G Protein-Coupled Receptors Desensitize and Downregulate EGF Receptors in Renal Mesangial Cells*

Jasjit S. Grewal‡, Louis M. Luttrell§, John R. Raymond‡§

Department of Medicine (Nephrology Division‡) Medical University of South Carolina, Charleston, South Carolina, 29425‡; the Research and Medical Specialty Services§ of the Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina, 29401; the Department of Medicine (Endocrinology Division§) Duke University Medical Center§, Durham, North Carolina, 27710; and the GRECC§ of the Durham Veterans Affairs Medical Center, Durham, North Carolina, 27710.

Correspondence: Room 829 CSB, Medical University of South Carolina, 171 Ashley Avenue, Charleston, S.C., 29425
phone 843-792-4122, fax 843-792-8399, e-mail raymondj@musc.edu

Key words not in the title: Mitogen, lysophosphatidic acid, bradykinin, 5-HT (serotonin, 5-hydroxytryptamine)

*This work was supported by the Department of Veterans Affairs (Merit Award to J.R.R.) the National Institutes of Health (DK52448 and DK54720 to J.R.R., and DK55524 to L.M.L.), a fellowship from the American Heart Association (to J.S.G.), and a laboratory endowment jointly supported by the M.U.S.C. Division of Nephrology and Dialysis Clinics, Incorporated (J.R.R.).
ABBREVIATIONS

EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EMSA, electrophoretic mobility shift assay; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; HEPES, N-[2-hydroxyethyl]piperazine-N’-[4-butanesulfonic acid]; PKC, protein kinase C; RTK, receptor tyrosine kinase.
ABSTRACT

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 (EGFR's) by various endogenous G protein-coupled receptors (GPCR's). GPCR's (5-HT2A, LPA, 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 CREB, NF-κB, and E2F), as well as subsequent rapid downregulation of cell surface EGFR's, and internalization and desensitization of the EGFR’s without change in the total cellular complement of EGFR’s. Internalization of the EGFR’s and the downregulation 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 b-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 PKC-dependent transactivation of EGFR’s. Moreover, GPCR’s induce profound desensitization of EGFR’s by a process associated with the loss of cell surface EGFR’s through clathrin-mediated endocytosis.
INTRODUCTION

Receptor tyrosine kinases (RTK’s) and G protein-coupled receptors (GPCR’s) are the two major families of receptors that convert extracellular signals into cellular physiological and mitogenic responses. Previously, the signals generated by RTK’s and GPCR’s were thought to be neatly compartmentalized, with very little cross-talk between or sharing of the signaling pathways. There is a new awareness that RTK’s like the EGF receptor, and GPCR’s 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 GPCR’s, using RTK’s themselves as signaling platforms (4-12). Thus, contrary to relatively recent dogma, it is now abundantly clear that RTK’s and GPCR’s engage in extensive cross talk with each other.

Just as there are similarities in the mechanisms that initiate the signaling pathways of GPCR’s and RTK’s, 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 GPCR’s and RTK’s 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 (GRK’s). 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 RTK’s desensitize GPCR’s. RTK’s can desensitize GPCR’s by phosphorylating the GPCR (13), by phosphorylating heterotrimeric G proteins (14), or by other mechanisms (15,16). It is also possible that GPCR’s could desensitize RTK’s, but little is known about this phenomenon.

Renal mesangial cells possess many mitogenic GPCR’s, including angiotensin II AT₁A (17), bradykinin B₂ (18,19), lysophosphatidic acid (20,21) and 5-hydroxytryptamine 5-HT₂A receptors (22). Mesangial cells also express RTK’s, 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 pre-treatment 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 EGF to initiate multiple signals including autophosphorylation of the EGF receptor (EGFR), phosphorylation of ERK, and regulation of transcription factor activities (NF-κB, E2F, CREB). Further, the desensitization pathway involves PKC, and results in dramatic internalization of native EGF 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.
MATERIALS AND METHODS

**Materials**-- Drugs and reagents were obtained from the following sources. 5-HT, bradykinin, lysophosphatidic acid, epidermal growth factor, and phorbol 12-myristate, 13-acetate were from Sigma (St. Louis, MO). Phospho-ERK antibodies were obtained from New England Biolabs (Beverly, MA). GF109203X (bisindolylmaleimide I) and protease inhibitors (4-(2-aminoethyl)-benzenesulphonyl fluoride, EDTA, ethylenediaminetetraacetic acid [EDTA], E-64, leupeptin, and aprotinin) were from Calbiochem (La Jolla, CA). 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 renal mesangial cells were obtained from cortical sections of kidneys from young 100-150 gram 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 incubated at 37°C in a humidified atmosphere of 95% air and 5% CO₂, and subcultured every 1-2 weeks by trypsinization until a pure culture of mesangial cells were obtained. These were plated at a density of 2-5 x 10⁴ cells/ml in RPMI media supplemented with 20% heat-inactivated fetal bovine serum and antibiotics (100 U/ml of penicillin and 100 µg/ml of streptomycin). Cells used were from passage numbers 6-16. HEK-293 cells were maintained in F12 medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum and 50 mg/ml gentamicin (Life Technologies), at 37°C in a humidified 5% CO₂ atmosphere.

