Considerable insight has been garnered on initial mechanisms of endocytosis of plasma membrane proteins and their subsequent trafficking through the endosomal compartment. It is also well established that ligand stimulation of many plasma membrane receptors leads to their internalization. However, stimulus-induced regulation of endosomal trafficking has not received much attention. In previous studies, we showed that sustained stimulation of protein kinase C (PKC) with phorbol esters led to sequestration of recycling endosomes in a juxtanuclear region. In this study, we investigated whether G-protein-coupled receptors that activate PKC exerted effects on endosomal trafficking. Stimulation of cells with serotonin (5-hydroxytryptamine (5-HT)) led to sequestration of the 5-HT receptor (5-HT2AR) into a Rab11-positive juxtanuclear compartment. This sequestration coincided with translocation of PKC as shown by confocal microscopy. Mechanistically the observed sequestration of 5-HT2AR was shown to require continuous PKC activity because it was inhibited by pretreatment with classical PKC inhibitor Gö6976 and could be reversed by posttreatment with this inhibitor. In addition, classical PKC autophosphorylation was necessary for receptor sequestration. Moreover inhibition of phospholipase D (PLD) activity and inhibition of PLD1 and PLD2 using dominant negative constructs also prevented this process. Functionally this sequestration did not affect receptor desensitization or resensitization as measured by intracellular calcium increase. However, the PKC- and PLD-dependent sequestration of receptors resulted in co-sequestration of other plasma membrane proteins and receptors as shown for epidermal growth factor receptor and protease activated receptor-1. This led to heterologous desensitization of those receptors and diverted their cellular fate by protecting them from agonist-induced degradation. Taken together, these results demonstrate a novel role for sustained receptor stimulation in regulation of intracellular trafficking, and this process requires sustained stimulation of PKC and PLD.

The protein kinase C (PKC) family of enzymes comprises 11 isoforms of serine/threonine kinases (1, 2) implicated in regulation of cell growth, differentiation, apoptosis, secretion, neurotransmission, and signal transduction (3–5). During the course of studying PKC, we showed that sustained stimulation of PKC with phorbol esters leads to translocation of classical PKC (cPKC) to a pericentrosomal region (6, 7). This sequestration was shown to be PLD-dependent (8, 9) and negatively regulated by ceramide formed from the salvage pathway (10). Ceramide inhibits autophosphorylation of cPKC, which was also found to be required for this novel translocation (11). Importantly sustained activation of cPKC also resulted in significant effects on recycling components and their sequestration to the same region, dubbed the pericentron (defined as the cPKC-dependent subset of recycling endosomes). On the other hand, components and markers of the endolysosomal compartment were not sequestered to the pericentron upon PKC stimulation (7). Functionally it was also shown that pericentron formation and sequestration of PKC requires clathrin-dependent endocytosis. Most importantly, formation of the pericentron is dynamic and reversible and requires continuous activation of PKC.

G-protein-coupled receptors (GPCRs) are the largest family of integral membrane receptors. They contain seven transmembrane domains (12), are coupled to heterotrimeric G-proteins, and are activated by a vast number of ligands. They regulate many cellular processes and serve as targets for at least half of the therapeutics currently present on the market. Upon agonist binding, conformational changes in the receptor lead to coupling with G-proteins (composed of α, β, and γ subunits). This leads to dissociation of α and βγ subunits that mediate downstream signaling (13). Interestingly PKC serves as one of the downstream targets of GPCRs. Thus, it became critical to determine whether persistent stimulation of receptors that couple to cPKC exerts effects on recycling endosomes. We focused on the serotonin (5-HT) 5-HT2A receptor (5-HT2AR)
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and the angiotensin II receptor (AT1AR) as two GPCRs that couple to \( \mathrm{G}_{\alpha}\), which in turn activates phospholipase \( \mathrm{C}\beta \) and then PKC (14, 15).

