RNA Editing Induces Variation in Desensitization and Trafficking of 5-Hydroxytryptamine 2c Receptor Isoforms*

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The 5-hydroxytryptamine2c receptor (5-HT2cR) is subjected to RNA editing, in the second intracellular loop, generating 14 different isoforms in human brain. This post-transcriptional event markedly alters the signaling properties of the receptor by reducing its ability to couple to G-proteins. Although the non-edited form of the receptor is essentially fully constitutively active, edited forms show lesser degrees of constitutive activity. We have used two extensively edited receptor isoforms, VGV and VSV, and the non-edited INI isoform to investigate how variations in constitutive receptor activity affect the trafficking and the interaction of these isoforms with components of the desensitization machinery in HEK 293 cells. We found that cell surface expression of the 5-HT2cR decreased in parallel with increased constitutive activity of the isoforms. The subcellular distribution of the various isoforms was dependent of their ability to interact with βarrestin2, which correlated with the constitutive activity level of each isoform. We observed that the agonist-independent interaction of βarrestin2 with constitutively active 5-HT2cR isoforms was reversed by inverse agonist treatments promoting receptor redistribution to the cell surface. Overexpression of a G-protein-coupled receptor kinase (GRK2) was able to stabilize the interaction of βarrestin2 with constitutively active 5-HT2cR isoforms even in the presence of inverse agonists. Taken together, our observations indicate that the constitutively active 5-HT2cR isoforms are spontaneously internalized in an agonist-independent manner. This endocytosis process is mediated by a GRK/βarrestin-dependent mechanism and is directly correlated with the constitutive activity status of the RNAedited receptor variants. Thus the ultimate physiological output of constitutively active receptors may be determined not only by their agonist-independent activity but also by their interactions with GRKs and βarrestin.

Following the well established paradigm, agonist-bound G-protein-coupled receptors (GPCRs) become substrates for G-protein-coupled receptor kinases (GRKs) that mediate phosphorylation, the agonist-occupied receptor is bound with high affinity by arrestin proteins (1–3). Arrestin binding un couples the receptor from its cognate G-protein (desensitization) resulting in receptor endocytosis mediated by clathrin-coated pits (4, 5). Internalized receptor can then be recycled back to the plasma membrane with highly variable kinetics (resensitization) or be degraded (down-regulation) or both. However, beyond this classic receptor theory, it has been found that some receptors can exhibit constitutive activity, defined as a specific molecular conformation of the receptor that is able to signal in an agonist-independent fashion (6–8). Most known spontaneously constitutively active GPCRs are constitutively phosphorylated, including the bradykinin B2 receptor (9, 10), the metabotropic glutamate receptor mGluR1 (11), the peripher al cannabinoid receptor (CB2) (12), the human cytomegalo virus US28 receptor (13), as well as the 5-hydroxytryptamine2c receptor (5-HT2cR) (14).

Serotonin 2c (previously 5-HT1c) receptors belong to the rhodopsin-like family of GPCRs and interact with Goαi to stimulate phospholipase Cβ (PLCβ) (15). The 5-HT2cR is known to exist naturally as several distinct protein isoforms secondary to RNA editing (16, 17). RNA editing is mediated by adenosine deaminases that act on double-stranded RNA to convert up to five adenosines residues to inosines residues within the pre-mRNA of the 5-HT2cR, creating non-synonymous changes in amino acids 156, 158, and 160 (16, 18). Interestingly, RNA editing takes place in the second intracellular loop region of the 5-HT2cR, starting two residues downstream of the DRY (Asp-Arg-Tyr) motif that is highly conserved among the GPCR family. This motif is suggested to play a critical role in agonist-induced receptor activation. Indeed, mutations of the aspartate residue in the DRY motif have been found to lead to constitutively active mutants of many GPCRs (19–22). The unedited version of the 5-HT2cR displays a large degree of constitutive activity essentially being unresponsive to further agonist stimulation. Importantly, RNA editing of the 5-HT2cR has been shown to create receptor isoforms that display lesser degrees of constitutive activity as induced by severely reducing the G-protein coupling efficiency (23).

Among the 14 5-HT2cR isoforms expressed in human brain, we have focused our study on three isoforms, the fully edited 5-HT2cR-VGV (Val156-Gly158-Val160) isoform displays the slowest level of constitutive activity and is essentially silent, β-arrestin; 5-HT, 5-hydroxytryptamine; 5-HT2cR, 5-hydroxytryptamine2c receptor; GFP, green fluorescent protein; YFP, yellow fluorescent protein; INI, Heteractis crispa red fluorescent protein; GRK, G-protein-coupled receptor kinase; PLC, phospholipase C; HA, hemagglutinin; MEM, minimal essential medium; ELISA, enzyme-linked immunosorbent assay.

