Functional Selectivity in Serotonin Receptor 2A (5-HT_{2A})
Endocytosis, Recycling, and Phosphorylation

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ABSTRACT

G protein-coupled receptor (GPCR) signaling is modulated by endocytosis and endosomal sorting of receptors between degradation and recycling. Differential regulation of these processes by endogenous ligands and synthetic drugs is a poorly understood area of GPCR signaling. Here, we describe remarkable diversity in the regulation of trafficking of GPCR induced by multiple ligands. We show that the serotonin receptor 2A (5-HT_{2A}), a prototypical GPCR in the study of functional selectivity at a signaling receptor, is functionally selective in endocytosis and recycling in response to five ligands tested: endogenous agonists serotonin (5-HT) and dopamine (DA), synthetic agonist 1-(2,5-dimethoxy-4-iodophenyl)-aminopropane (DOI), antagonist ketanserin, and inverse agonist and antipsychotic drug clozapine. Only four ligands (5-HT, DA, DOI, and clozapine) bring about receptor endocytosis. As we have earlier described with 5-HT and DA, there is ligand-specific requirement for protein kinase C (PKC) in endocytosis. We now show 5-HT_{2A} phosphorylation by PKC is necessary for 5-HT-mediated and DOI-mediated receptor endocytosis, but DA-mediated and clozapine-mediated internalization is not affected if PKC is inhibited. Internalized receptors are recycled to the cell surface, but there is variability in the time course of recycling. 5-HT- and DA-internalized receptors are recycled in 2.5 hours while agonist DOI and antagonist clozapine bring about recycling in 7.5 hours. Recycling in response to those ligands that require PKC activation to effect receptor endocytosis is dependent on receptor dephosphorylation by protein phosphatase 2A (PP2A). Thus, internalization and phosphorylation/dephosphorylation cycles may play a significant role in the regulation of 5-HT_{2A} by functionally and therapeutically important ligands.

Introduction

Serotonin (5-hydroxytryptamine; 5-HT) receptors are a family of G protein-coupled receptors (GPCRs) and ligand-gated ion channels found in the central and peripheral nervous systems where they mediate neurotransmission (Nichols and Nichola, 2008). 5-HT receptors influence various processes and are hence a primary target of several drugs, including many antidepressants, antipsychotics, anorectics, antiemetics, and hallucinogens (Nichols, 2004).

Several ligands bind to the serotonin 2A receptor (5-HT_{2A}), including biogenic amines such as dopamine (DA) and tryptamine as well as recreational drugs such as psilocybin and lysergic acid diethylamide (LSD) and therapeutic (anti-psychotic) drugs (Meltzer et al., 2003, 2012; Meltzer and Massey, 2011; Nichols, 2004). As seen with some GPCRs, ligands acting at the 5-HT_{2A} receptor differentially modulate intracellular transduction cascades, a phenomenon commonly called “functional selectivity” (Raote et al., 2007; Urban et al., 2007). Functional selectivity arises out of GPCR pleiotropy with respect to the signaling transduction cascades they couple to via interactions with different signaling proteins. Different ligands uniquely modulate sets of signaling pathways by stabilizing specific receptor conformations. Thus, ligands at a single receptor produce heterogeneous effects on the subsequent activation of signaling cascades with varying efficacies, which poses a challenge to classifying a ligand as an agonist or antagonist (Kenakin, 2011).

The effects of 5-HT_{2A} ligands, other than 5-HT, on subcellular transduction cascades downstream of receptor activation are minimally characterized; how they feed back to the receptor and affect its signaling and trafficking remains unknown. After ligand binding at the cell surface, the 5-HT_{2A} may be endocytosed and transiently sequestered in endosomes, the functional significance of which remains to be elucidated. 5-HT_{2A} internalization is associated with receptor desensitization in C6 glioma cells (Hanley and Hensler, 2002). However, internalization and desensitization are independent processes in human embryonic kidney 293 (HEK293) cells

ABBREVIATIONS: BSA, bovine serum albumin; DA, dopamine; DMEM, Dulbecco’s modified Eagle’s medium; DOI, 1-(2,5-dimethoxy-4-iodophenyl)-aminopropane; EGFP, enhanced green fluorescent protein; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase; HBSS, Hanks’ balanced salt solution; HEK293, human embryonic kidney 293 cells; 5-HT, 5-hydroxytryptamine serotonin; 5-HT_{2A}, 5-hydroxytryptamine receptor subtype 2A; LSD, lysergic acid diethylamide; MDMA, 3,4-methylenedioxymethamphetamine; OA, okadaic acid; PBS, phosphate-buffered saline; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PP2A, protein phosphatase 2A.
(Gray et al., 2001). On the basis of studies with other GPCRs (Gagnon et al., 1998; Kallal et al., 1998; Ko et al., 1999; Law et al., 1984; Tsao et al., 2001), receptor internalization and subsequent lysosomal targeting is believed to be the mechanism behind 5-HT2A downregulation by the antagonist clozapine.