Transfections were performed on 50-70% confluent monolayers in 100 mm dishes, using lipofectamine, lipofectin (Life Technologies) or FuGene™ 6 (Boehringer Mannheim, Indianapolis, IN). Empty vectors were added to transfections to keep the total mass of DNA added per dish constant within experiments. 48 h prior to studies, cells were placed in serum-free media supplemented with antibiotics and 0.1% bovine serum albumin.
Metabolic labeling of EGFR—Cells (~1x $10^7$) were grown in 100 mm culture dishes and washed twice with phosphate-free buffer (10 mM HEPES, pH 7.4, 137 mM NaCl, 3 mM KCl) and incubated in phosphate-free RPMI medium supplemented with 20 mM dextrose, 20 mM HEPES, pH 7.4, and 100 μCi $^{32}$P phosphoric acid for 4 h at 37°C. Cells were then treated with mitogens for 3 min with or without pre-treatment with inhibitors 30 minutes before stimulation. Cells were placed on ice, washed three times with ice-cold phosphate buffered saline, and lysed in a modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na$_3$VO$_4$, 1 mM NaF, 500 μM 4-(2-aminoethyl)-benzenesulphonyl fluoride, 500 μM EDTA, 1 μM E-64, 1 μM leupeptin, and 1 μg/ml of aprotinin). The cell lysates were rocked at 4°C for 1 hour, then centrifuged to remove insoluble debris. The lysates were then diluted to a protein concentration of 1-2 mg/ml, and 500 μl was used for immunoprecipitation using 5 μg of anti-EGFR monoclonal antibody. The mixture was rocked for 1 h at 4°C, then 20 μl of protein A- or protein G/A-agarose beads were added, and incubated on a rocker for another 20 min at 4°C. The immune complexes were isolated by centrifugation, washed three times with RIPA buffer, then dissociated from the agarose beads by adding Laemmli buffer. The samples heated to 90°C for 2 minutes, then loaded onto precast 4-20% polyacrylamide gels (Novex, San Diego, CA) and resolved under nonreducing conditions. The gels were dried and analyzed with a Phosphorimager.

Immunoblots—ERK immunoblots were performed essentially as described previously (28). The phospho-ERK antibody was used at 1:1000 dilution, whereas the control antibody, which recognizes equally well the phosphorylated and non-phosphorylated MAPK, was used at 1:500 dilution as per the manufacturer’s recommendations. After treatment, cells were scraped into Laemmli buffer, boiled for 3 min, and subjected to SDS-PAGE under reducing conditions with 4-20% pre-cast gels (Novex, San Diego, CA). After semi-dry transfer to polyvinylidene difluoride membranes, the membranes were blocked with a BLOTTO buffer (5% defatted dried milk in 10 mM Tris, 150 mM NaCl, 1% Tween-20, pH 8.0). The membranes were incubated overnight with the BLOTTO containing the phospho-ERK antibody. The membranes were
washed, then exposed to goat anti-rabbit alkaline phosphatase-conjugated IgG (1:1000) in BLOTTO for 1 hour, then washed again. Immunoreactive bands were visualized by a chemiluminescent method (CDP Star®, New England Biolabs) using pre-flashed Kodak X-AR film. For other immunoblots cell extracts were incubated with 5 µg/ml of anti-EGFR or anti-phosphotyrosine monoclonal antibodies and visualized as described above except the secondary antibody was a rabbit anti-mouse IgG alkaline phosphatase-conjugate.

**EGFR-GFP Plasmid Construction**—A bright green mutant of GFP, enhanced GFP (Clontech, Palo Alto, CA), was attached to the carboxyl terminus of human EGFR as previously described (29). This construct behaves like wild-type EGFR in assays of phosphorylation, protein-protein interactions, signal transduction, internalization and degradation (29).

**Down-regulation of EGFR’s by GPCR’s**—Cells grown in six-well plates were incubated with vehicle, EGF or 5-HT prior to incubation with various concentrations of EGF (1-100 ng/ml) for various times at 37°C. Monolayers were then washed twice with ice-cold Hank’s balanced salt solution (HBSS). Cells were then washed with cold acid wash buffer (50 mM glycine, 100 mM NaCl, pH 3.0) to dissociate bound EGF, followed by three cold HBBS washings. Cells were then incubated with 50 pM 125I-EGF for 90 minutes at 4°C in HEPES binding medium (RPMI 1640 with 40 mM HEPES, pH 7.4, 0.1% bovine serum albumin) in the continued presence of vehicle or 5-HT. Cells were then washed three times with HBBS and dissolved in 1 ml of 1M NaOH. The solubilized material was collected in scintillation vials and counted in a g-counter. Non-specific binding was determined in quadruplicate wells containing 100 ng/ml of unlabeled EGF and was subtracted from total binding to yield specific 125I-EGF binding at each time point. Data were analyzed using Prism 2.0 software (GraphPad Software, San Diego, CA).

