0026-895X/06/6901-207–215$20.00
MOLECULAR PHARMACOLOGY
Copyright © 2006 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 69:207–215, 2006

Vol. 69, No. 1
15396/3068165
Printed in U.S.A.

Activation of Adenylyl Cyclase by Endogenous G_s-Coupled Receptors in Human Embryonic Kidney 293 Cells Is Attenuated by 5-HT_7 Receptor Expression

Kjetil Wessel Andressen, Jens Henrik Norum, Finn Olav Levy, and Kurt A. Krobert

Department of Pharmacology, University of Oslo, Oslo, Norway

Received May 31, 2005; accepted September 27, 2005

ABSTRACT

Human 5-hydroxytryptamine$_7$ (5-HT_7) receptors display characteristics shared with receptors believed 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 the signaling of other similarly coupled G protein-coupled receptors by limiting their 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 human embryonic kidney (HEK) 293 cells. Using the ecdysone-inducible expression system, which allows for the 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 (EPR) receptors. βAR- and EPR-stimulated AC activity was attenuated by 5-HT_7 receptor expression in both membrane preparations and intact HEK293 cells. βAR- and EPR-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 protein kinase A activation, nor does it occur at the level of G protein activation because 1) βAR- and EPR-stimulated AC activity was not restored to control values when $G\alpha_s$ was overexpressed; and 2) β_1AR and β_2AR activation of $G\alpha_s$ was unaffected by the expression of 5-HT_7 receptors. In addition, overexpression of AC isoforms was unable to rescue βAR- and EPR-stimulated AC activity. Therefore, 5-HT_7 receptors probably 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.

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). The functional significance of 5-HT_7 splice variants, which differ only in the carboxyl 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 carboxyl termini (Bockaert et al., 2004). We have shown previously 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-$HT_{7(a)}$

This work was supported by The Norwegian Council on Cardiovascular Diseases, The Research Council of Norway, The Norwegian Cancer Society, Anders Jahre's Foundation for the Promotion of Science, The Novo Nordisk Foundation, The Family Blix Foundation, and grants from the University of Oslo.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.

doi:10.1124/mol.105.015396.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; AC, adenylyl cyclase; βAR, β-adrenergic receptor; EPR, prostanoid EP receptor; PKA, protein kinase A; H89, *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrobromide; GR113808, {1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl}methyl-1-methyl-1*H*-indole-3-carboxylate; CGP12177, (−)-4-(3-*t*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one; HEK, human embryonic kidney; HA, hemagglutinin; BRL24924, renzapride; 5-CT, 5-carboxamidotryptamine; ICYP, iodocyanopindolol; PgE_1, prostaglandin E_1; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; HRP, horseradish peroxidase; SB269970, (2*R*)-1-[(3-hydroxyphenyl)-sulfonyl]-2-[2-(4-methyl-1-piperidinyl)ethyl]pyrrolidine; SB207266, *N*-((1-butyl-4-piperidinyl)methyl)-3,4-dihydro-2*H*-(1,3)oxazino(3,2-*a*)indole-10-carboxamide; 8-OH-DPAT, 8-hydroxy-2-dipropylaminotetralin; GTPγS, guanosine 5′-[γ-thio]triphosphate; SR141716A, *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide hydrochloride.

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 proposed that properties governing 5-$HT_{7(a)}$ receptor activation of AC are consistent with a model which presumes that the 5-$HT_{7(a)}$ receptors are tightly associated with G protein, independent of agonist binding (Bruheim et al., 2003). For clarity, we use the term "preassociated" for this type of receptor-G protein association (i.e., presumed association of inactive receptor and G protein), whereas we reserve the 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). Although 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, because of 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.

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 an 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 the 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 the 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-$HT_{7(a,\ b,\ and\ d)}$ receptor expression on AC stimulation by the endogenous βAR and prostanoid EP receptors.

Materials and Methods

Materials. Serotonin, (−)isoproterenol, timolol, alprenolol, GDP, guanosine 5′-[γ-thio]triphosphate (GTPγS), and H89 were from Sigma-Aldrich (St. Louis, MO). Methiothepin (metitepine, 1-[10,11-dihydro-8-(methylthio)dibenzo[*b,f*]thiepin-10-yl]-4-methylpiperazine) maleate and 8-hydroxy-2-dipropylaminotetralin (8-OH-DPAT) hydrobromide were from Tocris Cookson Inc. (Bristol, UK). Prostaglandin E_1 was from Cayman Chemical (Ann Arbor, MI). Renzapride (BRL24924) hydrochloride, Zeocin, penicillin-streptomycin, G-418, ponasterone A, LipofectAMINE, and LipofectAMINE 2000 were from Invitrogen (Carlsbad, CA). Forskolin was from Calbiochem (San Diego, CA). Supersignal Dura West was from Pierce Chemical (Rockford, IL). Anti-pRas-GRF1 was from Cell Signaling Technology (Beverly, MA). Anti-HA-probe and Anti-$G\alpha_{s/olf}$ were from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep anti-rabbit IgG-HRP was from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Radiochemicals. [^{3}H]5-Carboxamidotryptamine (5-CT; 60–102 Ci/mmol), [^{3}H]GR113808 (84 Ci/mmol), (−)-3-[^{125}I]iodocyanopindolol (ICYP) (2000 Ci/mmol), [α-^{32}P]ATP (400 Ci/mmol), [2,8-^{3}H]cAMP (30–42 Ci/mmol), [^{3}H]CGP12177 (37 Ci/mmol), [N^6-methyl-^{3}H]mesulergine (87 Ci/mmol), and [γ-^{35}S]GTPγS (1033 Ci/mmol) were from GE Healthcare.