In this study, we show that sustained stimulation of those receptors led to their sequestration in a PKC- and PLD-dependent manner. Most importantly, this led to global sequestration of endosomes with profound effects on other membrane receptors. Epidermal growth factor receptor (EGFR) and protease activated receptor-1 (PAR-1) are known to be targeted into a degradative pathway upon their agonist treatment (16–18). Interestingly 5-HT induced co-sequestration of those receptors with 5-HT\(_{2A}\)R and protected them from degradation upon their own agonist treatment. The implications of these results on regulation of trafficking by GPCRs are discussed.

**EXPERIMENTAL PROCEDURES**

**Materials**—Minimal essential medium and high glucose Dulbecco’s modified Eagle’s medium were from Invitrogen. The HEK293 and C6 cell lines were purchased from American Type Culture Collection (Manassas, VA). 4\(\beta\)-Phorbol 12-myristate-13-acetate (PMA), G60976, and brefeldin A were purchased from Calbiochem. Agonist peptide (H-SFLLRN-NH\(_2\)) was from AnaSpec (San Jose, CA). Alexa Fluor 555-transferrin and Flu-3 were purchased from Molecular Probes (Eugene, OR). Anti-giantin antibody was from Covance Research Products, Inc. (Berkeley, CA). Anti-Rab11 and anti-green fluorescent protein (GFP) antibody were from Zymed Laboratories Inc./Invitrogen. Anti-EEA1 and anti-PKC\(\alpha\) antibody were from BD Biosciences. Anti-HA antibody was from Roche Applied Science. Anti-EGFR antibody was from Upstate Biotechnology (Lake Placid, NY). Polyclonal rabbit antibody specific for phosphorylated PKCa\(\beta\)I at Thr-638/641 was from Cell Signaling Technology (Danvers, MA). Anti-Lamp1 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-CD59 antibody was a generous gift from Dr. Stephen Tomlinson (Medical University of South Carolina, Charleston, SC). Serotonin, ketanserin, epidermal growth factor, and all other chemicals were from Sigma.

**Cell Culture**—HEK293 cells were maintained in minimal essential medium supplemented with 10% (v/v) fetal bovine serum in a 5% CO$_2$ incubator at 37 °C. Cells were passaged every 3–4 days to maintain cells in logarithmic growth. C6 glioma cells were grown in high glucose Dulbecco’s modified Eagle’s medium and maintained as above.

**Plasmids**—All recombinant DNA procedures were carried out following standard protocols. The wild type pBK-CMV-GFP-PKCBII was described previously (19). PKCBII-mCherry was described previously (11). The 5-HT\(_{2A}\)R-yellow fluorescent protein (YFP) receptor construct was generated by cloning the cDNA sequence of human 5-HT\(_{2A}\)R into XhoI and BamHI sites of EYFP-N1 vector (Clontech, BD Biosciences). The HA-PAR1 was a gift from Dr. JoAnn Trejo (University of California, San Diego, CA). The cells with stable expression of AT1AR-GFP were a gift from Dr. Thomas A. Morinelli (Medical University of South Carolina, Charleston, SC). The GFP-tagged Rab11 was kindly provided by Dr. Marino Zerial (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). The HA-tagged PLD1 and PLD2 wild type (WT) and mutants of PLD1 (K898R) and PLD2 (K758R) were a gift from Drs. Michael Frohman and Guangwei Du (Stony Brook University, New York, NY).

**Western Blotting**—Protein samples were boiled for 10 min in reducing SDS sample buffer and separated by 4–20% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes, blocked with PBS, 0.1% Tween 20 containing 5% nonfat dried milk; washed with PBS-Tween; and incubated overnight at 4 °C with rabbit polyclonal antibody for phospho-PKC\(\alpha\)/\(\beta\)I (1:1000), mouse monoclonal antibody for PKC\(\alpha\) (1:2000), sheep polyclonal antibody for EGFR (1:2000), or rabbit polyclonal antibody for GFP (1:1000) in PBS, 0.1% Tween 20 containing either 5% nonfat dried milk or 5% bovine serum albumin. The blots were washed with PBS-Tween and incubated with secondary antibody conjugated with horseradish peroxidase in PBS-Tween containing 5% nonfat dried milk. Detection was performed using enhanced chemiluminescence reagent (Pierce).

