Amplifications were performed in 50 μl of buffer containing 25 pmol of primers and 2.5 units of *Taq* polymerase (Promega) with a Hybaid Omnigene temperature cycler. Amplifications for α_{1B}-adrenergic receptor mRNA 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). Conditions used for the amplification of $G_{11}\alpha$ and $G_q\alpha$ were: 95 °C for 5 min, 60 °C for 30 s, 72 °C for 1 min (1 cycle); 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min (30 cycles); 95 °C for 30 s, 60 °C for 30 s, 72 °C for 5 min (1 cycle). 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

RNA was isolated from clones of Rat 1 fibroblasts stably transfected with cDNA species encoding either the wild-type hamster α_{1B}-adrenergic receptor or an altered form of this receptor (CAMα_{1B}-adrenergic receptor [5–7]) in which a fragment of the distal end of the third intracellular loop was replaced with the equivalent segment of the human β_2-adrenergic receptor. The RNA samples were subjected to RT–PCR with a primer pair designed to straddle the region altered to generate the CAMα_{1B}-adrenergic receptor. Such RT–PCR resulted in the expected generation of a fragment of 597 bp from both cell lines (Figure 1). The alteration in sequence to produce the CAMα_{1B}-adrenergic receptor introduces a specific site for the restriction enzyme *Stu*I that should allow the cleavage of the PCR product virtually in the centre to generate fragments of 292 and 305 bp. Treatment of the PCR product from the clone transfected with the CAMα_{1B}-adrenergic receptor cDNA resulted in all of the fragment being digested, whereas equivalent treatment with the PCR fragment derived from the cells transfected with the wild-type α_{1B}-adrenergic receptor cDNA had no effect (Figure 1). These results confirmed the identity of the expressed receptors and indicated that the entire cellular population of the CAMα_{1B}-adrenergic receptor cDNA transfected cells reflects the modified receptor. RT–PCR reactions with primers (see the Materials and methods section) designed to detect the presence of the pertussis toxin-insensitive G-proteins $G_q\alpha$ and $G_{11}\alpha$ demonstrated that both of these were expressed in both cell lines (results not shown).

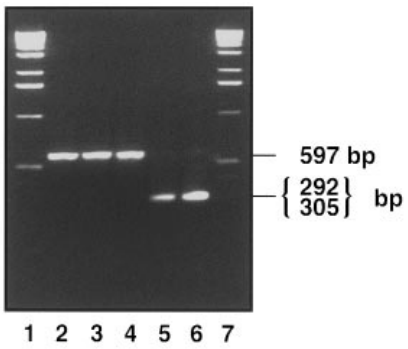

Figure 1 Cells transfected to express the wild-type or CAMα_{1B}-adrenergic receptor express only the expected form of the receptor

RNA isolated from wild-type (lanes 2 and 3) and CAM (lanes 4–6) α_{1B}-adrenergic receptor transfected cells was reverse-transcribed; PCR was then performed with primers (straddling the region of difference between the two forms of the receptor) designed to amplify a 597 bp fragment. Samples were then treated (lanes 3, 5 and 6) or not (lanes 2 and 4) with the restriction enzyme *Stu*I. Product derived from the CAM form of the receptor was cleaved to yield fragments of 305 and 292 bp (lanes 5 and 6), whereas that derived from the wild-type receptor was resistant to the enzyme (lane 3). Lanes 1 and 7 contained molecular-mass markers.

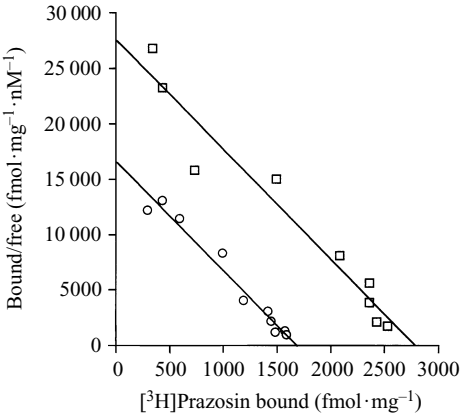

Figure 2 The wild-type receptor is expressed at higher levels than the CAMα_{1B}-adrenergic receptor

Membranes prepared from cells expressing either the wild-type α_{1B}-adrenergic receptor (□) or the CAMα_{1B}-adrenergic receptor (○) were analysed for their ability to bind a range of concentrations of [^{3}H]prazosin in a specific manner. In the experiment shown, the two forms of the receptor bound the ^{3}H-labelled ligand with very similar affinities (K_d 100 pM) but the level of expression of the wild-type receptor (2.8 pmol/mg of membrane protein) was greater than that of the CAM receptor (1.7 pmol/mg of membrane protein). Similar results were obtained in two further experiments.

