Agonist-Induced Desensitization and Phosphorylation of Human 5-HT_{1A} Receptor Expressed in Sf9 Insect Cells†

Canan G. Nebigil,‡,§ Maria N. Garnovskaya,‡,§ Shirley J. Casañas,‡,§ Janet G. Mulheron,§,|| Eric M. Parker,⊥ Thomas W. Gettys,▽ and John R. Raymond*,§

Departments of Medicine (Nephrology Section), Duke University, and Department of Veterans Affairs Medical Center, Durham, North Carolina 27705, Department of Medicine (Gastroenterology Section), Medical University of South Carolina, Charleston, South Carolina 29425, Department of Central Nervous System Biology, Bristol-Myers Squibb, Wallingford, Connecticut 06492, and Department of Oncology Drug Discovery, Bristol-Myers Squibb, Princeton, New Jersey 08543

Received May 3, 1995⊗

ABSTRACT: The human 5-HT_{1A} receptor was expressed in Sf9 insect cells to examine desensitization as manifested by agonist-induced uncoupling from G proteins and second messengers. New binding sites were detected after infection of cells with the 5-HT_{1A} receptor-bearing baculovirus. 5-HT_{1A} receptor agonists caused inhibition of cAMP accumulation that could be attenuated by specific receptor antagonists. Brief pretreatment with 5-HT resulted in (1) an uncoupling of receptor from G proteins as evidenced by a loss of high-affinity agonist binding sites and a diminished ability of the receptor to increase incorporation of AA-GTP into endogenous $G_{o\alpha}$-like G proteins, (2) a decreased ability of the receptor to inhibit cAMP accumulation, and (3) increased phosphorylation of the 5-HT_{1A} receptor on serine and threonine residues. Phosphorylation occurred in the presence of a number of cyclic nucleotide dependent kinase inhibitors, and desensitization of the cAMP response occurred in the presence of H-7 and also in cells with prolonged exposure to PMA. Both phosphorylation and desensitization were markedly attenuated by 100 nM and 1 μM heparin and demonstrated similar time courses and concentration−response relationships. Those results demonstrate a close association between agonist-induced desensitization and phosphorylation of the 5-HT_{1A} receptor in Sf9 cells through a pathway that mainly does not involve protein kinase A or C and might involve a G protein-linked receptor kinase.

Cloning studies have revealed a heretofore unsuspected diversity among the receptors for serotonin (5-hydroxytryptamine, 5-HT).[1] Nearly all of the cloned 5-HT receptors are coupled to G proteins, with the exception of the 5-HT_3 receptor, a serotonin-gated ion channel (Maricq et al., 1991). The genes/cDNA for at least a dozen subtypes of mammalian G protein-coupled (GCR) 5-HT receptors have been cloned (Hartig et al., 1992; Tecott & Julius, 1993), as have those for several closely related nonmammalian GCR-type 5-HT receptors (Hen, 1992). The existence of some others is supported by pharmacological studies. On the basis of pharmacological and genetic/molecular biological data, the GCR-type 5-HT receptors can be divided into three groups: the 5-HT_1 group, the 5-HT_2 group, and the 5-$HT_{4,5,6,7}$ group.[2] The 5-HT_1 group comprises at least five mammalian members including 5-HT_{1A}, 5-HT_{1B} (5-$HT_{1D\beta}$), 5-HT_{1D} (5-$HT_{1D\alpha}$), 5-HT_{1E}, and 5-HT_{1F} (5-$HT_{1E\beta}$) receptors. Those bear genetic and pharmacological similarities to several *Drosophila melanogaster* receptors and a receptor derived from the nervous system of the mollusc *Lymnaea stagnalis* (Hen, 1992; Sugamori et al., 1993). The existence of other closely related receptors from mammalian blood vessels, gut, and kidney has been documented by pharmacological means. The most typical features of the 5-HT_1 receptor group are their high affinity for 5-HT (*ca.* 1−100 nM) and an ability to inhibit the enzyme adenylyl cyclase primarily through pertussis toxin-sensitive G protein pools.

With the discovery of so many new players, one major issue in signal transduction over the last 3 years has been how specificity is conferred on the interactions of receptors with various combinations of G protein heterotrimers which "turn on" cellular signals. Important work for many groups has shown that a simple linear model between receptor and

† This work was supported in part by the NIH (NS30927 to J.R.R. and DK42486 to T.W.G.) and a Merit Award from the Department of Veterans Affairs (J.R.R.).

* Address for correspondence: Box 3459 Duke University Medical Center, Durham, NC 27710. Telephone: (919) 286-0411, ext. 7345. Telefax: (919) 286-6879.

‡ Denotes equal contribution by these three authors.

§ Duke University and Department of Veterans Affairs Medical Center.

|| Bristol-Myers Squibb, Princeton.

⊥ Bristol-Myers Squibb, Wallingford.

▽ Medical University of South Carolina.

⊗ Abstract published in *Advance ACS Abstracts,* August 15, 1995.

[1] Abbreviations: AA-GTP, [^{32}P]azidoanilidoguanosine triphosphate; βARK, β-adrenergic receptor kinase; CAMK II, Ca^{2+}- and calmodulin-dependent protein kinase type II; cAMP, adenosine 3′,5′-cyclic monophosphate; DMSO, Me_2SO_4; GRK, G protein-coupled receptor kinase; H-7, 1-(5-isoquinolonesulfonyl)-2-methylpiperazine; IBMX, isobutylmethylxanthine; PDBu, phorbol 12,13-dibutyrate; 5-HT, 5-hydroxytryptamine, or serotonin; NAN-190, 1-(2-methoxyphenyl)-4-[4-2-phthalimido)butyl]piperazine hydrobromide; PBS, isotonic phosphate-buffered saline solution; PKA, protein kinase A, or cAMP-dependent protein kinase; PKG, cGMP-dependent kinase; PKC, protein kinase C, or calcium- and phospholipid-dependent kinase; PMA, phorbol 12-myristate 13-acetate; Rp-cAMPS, Rp diastereomer of adenosine 3′,5′-cyclic monophosphothioate; Sp-cAMPS, Sp diastereomer of adenosine 3′,5′-cyclic monophosphothioate; (S)-UH-301, (S)-5-*F*-8-hydroxy-2-(dipropylamino)tetralin isomer; 8-OH-DPAT, (±)-8-hydroxydipropylaminotetralin.

