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Functional domains of the mouse β_3-adrenoceptor associated with differential G protein coupling

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Abstract

Alternative splicing of mouse β_3-adrenoceptor transcripts produces an additional receptor isoform (β_{3b}-adrenoceptor) with a C-terminus comprising 17 amino acids distinct from the 13 in the known receptor (β_{3a}-adrenoceptor). We have shown that the β_{3b}-adrenoceptor couples to both Gs and Gi while the β_{3a}-adrenoceptor couples only to Gs. To define the regions involved in this differential G protein coupling, we have compared wild type, truncated and mutant β_3-adrenoceptors. In CHO-K1 cells expressing β_3-adrenoceptors truncated at the splicing point, cyclic AMP accumulation with CL316243 increased by 59% following pre-treatment with pertussis toxin, suggesting that the C-terminal region of the β_{3a}-adrenoceptor inhibits coupling to Gi. We next utilized the cell penetrating peptide Tp10 to introduce peptides comprising the different C-terminal tail fragments into cells expressing β_{3a}-adrenoceptor, β_{3b}-adrenoceptor and the truncated β_3-adrenoceptor. Treatment with β_{3a}-Tp10 (1μM) caused cyclic AMP responses to CL316243 in the β_{3a}-adrenoceptor to become pertussis toxin-sensitive and display a 30% increase over control, whilst the other peptides did not affect any receptor. Mutation at a potential tyrosine phosphorylation site (Tyr392Ala β_{3a}-adrenoceptor) did not alter responses or pertussis toxin sensitivity relative to the parent receptor. Surprisingly, a Ser388Ala/Ser389Ala mutant β_{3b}-adrenoceptor became unresponsive to CL316243 while retaining an extracellular acidification rate response to SR59230A. Our findings suggest that the β_{3a}-adrenoceptor cannot couple to Gi because of conformational changes induced by a protein(s) that interacts with residues in the C-terminal tail, or because this protein(s) affects the intracellular localization of the β_{3a}-adrenoceptor.

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Introduction

The alternative splicing of transcripts encoding GPCRs has the potential to diversify the number of receptor subtypes beyond those encoded by distinct genes. Many GPCRs possess isoforms with differing C-terminal tails. These include the prostaglandin EP3 receptor (Namba et al., 1993), α_{1A}-adrenoceptor (Hirasawa et al., 1995; Chang et al., 1998), serotonin $5HT_4$ (Gerald et al., 1995) and $5HT_7$ receptors (Krobert et al., 2001) and the somatostatin $SSTR_2$ receptor (Vanetti et al., 1993). Although many splice variants share similar pharmacology, some show marked differences in signaling properties. For instance, four splice variants of the EP3 receptor couple to different subsets of G proteins (Namba et al., 1993), and 5-HT_4 splice variants display differential G-protein coupling (Pindon et al., 2002) and levels of constitutive activity (Claeysen et al., 1999).

The mouse β_3-adrenoceptor gene contains two introns, both of which undergo alternate splicing (van Spronsen et al., 1993; Granneman and Lahners, 1995; Evans et al., 1999; Hutchinson et al., 2002). One alternately spliced mRNA encodes the β_{3b}-adrenoceptor, with a C-terminal tail (-SSLLREPRHLYTCLGYP) differing from that found in the β_{3a}-adrenoceptor (-RFDGYEGARPFPT). The β_{3a}-adrenoceptor tail contains a putative tyrosine phosphorylation site and the β_{3b}-adrenoceptor tail a putative PKC phosphorylation site (Blom et al., 1999). Our previous studies have shown that while the splice variants display no significant pharmacological differences in binding affinity of [^{125}I] cyanopindolol or in affinity of competitors for this binding they do show differences in signaling properties (Hutchinson et al., 2002). Functional studies in CHO-K1 cells expressing either the β_{3a}- or the β_{3b}-adrenoceptor were carried out to examine extracellular acidification rate (ECAR), cyclic AMP accumulation and Erk1/2 phosphorylation responses. ECAR responses to all β_3-adrenoceptor agonists tested were greater in cells expressing β_{3a}-adrenoceptor compared to β_{3b}-adrenoceptor expressed at the same level. Pretreatment of cells

expressing β_{3b}-adrenoceptor but not those expressing β_{3a}-adrenoceptor with pertussis toxin caused an increase in maximal ECAR and cyclic AMP responses. Erk1/2 responses were unaffected by this treatment. The results suggested that in CHO-K1 cells the β_{3b}-adrenoceptor couples to both Gs and Gi whereas the β_{3a}-adrenoceptor couples solely to Gs. The increase in Erk1/2 phosphorylation following receptor activation was not dependent upon coupling of the receptors to G_i or the generation of cyclic AMP (Hutchinson et al., 2002). However, activated β_3-adrenoceptors can bind c-Src directly via 4 motifs (PXXP) present in the third intracellular loop and the C-terminus (Cao et al., 2000). Mutation of the proline residues in these motifs prevents both c-Src binding and Erk1/2 phosphorylation. In β_{3a}- or β_{3b}-adrenoceptor CHO-K1 cells Erk1/2 phosphorylation is inhibited by the c-Src inhibitor PP2 (Hutchinson et al., 2002).

To define the residues involved in differential G protein coupling of the β_{3a}-adrenoceptor and β_{3b}-adrenoceptor isoforms, we generated receptors mutated at key sites in the splice regions, and truncated receptors lacking either of the splice regions. To examine this further, we generated cell penetrating peptides (CPP) to introduce the spliced C-termini into cells expressing each of the β_{3a}-, β_{3b}-adrenoceptor and truncated receptor. The different C-termini of the β_{3a}-adrenoceptor and β_{3b}-adrenoceptor (p3a and p3b respectively) were covalently linked to a transport peptide, Transportan 10 (Tp10) (Pooga et al., 1998) via a disulfide bridge. Tp10 is a cell-penetrating peptide (CPP) used as a carrier for bioactive cargo (reviewed in Langel, 2002).

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Methods

Expression of the mouse β_{3a}- and β_{3b}-adrenoceptor and receptor mutants in CHO-K1 cells

Plasmids (pcDNA3.1+) carrying the coding region for each of the β_{3a}- and β_{3b}-adrenoceptor were as previously described (Hutchinson et al., 2002). Four mutants were created to examine the potential importance of residues in the C-terminus for G protein coupling. A construct for expression of the truncated mouse β_3-adrenoceptor was made by replacing a 570 bp Xho I /Xba I fragment from the β_{3a}-adrenoceptor plasmid with a 524 bp PCR fragment generated using the primers mb3.TF – 5' CGTCTATGCTCGAGTGTTCGTTGTGG 3' and mb3.TR – 5' CCGC TCTAGACCCCTATCTGTTGAGC 3' (restriction sites underlined). The Tyr392Ala β_{3a}-adrenoceptor mutant was made in the same way, by inserting a 561 bp PCR fragment generated using the forward primer mb3.TF and a reverse primer, 5' CGGTTCTAGACCCTTCACGTGGGAAACGGACGCGCACCTTCAGCGCCATC 3' containing an Xba I site. All PCR reactions were carried out as described before (Hutchinson et al., 2002), using Platinum Pfx High Fidelity DNA polymerase (Invitrogen). The β_{3b}-adrenoceptor mutant was made by QuikChange mutagenesis (Stratagene). Primers for this Ser388,389Ala mutant were, forward – 5' CCACCGCTCAACGCTGCCCTTCTTCGGGAACCC 3' and reverse – 5' GGGTTCCCGAAGAAGGGCAGCGTTGAGCGGTGG 3'. The complete insert and junctions with pcDNA3.1 were checked for each of the β_3-adrenoceptor constructs by DNA sequencing on both strands (Micromon, Monash University, Australia)

Chinese hamster ovary (CHO-KI) cells were grown in a 50:50 Dulbecco's Modified Eagle's Medium (DMEM)/Ham's F12 medium supplemented with 10% (v/v) foetal bovine serum (FBS), glutamine (2 mM), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37°C with 5% CO_2.