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constitutive activity of the receptor could lead to an agonist-independent endocytosis of serotonin receptors (Palcon) with 5 μg of receptor and 0.8 μg of βarrestin2-GFP or βarrestin1-GFP using a calcium phosphate co-precipitation method (32).

**RESULTS**

Co-immunoprecipitation—100-mm plates of HEK 293 cells were co-transfected with FLAG-5-HT2cR, βarr2-GFP, or βarr1-GFP and have shown that GRK2 can mediate desensitization of the 5-HT2cR (26, 27). However, whether the differential intrinsic signaling properties of the various 5-HT2cR isoforms may influence or be mediated by interaction with βarrestins and subsequently influence their relative cellular localization have not yet been examined. In this report, we have used these three edited variants of the human 5-HT2cR to investigate how various levels of constitutive activity, resulting from naturally occurring amino acids modifications, influence the cellular distribution of these GPCRs. Furthermore, we examined if the 5HT2cR internalization was βarr2-dependent and to what extent constitutive activity of the receptor could lead to an agonist-independent recruitment of the βarrestins. Thus, these various isoforms allow us to address the issue of whether constitutively active receptors are also constitutively desensitized and internalized.

**EXPERIMENTAL PROCEDURES**

**Materials**—Collagen solution, anti-FLAG M2-HRP antibody, anti-FLAG M2-agarose, 5-hydroxtryptamine hydrochloride, mesulergine hydrochloride N-[(1a,6-dimethylergolin-8-yl)N,N-dimethylsulfamide hydrochloride, SB260553 (N-3-pyridinyl-3,5-dihydro-5-methylbenzo[1,2-b:4,5-b]dipryrole-12H]carboxamide) hydrochloride, brefeldin A, and o-phenylenediamine dihydrochloride were obtained from Sigma-Aldrich Co. (St. Louis, MO). Living Colors A.v. peptide polyclonal antibody against the green fluorescent protein (GFP) was from BD Biosciences Clontech (Palo Alto, CA). Texas-Red transferrin was purchased from Molecular Probes (Eugene, OR). Cell culture reagents were obtained from Life Sciences. Monoclonal antibody against actin was from Chemicon International Inc. (Temecula, CA).

**Plasmid Constructs**—Construction of plasmids βarr2-2-GFP, βarr1-GFP, βarr2/GFP dominant negative mutant V54D, GRK2, and GRK2 dominant negative mutant [K220R] have been described previously (24). FLAG-tagged 5-HT2c receptor isoforms were constructed via polymerase chain reaction using a proofreading polymerase (Pfu, Stratagene). The following primers were used: βarr2-5, ACTGGAGCTCAAATGGGTGAAAAACCCGGAC, and βarr2-3, AGTGTGACCTCAAGTTGAGAACCCGGGAC; and βarr3-5, ACTGGTGACCTCAGAAGTTGCGTACACTAGTGC, and βarr3-3, CACACACTTACAGCAGTTACCTCCTCC. The PCR products were cloned into the Sacl-KpnI sites of pHCl-Red-N1 (Clontech). The Golgi-YFP expression vector labeling the trans-Golgi network was from Clontech. The CDNA vector encoding the Hα-tagged human Rab11a was a gift from SSG Ferguson. CDNA vectors for the 5-HT2cR-1N1, 5-HT2cR-VSV, and 5-HT2cR-VGV isoforms of the 5-HT2c receptor have been described previously (24). FLAG-tagged 5-HT2c receptor isoforms were constructed via polymerase chain reaction using a proofreading polymerase (Pfu, Stratagene). The following primers were used: βarr2-5, ATATGGTACATGTGGTTGTGAGAAGCCGGGAC, and βarr2-3, AGTGTGACCTCAAGTTGAGAACCCGGGAC; and βarr3-5, ACTGGTGACCTCAGAAGTTGCGTACACTAGTGC, and βarr3-3, CACACACTTACAGCAGTTACCTCCTCC. The PCR products were cloned into the Sacl-KpnI sites of pHCl-Red-N1 (Clontech). The sequences were confirmed using an automated ABI DNA sequencer.

