Different types of plasma membrane receptors engage in various forms of cross-talk. We used cultures of rat renal mesangial cells to study the regulation of EGF receptors (EGFRs) by various endogenous G protein-coupled receptors (GPCRs). GPCRs (5-hydroxytryptamine2A, lysophosphatidic acid, angiotensin AT1, bradykinin B2) were shown to transactivate EGFRs through a protein kinase C-dependent pathway. This transactivation resulted in the initiation of multiple cellular signals (phosphorylation of the EGFRs and ERK and activation of cAMP-responsive element-binding protein (CREB), NF-κB, and E2F), as well as subsequent rapid down-regulation of cell-surface EGFRs and internalization and desensitization of the EGFRs without change in the total cellular complement of EGFRs. Internalization of the EGFRs and the down-regulation of cell-surface receptors in mesangial cells were blocked by pharmacological inhibitors of clathrin-mediated endocytosis and in HEK293 cells by transfection of cDNA constructs that encode dominant negative β-arrestin-1 or dynamin. Whereas all of the effects of GPCRs on EGFRs were dependent to a great extent on protein kinase C, those initiated by EGF were not. These studies demonstrate that GPCRs can induce multiple signals through protein kinase C-dependent transactivation of EGFRs. Moreover, GPCRs induce profound desensitization of EGFRs by a process associated with the loss of cell-surface EGFRs through clathrin-mediated endocytosis.

Receptor tyrosine kinases (RTKs) and G protein-coupled receptors (GPCRs) are the two major families of receptors that convert extracellular signals into cellular physiological and mitogenic responses. Previously, the signals generated by RTKs and GPCRs were thought to be neatly compartmentalized, with very little cross-talk between or sharing of the signaling pathways. There is a new awareness that RTKs, such as the EGF receptor, and GPCRs possess the capacity for cross-talk during signal initiation and propagation. Cross-talk can take the form of using shared signaling pathways (1–3) or, for GPCRs, using RTKs themselves as signaling platforms (4–12). Thus, contrary to relatively recent dogma, it is now abundantly clear that RTKs and GPCRs engage in extensive cross-talk with each other.

Just as there are similarities in the mechanisms that initiate the signaling pathways of GPCRs and RTKs, there might also be similarities in the mechanisms by which those signals are terminated or desensitized. Indeed, there is a growing body of evidence that GPCRs and RTKs share mechanisms that regulate signal desensitization. Desensitization is a group of processes through which receptors or components of their signaling pathways become less responsive after previous exposures to receptor ligands. Homologous desensitization occurs when cells become unresponsive only to subsequent activation of the receptor that was previously stimulated. This type of desensitization is usually mediated by receptor specific kinases (GRKs). Heterologous desensitization refers to attenuation of one receptor system by another and is usually mediated by broad spectrum serine/threonine kinase such as protein kinases C and A.

A special form of heterologous desensitization may occur when RTKs desensitize GPCRs. RTKs can desensitize GPCRs by phosphorylating the GPCR (13), by phosphorylating heterotrimeric G proteins (14), or by other mechanisms (15, 16). It is also possible that GPCRs could desensitize RTKs, but little is known about this phenomenon.

Renal mesangial cells possess many mitogenic GPCRs, including angiotensin II AT1A (17), bradykinin B2 (18, 19), lysophosphatidic acid (20, 21), and 5-hydroxytryptamine (5-HT2A) receptors (22). Mesangial cells also express RTKs, which may participate in the proliferative phase of chronic renal failure (23) or in the recovery from renal failure (24). Mesangial cells possess an epidermal growth factor (EGF) receptor (25) that stimulates proliferative cascades in those cells (26). It is somewhat paradoxical that mesangial cells should express so many mitogenic receptors in that, under normal circumstances, proliferation is highly restrained within the confines of the glomerulus. This suggests that the responsiveness of mitogenic receptors must be rigidly controlled in mesangial cells. One mechanism through which rigid control of mitogenic signaling in mesangial cells might be exercised is desensitization.
In this study, we report that pretreatment of kidney mesangial cells with GPCR ligands (5-HT, bradykinin, lysophosphatidic acid) results in a PKC-dependent transactivation of EGFR followed by a profound decrease in the ability of EGFR to initiate multiple signals including autophosphorylation of the EGFR receptor (EGFR), phosphorylation of ERK, and regulation of transcription factor activities (NF-κB, E2F, CREB). Furthermore, the desensitization pathway involves PKC and results in a dynamic internalization of native EGFR receptors and transfected EGFR-GFP fusion proteins. Thus, preconditioning of cells by GPCR ligands may be a novel method to abrogate deleterious signals initiated by EGFR and other RTK.