Inositol phosphate generation in the absence of added agonist, measured with a Li^+ blockade accumulation assay, was approx. 2-fold higher in cells of the clone expressing the CAMα_{1B}-adrenergic receptor than in the wild-type receptor after the results had been modified to account for the level of incorporation of [^{3}H]inositol into cellular phospholipids, and approx. 3–4-fold higher when the generation of [^{3}H]inositol phosphates was not corrected in this manner (results not shown, but see Figure 4), as has previously been reported [16]. This was despite ligand-binding studies, with the specific binding of the α_1-adrenoceptor

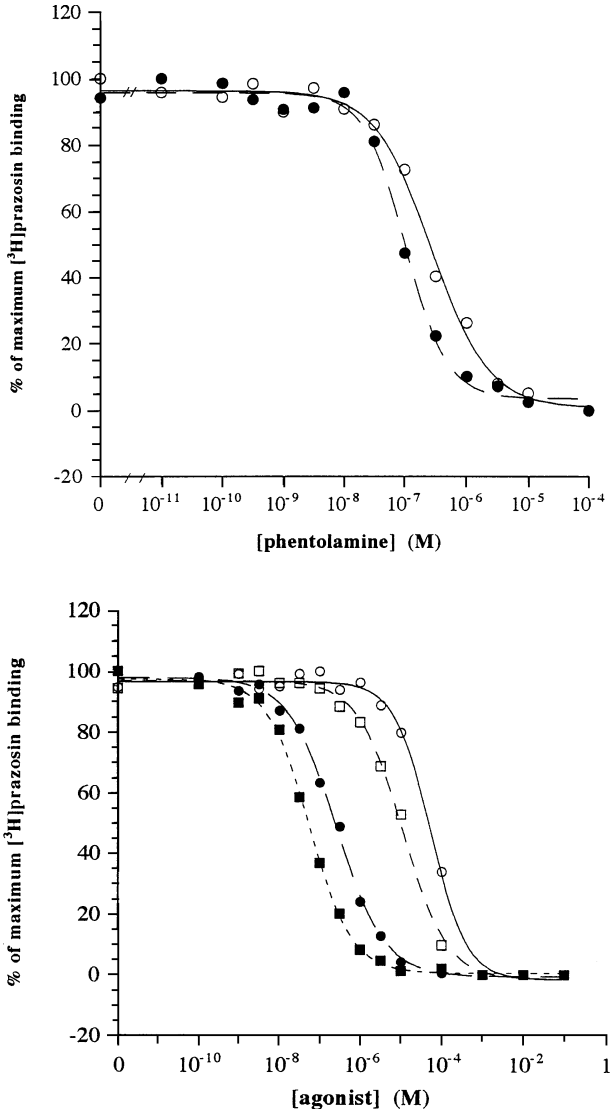

Figure 3 Antagonists bind the wild-type and CAMα_{1B}-adrenergic receptor with similar affinities, but agonists show substantially higher affinity for the CAM receptor

The specific binding of [^{3}H]prazosin (0.88 nM) to membranes of cells expressing the wild-type receptor (○,□) or the CAMα_{1B}-adrenergic receptor (●,■) was competed for by the antagonist phentolamine (upper panel) or by the agonists phenylephrine (lower panel; ○,●) and noradrenaline (lower panel; □,■). The results are taken from a single experiment representative of three performed.