**Intracellular Calcium Measurements**—Intracellular calcium release was measured by a fluorometric imaging plate reader (FLIPR) (Molecular Devices, Sunnyvale, CA). HEK293 cells were transfected and 24 h after transfection subcultured into 96-well flat bottom plates coated with poly-d-lysine (Greiner BioOne). After treatment, cells were loaded with the intracellular fluorescent calcium probe Fluo-3 (excitation, 488 nm; emission, 540 nm; Molecular Probes) in a loading buffer (Hanks’ balanced salt solution, 20 mm HEPES, 2.5 mm proprionate) for 1 h at 37 °C with or without pretreatments. After washing twice with loading buffer, cells were placed onto the FLIPR and exposed to agonists for 5 min. Increases in intracellular calcium were reflected by increases in detected fluorescence.

**Cleavable Biotin Recycling Assay**—After starvation, HEK293 cells overexpressing 5-HT\(_{2A}\)R-YFP were washed once with ice-cold PBS for 5 min. Next cells were incubated with 0.5 mg/ml (in PBS) sulfo-NHS-SS-biotin (Pierce) for 30 min on ice. After
incubation, excess biotin was quenched by washing with ice-cold 15 mM glycine. Next, cells were washed with prewarmed minimal essential medium, 0.1% bovine serum albumin containing 100 μg/ml cycloheximide and incubated for 1 h at 37 °C before treatment. All treatment conditions were done in duplicate. To measure total biotin associated with the cells, one set of samples was harvested immediately after treatment. To measure the internalized biotin, the second set of samples was stripped from cell surface biotin by washing three times for 5 min with ice-cold GSH cleavage buffer (50 mM GSH, 75 mM NaCl, 1 mM EDTA, 1% bovine serum albumin, 0.75% 10 N NaOH). Cell lysates were subjected to immunoprecipitation with streptavidin-agarose (Novagen, San Diego, CA) and analyzed by Western blot to detect 5-HT2AR-YFP. The amount of internalized receptor was determined as a percentage of total cell-associated biotin.

Statistics—Statistical significance was determined using one-way analysis of variance with Bonferroni post-test (GraphPad Prism, version 4). p values lower than 0.05 were considered to be statistically significant.

RESULTS

Continuous Exposure to Agonist Induces Sequestration of 5-HT2AR Receptors into the Rab11-positive Compartment—The agonist-induced internalization of the 5-HT2AR has been reported recently (20); however, the detailed trafficking of 5-HT2AR following continuous exposure to its natural ligand, 5-HT, has not yet been elucidated. To study the effects of 5-HT more closely, confocal microscopy studies were performed in HEK293 cells transiently transfected with 5-HT2AR-YFP. Immediately after agonist treatment (1 min), 5-HT2AR underwent endocytosis; however, continuous treatment with agonist (30 min to 2 h) induced redistribution of internalized 5-HT2AR and its sequestration in a perinuclear compartment (Fig. 1A and supplemental Fig. S1). Similar sequestration was observed for the AT1AR upon sustained ligand action (Fig. 1B). To study cellular localization of sequestered receptors, colocalization with markers of different compartments was performed. The sequestered 5-HT2AR colocalized with, but extended beyond, Rab11 (Fig. 1C), a marker of the perinuclear recycling compartment. There was no colocalization with Lamp1 (lysosomal marker) or giantin (Golgi marker). Brefeldin A, which disrupts the Golgi, did not inhibit 5-HT2AR sequestration (Fig. 1C). Taken together, sustained stimulation of receptors leads to their sequestration into a perinuclear recycling compartment.

Sequestration of 5-HT2AR Receptors Coincides with Sequestration of cPKC to the Pericentriol and Requires Sustained PKC Activity—A previous study implicated PKC in agonist-induced 5-HT2AR internalization (20), and our work has established a critical role for cPKC in the sequestration of recycling molecules into a subset of endosomes in the perinuclear region (6, 7). In addition, it was demonstrated previously that sustained activation of cPKC by PMA not only caused sequestration of recycling components but also led to a concomitant translocation of cPKCa and -βII but not -βI into the pericentriol (8). These findings prompted us to investigate whether the observed 5-HT-induced sequestration of receptors involves cPKC. In control cells, the 5-HT2AR was predominantly present on the cell surface, whereas cPKC localized in the cytosol. 5-HT induced rapid internalization of 5-HT2AR and a translocation of cPKC to the plasma membrane. More prolonged agonist treatment brought about the sequestration of the 5-HT2AR but also caused some co-sequestration of PKCβII (Fig. 2A).