**EXPERIMENTAL PROCEDURES**

Materials—Drugs and reagents were obtained from the following sources. 5-HT, bradykinin, lysophosphatidic acid, epidermal growth factor, and phosphor 12–myristate 13-acetate were from Sigma. Phospho-ERK antibodies were obtained from New England Biolabs (Beverly, MA). GF109203X (bisindolylmaleimide I) and protease inhibitors (4-(2-aminoethyl)-benzenesulfonyl fluoride, EDTA, E-64, leupeptin, and aprotinin) were from Calbiochem. Anti-phosphotyrosine antibody (PY99), protein A-agarose, and E2F oligonucleotides were from Santa Cruz Biotechnology (Santa Cruz, CA). NF-κB and CREB oligonucleotides were from Promega (Madison, WI).

**Cell Culture and Transfection**—Rat mesangial cells were obtained from cortical sections of kidneys from young 100–150-gram Harlan Sprague-Dawley rats using standard sieving techniques (27). The kidneys were harvested in accordance with a protocol reviewed and approved by the Institutional Animal Care and Use Committee of the Medical University of South Carolina. Cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO2 and were subcultured every 1–2 weeks by trypsinization until a pure culture of mesangial cells was obtained.

**Transfections** were performed on 50–70% confluent monolayers in 100-mm dishes, using LipofectAMINE, Lipofectin (Life Technologies), or FuGene™ 6 (Roche Molecular Biochemicals). Empty vectors were used as controls. Cells were transfected to keep the total mass of DNA per dish constant within experiments. 48 h prior to studies, cells were placed in or FuGene™ 6 (Roche Molecular Biochemicals). Empty vectors were transduced with 10% fetal bovine serum and 50 mM sucrose); and medium for chemical inhibition by con-
were grown on round coverslips by placing coverslips at the bottom of the wells in 6- or 12-well culture plates. After rinsing in PBS, adherent cells transfected with the EGFR-GFP fusion protein were identified by incubating with rhodamine-concanavalin A (10 μg/ml in PBS) for 2 min at 4 °C. Cells were rinsed with PBS several times and then fixed with 4% paraformaldehyde in PBS for 15 min followed by quenching the fixative with three 5-min washes with 50 mM NH₄Cl at room temperature. For single labeling of EGFR, cells on coverslips were fixed as described above and were then inverted onto 20 μl of fluorescein isothiocyanate-conjugated anti-EGFR antibody against an extracellular epitope of the receptor (1:100) dilution in PBS with 1% goat serum and incubated in the dark for 2 h at room temperature. Cells were rinsed four times with PBS supplemented with 1% goat serum for 10 min. Coverslips were then mounted on a slide with Slow-Fade medium (Molecular Probes, Eugene, OR) and sealed with Cytoseal (Electron Microscopy Sciences, Fort Washington, PA) solution before scanning under a confocal microscope (Olympus) with a 100× oil objective.

Analysis of Total Cellular Complement of EGFRs—Cells in 100 mm culture dishes were treated with 30 μg/ml cycloheximide or puromycin dihydrochloride for 1 h before treatment with 5-HT for different time periods, after which cells were washed and scraped into a modified radioimmune precipitation buffer as described above. Total EGFR protein was visualized by immunoprecipitation and immunoblotting as described above using anti-EGFR polyclonal antibody for immunoprecipitation and an anti-EGFR monoclonal antibody for immunoblotting.

RESULTS

Transactivation of EGFRs by the 5-HT₂A Receptor—Fig. 1 shows that when rat renal mesangial cells were treated with 1 μM 5-HT for 3 min, EGFRs became phosphorylated as detected by metabolic labeling and immunoprecipitation of EGFRs. The increase was dependent upon both the concentration of 5-HT (EC₅₀ = 160 nM) and time of incubation, peaking at 5–10 min. EGFR phosphorylation was blocked almost completely when cells were pretreated with the specific EGFR tyrosine kinase inhibitor AG1478 for 30 min prior to exposure to 5-HT. Thus, the 5-HT₂A receptor transactivates the EGFR through the intrinsic kinase activity of the EGFR in a manner already shown to occur with other GPCRs such as those for angiotensin II (6), carbachol, lysophosphatidic acid, and thrombin (12, 39). Because both ERK and PKC can induce phosphorylation of the EGFR (40), and because the 5-HT₂A receptor has been shown to activate both ERK and PKC (27, 41), we used inhibitors of ERK kinase (MEK1) and PKC to determine which of those intermediates might be involved in 5-HT-induced phosphorylation of the EGFR. Fig. 1c shows that a PKC inhibitor (5 μM GF109203X) greatly attenuated 5-HT-induced phosphorylation of the EGFR, whereas a MEK inhibitor (100 μM PD98059) did not. This concentration of PD98059 nearly completely attenuates ERK activation by the 5-HT₂A receptor in these cells as previously determined by us (27). Thus, PKC (and not MEK/ERK) seems to be involved in the transphosphorylation of the EGFR by the 5-HT₂A receptor in mesangial cells.