In addition to downregulation and desensitization, another consequence of receptor internalization could be the formation of a signaling endosome that modulates transduction pathways distinct from those regulated at the cell surface (Calebiro et al., 2009; Rosen et al., 2009). These processes could involve a reversion of covalent changes, such as receptor phosphorylation (after ligand binding), enabled by a change in the receptor’s local environment from the cell surface to an acidified endosome. Cycles of phosphorylation and dephosphorylation are important in GPCR trafficking, signaling, and subcellular targeting (Moro et al., 1993; Sibley et al., 1986). Phosphorylation enables receptors to interact with cellular machinery allowing for trafficking, resensitization, and the formation of a scaffold involved in signaling. While it is conventionally believed that kinases involved in GPCR phosphorylation are GRKs (Benovic et al., 1989; Lorenz et al., 1991), other kinases, including protein kinase C (PKC), have been shown to play a significant role (Blaukat et al., 2001; Pollok-Kopp et al., 2003).

Universally used readouts of signaling and regulation of canonical GPCRs such as the β-adrenoceptor, GRK-mediated receptor phosphorylation, and direct association with β-arrestin-2 are not involved in rat 5-HT2A regulation in HEK293 cells (Bhatnagar et al., 2001).

Although it was initially believed that the 5-HT2A receptor does not undergo phosphorylation, subsequent reports have demonstrated that phosphorylation by ribosomal S6 kinase 2 (Strachan et al., 2009) dramatically modulates 5-HT2A signaling (Sheffler et al., 2006; Strachan et al., 2010a,b). 5-HT2A phosphorylation at residues Ser291 and Thr386 by PKC has been shown in an in vitro assay (Turner and Raymond, 2005) and is hypothesized to play a role in modulating receptor signaling.

We have previously shown that PKC activation is necessary for 5-HT-mediated 5-HT2A internalization and is sufficient to bring about receptor endocytosis (Bhattacharyya et al., 2002, 2006). Subsequently, by showing that DA-mediated 5-HT2A endocytosis is independent of PKC activation, we described functional selectivity in 5-HT2A signaling (Bhattacharyya et al., 2006). These observations led us to the hypothesis that 5-HT2A functional selectivity extends to both proximal events in receptor signaling as well as distal events during receptor recycling.

Using five compounds that act via the 5-HT2A receptor—cognitive ligand (5-HT), synthetic agonist [1-(2,5-dimethoxy-4-iodophenyl)-aminopropane; DOI], partial-efficacy agonist (DA), antagonist (ketanserin), and inverse agonist (clozapine)—we show that different biochemical pathways are involved in receptor trafficking. As ligand-specific pathways of receptor regulation arise perforce from ligand-specific signaling via the 5-HT2A receptor, we use events in receptor trafficking as direct readouts of functional selectivity at the receptor. These readouts include receptor internalization, recycling, and the involvement of PKC in receptor trafficking.

Materials and Methods

Reagents, Expression Plasmids. Cloning of the rat-5-HT2A into pEGFP-N1 was described elsewhere (Bhattacharyya et al., 2002). All reagents unless otherwise mentioned were obtained from Sigma-Aldrich (St. Louis, MO).

Cell Lines, Transfection, and Stable Line Generation. HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) in 5% CO2 at 37°C, as described elsewhere (Bhattacharyya et al., 2002). All culture dishes, flasks, and other apparatuses were from Nalgene Nunc (Roskilde, Denmark) and Sarstedt AG and Co. (Nümbrecht, Germany). Generation of the SB1 cell population, expressing wild-type rat-5-HT2A–EGFP (enhanced green fluorescent protein), was described elsewhere (Bhattacharyya et al., 2002).