**Cell Culture and Transfection**—HEK 293 cells were grown in Eagle’s medium minimum essential medium with Earle’s salt (MEM) supplemented with 10% (v/v) fetal bovine serum and a 1:100 dilution of gentamicin reagent solution (Invitrogen). Cells were transiently transfected in collagen-coated 35-mm glass bottom dishes (MatTek, Ashland, MA). After 16 h, the medium was replaced with serum-free MEM and cells were starved for 5 h. Then HEK 293 cells were stimulated with 10 μM serotonin or 1 μM SB260553. Confocal microscopy was performed at 37 °C using a heated microscope stage at 100× magnification with a Zeiss laser-scanning microscope (LSM-510). Images were collected sequentially using single line excitation (488 nm) for GFP-labeled βarrestins or GFP-labeled 5-HT2cR. A double line excitation (488 and 568 nm) was used for detection of both GFP-tagged proteins and Texas Red conjugated transferrin and βarr2-RedFP. For detection of both GFP- and YFP-tagged proteins a broad excitation spectra (488–590 nm) was applied, and a deconvolution process was used to discriminate the respective emission spectra.

**Sequestration Assays**—Measurement of receptor surface expression by ELISA was performed under non-permeabilized conditions as follows. HEK 293 cells plated at a density of 3×10⁶ per 100-mm dish were transfected or not with FLAG-5-HT2cR cDNA augmented with empty vector CDNA to a total or 7 μg using a calcium phosphate protocol (32). The cells were trypsinized 18 h later and plated at a density of 125,000 cells per well in a 24-well plate and incubated in minimal essential media (MEM) containing 10% fetal bovine serum. For 48 h, the cells were serum-starved for 5 h before treatment with 10 μM serotonin or 1 μM SB260553 with or without 5 μM brefeldin A. After 30 or 60 min at 37 °C in a 5% CO₂ incubator the cells were washed out in ice-cold PBS and incubated in 1% bovine serum albumin in PBS for 30 min on ice. The cells were incubated on ice for 1 h with 4 μg/ml anti-FLAG M2-HRP antibody and wash three times with ice-cold 1% bovine serum albumin in PBS before being fixed in 3.7% paraformaldehyde in PBS for 15 min. The media was removed, and 750 μl of o-phenylenediamine dihydrochloride was added to each well. The reaction was stopped by 100 μl of HCl (3 N) before measurement of absorbance at 492 nm with a SmartSpec™3000 spectrophotometer (Bio-Rad Laboratories).

**Transferrin Uptake**—Transferrin uptake was carried out as described previously (33). Briefly, HEK 293 transfected cells, grown on collagen-coated 35-mm glass bottom dishes, were incubated for 1 h before transferrin treatment. Texas Red-conjugated transferrin was added to the cells to a final concentration of 55 μg/ml and incubated at 37 °C in a 5% CO₂ incubator. After 30 min the living cells were washed twice with warm MEM containing 10 μM serotonin before microscopy observation at 37 °C.

**Statistical Analysis**—Data are expressed as mean ± S.E. from the indicated number of experiments performed in triplicate. Statistical comparisons were performed using an unpaired Student’s t test.

**RESULTS**

**Cellular Localization and Trafficking of 5-HT2cR GFP-tagged Isoforms**—First we asked whether the RNA-editing process could affect the subcellular distribution of the three 5-HT2cR isoforms. For that purpose, the 5-HT2cR isoforms were tagged at the C terminus with the enhanced GFP and
transiently expressed in HEK 293 cells. In the absence of agonist, the 5-HT2cR-VGV-GFP isoform was expressed exclusively at the cell surface (Fig. 1A). After 7 min of serotonin stimulation at a saturating concentration (10 μM), the 5-HT2cR-VSV-GFP isoform underwent internalization and was clearly detected in intracellular vesicles (Fig. 1A). In contrast to the VSV isoform, the 5-HT2cR-INI-GFP chimera was detected mainly in large intracellular vesicles, but a faint signal could also be observed at the plasma membrane in a majority of cells (Fig. 1C, left panel). The agonist-independent internalization observed here most probably reflects the constitutive activity of this isoform. Stimulation of the 5-HT2cR-INI-GFP with serotonin did not induce any noticeable variation of the vesicular distribution of this GFP-tagged isoform, suggesting that the agonist can induce no further internalization of this isoform (Fig. 1C, middle panel). However, after treatment with the 5-HT2cR inverse agonist SB206553 (34) (1 μM) for 30 min at 37 °C, the 5-HT2cR-INI-GFP was largely detected at the plasma membrane suggesting recycling of the receptor (Fig. 1C, right panel). With respect to the 5-HT2cR-VSV-GFP isoform, we observed a cellular distribution intermediate between the lowest and the highest constitutively active 5-HT2cR-GFP isoforms. This isoform was detected mainly at the plasma membrane and at a lower degree in intracellular vesicles. Moreover, in response to serotonin stimulation, it was further internalized, whereas treatment with inverse agonist enhanced its plasma membrane localization (Fig. 1B).