[2] We use the nomenclature outlined in the Receptor & Ion Channel Nomenclature supplement of *Trends in Pharmacological Sciences* [(1994) *Trends Pharmacol. Sci. 15,* 25].

0006-2960/95/0434-11954$09.00/0

effector with an intervening G protein "on−off switch" does not adequately describe the complex paradigms contributing to signaling specificity. As important as mechanisms for turning on signals are mechanisms for turning signals off. The simplest mechanism for terminating a signal is to remove the agonist stimulus. However, under a scheme of that kind, the GCR-G protein−effector signaling components serve only as passive participants. Clearly, other mechanisms of attenuating signals in the continued presence of an agonist stimulus are desirable. Fortunately, nature has provided negative feedback mechanisms through a group of processes called desensitization. Many GCRs demonstrate rapid desensitization in the continued presence of agonist or another stimulus, manifested by uncoupling from second messengers (Sibley et al., 1987). In most instances, this rapid desensitization phenomenon depends on the posttranslational modification of the receptor by phosphorylation reactions (Sibley et al., 1987; Hausdorff et al., 1990; Huganir et al., 1990). A number of 5-HT receptors have been shown to undergo desensitization, including the 5-HT$_{2A}$ receptor (Pauwels et al., 1990; Kagaya et al., 1993), the 5-HT$_{1D\beta}$ receptor (Unsworth & Molinoff, 1992; Pleus & Bylund, 1992), and the 5-HT$_{1A}$ receptor (Blier & de Montigny, 1990; Raymond, 1991; Liu & Albert, 1991; Harrington et al., 1994). Regarding the 5-HT$_{1A}$ receptor, several groups have suggested a role for both protein kinase A (PKA, or cAMP-dependent protein kinase) and protein kinase C (PKC, or calcium- and phospholipid-dependent kinase) in desensitization of 5-HT$_{1A}$ receptor-mediated effects (Raymond, 1991; Liu & Albert; 1991; Harrington et al., 1994). However, the patterns of desensitization seemed to differ depending on the host cell used in the various studies.

As an initial effort to explore diverse patterns of regulation of 5-HT$_{1A}$ receptor function, we investigated the desensitization properties of 5-HT$_{1A}$ receptors in Sf9 insect cells. Using that model system, we have previously shown that the human 5-HT$_{1A}$ receptor activates a 40−41-kDa $G_{o\alpha}$-like G protein in Sf9 cells (Mulheron et al., 1994). In the current study, we examine cAMP second messenger modulation by the receptor (turning the signal on) and the effects of brief agonist pretreatment on the ability of the receptor to subsequently modulate cAMP levels (turning the signal off). Further, we also studied the effects of the same treatment on the phosphorylation state of the 5-HT$_{1A}$ receptor.

MATERIALS AND METHODS

Materials. [α-^{32}P]ATP (30 Ci/mmol), [γ-^{32}P]ATP (3000 Ci/mmol), and 5-[^{3}H]carboxamidotryptamine were from DuPont New England Nuclear, and 8-OH-[^{3}H]DPAT [8-OH-2-(di-*n*-propylamino)1,2,3,4-tetrahydronaphthalene] was from Research Products International (Mt. Prospect, IL). [^{32}P]-Azidoanilido-GTP was synthesized from [^{32}P]GTP (DuPont NEN) as described previously (Gettys et al., 1994; Mulheron et al., 1994). Cell culture supplies were from GIBCO (Grand Island, NY). *Spodoptera frugiperda* (Sf9) cells were obtained from Invitrogen. (*S*)-UH-301 was a gift of Dr. John Kebabian (Research Biochemicals Incorporated). All other reagents were of the highest quality available from Sigma (St. Louis, MO), Research Biochemicals Incorporated (Natick, MA), or Calbiochem (San Diego, CA).

Cell Culture. Insect cells were grown in monolayer or in suspension with serum-supplemented (10%) Grace's insect cell medium or with a defined non-serum supplemented medium (sf-900 II), each supplemented with gentamycin (100 μg/mL) at 26−28 °C in ambient atmosphere.