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 manufacturer's 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.

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; Cambrex Bio Science Walkersville, Inc., 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 analog G-418 (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 (American Type Culture Collection, Manassas, VA) were grown in

Dulbecco's modified Eagle's medium (Cambrex Bio Science Walkersville) 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 LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Cells were transfected with the following plasmids: human 5-$HT_{7(a)}$ (Krobert et al., 2001), human β_1AR or β_2AR, AC5, AC6, or AC7 (all in pcDNA3.1; all AC clones were generous gifts from Dr. Dermot M. F. Cooper, Department of Pharmacology, University of Cambridge, Cambridge, UK), human $G\alpha_{s(S)}$ or $G\alpha_{s(L)}$ (both in pcDNA3.1 obtained from the University of Missouri-Rolla cDNA Resource Center, http://www.cdna.org), control vector (pcDNA3.1), or full-length murine HA-Ras-GRF1 wild type (in pKH3 mammalian expression plasmid; 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. Twenty-four hours 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 described previously (Krobert et al., 2001). Radioligand binding assays for 5-HT_7, 5-$HT_{4(b)}$, and βAR were performed with 1.3 to 1.7 nM [^{3}H]5-CT, 0.2 to 0.5 nM [^{3}H]GR113808, or 20 to 100 pM (−)-3-[^{125}I]ICYP, respectively, as described previously (Krobert et al., 2001). B_{max} was estimated as described previously (Krobert et al., 2001) on the basis of 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 described previously (Krobert et al., 2001). Isoproterenol- and prostaglandin E_1 (PgE_1)-stimulated AC activities (performed in triplicates) are reported as the percentage 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 described previously (Clark and Knoll, 2002) with the following modifications: cells were trypsinized, pelleted, and resuspended in UltraCULTURE. Approximately 400,000 cells were plated in each well of a 96-well, round-bottomed microtiter plate and incubated with the hydrophilic compound [^{3}H]CGP12177 (10 nM) for 1 h at 4°C. Nonspecific binding was determined by the 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 per well 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-$HT_{7(a)}$ receptors, respectively) or hydrophilic 5-HT (100 μM). The incubation was carried out for 3 h at 13°C, which allows for equilibrium of ligand binding and inhibits 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 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 h before the experiment in 12-well plates (Falcon; BD Biosciences Discovery Labware, Bedford, MA). Cells were incubated with 0.5 mM 3-isobutyl-1-methylxantine (Sigma-Aldrich) for 10 min and stimulated with isoproterenol, PgE_1, or increasing concentrations of 5-HT for 5 min. The reaction was stopped by the 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 quadruplicate and are reported as stimulated cAMP content relative to cells not induced to express either 5-$HT_{7(a)}$ or 5-$HT_{4(b)}$ receptors (control groups). Increasing concentrations of 5-HT were 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 EC_{50}, and x is the concentration of agonist.

GTPγS Binding Assay Specific for Gα_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 described previously (Cussac et al., 2002). Membranes were preincubated 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 the addition of [^{35}S]GTPγS (0.3 nM in a final volume of 200 μl in 96-well optiplates; PerkinElmer Life and Analytical Sciences, Boston, MA). After 60-min incubation at room temperature, 20 μl of Nonidet P-40 (Sigma-Aldrich) was added (0.27% final concentration), and plates were incubated for 30 min under gentle agitation. Anti-$G\alpha_{s/olf}$ (10 μl; 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 antibodies (GE Healthcare) were added in a volume of 50 μl at a dilution indicated by the manufacturer, and the plates were incubated for 3 h with gentle agitation. The plates were then centrifuged (10 min, 1300g) immediately followed by radioactivity detection in a Topcount microplate scintillation counter (PerkinElmer). Nonspecific 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 with basal G_s activation.

