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Activation of adenylyl cyclase by endogenous G_s-coupled receptors in HEK293 cells is attenuated by 5-HT_7 receptor expression

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Running title: Attenuated signaling of endogenous G_s-coupled receptors

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Abbreviations: 5-HT, 5-hydroxytryptamine; AC, adenylyl cyclase; βAR, β-adrenergic receptor; EPR, prostanoid EP receptor; PKA, protein kinase A.

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Abstract

Human 5-HT_7 receptors display characteristics shared with receptors thought to form a tight physical coupling with G protein in the absence of ligand. Some receptors apparently preassociated with $G_{i/o}$ and $G_{q/11}$ are reported to inhibit signaling of other similarly coupled G protein-coupled receptors by limiting access to activate a common G protein pool. Therefore, we determined whether 5-HT_7 receptor expression was sufficient to limit signaling of endogenously expressed G_s-coupled receptors in HEK293 cells. Using the ecdysone-inducible expression system, which allows for titration of increasing receptor density in the same clonal cell line, we compared the effects of 5-$HT_{4(b)}$ and 5-$HT_{7(a,b,d)}$ receptor expression on adenylyl cyclase (AC) stimulation by the endogenous G_s-coupled β-adrenergic (βAR) and prostanoid EP receptors. βAR- and EP-stimulated AC activity was attenuated by 5-HT_7 receptor expression in both membrane preparations and intact HEK293 cells. βAR- and EP-stimulated AC activity was unaffected by expression of the G_s-coupled 5-HT_4 receptor. The mechanism of this heterologous desensitization seems independent of PKA activation; nor does it occur at the level of G protein activation since 1) βAR- and EP-stimulated AC activity was not restored to control values when $G_{\alpha s}$ was overexpressed and 2) β_1- and β_2AR activation of $G_{\alpha s}$ was unaffected by expression of 5-HT_7 receptors. In addition, overexpression of AC isoforms was unable to rescue βAR- and EP-stimulated AC activity. Therefore, 5-HT_7 receptors likely limit access and/or impede activation of AC by βAR and EP receptors. Although the 5-HT_7 receptor may preassociate with G protein and/or AC, the mechanism of this heterologous desensitization remains elusive.

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Introduction

Serotonin (5-Hydroxytryptamine, 5-HT) mediates its diverse physiological effects through at least 14 different receptor subtypes, of which 13 belong to the G protein-coupled receptor family (Hoyer *et al.*, 1994). Among the human 5-HT receptors, three different subtypes, 5-HT_4, 5-HT_6, and 5-HT_7, are coupled to G_s and at least the 5-HT_4 and 5-HT_7 receptors are expressed as several different functional splice variants (Gerald *et al.*, 1995; Heidmann *et al.*, 1997). Currently, the functional significance of 5-HT_7 splice variants, that differ only in the carboxy terminus (Heidmann *et al.*, 1997) remains unknown (Krobert *et al.*, 2001; Krobert and Levy, 2002), whereas, among the 5-HT_4 splice variants, constitutive activation of AC is dependent on the different carboxy termini (Bockaert *et al.*, 2004). Previously, we have shown that the 5-$HT_{4(b)}$ and 5-$HT_{7(a)}$ signaling properties differ fundamentally. The potency of 5-HT to stimulate AC increased with increasing receptor density in clones expressing 5-$HT_{4(b)}$ but not 5-$HT_{7(a)}$ receptors, even though 5-HT-stimulated AC activity in clones expressing 5-$HT_{7(a)}$ receptors had reached asymptotic levels (Bruheim *et al.*, 2003). This indicates that potency of 5-HT for stimulation of AC through the 5-$HT_{7(a)}$ receptor is independent of receptor-G_s stoichiometry. This is likely to be an inherent property of 5-$HT_{7(a)}$ receptor function, that distinguishes it from the 5-$HT_{4(b)}$ receptor. We have proposed that properties governing 5-$HT_{7(a)}$ receptor activation of AC is consistent with a model that presumes the 5-$HT_{7(a)}$ receptors are tightly associated with G protein, independent of agonist binding (Bruheim *et al.*, 2003). For clarity, we will use the term preassociated for this type of receptor-G protein association (i.e. presumed association of inactive receptor and G protein), whereas we will reserve the

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term precoupled for the association of active (but ligand-unoccupied) receptor and G protein (i.e. constitutive activity).

The cubic ternary complex model incorporates the existence of such an inactive receptor coupled to a G protein (Weiss *et al.*, 1996). Experimental support for the existence of receptors tightly associated (preassociated) to their respective G protein, in the absence of ligand, has been reported for the CB_1-cannabinoid receptor (Vasquez and Lewis, 1999; Mukhopadhyay *et al.*, 2000), the Mel_{1a} melatonin receptor (Roka *et al.*, 1999) and the vasoactive intestinal peptide $VPAC_1$ receptor (Shreeve 2002). Whereas the CB_1 receptor-$G_{\alpha i/o}$ association is sensitive to the destabilizing effect of guanine nucleotides (Mukhopadhyay *et al.*, 2000), high affinity agonist binding at the Mel_{1a} receptor is resistant to both the destabilizing effect of guanine nucleotides and pertussis toxin (Roka *et al.*, 1999). At the 5-HT_7 receptor, two groups have reported that a high proportion of recombinant human 5-$HT_{7(a)}$ receptors exist in the high affinity (presumably G protein-coupled) state (Adham *et al.*, 1998; Alberts *et al.*, 2001). The insensitivity of the high affinity agonist binding of the human 5-HT_7 receptor to the destabilizing effect of guanine nucleotides (Alberts *et al.*, 2001; Krobert *et al.*, 2001), is another indication the 5-HT_7 receptor and $G_{\alpha s}$ protein form a tight complex.

Expression of the $G_{i/o}$-coupled CB_1 receptor in superior cervical ganglia attenuated the ability of α_2AR and somatostatin receptors to activate $G_{i/o}$ and it was proposed that the CB_1 receptor, due to its preassociation with $G_{i/o}$, sequesters a proportion of the available G protein pool (Vasquez and Lewis, 1999). As a result, the available G protein pool is reduced, limiting activation by other $G_{i/o}$-coupled receptors and subsequently their respective signaling ability.

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The cubic ternary complex model also proposes the existence of a ligand-occupied inactive receptor coupled to G protein. Stabilization of an inactive CB_1 receptor-$G_{i/o}$ complex by the inverse agonist SR141716A inhibited insulin- and insulin-like growth factor 1-mediated activation of mitogen-activated protein kinase through $G_{i/o}$ (Bouaboula *et al.*, 1997). Likewise, purinergic receptor-stimulated $G_{q/11}$ activation and subsequent Ca^{2+} mobilization is attenuated in the presence of the guinea pig histamine H_1 receptor inverse agonist mepyramine, presumably by stabilization of a H_1 receptor-$G_{q/11}$ complex (Fitzsimons *et al.*, 2004). Taken together, these findings provide experimental evidence for a ligand-occupied inactive G protein-coupled state of the receptor. Furthermore, they indicate that an inactive receptor-G protein preassociation can limit access of other G protein-coupled receptors to activate a common G protein pool.

The primary objective of the current study was to determine whether 5-HT_7 receptors represented an example of a preassociated G_s-coupled receptor. Therefore, we tested whether 5-HT_7 receptor expression alone was sufficient to limit signaling of endogenously expressed G_s-coupled receptors in HEK293 cells. To test this hypothesis, we used the ecdysone-inducible expression system, which permitted reproducible expression of increasing receptor density in the same clonal cell line. Using this expression system, we compared the effects of 5-$HT_{4(b)}$ and 5-$HT_{7(a, b \text{ and } d)}$ receptor expression on AC stimulation by the endogenous βAR and prostanoid EP receptors.

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Materials and methods

Materials

Serotonin (5-Hydroxytryptamine hydrochloride), (-) isoproterenol, timolol, alprenolol, GDP, GTPγS (guanosine 5'-[γ-thio]triphosphate) and H89 (*N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrobromide) were from Sigma-Aldrich (St. Louis, MO, USA). Methiothepin (metitepine, 1-[10,11-Dihydro-8-(methylthio)dibenzo[b,f]thiepin-10-yl]-4-methylpiperazine) maleate and 8-OH-DPAT (8-hydroxy-2-dipropylaminotetralin) hydrobromide were from Tocris (Bristol, UK). Prostaglandin E_1 was from Cayman chemical company (Ann Arbor, MI, USA). Renzapride (BRL24924) hydrochloride was from Bethesda Research Laboratories (Bethesda, Md., USA). Zeocin™, penicillin-streptomycin, G418, ponasterone A, LipofectAMINE™ and LipofectAMINE2000™ were from Invitrogen (Leek, The Netherlands). Forskolin was from Calbiochem (San Diego, CA). Supersignal Dura West was from Pierce (Rockford, IL, USA). *Anti*-pRas-GRF1 was from Cell Signaling Technology (Beverly, MA, USA). *Anti*-HA-probe and *Anti*-$G_{\alpha s/olf}$ were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Sheep *anti*-rabbit IgG-HRP was from Amersham Biosciences (Buckinghamshire, UK).

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Radiochemicals

[^{3}H]5-CT (5-Carboxamidotryptamine) (60-102 Ci/mmol), [^{3}H]GR113808 ({1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl}methyl-1-methyl-1*H*-indole-3-carboxylate) (84 Ci/mmol), (-)-3-[^{125}I]iodocyanopindolol (CYP) (2000 Ci/mmol), [α-^{32}P]ATP (400 Ci/mmol) [2,8-^{3}H]cAMP (30-42 Ci/mmol), [^{3}H]CGP12177 (((-)-4-(3-t-butylamino-2-hydroxypropoxy)-[5,7-^{3}H]benzimidazol-2-one) (37 Ci/mmol), [N^{6}-methyl-^{3}H]mesulergine (87 Ci/mmol) and [γ-^{35}S]GTPγS (1033 Ci/mmol) were from Amersham Biosciences.