Expression of the Human 5-HT$_{1A}$ Receptor in Sf9 Cells. For most of the studies we used a baculovirus construct bearing the DNA of the human 5-HT$_{1A}$ receptor, which was created and characterized as described previously (Parker et al., 1994). For a few of the studies, we used a construct that incorporated an epitope recognition site (IBI Flag). Wild-type 5-HT$_{1A}$ receptor cDNA (Fargin et al., 1988) in the PBC12BI vector was amplified by PCR with mutagenic primers as follows. The 5′-primer was 5′-GGGCTAGC-CC**ATG**GACTACAAGGACGACGATGACGACAAG ˇ G-ATGTGCTCAGCCCTGG-3′, and the 3′-primer was GGGCTAGCCTCAGGCGGCAGAAGTTAC-5′. The oligonucleotides were designed to incorporate unique palindromic sites for NHE1 restriction enzyme (single underline), a start codon (bold), an amino-terminal Flag epitope (double underline), an enterokinase cleavage site (ˇ), and sufficient portions of the 5′- and 3′-coding regions of the 5-HT$_{1A}$ receptor to allow amplification. The PCR products were ligated into the NHE1 site of the pBlueBac vector. Orientation and correct sequence were confirmed by Sanger sequencing. The former construct yielded higher expression levels than the "flagged" construct and was used for all of the phosphorylation experiments. Virus was titered by the end point dilution method (Summers & Smith, 1987). Cells were examined 48−72 h after infection. Expression levels for the nontagged receptors were about 300−600 fmol/mg of protein (~2000−4000 sites per cell). The Flag-tagged receptors consistently attained much lower expression levels (~50−150 fmol/mg of protein, or 300−1000 sites per cell) than the untagged receptors. Probably because of the low level of expression, the flag-tagged receptors were not found to be suitable for detecting phosphorylated receptors in immunoprecipitates using either the anti-Flag M2 antibody (IBI) or specific anti-receptor sera. Therefore, the nontagged receptors were used for all of the phosphorylation studies and more than 90% of the desensitization studies. The baculovirus β_2-adrenergic receptor construct was obtained from Dr. Bob Lefkowitz (Pei et al., 1994).

Ligand Binding. Membranes were prepared by hypotonic lysis in ice-cold lysis buffer (5 mM Tris and 2 mM EDTA, pH 7.4) with 10 strokes of a glass-on-glass dounce and collected by centrifugation at 37000*g* for 20 min at 4 °C. Membranes were resuspended in 50 mM Tris, pH 7.4, supplemented with 8-OH-[^{3}H]DPAT or 5-[^{3}H]carboxamidotryptamine with or without 10 μM 5-HT as competing ligand, and incubated for 60 min at room temperature. Membranes were isolated by vacuum filtration through Whatman glass fiber filters and then subjected to scintillation counting.

Protein Determination. Proteins were measured by the method of Bradford using bovine serum albumin as the standard (Bradford, 1976).

Photoaffinity Labeling of Insect Cell G Proteins. Photoaffinity labeling of G proteins was performed using 50 μg of crude membrane protein exactly as described (Mulheron et al., 1994). In order to enhance agonist-induced labeling of AA-GTP, 10 μM GDP was included in the incubation mixture to suppress basal GTP binding in all experiments. Membranes derived from cells pretreated with either vehicle of 5-HT were preincubated at room temperature for 10 min with GDP in the presence or absence of 10 μM 5-HT in

50-μL volumes of 30 mM Hepes, pH 7.5, 100 mM NaCl, 100 mM EDTA, 1 mM benzamidine, 50 μM leupeptin, 5 mM $MgCl_2$, and 10 μM GDP. Then, 0.6 μCi of AA-GTP (3160 Ci/mmol) was added for an additional 10-min incubation. The membranes were collected by centrifugation at 13000*g* for 10 min and resuspended in the identical buffer lacking GDP but supplemented with 2 mM DTT. After exposure to ultraviolet light for 3 min at 4 °C, the reactions were stopped by addition of Laemmli buffer followed by 10% SDS−PAGE and autoradiography.

cAMP Assay. Cell monolayers were mechanically detached by striking the flasks 48−72 h after infection, incubated with gentle rocking for 30 min in PBS supplemented with 100 μM IBMX, and then aliquoted into tubes supplemented with the various concentrations of drugs. Assays were terminated after 5 or 30 min by centrifugation and removal of extracellular solutions from the cell pellet. Intracellular cAMP was released from cells either by acid lysis followed by neutralization to pH 5.8 or by sequential freeze−thaw−boil treatment. Samples were acetylated, and cAMP assays were performed by a standard radioimmunoassay using cAMP antibody and ^{125}I-labeled cAMP tracer synthesized in the laboratory of T.W.G. (Raymond et al., 1994).

Desensitization Assay. Cell monolayers were treated with vehicle or various doses of agents for various times in PBS supplemented with 100 μM IBMX, washed three times with PBS (room temperature), and then processed as above (Raymond, 1991). In desensitization assays, cells were exposed to test agents for 5 min to minimize the amount of time that might allow for resensitization. For experiments involving low molecular weight heparin as an inhibitor, cells were first permeabilized with streptolysin-O in very small volumes (<1 mL) to allow access of heparin to the intracellular compartment without grossly diluting (by egress from the cells) potentially important cellular mediators of desensitization.

Permeabilized Cell Phosphorylations. Cells were treated using a modification of a previously described protocol (Raymond, 1991). Detached cells ($\sim 1 \times 10^7$ per condition) were washed twice with KGG buffer (150 mM potassium glutamate, 10 mM Hepes, 5 mM EDTA, and 7 mM $MgCl_2$, pH 7.3) and then were permeabilized at room temperature by exposure to preactivated streptolysin-O (0.2 unit/mL) in KGG buffer for 10 min. The cells were washed once with unsupplemented KGG buffer and then resuspended in 500 μL of KGG supplemented with 2 mCi of [γ-^{32}P]ATP, 2.2 mM carrier-free ATP, and 5 mM glucose, followed by treatment with various agents. Because the permeabilization procedure allows ready diffusion of small molecules such as ATP across the plasma membrane, intracellular and extracellular concentrations of ions and other small molecules were assumed to be equal. After incubation with the various agents for 30 min at room temperature, the reactions were stopped by adding 10 mL of ice-cold KPE buffer (100 mM potassium gluconate, 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 5 mM orthovanadate, 5 mM EDTA, and 10 mM sodium fluoride, pH 7.3). Cells were washed in the same buffer twice by resuspension and centrifugation and then were disrupted by rapid freeze−thaw and with 10 strokes of a glass-on-glass dounce. The membranes were washed twice in the same buffer and then were suspended in solubilization buffer (1% SDS, 0.1% Triton X-100, 0.1% deoxycholate, 100 mM NaCl, 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 5 mM orthovanadate, 5 mM EDTA, 5 mM EGTA, and 10 mM sodium fluoride, pH 7.3, supplemented with 5 μg/mL each of leupeptin, soybean trypsin inhibitor, and benzamidine). Membranes were placed on ice and vortexed intermittently for 1 h, and then the mixture was centrifuged at 500000*g* for 30 min. Soluble supernatants were precleared by incubation for 1 h with a 1:100 dilution of preimmune rabbit serum and 20 μL of preswollen protein A−Sepharose 6-MB beads. The supernatants were then incubated at 4 °C overnight with 1:50 dilutions each of sera 5-HT_{1A}-TIL-4 and 5-HT_{1A}-TIL-2 (Raymond et al., 1989; Raymond et al., 1993). Receptor−immune complexes were precipitated with 20 μL of preswollen protein A−Sepharose 6-MB beads and washed twice in solubilization buffer and once in isotonic phosphate-buffered saline. Immune complexes were removed from the beads by adding Laemmli sample buffer and boiling for 5 min. Samples were then run on discontinuous 10% SDS−PAGE and visualized by autoradiography or by use of a PhosphorImager.