Similar observations were made in C6 glioma cells that harbor endogenous 5-HT2AR; when C6 cells were transfected with GFP-PKCBII and treated with 5-HT for 2 h, sequestration of PKC to the pericentriol was also observed (Fig. 2B). When cells were co-transfected with GFP-tagged Rab11 and PKCBII-mCherry, colocalization of PKC with Rab11 compartment was observed (Fig. 2B, lower panel).

Next the role of cPKC in the agonist-induced 5-HT2AR sequestration was evaluated. Pretreatment of cells with the
cPKC inhibitor Gö6976 prevented sequestration of 5-HT₂A receptors (Fig. 3A, PRE). It should be noted that following treatment with Gö6976 5-HT₂A receptors were detected in vesicular structures close to the plasma membrane that colocalized with the early endosomal marker EEA1 (Fig. 3A). In addition, to quantitate the amount of internalized receptor, a cleavable biotin recycling assay was performed. It was observed that after 2 h of 5-HT treatment, a significant amount of the receptor was internalized. Pretreatment of cells with Gö6976 only moderately decreased the amount of internalized receptor (Fig. 3B). Thus, cPKC appears to be required for sequestration of receptors and partially involved in internalization but not absolutely required for the latter.

To determine whether activation of PKC is sufficient to induce sequestration of 5-HT₂A receptors, cells were treated with the phorbol ester PMA. This treatment caused a robust translocation of both PKCβII and 5-HT₂A receptors to the pericentriol (Fig. 3C). Thus, activation of PKC is necessary and sufficient to induce the sequestration of GPCRs.

Next the effects of persistent ligand action on cPKC activity were determined by evaluating the phosphorylation of PKCα/βII on Thr-638/641. The results demonstrated that treatment of C6 and HEK293 cells with agonist caused an increase in the phosphorylation of Thr-638/641 that persisted for up to 2 h of treatment (Fig. 3D), thus demonstrating sustained activation of cPKC.

Importantly treatment of cells with the cPKC inhibitor following ligand-induced sequestration (Fig. 3A, POST) also caused dispersal of the sequestered 5-HT₂A receptors. Thus, sustained activity of cPKC is required for maintaining sequestration of receptors.

To further elucidate the role of cPKC phosphorylation in 5-HT₂A receptor sequestration, PKCβII autophosphorylation sites (Thr-641 and Ser-660) were mutated to alanine, thus generating a double alanine (DA) mutant of PKCβII. 5-HT stimulation of cells co-transfected with 5-HT₂A and WT PKCβII caused perinuclear sequestration of both 5-HT₂A and PKC (Fig. 3E). However, when cells were co-transfected with the 5-HT₂A receptor and the DA mutant of PKCβII, neither PKC nor the receptor was sequestered to the perinuclear region, although the DA mutant did translocate to the plasma membrane. When DA-PKCβII was expressed alone it showed a cytoplasmic distribution, typically observed for PKC in unstimulated cells (Fig. 3E, bottom panel). However, when coexpressed together with 5-HT₂A, there was a considerable amount of the DA-PKCβII localized to the membrane without agonist stimulation. It was previously shown that PKCβII mutated at S660A,T641A and mutated at both autophosphorylation sites (DA) does not dissociate efficiently from the membrane after activation (19). Because 5-HT₂A-YFP has a small amount of constitutive activity even in the absence of agonist, the results suggest that DA-PKCβII is activated in cells coexpressing 5-HT₂A. In
accordance with that, the 5-HT$_{2A}$ antagonist ketanserin completely prevented this basal localization of DA-PKC II to the plasma membrane (Fig. 3E, lower panel). It should be noted that although the DA mutant completely blocked 5-HT-induced 5-HT$_{2A}$R sequestration, a fraction of receptors was internalized and localized to the early endosomes (data not shown). Taken together, these results imply an important role for cPKC in the regulation of 5-HT$_{2A}$R sequestration into the pericentriol.
PLD Activity Is Required for Receptor Sequestration