Construction of expression vectors, establishing inducible EcR293 cell lines and transfection

Construction of expression vectors

The human 5-HT$_{4(b)}$ and 5-HT$_{7(a)}$ receptors were cloned and stably transfected into the inducible cell line EcR293 (Invitrogen) as described previously (Bruheim *et al.*, 2003). For expression of the human 5-HT$_{7(b)}$ and 5-HT$_{7(d)}$ receptors, previously cloned receptor cDNA (Krobert *et al.*, 2001) was excised from the plasmid pcDNA3.1 (Invitrogen) with *NheI* and *BamHI* and transferred to the expression vector pInd (Invitrogen). EcR293 cells were transfected with plasmid DNA (pInd containing human 5-HT$_{7(b)}$ or 5-HT$_{7(d)}$) using LipofectAMINE™ (Invitrogen) according to the manufacturers protocol.

Human β_1 and β_2 adrenoceptors were excised from the plasmid pAGA-2 (Levy *et al.*, 1993) with *EcoRI* and *XbaI* and transferred to pcDNA3.1.

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Selection of EcR293 cell lines stably expressing 5-HT$_{7(b)}$ or 5-HT$_{7(d)}$ receptors

EcR293 cells were cultured in 5-HT-free medium (UltraCULTURE™ general purpose serum-free medium (BioWhittaker, Walkersville, MD)), supplemented with L-glutamine (2 mM) penicillin (100 U/ml), streptomycin (100 μg/ml) and Zeocin™ (0.2 mg/ml)). Forty-eight hours after transfection, serial dilutions of transfected cells were plated in 96-well plates and the neomycin analogue G418 (0.2 mg/ml) (geneticin) was added. Limiting dilutions of isolated single colonies of cells transformed to the neomycin-resistant phenotype were performed to achieve single clonal cell lines. Single colonies were expanded and tested for ponasterone A-induced (10 μM for 24 h) expression of serotonin receptors by radioligand binding assay. For titration of receptor density, ponasterone A (0.1-10 μM) was added to the growth medium 24 h before conducting experiments.

Transfection of HEK293 or EcR293 cell lines

HEK293 cells (ATCC, Rockville, MD) were grown in Dulbecco's modified Eagle's medium (BioWhittaker) with 10 % fetal calf serum (EuroClone, Milano, Italy), penicillin (100 U/ml) and streptomycin (100 μg/ml). HEK293 or EcR293 cells inducibly expressing the 5HT$_{7(a)}$ receptor were transiently transfected with LipofectAMINE2000™ (Invitrogen) according to the manufacturers protocol. Cells were transfected with the following plasmids: human 5-HT$_{7(a)}$ (Krobert *et al.*, 2001), human β_1- and β_2AR, AC5, AC6 and AC7 (all in pcDNA3.1; all AC clones were generous gifts from Dr. Dermot MF Cooper, Department of Pharmacology, University of Cambridge, UK), human $G_{\alpha s(S)}$ and $G_{\alpha s(L)}$ (both in pcDNA3.1 obtained from the UMR cDNA Resource Center (www.cdna.org)), control vector (pcDNA3.1) or full-length murine HA-Ras-GRF1 wild type (in pKH3 mammalian expression plasmids)

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(Mattingly *et al.*, 1994; Mattingly and Macara, 1996), where indicated. After transfection, HEK293 cells were cultured in UltraCULTURE™, supplemented with L-glutamine (2 mM) penicillin (100 U/ml) and streptomycin (100 μg/ml), for 48 h. 24 h after transfection, EcR293 cells were induced with ponasterone A (10 μM) (where indicated) for an additional 24 h.

Membrane preparation, radioligand binding and adenylyl cyclase assay

Membranes were prepared as previously described (Krobert *et al.*, 2001). Radioligand binding assays for 5-HT_7, 5-$HT_{4(b)}$ and βAR were preformed with 1.3-1.7 nM [^{3}H]5-CT, 0.2-0.5 nM [^{3}H]GR113808 or 20-100 pM (-)-3-[^{125}I]CYP respectively, as previously described (Krobert *et al.*, 2001). B_{max} was estimated as described (Krobert *et al.*, 2001) based on a K_d value of 0.31 nM, 21 pM and 6.8 pM for [^{3}H]5-CT, [^{3}H]GR113808 and (-)-3-[^{125}I]CYP respectively. Adenylyl cyclase activity was measured and analyzed by determining conversion of [α-^{32}P]ATP to [^{32}P]cAMP in membranes as previously described (Krobert *et al.*, 2001). Isoproterenol- and Prostaglandin E_1 (PgE_1)-stimulated AC activities (performed in triplicates) are reported as percent activity relative to cells not expressing or not induced to express 5-HT_7 (or 5-$HT_{4(b)}$) receptors (control)).

Cell surface receptor binding

Cell surface βAR density was determined as previously described (Clark and Knoll, 2002) with the following modifications: Cells were trypsinized, pelleted and resuspended in UltraCULTURE™. Approximately 400,000 cells were transferred to

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96-well, round-bottom micro-titer plates and incubated with the hydrophilic compound [^{3}H]CGP12177 (10 nM) for 1 h at 4ºC. Non-specific binding was determined by inclusion of 1 μM alprenolol in parallel wells. Greater than 95% of cells remained intact after detachment as assessed by trypan blue staining. At the end of the incubation period, the plates were harvested in the same manner as for radioligand binding. B_{max} was estimated as described above using a K_d of 0.76 nM for [^{3}H]CGP12177. Determination of cell surface serotonin receptor density was performed similar to βAR density. Approximately 60,000 cells were incubated with the hydrophobic compounds [^{3}H]GR113808 (1 nM) or [^{3}H]mesulergine (90 nM) with or without the hydrophobic antagonists SB207266 (10 μM) or methiothepin (10 μM) (for the 5-$HT_{4(b)}$ and 5-$HT_{7(a)}$ receptor, respectively) or hydrophilic 5-HT (100 μM). The incubation was carried out for 3 h at 13ºC, which allows for equilibrium of ligand binding while inhibiting sequestration or the return of sequestered receptors to the cell surface (Hausdorff *et al.*, 1989). Plates were harvested as described above. The percentage of receptors on the cell surface was calculated as specific radioligand binding not displaced by the hydrophilic ligand. Specific cell surface receptor density was calculated as the difference between total radioligand binding and that resistant to displacement by the hydrophilic ligand and B_{max} was estimated as described above using a K_d of 21 pM and 9.2 nM for [^{3}H]GR113808 and [^{3}H]mesulergine, respectively.

cAMP accumulation

Cells were plated and subsequently induced (where indicated) 24 hours prior to the experiment in 12 well plates (Falcon). Cells were incubated with 0.5 mM 3-isobutyl-

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1-methylxantine (Sigma-Aldrich) for 10 min and stimulated with isoproterenol, PgE_1 or increasing concentrations of 5-HT for 5 minutes. The reaction was stopped by addition of trichloroacetic acid (Sigma-Aldrich) to a final concentration of 5%. cAMP content was determined by a radioimmunoassay as described previously (Skomedal *et al.*, 1980). Isoproterenol- and PgE_1-stimulated cAMP accumulation were performed in quadruplicates and are reported as stimulated cAMP content relative to cells not induced to express either $5\text{-}HT_{7(a)}$ or $5\text{-}HT_{4(b)}$ receptors (control groups). Increasing concentration of 5-HT was performed in duplicate and data were fit to the equation $Y=a+(b-a)x/(c+x)$ where a is basal cAMP accumulated, b is maximal cAMP accumulated stimulated by the agonist, c is EC50, and x is the concentration of agonist.

GTPγS binding assay specific for $G_{\alpha s}$

Agonist-stimulated G_s-protein activation was determined in membrane preparations by measuring the stimulation of [^{35}S]GTPγS binding coupled to an antibody capture-based scintillation proximity assay, as previously described (Cussac *et al.*, 2002). Membranes were pre-incubated for 30 min with indicated agonists in a buffer containing 20 mM HEPES, pH 7.4, 50 mM $MgCl_2$, 100 mM NaCl and 1 μM GDP. The reaction was started with addition of [^{35}S]GTPγS (0.3 nM in a final volume of 200 μl in 96-well optiplates (PerkinElmer Life Sciences)). After 60 min incubation at room temperature, 20 μl Nonidet P-40 (Sigma-Aldrich) was added (0.27 % final concentration) and plates were incubated for 30 min under gentle agitation. 10 μl *anti*-$G_{\alpha s/olf}$ (1.74 μg/ml final dilution) was then added to each well before an additional 30 min incubation period. Scintillation proximity assay beads coated with anti-rabbit

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antibodies (Amersham Biosciences (Buckinghamshire, UK)) were added in a volume of 50 µl at a dilution indicated by the manufacturer and the plates were incubated for 3h with gentle agitation. The plates were then centrifuged (10 min, 1300 g) immediately followed by radioactivity detection in a Topcount microplate scintillation counter (Packard). Non-specific binding was measured by parallel wells incubated with GTPγS (100 µM). Agonist-stimulated $G_{\alpha s}$ activation is reported as the fold increase in specific binding compared to basal-stimulated G_s activation.

Western blotting

EcR293 cells inducibly expressing the 5-$HT_{7(a)}$ receptors were cultured in 35 mm dishes and transfected with the indicated plasmids. Cells were stimulated as indicated, then washed and lysed in ice-cold cell lysis buffer (1% SDS, 1 mM Na_3VO_4, 50 mM Tris-HCl, pH 7.4 at RT), scraped with a Teflon cell scraper, sheared through a 25 GA syringe and immediately frozen in liquid N_2. The thawed cell lysates were cleared at 13,000 x g at 4°C and the protein concentrations in the supernatants were quantified using the BC assay quantitation kit (Uptima, Monticon, France) using BSA as a standard. Equal amounts of cell lysate proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were incubated with primary antibodies (*anti*-phospho-Ras-GRF1, 1:1000; *Anti*-HA-probe, 1:2000; *anti*-$G_{\alpha s/olf}$, 1:1000) in 5% non-fat dry milk in PBS with 0.05% Tween, and thereafter incubated with sheep *anti*-rabbit IgG HRP-conjugated secondary antibody. The immobilised HRP-conjugated secondary antibody were visualized with Supersignal

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Dura West extended duration chemiluminescent substrate and analyzed with a UVP BioChemie system.

Protein measurements

Protein concentration in membrane preparations were measured with the Micro BC Assay Reagent Kit (Uptima) using bovine serum albumin as a standard.

Statistics

Paired students-t-test was performed using GraphPad Prism 4.00 for Windows (GraphPad Software, San Diego, CA, USA).

