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Uncoupling and Endocytosis of 5-Hydroxytryptamine 4 Receptors

DISTINCT MOLECULAR EVENTS WITH DIFFERENT GRK2 REQUIREMENTS*

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The 5-hydroxytryptamine type 4 receptors (5-HT₄Rs) are involved in memory, cognition, feeding, respiratory control, and gastrointestinal motility through activation of a Gₛ/cAMP pathway. We have shown that 5-HT₄R undergoes rapid and profound homologous uncoupling in neurons. However, no significant uncoupling was observed in COS-7 or HEK293 cells, which expressed either no or a weak concentration of GRK2, respectively. High expression of GRK2 in neurons is likely to be the uncoupling for this difference because overexpression of GRK2 in COS-7 and HEK293 cells reproduced rapid and profound uncoupling of 5-HT₄R. We have also shown, for the first time, that GRK2 requirements for uncoupling and endocytosis were very different. Indeed, β-arrestin/dynamin-dependent endocytosis was observed in HEK293 cells without any need of GRK2 overexpression. In addition to this difference, uncoupling and β-arrestin/dynamin-dependent endocytosis were mediated through distinct mechanisms. Neither uncoupling nor β-arrestin/dynamin-dependent endocytosis required the serine and threonine residues localized within the specific C-terminal domains of the 5-HT₄R splice variants. In contrast, a cluster of serines and threonines, common to all variants, was an absolute requirement for β-arrestin/dynamin-dependent receptor endocytosis, but not for receptor uncoupling. Furthermore, β-arrestin/dynamin-dependent endocytosis and uncoupling were dependent on and independent of GRK2 kinase activity, respectively. These results clearly demonstrate that the uncoupling and endocytosis of 5-HT₄R require different GRK2 concentrations and involve distinct molecular events.

5-Hydroxytryptamine 4 receptors (5-HT₄Rs)¹ are widely expressed in the brain and at the periphery (1, 2). They are implicated in important physiological functions such as memory, cognition, feeding, respiratory control, and gastrointestinal motility (3–6). Among the G protein-coupled receptor (GPCR) genes, the 5-HT₄R gene is one of the largest (700 kb) (7, 8). To date, nine variants have been cloned in human, four in mouse, and three in rat. All of these variants differ at their C termini after a single position (Leu³⁵⁸). They share different sets of Ser and Thr residues in their specific domains and a common cluster of six Ser and Thr residues upstream of the splice site. All are putative sites for G protein receptor kinase (GRK)-mediated phosphorylation. 5-HT₄Rs are coupled to the Gₛ/cAMP/protein kinase A pathway (9). Other signal transduction pathways have also been reported, but mainly in heterologously transfected cells (2, 10, 11).

Twelve years ago, before the 5-HT₄R was cloned, we reported that activation of 5-HT₄R in colliculus neurons is followed by strong and rapid homologous desensitization unaffected by cAMP (12). Since this early work, very little additional information has been learned about the molecular mechanisms involved in the desensitization of this important receptor. Recently, Ponimaskin et al. (13) showed that 5-HT₄Rs are phosphorylated when heterologously expressed in insect cells. This phosphorylation is modulated by palmitoylation of Cys residues present in the C-terminal domain and is unrelated to the weak desensitization observed when the receptor is transfected in COS-7 cells.

GPCR activity represents a coordinated balance between molecular mechanisms governing receptor signaling, desensitization, and resensitization (14). The homologous desensitization of GPCR signaling is a multistep process, classically described by four main events (15–20). In the first step, GPCR is uncoupled from the G proteins following stimulation of the receptor by the agonist, resulting in attenuation of the primary response (i.e. second messenger production or channel regulation). In most cases, this process, often called “desensitization,” involves GRKs. However, in some cases, the kinase activity of GRKs is not required (21–25). Receptor/G protein uncoupling is due not only to phosphorylation of the receptor per se, but also to binding of β-arrestins (26–28). The second step is “endocytosis” of the receptor (29), which is often, but not always, β-arrestin-dependent (17, 30). The molecular characteristics involved in the interaction between the receptor and β-arrestin determine the particularities of endocytosis, intracellular trafficking, recycling, and resensitization (third step) or degradation (fourth step; also called “down-regulation”) (20, 31–34).

GPCRs have been divided into two classes based on their...
aptitude to bind β-arrestin (33). Class A receptors (e.g. μ-opioid, β2- and α1B-adrenergic, and dopamine D1 receptors) are defined by the presence of scattered Ser/Thr residues, rapid dissociation of arrestins, and rapid recycling back to the cell surface. Class B receptors (e.g. substance P, angiotensin 1a, and vasopressin-2 receptors) are defined by the presence of clustered Ser/Thr residues, formation of a stable complex with arrestins, and predominant targeting to lysosomes for degradation (down-regulation) (33). Although this model may be applied to many GPCR/arrestin interactions, there are exceptions. For example, a recent study by Tulipano et al. (35) showed that somatostatin 2a receptors belonging to class B, characterized by stable association with β-arrestin, are rapidly resensitized and recycled back to the plasma membrane, unlike other class B receptors.