Transfections were performed using Lipofectamine and the transfected cells selected in media containing 800 μg/ml G418, then maintained in media containing 400 μg/ml G418. Clonal cell lines were obtained by limiting dilution of mixed cell populations, and were expanded and analyzed by a single point [^{125}I]-(-) cyanopindolol (ICYP, 800 pM) binding screen. Suitable clones were grown further for a full saturation binding analysis. The clonal cell lines chosen for further characterization were maintained under 5% CO_2 at 37°C and passaged every 3-4 days. In experiments where cells were pre-treated with specific agents, concentration and time of treatment is indicated with the data.

Peptide synthesis

All peptides (Table 3) were assembled on an ABI 433 synthesiser using low-load MBHA-resin, tBoc-protected amino acids and DCC/HOBt activation. The p3b sequence contains a thiol-group on the endogenous Cys-residue that could easily be used to construct disulfides; however, it was necessary to add an N-terminal cysteine residue in the p3a sequence. Prior to cleavage an orthogonal fmoc-group on Lys^7 in the Tp10 sequence was removed, and tBoc-Cys(NPyS) was added using TBTU/HOBt activation. The peptides were then cleaved with HF containing 10% paracreosol for Tp10-N-Cys(NPyS) and 10% paracreosol/parathiocreosole for the others, followed by ether extraction and lyophilisation. The peptides were then purified on a reversed phase C18 HPLC column (Gyncotech) and the mass was confirmed by MALDI-TOF mass spectrometry (Applied Biosystems). The constructs were obtained by dissolving the peptides in degassed ddH_2O containing 0.1% trifluoroacetic acid and mixing them at 1:1,2 ratio (slight excess of the thiol-containing peptide) over night at room temperature. The final constructs were purified by semi-preparative HPLC as above.

Radioligand binding assay

Cell membranes were prepared as described earlier (Hutchinson et al., 2002) and saturation-binding experiments were performed as described earlier (Hutchinson et al., 2002). Briefly, homogenate (10-20μg protein) was incubated with ICYP (100-2000 pM) for 60 min at room temperature in the absence or presence of (-)-alprenolol (1 mM) to define non-specific binding. Reactions were terminated by rapid filtration through GF/C filters pre-soaked for 30 min in 0.5% (v v^{-1}) polyethyleneimine using a Packard Cell Harvester and radioactivity measured using a Packard Top Count. Experiments were performed in duplicate with *n* referring to the number of different membrane homogenate samples used.

Cyclic AMP accumulation studies

Cells ($1x10^4$ per well) were grown in 96-well plates in DMEM/Ham's F-12 medium containing 0.5% (v v^{-1}) FBS for 2 days. On the day of experiment, media was aspirated and appropriate drugs diluted in stimulation buffer (1mg ml^{-1} BSA, 0.5 mM IBMX, 0.5 M Hepes, pH 7.4 in Hank's balanced salt solution (HBSS)) added in a final volume of 100μl. After 30 min of incubation at 37˚C, media was removed and 100μl of lysis buffer (1 mg ml^{-1} BSA, 0.3% (v v^{-1}) Tween-20, 0.5 M Hepes, 0.5 mM IBMX, pH 7.4) added. Samples were rapidly frozen at -70°C to lyse cells prior to measurement of cyclic AMP.

To examine the effect of pertussis toxin, cells were treated with toxin (100 ng ml^{-1}) for 16 hr before stimulation with appropriate drugs. The effects of cell penetrating peptides (CPP) based on the C-terminus of the β_{3a}- and β_{3b}-adrenoceptor were examined by addition of 1μM of peptide for 30 min prior to stimulation of cells with CL316243 for 30 min. In experiments to examine the

possible coupling of the Ser388,389Ala β_{3b}-adrenoceptor mutant to Gi, cells were treated with CL316243 for 30 min followed by treatment with forskolin (10μM) for 30 min.

Cyclic AMP accumulation was measured utilising the cyclic AMP α–Screen Kit (Perkin-Elmer, Victoria, Australia). Samples were thawed and cyclic AMP standards (10pM to 1μM) were prepared in detection buffer (0.4 % (v v^{-1}) HBSS, 3 mM Hepes, 0.2% (v v^{-1}) Tween-20, 0.1% (v v^{-1}) BSA, pH 7.4) and 5μl of unknown samples or cyclic AMP standards were transferred into a white 384-well plate. 5μl of acceptor beads (anti-cyclic AMP acceptor beads diluted in detection buffer) were aliquoted to each well and incubated for 30 min in the dark. 15μL of donor beads (streptavidin donor beads diluted in detection buffer, 1M biotinylated cyclic AMP) solution was added to each well, the plate sealed and incubated in the dark overnight. cyclic AMP accumulation was detected utilising the Fusion^{TM} α microplate reader (Perkin-Elmer, Victoria, Australia). All results are expressed as the percentage of the forskolin response (10 μM) in a given experiment.

Cytosensor microphysiometer studies

The cytosensor microphysiometer (Molecular Devices Corp., California, U.S.A.) was used to measure β_3-adrenoceptor-mediated increases in ECAR as previously described (Hutchinson et al., 2002, 2005). Briefly, CHO-K1 cells expressing the β_3-adrenoceptor were seeded into 12 mm transwell inserts (Costar) at $5x10^5$ cells per cup and left to adhere overnight. On the day of experiment, cells were equilibrated for 2 h, and cumulative concentration-response curves to CL316243 or SR59230A constructed in paired sister cells with cells exposed to each

concentration of drug for 14 min. All drugs were diluted in modified RPMI1640. All results are expressed as a percentage of the maximal response to SR59230A in a given experiment.

Data analysis

All results are expressed as a mean ± s.e.mean of *n*. Data was analyzed using non-linear curve fitting (GraphPad PRISM version 4.0) to obtain pEC_{50} values (cytosensor microphysiometer and cyclic AMP experiments) or using a one-site fit to obtain K_D and B_{max} values (saturation experiments). Statistical significance was determined using 2-way ANOVA tests or Student's t-test. Probability values less than or equal to 0.05 were considered significant.