**Electrophoretic Mobility Shift Assay**—Oligonucleotides (E2F1, CREB or NF-κB transcription factor consensus binding sites) were end-labeled using T4 polynucleotide kinase and [γ32P]CTP. Nuclear extracts were prepared exactly as described (30), and the electrophoretic mobility
shift assay was modified from a previously published protocol (31). The reaction mixture was comprised of 10 µg of nuclear extracts, 1-2 µg of poly(dI-dC), 5 µl of 5× binding buffer (50 mM HEPES, pH 7.8, 5 mM spermidine, 15 mM MgCl₂, 36% glycerol, 3 mg/ml of bovine serum albumin and 15 mM DTT). This mixture was incubated on ice for 15 min, then 40,000-70,000 cpm of ³²P labeled oligonucleotide were added. The reaction mixture was incubated further for 15 min at room temperature. DNA-protein complexes were then resolved on 5% native polyacrylamide gels and quantified with a Phosphorimager after drying. For the CREB assays, cells were pre-treated with Ro20-1724 {4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone}, a selective cAMP-specific phosphodiesterase inhibitor. For competition assays, nuclear extracts were preincubated with unlabeled oligonucleotides at RT for 15 min before adding ³²P the labeled oligonucleotide. When possible, reactions were also carried out by using a ³²P labeled oligonucleotide carrying mutations in the consensus regions to check the specificity of the binding reaction.

Inhibition of Endocytosis– Assays for the inhibition of endocytosis were carried out as described previously by Jockers et al. (32). Cells that were serum-starved for 24 h in 6-well culture plates or 100 mm dishes were treated with various maneuvers that inhibit endocytosis including potassium depletion (33), hypertonic medium (34,35), concanavalin A (36), and monodansylcadaverine (37,38). Cells were incubated with cycloheximide (5 µg/ml) for 30 minutes prior to treatment with mitogens to prevent confounding effects of protein synthesis. Maneuvers to inhibit endocytosis were also performed for 30 minutes prior to experimentation coincident with cycloheximide. The compositions of the buffers used were as follow: potassium depletion buffer (20 mM HEPES, pH 7.4, 140 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, and 4.5 mg/l of dextrose); hypertonic medium (RPMI-1640, 0.5% bovine serum albumin, 4.5 g/l of dextrose, and 500 mM sucrose); and medium for chemical inhibition by conconavalin A (250 µg/ml) or 500 µM monodansylcadaverine (RPMI-1640, 0.5% bovine serum albumin, 4.5 g/l of dextrose). Cells were then shifted to 4°C, and cell surface EGFR’s were measured by radioligand binding.
Confocal Laser Scanning Microscopy— HEK293 or mesangial cells 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 decorated by incubating with rhodamine-concanavalin A (10µg/ml in PBS) for two min at 4°C. Cells were rinsed with PBS several times, 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 RT. For single labeling of EGFR, cells on coverslips were fixed as above, then were inverted onto 20 µl of FITC–conjugated anti-EGFR antibody raised 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 RT. Cells were rinsed 4× 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 Merlin" IX70, Melville, NY).

Analysis of Total cellular Complement of EGFRs  Cells in 100 mm culture dishes were treated with 30 mg/ml of cycloheximide or puromycin dihydrochloride for 1 h before treatment 5-HT for different time periods, after which cells were washed and scraped into a modified RIPA 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 EGFR’s by the 5-HT$_{2A}$ receptor.** Figure 1 shows that when rat renal mesangial cells were treated with 1 mM 5-HT for three minutes, EGFRs became phosphorylated as detected by metabolic labeling and immunoprecipitation of EGFRs. The increase was dependent upon both concentration of 5-HT (EC$_{50}$ = 160 nM) and time of incubation, peaking at 3-10 minutes. EGFR phosphorylation was almost completely blocked when cells were pretreated with the specific EGFR tyrosine kinase inhibitor, AG1478 for 30 minutes prior to exposure to 5-HT. Thus, the 5-HT$_{2A}$ receptor transactivates the EGFR through the intrinsic kinase activity of the EGFR, in a manner already shown to occur with other GPCR’s 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-HT$_{2A}$ 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. Figure 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-HT$_{2A}$ 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-HT$_{2A}$ receptor in mesangial cells.