Molecular processes for GPCR desensitization are pleiotropic and generally receptor-specific. Understandably, studies have been performed in heterologous cell lines because, unlike neuronal cultures, these systems are easily transfected and analyzed in molecular and biochemical terms. However, as recently reviewed, the signaling pathways of a given receptor are highly dependent on the cell line or even the subclone used (36, 37). The terminal domains, and full uncoupling could be obtained by

**EXPERIMENTAL PROCEDURES**

**Plasmids—**Plasmids pRK5-GRK2 and pRK5-GRK6 (where DN is dominant-negative) were kindly provided by Dr. S. Cotecchia (Faculté de Médecine, Université de Lausanne, Lausanne, Switzerland). Plasmid pRK5-GRK5 (constructed in the laboratory of Dr. R. J. Lefkowitz (Duke University Medical Center, Durham, NC)) was kindly provided by Dr. J. Pitcher (University College, London, UK). Plasmids pcDNA3-1-GRK4, pcDNA3-1-GRK6, and pcDNA3-1-β-arrestin-2-YFP were generously provided by Dr. M. Bouvier (University of Montreal, Montreal, Canada). pcDNA3-2-β-arrestin (319–418) was generated in the laboratory of Dr. J. L. Benovic (Duke University Medical Center, Durham, NC) was kindly provided by Dr. M. Bouvier. Plasmid pcDNA3-1-DN-dynamin (K44A) (generated by Dr. J. Pitcher (University College, London, UK)).

**Construction of Tagged 5-HT4R cDNAs—**Hemagglutinin (HA)-tagged 5-HT4R cDNAs in pRK5 were generated by Dr. M. A. McNiven (Mayo Clinic, Rochester, MN) and Rho-tagged 5-HT4R constructs were described previously (10). Briefly, constructs Δ546 and Δ358 were obtained by inserting a stop codon after residue 327, 346, or 358 in the 5-HT4R cDNA sequence using the QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands). The same kit was used to replace Ser or Thr residues between positions 347 and 355 with alanine residues to generate the Δ358A construct.

**Antibodies—**Anti-GRK4/5/6 monoclonal antibody was generated in the laboratory of Dr. R. J. Lefkowitz (41), who generously provided it. Anti-GRK2/3 polyclonal antibody was purchased from Tebu, Le Perray en Yvelines, France. Rabbit anti-β-arrestin antibody SG77 was purchased from CliniSciences, Montrouge, France. Mouse anti-c-Myc antibody 9E10 was a gift from Dr. B. Mouillak (Institut de Génomique Fonctionnelle, Montpellier, France). Mouse anti-Rho tag antibody was provided by Dr. S. Collins (Institut de Recherche en Biologie Humaine du CNRS, Brussels, Belgium) (40). Alexa Fluor 594-labeled secondary antibody was purchased from Invitrogen, Cergy-Pontoise, France. Horseradish peroxidase-conjugated anti-rabbit and anti-mouse antibodies were from Amersham Biosciences (Orsay, France).

**RNA Preparation and Real-time Quantitative Reverse Transcription-PCR—**Total RNA was extracted from colliculus neurons in primary cultures (33) and transfected with DNase I from a DNA-free™ kit (Ambion, Cambs, UK) according to the manufacturer’s instructions. RNA was then used to perform two-step reverse transcription-PCR. Briefly, 1 μg of DNA-free total RNA was reverse-transcribed using 200 units of Moloney murine leukemia virus reverse transcriptase (Invitrogen) in the presence of 2.5 μM random primers and 0.5 mM dNTP. The resulting cDNA (20 ng) was used as a template for real-time PCR using ABI PRISM 7000 with SYBR® Green PCR Master Mix, Applied Biosystems (Orsay, France). Standard curves were performed for each 5-HT4R variant by serial dilutions of the corresponding cDNA. Primers were designed with PrimerExpress™ software (Applied Biosystems). The sequences of all primers used are provided in Supplemental Table 1. PCR was performed in 10 μl in the presence of 300 nM specific primers. Thermal cycling parameters were 2 min at 50 °C and 1 min at 95 °C, followed by 40 cycles for 5 s at 95 °C and 1 min at 60 °C. Data were analyzed with ABI PRISM 7000 SDS software, and the threshold cycle (Ct) was calculated from each amplification plot. Standard curves (Ct value (y) versus log copy number (x)) were used to calculate the relative input amount of mRNA for each variant based on the Ct value (42). Results are expressed as a percentage of the most abundant variant (5-HT4A). Data are the means ± S.D. of duplicate determinations and are representative of three independent experiments.