Drugs and reagents

CL316243 was kindly supplied by Dr T. Nash (Wyeth-Ayerst). Drugs and reagents were purchased as follows: G418 (CalBiochem Corp, La Jolla, CA, U.S.A.); (-)-[^{125}I]-cyanopindolol (2200 Ci mmol^{-1}, NEN Life Science Products, Boston, MA, U.S.A.); pertussis toxin (Gibco BRL, Life Technologies, Gaithersburg, MD, U.S.A.); (-)-alprenolol, bacitracin, IBMX, polyethyleneimine, SR59230A (Sigma Chemical Company, St. Louis, MO, U.S.A.); aprotinin, leupeptin, pepstatin A (ICN, Costa Mesa, CA, U.S.A.). All cell culture media and supplements were obtained from Trace Biosciences (Castle Hill, NSW, Australia). Antibodies were obtained from Cell Signaling Technology (Beverly, MA, U.S.A.). All other drugs and reagents were of analytical grade.

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Results

Radioligand binding studies

Stably transfected cells were examined for levels of receptor expression in saturation experiments using [^{125}I] cyanopindolol. Expression levels and pK_D values for each individual β_3-adrenoceptor are shown in Table 1. All of the receptors studied had similar pK_D values, indicating that modifications to the C-terminus of either the β_{3a}- or β_{3b}-adrenoceptor had little or no effect on receptor affinity. Several clones were selected for each receptor to allow comparison at similar expression levels. In addition to the previously characterised β_{3a}- and β_{3b}-adrenoceptor (Hutchinson et al., 2002), saturation characteristics were determined for the truncated β_3-adrenoceptor, the Ser388,389Ala β_{3b}-adrenoceptor and the Tyr392Ala β_{3a}-adrenoceptor. Although expression levels for the Ser388,389Ala β_{3b}-adrenoceptor mutant were somewhat lower than for the other β_3-adrenoceptor variants it is known from previous studies (Hutchinson et al., 2002) that the G protein coupling properties of the receptors are retained over a wide range of expression levels.

Determination of the role of the C-terminus

Cyclic AMP responses to CL316243 in CHO-K1 cells expressing β_{3a}-adrenoceptors were unaffected by pre-treatment of cells with pertussis toxin (100 ng ml^{-1}, 16hr; Figure 2A, Table 2) whereas responses in cells expressing β_{3b}-adrenoceptor increased some 33% following pre-treatment with pertussis toxin (P<0.0001; Figure 2B, Table 2) confirming our previous results (Hutchinson et al., 2002). The pEC_{50} values for CL316243 at the β_{3a}- and β_{3b}-adrenoceptor were not significantly different and were not altered significantly by pertussis toxin pre-treatment

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(Table 2). The truncated β_3-adrenoceptor that lacks the C-terminal tail of either the β_{3a}- or β_{3b}-adrenoceptor splice variants behaved similarly to the β_{3b}-adrenoceptor and displayed pertussis toxin sensitivity (Figure 2C, Table 2). The maximal response to CL316243 was increased some 59% by pertussis toxin pre-treatment (P<0.0001; Table 2). This suggested that rather than the β_{3b}-adrenoceptor containing a motif that enables coupling to Gi, the C-terminus of the β_{3a}-adrenoceptor contains a motif that disables coupling to the inhibitory G protein. It should be noted that the responses observed here and in subsequent experiments result from β_3-adrenoceptor activation, as although CHO-K1 cells endogenously express low levels of β_2-adrenoceptors (Schonbrunn & Steffen, 2004), CL316,243 is a highly selective β_3-adrenoceptor agonist with antagonist actions at β_1- and β_2-adrenoceptor.

The effects of cell penetrating peptides based on the C-terminus of the β_3-adrenoceptor splice variants

CHO-K1 cells expressing β_{3a}-, β_{3b}- or the truncated β_3-adrenoceptor were treated with a number of different peptides as in indicated in Table 3. For all cell lines, the control treatment with the peptides p3a and p3b produced no significant alteration in basal cyclic AMP production or on responses to CL316243 (10nM), both in the presence or absence of pertussis toxin (Figure 3) compared to cells not treated with the peptides (the β_{3a}-adrenoceptor was still pertussis toxin insensitive and the β_{3b}-adrenoceptor or truncated β_3-adrenoceptor were still pertussis toxin sensitive). Thus the β_{3a}- and β_{3b}-adrenoceptor C-terminal tails alone are unlikely to penetrate cells and also have no discernable effect on the ability of any of the β_3-adrenoceptors to produce a cyclic AMP response.

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In contrast, treatment of cells expressing β_{3a}-adrenoceptor with the peptide Tp10-p3a (1μM) caused the cyclic AMP response to CL316243 to become pertussis toxin sensitive and display a 30% increase over control ($P<0.05$; Figure 3A). The same peptide had no effect on the pertussis toxin sensitivity of the β_{3b}- (Figure 3B) or the truncated β_3-adrenoceptor (Figure 3C). The other corresponding peptide Tp10-p3b (1μM) did not significantly affect responses of either of the pertussis toxin-sensitive receptors (β_{3b}- and truncated β_3-adrenoceptor) or the response of the β_{3a}-adrenoceptor (Figure 3). Neither Tp10-p3a or Tp10-p3b had any effect on basal cyclic AMP levels (Figure 3).

The effect of mutations in the C-terminus of β_{3a}- and β_{3b}-adrenoceptor on signaling properties

We generated a Tyr392Ala mutant β_{3a}-adrenoceptor to examine the possibility that phosphorylation of the β_{3a}-adrenoceptor tail contributes to its capacity to interfere with Gi coupling. In cells expressing this receptor, CL316243 increased cyclic AMP levels with pEC_{50} values similar to the wild-type β_{3a}-adrenoceptor (Figure 4). There were no significant changes in the cyclic AMP response between cells treated with vehicle and those with pertussis toxin (Figure 4, Table 2). Thus the Tyr392Ala mutant of the β_{3a}-adrenoceptor behaves like the parent receptor, and was not examined further for agonist-induced phosphorylation. A β_{3b}-adrenoceptor mutant (Ser388,389Ala) was used to determine if this putative phosphorylation site played a role in determining the type of G protein coupling. Surprisingly, although this mutant displayed similar binding characteristics (pK_D) to the wild type receptors and the other mutants (Table 1), it was not capable of stimulating cyclic AMP accumulation in response to CL316243 (Figure 5A). We also asked if this mutant could still couple to Gi by examining its ability to inhibit cyclic AMP production in response to forskolin (10μM). CL316243 had no effect at any concentration tested

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on cyclic AMP accumulation following stimulation by forskolin (Figure 5A). On the other hand, this receptor retained an ability to mediate a cytosensor response to SR59230A that must be independent of coupling to Gs or Gi (Figure 5B). SR59230A displayed similar potency at the Ser388,389Ala β_{3b}-adrenoceptor (pEC_{50} 6.81) and the wild type β_{3b}-adrenoceptor (pEC_{50} 6.88), but with a reduced maximal response (53.8%) (Figure 5B). In contrast, there was virtually no ECAR response to stimulation of the Ser388,389Ala β_{3b}-adrenoceptor with CL316243 compared to the wild type β_{3b}-adrenoceptor (Figure 5B).