Phosphoamino Acid Analysis of 5-HT_{1A} Receptor. Twenty million cells were preincubated for 30 min at room temperature in phosphate-free DMEM and then with the same medium supplemented with 20 mCi of $^{32}P_i$ for 60 min. Cells were gently pelleted at 200*g*, resuspended, and exposed to 10 μM 5-HT for 30 min. Cells were washed, lysed, and solubilized as previously described (Raymond, 1991). Receptors were purified by immunoprecipitation, run on 10% SDS−PAGE gel, and exposed to film. The receptor band was visualized after 2 days, excised, minced with a razor blade, and extracted from the gel by a modification derived from Boyle et al. (1991); extraction was done twice with 250 μL of 50 mM ammonium bicarbonate (pH 7.3) while homogenizing the gel slice with a hand-held tissue grinder (Kontes, Vineland, NJ) for 5 min. The slurry was transferred into a fresh screw-top tube, and residual gel bits were collected with one additional 250-μL rinse, which was added to the slurry along with 50 μL of β-mercaptoethanol and 10 μL of 10% SDS. The slurry was boiled for 10 min and then incubated at 37 °C for 2 h. The slurry was transferred to a polycarbonate tube and centrifuged at 500000*g* for 30 min. The supernatant was harvested and set aside. The pellet was washed with 250 μL of 50 mM ammonium bicarbonate, and the supernatant from this step was added to the other supernatant for a volume of 1 mL. To the supernatant was added 200 μL of trichloroacetic acid and 20 μg of RNase (a carrier protein); the mix was chilled on ice for 60 min and then centrifuged at 15000*g* for 10 min at 4 °C. The supernatant was discarded, and the precipitate was washed with 500 μL of ice-cold acetone.

The sample was divided in half, one portion being directly hydrolyzed in 1 M HCL at 110 °C for 6 h. The other portion was first hydrolyzed in 1 M NaOH for 24 h at 50 °C, a treatment that degrades phosphoserine and phosphothreonine, but not phosphotyrosine, according to Duclos et al. (1991). The sample was lyophilized twice to remove base and then subjected to HCl hydrolysis. Samples (as well as *O*-phospho-DL-serine, -threonine, and -tyrosine standards at 10 nmol each) were then chromatographed on cellulose plates in a solvent composed of isobutyric acid (5 parts) and 500 mM NH_4OH (3 parts) using an ascending "brick" technique as described (Duclos et al., 1991). A brown paper towel wick was taped to the top of the plate to allow the ascending separation to proceed for 18 h. Standards were visualized

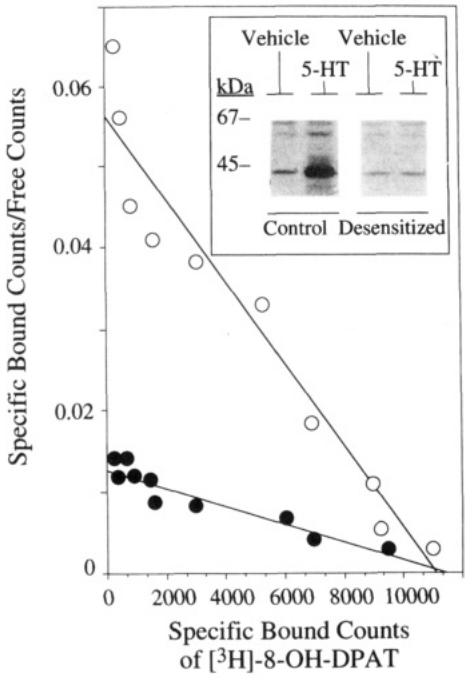

FIGURE 1: Uncoupling of human 5-HT$_{1A}$ receptor from G proteins in Sf9 cells after agonist pretreatment: A Scatchard plot derived from the saturation isotherm of binding of 8-OH-[^{3}H]DPAT to Sf9 cell membranes 72 h after infection with baculovirus bearing the DNA of the 5-HT$_{1A}$ receptor. Open circles (○) are from membranes derived from vehicle-treated cells, and closed circles (●) are from membranes obtained from cells pretreated for 30 min with 10 μM 5-HT. This experiment is representative of two done in triplicate with 8-OH-[^{3}H]DPAT. Similar results were obtained in two separate assays using 5-[^{3}H]carboxamidotryptamine. The inset depicts 10% SDS−PAGE of AA-GTP-labeling experiments of the 40−41-kDa $G_{o\alpha}$-like G protein in vehicle-treated and desensitized membranes (10 μM 5-HT for 30 min) in the absence or presence of an acute treatment with 10 μM coincident with AA-GTP incubation. Each lane contained 50 μg of membrane protein. This autoradiogram (representative of three experiments) was exposed to Kodak X-AR film for 36 h at −80 °C.

with an evenly applied ninhydrin spray and a heated hair drier, and radioactive bands were visualized by autoradiography.