Western Blotting. EcR293 cells inducibly expressing 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, and 50 mM Tris-HCl, pH 7.4, at room temperature), scraped with a Teflon cell scraper, sheared through a 25-gauge syringe, and immediately frozen in liquid N_2. The thawed cell lysates were cleared at 13,000g at 4°C, and the protein concentrations in the supernatants were quantified using the BC assay protein quantitation kit (Uptima, Monticon, France) using bovine serum albumin as a standard. Equal amounts of cell lysate proteins were separated by SDS-polyacrylamide gel electrophoresis (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% nonfat dry milk in phosphate-buffered saline with 0.05% Tween and thereafter incubated with sheep anti-rabbit IgG HRP-conjugated secondary antibody. The immobilized HRP-conjugated secondary antibody was visualized with Supersignal Dura West extended-duration chemiluminescent substrate and analyzed with a BioChemie system (UVP Inc., Upland, CA).

Protein Measurements. Protein concentration was measured with the Micro BC Assay Reagent Kit (Uptima) using bovine serum albumin as a standard.

Statistics. Paired Student's t test was performed using GraphPad Prism 4.00 for Windows (GraphPad Software, Inc., San Diego, CA).

Results

Increasing 5-$HT_{7(a)}$ Receptor Density Does Not Increase 5-HT Potency. We have reported previously 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} value) 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 more than 4-fold (Table 1). These data confirm the absence of a classic spare receptor effect in intact cells and indicate 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, consistent with a model in which 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 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 of protein) and higher (7.9 ± 0.1 pmol/mg of protein) receptor densities tested, eliciting a maximal response of 81 ± 1% and 75 ± 1%, respectively, of that obtained with the full agonist 5-HT (Fig. 1, top graph). At low 5-$HT_{4(b)}$ receptor density (0.72 ± 0.48 pmol/mg of 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 of protein; Fig. 1, bottom 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 accordance with data reported previously; Krobert and Levy, 2002). It is interesting that high 5-$HT_{7(a)}$ receptor density also inhibited 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;

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} value was calculated as described under *Materials and Methods*. Data shown are the mean ± S.E.M. from three (5-HT_7) and four (5-HT_4) experiments.

5-HT_7		5-HT_4	
Receptor Density	pEC_{50}	Receptor Density	pEC_{50}
pmol/mg protein		*pmol/mg protein*	
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*

* $p < 0.05$.

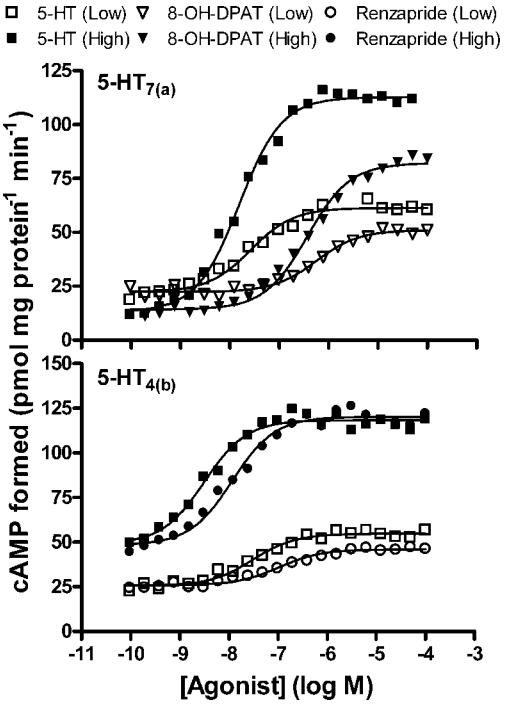

Fig. 1. Partial agonists become full agonists at high receptor densities at 5-HT_4 but not 5-HT_7 receptors. The top graph shows AC activity in response to increasing concentrations of 8-OH-DPAT and 5-HT in membranes from EcR293 cell lines expressing the 5-$HT_{7(a)}$ receptor at low (2.7 pmol/mg of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

Fujino et al., 2002). AC activity stimulated by isoproterenol and PgE_1, 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 femtomoles per milligram of 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 and D), even at receptor densities comparable with 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 nontransfected EcR293 cells with ponasterone 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 coexpressing 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.

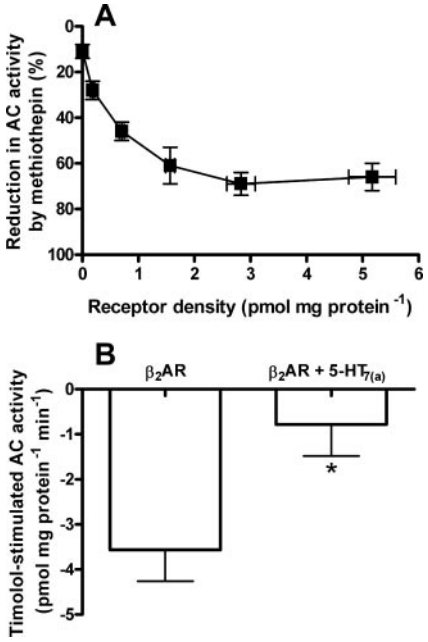

Fig. 2. Increasing 5-HT_7 receptor density increases its contribution to basal AC activity and inhibits constitutive activity of the β_2AR. A, effect of methiothepin (1 μM) on basal AC activity in membrane preparations of EcR293 cells expressing increasing 5-$HT_{7(a)}$ receptor densities. AC activity was measured as described under *Materials and Methods,* and data are presented as the percentage reduction of basal AC activity. Data shown are mean ± S.E.M. 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 ± S.E.M. of nine experiments. β_2AR receptor density was 2.2 ± 1.0 pmol/mg of protein in noninduced and 2.4 ± 1.1 pmol/mg of 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 of protein in noninduced and induced cells, respectively. *, timolol-mediated reduction of basal AC activity was significantly attenuated by 5-$HT_{7(a)}$ receptor expression ($p < 0.05$).