—It was shown previously that PLD activation was required for translocation of PKCα/βII and formation of the pericentron (8). To further define the mechanism of receptor sequestration, it became important to determine whether the PLD pathway was involved in agonist-induced 5-HT2AR sequestration. Inhibition of PLD with 1-butanol abolished the perinuclear sequestration of receptors; however, treatment with 2-butanol, a positional isomer of 1-butanol that does not inhibit PLD, was without effect (Fig. 4A). Although 1-butanol prevented 5-HT2AR sequestration, a significant portion of the receptor became located in the EEA1-positive early endosomes (Fig. 4A).

To further corroborate a role for PLD and to determine which PLD isoform is involved in receptor sequestration, cells were co-transfected with 5-HT2AR-YFP and wild type or dominant negative (KR) PLD1 or PLD2. Interestingly dominant negative versions of both isoforms inhibited sequestration of receptors, and receptors were present on the cell surface and in dispersed endosomes (Fig. 4B). Taken together, these results suggest that activity of both PLD isoforms is required for 5HT-induced 5-HT2AR sequestration into the pericentrum.

Inhibition of 5-HT2AR Sequestration Has No Effect on Desensitization or Resensitization of Intracellular Calcium Response to Agonist

—Because the above results implicate cPKC and PLD in sequestration of receptors, it became important to investigate whether sequestration played any role in receptor desensitization or resensitization. To study 5-HT2AR responsiveness to 5-HT, a FLIPR assay that measures changes in the concentration of cytoplasmic calcium was used. Interestingly the rates of 5-HT2AR desensitization or resensitization were not affected by overexpression of autophosphorylation-deficient (DA) or inactive (KR) forms of PKCβII (Fig. 5), implying that 5-HT2AR sequestration has no effect on receptor desensitization or resensitization as measured by changes in the concentration of cytoplasmic calcium.

5-HT Induces Global Sequestration of Molecules to Perinuclear Recycling Compartment and Can Affect Cellular Fate of Other Membrane Receptors

—Because sustained stimulation with 5-HT resulted in sequestration of both receptors and
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cPKC to the perinuclear region, it became critical to determine whether agonist treatment caused sequestration of other recycling molecules (e.g. CD59 and transferrin). Stimulation of cells with 5-HT induced significant co-sequestration of both receptors and CD59, a complement regulatory protein, as well as fluorescent transferrin into the perinuclear region (Fig. 6, A and B). Thus, sustained stimulation of receptors leads to sequestration of recycling molecules. Treatment with cPKC and PLD inhibitors efficiently blocked sequestration of both CD59 and transferrin in response to 5HT, suggesting that agonist-induced cPKC/PLD-dependent 5-HT2AR sequestration globally affects the intracellular trafficking of recycling membrane components.

The significance of 5-HT-induced formation of the pericentriol in regulation of trafficking and signaling of other membrane receptors was evaluated next, and for these studies, the effects of 5-HT on the endogenous EGFR were examined because PMA has been shown to regulate the trafficking of EGFR. Treatment with 5-HT led to the simultaneous sequestration of both 5-HT2AR and the EGFR in the perinuclear region (Fig. 7A). Moreover this translocation occurred in a cPKC- and PLD-dependent manner because it was inhibited by both Gö6976 and 1-butanol (Fig. 7A).

Interestingly it has been shown previously that activation of PKC with PMA diverts internalized EGFR from a degradative to a recycling pathway(s) (21). Thus, we wondered whether the 5-HT2AR displayed the capacity to regulate the fate of the EGFR. When cells were treated with epidermal growth factor, there was significant loss of the EGFR protein; however, pretreatment with either PMA or 5-HT prevented/ameliorated the loss of the EGFR (Fig. 7B). Importantly the protective effects of PMA and 5-HT were inhibited by 1-butanol (Fig. 7B), whereas the secondary alcohol (2-butanol) did not have any effect (data not shown).