The 5-HT₂A Receptor Stimulates Transcription Factors by Transactivation of EGFRs—Next, we examined the effects of 5-HT on the activation of three EGFR-stimulated transcription factors (E2F, CREB, and NF-κB). Fig. 2 shows that acute treatment with either 5-HT or EGF induced activation of all three transcription factors as assessed by electrophoretic mobility shift assay. Moreover, the stimulation of all three transcription factors by 5-HT could be attenuated by preincubation with AG1478. Similarly, AG1478 blocked 5-HT-induced phosphorylation of ERK in mesangial cells (not shown). Those results suggest that the 5-HT₂A receptor in mesangial cells activates transcription factors through the intermediary actions of the EGFR.

Pretreatment of Mesangial Cells with 5-HT Attenuates Multiple Subsequent EGFR Downstream Signals—To further explore the similarities between the effects of 5-HT and EGF on EGFR function, we next studied the effects of prior treatment with 5-HT on the activation of downstream signals by EGF.

Our rationale for those studies is that EGF treatment has been shown to desensitize the EGFR to subsequent activation by EGF. Thus, we hypothesized that 5-HT pretreatment might also desensitize the EGFR. Fig. 3 shows the results of studies in which EGF-induced ERK phosphorylation was assessed after pretreatment with vehicle or 5-HT. Those results clearly demonstrate that pretreatment of mesangial cells with 5-HT results in a marked attenuation of the ability of EGF to induce phosphorylation of ERK. We used a similar paradigm to assess the effects of prior treatment with 5-HT on the ability of EGF to activate the three transcription factors shown in Fig. 2. Those results are shown in Fig. 4, a–c. Pretreatment with 5-HT greatly reduced tran-
scription factor activation as reflected by their binding with respective labeled consensus *cis*-elements. The specificity of the interactions of the transcription factors with their consensus oligonucleotides was confirmed by competition with unlabeled oligonucleotides and mutant (nonbinding) oligonucleotides.

**Pretreatment of Mesangial Cells with GPCR Ligands Attenuates EGFR Autophosphorylation**—Figs. 3 and 4 show that multiple signals residing downstream from the EGFR can be attenuated by pretreatment with 5-HT, which suggested to us that desensitization of the EGFR most likely occurs at the level of the receptor itself. Therefore, we tested the effects of pretreatment with 5-HT on the ability of EGF to induce autophosphorylation of the EGFR. Fig. 5 shows that pretreatment of mesangial cells with 5-HT leads to a marked decrease in the ability of multiple concentrations of EGF to induce the phosphorylation of its receptor. The attenuation was consistent over a broad range of concentrations of EGF, suggesting that this effect may be relevant under physiological conditions.

If the effect of pretreatment of cells with 5-HT is truly important, we would expect that other GPCRs might also desensitize the EGFR. Indeed, attenuation of EGF-induced phosphorylation of EGFR was observed when cells were pretreated with other mitogenic GPCR ligands such as bradykinin and lysophosphatidic acid (Fig. 6, *a* and *b*) as well as angiotensin (not shown). Thus, the ability of GPCR to desensitize EGF-induced phosphorylation of EGFR is not limited to the 5-HT$_{2A}$ receptor. One of the major pathways that links G$_i$ and G$_q$-coupled receptors to mitogenic signals in mesangial and other cells involves PKC (3, 12, 27, 39). We therefore examined the effects of direct stimulation of PKC on the ability of EGF to induce phosphorylation of the EGFR. Fig. 6c shows that when cells were pretreated with 1 mM phorbol 12-myristate 13-acetate (PMA), the ability of EGF to induce tyrosine phosphoryl-
attenuates EGFR autophosphorylation. EGFR phosphorylation was measured as described in the legend for Fig. 1. Cells were treated with vehicle (gray bars) or 1 μM 5-HT (black bars) for 1 h prior to treatment for 3 min with various concentrations of EGF. The values of the bars represent the mean values obtained from three separate experiments ± standard errors. *, indicates p < 0.05 versus control; †, indicates p < 0.01 versus the values obtained from EGF without prior treatment with 5-HT (fifth bar from the left) as assessed using ANOVA and Fisher’s protected least significant difference post-hoc test for multiple comparisons.

