Activation of Adenylyl Cyclase by Endogenous Gs-Coupled Receptors in Human Embryonic Kidney 293 Cells Is Attenuated by 5-HT7 Receptor Expression

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ABSTRACT

Human 5-hydroxytryptamine2 (5-HT2) 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 Gq/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-HT7 receptor expression was sufficient to limit signaling of endogenously expressed Gs-coupled receptors in human embryonic kidney (HEK) 293 cells. Using the ec dyscylene-inducible expression system, which allows for the titration of increasing receptor density in the same clonal cell line, we compared the effects of 5-HT4(b) and 5-HT7(a,b,d) receptor expression on adenylyl cyclase (AC) stimulation by the endogenous Gs-coupled β-adrenergic (βAR) and prostanoid EP (EPR) receptors. βAR- and EPR-stimulated AC activity was attenuated by 5-HT7 receptor expression in both membrane preparations and intact HEK293 cells. βAR- and EPR-stimulated AC activity was unaffected by expression of the Gs-coupled 5-HT4 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 Gαs was overexpressed; and 2) β1AR and β2AR activation of Gαs was unaffected by the expression of 5-HT7 receptors. In addition, overexpression of AC isoforms was unable to rescue βAR- and EPR-stimulated AC activity. Therefore, 5-HT7 receptors probably limit access and/or impede activation of AC by βAR and EPR receptors. Although the 5-HT7 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-HT2a,b, 5-HT6, and 5-HT7, are coupled to Gs and at least the 5-HT4 and 5-HT7 receptors are expressed as several different functional splice variants (Gerald et al., 1995; Heidmann et al., 1997). The functional significance of 5-HT7 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-HT4 splice variants, constitutive activation of AC is dependent on the different carboxyl termini (Bockaert et al., 2004). We have shown previously that the 5-HT4(b) and 5-HT7(a) signaling properties differ fundamentally. The potency of 5-HT to stimulate AC increased with increasing receptor density in clones expressing 5-HT4(b), but not 5-HT7(a) receptors, even though 5-HT-stimulated AC activity in clones expressing 5-HT7(a) receptors had reached asymptotic levels (Bruheim et al., 2003). This indicates that potency of 5-HT for stimulation of AC through the 5-HT7(a) receptor is augmented by endogenously produced Gs-coupled receptors in HEK293 cells.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; AC, adenylyl cyclase; βAR, β-adrenergic receptor; EPR, prostanoid EP receptor; PKA, protein kinase A; H89, N-[2-(1-pyrrolidinyl)ethyl]-5-isoquinolinesulfonamide dihydrobromide; GR113808, [1-[(3-hydroxyphenyl)-((1-butyl-4-piperidinyl)methyl)-3,4-dihydro-2-(1,3)oxazino(3,2-)]indole-10-carboxylic acid (IC50); hemagglutinin; BRL24924, renzapride; 5-CT, 5-carboxamidotryptamine; ICYP, iodocyanopindolol; PGE1, prostaglandin E1; HEK, human embryonic kidney; HA, hemagglutinin; BRL24924, renzapride; 5-CT, 5-carboxamidotryptamine; ICYP, iodocyanopindolol; PGE1, prostaglandin E1; PAGIE, polyacrylamide gel electrophoresis; PVDV, polyvinylidene difluoride; HRP, horseradish peroxidase; SB269970, (2R,3S)-1-[(3-hydroxyphenyl)-[2-(4-methyl-1-piperidinyl)]ethyl]-5-isoquinolinesulfonamide dihydrobromide; SB207266, N-(1-butyl-4-piperidinyl) methyl-3,4-dihydro-2H-(1,3)oxazin-3,2-1)isoindole-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-1H-pyrazole-3-carboxamide hydrochloride.
receptor is independent of receptor-Gs stoichiometry. This is likely to be an inherent property of 5-HT7(a) receptor function that distinguishes it from the 5-HT4 Receptors. We proposed that properties governing 5-HT7(a) receptor activation of AC are consistent with a model which presumes that the 5-HT7(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 CB1-cannabinoid receptor (Vasquez and Lewis, 1999; Mukhopadhyay et al., 2000), the Mel1a melatonin receptor (Roka et al., 1999) and the vasoactive intestinal peptide VPAC1 receptor (Shreeve, 2002). Although the CB1 receptor-Gi/o association is sensitive to the destabilizing effect of guanine nucleotides (Mukhopadhyay et al., 2000), high-affinity agonist binding at the Mel1a receptor is resistant to both the destabilizing effect of guanine nucleotides and pertussis toxin (Roka et al., 1999). At the 5-HT7 receptor, two groups have reported that a high proportion of recombinant human 5-HT7(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-HT7 receptor to the destabilizing effect of guanine nucleotides and pertussis toxin (Alberts et al., 2001; Krobert et al., 2001) is another indication the 5-HT7 receptor and Gαi protein form a tight complex.