Internalization Assay. The internalization assay was performed as previously described elsewhere (Bhattacharyya et al., 2010). In brief, the cells were plated on poly-DL-ornithine-coated glass coverslips at a density of 40,000 to 60,000 cells per dish. At 16 hours after plating, the cells were incubated in serum-free DMEM for 24 hours. The cells were washed and incubated with 100 μg/ml cycloheximide for 6 hours. This treatment inhibits protein synthesis and allows sufficient time for already synthesized receptor traffic to the cell surface. Most cells in this point show EGFP-associated fluorescence at the cell membrane with minimal intracellular fluorescence. Cells were then stimulated with a ligand at the described concentration at 37°C in 5% CO2 for 15 minutes. During the last 5 minutes of the incubation, the cells were exposed to 10 μg/ml human transferrin tagged to Alexa 568 (Invitrogen) in DMEM as an endocytic tracer. Surface-associated transferrin was then stripped with ice-cold ascorbate buffer at pH 4.5 and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature. Receptor internalization in the cells was confirmed by the appearance of a subcellular pool of fluorescence from the internalized receptors, whereas the control (unstimulated) cells had fluorescence associated primarily with the plasma membrane.

If they were used, the specific inhibitors of intracellular molecules (sphingosine, okadaic acid) were added 15 minutes before stimulation of the receptor-expressing cells with a ligand, unless otherwise mentioned. The internalization assay was then performed as already described but in the continued presence of the inhibitor.

Recycling Assay. The SB1 cells, grown in 35-mm coverslip dishes, were treated starved for 24 hours and treated with cycloheximide (100 μg/ml) to clear the internal fluorescence, as already mentioned in the description of the internalization assay. Subsequently, 10 μM 5-HT, 10 μM DA, 100 nM DOI, or 100 nM clozapine was applied for 15 minutes. The cells were then washed free of the ligand and were incubated for various time periods in the absence of ligand or serum and in the continued presence of cycloheximide. Five minutes before fixation, the cells were treated with Alexa 568–tagged transferrin. The surface transferrin was stripped using ice-cold ascorbate buffer (pH 4.5), and then the cells were fixed in ice-cold 4% paraformaldehyde for 20 minutes and imaged.

As in the internalization assay, any inhibitors used (sphingosine, okadaic acid) were added 15 minutes before the stimulation of the receptor-expressing cells with a ligand. Recycling assays were then performed (as already described) in the continued presence of the inhibitor unless otherwise noted.

ELISA-Based Quantification of Receptor Recycling. The SB1 cells were grown in poly-DL-ornithine-coated wells on a 96-well plate initially in DMEM supplemented with 10% FBS. At 24 hours after plating, the medium was changed to DMEM supplemented with 10% serum, dialyzed against PBS. The cells were maintained in this medium for 48 hours. Ligands were then added for 15 minutes at 37°C. The cells were subsequently washed three times and incubated in DMEM supplemented with 10% dialyzed FBS for different periods, ranging from 0 minutes to 7.5 hours. At each time point, the cells were fixed for 8 minutes on ice in ice-cold 2% paraformaldehyde (PFA,
nonpermeabilizing conditions). After fixation, all dishes were stored at 4°C in M1 buffer (20 mM HEPES, 1.3 mM CaCl$_2$, 2 mM MgCl$_2$, 150 mM NaCl, and 5 mM KCl, pH 7.4) and were processed together. The cells were then blocked in 0.2% bovine serum albumin (BSA) in M1 on ice for 1 hour, followed by application of 1:200 dilution of extracellularly directed anti-5-HT$_{2A}$ antibody from Calbiochem (San Diego, CA) or Neuromics (Edina, MN) in M1, overnight at 4°C. Cells were washed and incubated with 1:500 diluted goat-anti-rabbit horseradish peroxidase (HRP) secondary antibody (Molecular Probes, Eugene, OR) for 1 hour on ice. A colorimetric reaction was developed by the addition of TMB/H$_2$O$_2$ for 90 seconds at room temperature, followed by adding an equal volume of 1 N H$_2$SO$_4$. Colorimetric measurement was done on an enzyme-linked immunosorbent assay (ELISA) plate reader at 450 nm from Bio-Rad Laboratories (Hercules, CA). The graph plotted represents the mean of at least three independent experiments; in each, the ligand treatment was performed in 8 to 16 wells of the 96-well plate.