Statistical Analyses. Paired one-tailed *t*-tests were used to compare the data sets. A modified Bonferroni was used to correct for multiple comparisons as described in the appropriate figure captions to allow us to maintain a 95% confidence interval.

RESULTS

Uncoupling of Human 5-HT$_{1A}$ Receptor from G Proteins in Sf9 Cells after Agonist Pretreatment. We used two separate techniques to document that agonist pretreatment uncouples the 5-HT$_{1A}$ receptor from G proteins native to the Sf9 cell. Saturation plots of binding of the agonist ligands 8-OH-[^{3}H]DPAT and 5-[^{3}H]carboxamidotryptamine ($n = 2$ in triplicate for each ligand) to Sf9 cell membranes 72 h after infection with baculovirus bearing the DNA of the 5-HT$_{1A}$ receptor were constructed (Figure 1). Nonlinear regression analysis revealed calculated K_d values of 3.1 and 3.4 nM for 8-OH-[^{3}H]DPAT and 3.6 and 3.6 nM for [^{3}H]-carboxamidotryptamine. All plots were fit best to a single site. After pretreatment of intact cells for 30 min with 10 μM 5-HT, the K_d values were increased to 12.9 and 15.1 nM for 8-OH-[^{3}H]DPAT, and 9.9 and 8.2 nM for [^{3}H]-carboxamidotryptamine. That shift was similar to the shift induced by GTPγS treatment under the same experimental conditions (Mulheron et al., 1994). The shift in the K_d indicates a decrease in the affinity of both agonists for the receptor, as would be anticipated when the ternary complex of agonist−receptor−G protein is disrupted by uncoupling of the G protein from the receptor. The total number of binding sites was unchanged with 5-HT pretreatment in each set of experiments. Further support of agonist-induced uncoupling of the 5-HT$_{1A}$ receptor from G proteins was provided by AA-GTP-labeling experiments of the endogenous Sf9 cell 40−41-kDa $G_{o\alpha}$-like G protein in vehicle-treated and desensitized membranes (Figure 1, inset). In membranes prepared from cells exposed to vehicle, acute treatment with 10 μM 5-HT coincident with the photoactivatable cross-linker GTP analog, AA-GTP, produced a marked increase in the photoincorporation of AA-GTP into the G protein band (Mulheron et al., 1994) (Figure 1). The results are as expected if agonist-activated 5-HT$_{1A}$ receptor increases the affinity of AA-GTP for the G protein. In contrast, brief agonist pretreatment of cells abolished the ability of 5-HT to increase labeling of the G protein(s). Thus, two lines of experimentation, one measuring the effect of the G protein on receptor function (ligand binding) and the other measuring the effect of receptor on G protein function (AA-GTP binding), suggest that agonist pretreatment uncouples the 5-HT$_{1A}$ receptor from G proteins in Sf9 cells.

Uncoupling of the Human 5-HT$_{1A}$ Receptor from Second Messenger in Sf9 Cells after Agonist Pretreatment. We next examined whether agonist pretreatment would also attenuate the ability of the 5-HT$_{1A}$ receptor to modulate second messengers in Sf9 cells. We chose to test the ability of the 5-HT$_{1A}$ receptor to inhibit cAMP accumulation, the prototypical second messenger of 5-HT$_1$ receptors (Tecott & Julius, 1993). Two groups have recently shown that several other receptors could inhibit cAMP accumulation or adenylyl cyclase activity in Sf9 cells (Oker-Bloom et al., 1993; Ng et al., 1993). Figure 2A shows that, in Sf9 cells, the 5-HT$_{1A}$ receptor is able to inhibit cAMP accumulation induced by 10 μM forskolin. Two agonists (8-OH-DPAT in Figure 2A and 5-HT in Figure 2B,C) of the receptor inhibited cAMP accumulation by ~50−60% in a concentration-dependent manner. Two selective competitive antagonists of the receptor, NAN-190 and (*S*)-UH-301, produced the expected rightward shifts in the inhibitory concentration−response curves of 8-OH-DPAT (Figure 2A). In control cells infected with the DNA of the β_2-adrenergic receptor (Pei et al., 1994), 5-HT and 8-OH-DPAT had no effect on cAMP accumulation.

Desensitization of the Inhibition of cAMP Accumulation. Figure 2B,C shows that agonist pretreatment resulted in a concentration- and time-dependent attenuation of the ability of the 5-HT$_{1A}$ receptor to inhibit cAMP accumulation. The effect was maximal at 10 μM 5-HT and after 30 min of pretreatment. After 30 min of pretreatment with concentrations <10 μM 5-HT, there were no statistically significant effects on the IC$_{50}$ values or percent inhibition, although there was a clear trend toward higher IC$_{50}$ values (less potency) in all conditions of 5-HT pretreatment. In contrast, the maximal inhibition of cAMP accumulation was reduced in a highly significant manner with 10 μM 5-HT pretreatment. Values for those experiments (expressed as IC$_{50}$, percent inhibition, number of times performed, and P *vs* control for percent inhibition) were as follows: control = 7 ± 2 nM, 63 ± 5%, 4; 250 nM 5-HT × 30 min = 17 ± 6 nM, 47 ±