EGFR phosphorylation was measured as described in the legend for Fig. 1. Cells were treated with vehicle, 100 nM bradykinin (BK), 100 nM lysophosphatidic acid (LPA), or 1 μM PMA for 1 h prior to treatment for 3 min with various concentrations of EGF. The values of the bars represent the mean values obtained from three separate experiments ± standard errors. *, indicates p < 0.05 versus control; †, indicates p < 0.01 versus the values obtained from EGF without prior treatment with bradykinin, lysophosphatidic acid, or PMA as assessed using ANOVA and Fisher’s protected least significant difference post-hoc test for multiple comparisons.

Fig. 5. Pre-exposure of mesangial cells to 5-HT attenuates EGFR autophosphorylation. EGFR phosphorylation was measured as described in the legend for Fig. 1. Cells were treated with vehicle (gray bars) or 1 μM 5-HT (black bars) for 1 h prior to treatment for 3 min with various concentrations of EGF. The values of the bars represent the mean values obtained from three separate experiments ± standard errors. *, indicates p < 0.05 versus control; †, indicates p < 0.01 versus the values obtained from EGF without prior treatment with 5-HT (fifth bar from the left) as assessed using ANOVA and Fisher’s protected least significant difference post-hoc test for multiple comparisons.

Fig. 6. Pre-exposure of mesangial cells to multiple GPCR ligands attenuates EGFR autophosphorylation. EGFR phosphorylation was measured as described in the legend for Fig. 1. Cells were treated with vehicle, 100 nM bradykinin (BK), 100 nM lysophosphatidic acid (LPA), or 1 μM PMA for 1 h prior to treatment for 3 min with various concentrations of EGF. The values of the bars represent the mean values obtained from three separate experiments ± standard errors. *, indicates p < 0.05 versus control; †, indicates p < 0.01 versus the values obtained from EGF without prior treatment with bradykinin, lysophosphatidic acid, or PMA as assessed using ANOVA and Fisher’s protected least significant difference post-hoc test for multiple comparisons.

Fig. 7. Pre-exposure of mesangial cells to 5-HT or EGF diminishes cell-surface 125I-EGF binding. Cells were pretreated with 300 nM 5-HT, 3 μM 5-HT, or 20 ng/ml EGF for various time periods (10–60 min) followed by acid washing. Cell-surface 125I-EGF binding was then measured as described under “Experimental Procedures.” The plot shown in this figure is derived from one experiment performed in duplicate, which is representative of three that showed similar results.

Fig. 8 (panels A–D) shows the results of experiments in which cell-surface receptors were visualized in nonpermeabilized cells with a fluorescein isothiocyanate-conjugated anti-EGFR antibody (raised against an extracellular epitope of the EGFR). This method was used to visualize surface receptors on mesangial cells after incubation with vehicle (panel A), 300 nM 5-HT for 20 min (panel B) or 60 min (panel C), or EGF (20 ng/ml) for 60 min (panel D). The results show that there was a marked decrease in cell-surface EGFR after incubation with either 5-HT or EGF. Thus these two methods clearly demonstrate that preincubation with 5-HT or EGF reduces the number of cell-surface EGFRs in mesangial cells. Those studies cannot, however, distinguish between a redistribution of EGFRs to intracellular compartments and a loss of total EGFRs (from increased degradation or decreased synthesis). Thus, we used an EGFR-GFP fusion protein to assess whether 5-HT and EGF could induce a redistribution of EGFR within mesangial cells. This construct has already been used to demonstrate that EGF causes the EGFR-GFP fusion protein to internalize in a similar manner to wild-type EGFR in HEK293 and NIH 3T3 cells (29). We transiently transfected HEK293 cells with the EGFR-GFP construct and also with cDNA encoding the human 5-HT2A receptor. Fig. 8, E–H, shows that both EGF and 5-HT induced redistribution of EGFR-GFP away from the cell surface and into a nuclear or perinuclear locale in HEK293 cells. Representative photomicrographs are shown for treatment with vehicle (panel E), with 300 nM 5-HT for 20 min (panel F) or 60 min (panel G), or with EGF (20 ng/ml) for 60 min (panel H). The red areas show the plasma membrane identified by rhodamine-concanavalin A after fixation of the cells, whereas the green areas represent the EGFR-GFP fusion protein (42). The yellow areas indicate superimposition of the red and green signals. We used a computer algorithm to provide a semiquantitative assessment (Lux units) of the subcellular localization of the EGFR-GFP fusion protein in HEK293 cells (Fig. 9). Those results showed that most of the fusion protein...
was located on or near the plasma membrane in quiescent cells, whereas the cell-surface receptors were reduced by ~75% after stimulation with either EGF or 5-HT.