Quantification of 5-HT2cR Isoform Cell Surface Expression—We next sought to quantify, by ELISA, the respective proportion of each N terminus FLAG-tagged 5-HT2cR isoforms internalized in the presence of agonist or recycled back to the plasma membrane in the presence of inverse agonist. First, we measured the cell surface expression of the FLAG-5-HT2cR-VSV-GFP isoform transiently expressed in HEK 293 cells. After 30 min of 10 μM serotonin stimulation we observed a 38% decrease of FLAG-5-HT2cR-VSV cell surface expression (Fig. 2A). No significant variation of cell surface receptor expression was observed in cells treated with 1 μM SB206553 for 30 min. These findings suggest that the FLAG-5-HT2cR-VSV isoform was expressed at the cell surface and internalized upon agonist stimulation. We observed that the agonist and inverse agonist treatments for 30 min at 37 °C of cells expressing the FLAG-5-HT2cR-VSV resulted in a 29% decrease but a 1.8-fold increase of cell surface expression of this isoform respectively, suggesting that serotonin can induce internalization of cell surface receptors but that not all the receptors are expressed at the plasma membrane in the absence of stimulation (Fig. 2B). Regarding the cellular distribution of the FLAG-5-HT2cR-INI isoform transiently expressed in HEK 293 cells no significant agonist effects were observed (data not shown). Moreover, 30 and 60 min of 1 μM SB206553 exposure, resulted in a 2-fold and a 2.5-fold increase of cell surface receptor expression, respectively (Fig. 2C). These results suggest a dynamic recycling of intracellular receptor pools to the plasma membrane, and small differences of cell surface receptor detected between 30 and 60 min of inverse agonist treatment indicates that most of the internalized receptors were recycled back to the membrane after 30 min. Altogether, these findings confirm our cellular visualization data regarding the intracellular trafficking of the 5-HT2cR-GFP isoforms and indicate that RNA editing induces a progressive loss of constitutive endocytosis of the unedited 5-HT2cR isoform.
Agonist-independent Endocytosis of Serotonin Receptors

Investigation of βarr2 Translocation to the 5-HT2cR Isoforms—Next we examined whether the 5-HT2cR could induce a cellular relocalization of βarr2s. To assess this hypothesis, we used HEK 293 cells transiently co-transfected with βarr2-GFP or βarr1-GFP chimeras and each of the three 5-HT2cR isoforms. In cells expressing the 5-HT2cR-VSV isoform, which best responds to agonist, the βarr2-GFP was found in the cytosol. However, a slight punctated signal could still be detected at the plasma membrane indicating a minor degree of constitutive activity of this isoform and highlighting the sensitivity of the βarr2 translocation assay. After serotonin stimulation, the βarr2-GFP was recruited to the plasma membrane (Fig. 3A, upper panels). By contrast, in cells expressing the constitutively active 5-HT2cR-INI isoform, the βarr2-GFP was localized at the plasma membrane as well as on large vesicles, suggesting a constitutive βarr2 recruitment and receptor internalization (Fig. 3A, lower panels). As expected, stimulation of this isoform with serotonin did not promote any apparent changes in the βarr2-GFP distribution. In cells transiently transfected with the partially responsive 5-HT2cR-VSV isoform, the distribution of the βarr2-GFP was similar to that observed with the 5-HT2cR-INI isoform, but recruitment of the βarr2-GFP from the cytosol to the plasma membrane can be further enhanced in the presence of serotonin (Fig. 3A, middle panels). Interestingly, when either the 5-HT2cR-VSV or -INI isoform was expressed, βarr1-GFP was mostly distributed in the cytoplasm. Furthermore, under identical experimental conditions, βarr1-GFP did not appear to redistribute upon serotonin stimulation (Fig. 3B). Nonetheless, in some cells a faint punctated βarr1-GFP signal was detected at the plasma membrane, in the absence of agonist, regardless of the 5-HT2cR isoform expressed. Among the tested isoforms, the 5-HT2cR-VGV was the only one capable of inducing a slight βarr1-GFP translocation in response of agonist stimulation (Fig. 3B). Altogether, these results suggest that βarrs are minimally interacting with the 5-HT2cR ISMs.