**Epifluorescence Microscopy.** Imaging was performed with a Nikon Eclipse T2000E epifluorescence microscope (Tokyo, Japan) using a 20×/NA 0.75 for live imaging and 60×/NA 1.4 oil immersion objective. Fluorescence was visualized using bandpass filter sets from Chroma Technologies (Rockingham, NC): exciter 500 nm/20, dichroic 568 nm–575 nm/30, and emitter 515 nm long pass, and exciter 545 nm/30, dichroic 620 nm/60, emitter 575 nm long pass for detecting GFP and Alexa 568–tagged human transferrin, respectively. Images were acquired using a Photomicrotic Cascade II 512 EM-CCD camera (Photometrix, Tucson, AZ) with Image Pro-Plus AMS software (Media Cybernetics, Bethesda, MD).

**Ca$^{2+}$ Imaging.** The SB1 cells were plated on poly-lysine–ornithine–coated glass coverslip dishes at a density of 40,000–60,000 cells per dish in DMEM supplemented with 10% FBS. The cells were incubated in serum-free DMEM overnight to eliminate the extracellular 5-HT and then were loaded with 2.5 μM Rhod-2, AM (Invitrogen) with Pluronic F-127 (Invitrogen) (1:1) in Hank’s balanced salt solution (HBSS, with Ca$^{2+}$ and Mg$^{2+}$) on ice for 1 hour, washed with HBSS, and left for an hour for Rhod-2, AM ester to be hydrolyzed in the cell. The cells were washed three times with HBSS (without Ca$^{2+}$ and Mg$^{2+}$).

To observe receptor activation, the changes in fluorescence before and after the addition of 5-HT were monitored. To confirm that ketanserin bound to the receptor, cells were pretreated with ketanserin (10 nM) before adding 5-HT (10 μM). The addition of the ligand and imaging of the changes in fluorescence associated with Rhod-2, AM were performed at 37°C in a heated and humidified chamber (model INU-02-WELS; Tokai Hit, Shizuoka-ken, Japan). Images were acquired at 5-second intervals for 3 minutes.

**Data Analysis.** The receptor internalization was quantified as previous described elsewhere (Bhattacharya et al., 2010; Kalia et al., 2006) using a modified version of a quantitative colocalization routine implemented in MATLAB (MathWorks, Natick, MA). A set of two corresponding images was taken of a field of cells to record EGFP (receptor-associated) and Alexa Fluor 568 (transferrin) signals separately. A top-hat algorithm was used to perform local background subtraction on both images using a disk of radius 5 pixels. In-focus endosomes were identified in the transferrin image using iterative thresholding and intensity-dependent trimming. In brief, initial thresholds were set by inspection. At every iterative step (going from 0.1 of maximal object intensity to 0.5, step size = 0.1), connected nonzero pixels were labeled as individual objects (putative endosomes). Objects smaller than 3 pixels or larger than 500 pixels were discarded. The ratio of intensity of the EGFP signal colocalized with transferrin endosomes to total EGFP signal was calculated (colocalization ratio). We analyzed 24 such pairs of images as a set. The averaged values from each such set of images from an experimental condition were normalized to values from the control cells that had not been exposed to any ligand. This was repeated a total of three times, resulting in 72 pairs of images. Statistical comparisons were made using the Mann-Whitney U test.

**Results.**

Some Ligands That Bind to 5-HT$_{2A}$ Bring about Its Internalization. We looked at receptor endocytosis in response to the five ligands (Fig. 1) known to bind to the 5-HT$_{2A}$ receptor. As we have previously shown, 5-HT and DA bring about 5-HT$_{2A}$ internalization in SB1 cells (HEK293 cells stably expressing 5-HT$_{2A}$-EGFP) (Bhattacharyya et al., 2002, 2006); the inverse agonist clozapine also causes receptor endocytosis (Willins et al., 1999). Further, we now show that the synthetic agonist DOI also brings about receptor internalization. The antagonist ketanserin, however, does not effect receptor internalization.