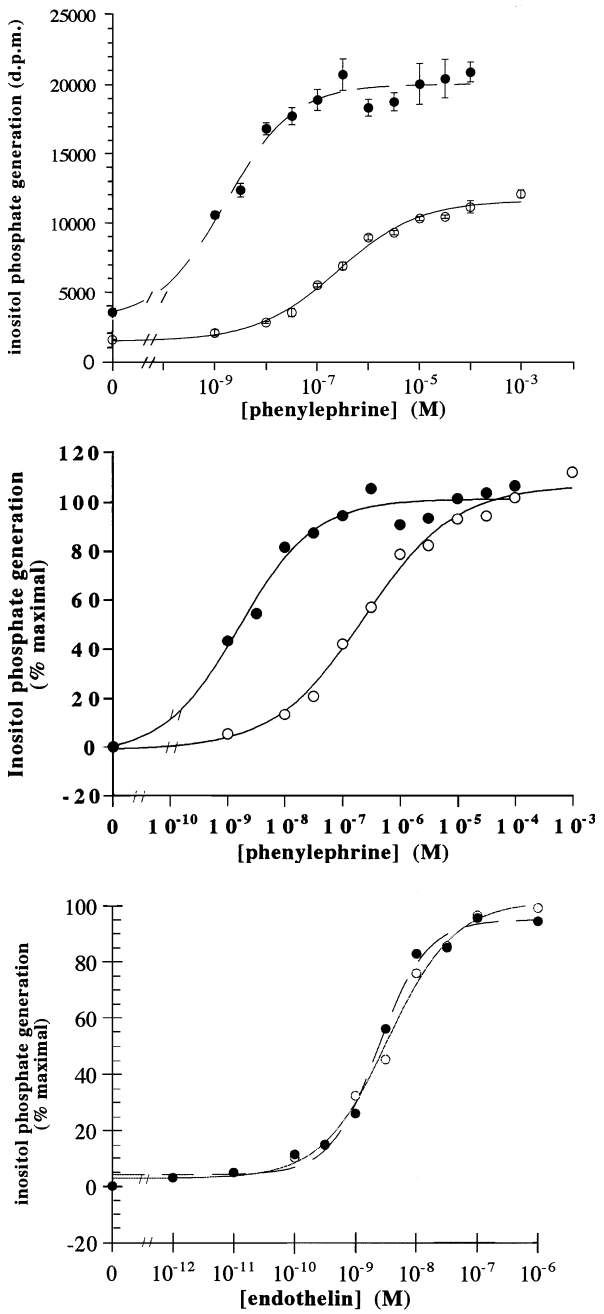

Figure 4 Phenylephrine, but not endothelin 1, stimulates inositol phosphate generation to both greater levels and more potently in cells expressing the CAMα_{1B}-adrenergic receptor than in those expressing the wild-type receptor

Cells expressing the wild-type receptor (○) or the CAMα_{1B}-adrenergic receptor (●) were labelled with [^{3}H]inositol and stimulated with either various concentrations of phenylephrine (top and middle panels) or endothelin 1 (bottom panel). The generation of ^{3}H-labelled inositol phosphates was then measured as described in the Materials and methods section. In the experiment shown in the top and middle panels, the EC_{50} and Hill coefficient for phenylephrine stimulation of inositol phosphate production in the cells expressing CAMα_{1B}-adrenergic receptor were 1.6 nM and 0.55; in the cells expressing wild-type receptor they were 240 nM and 0.65 respectively. In the middle panel the results for phenylephrine in the top panel are presented as the percentage of the maximal effect achieved. Similar results were obtained in two further experiments. The EC_{50} and Hill coefficients for endothelin 1 stimulation of inositol phosphate generation, as shown in the bottom panel, were 3.4 nM and 0.8 for the cells expressing wild-type α_{1B}-adrenergic receptor, and 2.4 nM and 1.1 respectively in the cells expressing CAMα_{1B}-adrenergic receptor. Similar results were obtained in two further experiments with each agonist (see text for details).

antagonist [^{3}H]prazosin, which demonstrated that the CAMα_{1B}-adrenergic receptor was expressed at lower levels than the wild-type receptor (Figure 2). Over a series of experiments levels of the CAMα_{1B}-adrenergic receptor were 79.3 ± 12.5% of those of the wild-type receptor (mean $\pm$ S.E.M.; $n = 10$). The estimated affinities for [^{3}H]prazosin binding to the wild-type and the CAMα_{1B}-adrenergic receptor were, however, very similar ($K_d = 86 \pm 15$ pM at the wild-type receptor and 96 ± 19 pM at the CAM receptor; means $\pm$ S.E.M.; $n = 3$ in each case). Competition for the binding of [^{3}H]prazosin by the α_1-antagonist phentolamine to the wild-type ($K_i = 21 \pm 1$ nM) and the CAM ($K_i = 11 \pm 1$ nM) α_{1B}-adrenergic receptors (means $\pm$ S.E.M.; $n = 3$) was achieved with similar affinities (Figure 3, upper panel), but the potencies of the