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Results

Increasing 5-HT$_{7(a)}$ receptor density does not increase 5-HT potency

We have previously reported that the potency of 5-HT to stimulate AC increased with increasing receptor density in membrane preparations from EcR293 cell lines induced to express the 5-HT$_{4(b)}$ receptor but not the 5-HT$_{7(a)}$ receptor (Bruheim *et al.*, 2003). Our first objective was to determine whether intact cells displayed the same phenomenon to eliminate the possibility that this finding was an artifact of membrane preparations. The potency of 5-HT (pEC_{50}) to stimulate AC was examined in intact EcR293 cells expressing either low or high densities of 5-HT$_{4(b)}$ or 5-HT$_{7(a)}$ receptors. As shown in table 1, the potency of 5-HT was increased (leftward-shift of 0.8 in pEC_{50}) only in EcR293 cells expressing high densities of the 5-HT$_{4(b)}$ receptor. In contrast, the potency of 5-HT was unchanged in EcR293 cells expressing high densities of the 5-HT$_{7(a)}$ receptor, even though 5-HT$_{7(a)}$ receptor density exceeded that of 5-HT$_{4(b)}$ by greater than 4-fold (Table 1). This data confirms the absence of a classical spare receptor effect in intact cells and indicates that this property is an inherent characteristic of 5-HT$_{7(a)}$ receptor function *in vivo*.

Partial agonists become full agonists with increasing receptor density at 5-HT$_{4(b)}$ but not 5-HT$_{7(a)}$ receptors

We have proposed that the potency of 5-HT for stimulation of AC through the 5-HT$_{7(a)}$ receptor is independent of receptor-G_s stoichiometry and is consistent with a model where the 5-HT$_{7(a)}$ receptors are tightly associated with G protein, independent of agonist binding (Bruheim *et al.*, 2003). In such a system, partial agonists are not expected to become full agonists in the presence of spare receptors. Therefore, to

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confirm and extend support for the existence of a stable complex between inactive 5-$HT_{7(a)}$ receptors and G proteins, we determined the efficacy of 8-OH-DPAT and renzapride, agonists at the 5-HT_7 and 5-HT_4 receptors, respectively, at low and high receptor density in EcR293 cells. At the 5-$HT_{7(a)}$ receptor, 8-OH-DPAT remained a partial agonist at both the lower (3.6 ± 1.0 pmol/mg protein) and higher (7.9 ± 0.1 pmol/mg protein) receptor densities tested, eliciting a maximal response 81 ± 1% and 75 ± 1% respectively of that obtained with the full agonist 5-HT (Fig. 1 upper graph). At low 5-$HT_{4(b)}$ receptor density (0.72 ± 0.48 pmol/mg protein), renzapride displayed partial agonist activity, eliciting a maximal response 87 ± 3% of that obtained with the full agonist 5-HT. However and in contrast to the 5-$HT_{7(a)}$ receptor, the efficacy of renzapride was equal (100 ± 1%) to the full agonist 5-HT at high receptor densities (3.8 ± 0.1 pmol/mg protein; Fig. 1 lower graph).

Contributions of the 5-HT_7 receptor to basal AC activity

Assuming a high proportion of 5-HT_7 receptors form a stable association with G protein in the absence of agonist, and given the high constitutive activity of 5-HT_7 receptors (Krobert and Levy, 2002), it may be hypothesized that 5-HT_7 receptors would account for a larger percentage of basal AC activity (constitutive AC activity) at increasing 5-HT_7 receptor density. To test this hypothesis, we determined the efficacy of the full inverse agonist methiothepin at increasing 5-$HT_{7(a, b and d)}$ receptor density. As shown in Fig. 2A, the reduction of basal AC activity mediated by methiothepin increased with increasing receptor density reaching an asymptote where basal AC activity was reduced by 65% at the highest receptor densities. This effect of methiothepin was observed in every clone tested, irrespective of whether there was a corresponding increase in basal AC activity with increasing 5-HT_7 receptor density (in

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accordance with previously reported data (Krobert and Levy, 2002)). Interestingly, high 5-$HT_{7(a)}$ receptor density also inhibited β_2-adrenoceptor (β_2AR) constitutive AC activation (Fig. 2B) in EcR293 cells revealed by the inverse agonist timolol (Chidiac *et al.*, 1994). These data indicate that the 5-$HT_{7(a)}$ receptor may limit access of the β_2AR to G protein.

5-HT_7 receptor expression attenuates endogenous G_s-coupled receptor AC activation

Given that high 5-HT_7 receptor density abolished β_2AR constitutive AC activation, we next determined whether 5-HT_7 receptor expression modified ligand-mediated AC activation by endogenous G_s-coupled receptors. βAR and prostanoid EP receptors (EPR) both couple via $G_{\alpha s}$ to activate AC and both are endogenously expressed in HEK293 cells (Friedman *et al.*, 2002; Fujino *et al.*, 2002). Isoproterenol- and PgE_1-stimulated AC activity (acting on βAR and EPR respectively) was attenuated with increasing 5-HT_7 receptor density both in membrane preparations (Fig. 3A) and intact cells (Fig. 3B), irrespective of the splice variant expressed (all three splice variants were equally effective). Isoproterenol- and PgE_1-stimulated AC activity was reduced by 5-HT_7 densities as low as a few hundred fmol/mg protein and approximately 75% by the highest 5-HT_7 receptor densities examined. In contrast, isoproterenol- and PgE_1-stimulated AC activity was not reduced in cells expressing the 5-$HT_{4(b)}$ receptor (Fig. 3 C, D), even at receptor densities comparable to those that gave ~50% inhibition by the 5-HT_7 receptor in membrane preparations. Rather, high 5-$HT_{4(b)}$ receptor density modestly increased isoproterenol- and PgE_1-stimulated AC activity in membrane preparations. Incubation of non-transfected EcR293 cells with ponasterone

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A did not modify either isoproterenol- or PgE_1-stimulated AC activity (data not shown).

β-adrenoceptor cell surface receptor density is not modified by 5-HT_4 or 5-HT_7 receptor expression

The 5-HT_7 receptor splice variants display varying degrees of constitutive internalization in the absence of ligand (Guthrie *et al.*, 2005). This property may promote endocytosis or limit cell surface expression of endogenous βAR and EPR. To determine whether a reduction of endogenous cell surface receptors accompanied the reduced activation of AC, we measured the effect of 5-$HT_{4(b)}$ and 5-$HT_{7(a)}$ receptor expression upon cell surface βAR density. A high percentage of 5-$HT_{4(b)}$ and 5-$HT_{7(a)}$ receptors (64 ± 4 and 70 ± 6 respectively) are on the cell surface of EcR293 cells induced to express high 5-HT receptor densities (Table 2). These values are similar to that reported by Guthrie *et al.* (2005) in HEK293 cells. High density expression of 5-HT_4 or 5-HT_7 receptors did not modify the density of endogenous βAR on the cell surface of EcR293 cells (Table 2) or in EcR293 cells co-expressing transiently transfected β_2ARs (data not shown). These data indicate that a reduction of cell surface βAR and EPR is not mediating the attenuated βAR and EPR activation of AC.

Mechanism of attenuated endogenous G_s-coupled receptor AC activation is PKA independent

To determine if high constitutive activity of the 5-HT_7 receptor, through sustained activation of AC and subsequent activation of protein kinase A (PKA), mediated the heterologous desensitization of the endogenous βAR and EPR, we inhibited PKA activity with H89. As shown in Fig. 4A, isoproterenol- and PgE_1-stimulated AC

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activities in EcR293 cells expressing 5-$HT_{7(a)}$ receptors remained similarly attenuated both in the presence and absence of H89. However, 5-HT-induced PKA-dependent phosphorylation of Ser916 on Ras-GRF1 was inhibited by H89 in EcR293 cells expressing 5-$HT_{7(a)}$ receptors (Fig. 4B), indicating that H89 inhibits PKA activity under these experimental conditions. Therefore, it is unlikely that the classical PKA-dependent mechanism of heterologous desensitization is mediating the attenuation of signaling of the endogenous βAR and EPR.

5-HT_7 receptors limit the ability of endogenous βAR and prostanoid EP receptors to activate AC, but not $G_{\alpha s}$

We have proposed that a strong physical (pre)association of the 5-HT_7 receptor with $G_{\alpha s}$ in the absence of ligand accounts for the atypical properties of 5-HT_7 receptor function. If the endogenous βAR and EPR utilize the same pool of $G_{\alpha s}$ as 5-HT_7 receptors, and 5-HT_7 receptors preassociate with $G_{\alpha s}$, access of βAR and EPR to $G_{\alpha s}$ may be impeded as 5-HT_7 receptor density increases. To determine whether the amount of $G_{\alpha s}$ was limiting, we overexpressed $G_{\alpha s(S\ or\ L)}$ protein together with 5-$HT_{7(a)}$ receptors. Overexpression of $G_{\alpha s}$ had no effect on isoproterenol- and PgE_1-stimulated AC activities whether tested by $G_{\alpha s(S\ or\ L)}$ overexpression in EcR293 cells induced to express 5-$HT_{7(a)}$ receptors (Fig. 5A) or tested by co-transfection of HEK293 cells by 5-$HT_{7(a)}$ and $G_{\alpha s(S\ or\ L)}$ (Fig. 5B). In both systems, isoproterenol- and PgE_1-stimulated AC activity remained attenuated by 5-HT_7 receptor expression. Interestingly, isoproterenol-stimulated βARs were able to activate $G_{\alpha s}$ equally well in the presence or absence of 5-HT_7 receptors as revealed by GTPγS binding (Fig. 6A). The fact that βARs are able to activate $G_{\alpha s,}$ while βAR activation of AC remained attenuated in the presence of 5-$HT_{7(a)}$ receptors (Fig. 6B), suggests that access and/or availability of AC

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to activated $G_{\alpha s}$ is limiting. To determine whether the amount of AC was limiting, we overexpressed AC5, a subtype of AC shown to be activated by the 5-$HT_{7(a)}$ receptor in HEK293 cells (Baker *et al.*, 1998). Isoproterenol- and PgE_1-stimulated AC activities in EcR293 cells induced to express 5-HT_7 receptors were not altered by overexpressing AC5 (Fig. 7A), even though forskolin-stimulated AC activity increased two-fold (Fig. 7B), indicating that AC5 was properly expressed in the membrane preparations tested. Likewise, overexpression of AC6 (previously shown to be activated by βARs in HEK293 cells (Krupinski *et al.*, 1992)) or AC7 did not rescue isoproterenol- and PgE_1-stimulated AC activities (data not shown).