**Cell Cultures and Transfection—**Primary cultures of colliculus neurons were prepared as described previously (43). Briefly, cells dissociated from the colliculi of 14–15-day-old Swiss mouse embryos were plated in serum-free medium in 12-well culture dishes (0.8 × 106 cells/dish). Cultures were maintained for 6 days at 37 °C in a humidified atmosphere of 5% CO2, 95% H2O, and air. Colliculus neurons were nucleofected using a mouse neuron Nucleofector™ kit (Axama Biosystems, Koenl, Germany). One Nucleofection sample contains 5 × 106 cells, 3 μg of HA-tagged or Rho-tagged 5-HT4R cDNA in pRK5, and 100 μl of mouse neuron Nucleofector solution according to the manufacturer's instructions. COS-7 and HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% dialyzed fetal calf serum and antibiotics. They were transfected to 60–70% confluence by electroporation as described previously (10). Colliculus neurons and transfected COS-7 and HEK293 cells were processed for subsequent experiments: desensitization, immunofluorescence, Western blotting, and cell-surface enzyme-linked immunosorbent assay (ELISA).

**Determination of cAMP Production in Transfected Cells—**COS-7 or HEK293 cells were transfected with the appropriate cDNA and plated in 24-well plates (70,000 cells/well). Twenty-four hours post-transfection, cells were harvested, and cell lysates were subjected to a luciferase assay (ECL™, Amersham Pharmacia, Little Chalfont, UK). The amount of luciferase activity was proportional to the amount of cAMP produced.
Uncoupling of 5-HT₄R-stimulated cAMP Production in Colliculus Neurons—To analyze receptor desensitization, neurons endogenously expressing 5-HT₄Rs (prepared as described above) were preincubated with 10 μM 5-HT for the indicated time periods (0–2 h). After three washes with culture medium, cAMP accumulation was started by addition of culture medium containing 5-HT (30 μM), isobutylmethylxanthine (1 mM), and forskolin (0.1 μM), and the accumulation was measured for 5 min. Quantification of cAMP production was performed as described under “Determination of cAMP Production in Transfected Cells.”

Uncoupling of 5-HT₄R-stimulated cAMP Production in the Heterologous System—COS-7 or HEK293 cells were transfected with the indicated plasmid constructs (wild-type (WT) or mutant) either alone or in combination with GRKs as indicated in the figure legends. Cells were seeded at 0.5 × 10⁶ cells/ml on a 24-well plate 1 day prior to the experiment. To analyze receptor uncoupling, cells expressing 5-HT₄R in the presence or absence of GRKs were incubated with 5-HT (10 μM) for different time periods (uncoupling period). Subsequently, the cells were washed three times with HEPEs-buffered saline, and cAMP accumulation was initiated by addition of HEPEs-buffered saline containing 5-HT (10 μM) with 1-ascorbic acid (0.1 mM) and the phosphodiesterase inhibitor Ro 20-1724 (0.1 mM). The accumulation was measured for 5 min. Quantification of cAMP production was performed as described under “Determination of cAMP Production in Transfected Cells.”

Intact Cell 5-HT₄R Phosphorylation—To measure phosphorylation of Rh-tagged 5-HT₄R in intact cells, transiently transfected COS-7 cells were plated at a density of 5 × 10⁶ cells/dish on 100-mm dishes. The next day, the cells were washed with serum- and phosphate-free DMEM and then labeled with 150 μCi/ml [γ³²P]orthophosphate for 90 min at 37 °C. Labeled cells were stimulated or not with 10 μM 5-HT for 10 min as indicated in the figure legends. Statistical analysis was carried out with the Student’s t test using GraphPad Prism 3.0 software. p values <0.05 were considered as statistically significant.

RESULTS

The Rapid and Potent Uncoupling of 5-HT₄R in Colliculus Neurons Is Not Observed in the COS-7 or HEK293 Cell Line—Previous work in our laboratory revealed a rapid homologous desensitization of 5-HT₄R in mouse colliculus neurons (12). Indeed, preincubation of mouse colliculus neurons with 5-HT (10 μM) for different periods of time (0–60 min), called the “uncoupling period” (Fig. 1A), drastically reduced the accumulation of cAMP measured during a subsequent 5-min incubation in the presence of 5-HT (10 μM), isobutylmethylxanthine (1 mM), and forskolin (0.1 μM). After a 5-min uncoupling period, the maximum stimulation was already reduced by 62%, and after 60 min, by ~90% (Fig. 1A).

In an effort to better understand the molecular mechanisms involved in this process, we first wanted to identify the relative expression levels of the various isoforms endogenously present in these neuronal cells. Four 5-HT₄R splice variants (5-HT₄aR, 5-HT₄bR, 5-HT₄cR, and 5-HT₄dR) have been cloned in mouse colliculus neurons (10). Using real-time quantitative reverse transcription-PCR with total colliculus neuronal mRNA, we found that three of the four splice variants were significantly expressed (5-HT₄aR, 5-HT₄bR, and 5-HT₄cR), as shown in Fig. 1B, with the expression of mouse 5-HT₄dR being the greatest.