PAR-1 provides an example of a GPCR that is rapidly internalized and degraded following its activation (17, 18), and thus, it was chosen for additional study. First we confirmed that treatment with the PAR-1 agonist peptide (SFLLRN) led to significant degradation of PAR-1, whereas PMA caused sequestration of PAR-1 as analyzed by confocal microscopy (Fig. 8A). Most interestingly, however, treatment of cells with 5-HT led to co-sequestration of both receptors in a cPKC- and PLD-dependent manner, and when cells were pretreated with 5-HT, SFLLRN did not induce degradation (Fig. 8B). Taken together, these results indicate that sustained stimulation with 5-HT changes the cellular fate of membrane receptors that are normally targeted for degradation.

DISCUSSION

The results from this study reveal dramatic effects of sustained stimulation of cPKC by GPCRs on the trafficking of several proteins leading to their sequestration in the perinuclear region. It is shown that

FIGURE 5. Inhibition of 5-HT2AR sequestration has no effect on desensitization or resensitization of intracellular calcium increase. HEK293 cells co-transfected with 5-HT2AR-YFP and WT, kinase-dead (KR), or DA PKCβII were treated with 10 μM 5-HT for the indicated time points (A) or treated with 10 μM 5-HT for 2 h, washed, and incubated in 5-HT-free medium for the indicated time points (B). Cells were then loaded with the intracellular fluorescent calcium probe Fluo-3, placed into a FLIPR, and exposed to 10 μM 5-HT for 5 min. Data shown are presented as percentage of control (calcium increase following treatment with 10 μM 5-HT for 5 min) and are representative of three independent experiments. AU, arbitrary units.

FIGURE 6. 5-HT induces global sequestration of molecules to the pericentriol. A, HEK293 cells overexpressing 5-HT2AR-YFP (green) were treated with 10 μM 5-HT for 2 h with or without 1-3 μM Gö6976 or 15-min 0.4% 1-butanol (1-BUT) pretreatment (PRE). Cells were then fixed and stained with anti-CD59 antibody (red). B, HEK293 cells were treated as above in the presence of 10 μg/ml transferrin-Alexa Fluor 555 (AF-Tf) (red). The bar represents 10 μm.
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sustained stimulation of G_12/13-coupled GPCRs leads to their sequestration and trafficking. This sequestration was cPKC-dependent and required continuous cPKC activation and PLD activity. Importantly, receptor stimulation and sequestration globally affected cellular trafficking by sequestration of recycling endosomes and changed the cellular fate of EGFR and PAR-1 by diverting their trafficking and thus protecting them from degradation (Fig. 9).

Many studies have implicated PKC in the internalization of specific receptors, transporters, and channels (22–32). Some of these studies have focused on direct phosphorylation of these molecules by PKC. Although the current studies do not rule out a role for direct phosphorylation by PKC in mediating specific effects, the results point to a more global cPKC-dependent process by which sustained activation of cPKC regulates endosomal trafficking. Sustained agonist treatment led to activation of cPKC and resulted in receptor and cPKC sequestration in a PKC-dependent subset of the Rab11 compartment, the pericentrum. This sequestration was dependent on continuous cPKC activity. Pretreatment with cPKC inhibitor prevented receptors sequestration, whereas posttreatment led to dispersal of sequestered receptors. It needs to be emphasized that internalization of receptors was not completely blocked because some receptors were still observed in early endosomes. Similarly internalization of the biotinylated receptor was only partially inhibited when cells were pretreated with cPKC inhibitor.

The observed process also requires PLD as a further downstream target of cPKC activation, suggesting that either phosphatidic acid (the product of the PLD reaction) or subsequent lipid metabolites are required for this novel endosomal trafficking. It was shown previously that PLD2 was required for endocytosis of angiotensin receptor (33). Activity of PLD2 was also shown to be important for endocytosis, desensitization, and resensitization of μ-opioid receptor, and it was shown to directly interact with this receptor (34, 35). It is shown here that both PLD1 and PLD2 were involved in sequestration of 5-HT_2A receptor. Because PLD1 and PLD2 have different cellular localization (33) it is possible that they control different steps of the observed sequestration. PLD2 localized mainly on the cell surface (33), whereas PLD1 was shown to be present in recycling endosomes and to colocalize with sequestered PKC (9). It can be speculated that PLD2 is responsible for the initial step, whereas PLD1 is responsible for the distal steps of sequestration, but this idea has to be investigated further. It cannot be excluded that there is a direct interaction between one or both isoforms of PLD and receptors during the sequestration. Indeed colocalization of 5-HT_2A receptor with PLD2 was observed in control cells, whereas colocalization with PLD1 was observed upon receptor sequestration.