The 5-HT$_{2A}$ receptor stimulates transcription factors by transactivation of EGFR’s. Next, we examined the effects of 5-HT on the activation of three EGFR-stimulated transcription factors (E2F, CREB, and NF-κB). Figure 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 (EMSA). 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-HT$_{2A}$
receptor in mesangial cells activates transcription factors through the intermediary actions of the EGFR.

Pre-treatment 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 pre-treatment might also desensitize the EGFR. Figure 3 shows the results of studies in which EGF-induced ERK phosphorylation was assessed after pre-treatment with vehicle or 5-HT. Those results clearly demonstrate that pre-treatment 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 Figure 2. Those results are shown in Figures 4a-c. Pre-treatment with 5-HT greatly reduced transcription 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 (non-binding) oligonucleotides.

Pre-treatment of mesangial cells with GPCR ligands attenuates EGFR autophosphorylation. Figures 3 and 4 show that multiple signals that reside downstream from the EGFR can be attenuated by pre-treatment with 5-HT. This suggested to us that the desensitization of the EGFR most likely occurs at the level of the receptor itself. Therefore, we tested the effects of pre-treatment with 5-HT on the ability of EGF to induce autophosphorylation of the EGFR. Figure 5 shows that pre-treatment 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 pre-treatment of cells with 5-HT is truly important, we would expect that other GPCR’s might also desensitize the EGFR. Indeed, attenuation of EGF-induced phosphorylation of the EGFR was observed when cells were pre-treated with other mitogenic GPCR ligands such as bradykinin and lysophosphatidic acid (Figures 6a, 6b) and 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. Figure 6c shows that when cells were pre-treated with 1 µM phorbol 12-myristate, 13-acetate (PMA), the ability of EGF to induce tyrosine phosphorylation of the EGFR was reduced by at least 60%. These results suggest that PKC is involved in both transactivation and desensitization of the EGFR by GPCR’s.

**GPCR-induced EGFR desensitization is associated with EGFR internalization.** One potential mechanism through which the EGFR could be desensitized is by internalization such that cell surface EGFR’s available for binding by EGF would be diminished. Thus, we measured cell surface EGFR’s by ligand binding and by confocal microscopy. For ligand binding, cells were treated for one hour with vehicle, EGF or 5-HT, subjected to acid wash, and then cell surface $^{125}$I-EGF binding was measured. Figure 7 shows that preincubation with either 5-HT (300 nM or 3 µM) or EGF (20 ng/ml) resulted in a marked down-regulation of cell surface $^{125}$I-EGF binding, which was nearly complete by 10 min. of pre-incubation. This down-regulation of binding could either be due to decreased numbers of cell surface receptors or to decreased affinity of the cell surface receptors for EGF.

Figure 8 (Panels a-d) show results of experiments in which cell surface receptors were visualized in non-permeabilized cells with a FITC–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 (a), 300 nM 5-HT for 20 min (b) or 60 min (c), or with EGF (20 ng/ml) for 60 min (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 EGFR’s in mesangial cells. Those studies cannot, however, distinguish between a redistribution of EGFR’s to intracellular compartments and a loss of total EGFR’s (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 HEK 293 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. Figure 8 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 (panels e-h). Representative pictures are shown for treatment with vehicle (e), 300 nM 5-HT for 20 min (f) or 60 min (g), or with EGF (20 ng/ml) for 60 min (h). The red areas show the plasma membrane decorated by rhodamine-conconavalin-A after fixation of the cells, whereas the green areas represent the EGFR-GFP fusion protein (42). Yellow areas indicate superimposition of the red and green signals. We used a computer-algorithm to provide a semi-quantitative assessment (Lux units) of the subcellular localization of the EGFR-GFP fusion protein in HEK293 cells (Figure 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 H75% after stimulation with either EGF or 5-HT.

5-HT-induced EGFR down-regulation involves PKC. 5-HT activates PKC in mesangial cells (43), and it has been shown to mediate both PKC-dependent (27,44) and –independent effects (45) in those cells (see Figure 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. Figure 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 intact mesangial cells after 60 minutes of incubation. Incubation with 3 µM GF109203X nearly completely blocked the down-regulation
of the EGFR induced by 5-HT, but was ineffective in blocking EGF-induced down-regulation. These data correlate well with those in Figure 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 downregulation requires EGFR internalization.** Downregulation of cell surface receptors can involve receptor internalization, degradation, or both. In order to study whether internalization of the EGFR is a component of the GPCR-induced downregulation 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 β-arrestin and dynamin GTPase. Dynamin is required for clathrin-mediated endocytosis, and a dominant negative version of dynamin (K44A dynamin) has been previously been used to block internalization of both GPCR’s and RTK’s (46,47). A peptide fragment of β-arrestin 1 (β-arrestin1 319-418) has been previously demonstrated to block GPCR-induced clathrin-mediated endocytosis [46,79] because it binds clathrin cages but not GPCRs. Figure 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 minutes of treatment. Those results support a probable role for endocytosis in 5-HT-induced downregulation of EGFR’s in HEK293 cells.