5-HT-induced EGFR Down-regulation Involves PKC—5-HT activates PKC in mesangial cells (49), and it has been shown to mediate both PKC-dependent (27, 44) and -independent effects (45) in those cells (see Fig. 1). We performed experiments using a specific PKC inhibitor (GF109203X) to establish a role for PKC in the down-regulation of cell-surface EGFR by 5-HT. Fig. 10 shows that in the absence of any inhibitor, both 5-HT and EGF resulted in a marked down-regulation of 125I-EGF binding to mesangial cells with a fluorescein isothiocyanate-conjugated anti-EGFR antibody (raised against an extracellular epitope of the EGFR). This method was used to visualize cell-surface receptors on mesangial cells after incubation with vehicle (A), 300 nM 5-HT for 20 min (B) or 60 min (C), or EGF (20 ng/ml) for 60 min (D). Panels E–H show the results obtained when an EGFR-GFP fusion protein was expressed by transient transfection of an EGFR-GFP construct and also with cDNA encoding the human 5-HT2A receptor into HEK293 cells. This second method was used to identify the EGFR-GFP fusion protein on mesangial cells after incubation with vehicle (E), 300 nM 5-HT for 20 min (F) or 60 min (G), or EGF (20 ng/ml) for 60 min (H). The red areas show the plasma membrane identified by rhodamine-concanavalin A after fixation of the cells, whereas the green areas represent the EGFR-GFP fusion protein. The yellow areas indicate superimposition of the red and green signals. The confocal micrographs are representative of three separate experiments.

Blocking EGF-induced down-regulation. These data correlate well with those in Fig. 1c, which show that GF109203X blocks 5-HT-induced transphosphorylation of the EGFR. These data are also in keeping with a mechanism of action of PKC that occurs upstream of EGFR activation.

GPCR-induced EGFR Down-regulation Requires EGFR Internalization—Down-regulation of cell-surface receptors can involve receptor internalization, degradation, or both. To study whether internalization of the EGFR is a component of the GPCR-induced down-regulation of cell-surface EGFRs, we transfected HEK293 cells with cDNAs encoding the EGFR-GFP fusion protein, the human 5-HT2A receptor, and dominant negative forms of both β-arrestin and dynamin GTPase. Dynamin is required for clathrin-mediated endocytosis, and a dominant negative version of dynamin (K44A dynamin) has been used previously to block internalization of both GPCRs and RTKs (46, 47). A peptide fragment of β-arrestin 1, β-arrestin1-(319–418), has been demonstrated previously to block GPCR-induced clathrin-mediated endocytosis (46, 79) because it binds clathrin cages but not GPCRs. Fig. 11 shows that β-arrestin1-(319–418) (panels C and D) and K44A dynamin (panels E and F) effectively prevent the 5-HT-induced endocytosis of EGFR in HEK293 cells as determined by confocal microscopy. Red indicates the decoration of the cell surface (post-fixation and treatment) by rhodamine-concanavalin A. The green signal is generated by the EGFR-GFP. Areas of overlap are indicated in yellow. In mock-transfected cells, most of the EGFR-GFP leaves a predominantly plasma membrane location and internalizes into intracellular compartments that seem to include the nucleus. In cells transfected with either of the dominant negative constructs, little 5-HT-induced internalization is seen after 60 min of treatment. Those results support a probable role for endocytosis in 5-HT-induced down-regulation of EGFRs in HEK293 cells.

We also exposed rat mesangial cells to 5-HT in the presence and absence of various chemical inhibitors of endocytosis, including conacainavlin A (ConA), monodansylcadaverine, and potassium depletion. Because no specific inhibitors of endocytosis are available, we had to use these multiple approaches to block endocytosis. Fig. 12a shows that these maneuvers attenuated the down-regulation of the EGFR induced by 5-HT (similar results of low temperature are not shown). We obtained
post-fixation and treatment) by rhodamine-concanavalin A. The green signal is generated by the EGFR-GFP. Areas of overlap are indicated in yellow. The 5HT2A receptor was not visualized, but 5-HT did not induce internalization in HEK293 cells. Cells were transfected with cDNAs encoding both the 5-HT2A receptor and EGFR-GFP in addition to empty vector (A and B) or cDNAs encoding dominant interfering mutants of β-arrestin (C and D) or dynamin (E and F) as described under “Experimental Procedures.” The cell surfaces were then identified with concanavalin A-rhodamine, and then the cells were fixed and subjected to confocal microscopy. Red indicates the decoration of the cell surface (post-fixation and treatment) by rhodamine-concanavalin A. The green signal is generated by the EGFR-GFP. Areas of overlap are indicated in yellow. The 5HT2A receptor was not visualized, but 5-HT did not induce internalization of the EGFR absent transfection with the 5-HT2A receptor (not shown).