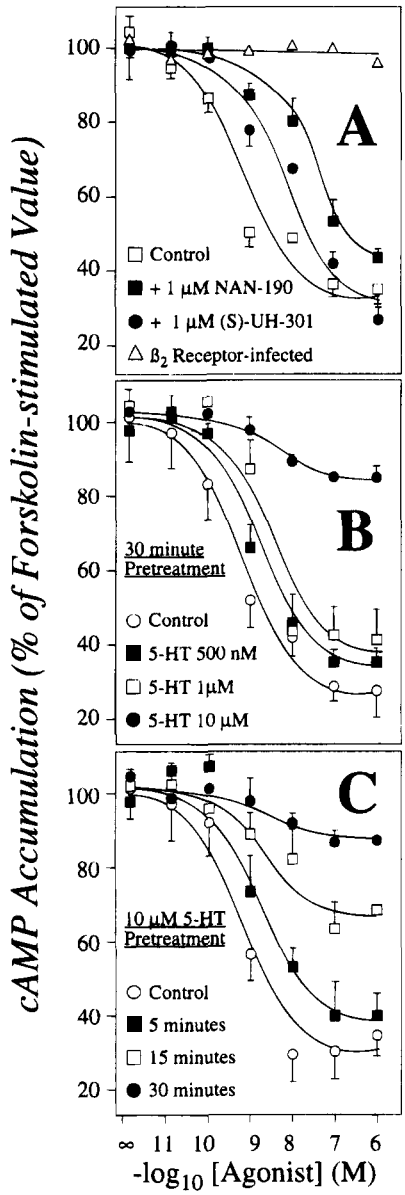

FIGURE 2: Time- and concentration-dependent agonist-mediated attenuation of cAMP inhibition by the 5-HT_{1A} receptor. Panel A depicts concentration−response curves for the effects of 8-OH-DPAT on forskolin (10 μM)-stimulated cAMP accumulation in intact Sf9 cells 72 h after infection with baculovirus bearing the DNA of the 5-HT_{1A} receptor unless otherwise noted. Triangles (△) are values from experiments performed on cells infected with baculovirus bearing the DNA of the β_2-adrenergic receptor. Points represent the absence (□) or presence of competing specific antagonists, 1 μM NAN-190 (■) or 1 μM (S)-UH-301 (●). Basal values ranged from ~50 to 250 pmol/25 000 cells/30 min, and stimulated values, from ~500 to 2500 pmol/25 000 cells/30 min. Panel B shows the effects of 30 min of pretreatment of intact cells with various concentrations of 5-HT on the subsequent ability of 5-HT to inhibit cAMP accumulation induced by 10 μM forskolin. Those experiments were repeated at least four times in triplicate. Panel C shows the effect of pretreatment with 10 μM 5-HT for various time periods on the subsequent ability of 5-HT to inhibit cAMP accumulation. Those experiments were repeated at least three times in triplicate. Complete data are presented in the text. Basal values ranged from ~10 to 50 pmol/25 000 cells/5 min, and stimulated values, from ~50 to 250 pmol/25 000 cells/5 min for panels B and C.

13%, 5, 0.45; 500 nM 5-HT × 30 min = 297 ± 2 nM, 43 ± 9%, 6, 0.40; 1 μM 5-HT × 30 min = 48 ± 9 nM, 57 ± 6%, 13, 0.44; 5 μM 5-HT × 30 min = 121 ± 69 nM, 55 ± 13%, 8, 0.08; 10 μM 5-HT × 30 min = 59 ± 33 nM, 14 ± 5%, 4, 0.0002. For those experiments, paired, one-tailed *t*-tests were used; correction for multiple comparisons by reverse Bonferonni resulted in a *P* value of 0.01 = 95% confidence.

The desensitization effect of 10 μM 5-HT was seen after incubations as brief as 15 min and persisted for up to 60 min of pretreatment. Values for those experiments (expressed as IC_{50}, percent inhibition, number of times performed, and *P vs* control for percent inhibition) were as follows: control = 5 ± 3 nM, 66 ± 4%, 4; 10 μM 5-HT × 15 min = 25 ± 14 nM, 33 ± 7%, 4, 0.003; 10 μM 5-HT × 30 min = 75 ± 13 nM, 17 ± 5%, 7, 0.0003; 10 μM 5-HT × 60 min = 182 ± 43 nM, 15 ± 7%, 7, 0.0003. For those experiments, paired, one-tailed *t*-tests were used; correction for multiple comparisons by reverse Bonferonni resulted in a *P* value of 0.013 = 95% confidence.

Effects of Some Inhibitors on Agonist-Induced Desensitization. Because previous studies have stressed the importance of PKC on the desensitization of 5-HT_{1A} receptors in a number of mammalian cells (Raymond, 1991; Liu & Albert, 1991; Harrington et al., 1994), we investigated the role of PKC on the agonist-induced desensitization of the receptor in Sf9 cells. If the desensitization directly involved a PKC pathway, phorbol esters such as PDBu should be able to mimic the effects of 5-HT to induce desensitization of the receptor. Pretreatment of cells with 1 μM PDBu caused a slight statistically insignificant decrease in the maximal inhibition of cAMP [66 ± 9% in control cells *vs* 51 ± 13% in PDBu treated cells (n = 8 in triplicate; P = 0.09)] with a slight decrease in the potency of the response, which was also not statistically significant [6 ± 3 nM in control cells *vs* 20 ± 14 in PDBu-treated cells (Figure 3)]. A higher dose of PDBu (10 μM) was used in three studies and showed no statistically significant desensitization (22 ± 10 nM and 61 ± 13% inhibition of cAMP accumulation). In addition to the studies designed to activate PKC with phorbol esters, we used two strategies designed to block the action of PKC. First, we pretreated cells for 1 h with 10 μM of the nonspecific kinase inhibitor, H-7. Under that condition, there was still statistically significant desensitization of the maximal inhibition of cAMP (46 ± 8% in control cells *vs* 26 ± 13% in cells pretreated with 10 μM 5-HT, n = 5 in triplicate, P = 0.01) without a statistically significant change in the IC_{50} values. Second, we repeated the studies in cells which had been treated overnight with 16 μM PMA, a treatment which downregulates cellular PKC content by accelerating its degradation. Despite the prolonged treatment with PMA, cells still exhibited agonist-induced desensitization as evidenced by a statistically significant decrease in the maximal inhibition of cAMP accumulation (66 ± 9% in cells treated with PMA alone *vs* 18 ± 6% in cells treated with prolonged PMA followed by 10 μM 5-HT for 30 min, n = 3, P = 0.0002). There was not a statistically significant change in the IC_{50} values (6 ± 2 nM in cells treated with PMA alone *vs* 68 ± 80 nM in 5-HT-treated cells). In aggregate, those three approaches do not support a major role for PKC in the agonist-induced desensitization of the 5-HT_{1A} receptor in Sf9 cells.