We also exposed rat mesangial cells to 5-HT in the presence and absence of various chemical inhibitors of endocytosis, including conconavalin A (con-A), monodansylcadaverine, and potassium depletion. Because no specific inhibitors of endocytosis are available, we had to use these multiple approaches to block endocytosis. Figure 12a shows that these maneuvers
attenuated the downregulation of the EGFR induced by 5-HT (similar results of low temperature are not shown). We obtained similar results when endocytosis was blocked by low temperature or exposure to hypertonic sucrose (not shown). Figure 12b shows that incubation with cycloheximide (30 µg/ml) for one hour did not impair the downregulation of cell surface EGFR by 5-HT, ruling out a significant contribution of EGFR protein synthesis in this effect. Thus, these studies demonstrate that internalization of the EGFR is a key component of its downregulation by 5-HT. However, these studies do not demonstrate whether the functional desensitization of the EGFR induced by 5-HT also requires internalization.

Effect of 5-HT pre-treatment 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. Figure 13 illustrates experiments in which immunoblots were performed from whole cell lysates after incubation with 1 µM 5-HT for up to 150 minutes 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, only diminishing by 25% at 60 minutes. Thus, within the time frame of desensitization of the EGFR by 5-HT, there is only a small decline in the total number of EGFR’s. After 60 minutes, 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 GPCR’s can transactivate EGFR’s through a PKC-dependent pathway. This transactivation results in the initiation of multiple cellular signals, as well as subsequent internalization and desensitization of the EGFR’s. What is new about this work is that we show that activation of GPCR’s 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 EGFR’s. Desensitization of the EGFR can be initiated by several GPCR’s (5-HT2A, LPA, angiotensin AT1, bradykinin B2) that classically couple to Gq-type G proteins. Desensitization can also be mimicked by chemical activation of PKC by PMA. Activation of the 5-HT2A receptor desensitizes a number of EGFR signals, including EGFR autophosphorylation, phosphorylation of ERK, and activation of transcription factors (CREB, NF-κB, and E2-F). Internalization and downregulation 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 EGFR’s by a process associated with the loss of cell surface EGFR’s through internalization. Our data also show that the 5-HT2A receptor transactivates the EGFR in a manner already shown to occur with other GPCR’s such as those for angiotensin II (6), carbachol, lysophosphatidic acid, thrombin (12,39), and for the β2 adrenergic receptor (48). The process of EGFR induced by EGF and GPCRs are distinct in that the GPCR signal is PKC-dependent whereas the EGF signal is not. These relationships are depicted in Figure 14.

Although our data implicate PKC in the activation and desensitization of the EGFR induced by GPCR’s, the mechanism of that process is undefined. Prenzel et al. showed that release of heparin-bound EGF by a membrane-bound metalloproteinase-like enzyme mediated some of the effects of GPCR’s to activate EGFR’s (11). This enzyme resembled zinc-dependent proteases called ADAM’s (cell surface proteins that contain a disintegrin and metalloprotease 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-HT2A
receptor-induced phosphorylation of EGFR’s (not shown). Thus, those studies do not support a role for a PKC-activated membrane-bound metalloproteinase-like enzyme in the GPCR-induced activation of EGFR’s 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, Beguinot et al. demonstrated that PMA could induce internalization of EGFR’s and a transient decrease in cell surface $^{125}$I-EGF binding without inducing degradation of the EGFR’s (57). Others, however, have suggested that PKC-dependent phosphorylation enhances and stabilizes EGFR levels and/or signaling (58-62). PKC-a 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-a 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 GPCR’s 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 GPCR’s.

Some have suggested that PKC-mediated effects on EGFR’s 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. showed evidence that PKC could alter the affinity of EGF for the EGFR without down-regulating the receptor (65). Kaji et al. showed that PKC decreased the affinity of the EGFR’s for EGF without changing receptor number or by inducing internalization (67). In contrast, PKC-a was shown to reduce EGFR numbers without changing the affinity of EGF for the EGFR (63).

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). Veheijden et al. showed that PKC inhibits EGFR tyrosine kinase activity without changing receptor dimerization (68). 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 either phosphorylation of either Thr-654 or Thr-669 to down-regulation of the EGFR (69). On the other hand, Bowen et al showed that phosphorylation of Thr-654 blocked mitogenic stimulation by the EGFR (70). 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 GPCR’s occurs. The evidence for this is that pre-treatment of mesangial cells with 5-HT results in 1) a decrease in cell surface $^{125}$I-EGF binding, 2) a translocation of an EGFR-GFP fusion protein away from the plasma membrane, and 3) multiple inhibitors of clathrin mediated endocytosis prevent GPCR-driven internalization of the EGFR’s. Moreover, 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 minutes of pre-treatment with 5-HT appears to be largely independent of protein degradation because the amount of total cellular immunoreactive EGFR’s decreases by only about 25%.