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Discussion

The primary finding of this study is that expression of the G_s-coupled 5-HT_7 receptor attenuates AC activation by βAR and EPR, G_s-coupled receptors endogenously expressed in both HEK293 cells and EcR293 cells. The presence of 5-HT_7 receptors also attenuated βAR constitutive and ligand-stimulated AC activity when βAR were overexpressed. The degree of inhibition was dependent on the density of 5-HT_7 receptor expression. However, βAR and EPR did partially activate AC even at the highest 5-HT_7 receptor densities. We argue that this effect is not simply a consequence of high receptor expression. First, βAR and EPR activation of AC was reduced by 5-HT_7 receptor densities as low as ~150 fmol/mg protein (βAR-stimulated AC activity = 79 ± 5 % of control, EPR-stimulated AC activity = 83 ± 5 % of control, both $p < 0.05$ vs. control). These levels are similar to those reported in membranes from guinea pig brain and are not substantially higher than those observed in rat and human brain (~30 and 70 fmol/mg respectively (Thomas *et al.*, 2002)). Second, βAR and EPR AC activation was unchanged by expression of the G_s-coupled 5-HT_4 receptor under similar high receptor densities and conditions. Therefore, we conclude that this effect is an intrinsic property of 5-HT_7 receptors and not due solely to overexpression of 5-HT_7 receptors. Whereas, similar to our findings, $G_{i/o}$-coupled CB_1 receptor expression attenuated the ability of α_2AR and somatostatin receptors to activate downstream effectors of $G_{i/o}$ (Vasquez and Lewis, 1999), to our knowledge, this would be the first documentation of this effect in G_s-coupled receptors.

Several findings in this study indicate that constitutive activation of AC, and subsequent activation of PKA by 5-HT_7 receptors, does not mediate the heterologous desensitization of βAR and EPR. First and foremost, the 5-$HT_{4(b)}$ receptor also

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constitutively activates AC, as the 5-$HT_{4(b)}$ inverse agonist SB207266 reduced basal cAMP accumulation (51 ± 2 % reduction by 10 μM SB207266) in intact EcR293 cells induced to express 5-$HT_{4(b)}$ receptors (1.54 ± 0.07 pmol/mg protein, n = 3). Expression of 5-HT_4 in numerous cell lines has revealed that 5-HT_4 receptors have a high constitutive activity even at low and physiological levels (Bockaert *et al.*, 2004). Additionally, mouse 5-$HT_{4(b)}$ receptors constitutively activate AC in intact COS-7 cells to levels equivalent to the human constitutively active mutant β_2AR (Claeysen *et al.*, 1999). Therefore, attenuation of isoproterenol- and PgE_1-stimulated AC activity would also be expected in the presence of 5-$HT_{4(b)}$ receptors, if constitutive activation of AC was the key determinant mediating PKA-dependent heterologous desensitization. However, as shown in Fig. 3 C, D, expression of the 5-$HT_{4(b)}$ receptor did not inhibit isoproterenol- or PgE_1-stimulated AC activity. Second, attenuation of the isoproterenol- and PgE_1-stimulated AC activity was not affected when 5-HT_7 constitutive AC activity was blocked by the presence of 5-HT_7 inverse agonists (seven inverse agonists tested; methiothepin, clozapine, metergoline, spiperone, SB269970, methysergide and mesulergine; data not shown)). This indicates that the pool of $G_{\alpha s}$ contributing to 5-HT_7 constitutive activity is not involved in attenuation of βAR- or EPR-stimulated AC activity. Third, isoproterenol- and PgE_1-stimulated AC activity was not restored to control values when PKA activity was inhibited by H89 (Fig. 4). Fourth, βAR activation of $G_{\alpha s}$ was not attenuated (Fig. 6), as would be expected if βAR were desensitized through the classical mechanisms.

Alternatively, the ability of the endogenous receptors to access $G_{\alpha s}$ may be impeded by 5-HT_7 receptor expression. Vasquez and Lewis (1999) propose that the CB_1 receptor, due to its preassociation with $G_{i/o}$ in the absence of ligand, sequesters a proportion of the available G protein pool. As a result, the available G protein pool is

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reduced, limiting activation by other $G_{i/o}$-coupled receptors. In support, Vasquez and Lewis (1999) have demonstrated that expression of the $G_{i/o}$-coupled CB_1 receptor in superior cervical ganglia attenuated the ability of α_2AR and somatostatin receptors to activate $G_{i/o.}$ In order to presume that 5-HT_7 receptors are sequestering and limiting access to G protein, it is a prerequisite to demonstrate that the 5-HT_7 receptor is similarly preassociated with G_s.

In fact, such a basis exists, since 5-HT_7 receptors (as opposed to the 5-$HT_{4(b)}$ receptor) exhibit multiple properties similar to other G protein-coupled receptors known to form a tight complex with G protein in the absence of ligand (Vasquez and Lewis, 1999; Mukhopadhyay *et al.*, 2000; Roka *et al.*, 1999; Shreeve, 2002). For example, a very high fraction of 5-HT_7 receptors exist in a high affinity agonist binding state, which is insensitive to the destabilizing effect of guanine nucleotides (Alberts *et al.*, 2001; Krobert *et al.*, 2001). In addition, the mode of G protein coupling of the 5-HT_7 receptor is incongruent with the predictions of the operational model of agonism (Black and Leff, 1983), because the 5-$HT_{7(a)}$ receptor does not display a classical spare receptor phenomenon (Bruheim *et al.*, 2003). In the present studies, using intact EcR293 cells, we confirm that the potency of 5-HT remains unchanged at high 5-$HT_{7(a)}$ receptor densities, whereas the potency of 5-HT increases at high 5-$HT_{4(b)}$ receptor density, in accordance with our observations in cell membranes (Bruheim *et al.*, 2003). Furthermore, we show that the efficacy of the 5-HT_7 partial agonist 8-OH-DPAT remains unchanged relative to 5-HT at high 5-HT_7 receptor densities, extending support for the absence of a spare receptor phenomenon. In contrast, the 5-HT_4 partial agonist renzapride becomes a full agonist relative to 5-HT at high receptor densities (Fig.1) as expected in the presence of spare receptors, in accordance with the operational model of agonism. On the basis of these data, we

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have proposed that the potency of 5-HT for stimulation of AC through the 5-$HT_{7(a)}$ receptor is independent of receptor-G_s stoichiometry. This is consistent with a model where the inactive conformational state of 5-$HT_{7(a)}$ receptors are tightly associated with G protein (possibly complexed in a fixed stoichiometry), independent of agonist binding (Bruheim *et al.*, 2003). This is in contrast to the 5-$HT_{4(b)}$ receptor, which may associate with G protein independent of agonist binding, only or primarily when in the active conformational state. We propose that these characteristics distinguish the 5-HT_7 from the 5-$HT_{4(b)}$, and may be related to the ability of 5-HT_7 receptors to attenuate signaling through other G_s-coupled receptors. It is well established that the 5-HT_7 receptor has a high constitutive activity, measured as AC activity (Krobert and Levy, 2002), a property that by definition gives rise to the active conformational state of the 5-HT_7 receptor coupled with G_s. In fact, we show that 5-HT_7 accounts for an increasing percentage of basal AC activation with increasing receptor density (accounting for up to 65% of total basal AC activity), at the expense of other G_s-coupled receptors (Fig. 2). Taken together, these findings support the concept of a tight association between both the inactive and active conformational state of the 5-HT_7 receptor and G_s protein.

Although the property of preassociation between the 5-HT_7 receptor and G_s protein may contribute to the attenuation of isoproterenol- and PgE_1-stimulated AC activity, it is unlikely it does so by sequestering or limiting access to a common G_s pool for the following reasons: 1) Overexpression of $G_{\alpha s}$ in the presence of high 5-HT_7 receptor density did not restore isoproterenol- and PgE_1- stimulated AC activity to control levels (Fig. 5). 2) β_1- and β_2AR activation of $G_{\alpha s}$ is unaffected by high expression of 5-HT_7 receptors (Fig. 6), indicating that βAR can access, couple to and activate a pool of $G_{\alpha s}$. The primary implication of these findings is that the mechanism

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of heterologous desensitization is not occurring at the level of G protein activation. Therefore, it is unlikely that traditional desensitization mechanisms (*i.e.* G protein-coupled receptor kinase and PKA-dependent phosphorylation) are underlying the 5-HT_7-mediated effect, since these mechanisms result in the uncoupling of receptor from G protein. Furthermore, the mechanism employed by the 5-HT_7 receptor differs from that of the CB_1 receptor, as overexpression of $G_{i/o}$ rescued the ability of the endogenous α_2AR and somatostatin receptors to activate $G_{i/o}$ (Vasquez and Lewis, 1999). The fact that β_1- and β_2AR activation of AC remains attenuated (Fig. 6B), even though they can activate $G_{\alpha s}$ (Fig. 6A), indicates that the 5-HT_7 receptor in some way limits access to or impedes activation of AC directly. As we have previously proposed, 5-HT_7 receptor activation of AC conforms to a model assuming a preassociated signaling complex that includes G protein and AC (Bruheim *et al.*, 2003). Therefore, the amount of AC available for activation by $G_{\alpha s}$ may become the limiting component, since the approximate molar ratio of receptor/G protein/AC has been estimated as 1:200:3 (Alousi *et al.*, 1991; Post *et al.*, 1995). However, our data indicate that the amount of AC is also not the limiting component, since overexpression of AC5, AC6 or AC7 (AC isoforms known to interact with βAR and 5-HT_7 receptors (Krupinski *et al.*, 1992; Baker *et al.*, 1998)) did not rescue isoproterenol- or PgE_1-stimulated AC activity (data shown only for AC5 in Fig. 7). In certain cell types, βAR, but not EPR activation of AC was elevated when overexpressing AC6, presumably because AC6 co-localized only in the microdomain containing the βAR (Ostrom *et al.*, 2000). This suggests that the newly synthesized AC may not be accessible by βAR and EPR in EcR293 cells, possibly due to compartmentation of AC and the receptors into different microdomains. Alternatively, βAR and EPR have access to AC, however, the presence of 5-HT_7 receptors somehow

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impedes the ability of these receptors to activate AC. Further study is needed to determine this elusive and potentially novel mechanism of heterologous desensitization mediated by 5-HT_7 receptor expression.