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Discussion

In the β_2-adrenoceptor, structural determinants for Gs coupling reside in the second and third intracellular loops and in helix 8, located in the proximal C-terminal tail (O'Dowd et al., 1988; Cheung et al., 1989). These regions are well conserved between the three β-adrenoceptors, suggesting that all subtypes share common determinants for coupling to Gs. In contrast, the β-adrenoceptors all have the capacity for Gi coupling, but the amino acid determinants and cell-type dependence vary. When expressed in CHO-K1 or HEK 293 cells, both the β_1- and β_2-adrenoceptor undergo agonist-dependent phosphorylation and a consequent increase in Gi coupling (Martin et al., 2004). In cardiac myocytes, on the other hand, interaction with Gi accompanies receptor endocytosis. Interaction of the β_2-adrenoceptor with Gi is dependent on a functional PDZ motif at the receptor C-terminus (DSLL, (Xiang and Kobilka, 2003), whereas the β_1-adrenoceptor C-terminal PDZ motif (ESKV) prevents receptor internalization and Gi coupling (Xiang et al., 2002). Neither the β_{3a}- nor the β_{3b}-adrenoceptor has a known consensus PDZ motif at the C-terminus, though our findings indicate that this region in the β_{3a}-adrenoceptor is important for protein-protein interactions.

Coupling of the β_3-adrenoceptor to Gi and resultant inhibition of cyclic AMP accumulation has been reported in primary white and brown adipocytes and in 3T3-F442A adipocytes that express endogenous β_3-adrenoceptors (Chaudhry et al., 1994; Soeder et al., 1999; Lindquist et al., 2000). Gi coupling has also been implicated in β_3-adrenoceptor mediated phosphorylation of Erk1/2, although findings vary. Pre-treatment of mouse 3T3-F442A and C3H10T1/2 adipocytes with

pertussis toxin inhibits β_3-adrenoceptor mediated Erk1/2 phosphorylation (Soeder et al., 1999; Cao et al., 2000), whereas mouse 3T3-L1 adipocytes are not pertussis toxin-sensitive (Mizuno et al., 2000). In primary cultures of mouse brown adipocytes, Erk1/2 phosphorylation occurs exclusively via Gs activated pathways (Lindquist et al., 2000). pertussis toxin inhibits Erk1/2 phosphorylation in CHO-K1 and HEK 293 cells expressing the human β_3-adrenoceptor (Gerhardt et al., 1999; Soeder et al., 1999), but not in CHO-K1 cells expressing the mouse β_3-adrenoceptor (Hutchinson et al., 2002). These inconsistencies indicate that the predominant Erk1/2 phosphorylation pathway is particularly receptor- and cell type-dependent.

The discovery of introns in the β_3-adrenoceptor gene (Granneman et al., 1992) sparked a search for multiple mRNAs encoding functional β_3-adrenoceptors (Granneman et al., 1992; Bensaid et al., 1993; Granneman et al., 1993). We found two splice variants of the mouse β_3-adrenoceptor (Evans et al., 1999) that have different properties, the β_{3b}-adrenoceptor coupling to Gs and Gi, but the β_{3a}-adrenoceptor coupling only to Gs (Hutchinson et al., 2002). To define the residues involved in this differential coupling, we generated truncated receptors lacking either splice region. CL316243-stimulated cyclic AMP accumulation in cells expressing the truncated β_3-adrenoceptor was increased 59% by pre-treatment with pertussis toxin, indicating that like the β_{3b}-adrenoceptor, this receptor is able to couple to Gi. Hence the lack of Gi coupling displayed by the β_{3a}-adrenoceptor must be due to interference by the C-terminal tail. This could happen in three ways; (i) amino acid(s) in the unique C-terminus undergo intramolecular interaction with residues in the Gi coupling domains, (ii) amino acid(s) in the C-terminus bind to a separate protein or complex that causes steric hindrance or a conformational change that favours coupling

to Gs over Gi, or (iii) binding of a protein or complex results in localization of the receptor to a cellular compartment where it cannot couple to Gi.

We sought to distinguish intramolecular from intermolecular interactions by using peptides corresponding to the unique β_{3a}- and β_{3b}-adrenoceptor C-termini. To deliver these peptides, the different C-termini were linked via a disulphide bridge to the transport peptide Tp10, a deletion analog of transportan (Pooga et al., 1998). Transportan was originally made as a chimera of the neuropeptide Galanin(1-12) and the wasp venom mastoparan. Although mastoparan is a G-protein activator (Higashijima et al., 1988), transportan is much less active than mastoparan, and Tp10 is inactive (Soomets et al., 2000). These tools were used to examine the responses of CHO-K1 cells expressing β_{3a}-adrenoceptor, β_{3b}-adrenoceptor and the truncated β_3-adrenoceptor to the β_3-adrenoceptor agonist CL316,243.

When Tp10-p3 conjugates enter the cell, the reductive environment reduces the disulphide bond within minutes, releasing the C-terminal peptides and also Tp10 as monomers (Hallbrink et al., 2001). Treatment of cells with the disulphide construct Tp10-p3a caused the cyclic AMP responses to CL316243 in the β_{3a}-adrenoceptor to become pertussis toxin sensitive and display a 30% increase over control. The corresponding Tp10-p3b peptide had no significant effect on responses to CL316243 by any of the β_3-adrenoceptors. In particular, Tp10-p3b did not cause the β_{3a}-adrenoceptor response to become pertussis toxin-sensitive, indicating that the Tp10 peptide *per se* does not affect G-protein coupling. The observed effect of the Tp10-p3a peptide on the pertussis toxin-sensitivity of the β_{3a}-adrenoceptor also demonstrates that the introduction of an N-terminal cysteine in the conjugated p3a does not impair the ability of this peptide to alter

signaling by the β_{3a}-adrenoceptor. Furthermore it does not cause the peptide to affect signaling by the β_{3b}-adrenoceptor or the truncated β_3-adrenoceptor.

Intramolecular binding within the β_{3a}-adrenoceptor can be ruled out by our finding that the Tp10-p3a peptide restored the ability of this receptor to couple to Gi. If the peptide were competing with the C-terminus for binding to site(s) within the receptor, it would suppress the Gi interaction rather than enhance it. Thus our data are consistent with the view that the Tp10-p3a peptide competes with the endogenous β_{3a}-adrenoceptor C-terminus for binding of a separate protein or complex. It seems unlikely that this binding causes steric hindrance to interactions with Gi but not Gs, as the amino acids involved in G protein coupling are thought to be within common intracellular domains, namely the second and third loops and the proximal C-terminal tail (O'Dowd et al., 1988; Marin et al., 2000). It is possible, though, that binding of other proteins to the β_{3a}-adrenoceptor produces an allosteric effect, promoting a receptor conformation that has low affinity for Gi but unchanged affinity for Gs. An alternative hypothesis is that binding of protein(s) such as caveolin or other scaffolding proteins to the β_{3a}-adrenoceptor C-terminus localizes the receptor to membrane microdomains or intracellular compartments where it cannot couple to Gi. By analogy, the binding of proteins to the β_1-adrenoceptor C-terminal PDZ motif prevents internalization of the receptor and Gi coupling in cardiac myocytes (Xiang et al., 2002).