To determine whether the potent and rapid uncoupling observed in neurons also occurs in a heterologous cell system and whether one of the splice variants specifically undergoes more rapid uncoupling, we transfected COS-7 cells with each of these variants. Interestingly, as shown in Fig. 1C, none of the splice variants were dramatically uncoupled following agonist stimulation in COS-7 cells. The time courses of desensitization were very slow, and a 60-min preincubation with 5-HT (10 μM) reduced the maximum agonist stimulation by only 20%. Similar results were obtained with HEK293 cell lines both when transiently transfected (Fig. 1D) or when stably expressing 5-HT₄R (data not shown). Therefore, unlike the β₂-adrenergic or dopamine D₁ receptor (28, 44), the 5-HT₄R splice variants did not undergo a rapid loss of response in these heterologous cell lines.
expression systems. Obviously, some key elements of 5-HT₄R uncoupling were missing.

**GRK2 Is Likely to Be the Kinase Involved in the Rapid and Potent Uncoupling in Colliculus Neurons**—It is well known that GRKs are often an important component in receptor/G protein uncoupling process; therefore, we analyzed the nature of the endogenous GRKs expressed in colliculus neurons. We found that only GRK2 was expressed at high density, whereas GRK4/5/6 expression remained undetectable. A very low density expression of endogenous GRKs was detected in COS-7 cells with each of the four mouse splice variants that are expressed endogenously in colliculus neurons (5-HT₄(a)R, 5-HT₄(b)R, 5-HT₄(e)R, and 5-HT₄(f)R) in combination with GRK2. To determine whether one of the splice variants specifically undergoes more rapid uncoupling, we transfected COS-7 cells with each of the four mouse splice variants that are expressed in colliculus neurons (5-HT₄(a)R, 5-HT₄(b)R, 5-HT₄(e)R, and 5-HT₄(f)R) in combination with GRK2. The 5-HT/GRK2-mediated attenuation of 5-HT₄R splice variant-stimulated cAMP formation was examined. Despite a significant difference in the number of potential phosphate acceptor sites after the splice site (Leu358) (four in 5-HT₄(a)R, six in 5-HT₄(b)R, one in 5-HT₄(e)R, and none in variant 5-HT₄(f)R) (Fig. 3A), a similar uncoupling was observed (Fig. 3B). The absence of a role for these residues in 5-HT₄R uncoupling was confirmed by the fact that a receptor mutant (Δ358) truncated at the level of the Leu358 splice site was similarly uncoupled (Fig. 4C).

Ser/Thr clusters (three or four consecutive Ser/Thr residues) have been reported previously to be better potential phosphate acceptor sites for GRK than scattered Ser/Thr residues (33). In
Fig. 2. Effect of overexpression of GRKs on 5-HT₄R uncoupling. A, GRKs from COS-7 cells and colliculus neurons were subjected to Western blot analysis. COS-7 cells were transiently transfected with 1 μg of each GRK cDNA (GRK2/4/5/6). The day after transfection, the levels of GRK overexpression in COS-7 cells were compared with the levels of endogenous GRKs expressed in COS-7 cells and colliculus neurons by Western blotting of equal amounts of whole cell lysates. The monoclonal antibodies used to develop immunoreactive bands do not differentiate between GRK2 and GRK3 or between GRK4/5 and GRK6. The Western blot shown is representative of three independent experiments.

B, 5-HT₄(a)R-mediated cAMP formation was differentially affected by GRKs. COS-7 cells were transiently transfected with 5-HT₄(a)R (100 ng of cDNA) alone or in combination with GRK2, GRK4, GRK5, or GRK6 (1 μg of each GRK cDNA). The day after transfection, cells were stimulated for 5 min at 37 °C with increasing concentrations of 5-HT in the presence of a phosphodiesterase inhibitor (Ro 20-1724). Data are expressed as a percentage of the maximum cAMP response produced in cells in which the receptor was expressed alone. Basal and 5-HT-stimulated cAMP levels of receptor phosphorylation when expressed alone or in the presence of the kinase-defective GRK2(K220R) mutant, in which the kinase activity was disrupted by site-directed mutagenesis. GRK2(K220R) was as efficient as and only slightly less potent than native GRK2 in 5-HT₄R uncoupling (Fig. 5, A and B). The fact that GRK2 uses a phosphorylation-independent mechanism to trigger 5-HT₄R uncoupling was confirmed by the presence of a marked increase in 5-HT-dependent phosphorylation of 5-HT₄R when expressed with GRK2 and, in contrast, the absence of receptor phosphorylation when expressed alone or in the presence of the kinase-defective GRK2(K220R) mutant (Fig. 5C).

5-HT₄R Uncoupling and Endocytosis Require Different GRK2 Expression Levels—To determine whether the level of GRK2 is important for 5-HT₄R endocytosis in transiently transfected cells, we examined the agonist-induced loss of surface HA-tagged 5-HT₄R in HEK293 cells in the presence and absence of GRK2 overexpression. The amount of HA-tagged 5-HT₄R remaining on the cell surface was quantified by ELISA (Fig. 6A) and used as a measure of receptor internalization. In HEK293 cells, agonist induced a 36% loss of surface receptor after a 30-min exposure. To our surprise, overexpression of GRK2 did not potentiate the decrease in surface 5-HT₄R (Fig. 6A). These observations suggest that endogenous levels of GRK2 are insufficient for rapid uncoupling, but are sufficient for agonist-induced receptor internalization.