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Footnotes

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Figure legends

Fig. 1 Partial agonists become full agonists at high receptor densities at 5-HT_4, but not 5-HT_7 receptors. The upper graph shows AC activity in response to increasing concentrations of 8-OH-DPAT and 5-HT in membranes from EcR293 cell lines expressing the 5-$HT_{7(a)}$ receptor at low (2.7 pmol/mg protein - open symbols) and high (7.8 pmol/mg protein - closed symbols) receptor density. The lower graph shows AC activity in response to increasing concentrations of renzapride and 5-HT in membranes from EcR293 cell lines expressing the 5-$HT_{4(b)}$ receptor at low (0.24 pmol/mg protein - open symbols) and high (3.7 pmol/mg protein - closed symbols) receptor density. AC activity was measured as described in methods and the data shown are representative of that obtained from three independent experiments.

Fig. 2 Increasing 5-HT_7 receptor density increases its contribution to basal AC activity and inhibits constitutive activity of the β_2AR.

A) Effect of methiothepin (1 μM) on basal AC activity in membrane preparations of EcR293 cells expressing increasing 5-$HT_{7(a)}$ receptor densities. AC activity was measured as described in Materials and methods and data are presented as percent reduction of basal AC activity. Data shown are mean ± SEM of six experiments from two independent EcR293 clones expressing 5-$HT_{7(a)}$ receptors. Similar data were obtained with clones expressing the 5-$HT_{7(b)}$ and the 5-$HT_{7(d)}$ receptors (data not shown).

B) Effect of timolol (10 μM) on basal AC activity in membrane preparations of EcR293 cells transiently expressing β_2AR in the presence or absence of 5-$HT_{7(a)}$ receptors. Data presented are mean ± SEM of nine experiments. β_2AR receptor

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density was 2.2 ± 1.0 pmol/mg protein in non-induced and 2.4 ± 1.1 pmol/mg protein in EcR293 cell membranes induced to express 5-$HT_{7(a)}$ receptors. 5-$HT_{7(a)}$ receptor density was 0.011 ± 0.004 and 11.1 ± 1.4 pmol/mg protein in non-induced and induced cells, respectively. * - Timolol-mediated reduction of basal AC activity was significantly attenuated by 5-$HT_{7(a)}$ receptor expression ($p < 0.05$).

Fig. 3 Expression of 5-HT_7 receptors attenuates endogenous G_s-coupled receptor signaling. The figure shows isoproterenol- (solid triangles; Iso) and PgE_1- (solid circles) (both 10 μM) stimulated AC activity in EcR293 cell membranes (A and C) or cAMP accumulation in intact EcR293 cells (B and D) induced to express increasing densities of either 5-HT_7 (A and B) or 5-$HT_{4(b)}$ (C and D) receptors. All data are expressed as isoproterenol- and PgE_1-stimulated AC activity above basal as percent of control (uninduced cells). Total cAMP accumulated was measured after 5 min stimulation with isoproterenol or PgE_1 in intact cell experiments. Data shown for 5-HT_7 receptor membranes are collapsed across the three splice variants since there was no difference between the splice variants. Data are mean ± SEM of 10 experiments obtained from two 5-HT_7 clonal cell lines for each splice variant (A), seven cAMP accumulation experiments from two 5-$HT_{7(a)}$ clonal cell line (B), eight experiments collapsed from two 5-$HT_{4(b)}$ clonal cell lines (C), or six cAMP accumulation experiments from one 5-$HT_{4(b)}$ clonal cell line (D).

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Fig. 4 PKA activation is not the mechanism of attenuation of G_s-coupled receptor responses

A) Isoproterenol- or PgE_1-stimulated (both at 10 μM) AC activity of EcR293 cell membranes induced to express 5-$HT_{7(a)}$ receptors in the presence or absence of the PKA inhibitor H89. EcR293 cells were incubated with either ponasterone A (10 μM: induced group) or vehicle (ethanol: non-induced group; control). Sister plates of induced and non-induced cells were incubated with H89 (20 μM) or vehicle (50% ethanol) for 3 x 8 h (24 h total incubation). Data shown are AC activity in membrane preparations as a percent of the non-induced group and are mean ± SEM from four experiments. 5-$HT_{7(a)}$ receptor density was 4.4 ± 0.8 pmol/mg protein in vehicle and 4.2 ± 0.7 pmol/mg protein in H89-treated groups. B) EcR293 cells were transfected with HA-Ras-GRF1 24 h prior to inducing 5-$HT_{7(a)}$ receptor expression. During incubation with ponasterone A (10 μM) the cells were co-incubated 3 x 8 h with 20 μM H89 or vehicle. After the 24 h induction period, cells were stimulated with 10 μM 5-HT or vehicle for 5 min, lysed, and proteins were separated on 6% SDS-PAGE, electroblotted to PVDF membrane and probed with *anti*-pRas-GRF1 (upper panel). Total HA-Ras-GRF1 was detected with *anti*-HA (lower panel). The blot shown is representative of three experiments.

Fig. 5 Overexpression of $G_{\alpha s}$ does not rescue βAR- or EPR-stimulated AC activity

The figure shows isoproterenol- (Iso) and PgE_1-stimulated AC activity (both at 10 μM) in membranes from EcR293 cells induced to express 5-$HT_{7(a)}$ receptors (A) or HEK293 cells transiently expressing 5-$HT_{7(a)}$ receptors (B) in the presence or absence

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of transient $G_{\alpha s(S\ or\ L)}$ overexpression. Data shown are collapsed from experiments with $G_{\alpha s(S)}$ and $G_{\alpha s(L)}$ as no significant differences were noted. A) 24 h prior to 5-$HT_{7(a)}$ receptor induction by ponasterone A or vehicle, EcR293 cells were transfected with $G_{\alpha s(S\ or\ L)}$ or control vector (pcDNA3.1). AC activity was assayed 24 h after induction of 5-$HT_{7(a)}$ receptor expression, 48 h after transfection of $G_{\alpha s}$. Data are mean ± SEM of four experiments and are reported as percent of control (non-induced EcR293 cells). (B) HEK293 cells were transiently co-transfected with $G_{\alpha s(S\ or\ L)}$ or control vector (pcDNA3.1) and 5-$HT_{7(a)}$ or control vector (pcDNA3.1) and grown for 48 h prior to AC assay. Data are mean ± SEM of four experiments and are reported as percent of control (HEK293 cells not transfected with 5-$HT_{7(a)}$). 5-$HT_{7(a)}$ receptor density was 18 ± 3 and 5.9 ± 2.4 pmol/mg protein in A and B, respectively. C) EcR293 cells transiently transfected with $G_{\alpha s(S\ or\ L)}$ (where indicated) and induced to express 5-$HT_{7(a)}$ receptors (where indicated) were lysed and proteins separated on 10 % SDS-PAGE, electroblotted to PVDF membranes and probed with *anti*-$G_{\alpha s/olf}$.

Fig. 6 βAR activation of $G_{\alpha s}$ is unaffected by 5-HT_7 receptor expression

A) Isoproterenol-stimulated (10 μM) [^{35}S]GTPγS binding at $G_{\alpha s}$ ($G_{\alpha s}$ was isolated by an antibody capture technique as described in Materials and methods) was assayed in membrane preparations from EcR293 cells induced to express 5-$HT_{7(a)}$ receptors (where indicated) and co-expressing transiently transfected β_1AR, β_2AR or control (pcDNA3.1). [^{35}S]GTPγS binding is presented as fold increase of basal. B) isoproterenol- (10 μM) stimulated AC activity (pmol above basal) in the same EcR293 cells membrane preparations as above. Receptor density was 0.37 ± 0.09 and 0.80 ± 0.37 pmol/mg protein for the β_1AR and β_2AR, respectively and the presence of

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5-$HT_{7(a)}$ receptors (9.1 ± 1.8 pmol/mg protein) did not alter βAR densities. Data shown are mean ± SEM of four experiments performed in triplicate.

Fig. 7 Overexpression of adenylyl cyclase does not rescue βAR- or EPR-stimulated AC activity

A) Isoproterenol- (Iso) and PgE_1-stimulated AC activity (both at 10 μM) in membranes from EcR293 cells induced to express 5-$HT_{7(a\ or\ b)}$ receptors in the presence or absence of transient AC5 overexpression. Data are reported as percent of control (non-induced EcR293 cells). B) Forskolin- (100 μM) stimulated AC activity in membranes from EcR293 cells induced to express 5-$HT_{7(a\ or\ b)}$ receptors (where indicated) in the presence or absence (control vector; pcDNA3.1) of transient AC5 overexpression. Data are mean ± SEM of five experiments. 5-HT_7 receptor density was 6.7 ± 2.3 and 6.1 ± 2.1 pmol/mg protein in induced cells without or with AC5 overexpression, respectively.

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Table 1 Effect of receptor density on potency of 5-HT at 5-HT$_{4(b)}$ and 5-HT$_{7(a)}$ receptors. The amount of cAMP accumulated during 5 min exposure to increasing concentrations of 5-HT (8 pM to 50 µM) was measured in intact EcR293 cells expressing either the 5-HT$_{4(b)}$ or 5-HT$_{7(a)}$ receptor at low and high receptor densities. cAMP levels were measured and the pEC_{50} was calculated as described in materials and methods. Data shown are the mean ± S.E.M. from three (5-HT$_7$) and four (5-HT$_4$) experiments. * - $p < 0.05$

5-HT$_7$		5-HT$_4$	
Receptor density (pmol/mg protein)	pEC_{50}	Receptor density (pmol/mg protein)	pEC_{50}
0.59 ± 0.27	7.3 ± 0.36	0.51 ± 0.06	8.14 ± 0.18
8.8 ± 2.3	7.3 ± 0.16	1.8 ± 0.2	8.93 ± 0.35*

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Table 2 Effect of 5-HT$_{4(b)}$ and 5-HT$_{7(a)}$ receptor expression on β-adrenoceptor cell surface receptor density. The density of β-adrenoceptors and serotonin receptors at the cell surface was determined in EcR293 cells induced to express either 5-HT$_{4(b)}$ or 5-HT$_{7(a)}$ receptors. Cell surface receptor density was determined as described in methods. Data shown are the mean ± S.E.M. from four experiments and are expressed as fmol/mg protein. N.D. – not detectable.