To examine sites in the C-terminal tail that might inhibit or modulate Gi coupling, we asked whether signaling is altered by mutation of amino acids that constitute putative phosphorylation sites. The affinity of β-adrenoceptor ligands for the mutant receptors was unaltered, consistent with their identical sequences in the transmembrane domains that form the β_3-adrenoceptor

ligand-binding pocket (Guan et al., 1995; Granneman et al., 1998; Gros et al., 1998). The β_{3a}-adrenoceptor C-terminal tail has a Tyr residue that may represent a target site for protein tyrosine kinases (Pinna & Ruzzene, 1996). We hypothesized that phosphorylation of the β_{3a}-adrenoceptor C-terminal tail may facilitate the protein interactions deduced from our CPP experiments. However, the mutant Tyr392Ala receptor still lacked the capacity for Gi coupling. Thus the identity of C-terminal amino acids conferring the β_{3a}-adrenoceptor phenotype remains unknown, as there are no other consensus phosphorylation sites or currently recognized motifs present.

We also examined a mutant β_{3b}-adrenoceptor in which Ser388 and Ser 389 were mutated to Ala residues. Serine and threonine residues in the C-terminal tail are determinants for desensitization of GPCRs (Hausdorff et al., 1991). Unlike the β_{3a}-adrenoceptor tail, the β_{3b}-adrenoceptor tail contains two serine residues which hypothetically form part of a PKC consensus site $(R/K)_{1-3}$-$(X)_{0-2}$-$S/T(X)_{0-2}$-$(R/K)_{1-3}$. Surprisingly, the Ser388,389Ala β_{3b}-adrenoceptor was unable to couple to Gs or Gi, indicating a significant conformational change in this receptor relative to the wild type β_{3b}-adrenoceptor. It also displayed virtually no response to CL 316243 in the cytosensor. This is not, however, a “dead” receptor, as it produced an ECAR response to SR59230A with similar potency to the wild type receptor. The ECAR response is mediated by β_3-adrenoceptors and antagonised by the neutral antagonist bupranolol and provides further evidence that SR59230A can activate cell signaling distinct from the Gs-cyclic AMP pathway (Hutchinson et al., 2005). Our recent studies indicate that changes in ECAR in response to CL316243 are mediated by cyclic AMP but those to SR59230A may in addition involve p38 MAP kinase (Sato *et al.*, BPS proceedings 2005)

In conclusion, our results indicate that both the β_{3a}- and β_{3b}-adrenoceptor are inherently capable of coupling to Gs and Gi, but that the β_{3a}-adrenoceptor is restrained from coupling to Gi by interaction of residues in the C-terminus with other protein(s). The difference between β_{3a}-adrenoceptor and β_{3b}-adrenoceptor signaling cannot be altered by mutation of a residue potentially involved in Tyr phosphorylation (Tyr392 β_{3a}-adrenoceptor). Competition for proteins interacting with the β_{3a}-adrenoceptor by an internalised p3a-peptide allows coupling of the β_{3a}-adrenoceptor to Gi as seen for the β_{3b}- or truncated β_3-adrenoceptor. The use of cell-permeable peptides corresponding to a particular receptor domain has provided a valuable approach to distinguishing between intra- and intermolecular interactions.

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Footnotes

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Legends for Figures

Figure 1: Model of the C-terminal region of the mouse β_{3a}- and β_{3b}-adrenoceptor. Letters represent the one letter amino acid code. Cys358 represents the palmitoylation site found in all β-adrenoceptor subtypes, Asn387 is the splice site for the β_{3a}- and β_{3b}-adrenoceptors and the termination site in the truncated β_3-adrenoceptor. In the β_{3a}-adrenoceptor, Tyr392 represents a high probability tyrosine phosphorylation site, and in the β_{3b}-adrenoceptor, Ser388,389 represents a putative PKC phosphorylation site.

Figure 2: The β_3-adrenoceptor agonist CL316243 produced concentration-dependent increases in cyclic AMP accumulation in cells expressing (A) β_{3a}-adrenoceptor, (B) β_{3b}-adrenoceptor, or (C) truncated β_3-adrenoceptor. Pertussis toxin treatment (100 ng ml^{-1} 16 h) significantly increased responses to CL316243 ($p<0.05$) in cells expressing the β_{3b}- and truncated β_3-adrenoceptor but not the β_{3a}-adrenoceptor. Values represent mean ± s.e.mean from 4 individual experiments each point performed with duplicate repeats. The results are expressed as a percentage of the forskolin response.

Figure 3: The effect of cell penetrating peptides on cyclic AMP accumulation in response to stimulation of β_3-adrenoceptors in CHO-K1 cells expressing (A) β_{3a}-adrenoceptor, (B) β_{3b}-adrenoceptor, and (C) truncated β_3-adrenoceptors. Clear histograms represent basal levels and filled histograms cyclic AMP levels after exposure to CL316243 (10nM, 30 min) in the absence of (-) or after treatment (+) with pertussis toxin (100 ng ml^{-1} 16 h). In control experiments (Ctl), responses of β_{3a}-adrenoceptors were unaffected by pertussis toxin pretreatment but those to β_{3b}-

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adrenoceptor or truncated β_3-adrenoceptor were enhanced. Peptides (1μM) consisting of the C-terminal tail of the β_{3a} (p3a), β_{3b} (p3b) or β_{3b}- linked to the cell permeable peptide (Tp10-p3b) did not affect the pattern of response whereas treatment with the β_{3a}- linked to the cell permeable peptide (Tp10-p3a) changed the profile of sensitivity of the β_{3a}-adrenoceptor so that it became pertussis toxin sensitive (* $P<0.05$) to resemble the β_{3b}-adrenoceptor and truncated β_3-adrenoceptor. Values represent mean ± s.e.mean obtained from 4 experiments each point performed with duplicate repeats. The results are expressed as a percentage of the forskolin response.

Figure 4: Concentration-dependent cyclic AMP accumulation responses to CL316243 for the β_{3a}-adrenoceptor mutant Tyr392Ala, demonstrating no significant difference between control and cells treated with pertussis toxin. Values represent mean ± s.e.mean from 4 individual experiments performed in duplicate. The results are expressed as a percentage of the forskolin response.

Figure 5: A) cyclic AMP accumulation in the β_{3b}-adrenoceptor mutant Ser388,389Ala. There was no cyclic AMP accumulation following treatment with CL316243 suggesting an inability to couple to Gs. Increased cyclic AMP levels in response to the direct activation of adenylate cyclase by forskolin were not reduced by pre-incubation of cells with CL316243 suggesting that the mutant was also unable to couple to Gi. The pattern of activity was not altered by pre-incubation of cells with pertussis toxin (100ng ml^{-1}; 16hr). Values represent mean ± s.e.mean obtained from 4 individual experiments, each point performed in duplicate. The results are expressed as a percentage of the forskolin response. B) ECAR responses in the cytosensor

microphysiometer in the WT β_{3b}-adrenoceptor and the Ser388,389Ala β_{3b}-adrenoceptor. In the WT β_{3b}-adrenoceptor both CL316,243 and SR59230A were full agonists. In contrast the Ser388,389Ala β_{3b}-adrenoceptor showed virtually no response to CL316,243 but was not totally functionally impaired since responses to SR59230A were obtained with similar potency to the WT receptor. Values represent mean ± s.e.mean obtained from 4-9 experiments. Data is presented as a percentage of the maximal response to the highest concentration of SR59230A in the WT β_{3b}-adrenoceptor.