Representative images of receptor-associated EGFP fluorescence show the SB1 cells cleared of internal 5-HT$_{2A}$-EGFP–associated fluorescence by serum starvation for 24 hours and treatment with cycloheximide (100 μg/ml) for 6 hours (Fig. 2A, D, and G). The cells were then incubated with a ligand for 15 minutes. The extent to which different concentrations of the four ligands brought about receptor internalization was characterized by ELISA using an antibody targeted against an extracellular portion of the 5-HT$_{2A}$ receptor (Supplemental Fig. 1). For all subsequent experiments, the concentrations of the ligands chosen were those that were well above those that caused maximal receptor internalization. The concentration of clozapine used was 10% that seen at steady-state levels in the blood in patients being treated with the drug (Miller et al., 1994; Perry et al., 1991; Potkin et al., 1994). 5-HT$_{2A}$ endocytosis (Fig. 2) was assayed for using 10 μM 5-HT (Fig. 2B), 10 μM DA (Fig. 2C), 100 nM DOI (Fig. 2D), 100 nM clozapine (Fig. 2F), and 100 nM ketanserin (Fig. 2H). 5-HT, DA, DOI, and clonazapine all induced robust internalization, but ketanserin brought about no detectable 5-HT$_{2A}$ endocytosis.

As we have described elsewhere (Bhattacharyya et al., 2002), PKC-activation by a phorbol ester (PMA) is sufficient to bring about 5-HT$_{2A}$ endocytosis (Supplemental Fig. 2).

After internalization, the receptor localized to transferrin–labeled endosomes. We used this as a method of quantification of receptor internalization (Fig. 2I), measuring the ratio of endosome-associated EGFP to the total EGFP and normalizing this to vehicle-treated (control) cells (see Materials and Methods). The process of 5-HT$_{2A}$ endocytosis has been shown to be clathrin dependent and arrestin independent in HEK293 cells (Bhatnagar et al., 2001).

As confirmation that ketanserin did bind to the 5-HT$_{2A}$ receptor, we assayed its ability to inhibit 5-HT-mediated intracellular Ca$^{2+}$ changes. In SB1 cells, 5-HT increases cytosolic Ca$^{2+}$ via an IP$_3$-mediated pathway (Bhattacharyya et al., 2002).

**Fig. 1.** 5-HT$_{2A}$ ligands used in this study, agonists or partial agonists 5-HT, DOI, dopamine, neutral antagonist ketanserin, and inverse agonist clozapine. (All images have been modified from Wikipedia.)
et al., 2002). Preincubating SB1 cells with 10 nM ketanserin for 15 minutes completely abrogates any Ca$^{2+}$ changes induced by 10 μM 5-HT (data not shown).

**PKC Activation Is Required for Some, but Not All, Ligands, To Bring about 5-HT$_{2A}$ Internalization.** As previously described elsewhere, PKC activation is required for internalization mediated by 5-HT but not DA (Bhattacharyya et al., 2006). We also have observed that DOI but not clozapine requires PKC to bring about internalization of 5-HT$_{2A}$. SB1 cells, cleared of internal fluorescence (Fig. 3I), were treated with either ligand alone (Fig. 3, A–D) or first incubated with sphingosine (10 μM), an inhibitor of PKC activation, for 15 minutes (Fig. 3, E–H). Afterward, 5-HT, DA, DOI, and clozapine (100 nM) were added in the continued presence of sphingosine. In accordance with previous observations, sphingosine inhibited 5-HT-mediated receptor internalization almost entirely (Fig. 3E), but not DA-mediated internalization (Fig. 3F). Further, DOI-mediated 5-HT$_{2A}$ internalization was also completely prevented (Fig. 3G). Inverse agonist clozapine, however, effected receptor trafficking independent of PKC inhibition (Fig. 3H). We quantified the effects of PKC activation (using PMA) and inhibition (Fig. 3J); the gray bars represent internalization without sphingosine, and the black bars represent levels of internalization when cells were preincubated in sphingosine.

**Abrogation of a Putative PKC Phosphorylation Site (S291) in the 5-HT$_{2A}$ Receptor Inhibits Receptor Internalization Mediated by 5-HT and DOI but Not Clozapine or Dopamine.** As PKC plays a role in receptor trafficking, we hypothesized there could be a PKC-dependent phosphorylation of the receptor. Using various prediction algorithms online (Amanchy et al., 2007; Blom et al., 1999, 2004; Blom et al., 2004; Xue et al., 2006), we identified four putative PKC phosphorylation sites on the receptor. Individually mutating each of these sites, we identified one site, serine 291, which when altered to an alanine (5-HT$_{2A}$-S291A-EGFP) abolished PKC-mediated internalization. This residue within a peptide segment of the receptor was also shown to be phosphorylated by PKC in an in vitro assay (Turner and Raymond, 2005). HEK293 cells stably expressing the 5-HT$_{2A}$-S291A-EGFP receptor were cleared of internal fluorescence and treated with each of the four ligands mentioned (Fig. 4, A–D). This single-point mutation completely inhibited 5-HT$_{2A}$ internalization by 5-HT (Fig. 4A) and DOI (Fig. 4C) but behaved exactly as the wild type vis-à-vis its internalization by DA (Fig. 4B) and clozapine (Fig. 4D).