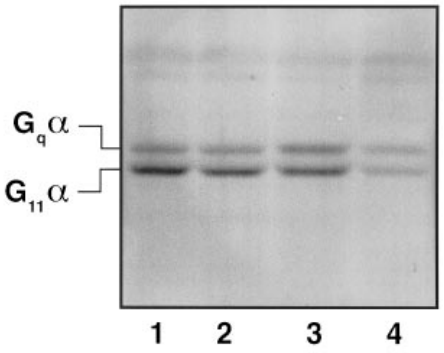

Figure 5 Membrane levels of $G_q\alpha$ and $G_{11}\alpha$ are decreased by phenylephrine treatment of cells expressing wild-type or CAMα_{1B}-adrenergic receptor, but the effect is greater in the cells expressing the CAM receptor

Membranes were isolated from cells expressing either the wild-type receptor (lanes 1 and 2) or the CAMα_{1B}-adrenergic receptor (lanes 3 and 4) after treatment with either vehicle (lanes 1 and 3) or 100 μM phenylephrine (lanes 2 and 4) for 16 h. The samples were then resolved by SDS/PAGE under conditions that can resolve $G_q\alpha$ from $G_{11}\alpha$ (see the Materials and methods section) and the presence of these polypeptides was detected with antiserum CQ [13], which identifies the C-terminal decapeptide that is completely conserved in these two G-proteins. Similar results were obtained on three separate sets of membrane preparations.

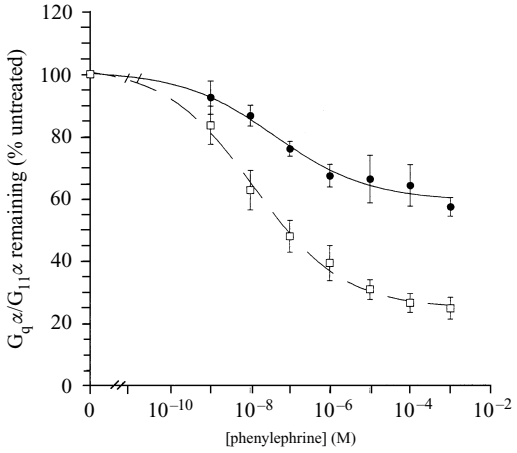

Figure 6 More pronounced down-regulation of $G_q\alpha/G_{11}\alpha$ is produced by the CAM than the wild-type α_{1B}-adrenergic receptor at all concentrations of phenylephrine

Cells expressing either the wild-type receptor (●) or the CAMα_{1B}-adrenergic receptor (□) were exposed to various concentrations of phenylephrine for 16 h. Membranes were prepared, resolved by SDS/PAGE under conditions that do not resolve $G_q\alpha$ from $G_{11}\alpha$ and immunoblotted to detect the combined total levels of these two G-proteins. Such immunoblots were scanned; the results are presented as means ± S.E.M. ($n = 3$, derived from independent experiments on separate membrane preparations).

agonists phenylephrine and noradrenaline to compete for the binding of [^{3}H]prazosin were approx. 160-fold higher at the CAMα_{1B}-adrenergic receptor [IC_{50} (corrected for radioligand occupancy] for noradrenaline, 5.3 ± 0.2 nM; for phenylephrine, 24 ± 2 nM) than at the wild-type receptor [IC_{50} (corrected for radioligand occupancy) for noradrenaline, 930 ± 50 nM; for phenylephrine, 4.6 ± 0.3 μM] (means ± S.E.M.; $n = 3$ in all cases) (Figure 3, lower panel). Stimulation of inositol phosphate generation from [^{3}H]inositol-labelled cells by the agonists noradrenaline (EC_{50} 5.3 ± 0.6 nM compared with 770 ± 60 nM) and phenylephrine (EC_{50} 5.1 ± 0.4 nM compared with 590 ± 10 nM) (means ± S.E.M.; $n = 3$) was also more than 100-fold more potent in cells of the clone expressing the CAMα_{1B}-adrenergic receptor than in those expressing the wild-type receptor (Figure 4, top and middle panels). Furthermore, the maximal stimulation of inositol phosphate production by phenylephrine was substantially greater (170 ± 20 %, mean ± S.E.M.; $n = 3$) at the CAMα_{1B}-adrenergic receptor (Figure 4, top panel). These effects could be clearly attributed to the specific forms of the α_{1B}-adrenergic receptor expressed and not simply to some trivial example of clonal variation, because inositol phosphate generation in response to endothelin 1, acting at the endogenously expressed ET_A endothelin receptor, was similar both in maximal effect (in the CAMα_{1B}-adrenergic-receptor-expressing cells endothelin 1 produced 108.6 ± 5.8 % of the inositol phosphate response observed in the cells expressing the wild-type receptor) and in the potency of the ligand (EC_{50} 4.8 ± 2.1 nM at the CAMα_{1B}-adrenergic receptor and 3.0 ± 1.2 nM at the wild-type receptor; mean ± S.E.M.; $n = 3$ in each case) (Figure 4, bottom panel).