Expression of the Gi/o-coupled CB1 receptor in superior cervical ganglia attenuated the ability of α2AR and somatostatin receptors to activate Gαi, and it was proposed that the CB1 receptor, because of its preassociation with Gαi, 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 Gαi-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 CB1 receptor-Gi/o complex by the inverse agonist SR141716A inhibited insulin- and insulin-like growth factor 1-mediated activation of mitogen-activated protein kinase through Gαi (Bouaboula et al., 1997). Likewise, purinergic receptor-stimulated Gαq11 activation and subsequent Ca2+ mobilization is attenuated in the presence of the guinea pig histamine H1 receptor inverse agonist mepyramine, presumably by stabilization of an H1 receptor-Gαq11 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-HT7 receptors represented an example of a preassociated Gαi-coupled receptor. Therefore, we tested whether 5-HT7 receptor expression alone was sufficient to limit the signaling of endogenously expressed Gαi-coupled receptors in HEK293 cells. To test this hypothesis, we used the edcsynase-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-HT7(b) and 5-HT7(a, b, and d) receptors on AC stimulation by the endogenous βAR and prostanoid EP receptors.

Materials and Methods

Materials. Serotonin, (-)-isoproterenol, timolol, alprenolol, GDP, guanosine 5’-[(y-thio)triphosphate (GTPγS), and H89 were from Sigma-Aldrich (St. Louis, MO). Methiothepin (metitepine, 1-[10,11-dihydro-8-(methylthio)dibenzo[b,7]thiepin-10-yl]-4-methylpiperazine) maleate and 8-hydroxy-2-dipropylaminotetralin (8-OH-DOAT) hydrobromide were from Tocris Cookson Inc. (Bristol, UK). Prostaglandin E2 was from Cayman Chemical (Ann Arbor, MI). Renzapride (BRL24924) hydrochloride, Zecol, penicillin-streptomycin, G-418, Zeocin, penicillin-streptomycin, and forskolin was from Calbiochem (San Diego, CA). Supersignal Dura West was from Pierce Chemical (Rockford, IL). Anti-phos-GRF1 was from Cell Signaling Technology (Beverly, MA). Anti-HA-probe and Anti-Gαi/αo/αd were from Santa Cruz Biotechnology (Santa Cruz, CA). Sheep anti-rabbit IgG-HRP was from GE Healthcare (Little Chalfont, Buckinghamshire, UK).

Radiochemicals. [3H]5-Cardboximidotryptamine (5-CT; 60–102 Ci/mmol), [3H]H11002 (200 Ci/mmol), [3H]H9262 (60 Ci/mmol), [3H]H9253 (100 Ci/mmol), [3H]H9251 (37 Ci/mmol), [3H]GMP12177 (37 Ci/mmol), [3H]H9253 (87 Ci/mmol), and [3H]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-HT7(b) and 5-HT7(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-HT7(b) and 5-HT7(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 pLd (Invitrogen). EcR293 cells were transfected with plasmid DNA [pld containing human 5-HT7(b) or 5-HT7(d)] using LipofectAMINE (Invitrogen) according to the manufacturer’s protocol.