Characterization of 5-HT2cR-INI-containing Vesicles—To investigate whether the constitutively activated 5-HT2cR-INI isoform traffic with βarrs, their relative cellular distributions were studied in HEK 293 cells by confocal fluorescence microscopy using a GFP-tagged 5-HT2cR-INI isoform and an Hc-Red-tagged βarr2. We observed that 5-HT2cR-INI-GFP and βarr2-HcRed fully co-localized in large vesicles in absence of agonist (Fig. 4A, upper panels). To ensure that this phenomenon was reflecting a continuous endocytosis-recycling cycle of the constitutively active receptor, we used Texas Red-labeled transferrin, which is well characterized as a marker for recycling compartments (35, 36). We observed that the βarr2-GFP as well as the 5-HT2cR-INI-GFP co-localized with transferrin-containing vesicles (Fig. 4, B and C). To identify the intracellular compartment containing the 5-HT2cR-INI, we compared the distribution pattern of the 5-HT2cR-INI-GFP and HA-tagged rab11a, a well established marker of the pericentriolar recycling endosome (37). We observed that most of the 5-HT2cR-INI-GFP co-localize with rab11a in absence of stimulation (Fig. 5A). In addition, this receptor also co-localized partially with the early endosome marker rab5-YFP (data not shown). Altogether, these data suggest that the 5-HT2cR-INI-βarr2 complexes are present in the same endosomal vesicles and that the 5-HT2cR-INI isoform undergoes constitutive endocytosis.

We next asked if the inverse agonist-induced plasma membrane redistribution of constitutively active 5-HT2cR-INI isoform (Figs. 1 and 2) was the result of the mobilization of endosomal pool of receptors or from newly synthesized receptors. We compared the relative cellular distributions of vesicles containing the 5-HT2cR-INI isoform and the Golgi membranes in HEK 293 cells by confocal fluorescence microscopy. We observed no co-localization between the 5-HT2cR-INI-GFP and the YFP-tagged Golgi protein (Golgi-YFP) (Fig. 5B, upper panels) or between the βarr2-GFP and the Golgi-YFP in the presence of the 5-HT2cR-INI (Fig. 5B, lower panels). We also investigated, by ELISA, whether brefeldin A, a commonly used inhibitor of protein secretion (38), would influence the cell surface expression of the FLAG-5-HT2cR-INI isoform (Fig. 2C). After 1 μM SB206553 treatment for 30 min we observed no significant impact of the brefeldin A (5 μM) on the FLAG-5-HT2cR-INI cell surface redistribution (Fig. 5C). However, control experiments demonstrated the disassembly of the Golgi complex after treatment with brefeldin A (5 μM) (Fig. 5D).
These findings suggest that the constitutively active 5-HT2cR-INI isoform was mobilized from endosomal compartments back to the cell surface upon inverse agonist treatment and that this phenomenon was independent of the secretion of newly synthesized 5-HT2cR-INI isoform.

**Effect of Inverse Agonists on**
**Arrrestin-2 Cellular Trafficking with the 5-HT2cR Isoforms**—Based on the observation that βarr2-GFP was spontaneously translocated in cells transiently transfected with constitutively active 5-HT2cR isoforms, we hypothesized that treatment with an inverse agonist would result in the cytosolic redistribution of βarr2-GFP. Indeed, we have observed that 7-min treatment with a saturating concentration (1 μM) of SB206553 fully reversed the constitutive translocation of the βarr2-GFP co-expressed with the 5-HT2cR-VSV or -INI isoforms, as the βarr2-GFP was almost completely redistributed from a vesicular-plasma membrane localization to the cytosol (Fig. 6, A and B, upper panels). We also observed that 1 μM mesulergine, another 5-HT2cR inverse agonist, was able to reverse the βarr2-GFP membrane distribution induced by the constitutively active 5-HT2cR-INI isoform (data not shown). In line with this, we observed that the spontaneous association of βarr2-GFP with the constitutively active 5-HT2cR isoforms, assessed by co-immunoprecipitation, was reversed by inverse agonist treatment (Fig. 6, E and F). These results suggest that the inverse agonist binding induces a conformational change of the receptor isoforms that are no longer capable of interacting with βarrrestin2.