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Table 1: ICYP saturation binding parameters for mutant and native mouse β_{3a}- and β_{3b}-adrenoceptors expressed in CHO-K1 cells. Parameters derived from nonlinear regression analysis represent the mean ± s.e. mean (n=4).

Membrane source	**pK_D [a]**	**B_{max} [b] (fmol/mg protein)**
β_{3a}-adrenoceptors	9.00 ± 0.28	1148 ± 241
β_{3b}-adrenoceptors	8.85 ± 0.05	1309 ± 128
Truncated β_3-adrenoceptors	8.71 ± 0.12	1224± 105
Ser388,389Ala β_{3b}-adrenoceptor	8.68 ± 0.05	627 ± 23
Tyr392Ala β_{3a}-adrenoceptor	8.79 ± 0.10	1046 ± 186

[a] Negative logarithm of the radioligand equilibrium dissociation constant

[b] Maximal density of binding sites

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Table 2: Agonist potency (pEC_{50}) and maximal responses for effects on cyclic AMP accumulation in CHO-K1 cells expressing the mouse β_3-adrenoceptor and its mutants following stimulation with CL316243. Maximum responses are expressed as a fraction of that to CL316,243 in the absence of pertussis toxin (PTX) pretreatment. Values are mean ± s.e.mean of n experiments.

CHO-K1 β_3-adrenoceptor	**pEC_{50}**	**Maximal response / Maximal response + PTX**	**n**
β_{3a}-adrenoceptors	9.92 ± 0.12	1.00 ± 0.09	4
+ pertussis toxin	9.98 ± 0.07	1.04 ± 0.08	4
β_{3b}-adrenoceptors	10.01 ± 0.14	1.00 ± 0.09	4
+ pertussis toxin	9.95 ± 0.08	1.33 ± 0.08***	4
Truncated β_3-adrenoceptors	9.96 ± 0.12	1.00 ± 0.11	4
+ pertussis toxin	9.93 ± 0.06	1.59 ± 0.10***	4
Ser388,389Ala β_{3b}-adrenoceptor	n/a	n/a	4
+ pertussis toxin	n/a	n/a	4
Tyr392Ala β_{3a}-adrenoceptor	9.68 ± 0.02	1.00 ± 0.10	4
+ pertussis toxin	9.83 ± 0.03	0.98 ± 0.10	4

***Indicates P<0.0001 determined by two-way ANOVA of concentration-response curves produced β_{3b}-adrenoceptors and mutant cells treated or untreated with pertussis toxin.

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Table 3 Peptides based on the C-terminal region of the β_{3a}- and β_{3b}-adrenoceptor used to examine β_3- adrenoceptor signaling

β_{3a}-adrenoceptor C-terminus (p3a)	**Cys-RFDGYEGARPFPT-*NH_2***
β_{3b}-adrenoceptor C-terminus (p3b)	**SSLLREPRHLYTCLGYP-*NH_2***
Tp-10 β_{3a}-adrenoceptor C-terminus (Tp10-p3a)	**AGYLLGK(εCys)INLKALAALAKKIL-*NH_2*** ***SS*** **Cys-RFDGYEGARPFPT-*NH_2***
Tp-10 β_{3b}-adrenoceptor C-terminus (Tp10-p3b)	**AGYLLGK(εCys)INLKALAALAKKIL-*NH_2*** ***SS*** **SSLLREPRHLYTCLGYP-*NH_2***