Human β1 and β2 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-HT7(b) or 5-HT7(a) 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 was transfected 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, Milan, Italy), penicillin (100 U/ml), and streptomycin (100 μg/ml). HEK293 or EcR293 cells inducibly expressing the 5-HT7(a) receptor were transiently transfected with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's protocol. Cells were transfected with the following plasmids: human 5-HT7(a) (Krobert et al., 2001), human βAR or β2AR, 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 Goαs or Goαi (both in pcDNA3.1 obtained from the University of Missouri-Rolla cDNA Resource Center, http://www.cdna.org), control vector (pcDNA3.1), and 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 1-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 poneasterone 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-HT7, 5-HT4(b), and 5-HT3 receptors were performed with 1.3 to 1.7 nM [3H]5-HT-CT, 0.2 to 0.5 nM [3H]GR113808, or 20 to 100 pM (∼3-[125I]ICYP, respectively, as described previously (Krobert et al., 2001). Bmax was estimated as described previously (Krobert et al., 2001) on the basis of a Kd value of 0.31 nM, 21 pM, and 6.8 pM for [3H]5-HT-CT, [3H]GR113808, and (∼3-[125I]ICYP, respectively. Adenylyl cyclase activity was measured and analyzed by determining conversion of [32P]ATP to [32P]cAMP in membranes, as described previously (Krobert et al., 2001). Isoproterenol- and prostaglandin E1 (PE, stimulated-activated cAMP activities (performed in triplicates) are reported as the percentage activity relative to cells not expressing or not induced to express 5-HT7(b) or 5-HT4(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 probe, 1:2000; anti-Gαi/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 membranes were incubated with primary antibodies (anti-phospho-Ras-GRF1, 1:1000; anti-HA-probe, 1:100) where indicated plasmids. Cells were stimulated as indicated, then washed and lysed in ice-cold cell lysis buffer (1% SDS, 1 mM Na2VO4, 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 N2. 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, Monticoon, 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-Gαia/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 (UVIP 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-HT7(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

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 PE2E-stimulated cAMP accumulation were performed in quadruplicate and are reported as stimulated cAMP content relative to cells not induced to express either 5-HT7(a) or 5-HT4(b) receptors (control groups). Increasing concentrations of 5-HT were performed in duplicate, and data were fit to the equation Y = a + (b - a)/(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 Goαi.

Agonist-stimulated Gαi-protein activation was determined in membrane preparations by measuring the stimulation of [35S]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 MgCl2, 100 mM NaCl, and 1 μM GDP. The reaction was started with the addition of [35S]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-Gαia (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, 1300 g) 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 Goαi activation is reported as the fold increase in specific binding compared with basal Goαi activation.

Western Blotting.

EcR293 cells inducibly expressing 5-HT7(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 Na2VO4, 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 N2. 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, Monticoon, 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-Gαia/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 (UVIP Inc., Upland, CA).

Protein Measurements.

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Statistics.

Paired Student’s t test was performed using GraphPad Prism 4.00 for Windows (GraphPad Software, Inc., San Diego, CA).
Partial Agonists Become Full Agonists with Increasing Receptor Density at 5-HT4(b) but Not 5-HT7(a) Receptors. We have proposed that the potency of 5-HT for stimulation of AC through the 5-HT7(a) receptor is independent of receptor-Gs stoichiometry, consistent with a model in which the 5-HT7(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-HT7(a) receptors and G proteins, we determined the efficacy of 8-OH-DPAT and renzapride, agonists at the 5-HT7 and 5-HT4 receptors, respectively, at low and high receptor densities in EcR293 cells. At the 5-HT7(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-HT7(a) receptor density (0.72 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-HT7(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-HT7 Receptor to Basal AC Activity. Assuming a high proportion of 5-HT7 receptors form a stable association with G protein in the absence of agonist, and given the high constitutive activity of 5-HT7 receptors (Krobert and Levy, 2002), it may be hypothesized that 5-HT7 receptors would account for a larger percentage of basal AC activity (constitutive AC activity) at increasing 5-HT7 receptor density. To test this hypothesis, we determined the efficacy of the full inverse agonist methiothepin at increasing 5-HT7(a,b) and 5-HT7(c,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-HT7 receptor density (in accordance with data reported previously; Krobert and Levy, 2002). It is interesting that high 5-HT7(a) receptor density also inhibited β2AR constitutive AC activation (Fig. 2B) in EcR293 cells, revealed by the inverse agonist timolol (Chidiac et al., 1994). These data indicate that the 5-HT7(a) receptor may limit access of the β2AR to G protein.