FIG. 11. Effects of blocking clathrin-mediated endocytosis with chemical inhibitors on 5-HT2A receptor-induced EGFR-GFP internalization in HEK293 cells. Cells were transfected with cDNAs encoding both the 5-HT2A receptor and EGFR-GFP in addition to empty vector (A and B) or cDNAs encoding dominant interfering mutants of β-arrestin (C and D) or dynamin (E and F) as described under “Experimental Procedures.” The cell surfaces were then identified with concanavalin A-rhodamine, and then the cells were fixed and subjected to confocal microscopy. Red indicates the decoration of the cell surface (post-fixation and treatment) by rhodamine-concanavalin A. The green signal is generated by the EGFR-GFP. Areas of overlap are indicated in yellow. The 5HT2A receptor was not visualized, but 5-HT did not induce internalization of the EGFR absent transfection with the 5-HT2A receptor (not shown).

FIG. 12. Effects of blocking clathrin-mediated endocytosis with chemical inhibitors on 5-HT2A receptor-induced EGFR-GFP internalization in mesangial cells. Rat renal mesangial cells were exposed to 1 μM 5-HT in the presence and absence of various inhibitors of clathrin-mediated endocytosis, including potassium depletion buffer, ConA, and monodansylcadaverine (MDC). Then cell-surface 125I-EGF binding was measured as described under “Experimental Procedures.” Panel a shows that these maneuvers attenuated the desensitization of the EGFR induced by 5-HT. Panel b shows that incubation with cycloheximide (CHX, 30 μg/ml) for 1 h did not impair the down-regulation of cell-surface EGFR by 5-HT. The values presented are derived from the means ± standard errors from three experiments performed in duplicate. *, indicates p < 0.05 versus control as assessed using ANOVA and the Bonferroni-Dunn test for multiple comparisons.

Effects of 5-HT Pretreatment on Total Immunoreactive EGFR Protein—The next question to ask was whether GPCR activation leads only to internalization of EGFR (removal from the cell surface), or whether a component of reduction of the total complement of receptors within the cell is involved. Fig. 13 illustrates experiments in which immunoblots were performed from whole cell lysates after incubation with 1 μM 5-HT for up to 150 min in the presence of cycloheximide. Those experiments show that the total cellular complement of EGFR is markedly reduced by treatment with 5-HT despite the presence of cycloheximide. Cycloheximide alone had no effect on the amount of EGFR immunoreactivity in whole cell lysates (not shown). The initial decline in EGFR immunoreactivity is very gradual, diminishing only by 25% at 60 min. Thus, within the time frame of desensitization of the EGFR by 5-HT, there is only a small decline in the total number of EGFRs. After 60 min, the immunoreactivity drops off sharply. If the down-regulation of the total amount of cellular EGFR does not involve alterations in protein synthesis, then the degradation of EGFR is likely accelerated by incubation with GPCR ligands. Thus, the initial desensitization of the EGFR by the 5-HT2A receptor appears to be related to internalization of the EGFR, whereas later effects may be due to degradation of the EGFR.

FIG. 13. Effects of pretreatment of mesangial cells with 5-HT on the levels of immunoreactive EGFRs. Mesangial cells were treated with 1 μM 5-HT for the indicated times, and then cells were scraped into Laemmli buffer and heated to 90 °C for 2 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 4–20% polyacrylamide gels (Novex) and resolved under nonreducing conditions. Immunoblots were then performed with an anti-EGFR antibody as described under “Experimental Procedures.” The insert is representative of three identical experiments. Values for each time point were determined by densitometry and represent the means ± standard errors from three experiments.

in this effect. Thus, these studies demonstrate that internalization of the EGFR is a key component of its down-regulation by 5-HT. However, these studies do not demonstrate whether the functional desensitization of the EGFR induced by 5-HT also requires internalization.