To analyze the nature of the endocytosis, we followed the ability of c-Myc-tagged 5-HT₄(a)R constructs to associate with β-arrestin-2-YFP and to undergo endocytosis. We used a β-arrestin-2-YFP translocation assay and fluorescence microscopy in HEK293 cells. Cell-surface receptors were prelabeled with primary antibodies prior to agonist stimulation. In the absence of agonist, β-arrestin-2 was distributed throughout the cytoplasm in cells expressing the receptor, whereas c-Myc-tagged 5-HT₄(a)R was observed at the plasma membrane. Following a 15-min stimulation with 5-HT, an extensive co-localization of both β-arrestin-2-YFP and the c-Myc-tagged receptor appeared as punctate fluorescence labeling of endocytic vesicles (Fig. 6B). After a 45-min period of agonist exposure, a change in the
pattern of staining was observed, *i.e.* the c-Myc-5-HT$_{4a}$R-$\beta$-arrestin-2-YFP complexes translocated to a perinuclear compartment (Fig. 6B).

Given the important roles that phosphorylated Ser and Thr play in promoting receptor-$\beta$-arrestin interaction (33), we examined whether some specific Ser/Thr residues in the 5-HT$_{4}$R
C-terminal domain are involved in 5-HT4R/β-arrestin-2 association and endocytosis. We began by analyzing and comparing the endocytosis of the 5-HT4aR, 5-HT4bR, and 5-HT4eR splice variants as well as the endocytosis of the Δ358 mutant, which shares the sequence common to all of the variants. As shown in Fig. 6B, all of the variants, as well as the Δ358 mutant, exhibited similar endocytosis kinetics and the ability to associate with β-arrestin-2. Consequently, Ser/Thr residues scattered over specific C-terminal domains of the 5-HT4aR, 5-HT4bR, and 5-HT4eR splice variants (Fig. 3A) do not seem to be implicated in the 5-HT4R association with β-arrestin-2 and endocytosis (Fig. 6B). When cells were incubated for 15 min in the presence of agonist, washed extensively, and subjected to an additional incubation in the absence of agonist for 6 h, all of the 5-HT4R splice variants that formed stable complexes with β-arrestin translocated to endosomes in a perinu-
Molecular Events in the Uncoupling and Endocytosis of 5-HT₄Rs

**Fig. 7. β-Arrestin-2 is not recruited by the truncated 5-HT₄R mutants lacking the Ser/Thr cluster within the C-terminal domain.** HEK293 cells were transiently cotransfected with 600 ng of c-Myc-Δ358, c-Myc-Δ358Ala, or c-Myc-Δ346 in combination with 1 µg of GRK2 and 0.3 µg of β-arrestin-2-YFP. Intact cells were immunostained with anti-c-Myc antibody before activation of 5-HT₄R. The upper panels show the distribution of β-arrestin-2-YFP and c-Myc-tagged 5-HT₄R either before (0 min; control) or after (15 and 45 min) addition of 30 µM 5-HT to the culture medium at 37 °C. The lower panels show cells that were stimulated with agonist for 15 min and then extensively washed and subjected to an additional incubation in agonist-free medium for 6 h. Cells were fixed after treatments. Immunoreactivity was revealed with Alexa Fluor 594-conjugated secondary antibodies. Fluorescence microscopy was then used to visualize the redistribution of the antibody-labeled receptor and β-arrestin-2-YFP. The prominent co-localization of activated c-Myc-Δ358 with β-arrestin-2-YFP, whereas mutants lacking the Ser/Thr cluster upstream of the Leu³⁵⁸ splice site (Δ358Ala and Δ346) did not co-localize with β-arrestin-2-YFP.

| Receptors         | WT 5-HT₄R | Δ358 | Δ358Ala | Δ346 |
|-------------------|-----------|------|---------|------|
| Basal             | 100       | 75   | 70      | 65   |
| 5-HT 30 min       | 75         | 50   | 45      | 40   |

**Fig. 8. Dominant-negative β-arrestin-(319–418) and dominant-negative dynamin(K44A) inhibit agonist-induced internalization of both truncated 5-HT₄R and C-terminal mutants.** HEK293 cells were transiently cotransfected with GRK2 and the c-Myc-tagged WT receptor, Δ358, Δ358Ala, or Δ346 (100 ng of each plasmid) in combination with either dominant-negative β-arrestin-(319–418) (Bar (319–418); 1 µg) or dominant-negative dynamin(K44A) (Dyn K44A; 1 µg). Cells were pretreated (dark gray bars) or not (light gray bars) for 30 min at 37 °C. Cells were then fixed, and the amount of receptor remaining on the cell surface was quantified by ELISA. Results shown are the means ± S.E. of four separate experiments performed in triplicate. * p < 0.01 (significantly different from the corresponding WT receptor and Δ358 mutant in the absence of the dominant-negative proteins).