5-HT7 Receptor Expression Attenuates Endogenous Gs-Coupled Receptor AC Activation. Given that high 5-HT7 receptor density abolished β2AR constitutive AC activation, we next determined whether 5-HT7 receptor expression modified ligand-mediated AC activation by endogenous Gs-coupled receptors. βAR and prostanoid EP receptors (EPR) both couple via Goβγ to activate AC and both are endogenously expressed in HEK293 cells (Friedman et al., 2002;
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Fujino et al., 2002). AC activity stimulated by isoproterenol and PgE1, acting on βAR and EPR, respectively, was attenuated with increasing 5-HT7 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 PgE1-stimulated AC activity was reduced by 5-HT7 density as low as a few hundred femtomoles per milligram of protein and approximately 75% by the highest 5-HT7 receptor densities examined. In contrast, isoproterenol- and PgE1-stimulated AC activity was not reduced in cells expressing the 5-HT7(b) receptor (Fig. 3, C and D), even at receptor densities comparable with those that gave ~50% inhibition by the 5-HT7 receptor in membrane preparations. Rather, high 5-HT7(b) receptor density modestly increased isoproterenol- and PgE1-stimulated AC activity in membrane preparations. Incubation of nontransfected EcR293 cells with onasterone A did not modify either isoproterenol- or PgE1-stimulated AC activity (data not shown).

**β-Adrenoceptor Cell Surface Receptor Density Is Not Modified by 5-HT1 or 5-HT7 Receptor Expression.** The 5-HT7 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-HT7(b) and 5-HT7(a) receptor expression upon cell-surface βAR density. A high percentage of 5-HT7(b) and 5-HT7(a) receptors (64 ± 4 and 70 ± 6, respectively) are on the cell surface of EcR293 cells induced to express high 5-HT7 receptor densities (Table 2). These values are similar to that reported by Guthrie et al. (2005) in HEK293 cells. High-density expression of 5-HT7 or 5-HT7 receptors did not modify the density of endogenous βAR on the cell surface of EcR293 cells (Table 2) or in EcR293 cells expressing transiently transfected βARs (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 Gs-Coupled Receptor AC Activation Is Protein Kinase A-Independent.** To determine whether high constitutive activity of the 5-HT7 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 PgE1-stimulated AC activities in EcR293 cells expressing 5-HT7(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-HT7(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-HT7 Receptors Limit the Ability of Endogenous βAR and Prostanoid EP Receptors to Activate AC but Not Gaα5.** We have proposed that a strong physical (pre)association of the 5-HT7 receptor with Gaα5 in the absence of ligand accounts for the atypical properties of 5-HT7 receptor function. If the endogenous βAR and EPR use the same pool of Gaα5 as 5-HT7 receptors, and 5-HT7 receptors preassociate with Gaα5, access of βAR and EPR to Gaα5 may be impeded as 5-HT7 receptor density increases. To determine whether the amount of Gaα5 was limiting, we overexpressed Gaα5(S or L) protein together with 5-HT7(a) receptors. Overexpression of Gaα5 had no effect on isoproterenol- and PgE1-stimulated AC activities, whether tested by Gaα5(S or L) overexpression in EcR293 cells induced to express 5-HT7(a) receptors (Fig. 5A) or tested by cotransfection of HEK293 cells by 5-HT7(a) and Gaα5(S or L) (Fig. 5B). In both systems, isoproterenol- and PgE1-stimulated AC activity remained attenuated by 5-HT7 receptor expression. It is interesting that isoproterenol-stimulated βARs were able to activate Gaα5 equally well in the presence or absence of 5-HT7 receptors, as revealed by GTPγS binding (Fig. 6A). The fact that βARs are able to activate Gaα5, whereas βAR activation of AC remained attenuated in the presence of 5-HT7(a) receptors (Fig. 6B), suggests that access and/or availability of AC to activated Gaα5 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-HT7(a) receptor in HEK293 cells (Baker et al., 1998). Isoproterenol- and PgE1-stimulated AC activities in EcR293 cells induced to express 5-HT7 receptors were not

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**Fig. 2.** Increasing 5-HT7 receptor density increases its contribution to basal AC activity and inhibits constitutive activity of the β2AR. A. Effect of methiothepin (1 μM) on basal AC activity in membrane preparations of EcR293 cells expressing increasing 5-HT7 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 show mean ± S.E.M. of six experiments from two independent EcR293 clones expressing 5-HT7 receptors. Similar data were obtained with clones expressing the 5-HT7b and the 5-HT7a receptors (data not shown). B. Effect of timolol (10 μM) on basal AC activity in membrane preparations of EcR293 cells transiently expressing β2AR in the presence or absence of 5-HT7 receptors. Data presented are mean ± S.E.M. of nine experiments. β2AR receptor density was 2.2 ± 0.1 pmol/mg of protein in noninduced and 2.4 ± 1.1 pmol/mg of protein in EcR293 cell membranes induced to express 5-HT7a receptors. 5-HT7a 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-HT7a receptor expression (p < 0.05).
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 PgE1-stimulated AC activities (data not shown).