Sustained exposure of the two clones to a concentration of phenylephrine (100 μM) that resulted in maximal generation of [^{3}H]inositol phosphates in both cell lines resulted in a decrease in cellular levels of some combination of the α subunits of the phosphoinositidase C-linked, pertussis toxin-insensitive, G-proteins G_q and G_{11} when immunoblotting experiments were performed with SDS/PAGE [10 % (w/v) acrylamide], conditions that are unable to resolve these two polypeptides (results not shown). Resolution of these two G-protein α subunits by SDS/PAGE [10 % (w/v) acrylamide] that incorporated 6 M urea into the resolving gel [10] indicated that cellular steady-state levels of $G_{11}\alpha$ were higher than those of $G_q\alpha$ but that very similar levels of each of these G-proteins were present in membranes of untreated wild-type receptor and CAMα_{1B}-adrenoceptor-expressing cells (Figure 5). Phenylephrine treatment resulted in a down-regulation of both of these polypeptides in cells expressing both the wild-type receptor and the CAMα_{1B}-adrenoceptor (Figure 5). Importantly, however, the maximal degree of $G_q\alpha$ and $G_{11}\alpha$ down-regulation that could be achieved in response to phenylephrine treatment was substantially greater in the clone expressing the CAMα_{1B}-adrenoceptor (Figures 5 and 6). Furthermore, sustained exposure of wild-type and the CAMα_{1B}-adrenoceptor-expressing cells to various concentrations of phenylephrine indicated that the enhanced capacity to cause down-regulation of these two G-proteins was observed at all concentrations of the agonist (Figure 6). However, in contrast with the differences in affinity of phenylephrine at the wild-type and CAMα_{1B}-adrenoceptor measured in binding assays and for inositol phosphate generation, the potency of phenylephrine to cause down-regulation of G_q/G_{11} was only slightly greater in cells expressing the CAMα_{1B}-adrenoceptor (15 ± 11 nM) than in those expressing the wild-type α_{1B}-adrenoceptor (65 ± 18 nM) (means ± S.E.M.; $n = 3$ in each case) (Figure 6).

Time courses of phenylephrine-induced $G_q\alpha/G_{11}\alpha$ down-regulation (Figure 7) demonstrated that the degree of loss of these G-proteins was more pronounced at all time points in the cells expressing the CAMα_{1B}-adrenoceptor than in the cells expressing the wild-type receptor.

Little difference in the turnover of $G_q\alpha/G_{11}\alpha$ could be observed between the wild-type and cells expressing the CAMα_{1B}-adrenergic receptor in the absence of agonist ligand (Figure 8). Half-lives for these G-proteins, as assessed in ^{35}S-labelled amino acid

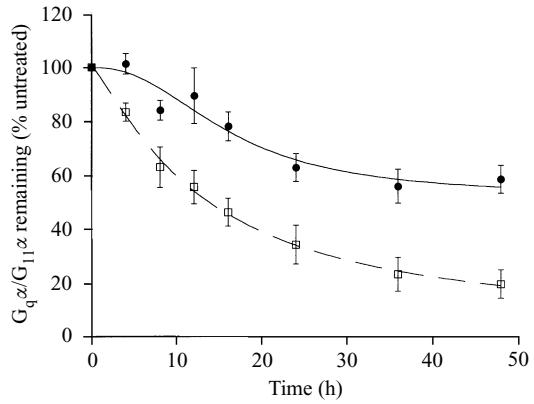

Figure 7 Time courses of phenylephrine-induced $G_q\alpha/G_{11}\alpha$ down-regulation in cells expressing the CAM or the wild-type α_{1B}-adrenergic receptor

Cells expressing either the wild-type receptor (●) or the CAMα_{1B}-adrenergic receptor (□) were exposed to 100 μM phenylephrine for various periods. Membranes were prepared, resolved by SDS/PAGE under conditions that do not resolve $G_q\alpha$ from $G_{11}\alpha$ and immunoblotted to detect the combined total levels of these two G-proteins. Such immunoblots were scanned; the results are presented as means ± S.E.M. ($n = 3$, derived from independent experiments on separate membrane preparations).