**Effect of GRK2 on**
**Arrrestin-2 Cellular Trafficking with the 5-HT2cR Isoforms**—It is well established that for most GPCRs, βarrrestin recruitment is agonist-dependent but significantly enhanced by GRK (39, 40). Furthermore, GRK2 has been shown to impair 5-HT2cR-mediated PLC activation (26, 27). Consequently, we next investigated whether GRK2 overexpression could modulate βarr2-GFP translocation in cells expressing the 5-HT2cR isoforms. In the absence of receptor activation, βarr2-GFP was evenly distributed throughout the cytoplasm of HEK 293 cells co-expressing the 5-HT2cR-VGV isoform (Fig. 6A, upper panel). However, we observed that GRK2 overexpression led to a basal enhancement of the membrane localization of βarr2-GFP in cells co-expressing this isoform (Fig. 6A, lower panels). Co-immunoprecipitation experiments established that the basal interaction of βarr2-GFP with the FLAG-5-HT2cR-VGV isoform was enhanced by 1.9-fold in the presence of GRK2 expression (Fig. 6D). We found that GRK2 overexpression not only induced a more robust membrane localization of the βarr2-GFP in cells transfected by the 5-HT2cR-VSV isoform but also seems to delay, without totally preventing, the βarr2-GFP cytoplasmic redistribution in the presence of inverse agonist (Fig. 6B, lower panels). On the other hand, GRK2 overexpression totally prevented the inverse agonist-mediated βarr2-GFP cytosolic redistribution in cells expressing the 5-HT2cR-INI isoform, indicating an increased affinity of βarr2-GFP for this receptor (Fig. 6C, lower panels). This observation was corroborated by co-immunoprecipitation of the FLAG-tagged 5-HT2cR-INI isoform with the βarr2-GFP in the presence of inverse agonist (Fig. 6E, left panel). In line with this, co-expression of a dominant negative GRK2, GRK2[K220R], resulted in a significant cytosolic redistribution of βarr2-GFP (Fig. 6F) and greatly impaired the co-immunoprecipitation of βarr2-GFP with the FLAG-tagged 5-HT2cR-INI isoform in the absence of inverse agonist (Fig. 6E, right panel), whereas GRK2 co-expression did not seem to induce any obvious change (Fig. 6, E [left panel] and F). These results are consistent with a previous study showing the impact of GRK2[K220R] on the 5-HT2cR inositol phosphate signaling (27) and suggest that desensitization of 5-HT2cR isoforms leading to βarr2 recruitment can be mediated by GRK2.

**Fig. 3.** 5-HT2cR mediated translocation of βarr2-GFP and βarr1-GFP. Fluorescence confocal microscope images of live HEK 293 cells that were transiently transfected with 5-HT2cR-VGV, -VSV, or -INI isoforms (5 μg) and βarr2-GFP (0.8 μg) (A) or βarr1-GFP (0.8 μg) chimeras (B) are shown. The distribution of βarr2-GFP and βarr1-GFP fluorescence was visualized with the confocal microscope before (0 min) and after (7 min) treatment with 10 μM serotonin. The confocal images shown are representative of three independent experiments. Bar, 10 μm.
GRK2 Dominant Negative Effect on 5-HT2cR Isoforms Cell Surface Expression—Based on the observation that GRK2 and GRK2 dominant negative K220R overexpression were able to modulate the interaction 5-HT2cR/βarr2, we have examined the effect of GRK2[K220R] on the cell surface expression of the two 5-HT2cR isoforms (VGV and INI), which display the most radically different phenotypes. When GRK2[K220R] was co-expressed, we noticed that the FLAG-5-HT2cR-VGV remained at the cell surface even after 30 min of 10 μM serotonin stimulation indicating that the endocytosis of the FLAG-5-HT2cR-VGV isoform was prevented by the GRK2[K220R] expression (Fig. 7A). In cells transfected with the FLAG-5-HT2cR-INI we noticed that GRK2[K220R] co-expression promoted a 2.2-fold increase in cell surface receptor expression in the absence of stimulation (Fig 7B) similar to what was observed with the inverse agonist (Fig. 2C). Representative confocal images of the cellular distribution of the 5-HT2cR-INI GFP in the presence or absence of GRK[K220R] confirm that the receptor internalization was markedly reduced by this dominant negative mutant (Fig. 7C). We also assessed the impact of the βarr2 dominant negative, βarr2[V54D], which is known to inhibit arrestin-dependent GPCR internalization presumably by interfering with receptor recruitment to clathrin-coated pits (5). We found that βarr2[V54D] co-expression induced a 2-fold increase of the cell surface distribution of the constitutively active FLAG-5-HT2cR-INI isoform (Fig. 7B). The co-expression of the βarrestin and GRK2 mutants did not noticeably affect the amount of receptors expressed under these conditions as determined by immunodetection of the FLAG-tagged receptor by Western blotting (data not shown). These results demonstrate that GRK2[K220R] is able to prevent both the agonist-dependent and constitutive endocytosis of the 5-HT2cR isoforms and that βarrestin is required for constitutive endocytosis of the FLAG-5-HT2cR-INI isoform.