Discussion

The primary finding of this study is that expression of the Gs-coupled 5-HT7 receptor attenuates AC activation by βAR and EPR, Gs-coupled receptors expressed endogenously in both HEK293 cells and EcR293 cells. The presence of 5-HT7 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-HT7 receptor expression. However, βAR and EPR did partially activate AC even at the highest 5-HT7 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-HT7 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 Gs-coupled 5-HT4 receptor under similar high receptor densities and conditions. Therefore, we conclude that this effect is an intrinsic property of 5-HT7 receptors and not solely caused by receptor overexpression. Whereas, similar to our findings, Gs0-coupled CB1 receptor expression attenuated the ability of α2AR and somatostatin receptors to activate downstream effectors of Gi/o (Vasquez and Lewis, 1999); to our knowledge, this would be the first documentation of this effect in Gs-coupled receptors.

Several findings in this study indicate that constitutive activation of AC and subsequent activation of PKA by 5-HT7 receptors do not mediate the heterologous desensitization of βAR and EPR. First and foremost, the 5-HT7 receptor also constitutively activates AC, because the 5-HT7 inverse agonist SB207266 reduced basal cAMP accumulation (51 ± 2% reduction by 10 μM SB207266) in intact EcR293 cells induced to express 5-HT4 receptors (1.54 ± 0.07 pmol/mg of protein, n = 3). Expression of 5-HT7 in numerous cell lines has revealed that 5-HT7 receptors have a high constitutive activity even at low and physiological levels (Bockaert et al., 2004). In addition, mouse 5-HT4 receptors constitutively activate AC in intact COS-7 cells to levels equivalent to the human constitutively active mutant β3AR (Claeysen et al., 1999). Therefore, attenuation of isoproterenol- and PgE1-stimulated AC activity would also be expected in the presence of 5-HT7 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-HT7 receptor did not inhibit isoproterenol- or PgE1-stimulated AC activity. Second, attenuation of the isoproterenol- and PgE1-stimulated AC activity was not affected when 5-HT7 constitutive AC activity was blocked by the presence of 5-HT7 inverse agonists (seven inverse agonists tested: methiothepin, clozapine, metergoline, spiperone, SB269970, methysergide, and mesulergine; data not shown). This indicates that the pool of Gsα contributing to 5-HT7 constitutive activity is not involved in attenuation of βAR- or EPR-stimulated AC activity. Third, isoproterenol- and PgE1-stimulated AC activity was not restored to control values when PKA activity was inhibited by H89 (Fig. 4).

![Fig. 3.](https://example.com/image3.png)

**Fig. 3.** Expression of 5-HT7 receptors attenuates endogenous Gs-coupled receptor signaling. The figure shows isoproterenol- (△, Iso) and PgE1- (○) stimulated AC activity and basal CAMP accumulation in intact EcR293 cells (B and D) induced to express increasing densities of 5-HT7 receptors. All data are expressed as percentage of control (uninduced cells). Total CAMP accumulation was measured after 5-min stimulation with isoproterenol or PgE1, in intact cell experiments. Data shown for 5-HT7, 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-HT7, clonal cell lines for each splice variant (A), seven CAMP accumulation experiments from two 5-HT7, clonal cell lines (B), eight experiments collapsed from two 5-HT7, clonal cell lines (C), or six CAMP accumulation experiments from one 5-HT7, 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 Goi may be impeded by 5-HT7 receptor expression. Vasquez and Lewis (1999) propose that the CB1 receptor, because of its preassociation with Goi 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 Goi-coupled receptors. In support, Vasquez and Lewis (1999) have demonstrated that expression of the Goi-coupled CB1 receptor in superior cervical ganglia attenuated the ability of α2AR and somatostatin receptors to activate Giβ. To presume that 5-HT7 receptors are sequestering and limiting access to G protein, it is a prerequisite to demonstrate that the 5-HT7 receptor is similarly preassociated with Giβ.