To investigate whether the observed differences in receptor endocytosis would differentially affect the recycling of the receptors, cells expressing either the Δ346 or Δ358Ala mutant, both of which are unable to interact with β-arrestin, were incubated with agonist for 15 min and then subjected to an additional incubation in the absence of agonist for 6 h. As shown in Fig. 9A, in the absence of any stimulation, 5-HT₄Rs were strictly expressed on the membrane. Indeed, no labeling was observed in orthogonal sections of the neurons in contrast, 5-HT induced a significant change in cell-surface expression. An unequivocal labeling of endocytosed receptors was noticeable in both the Z section and orthogonal sections of the neurons.

A more quantitative assessment of 5-HT₄R endocytosis was carried out by ELISA. A 30-min stimulation induced a loss of 34% of surface receptors (Fig. 9B). In addition, Fig. 9B clearly shows that 5-HT₄R endocytosis in colliculus neurons was largely reduced by dominant-negative β-arrestin-(319–418) and dominant-negative dynamin(K44A). As in HEK293 cells, WT 5-HT₄R endocytosis in colliculus neurons was β-arrestin/dynamin-dependent.
splice variants within the C-terminal domain to traffic with and the phosphorylation of Ser/Thr clusters common to all opposed to its uncoupling, requires the kinase activity of GRK2 transiently transfected with 2.5 endocytosis in colliculus neurons.

Molecular Events in the Uncoupling and Endocytosis of 5-HT_4Rs

Three main conclusions can be drawn from these experiments. First, the common cluster of Ser and Thr residues upstream of the Leu^{27932} splice site is an absolute requirement for normal β-arrestin/dynamin-dependent endocytosis and trafficking. This indicates that the endocytosis of 5-HT_4R, as opposed to its uncoupling, requires the kinase activity of GRK2 and the phosphorylation of Ser/Thr clusters common to all splice variants within the C-terminal domain to traffic with β-arrestin. Second, in the absence of Ser/Thr clusters, the receptor is still endocytosed, but in a β-arrestin/dynamin-independent manner. Third, β-arrestin is not the only trafficking protein able to control receptor internalization of 5-HT_4R.

The Kinase-deficient GRK2 Mutant Suppresses β-Arrestin-dependent Endocytosis and Promotes β-arrestin-independent Endocytosis—We next determined whether the overexpression of GRK2(K220R) in HEK293 cells could block or induce endocytosis of the WT receptor. After a 30-min stimulation with 5-HT, the endocytosis of 5-HT_4R was greater when GRK2(K220R) was expressed (Fig. 10A) instead of the native GRK2 kinase (Fig. 6A). Note that, in the presence of GRK2(K220R), dominant-negative β-arrestin-(319–418) was almost inactive in reducing endocytosis (Fig. 10A). This is also directly visualized in Fig. 10B. In the presence of GRK2(K220R), β-arrestin-dependent endocytosis of 5-HT_4R was not observed. In contrast, the endocytosis of 5-HT_4R was clearly present and distributed in vesicles in which β-arrestin was absent (Fig. 10B, right panels). These results support the conclusion that kinase activity and Ser/Thr clusters are required for normal β-arrestin/dynamin-dependent endocytosis and trafficking of 5-HT_4R.

FIG. 9. 5-HT induces 5-HT_4R/β-arrestin- and dynamin-dependent endocytosis in colliculus neurons. A, colliculus neurons were transiently transfected with 2.5 μg of Rho-tagged 5-HT_4R using the mouse neuron Nucleofector<sup>®</sup> kit. Intact cells were immunostained with anti-Rho antibody before activation of 5-HT_4R. The distribution of Rho-tagged 5-HT_4R is shown either before (0 min) or after (30 min) addition of 10 μM 5-HT to the culture medium at 37 °C. Immunoreactivity was revealed with Alexa Fluor 488-conjugated secondary antibodies. Confocal microscopy was then used to visualize the redistribution of antibody-labeled receptors. B, colliculus neurons were transiently transfected with Rho-tagged 5-HT_4R (2.5 μg) in combination with either dominant-negative β-arrestin-(319–418) (parr 319–418; 2 μg), or dominant-negative dynamin(K44A) (Dyn K44A; 2 μg). Neurons were pretreated (light gray bars) or not (dark gray bars) for 30 min at 37 °C with 10 μM 5-HT. Neurons were then fixed, and the amount of receptor remaining on the cell surface was quantified by ELISA. Results shown are the means ± S.E. of two separate experiments performed in sextuplicate. *, p < 0.01 (significantly different from the corresponding cells before 5-HT treatment).