Mechanism of Attenuated Endogenous G_s-Coupled Receptor AC Activation Is Protein Kinase A-Independent. To determine whether 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 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 (Norum et al., 2005) 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 a 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 use 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 $G\alpha_{s(S\text{ or }L)}$ 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 $G\alpha_{s(S\text{ or }L)}$ overexpression in EcR293 cells induced to express 5-$HT_{7(a)}$ receptors (Fig. 5A) or tested by cotransfection of HEK293 cells by 5-$HT_{7(a)}$ and $G\alpha_{s(S\text{ or }L)}$ (Fig. 5B). In both systems, isoproterenol- and PgE_1-stimulated AC activity remained attenuated by 5-HT_7 receptor expression. It is interesting that 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 $G\alpha_s$, whereas 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 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 2-fold (Fig. 7B), indicating that AC5 was properly expressed in the membrane preparations tested. Likewise, overexpression of AC6 (shown previously 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).

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 expressed endogenously 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 of protein (βAR-stimulated AC activity = 79 ± 5% of control, EPR-stimulated AC activity = 83 ± 5% of control, both $p < 0.05$ versus 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 solely caused by receptor overexpression. 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 do not mediate the heterologous desensitization of βAR and EPR. First and foremost, the 5-$HT_{4(b)}$ receptor also constitutively activates AC, because 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 of 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). In addition, 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 and 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

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 under *Materials and Methods*. Data shown are the mean ± S.E.M. from four experiments.

EcR293 Clone	βAR Density		5-HT Receptor Density	
	Noninduced	Induced	Noninduced	Induced
	fmol/mg protein			
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

N.D., not detectable.

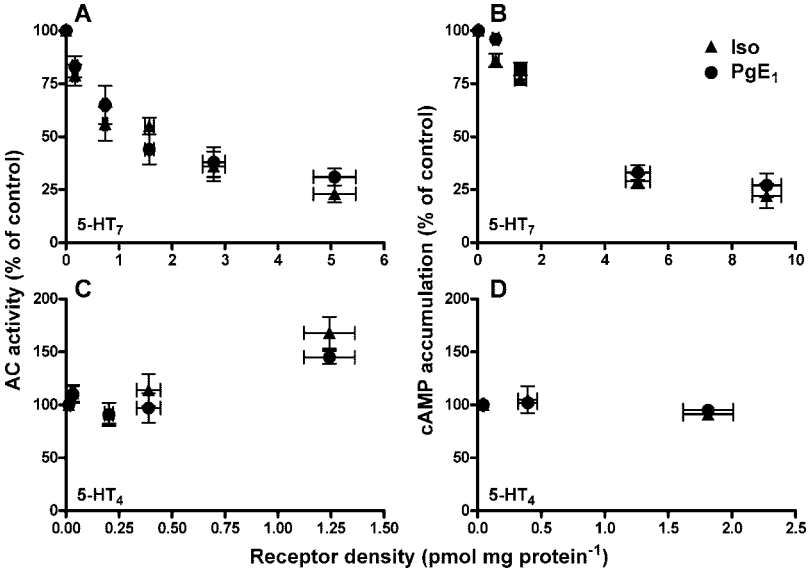

Fig. 3. Expression of 5-HT_7 receptors attenuates endogenous G_s-coupled receptor signaling. The figure shows isoproterenol- (▲, Iso) and PgE_1- (●) (both 10 μM) stimulated AC activity in membranes of EcR293 cells (A and C) or cAMP accumulation in intact EcR293 cells (B and D) induced to express increasing 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 or cAMP accumulation above basal as a percentage 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 because there was no difference between the splice variants. Data are mean ± S.E.M. 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 lines (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).

would be expected if βAR were desensitized through the classic mechanisms.