In fact, such a basis exists, because 5-HT7 receptors (as opposed to the 5-HT4(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-HT7 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-HT7 receptor is incongruent with the predictions of the operational model of agonism (Black and Leff, 1983), because the 5-HT7 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-HT7 receptor density, whereas the potency of 5-HT increases at high 5-HT receptor density, in accordance with our observations in cell membranes (Bruheim et al., 2003). Furthermore, we show that the efficacy of the 5-HT7 partial agonist 8-OH-DPAT remains unchanged relative to 5-HT at high 5-HT7 receptor densities, extending support for the absence of a spare receptor phenomenon. In contrast, the 5-HT4 partial agonist ren-

**Fig. 4.** PKA activation is not the mechanism of attenuation of Gs-coupled receptor responses. A, isoproterenol- or PgE1-stimulated (both at 10 μM) AC activity in membranes of EcR293 cells induced to express 5-HT7 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 (ethanol) for 3 x 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-HT7 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-HT7 receptor expression. During incubation with ponasterone A (10 μM), the cells were incubated 3 x 8 h with 20 μM H89 or vehicle. After the 24-h incubation period, cells were stimulated with 10 μM 5-HT or vehicle for 5 min, lysed, and proteins were separated on a 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 Goa, does not rescue βAR- or EPR-stimulated AC activity. The figure shows isoproterenol- (Iso) and PgE1-stimulated AC activity (both at 10 μM) in membranes from EcR293 cells induced to express 5-HT7 receptors (A) or HEK293 cells transiently expressing 5-HT7 receptors (B) in the presence or absence of transient Goa or control Goa (C). AC activity was assayed 24 h after induction of 5-HT7 receptor induction by ponasterone A or vehicle, EcR293 cells were transfected with Wt or control (pcDNA3.1). AC activity was assayed 24 h after induction of 5-HT7 receptor expression, 48 h after transfection of Goa. Data are mean ± S.E.M. of four experiments and are reported as a percentage of control (noninduced EcR293 cells). C, HEK293 cells were transiently cotransfected with Goa or control vector (pcDNA3.1) and 5-HT7 receptors (where indicated) 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). Data are mean ± S.E.M. from four experiments and are reported as a percentage of control (HEK293 cells).
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-HT7(a) receptor is independent of receptor-Gs stoichiometry. This is consistent with a model in which the inactive conformational state of 5-HT7(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-HT4(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-HT7 from the 5-HT4(b) and may be related to the ability of 5-HT7 receptors to attenuate signaling through other Gs-coupled receptors. It is well established that 5-HT7 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 Gs-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-HT7 receptor and Gs protein.

Although the property of preassociation between the 5-HT7 receptor and Gs protein may contribute to the attenuation of isoproterenol- and Pge1-stimulated AC activity, it is unlikely to do so by sequestering or limiting access to a common Gs pool for the following reasons: 1) overexpression of Gαs in the presence of high 5-HT7 receptor density did not restore isoproterenol- and Pge1-stimulated AC activity to control levels (Fig. 5); 2) β1- and β2AR activation of Gαs is unaffected by high expression of 5-HT7 receptors (Fig. 6), indicating that βAR can access, couple to, and activate a pool of Gαs. 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-HT7-mediated effect, because these mechanisms result in the uncoupling of receptor from G protein. Furthermore, the mechanism used by the 5-HT7 receptor differs from that of the CB1 receptor, because overexpression of Gαo rescued the ability of the endogenous α2AR and somatostatin receptors to activate Gαo (Vasquez and Lewis, 1999). The fact that β1- and β2AR activation of AC remains attenuated (Fig. 6B), even though they can activate Gαs (Fig. 6A), indicates that the 5-HT7 receptor in some way limits access to or impedes activation of AC directly. As we have proposed previously, 5-HT7 receptor activation of AC conforms to a model assuming a preassociated signaling complex that includes G protein.

![Fig. 6. βAR activation of Gαs is unaffected by 5-HT7 receptor expression.](image)

![Fig. 7. Overexpression of adenylyl cyclase does not rescue βAR- or EPR-stimulated AC activity.](image)
and AC (Bruheim et al., 2003). Therefore, the amount of AC available for activation by Goα 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-HT7 receptors; Krupinski et al., 1992; Baker et al., 1998) did not rescue isoprotrenol- or PgE2-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-HT7 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-HT7 receptor expression.

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References


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