To control GPCR signaling initiated by agonist, receptors undergo desensitization, a key process that prevents pathologies that would result from sustained receptor signaling. This can occur through several mechanisms (36). It can be accomplished through phosphorylation of GPCRs by GPCR kinases (37) that leads to binding of another group of proteins, arrestins (38). This uncouples GPCRs from G-proteins and facilitates receptor internalization. Receptor trafficking is also important for regulation of their activity (39). Internalization of 5-HT_2A receptor was shown to be important in receptor desensitization (40, 41) in some cell types. Published studies suggest that the role of

**FIGURE 7. Effect of 5-HT-induced sequestration on fate of EGFR.** A, HEK293 cells transfected with 5-HT_2A-R-YFP (green) were treated with 10 μM 5-HT for 2 h with or without 1 h 3 μM G06976 or 15 min 0.4% 1-butanol (1-BUT) pretreatment (PRE). After fixation and permeabilization, immunofluorescence against endogenous EGFR (red) was performed, and cells were analyzed by confocal microscopy. The bar represents 10 μm. B, HEK293 cells transfected with 5-HT_2A-R-YFP were treated with 2 ng/ml epidermal growth factor (E), 100 nm PMA (P), 10 μM 5-HT for 3 h (SHT) with or without 1-h pretreatment with 100 nm PMA (P+E) or 10 μM 5-HT (SHT+E). To inhibit PLD, cells were pretreated with 0.4% 1-butanol for 15 min. After treatment cells were harvested and lysed. Levels of endogenous EGFR were determined by Western blot. Blots were stripped and reprobed for β-actin to normalize for loading. Insets are representative of five independent experiments. Results are means ± S.E. (n = 5). *** indicates a p value < 0.001 for PMA + epidermal growth factor versus epidermal growth factor, and ** indicates a p value < 0.01 for 5-HT + epidermal growth factor versus epidermal growth factor.
PKC in 5-HT_{2A}R desensitization seems to be variable depending on the cell type (40, 42–45). In our hands sequestration did not exert significant effects on receptor desensitization or resensitization possibly due to the fact that receptor internalization was not completely inhibited. Although sequestration of receptors does not appear to be important for desensitization or resensitization as measured by intracellular calcium increase, it cannot be excluded that other receptor functions are modulated by this process. Because those functions can be unique for specific receptors they have to be studied carefully in appropriate model systems.

It was observed that sustained receptor stimulation led to not only sequestration of receptors but also to sequestration of other molecules from the cell surface. Functionally this may exert significant effects on the availability of recycling proteins at the plasma membrane and their specific functions. Interestingly it was shown previously that 5-HT inhibits voltage-gated potassium currents via internalization of Kv1.5 (30). Current studies implicate a role of 5-HT in changing the cellular fate of EGFR and PAR-1. It was shown previously that activation of PKC with PMA leads to internalization of EGFR without its degradation (21, 46). Here it was shown that sustained stimulation of GPCRs with agonist leads to a similar effect, which is of importance because defects in trafficking of EGFR have been implicated in cell transformation and oncogenesis (47).

Similarly we showed that sustained 5-HT treatment protected PAR-1 from agonist-induced degradation. This is consistent with previously made observations that PMA leads to heterologous desensitization of PAR-1 (48).

In conclusion, the results from this study demonstrate that sustained stimulation of cPKC by GPCRs leads to cPKC- and PLD-dependent sequestration of these receptors to a cPKC-dependent subset of recycling compartment (pericentrion) and colocalization with Rab11 perinuclear recycling endosomes. Concomitant sequestration of other cell surface receptors (e.g. PAR-1 and EGFR) that are normally targeted for degradation was observed.

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