DISCUSSION

In this report, we took advantage of naturally occurring amino acid modifications in the second intracellular loop of the 5-HT2cR to investigate the cellular distribution of fully responsive versus constitutive active isoforms of a GPCR. We used these isoforms to assess if constitutively active isoforms could be spontaneously desensitized by the GRK2/βarrestins system.

We provide evidence that the fully edited 5-HT2cR-VGV isoform, which displays the lowest level of constitutive activity, was fully expressed at the cell surface under basal conditions and was rapidly internalized (5–10 min) in the presence of...
agonist. In contrast, the 5-HT2cR-VSV and -INI isoforms were constitutively internalized proportionally to their level of constitutive activity. The constitutively activated 5-HT2cR-INI was mainly detected in endocytic vesicles, suggesting that the rate of constitutive endocytosis of this isoform was faster than its recycling rate, allowing the endosomal accumulation of the 5-HT2cR-INI. The 5-HT2cR-VSV, which displays an intermediate level of constitutive activity, was detected both at the cell surface and in intracellular vesicles. This distribution suggests an intermediate level of constitutive endocytosis resulting in equilibrium between cell surface and internalized receptors.

Most GPCRs are internalized by arrestin-dependent mechanisms, where the ability of arrestins to dissociate from a sequestered receptor dictates the rate at which the receptor is resensitized and recycled back to the cell surface (41). We observed that, in correlation with their degree of constitutive activity, the 5-HT2cR isoforms are internalized by an arrestin2-dependent mechanism and that 5-HT2cR inverse agonist treatment causes the cytoplasmic redistribution of βarr2-GFP and the concomitant externalization of the constitutively activated 5-HT2cR isoforms to the plasma membrane. Furthermore, the βarr2 dominant negative βarr2[V54D] expression also induced the surface redistribution of the 5-HT2cR-INI. These observations suggest that 5-HT2cRs are internalized by a βarr2-dependent mechanism and that inverse agonists either induce the dissociation of 5-HT2cR/βarr2 complexes, resulting in the depletion of intracellular pool of constitutively activated receptors, or prevent the recycled receptors from being constitutively phosphorylated and rapidly internalized, or both. The results of βarr2-GFP translocation to the various 5-HT2cR isoforms also suggest that, regardless of the isoform, βarr2 has a higher affinity for these receptors than βarr1. We observed that the 5-HT2cR-VGV isoform was the only isoform capable of recruiting βarr1-GFP. The apparent lack of interactions of the VSV and INI isoforms with βarr1 may actually reflect the fact that these isoforms must already be in complexes with endogenous βarr2, and βarr1-GFP does not have sufficient affinity to
Fig. 6. Effect of SB206553 and overexpressed GRK2 on redistribution of βarr2-GFP to the 5-HT2cRs in HEK 293 cells. The effect of overexpressed GRK2 on the inverse agonist-mediated cytosolic redistribution of βarr2-GFP from the 5-HT2cR isoforms in HEK 293 cells is shown. Cells were transiently transfected with 5-HT2cR-VGV, -VSV, or -INI (5 μg) and βarr2-GFP (0.8 μg) with or without overexpressed GRK2 (0.8 μg) before and after 7 min of 1 μM SB206553 treatment. A, βarr2-GFP translocation to the 5-HT2cR-VGV isoform in cells overexpressing GRK2 was more robust but still can be reversed by the inverse agonist. B, GRK2 overexpression did not modify the distribution of βarr2-GFP in cells.
expressing the 5-HT2cR-VSV isoform but impaired the inverse agonist effect. C, the cytosolic redistribution of βarr2-GFP from the constitutively active 5-HT2cR-INI isoform induced by the inverse agonist is shown in the upper panels. Lower panels illustrate that this phenomenon was impaired in cells containing overexpressed GRK2. D, the impact of GRK2 overexpression on the co-immunoprecipitation of βarr2-GFP with the FLAG-tagged 5-HT2cR-VSV isoform is shown. E, co-immunoprecipitation of βarr2-GFP with the FLAG-tagged 5-HT2cR-INI in HEK 293 cells. In the left panel, the basal interaction of βarr2-GFP with the 5-HT2cR-INI (lane 2) was reversed by 1 μM SB206553 for 30 min at 37 °C (lane 3) but not when GRK2 was overexpressed (lane 4). In the right panel, the overexpression of the GRK2 dominant negative [K220R] (lane 3) impairs the basal interaction of the βarr2-GFP with the 5-HT2cR-INI (lane 2). The Western blots shown are representative of three independent experiments. F, impact of GRK2 (upper image) or GRK2[K220R] (lower image) overexpression on the βarr2-GFP distribution is shown in cells expressing the 5-HT2cR-INI isoform. Bar, 10 μm.