On the other hand, 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, because of 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 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 $G_{i/o}$. 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, because 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 (Roka et al., 1999; Vasquez and Lewis, 1999; Mukhopadhyay et al., 2000; 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 classic 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 density, 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 ren-

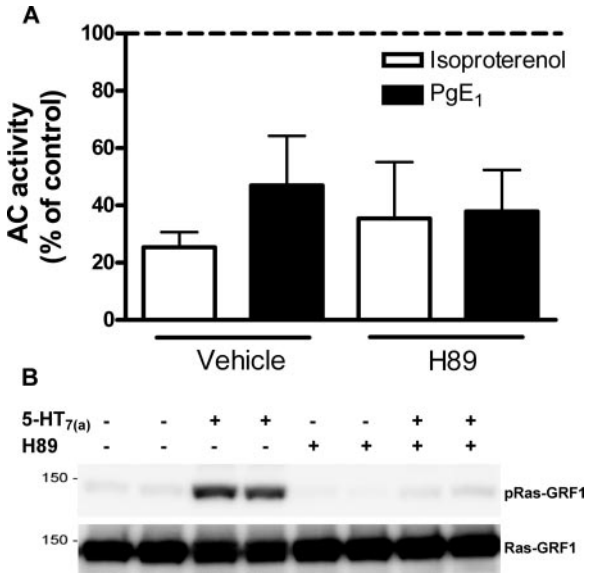

Fig. 4. PKA activation is not the mechanism of attenuation of G_s-coupled receptor responses. A, isoproterenol- or PgE_1-stimulated (both at 10 μM) AC activity in membranes of EcR293 cells induced to express 5-$HT_{7(a)}$ receptors in the presence or absence of the PKA inhibitor H89. EcR293 cells were incubated with either ponasterone A (10 μM, induced group) or vehicle (ethanol, noninduced group; control). Sister plates of induced and noninduced cells were incubated with H89 (20 μM) or vehicle (50% ethanol) for 3 × 8 h (24 h total incubation). Data shown are AC activity in membrane preparations as a percentage of the noninduced group and are mean ± S.E.M. from four experiments. 5-$HT_{7(a)}$ receptor density was 4.4 ± 0.8 pmol/mg of protein in vehicle and 4.2 ± 0.7 pmol/mg of protein in H89-treated groups. B, EcR293 cells were transfected with HA-Ras-GRF1 24 h before inducing 5-$HT_{7(a)}$ receptor expression. During incubation with ponasterone A (10 μM), the cells were coincubated 3 × 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 (top). Total HA-Ras-GRF1 was detected with anti-HA (bottom). The blot shown is representative of three experiments.

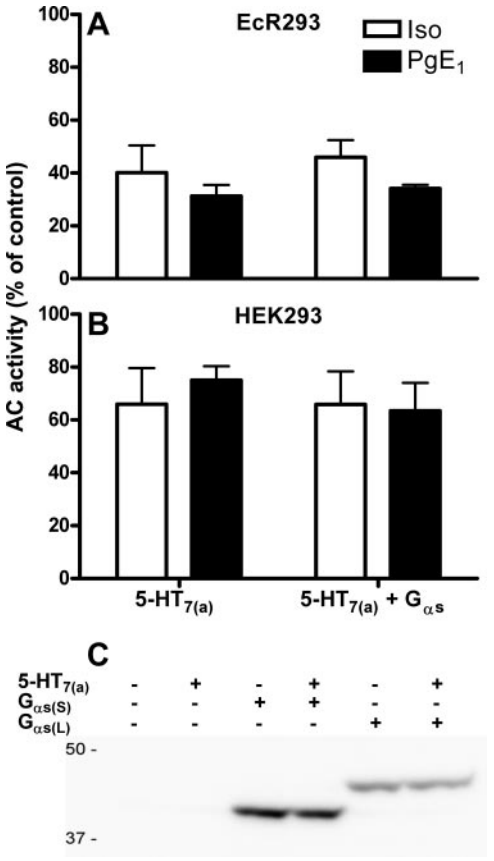

Fig. 5. Overexpression of $G\alpha_s$ does not rescue βAR- or EPR-stimulated AC activity. The figure shows isoproterenol- (Iso) and PgE_1-stimulated AC activity (both at 10 μM) in membranes from EcR293 cells induced to express 5-$HT_{7(a)}$ receptors (A) or HEK293 cells transiently expressing 5-$HT_{7(a)}$ receptors (B) in the presence or absence of transient $G\alpha_{s(S\text{ or }L)}$ overexpression. Data shown are collapsed from experiments with $G\alpha_{s(S)}$ and $G\alpha_{s(L)}$, because no significant differences were noted. A, 24 h before 5-$HT_{7(a)}$ receptor induction by ponasterone A or vehicle, EcR293 cells were transfected with $G\alpha_{s(S\text{ or }L)}$ 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 ± S.E.M. of four experiments and are reported as a percentage of control (noninduced EcR293 cells). B, HEK293 cells were transiently cotransfected with $G\alpha_{s(S\text{ or }L)}$ or control vector (pcDNA3.1) and 5-$HT_{7(a)}$ [or control vector (pcDNA3.1)] and were grown for 48 h before AC assay. Data are mean ± S.E.M. of four experiments and are reported as a percentage 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 of protein in A and B, respectively. C, EcR293 cells transiently transfected with $G\alpha_{s(S\text{ or }L)}$ (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-$G\alpha_{s/olf}$.