We used several distinct maneuvers to block GPCR-induced internalization of EGFRs including concanavalin A (con-A), monodansylcadaverine, hypertonic medium, potassium depletion, low temperature, and dominant interfering constructs of β-arrestin and dynamin. Because no specific inhibitors of endocytosis are available, we had to use these multiple approaches to block endocytosis. Con-A is a lectin that binds selectively to glycoprotein-associated terminal mannose residues, and blocks their lateral movement in many cell types (36). Monodansylcadaverine blocks clathrin-mediated internalization proximal to endocytic vesicles (37,38). Hyperosmolarity interferes with clathrin-mediated endocytosis by preventing the
formation of clathrin-coated pits (35,71). Potassium depletion interferes with clathrin-mediated endocytosis by preventing 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 been previously been used to block internalization of both GPCR’s and RTK’s (46,47). A peptide fragment of β-arrestin 1 (β-arrestin1 (319-418) has been previously 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 GPCR’s.

In our experiments, we observed that con-A significantly lowered the basal level of 125I-EGF binding (Figure 12). The explanation for this effect is most likely con-A-induced proteolytic cleavage of the EGFR, as recently described by Tang et al. in vascular smooth muscle cells (75). That group also showed evidence 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, & Tyr1183) and two minor (Tyr992 & Tyr1086) autophosphorylation sites (76,77), and binding/activation sites for phospholipase C-γ, adapter protein 2 (78), and Shc (79). The proteolytic effect of con-A on EGFR does not universally attenuate EGFR functions, however. In NIH3T3 cells, con-A does not affect EGFR functions such as EGF binding or proximal signals like the activation of PLC-γ or sodium-proton exchange activity (80,81).

Although our work has not yet delineated the precise molecular mechanisms through which PKC and clathrin-mediated endocytosis conspire to rapidly desensitize EGFR’s after activation of GPCR’s, the existence of this process defines a novel form of cross talk between GPCR’s and EGFR’s. Thus, GPCR’s can both transactivate and desensitize EGFR’s in kidney mesangial cells. The implication of these findings is that GPCR activation in some settings may precondition cells to become unresponsive to subsequent challenge with mitogens like EGF that bind to and signal through RTK’s. Further work will be needed to 1) define the molecular
mechanisms of this process, 2) determine whether GPCR’s linked to other G proteins (G<sub>S</sub>, G<sub>i</sub>) can desensitize the EGFR, 3) determine whether GPCR’s can desensitize other RTK’s, and 4) establish whether this process occurs in other cell types.
ACKNOWLEDGMENTS

We thank Drs. Alexander Sorkin and Royston Carter (University of Colorado) for providing the plasmid encoding the EGFR-GFP fusion protein, and for helpful suggestions.
FIGURE LEGENDS

Figure 1. Transactivation of the EGFR by the 5-HT$_{2A}$ receptor in rat renal mesangial cells is PKC-dependent. Cells were pre-loaded with $^{32}$P orthophosphoric acid, following which EGFR’s were isolated by immunoprecipitation as described in Materials and Methods. A. To determine the concentration-dependent phosphorylation of EGFR, cells were treated with various concentrations of 5-HT for 3 minutes. B. To determine the time-dependent phosphorylation of EGFR, cells were treated with 1 µM 5-HT for various periods of time from 1-10 minutes in the presence or vehicle (black bars) or 10 µM AG1478 (hatched bars). Inset shows representative autoradiographs with (+) or without (-) AG1478. C. Cells were preincubated with inhibitors (100 µM GF109203X or 5 µM PD98059) for 30 minutes prior to treatment with 1 µM 5-HT for 3 minutes. Inset shows representative autoradiographs with and without inhibitors. Experiments were repeated at least three times. Error bars represent means ± standard errors. * indicates $p < .05$ vs control, and † indicates $p < 0.01$ vs 5-HT without blocker as assessed using ANOVA and Fisher’s protected least significant difference post-hoc test for multiple comparisons. Similar results were found after immunoprecipitation of EGFR’s followed by phosphotyrosine immunoblotting (not shown).

Figure 2. 5-HT induces activation of transcription factors via EGFR activation. Electrophoretic mobility shift assays were performed to assess the activities of E2F, CREB, and NF-κB after stimulation of the cells with 5-HT or EGF. Some cells were preincubated with 10 µM AG1478 for 30 minutes prior to treatment with 1 µM 5-HT or EGF, 10 ng/ml. Experiments were repeated at least three times.