The results suggest that RNA editing taking place two residues downstream of the DRY motif present in the second intracellular loop of the 5-HT2cR may directly influence βarrestin affinity and selectivity. This hypothesis is supported by the fact that an Arg to Gly mutant in the DRY motif of the N-formyl peptide receptor was no longer able to bind G-protein nor the βarrestin2, even if it did become phosphorylated in response to agonist (42). These data suggest that the highly conserved DRY motif is important for phosphorylation-dependent arrestin binding as well as G-protein activation.

βarrestins are thought to bind with high affinity to agonist-activated GPCRs that have been phosphorylated by GRKs. In the present study, we demonstrate that the basal recruitment of βarr2-GFP to the 5-HT2cR-VSV isoform can be enhanced by GRK2 overexpression, most likely due to the low level of constitutive activity of this isoform. Moreover, GRK2 overexpression totally prevents the 5-HT2cR-INI constitutive activity of this isoform. Moreover, GRK2 overexpression, most likely due to the low level of constitutive activity of this isoform. This hypothesis is supported by the fact that an Arg to Gly mutant in the DRY motif of the N-formyl peptide receptor was no longer able to bind G-protein nor the βarrestin2, even if it did become phosphorylated in response to agonist (42). These data suggest that the highly conserved DRY motif is important for phosphorylation-dependent arrestin binding as well as G-protein activation.

Taken together, these results suggest that the efficiency of interaction of βarr2-GFP with the 5-HT2cR-INI was enhanced by GRK2-mediated phosphorylation of the receptor. These findings also imply that GRK2 was able to recognize the constitutively active conformation of the 5-HT2cR isoforms in the absence of agonist.

Although RNA editing has been shown to greatly affect the signaling properties of the 5-HT2cR, we report that it also dictates the cellular distribution of the receptor and directly influences the interaction of the receptor with the components of the desensitization process. In addition, our data suggest that this RNA editing, which takes place just after the DRY motif, is important for the differential interaction of βarr2-GFP with the 5-HT2cR isoforms. Nevertheless, the amino acid modifications in the second intracellular loop are likely not the only

The upper panels illustrate that the phenomenon was impaired in cells containing overexpressed GRK2. D, the impact of GRK2 overexpression on the co-immunoprecipitation of βarr2-GFP with the FLAG-tagged 5-HT2cR-VSV isoform is shown. E, co-immunoprecipitation of βarr2-GFP with the FLAG-tagged 5-HT2cR-INI in HEK 293 cells. In the left panel, the basal interaction of βarr2-GFP with the 5-HT2cR-INI (lane 2) was reversed by 1 μM SB206553 for 30 min at 37 °C (lane 3) but not when GRK2 was overexpressed (lane 4). In the right panel, the overexpression of the GRK2 dominant negative [K220R] (lane 3) impairs the basal interaction of the βarr2-GFP with the 5-HT2cR-INI (lane 2). The Western blots shown are representative of three independent experiments. F, impact of GRK2 (upper image) or GRK2[K220R] (lower image) overexpression on the βarr2-GFP distribution is shown in cells expressing the 5-HT2cR-INI isoform. Bar, 10 μm.
elements regulating the recruitment of βarr2-GFP, because the GRK2 overexpression was capable of irreversibly blocking the 5-HT2cR-INI-βarr2-GFP interaction. This is supported by the constitutive activation of the 5-HT2cR resulting from the tyrosine mutation in the NPXXY motif present at the end of the transmembrane segment VII (43). Interestingly, the GRK2 effect described in this study supports the hypothesis that GRK can recognize the GPCR-activated status independently of agonist binding.

In conclusion, this study provides insights on the molecular determinants regulating the spontaneously desensitization and endocytosis of a constitutively active GPCR. Identification of such mechanisms is important, because a variety of human diseases results from naturally occurring mutations leading to constitutively activeGPCRs, such as retinitis pigmentosa (rhodopsin mutants) (44), hyperthyroidism (thyrotropin receptor mutants) (45), and suggest that novel pharmacological approaches designed to modulate these processes may find utility in treating such disorders.

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