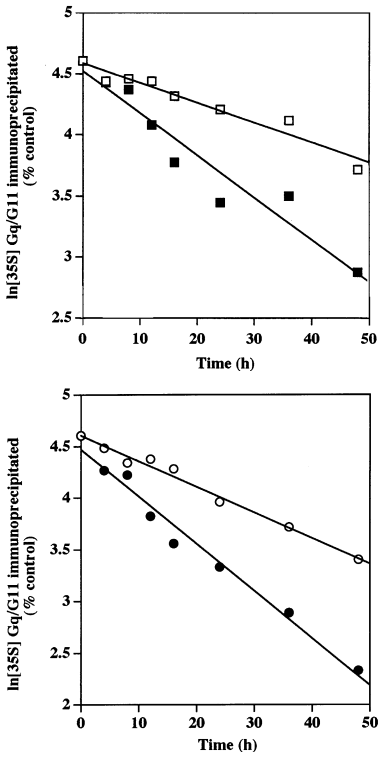

Figure 8 Phenylephrine treatment of cells expressing wild-type receptor or CAMα_{1B}-adrenergic receptor accelerates the rate of degradation of $G_q\alpha/G_{11}\alpha$

Cells expressing wild-type receptor (upper panel) or CAMα_{1B}-adrenergic receptor (lower panel) were labelled with Tran^{35}S-label as described in the Materials and methods section. The radiolabel was removed and the cells were maintained in the presence (■,●) or absence (□,○) of 100 μM phenylephrine for various periods. Samples were taken and $G_q\alpha$ and $G_{11}\alpha$ were immunoprecipitated with antiserum CQ. The immunoprecipitates were resolved by SDS/PAGE under conditions that do not resolve the two G-proteins and subsequently exposed to a phosphorimager plate for 12 h. The radiolabel remaining in $G_q\alpha/G_{11}\alpha$ was then quantified. The results are presented as best fits to a mono-exponential function from one of three independent experiments performed (see text for details).

pulse–chase experiments, were adequately described by a mono-exponential function, being between 33 and 36 h in individual experiments. As expected from previous studies [10], the rate of degradation of $G_q\alpha/G_{11}\alpha$ in the cells expressing the wild-type α_{1B}-adrenergic receptor was accelerated by the presence of 100 μM phenylephrine (estimated $t_{\frac{1}{2}}$ 21.1 ± 2.7 h). A similar effect of phenylephrine was observed in the cells expressing the CAMα_{1B}-adrenoceptor (Figure 8), except that the rate of degradation ($t_{\frac{1}{2}}$ 14.1 ± 1.6 h) (means ± S.E.M.; $n = 3$ in each case) was somewhat more pronounced than in the cells expressing the wild-type receptor.

DISCUSSION

Modifications of GPCRs that result in the receptor displaying agonist-independent second-messenger regulatory capacity have been observed both in designed mutations in the laboratory [3–7] and in spontaneous mutations [17–20] within the human population. GPCRs of this form have been termed CAM receptors, and much recent interest has centred on the degree to which these provide useful models, at least in certain aspects, of the conformational changes that must occur on the binding of agonists to wild-type receptors to allow efficient transmission of a signal via activation of heterotrimeric G-proteins [21]. Evidence, derived initially from observations that varying degrees of overexpression of wild-type GPCRs resulted in an increase in basal activity (i.e. in the absence of agonist) of the effector system coupled to that receptor and that there was a strong correlation between the level of expression of the GPCR and the amount of effector regulation [22–24], has indicated that adrenergic GPCRs must have the capacity to exist and spontaneously interconvert between a minimum of two (and possibly a continuum of) states, an inactive ground-state conformation R and an active conformation R* [22,25–27].