These data were in accordance with our observations regarding the effects of inhibition of PKC activation on ligand-mediated receptor internalization. The endocytic process is unaffected in the case of those ligands that did not require PKC activation to bring about receptor trafficking, but ligands that did require PKC activation were unable to effect internalization of the 5-HT$_{2A}$-S291A.

Ligand-independent 5-HT$_{2A}$ internalization can be brought about by activation of PKC (Bhattacharyya et al., 2002, 2006) using PMA. If receptor phosphorylation by PKC was required to initiate 5-HT$_{2A}$ trafficking, we predicted that PKC-phosphorylation-deficient 5-HT$_{2A}$-S291A would be insensitive to PMA-mediated internalization. Consistent with our prediction,
when PKC was activated using 4 nM PMA, the modified receptor showed no detectable internalization (Fig. 4E).

**Time Course of 5-HT_{2A} Recycling Is Ligand Dependent.** We proceeded to follow 5-HT_{2A} to determine its postendocytic fate. SB1 cells were treated with ligand for 15 minutes and kept at 37°C for various times, then fixed and imaged. As described earlier, receptors internalized in response to both 5-HT and DA were completely recycled in 2.5 hours (Bhattacharyya et al., 2006). The cells treated with 5-HT (Fig. 5A) showed the presence of an intracellular pool of receptors 2 hours after ligand washout (Fig. 5B). This intracellular receptor is localized to transferrin-positive endosomes (data not shown) but by 2.5 hours, all receptor-associated fluorescence was membrane localized (Fig. 5C). Similar results were seen with DA (Fig. 5, D–F).

Receptor recycling was also observed when receptor-expressing cells were treated with the synthetic ligand agonist DOI (Fig. 5, G–I). Again, SB1 cells were pulsed for 15 minutes with the ligand and chased for different time points up to 8 hours. Instead of the 2.5 hours taken for receptors to recycle when 5-HT or DA was used, receptors were resident in transferrin-labeled endosomes for 5 hours extra (Fig. 5, H and K). Recycling of DOI-internalized receptors was complete in 7.5 hours. Receptor recycling was quantified for 5-HT, DA, DOI, and ketanserin by ELISA (Fig. 6M).

A simplistic explanation that could account for shorter 5-HT_{2A} recycling times in response to 5-HT or DA is their relatively quick degradation in comparison with clozapine or DOI. To rule this out, we quantified internalization/recycling in the presence of an antioxidant (ascorbic acid) to reduce DA oxidation. The 5-HT_{2A} recycling brought about by 5-HT and DA was unaffected by the presence of 2 mM ascorbic acid (Supplemental Fig. 3). In addition, the time taken for 5-HT_{2A} recycling in response to clozapine was independent of the concentration of ligand used. All concentrations tested, from 4 nM to 10 μM clozapine, brought about recycling in 7.5 hours (data not shown).
Protein Phosphatase 2A Is Required for Receptor Recycling in the Case of Ligands That Bring about PKC-Dependent Receptor Internalization. As PKC activation and hence potential receptor phosphorylation are important steps in 5-HT2A internalization by 5-HT and DOI, we checked for the involvement of enzymes active in the endosome that would be required to return receptors to a form that is competent to recycle by dephosphorylating them. Inhibiting endosome acidification using ammonium chloride (2 mM) inhibited recycling of receptors that had been internalized by 5-HT (Supplemental Fig. 4, B–D) or by PKC activation (PMA 4 nM, data not shown) but not for receptors internalized by dopamine (Supplemental Fig. 4, E–G).