In contrast, desensitization was attenuated by preincubation of permeabilized cells with heparin at either 100 nM or 1 μM (Figure 3). Values for those experiments (expressed as IC_{50}, percent inhibition, and number of times performed), all of which were performed in streptolysin-O-permeabilized cells, were as follows: control = 3 ± 2 nM, 58 ± 5%, 8;

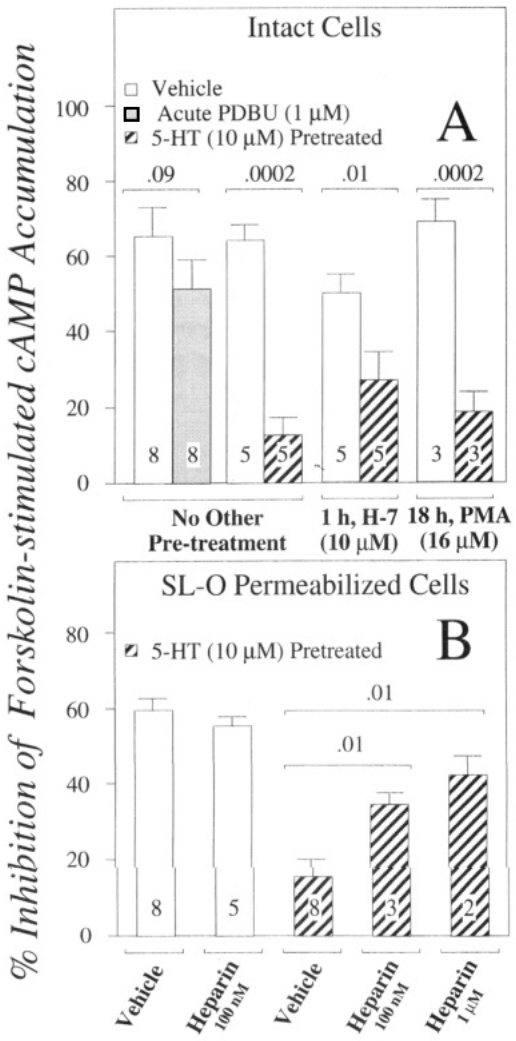

FIGURE 3: Heparin sensitivity of, and probable lack of involvement of PKC in, agonist-induced desensitization of 5-HT$_{1A}$ receptor in Sf9 cells. Accumulation of cAMP was measured in intact cells for 5 min. Panel A: Cells were exposed to a number of pharmacological manipulations, as indicated, and then to vehicle (open bars), 1 μM PDBu (gray bar), or 10 μM 5-HT (hatched bars) for 30 min prior to assays. Panel B: Results from experiments performed in cells permeabilized with streptolysin-O. Cells were concurrently incubated with streptolysin-O (SL-O) and heparin or vehicle for 30 min prior to adding 10 μM 5-HT (hatched bars) or vehicle (open bars) for another 30 min. Intracellular cAMP was measured after 5 min, and maximal ability to inhibit forskolin-stimulated cAMP accumulation was determined as in panel A. For both panels, the maximal ability to inhibit cAMP accumulation was calculated by nonlinear regression analysis of concentration–response curves generated by subsequent treatment with various concentrations of 5-HT (10^{-10} to 10^{-4} M). The number of repetitions of each experiment performed in triplicate is indicated within each bar. Paired one-tailed *t*-tests were performed to compare the respective conditions as indicated. The *P* values are presented above each pair. Using a reverse Bonferonni calculation, 95% confidence corresponds to $P = 0.012$ for panel A and $P = 0.025$ for panel B.

heparin alone = 10 ± 5 nM, 54 ± 3%, 5; pretreated with 10 μM 5-HT × 30 min = 623 ± 148 nM, 15 ± 5%, 8; 5-HT + 100 nM heparin = 259 ± 32 nM, 35 ± 4%, 4; 5-HT + 100 nM heparin = 59 nM, 41%, 2. Values for heparin-attenuated (at both doses) 5-HT-induced desensitization were significantly different from 5-HT pretreatment alone in terms of both IC_{50} and percent inhibition ($p < 0.01$ for all). Heparin appeared to have an intracellular site of action, because treatment of nonpermeabilized cells with

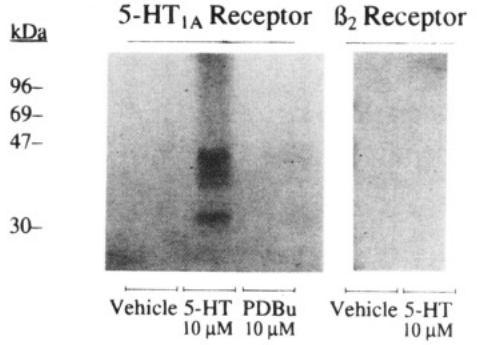

FIGURE 4: Agonist-induced phosphorylation of the human 5-HT$_{1A}$ receptor in Sf9 cells. Cells were permeabilized with streptolysin-O and then phosphorylation and immunoprecipitation experiments were performed as in Materials and Methods. Cells were treated for 30 min with vehicle, 10 μM 5-HT, or 10 μM PDBu. Phosphorylated 5-HT$_{1A}$ receptors were isolated by immunoprecipitation and 10% SDS–PAGE. Essentially the same results were obtained in four different experiments. This gel was exposed to Kodak X-AR film for 5 days at −80 °C.

heparin did not prevent 5-HT-induced desensitization (n = 5, not shown).