zapride 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. From these data, 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. This is consistent with a model in which the inactive conformational state of 5-$HT_{7(a)}$ receptors is 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 to do 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 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, because these mechanisms result in the uncoupling of receptor from G protein. Furthermore, the mechanism used by the 5-HT_7 receptor differs from that of the CB_1 receptor, because 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 proposed previously, 5-HT_7 receptor activation of AC conforms to a model assuming a preassociated signaling complex that includes G protein

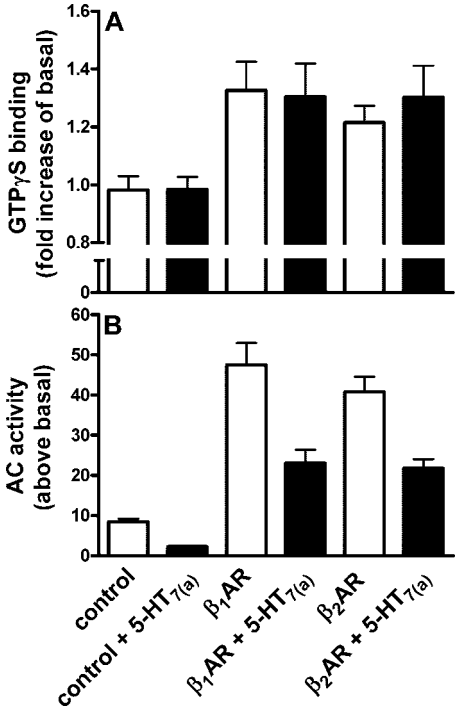

Fig. 6. βAR activation of $G\alpha_s$ is unaffected by 5-HT_7 receptor expression. A, isoproterenol-stimulated (10 μM) [^{35}S]GTPγS binding at $G\alpha_s$ ($G\alpha_s$ was isolated by an antibody capture technique as described under *Materials and Methods*) was assayed in membrane preparations from EcR293 cells induced to express 5-$HT_{7(a)}$ receptors (where indicated) and coexpressing 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 (picomoles per milligram of protein per minute above basal) in the same EcR293 cell membrane preparations as above. Receptor density was 0.37 ± 0.09 and 0.80 ± 0.37 pmol/mg of protein for the β_1AR and β_2AR, respectively, and the presence of 5-$HT_{7(a)}$ receptors (9.1 ± 1.8 pmol/mg of protein) did not alter βAR densities. Data shown are mean ± S.E.M. of four experiments performed in triplicate.

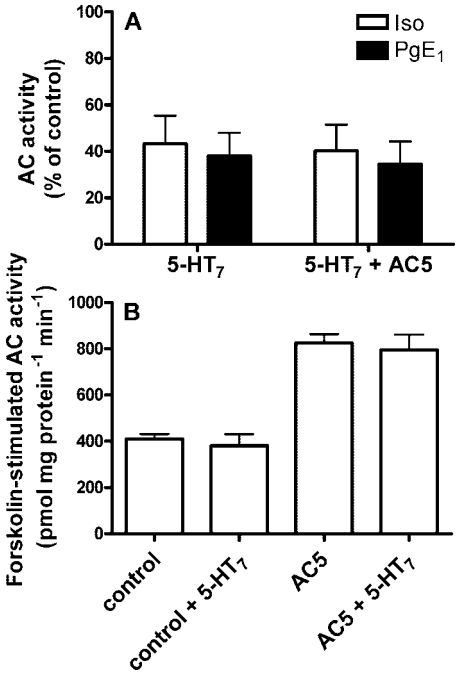

Fig. 7. Overexpression of adenylyl cyclase does not rescue βAR- or EPR-stimulated AC activity. A, isoproterenol- (Iso) and PgE_1-stimulated AC activity (both at 10 μM) in membranes from EcR293 cells induced to express 5-$HT_{7(a\ or\ b)}$ receptors in the presence or absence of transient AC5 overexpression. Data are reported as a percentage of control (noninduced 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 ± S.E.M. of five experiments. 5-HT_7 receptor density was 6.7 ± 2.3 and 6.1 ± 2.1 pmol/mg of protein in induced cells without or with AC5 overexpression, respectively.

and AC (Bruheim et al., 2003). Therefore, the amount of AC available for activation by $G\alpha_s$ may become the limiting component, because 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, because 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 colocalized 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 because of compartmentalization of AC and the receptors into different microdomains. On the other hand, βAR and EPR have access to AC; however, the presence of 5-HT_7 receptors somehow 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.

Acknowledgments

We are grateful to Dr. Dermot M. F. Cooper (University of Cambridge, Cambridge, UK) for providing the plasmids encoding AC5, AC6, and AC7 and Dr. Raymond R. Mattingly (Wayne State University, Detroit, MI) for providing the plasmids encoding Ras-GRF1.