The pH-sensitive, endosome-localized enzymes that may have a role in the process of receptor recycling internalized in a phosphorylation-dependent manner, including protein phosphatase 2A (PP2A) (Krueger et al., 1997). We inhibited PP2A activity using the specific inhibitor okadaic acid at 10 nM. The cells were treated with 5-HT (Fig. 6A), DA (Fig. 6D), DOI (Fig. 6G), or clozapine (Fig. 6J). Again, the cells were observed for 2.5 hours (5-HT and DA) in the absence (Fig. 6, B and E) or presence (Fig. 6, C and F) of okadaic acid. With DOI treatment, the cells were observed for 7.5 hours in the absence (Fig. 6H) or presence (Fig. 6I) of okadaic acid. In accordance with our observations thus far, the receptors that internalized via a PKC-dependent mechanism (in response to 5-HT and DOI) also required PP2A activity to recycle, and those internalized by DA and clozapine resisted treatment by the PP2A inhibitor. A quantitative measure inhibition of recycling by PP2A is included in Fig. 6M.

A qualitative description of 5-HT2A recycling in response to clozapine seemed to show that PP2A has no role in receptor recycling as recycling appears complete within 7.5 hours in the absence (Fig. 6K) or presence (Fig. 6L) of okadaic acid.

As an estimate of the critical time period during which PP2A might be acting to dephosphorylate the receptor, we treated SB1 cells with 5-HT and added okadaic acid at different times after this treatment. Up to 60 minutes after addition of 5-HT, PP2A inhibition was able to block 5-HT2A recycling. At 90 minutes after addition of the ligand, however, okadaic acid treatment had no effect on recycling. It would seem that receptor dephosphorylation is complete between 60 and 90 minutes after endocytosis (Supplemental Fig. 5).
Recycling of PMA-Internalized 5-HT2A Is Also PP2A Dependent. When SB1 cells cleared of internal fluorescence (Fig. 7A) were incubated in 4 nM PMA to activate PKC, wild-type receptor was internalized (Fig. 7B), and receptor recycling was complete in 2.5 hours (Fig. 7C) in a manner similar to that seen with activation of the receptor by 5-HT. Again, we predicted receptor recycling after PMA-mediated internalization would be PP2A dependent. In agreement with this prediction, incubation of cells with 10 nM okadaic acid inhibited receptor recycling after internalization using PMA (Fig. 7D).

Discussion

Although functional selectivity in receptor signaling has been known for the better part of two decades (Berg et al., 1998; Clarke and Bond, 1998; Ghanouni et al., 2001; Gonzalez-Maeso et al., 2003; Urban et al., 2007), our appreciation of how this feeds back to modulating the receptor itself is limited. By use of 5-HT2A, we have studied the intracellular sorting of a GPCR after the interaction of its cognate ligand 5-HT and four other ligands: partial-efficacy agonist DA, synthetic agonist DOI, antagonist ketanserin, and inverse agonist clozapine. Ligand-specific regulation of 5-HT2A trafficking must arise as a direct result of functionally selective signaling at the receptor. With the time course of recycling and the involvement of PKC-mediated receptor phosphorylation in trafficking as functional readouts, the results described herein have been used to characterize functional selectivity at the 5-HT2A receptor and to describe the tightly controlled regulation of GPCR endocytosis and recycling by multiple ligands.

In brief, using endogenous or therapeutically significant GPCR-ligand interactions, we have shown that 1) every ligand tested elicited a unique or “functionally selective” set of behaviors at the 5-HT2A receptor, 2) GPCR functional selectivity acts at both proximal and distal signaling events and can feed back to the receptor by modulating its spatiotemporal functioning, and 3) 5-HT2A may be phosphorylated in a ligand-dependent manner and that this PKC-mediated phosphorylation acts as a regulatory step in 5-HT2A trafficking.

Four of five ligands tested (5-HT, DA, DOI, and clozapine) brought about receptor internalization and subsequent receptor recycling. As the inverse agonist clozapine caused receptor endocytosis, it is clear that 5-HT2A endocytosis was independent of receptor activation at its canonical intracellular transduction cascade: IP3-mediated Ca2+ release from the endoplasmic reticulum. The lack of 5-HT2A endocytosis in response to ketanserin was in accordance with previous observations in two cell lines where treatment of 5-HT2A-expressing cells with a 10-minute pulse of ketanserin brought about little to no receptor internalization (Berry et al., 1996; Hanley and Hensler, 2002). Likewise, clozapine too has been shown to induce 5-HT2A endocytosis in multiple cell lines and even in vivo (Bhatnagar et al., 2001; Willins et al., 1998, 1999).