FIG. 10. The kinase-deficient GRK2(K220R) mutant induces 5-HT_4R/β-arrestin-2-independent endocytosis. A, the relative cell-surface expression of HA-tagged 5-HT_4R (100 ng) transfected in HEK293 cells in combination with 2.5 μg of GRK2(K220R) was determined before and after stimulation with 5-HT (30 μM) for 5 and 30 min by ELISA and in the absence and presence of dominant-negative β-arrestin-(319–418) as described under “Experimental Procedures.” Data represent the means ± S.E. of four independent experiment. *, p < 0.01 (significantly different from the corresponding cells before 5-HT treatment). B, HEK293 cells transiently expressing c-Myc-tagged 5-HT_4R in combination with β-arrestin-2-YFP and 1 μg of GRK2 (left panels) or 2.5 μg of GRK2(K220R) (right panels) were incubated in the absence (control) or presence of 30 μM 5-HT for 15 or 45 min at 37 °C or for 15 min with agonist and then extensively washed and subjected to an additional incubation in agonist-free medium for 6 h. Cells were fixed and processed for immunofluorescence microscopy. These images are representative of many cells examined in at least three independent experiments. Fluorescence microscopy was then used to visualize the redistribution of antibody-labeled receptors, and β-arrestin-2-YFP was visualized. Note the prominent co-localization of activated c-Myc-tagged 5-HT_4R with β-arrestin-2-YFP when GRK2 was coexpressed and the total absence of co-localization when the kinase-deficient GRK2(K220R) mutant was coexpressed.
It is important to study the molecular mechanisms of signal transduction in native tissues because they can be substantially different from those in heterologous cell lines, as shown recently (37, 45, 46). This is particularly important for studying desensitization of GPCRs, which are extremely dependent on the specific molecular and cellular characteristics of the receptor as well as the cell in which they are expressed. This has been recently reported for γ-aminobutyric acid type B receptors (25), which were desensitized in cerebellar granule cells neurons, but not in HEK293 cells. The reason is that GRK4, which is required for γ-aminobutyric acid type B receptor desensitization, is absent in HEK293 cells. Similarly, we have shown here that the rapid and marked uncoupling of 5-HT₄Rs observed in colliculus neurons was not observed when the receptor was expressed in COS-7 or HEK293 cells. One of the main reasons is probably the absence of significant expression of GRK2 in COS-7 cells and only modest expression in HEK293 cells (30, 47). In contrast, colliculus neurons expressed a high concentration of GRK2, and no other GRK was significantly expressed in these neurons. The main role for GRK2 in 5-HT₄R uncoupling was confirmed by the fact that overexpression of GRK2 in COS-7 and HEK293 cells mimicked the 5-HT₄R uncoupling characteristics observed in neurons. However, GRK5 was less active, and both GRK4 and GRK6 were inactive. The GRK2-mediated uncoupling of 5-HT₄Rs was also phosphorylation-independent. Indeed, no difference in GRK2-mediated uncoupling was observed between the different 5-HT₄R splice variants, which differ in the number of Ser and Thr residues present in their specific C-terminal domains. Uncoupling remained unaffected when the cluster of Ser and Thr residues localized upstream of the Leu³⁵⁸³ splice site was removed by truncation or mutated to Ala. Finally, full uncoupling was observed when the kinase-deficient GRK2 mutant was expressed. There are several reports showing phosphorylation-independent reduction of GPCR agonist response by kinase-deficient GRK2 or GRK4 (21, 23, 25). However, only a few studies have reported the role of kinase-deficient GRK in the classical agonist-promoted desensitization paradigm (24, 25, 48). Interestingly, two recent studies, one concerning γ-aminobutyric acid type B receptors and GRK4 (25) and the other concerning m₁ muscarinic receptors and GRK2 (46), demonstrated a kinase-independent role of GRKs in neurons. To date, the mechanism by which GRK2 induces 5-HT₄R uncoupling in a phosphorylation-independent manner remains to be studied. We can only speculate based on the known properties of GRK2. This kinase has been proposed to interact with many signaling proteins capable of influencing signaling efficacy. These include Go protein(s) (mainly Goq) via the N-terminal RGS (regulator of G protein signaling) domain of GRK2, Gβγ and phosphatidylinositol diphosphate via the C-terminal pleckstrin homology domain of GRK2, clathrin, GIT1 (a member of the GRK-interacting protein family), caveolin, phosphoinositide 3-kinase-α and -γ, and Ca²⁺ sensor protein (reviewed in Ref. 49). Many of these interactions, especially with Gβγ, are probably involved in the kinase-independent uncoupling of 5-HT₄Rs by GRK2.

Following the early work of Lefkowitz and co-workers (31), Su et al. (29) were the first to demonstrate that functional uncoupling precedes receptor migration to a buoyant fraction (likely endocytic vesicles). Since these early days, much data have indicated that uncoupling and endocytosis are distinct steps (50–52). However, dissociating the two events kinetically or in terms of molecular events is not always easy. Here, we have shown that the uncoupling and endocytosis of 5-HT₄Rs require very different GRK2 expression levels and distinct molecular events. Indeed, although the modest endogenous expression level of GRK2 in HEK293 cells was sufficient to obtain endocytosis of 5-HT₄Rs that was both β-arrestin- and dynamin-dependent, it was totally insufficient to achieve the marked 5-HT₄R uncoupling observed in neurons. There is also another clear difference; uncoupling was independent of phosphorylation, whereas 5-HT₄R/β-arrestin-dependent endocytosis required the C-terminal Ser/Thr cluster as well as the kinase activity of GRK2. Indeed, we clearly showed that the kinase-deficient GRK2 mutant inhibited 5-HT₄R/β-arrestin-dependent endocytosis, but promoted 5-HT₄R/β-arrestin-independent endocytosis. Thus, phosphorylation of the 5-HT₄R C-terminal domain seems to be absolutely necessary for β-arrestin recruitment. This model contrasts with those of the dopamine D₁ receptor, in which phosphorylation of Ser/Thr permits access of arrestin to its receptor-binding domain rather than creating an arrestin-binding site per se (28). However, in our study, the Δ346 mutant, whose i₁ loop is easily accessible, did not associate with β-arrestin, whereas the Δ358 mutant, which possesses the Ser/Thr cluster, was always able to interact with β-arrestin. These results confirm that the Ser/Thr cluster in the 5-HT₄R C-terminal domain is necessary for β-arrestin association. However, our results do not demonstrate that the Ser/Thr cluster is “the binding site” for β-arrestin. This phosphorylated Ser/Thr cluster could be necessary for the proper conformation of the receptor to allow appropriate β-arrestin binding.