In efforts to define elements of α_1-adrenergic receptors responsible for the activation of pertussis toxin-insensitive G-proteins, Cotecchia and co-workers generated a series of receptor chimaeras in which different elements of the third intracellular loop of the hamster α_{1B}-adrenergic receptor were replaced by equivalent sequences from the human β_2-adrenoceptor [5–7]. One of these chimaeras, which was derived by replacement of residues 288–294 of the α_{1B}-adrenergic receptor with the corresponding sequence of the β_2-adrenoceptor, caused a triple mutation ($Ala^{293} \rightarrow Leu$, $Lys^{290} \rightarrow His$, $Arg^{288} \rightarrow Lys$) and resulted in the expressed receptor displaying constitutive activation of inositol phosphate generation and a capacity to act as a proto-oncogene, as measured by an ability to induce focus formation in fibroblasts transfected with this construct [7]. This is the CAMα_{1B}-adrenergic receptor used in the present study. Although the CAMα_{1B}-adrenergic receptor was inherently able to produce this effect, addition of agonist potentiated this effect greatly, indicating that this form of the receptor did not function in a fully agonist-independent manner [7]. Similarly, although the degree of agonist-independent inositol phosphate generation produced by the CAMα_{1B}-adrenergic receptor is markedly greater than that by the wild-type receptor, the CAM receptor still produces a further robust stimulation on addition of agonist [16].

We have recently reported that sustained agonist treatment of Rat 1 fibroblasts transfected to express high levels of each of the wild-type $\alpha_{1A/D}$-, α_{1B}- and α_{1C}-adrenergic receptors results in a substantial down-regulation of the phosphoinositidase C-linked, pertussis toxin-insensitive G-proteins $G_q\alpha$ and $G_{11}\alpha$ [10], and in the present study we have explored whether the CAM-mutant of the α_{1B}-adrenergic receptor has the capacity to act more effectively

in this regard than the wild-type receptor. This is clearly true (Figures 5 and 6). Addition of 100 μM phenylephrine produced a markedly greater degree of G-protein down-regulation in the cells expressing the CAMα_{1B}-adrenergic receptor than in those expressing the wild-type receptor (Figure 5). This was despite the fact that the CAMα_{1B}-adrenergic receptor was expressed at somewhat lower steady-state levels than the wild-type receptor (Figure 2). We paid particular attention to this point by routinely performing [^{3}H]prazosin-binding studies on membranes isolated from the control cells in such experiments (i.e. without agonist or at zero time points) to confirm the levels of expression in specific experiments. As we have shown previously for the wild-type α_{1B}-adrenergic receptor [10], down-regulation of $G_q\alpha$ and $G_{11}\alpha$ by phenylephrine occupancy of the CAMα_{1B}-adrenergic receptor seemed to be relatively non-selective for these two G-proteins, indicating equivalent interactions of these with the activated receptor [9].

Although the CAMα_{1B}-adrenergic receptor did result in the generation of higher levels of inositol phosphates in the absence of agonist than in the wild-type receptor, this was also true in response to maximally effective concentrations of agonist (Figure 4), confirming earlier results [16]. Indeed, the basal inositol phosphate generation from both receptor variants was only a fraction of that produced in response to agonist. Inositol phosphate generation in response to maximally effective concentrations of phenylephrine or noradrenaline was approx. 2-fold higher in the cells expressing CAMα_{1B}-adrenergic receptor than in those expressing the wild-type receptor. In contrast, there was little difference in the maximal ability of endothelin 1 to stimulate inositol phosphate generation in the two cell lines. These results indicate that it is an inherent property of the CAMα_{1B}-adrenergic receptor, rather than some undefined element of clonal cell line variation, that was responsible for the greater response to α_1-agonists. There were certainly no higher steady-state levels of $G_q\alpha$ or $G_{11}\alpha$ in the cells expressing CAMα_{1B}-adrenergic receptor (Figure 5) and thus it seems likely that the CAMα_{1B}-adrenergic receptor is able to activate these G-proteins with higher stoichiometry than the wild-type receptor. As we, and others, have previously reported, the sustained presence of agonist is required to cause the down-regulation of G_q and G_{11} via the wild-type α_{1B}-adrenergic receptor (and indeed for other phosphoinositidase C-coupled receptors [28–30]) and activating mutants of G-proteins have shorter half-lives than their native counterparts (see, for example, [31]). An enhanced G-protein activation stoichiometry would therefore be fully consistent with the observed greater degree of down-regulation of these G-proteins by agonist occupation of the CAMα_{1B}-adrenergic receptor. Although the EC_{50} for agonist stimulation of inositol phosphate generation at the wild-type receptor was more than 100-fold that at the CAMα_{1B}-adrenoceptor, this was not true when agonist-mediated $G_q\alpha/G_{11}\alpha$ down-regulation was measured. In this assay the measured difference in EC_{50} was only approx. 4–5-fold. We do not have a ready explanation for these differences but it should be remembered that agonist occupation of the wild-type receptor resulted in a relatively small percentage down-regulation of the G-proteins, making estimation of the EC_{50} value substantially more difficult to quantify reliably than in the other endpoints measured.