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Figure 1

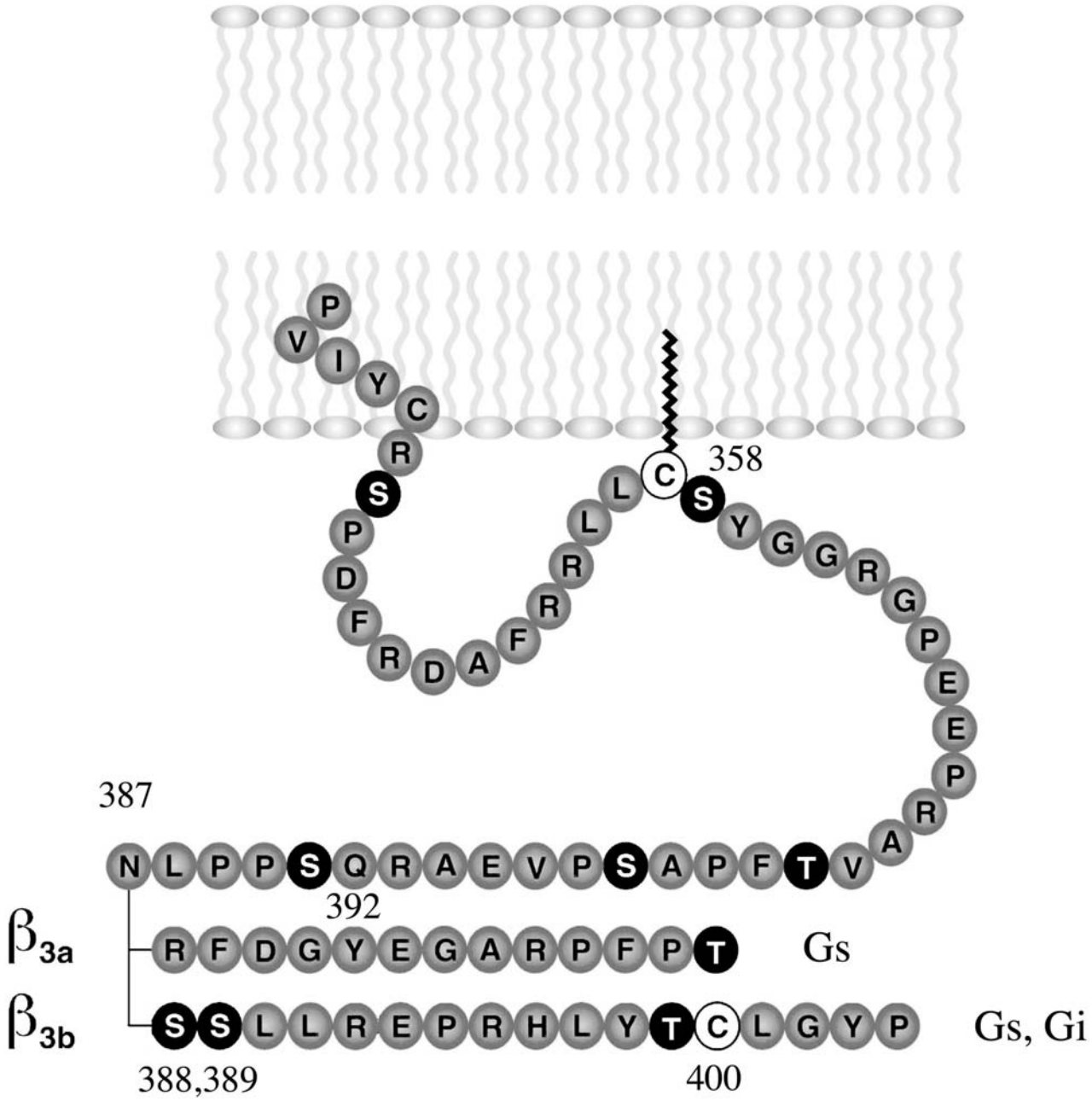

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Figure 2

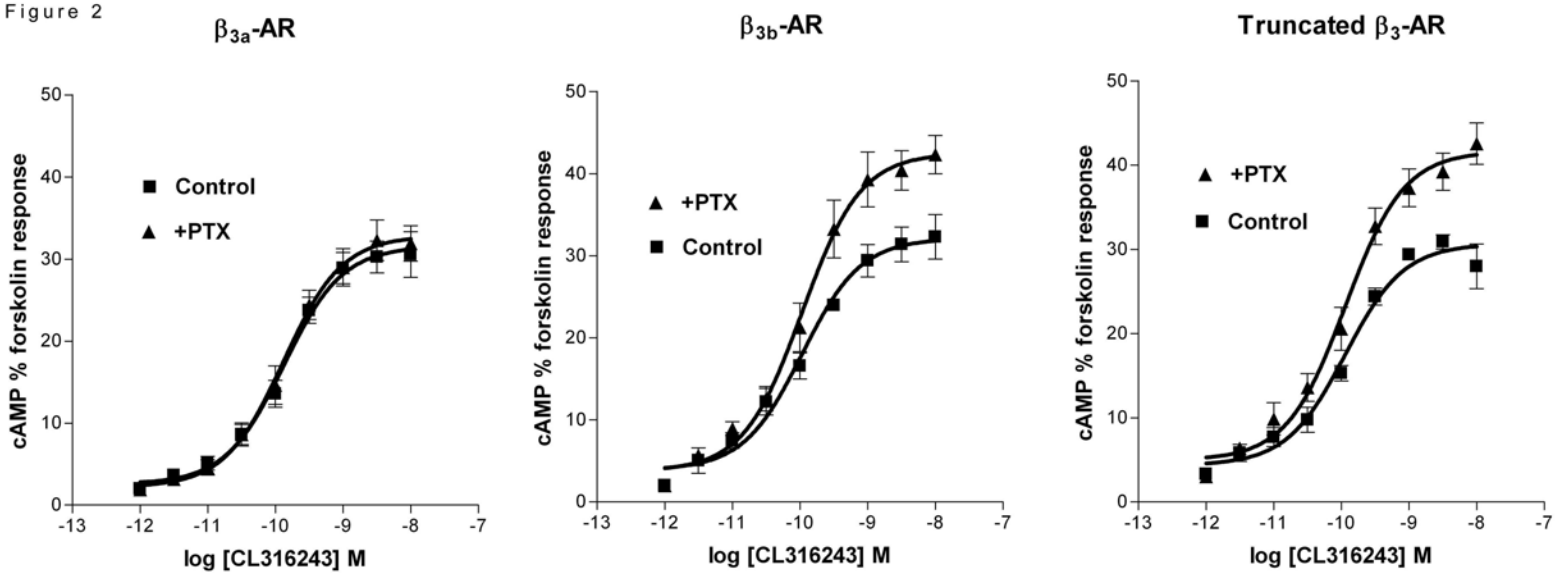

Figure 3

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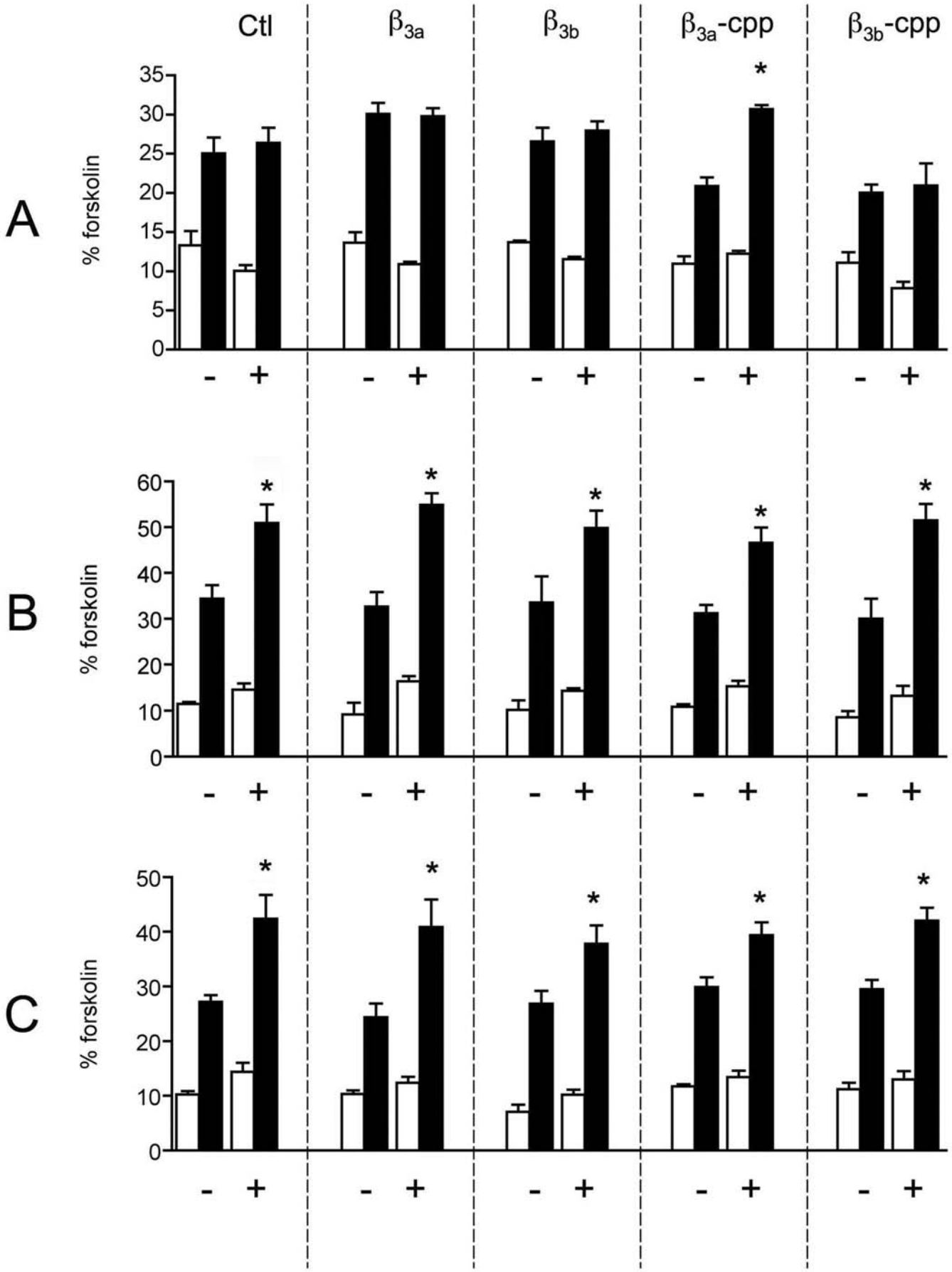