	βAR density		5-HT receptor density	
EcR293 clone	Non-induced	Induced	Non-induced	Induced
5-HT$_{4(b)}$	7.2 ± 2.0	8.1 ± 1.7	73 ± 17	3800 ± 900
5-HT$_{7(a)}$	6.9 ± 2.0	7.8 ± 2.2	N.D.	8600 ± 2200

Figure 1

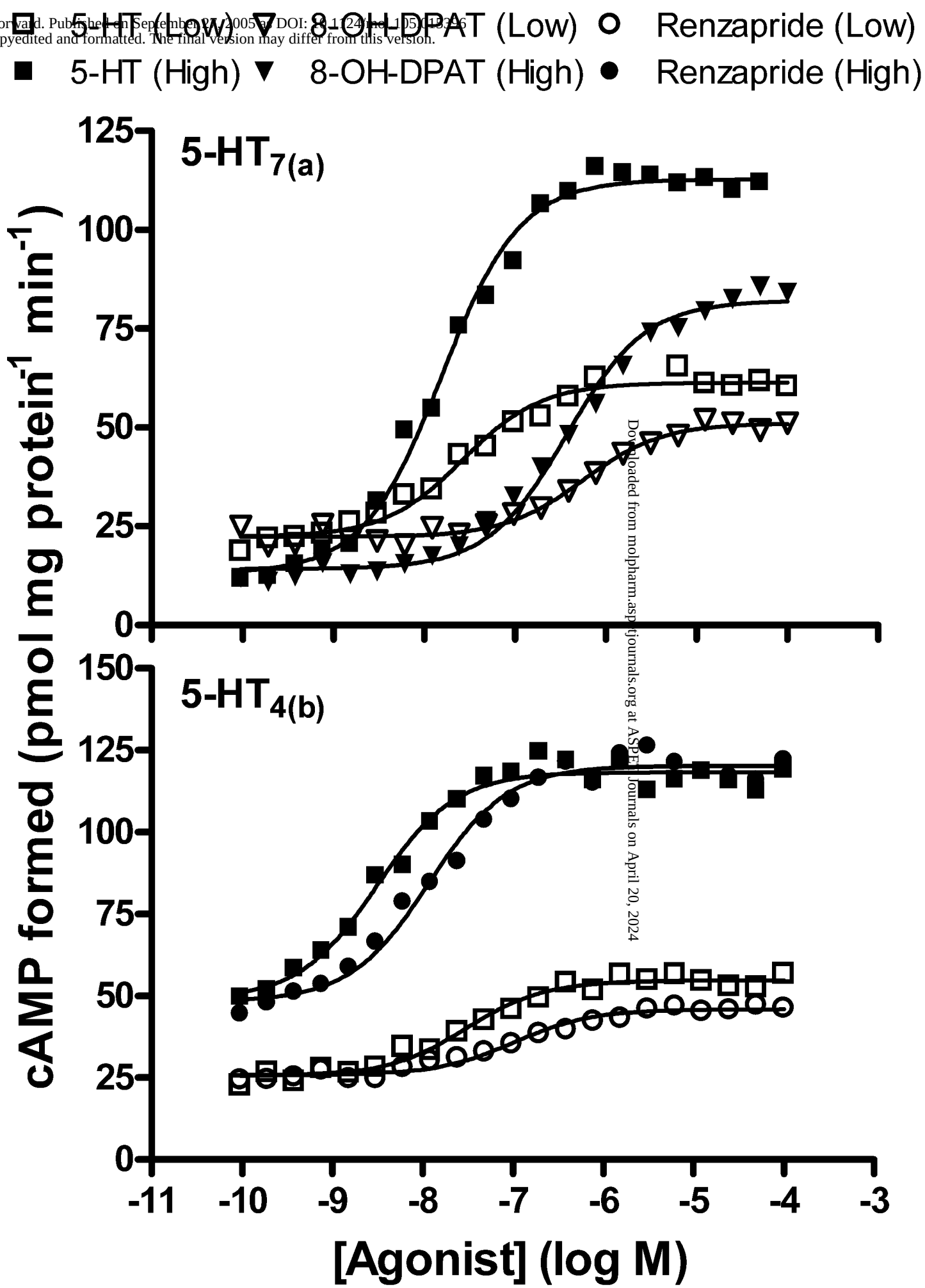
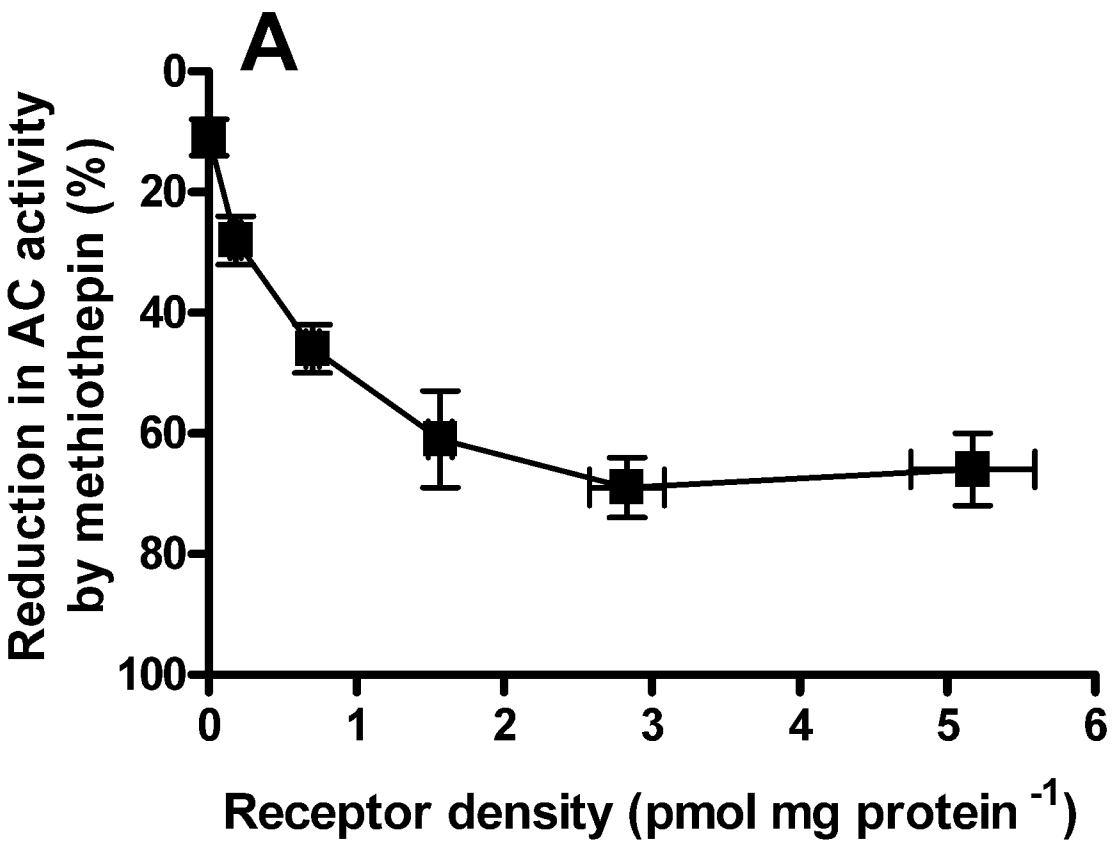


Figure 2


A
Reduction in AC activity by methiothepin (%)
0
20
40
60
80
100
0
1
2
3
4
5
6
Receptor density (pmol mg protein -1)

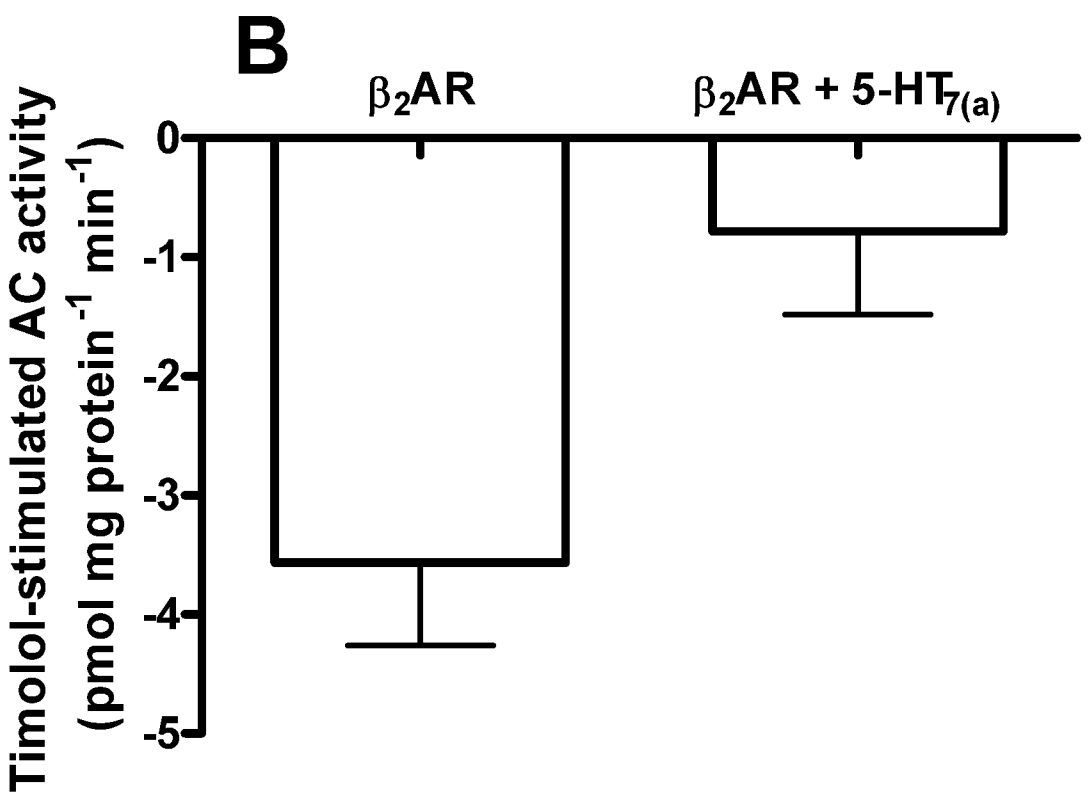

B
β2AR
β2AR + 5-HT7(a)
Timolol-stimulated AC activity (pmol mg protein-1 min-1)
0
-1
-2
-3
-4
-5