We also examined whether the expression of the CAMα_{1B}-adrenergic receptor would result in an agonist-independent down-regulation of these G-proteins. Although this is impossible to assess fairly in different clones of cells, we did not detect substantially lower steady-state levels of $G_q\alpha$ and $G_{11}\alpha$ in the cells expressing CAMα_{1B}-adrenergic receptor. However, given the difficulties of accurately measuring small alterations by the use of relative intensity immunoblotting and, as noted above, the still relatively small degree of phosphoinositidase C (and thus G-protein) activation produced by the CAMα_{1B}-adrenergic receptor in the absence of agonist compared with that achieved in the presence of agonist, it is perhaps to be expected that very small (and perhaps technically undetectable) alterations are the most that could be expected in the absence of agonist.

Further support for the contention that the CAM form of the α_{1B}-adrenergic receptor provides only limited signalling capacity in the absence of agonist was derived from examining the rate of turnover of $G_q\alpha/G_{11}\alpha$ in the cells. Analysis of immunoprecipitated $G_q\alpha/G_{11}\alpha$ after pulse-labelling with Tran^{35}S-label and a chase performed in the absence of phenylephrine indicated the half-life of these proteins, which was between 33 and 37 h, to be very similar in the two cell lines. No differences could be detected between cells harbouring the wild-type or the CAMα_{1B}-adrenergic receptor. This value is also very similar to results we have generated previously for the half-life of $G_q\alpha/G_{11}\alpha$ on a completely independent series of clones derived from Rat 1 fibroblasts [10]. Addition of a maximally effective concentration of phenylephrine during the chase phase of the experiments resulted in an acceleration in the rate of degradation of $G_q\alpha/G_{11}\alpha$ in cells expressing both the wild-type and the CAMα_{1B}-adrenergic receptor. The acceleration in the rate of degradation of these G-proteins due to agonist occupancy of the wild-type α_{1B}-adrenergic receptor in this study (the measured mean half-life in the presence of phenylephrine was approx. 21 h) was not as great as noted previously; this is entirely consistent with a smaller degree of down-regulation of the G-proteins produced by the receptor than that reported by Wise et al. [10]. The acceleration in the rate of degradation of $G_q\alpha/G_{11}\alpha$ was more pronounced with agonist occupancy of the CAMα_{1B}-adrenergic receptor (the measured half-life in the presence of 100 μM phenylephrine was 14 h) and this might be primarily responsible for the greater degree of $G_q\alpha/G_{11}\alpha$ down-regulation produced by agonist treatment of the cells expressing CAMα_{1B}-adrenergic receptor. However, this difference seems too limited to account, in isolation, for the very marked differences in G-protein regulation observed.

The results reported here confirm previous reports that this CAMα_{1B}-adrenergic receptor displays a degree of agonist-independent signalling capacity that is greater than that of the wild-type receptor. However, the CAM receptor is far from being fully agonist-independent and indeed is able to cause greater maximal output from the $G_q\alpha/G_{11}\alpha$ to the phosphoinositidase C pathway than equivalent levels of the wild-type α_{1B}-adrenergic receptor on addition of agonist (Figure 4). As steady-state basal levels of $G_q\alpha$ and $G_{11}\alpha$ are not appreciably different in the cell lines expressing the two forms of the receptor, and the phosphoinositidase C response of the endogenously expressed endothelin ET_A receptor is also not different in the cells expressing wild-type or CAMα_{1B}-adrenergic receptors, the differences in signalling effectiveness of the two receptor variants are not due to trivial differences in the individual cell lines. We have previously established that, for a range of wild-type receptors expressed in various cellular backgrounds, agonist-dependent G-protein down-regulation is limited to the G-proteins activated by a receptor [28,29,32,33]. Furthermore, it reflects a measure of sustained activation of the G-protein pool. The extent of the effect must be dependent on the fraction of the appropriate G-protein pool that is activated by the receptor because high-level receptor expression is substantially more effective at producing G-protein down-regulation than is low-level expression [32,33], even in situations in which the lower level of receptor is capable of causing maximal activation of second-messenger generation owing to the effector enzyme being the limiting component for

the output of the signalling cascade [33,34]. The key difference in the present study is thus likely to reflect a greater stoichiometry of activation of the G-proteins by the CAM version of the α_{1B}-adrenergic receptor compared with the wild-type receptor.

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