Effects of 5-HT Pretreatment on Total Immunoreactive EGFR Protein—The next question to ask was whether GPCR activation leads only to internalization of EGFR (removal from the cell surface), or whether a component of reduction of the total complement of receptors within the cell is involved. Fig. 13 illustrates experiments in which immunoblots were performed from whole cell lysates after incubation with 1 μM 5-HT for up to 150 min in the presence of cycloheximide. Those experiments show that the total cellular complement of EGFR is markedly reduced by treatment with 5-HT despite the presence of cycloheximide. Cycloheximide alone had no effect on the amount of EGFR immunoreactivity in whole cell lysates (not shown). The initial decline in EGFR immunoreactivity is very gradual, diminishing only by 25% at 60 min. Thus, within the time frame of desensitization of the EGFR by 5-HT, there is only a small decline in the total number of EGFRs. After 60 min, the immunoreactivity drops off sharply. If the down-regulation of the total amount of cellular EGFR does not involve alterations in protein synthesis, then the degradation of EGFR is likely accelerated by incubation with GPCR ligands. Thus, the initial desensitization of the EGFR by the 5-HT2A receptor appears to be related to internalization of the EGFR, whereas later effects may be due to degradation of the EGFR.

DISCUSSION

These studies demonstrate that GPCRs can transactivate EGFRs through a PKC-dependent pathway. This transactivation results in the initiation of multiple cellular signals, as well as subsequent internalization and desensitization of the EGFRs. What is new about this work is that we show that activation of GPCRs can profoundly desensitize a prototypical RTK, the EGFR. The effect is rapid, being manifested within minutes, and is associated with a rapid internalization of cell-surface EGFRs. Desensitization of the EGFR can be initiated by several GPCRs (5-HT2A, lysophosphatidic acid, angiotensin
AT$_1$, bradykinin B$_2$) that classically couple to $G_q$-type G proteins. Desensitization can also be mimicked by chemical activation of PKC by PMA. Activation of the 5-HT$_{2A}$ receptor desensitizes a number of EGFR signals, including EGFR auto-phosphorylation, phosphorylation of ERK, and activation of transcription factors (CREB, NF-$\kappa$B, and E2F). Internalization and down-regulation of cell-surface EGFRs induced by 5-HT (but not EGF) can also be blocked by pharmacological inhibition of PKC. Thus, GPCRs can induce desensitization of EGFRs by a process associated with the loss of cell-surface EGFRs through internalization. Our data also show that the 5-HT$_{2A}$ receptor transactivates the EGFR in a manner already shown to occur with other GPCRs such as those for angiotensin II (6), carbachol, lysophosphatidic acid, thrombin (12, 39) and for the $\beta_2$ adrenergic receptor (48). The process of EGFR induced by EGF and GPCRs is distinct in that the GPCR signal is PKC-dependent whereas the EGF signal is not. These relationships are depicted in Fig. 14.

Although our data implicate PKC in the activation and desensitization of the EGFR induced by GPCRs, the mechanism of that process is undefined. Prenzel et al. (11) showed that release of heparin-bound EGF by a membrane-bound metalloproteinase-like enzyme mediated some of the effects of GPCRs to activate EGFRs. This enzyme resembled zinc-dependent proteases called ADAMs (cell-surface proteins that contain a disintegrin and metalloproteinase domain), some of which can be activated by PKC (49, 50). We tested this possibility by incubating cells with three different inhibitors of metalloproteinases, MMP-3 inhibitors I and II (Calbiochem) and BB94 (Bristol Biotech). None of those inhibitors had any effect on 5-HT$_{2A}$ receptor-induced phosphorylation of EGFRs (not shown). Thus, those studies did not support a role for a PKC-activated membrane-bound metalloproteinase-like enzyme in GPCR-induced activation of EGFRs in rat renal mesangial cells.

Previous evidence that PKC is involved in transactivation of the EGFR is variable (10, 51). Some studies have suggested potential roles for PKC in the negative regulation of EGFR signaling (52–56). In fact, Baguinot et al. (57) demonstrated that PMA could induce internalization of EGFRs and a transient decrease in cell-surface $^{125}$I-EGF binding without inducing degradation of the EGFRs. Others, however, have suggested that PKC-dependent phosphorylation enhances and stabilizes EGFR levels and/or signaling (58–62). PKC-$\alpha$ was shown to associate with EGFR and to increase its phosphorylation in transfected HEK293 and NIH3T3 cells (54). The authors hypothesized that PKC-mediated EGFR phosphorylation played a key role in EGFR internalization. In that regard, it is tempting to speculate that such a mechanism could link our finding that both PKC and EGFR internalization mediate the desensitization process. Another group showed that PKC-$\alpha$ reduces EGFR numbers without changing the affinity of EGF for the EGFR (63). What separates our current report from the previous work described in this paragraph is that we used endogenous prototypical $G_q$-coupled GPCRs to activate PKC, whereas the other studies almost exclusively used chemical activation of PKC to study its effects on EGFR functions. Moreover, we have demonstrated a clear-cut desensitization of several signals that emanate from the EGFR by multiple different GPCRs.