Studies have shown that rat 5-HT2A is not phosphorylated by GRK as are many other GPCRs (Gray et al., 2001). Given the lack of GRK involvement, it has been believed that 5-HT2A endocytosis occurs independently of ligand-mediated phosphorylation. It has been difficult to probe for and identify 5-HT2A phosphorylation (Sheffler et al., 2006), although it has been identified that ribosomal S6 kinase phosphorylates the receptor and modifies its signaling. PKC has also been shown to phosphorylate 5-HT2A at two specific residues in vitro and could regulate intracellular signaling via the receptor (Turner and Raymond, 2005; Turner et al., 2007). We chose to better characterize the effects of previously described PKC-mediated phosphorylation of the 5-HT2A. We observed the effect of this phosphorylation at one of these residues, serine 291, and its effect on modulation of receptor trafficking. We have demonstrated a ligand-dependent requirement for PKC phosphorylation at this residue to bring about endocytosis and concordantly for PP2A to allow for receptor dephosphorylation and recycling. Those ligands that require PKC activation to bring about receptor internalization also require PP2A-mediated dephosphorylation before receptor recycling can occur. Mutation of the serine 291 to an alanine to prevent phosphorylation by PKC mimicked the effect of PKC-inhibition on internalization. Rat 5-HT2A is thus a useful model GPCR to study GRK-independent, PKC-dependent regulation of receptor endocytosis and recycling.

The physiologic functions of 5-HT2A trafficking/recycling have not been identified, but it is clear that different ligands display functional selectivity at the receptor in terms of events proximal to receptor-ligand binding. Here, we show that distal effects—recycling over 2.5 to 7.5 hours—are also differentially modulated in a ligand-dependent manner.

Conventionally, GPCR recycling has been thought to be a nonregulated process. It is only recently that some processes involved specifically in the regulation of receptor recycling...
have been described. Our description of ligand-dependence in the time course of receptor recycling provides evidence that recycling is a tightly regulated, functionally significant process. 5-HT and DA bring about complete receptor recycling in 2.5 hours, but DOI and clomipramine treatment cause the receptor to be resident in transferrin-positive endosomes for 7.5 hours. Identification of ligand-specific signaling that defines the time course of receptor recycling would be critical for a better understanding of how drugs and other synthetic ligands act at the receptor.

In vivo, when an antipsychotic drug such as clozapine is administered, 5-HT2A would be exposed to both endogenous ligands as well as the antipsychotic drug. Regulation of intracellular trafficking of a single receptor with two disparate time courses of recycling in response to two structurally and functionally different ligands is completely uncharacterized. A recent report describes two separate GPCRs in receptor-dependent sorting of GPCRs from the same endosome (Puthevenedu et al., 2010). Functional selectivity in 5-HT2A trafficking with such widely varying time courses of recycling could offer the ideal model system to study the sorting of a single GPCR from a single endosome in response to multiple ligands. It would appear that (for 5-HT and DOI) phosphorylation/desensitization do not dictate the dynamics of recycling but are essential processes. In other words, these processes are not rate-limiting steps during 5-HT2A recycling. For clozapine and dopamine, however, phosphorylation/desensitization (by PKC) do not seem to play a role in recycling.

Given that 5-HT2A desensitization is independent of receptor endocytosis in HEK293 cells (Gray et al., 2001), it is unknown what functions internalization may mediate. It is probable that the receptor when localized to endosomes interacts with a different set of molecular players than those at the cell membrane and exhibits an altered behavior from that at the surface. This modulation of intracellular cascades—endoosomal signaling or the formation of a “signalsome” (Calebiro et al., 2009; Rosen et al., 2009)—has conventionally been associated with β-arrestin-2, a molecule involved in receptor internalization as well as functioning as a scaffold for MAPK signaling (Tolgo et al., 2003), probably in intracellular locations. Since rat 5-HT2A receptor endocytosis is arrestin independent in HEK cells, the receptor could serve as a good model to study endosomal signaling that does not involve arrestin. Studies with the μ-opioid receptor show an inverse correlation between the ability of a ligand to induce endocytosis and its propensity to promote physiological tolerance (Jordan and Devi, 1999; Rozenfeld and Devi, 2011; Whistler, 2012; Whistler et al., 1999). A similar characterization of the functional significance of ligand-specific modulation of internalization and recycling of the 5-HT2A should reveal interesting modes of regulation of GPCR function.

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**Performed data analysis:** Raote, Bhattacharrya, Panicker.

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