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Figure 4

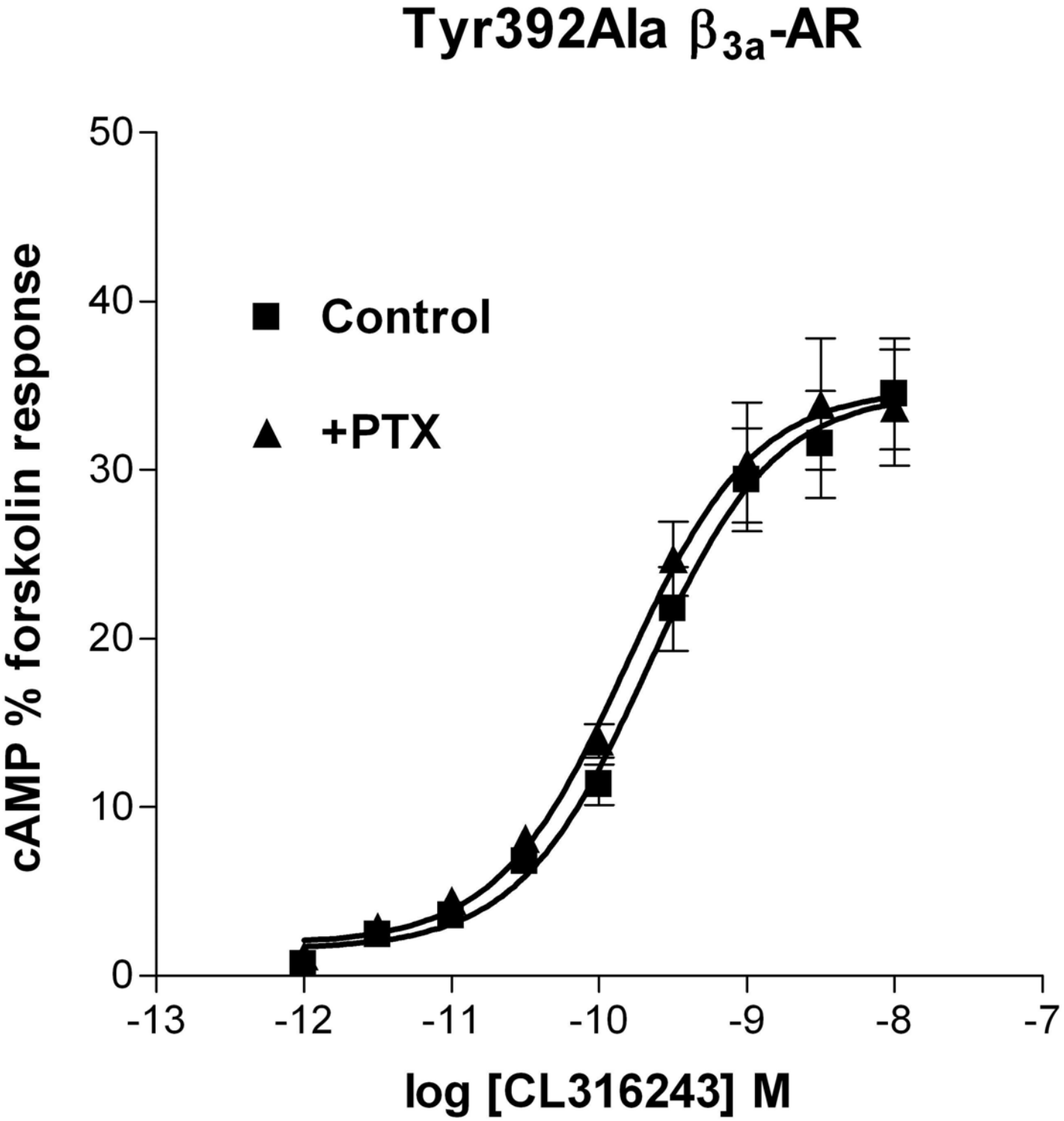

Figure 5

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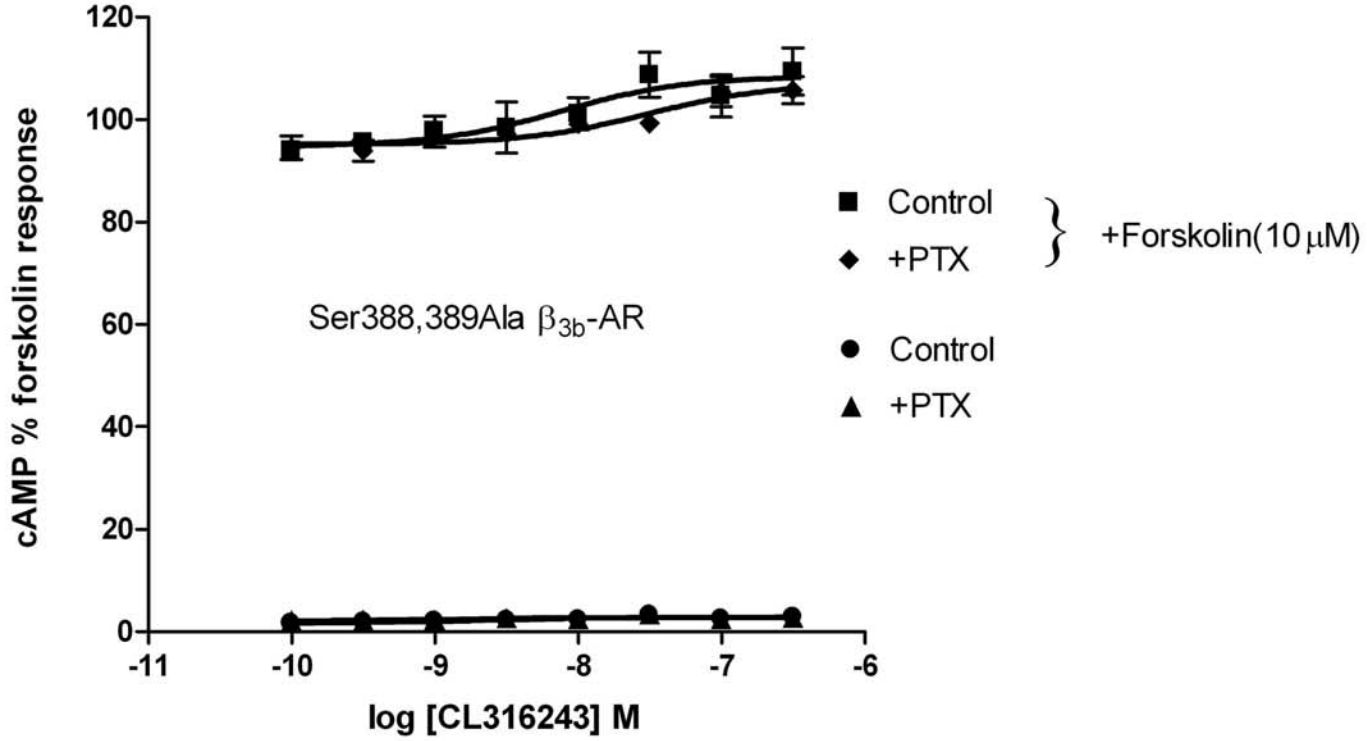
A



B