Figure 3. Pre-exposure of mesangial cells to 5-HT attenuates EGF-induced ERK phosphorylation. ERK phosphorylation was measured using a phosphorylation state-specific antibody as described under Materials and Methods. Cells were treated with vehicle or 1 µM 5-
HT for one hour prior to treatment for 3 minutes with various concentrations of EGF. Panel A shows representative autoradiographs from one of the experiments. The values of the bars in Panel B represent the mean values obtained from three separate experiments ± standard errors. * indicates $p < .05$ vs control, and † indicates $p < .05$ vs the values obtained from the same concentrations of EGF without prior treatment with 5-HT as assessed using ANOVA and the Bonferroni-Dunn test for multiple comparisons.

Figure 4. Pre-exposure of mesangial cells to 5-HT attenuates activation of multiple transcription factors by EGF. Transcription factor assays were performed using an electrophoretic mobility shift method as described in Materials and Methods. Cells were pre-treated for 20 minutes or one hour with vehicle or 1 µM 5-HT, after which nuclear extracts were incubated with radioactive oligonucleotides in the presence or absence of competing unlabeled oligonucleotides. The values of the bars represent the mean values obtained from three separate experiments ± standard errors. * indicates $p < .05$ vs control, and † indicates $p < .01$ vs 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. Panels A and B contain representative inserts from one of the experiments.

Figure 5. Pre-exposure of mesangial cells to 5-HT attenuates EGFR autophosphorylation.

EGFR phosphorylation was measured as described in the legend of Figure 1. Cells were treated with vehicle (light bars) or 1 µM 5-HT (dark bars) for one hour prior to treatment for 3 minutes with various concentrations of EGF. The values of the bars represent the mean values obtained from three separate experiments ± standard errors. * indicates $p < .05$ vs control, and † indicates $p < .01$ vs 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.

Figure 6. Pre-exposure of mesangial cells to multiple GPCR ligands attenuates EGFR
autophosphorylation. EGFR phosphorylation was measured as described in the legend of Figure 1. Cells were treated with vehicle, 100 nM bradykinin (BK), 100 nM lysophosphatidic acid (LPA) or 1 µM PMA for one hour prior to treatment for 3 minutes with various concentrations of EGF. The values of the bars represent the mean values obtained from three separate experiments ± standard errors. * indicates $p < .05$ vs control, and † indicates $p < .01$ vs the values obtained from EGF without prior treatment with BK, LPA, or PMA as assessed using ANOVA and Fisher’s protected least significant difference post-hoc test for multiple comparisons.

Figure 7. Pre-exposure of mesangial cells to 5-HT or EGF diminishes cell surface $^{125}$I-EGF binding. Cells were pre-treated with 300 nM 5-HT, 3 µM 5-HT or 20 ng/ml of EGF for various time periods (10-60 minutes), followed by acid washing. Cell surface $^{125}$I-EGF binding was then measured as described under Materials and Methods. The plot shown in this figure is derived from one experiment performed in duplicate, which is representative of three that showed similar results.

Figure 8. Microscopic evidence for the loss of cell surface EGF receptors after pre-exposure to 5-HT. Panel A-D show results of a representative micrograph experiment (one of three) in which cell surface receptors were visualized in non-permeabilized mesangial cells with a FITC–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 (A), 300 nM 5-HT for 20 min (B) or 60 min (C), or with EGF (20 ng/ml) for 60 min (D). Panels E-H show 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-HT$_{2A}$ receptor into HEK 293 cells. This second method was used to 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 with EGF (20 ng/ml) for 60 min (H). The red areas show the plasma membrane decorated by rhodamine-concanavalin-A after fixation of the cells, whereas the green areas represent the EGFR-GFP fusion protein. Yellow areas indicate superimposition of the red and
green signals. The confocal micrographs are representative of three separate experiments.

Figure 9. Semi-quantitative analysis of the subcellular location of the EGFR-GFP fusion protein after treatment with 5-HT. Microscopic fields derived from the experiments described in Figure 8, panels E-H, were subjected to analysis by computer algorithm. The relative intensities of Con-A rhodamine (cell surface-hatched bars), EGFR-GFP on the cell surface (white bars), and EGFR-GFP in the nucleus (dark bars) were expressed as lux units.

Figure 10. Effects of inhibition of PKC on 5-HT-induced down-regulation of cell-surface 125I-EGF binding to mesangial cells. Cells were treated with vehicle or 3 µM GF109203X for 30 minutes, then with 1 µM 5-HT (dark bar), 10 ng/ml of EGF (hatched bar), or vehicle (white bar) for one hour prior to measurement of cell surface 125I-EGF binding as described under Materials and Methods. The values presented are derived from the means ± standard errors from three experiments performed in duplicate. * indicates $p < .05$ vs control as assessed using ANOVA and the Bonferroni-Dunn test for multiple comparisons.