Following overexpression of the kinase-deficient GRK2 mutant, the receptor could be endocytosed, but in a β-arrestin/dynamin-independent manner. Several GPCRs are known to require β-arrestins for endocytosis. These include the metabotropic glutamate type 1d receptor (23), the secretin receptor (53), 5-HT₄AR (54), protease-activated receptor-1 (55), the pentacyclin receptor (56), the formyl peptide receptor (57), the m2 muscarinic receptor (58), leukotriene B₄ receptor-1 (30), and the neuropeptide Y1 receptor (59). GRK2 associated with the 5-HT₄Rs may directly interact with clathrin via a clathrin box (60), which is present in the C-terminal region of GRK2. Further work is needed to investigate this possible mechanism.

Whatever the theoretical interest of the β-arrestin-independent endocytosis, it is important to note that it is the β-arrestin/dynamin-dependent endocytosis that is of physiological significance. Indeed, β-arrestin-independent endocytosis was almost unobserved with WT 5-HT₄R in HEK293 cells. Moreover, 70% of 5-HT₄R endocytosis in colliculus neurons was blocked either by dominant-negative β-arrestin or by dominant-negative dynamin. Thus, β-arrestin and dynamin control 5-HT₄R endocytosis in neurons.
they have been shown to be clearly implicated in other GPCR systems (62, 63).

Several important physiological effects have been attributed to 5-HT₄Rs. A recent study provides evidence that stress-induced hypogonadism and novelty-induced exploratory activity are attenuated in 5-HT₄R knockout mice (4). Moreover, Lucas et al. (64) reported the existence of a key function for 5-HT₄R in the complex network linking the medial prefrontal cortex to the dorsal raphe neurons. They proposed that 5-HT₄Rs exert a tonic and positive influence on a subpopulation of 5-HT neurons.

In the enteric nervous system, 5-HT₄Rs, which are presynaptic, have been shown to enhance transmitter levels and thus to strengthen neurotransmission in prokinetic pathways (6). 5-HT₄Rs also regulate respiratory neurons (5).

All of these recent findings favor an intriguing role for 5-HT₄R in the fine-tuning of neuronal activity. Therefore, it is important to understand how 5-HT₄R responsiveness can be regulated to design efficacious drugs controlling these functions. In view of the present data, it is perhaps not surprising that only partial agonists have been selected as therapeutic drugs acting on 5-HT₄Rs. Metoclopramide, cisapride, and tegaserod are used to treat gastrointestinal diseases, and SL650155 is the only 5-HT₄R ligand in development for brain disorders (65). Obviously, a complete study of the action of agonists (full and partial), antagonists, and inverse agonists on 5-HT₄R uncoupling and endocytosis has to be done.

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Uncoupling and Endocytosis of 5-Hydroxytryptamine 4 Receptors: DISTINCT MOLECULAR EVENTS WITH DIFFERENT GRK2 REQUIREMENTS
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**Supplementary Table 1: Primers used for quantitative PCR.** For each gene are indicated the Genbank accession number of the reference sequence used (Ref Seq), the nucleotide numbers in this sequence at which the primers start, and the sequences of forward and reverse primers.

| cDNA  | Ref Seq | Forward primer       | Reverse primer       |
|-------|---------|----------------------|----------------------|
| m5-HT4(a) | Y09587 | m5-HT4(a)-1056F TGGATCCACCCATGTACTAAGGT | m5-HT4(a)-1109R GTGTATGGGCAATTTCCTCCAGTT |
| m5-HT4(b) | Y09585 | m5-HT4(b) - 1028F CTGTCCCCTGTCAACCACA | m5-HT4(b) - 1092R CTCTCCCACCTGGCCACCA |
| m5-HT4(e) | Y09588 | m5-HT4(e) - 1028F CTGTCCCCTGTCAACCACA | m5-HT4(e) - 1086R AACAGGTCTATTGCAGGAGAGC |
| m5-HT4(f) | AJ011369 | m5-HT4(e) - 987F TGATGATGAGCGCTACAAAAGAC | m5-HT4(f) - 1066R GACGGGAACAGGTCTATTAGTACATG |