References

Adham N, Zgombick JM, Bard J, and Branchek TA (1998) Functional characterization of the recombinant human 5-hydroxytryptamine$_{7(a)}$ receptor isoform coupled to adenylate cyclase stimulation. *J Pharmacol Exp Ther* **287:**508–514.

Alberts GL, Chio CL, and Im WB (2001) Allosteric modulation of the human 5-HT_{7A} receptor by lipidic amphipathic compounds. *Mol Pharmacol* **60:**1349–1355.

Alousi AA, Jasper JR, Insel PA, and Motulsky HJ (1991) Stoichiometry of receptor-G_s-adenylate cyclase interactions. *FASEB J* **5:**2300–2303.

Baker LP, Nielsen MD, Impey S, Metcalf MA, Poser SW, Chan G, Obrietan K, Hamblin MW, and Storm DR (1998) Stimulation of type 1 and type 8 Ca^{2+}/calmodulin-sensitive adenylyl cyclases by the G_s-coupled 5-hydroxytryptamine subtype 5-HT_{7A} receptor. *J Biol Chem* **273:**17469–17476.

Black JW and Leff P (1983) Operational models of pharmacological agonism. *Proc R Soc Lond B Biol Sci* **220:**141–162.

Bockaert J, Claeysen S, Compan V, and Dumuis A (2004) 5-HT_4 receptors. *Curr Drug Targets CNS Neurol Disord* **3:**39–51.

Bouaboula M, Perrachon S, Milligan L, Canat X, Rinaldi-Carmona M, Portier M, Barth F, Calandra B, Pecceu F, Lupker J, et al. (1997) A selective inverse agonist for central cannabinoid receptor inhibits mitogen-activated protein kinase activation stimulated by insulin or insulin-like growth factor 1. Evidence for a new model of receptor/ligand interactions. *J Biol Chem* **272:**22330–22339.

Bruheim S, Krobert KA, Andressen KW, and Levy FO (2003) Unaltered agonist potency upon inducible 5-$HT_{7(a)}$ but not 5-$HT_{4(b)}$ receptor expression indicates agonist-independent association of 5-$HT_{7(a)}$ receptor and G_s. *Receptors Channels* **9:**107–116.

Chidiac P, Hebert TE, Valiquette M, Dennis M, and Bouvier M (1994) Inverse agonist activity of β-adrenergic antagonists. *Mol Pharmacol* **45:**490–499.

Claeysen S, Sebben M, Becamel C, Bockaert J, and Dumuis A (1999) Novel brain-specific 5-HT_4 receptor splice variants show marked constitutive activity: role of the C-terminal intracellular domain. *Mol Pharmacol* **55:**910–920.

Clark RB and Knoll BJ (2002) Measurement of receptor desensitization and internalization in intact cells. *Methods Enzymol* **343:**506–529.

Cussac D, Newman-Tancredi A, Duqueyroix D, Pasteau V, and Millan MJ (2002) Differential activation of Gq/11 and Gi_3 proteins at 5-hydroxytryptamine$_{2C}$ receptors revealed by antibody capture assays: influence of receptor reserve and relationship to agonist-directed trafficking. *Mol Pharmacol* **62:**578–589.

Fitzsimons CP, Monczor F, Fernandez N, Shayo C, and Davio C (2004) Mepyramine, a histamine H_1 receptor inverse agonist, binds preferentially to a G protein-coupled form of the receptor and sequesters G protein. *J Biol Chem* **279:**34431–34439.

Friedman J, Babu B, and Clark RB (2002) β_2-Adrenergic receptor lacking the cyclic AMP-dependent protein kinase consensus sites fully activates extracellular signal-regulated kinase 1/2 in human embryonic kidney 293 cells: lack of evidence for G_s/G_i switching. *Mol Pharmacol* **62:**1094–1102.

Fujino H, West KA, and Regan JW (2002) Phosphorylation of glycogen synthase kinase-3 and stimulation of T-cell factor signaling following activation of EP_2 and EP_4 prostanoid receptors by prostaglandin E_2. *J Biol Chem* **277:**2614–2619.

Gerald C, Adham N, Kao HT, Olsen MA, Laz TM, Schechter LE, Bard JA, Vaysse PJ, Hartig PR, Branchek TA, et al. (1995) The 5-HT_4 receptor: molecular cloning and pharmacological characterization of two splice variants. *EMBO (Eur Mol Biol Organ) J* **14:**2806–2815.

Guthrie CR, Murray AT, Franklin AA, and Hamblin MW (2005) Differential agonist-mediated internalization of the human 5-hydroxytryptamine 7 receptor isoforms. *J Pharmacol Exp Ther* **313:**1003–1010.

Hausdorff WP, Bouvier M, O'Dowd BF, Irons GP, Caron MG, and Lefkowitz RJ (1989) Phosphorylation sites on two domains of the β_2-adrenergic receptor are involved in distinct pathways of receptor desensitization. *J Biol Chem* **264:**12657–12665.