Some have suggested that PKC-mediated effects on EGFRs include reductions of high affinity binding sites and tyrosine autophosphorylation (64), although others were not able to demonstrate that PKC was involved in down-regulating EGFR (65, 66). Harada et al. (65) showed evidence that PKC could alter the affinity of EGF for the EGFR without down-regulating the receptor. Kaji et al. (67) showed that PKC decreased the affinity of the EGFRs for EGF without changing receptor number or by inducing internalization. In contrast, PKC-$\alpha$ was shown to reduce EGFR numbers without changing the affinity of EGF for the EGFR (68).

Studies on the roles of specific serine/threonine phosphorylation sites of the EGFR have had a similar lack of consensus. The EGFR can be phosphorylated on Thr$^{654}$ by PKC (66) and on Thr$^{669}$ by ERK (40). Phosphorylation of Thr$^{654}$ was shown to decrease high affinity EGF binding to the EGFR, but this residue was not involved in PKC-mediated down-regulation of the EGFR (64). Verheijden et al. (68) showed that PKC inhibits EGFR tyrosine kinase activity without changing receptor dimerization. One group suggested that PKC- and ERK-dependent phosphorylation of the EGFR receptor does not mediate desensitization of the EGFR (69). PKC can phosphorylate the EGFR at Thr$^{654}$ (66), but one group could not link phosphorylation of either Thr$^{654}$ or Thr$^{669}$ to down-regulation of the EGFR (69). On the other hand, Bowen et al. (70) showed that phosphorylation of Thr$^{654}$ blocked mitogenic stimulation by the EGFR. Another group showed that phosphorylation of Thr$^{654}$ inhibits ligand-induced internalization and down-regulation of the EGFR (58).

Internalization of the EGFR through clathrin-coated pits appears to be the major process through which desensitization of the EGFR by GPCRs occurs. The evidence for this is that pretreatment of mesangial cells with 5-HT results in 1) a decrease in cell-surface $^{125}$I-EGF binding, 2) a translocation of an immunoreactive EGFR, and 3) multiple inhibitors of clathrin-mediated endocytosis preventing GPCR-driven internalization of the EGFRs. Moreover, the blockade of endocytosis prevents desensitization of the EGFR. Desensitization appears to be independent of protein synthesis, because the studies were performed in the presence of inhibitors of protein synthesis. Desensitization in the first 60 min of pretreatment with 5-HT appears to be largely independent of protein degradation because the amount of total cellular immunoreactive EGFRs decreases by only about 25%.

We used several distinct maneuvers to block GPCR-induced internalization of EGFRs including ConA, monodansylcadaverine, hypertonic medium, potassium depletion, low temperature, and dominant interfering constructs of $\alpha$-arrestin and $\beta$-arrestin. None of those inhibitors had any effect on 5-HT$_{2A}$ receptor-induced phosphorylation of EGFRs (not shown). Thus, those studies did not support a role for a PKC-activated membrane-bound metalloproteinase-like enzyme in GPCR-induced activation of EGFRs in rat renal mesangial cells.
with clathrin-mediated endocytosis by preventing the formation of clathrin-coated pits (33, 72). Incubation in hypertonic medium prevents formation of clathrin-coated pits (34). Dynamin is required for clathrin-mediated endocytosis, and a dominant negative version of dynamin (K44A dynamin) has previously been used to block internalization of both GPCRs and RTKs (46, 47). A peptide fragment of β-arrestin1, β-arrestin1-319–418, has previously been demonstrated to block GPCR-induced clathrin-mediated endocytosis (46, 79), presumably because it binds clathrin cages, but is unable to bind to receptors (73, 74). Thus, the effectiveness of multiple strategies to block endocytosis supports a role for clathrin-mediated endocytosis of the EGFR in its desensitization by GPCRs.

In our experiments, we observed that ConA significantly lowered the basal level of 125I-EGF binding (Fig. 12). The explanation for this effect is most likely ConA-induced proteolytic cleavage of the EGFR, as recently described by Tang et al. (75) in vascular smooth muscle cells. That group also showed that the cleavage event involved mainly the carboxyl terminus of the EGFR and did not interfere with 125I-EGF binding. This portion of the EGFR contains three major (Tyr1068, Tyr1148, and Tyr1183) and two minor (Tyr992 and Tyr1086) autophosphorylation sites (76, 77) and binding/activation sites for phospholipase C-γ, adapter protein 2 (78), and Shc (79). The proteolytic effect of ConA on EGFR does not universally attenuate EGFR functions, however. In NIH3T3 cells, ConA does not affect EGFR functions such as EGF binding or tyrosine phosphatase activity of the EGFR-GFP fusion protein and for helpful suggestions.

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