Figure 11. Effects of blocking clathrin-mediated endocytosis with cDNA constructs on 5-HT$_{2A}$ receptor-induced EGFR-GFP internalization in HEK293 cells. Cells were transfected with cDNA’s encoding both the 5-HT$_{2A}$ receptor and EGFR-GFP in addition to empty vector (panels A and B) or cDNA’s encoding dominant interfering mutants of b-arrestin (panels C and D) or dynamin (panels E and F) as described under Materials and Methods. The cell surfaces were then decorated 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 5HT$_{2A}$ receptor was not visualized, but 5-HT did not induce internalization of the EGFR absent transfection with the 5-HT$_{2A}$ receptor (not shown).
Figure 12. Effects of blocking clathrin-mediated endocytosis with chemical inhibitors on 5-HT$_{2A}$ receptor-induced EGFR-GFP internalization in mesangial cells. Rat renal mesangial cells were exposed 1 µM 5-HT in the presence and absence of various inhibitors of clathrin-mediated endocytosis, including potassium depletion buffer, concanavalin A (con-A), and monodansylcadaverine (MDC). Then, cell surface $^{125}$I-EGF binding was measured as described under Materials and Methods. Panel A shows that these maneuvers attenuated the desensitization of the EGFR induced by 5-HT. Panel B shows that incubation with cycloheximide (30 µg/ml) for one hour 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 $p < .05$ vs control as assessed using ANOVA and the Bonferroni-Dunn test for multiple comparisons.

Figure 13. Effects of pre-treatment of mesangial cells with 5-HT on the levels of immunoreactive EGFR’s. Mesangial cells were treated with 1 µM 5-HT for the indicated times, and then cells were scraped into Laemmli buffer, heated to 90° C for 2 minutes. Proteins were separated by SDS-PAGE on 4-20% polyacrylamide gels (Novex, San Diego, CA) and resolved under nonreducing conditions. Immunoblots were then performed with an anti-EGFR antibody as described in the Materials and Methods section. The insert is representative of three identical experiments. Values for each time point were determined by densitometry, and represent the means ± standard errors for the three experiments.

Figure 14. Schematic depiction of GPCR regulation of EGFR. This scheme shows that GPCR’s regulate various EGFR functions through a PKC-dependent pathway.
271(49), 31098-31105


**Figure 1**

**Panel A**
- Time (minutes): 0, 1, 2, 3, 5, 10
- **Legend:**
  - Vehicle
  - 5-HT
  - PD98059
  - GF109203X

**Panel B**
- EGF Phosphorylation (% of Control)
- [5-HT] (µM): 0, EGF, 0.1, 0.3, 1.0, 3.0
- **Legend:**
  - Vehicle
  - EGF
  - 5-HT
  - + AG1478

**Panel C**
- **Legend:**
  - Vehicle
  - 5-HT
- **Legend with Symbols:**
  - * by guest on September 1, 2017 http://www.jbc.org/ Downloaded from

**Grewal et al., Figure 1**
Grewal et al., Figure 2
Grewal et al., Figure 3
Grewal et al., Figure 4
EGFR Phosphorylation (% of Control)

Grewal et al., Figure 5

EGF (ng/ml) 0 1 3 10 30 100
Vehicle
+ 5-HT

* * * * ＊ ＊ ＊ ＊
Grewal et al., Figure 6
Grewal et al., Figure 7
Grewal et al., Figure 8
Grewal et al., Figure 9
$^{125}$I-EGF Binding (% of control)

- **Control**
  - Vehicle
  - 1 µM 5-HT
  - 1 µM 5-HT with EGF (10 ng/ml)

- **GF109203X**
  - Vehicle

* indicates statistical significance.
Vehicle  5-HT x 60 min

Mock

β-Arrestin1 (319-418)

K44A Dynamin

Grewal et al., Figure 11
125I-EGF Binding (% of control)

KCl Depletion

Con-A

MDC

No manipulation

Endocytosis Inhibitor Alone + 1 µM 5-HT

Endocytosis Inhibitor

Vehicle CHX

1 µM 5-HT

Control

*
EGFR in Whole Cell Lysates (% of control)

Time of Incubation with 1 µM 5-HT in minutes

Grewal et al. Figure 13
EGF

GPCR → PKC → ? → EGFR

Internalization          Signals

Signals                Desensitization

Downregulation

Grewal et al., Figure 14
G protein-coupled receptors desensitize and downregulate EGF receptors in renal mesangial cells
Jasjit S. Grewal, Louis M. Luttrell and John R. Raymond

J. Biol. Chem. published online May 22, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103578200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at
http://www.jbc.org/content/early/2001/05/22/jbc.M103578200.citation.full.html#ref-list-1