Heidmann DE, Metcalf MA, Kohen R, and Hamblin MW (1997) Four 5-hydroxytryptamine$_7$ (5-HT_7) receptor isoforms in human and rat produced by alternative splicing: species differences due to altered intron-exon organization. *J Neurochem* **68:**1372–1381.

Hoyer D, Clarke DE, Fozard JR, Hartig PR, Martin GR, Mylecharane EJ, Saxena PR, and Humphrey PPA (1994) International union of pharmacology classification of receptors for 5-hydroxytryptamine (serotonin). *Pharmacol Rev* **46:**157–203.

Krobert KA, Bach T, Syversveen T, Kvingedal AM, and Levy FO (2001) The cloned human 5-HT_7 receptor splice variants: a comparative characterization of their pharmacology, function and distribution. *Naunyn-Schmiedeberg's Arch Pharmacol* **363:**620–632.

Krobert KA and Levy FO (2002) The human 5-HT_7 serotonin receptor splice variants: constitutive activity and inverse agonist effects. *Br J Pharmacol* **135:**1563–1571.

Krupinski J, Lehman TC, Frankenfield CD, Zwaagstra JC, and Watson PA (1992) Molecular diversity in the adenylylcyclase family. Evidence for eight forms of the enzyme and cloning of type VI. *J Biol Chem* **267:**24858–24862.

Levy FO, Zhu X, Kaumann AJ, and Birnbaumer L (1993) Efficacy of β_1-adrenergic receptors is lower than that of β_2-adrenergic receptors. *Proc Natl Acad Sci USA* **90:**10798–10802.

Mattingly RR and Macara IG (1996) Phosphorylation-dependent activation of the Ras-GRF/CDC25^{Mm} exchange factor by muscarinic receptors and G-protein βγ subunits. *Nature (Lond)* **382:**268–272.

Mattingly RR, Sorisky A, Brann MR, and Macara IG (1994) Muscarinic receptors transform NIH 3T3 cells through a Ras-dependent signalling pathway inhibited by the Ras-GTPase-activating protein SH3 domain. *Mol Cell Biol* **14:**7943–7952.

Mukhopadhyay S, McIntosh HH, Houston DB, and Howlett AC (2000) The CB_1 cannabinoid receptor juxtamembrane C-terminal peptide confers activation to specific G proteins in brain. *Mol Pharmacol* **57:**162–170.

Norum JH, Méthi T, Mattingly RR, and Levy FO (2005) Endogenous expression and protein kinase A-dependent phosphorylation of the guanine nucleotide exchange factor Ras-GRF1 in human embryonic kidney 293 cells. *FEBS J* **272:**2304–2316.

Ostrom RS, Violin JD, Coleman S, and Insel PA (2000) Selective enhancement of β-adrenergic receptor signaling by overexpression of adenylyl cyclase type 6: colocalization of receptor and adenylyl cyclase in caveolae of cardiac myocytes. *Mol Pharmacol* **57:**1075–1079.

Post SR, Hilal-Dandan R, Urasawa K, Brunton LL, and Insel PA (1995) Quantification of signalling components and amplification in the β-adrenergic-receptor-adenylate cyclase pathway in isolated adult rat ventricular myocytes. *Biochem J* **311 (Pt 1):**75–80.

Roka F, Brydon L, Waldhoer M, Strosberg AD, Freissmuth M, Jockers R, and Nanoff C (1999) Tight association of the human Mel_{1a}-melatonin receptor and G_i: precoupling and constitutive activity. *Mol Pharmacol* **56:**1014–1024.

Shreeve SM (2002) Identification of G-proteins coupling to the vasoactive intestinal peptide receptor $VPAC_1$ using immunoaffinity chromatography: evidence for precoupling. *Biochem Biophys Res Commun* **290:**1300–1307.

Skomedal T, Grynne B, Osnes JB, Sjetnan AE, and Øye I (1980) A radioimmunoassay for cyclic AMP (cAMP) obtained by acetylation of both unlabeled and labeled (^{3}H-cAMP) ligand, or of unlabeled ligand only. *Acta Pharmacol Toxicol* **46:**200–204.

Thomas DR, Atkinson PJ, Hastie PG, Roberts JC, Middlemiss DN, and Price GW (2002) [^{3}H]-SB-269970 radiolabels 5-HT_7 receptors in rodent, pig and primate brain tissues. *Neuropharmacology* **42:**74–81.

Vasquez C and Lewis DL (1999) The CB1 cannabinoid receptor can sequester G-proteins, making them unavailable to couple to other receptors. *J Neurosci* **19:**9271–9280.

Weiss JM, Morgan PH, Lutz MW, and Kenakin TP (1996) The cubic ternary complex receptor occupancy model. I. Model description. *J Theor Biol* **178:**151–167.

Address correspondence to: Dr. Finn Olav Levy, Department of Pharmacology, University of Oslo, P.O. Box 1057 Blindern, N-0316 Oslo, Norway. E-mail: f.o.levy@medisin.uio.no