Figure 3

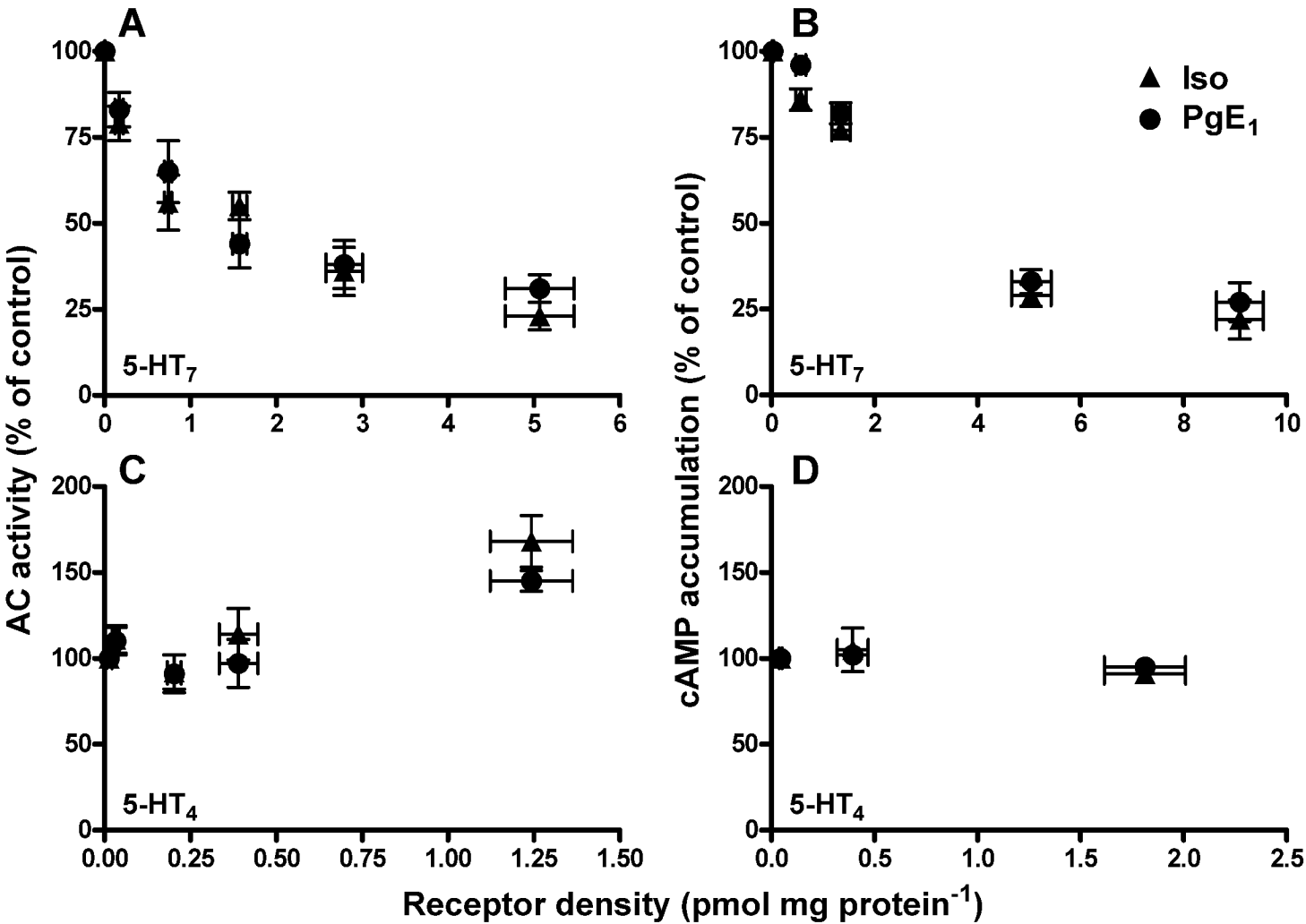

A
B
C
D
Iso
PgE1
5-HT7
5-HT7
5-HT4
5-HT4
AC activity (% of control)
cAMP accumulation (% of control)
Receptor density (pmol mg protein-1)

Figure 4

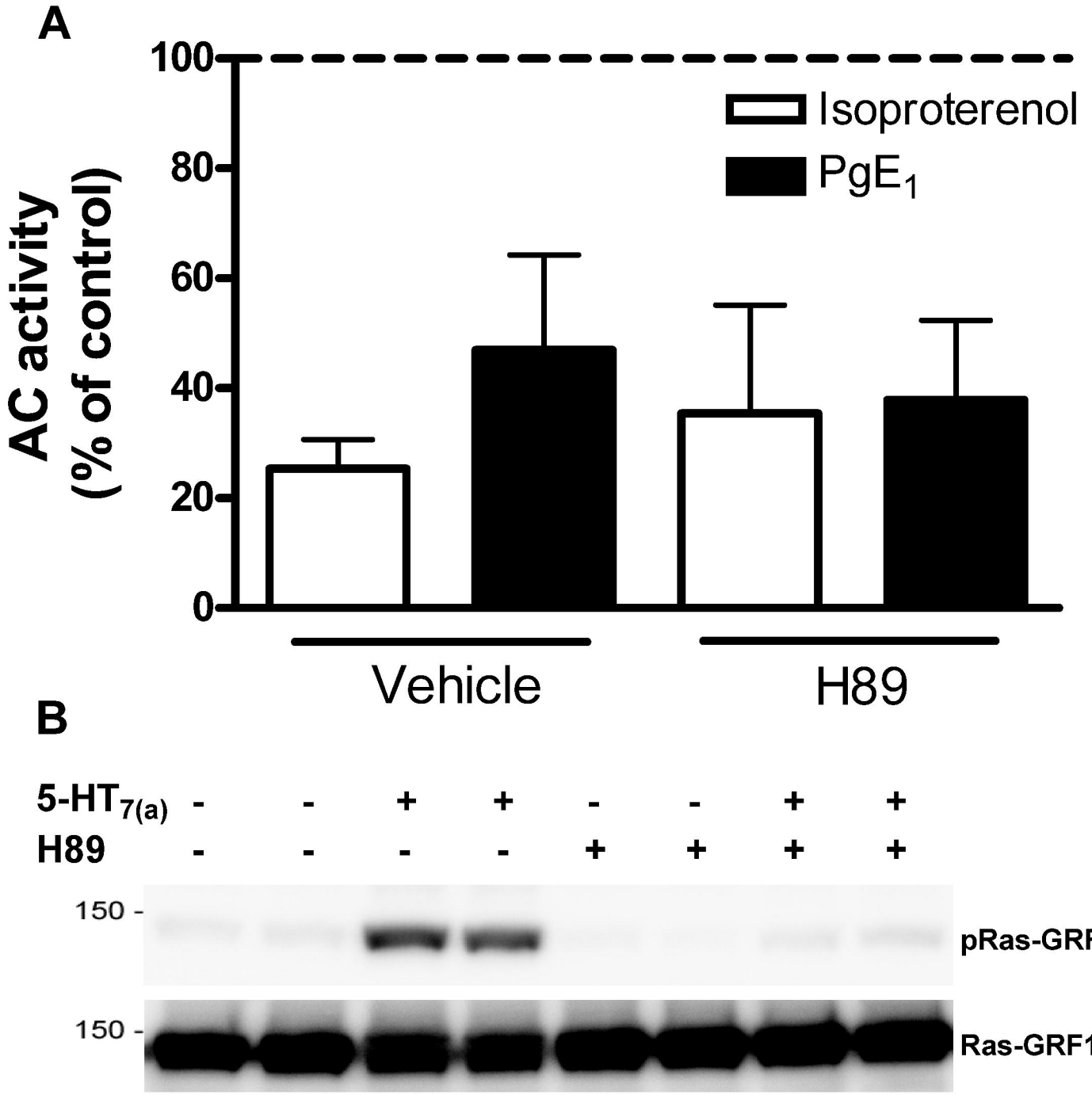

A
100
80
60
40
20
0
AC activity
(% of control)
Isoproterenol
PgE1
Vehicle
H89
B
5-HT7(a) - - + + - - + +
H89 - - - - + + + +
150 -
pRas-GRF1
150 -
Ras-GRF1

Figure 5

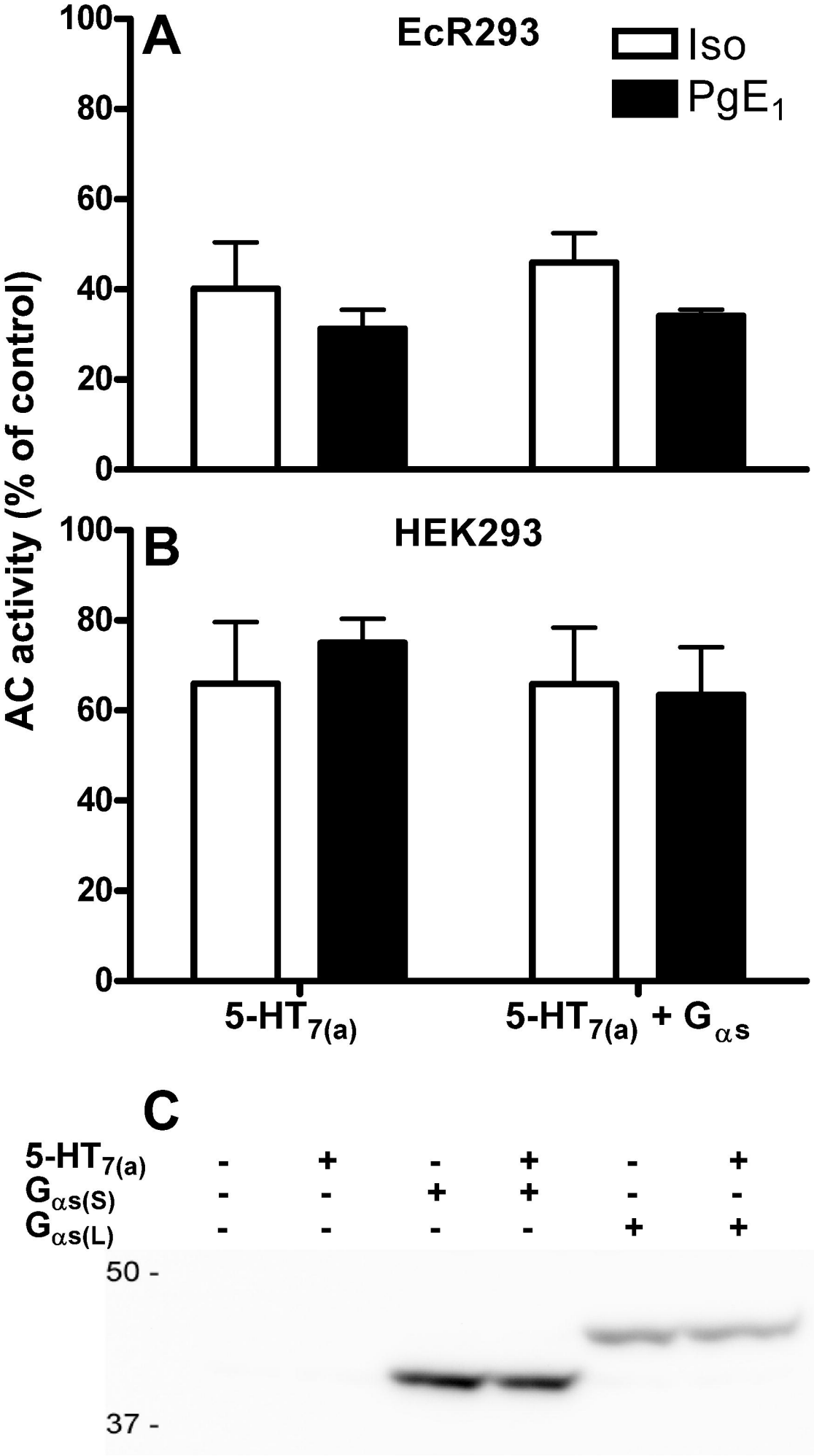


Figure 6

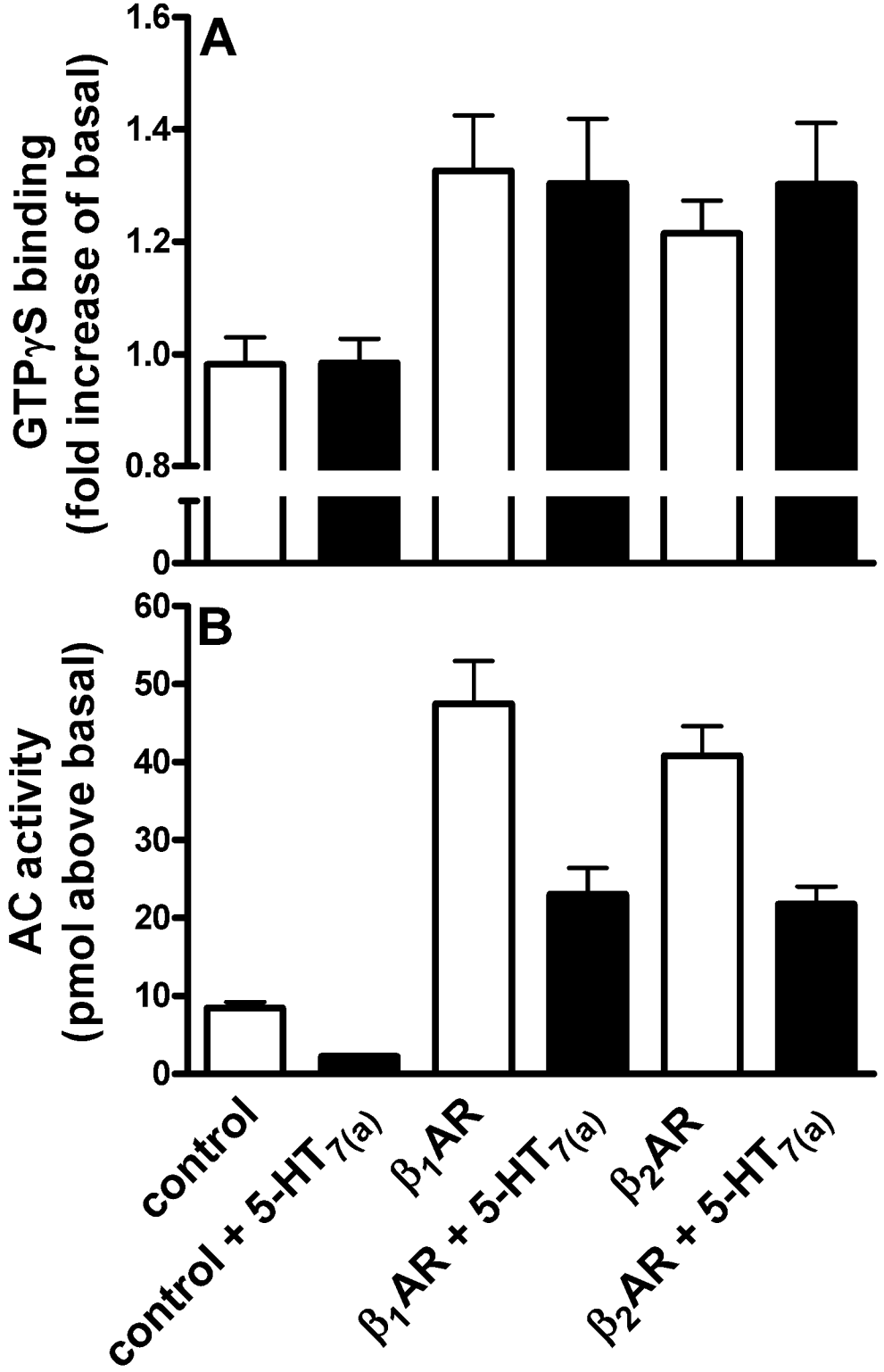
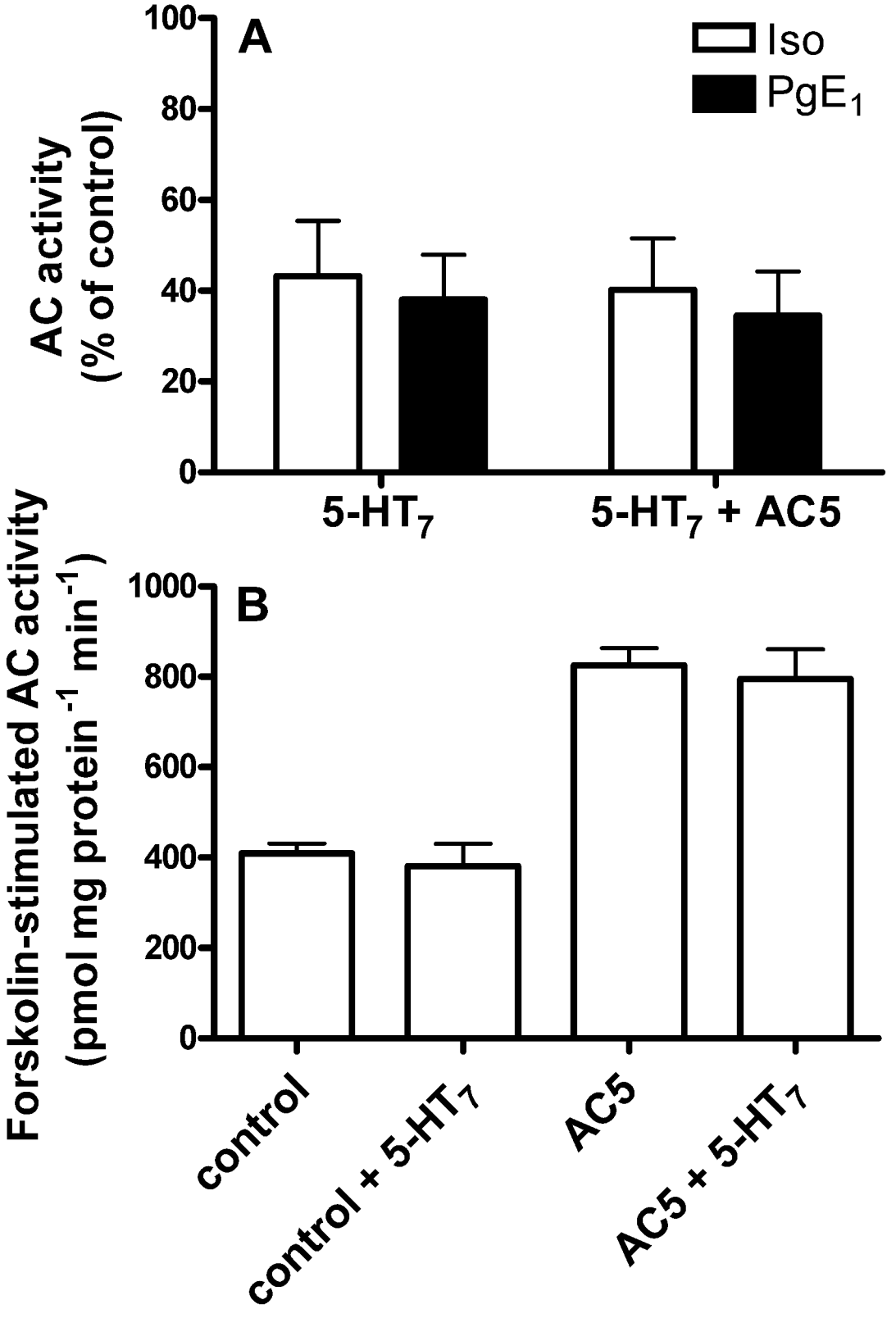

A
GTPγS binding
(fold increase of basal)
1.6
1.4
1.2
1.0
0.8
0
B
AC activity
(pmol above basal)
60
50
40
30
20
10
0
control
control + 5-HT7(a)
β1AR
β1AR + 5-HT7(a)
β2AR
β2AR + 5-HT7(a)

Figure 7


A
Iso
PgE1
AC activity
(% of control)
100
80
60
40
20
0
5-HT7
5-HT7 + AC5
B
Forskolin-stimulated AC activity
(pmol mg protein-1 min-1)
1000
800
600
400
200
0
control
control + 5-HT7
AC5
AC5 + 5-HT7