Agonist-Induced Phosphorylation of the 5-HT$_{1A}$ Receptor in Sf9 Cells. We next studied the ability of agonist treatment to induce phosphorylation of the 5-HT$_{1A}$ receptor in Sf9 cells. Receptors were isolated using immunoprecipitation with two antipeptide sera raised against sequences within the putative third cytosolic loop of the human 5-HT$_{1A}$ receptor as previously described (Raymond, 1991). The ability of those antibodies to immunoprecipitate liganded and phosphorylated human and rat 5-HT$_{1A}$ receptors has been documented previously, as well as lack of cross-reactivity with several other closely related 5-HT receptor types (Fargin et al., 1989; Raymond et al., 1989, 1993; Raymond, 1991; Parker et al., 1994). Immunoprecipitates were analyzed by 10% discontinuous SDS–PAGE followed by autoradiography or imaging with a PhophorImager. Figure 4 shows that treatment with 10 μM 5-HT resulted in a marked increase in the incorporation of radioactivity into a band of ~44 kDa, similar in size to immunoblotted receptors expressed in the same cells (Mulheron et al., 1994; Parker et al., 1994). Smaller labeled products may have been the result of receptor degradation, although that hypothesis was not tested. In contrast, 10 μM PDBu induced only a very slight increase in labeling, suggesting that the agonist-induced phosphorylation probably occurs independent of PKC. There was no specific labeling in β_2-adrenergic receptor infected cells.

The concentration–response relationship of 5-HT to phosphorylation closely paralleled that of desensitization, with phosphorylation being apparent at 1 μM 5-HT and maximal at 10 μM 5-HT (Figure 5A). The time course of 5-HT-induced phosphorylation was also similar to that for desensitization, being maximal between 15 and 30 min (Figure 5B).

Because likely candidate kinases (GRK, PKC) are serine–threonine kinases, it was important to document that the 5-HT$_{1A}$ receptor was phosphorylated on those residues. Phosphoamino acid analysis of immuno-purified phosphorylated receptors is presented in Figure 6. Comigration with authentic phosphoamino acid standards was consistent with phosphoserine and phosphothreonine, although definite conclusions about the relative amount of each phosphoamino acid should not be made because of the differing sensitivity of those residues to acid hydrolysis (Duclos et al., 1991).

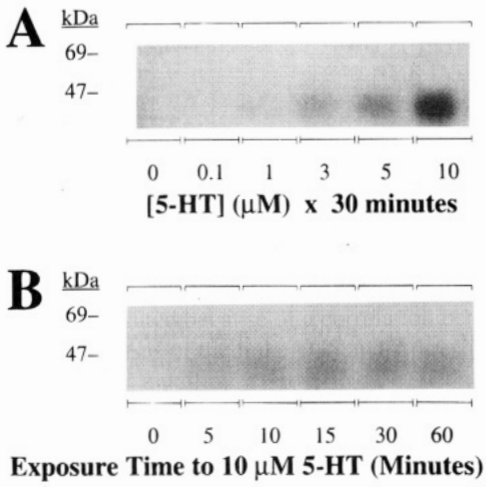

FIGURE 5: Time and concentration dependence of phosphorylation of the human 5-HT_{1A} receptor in Sf9 cells. Experiments were performed as described in the caption to Figure 4, except that preincubation time with 5-HT (10 μM) was varied (panel A), or that concentration of 5-HT was varied during 30-min incubations (panel B). The experiments shown are representative of two with very similar results. The gels were exposed to autoradiography film for 14 days (panel A) and 21 days (panel B) at −80 °C.

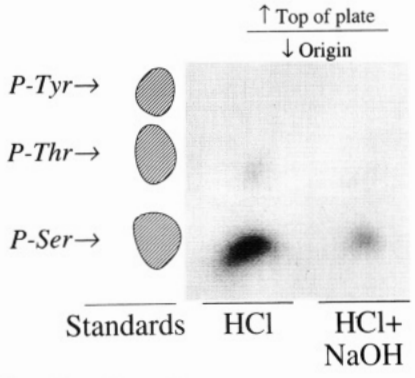

FIGURE 6: Phosphoamino acid analysis of phosphorylated 5-HT_{1A} receptor. Extended one-dimensional thin-layer chromatography was performed as described under Materials and Methods. Areas of the standards visualized by ninhydrin are depicted on the left (hatched areas). Horizontal line at the top indicates the top of the TLC plate; the origin was 17 cm below that line. The radioactive spots were visualized by autoradiography (42 days at −80 °C). This experiment was performed once.

Further support for the identity of the radioactive spots as phosphothreonine and phosphoserine is demonstrated by the sensitivity of both species to mild alkaline hydrolysis (Figure 6), a feature that distinguishes them from phosphotyrosine (Duclos et al., 1991).

In order to investigate more completely the potential role of PKC in the phosphorylation of the receptor by 5-HT, a number of inhibitors of common second messenger kinases were used in concert with 5-HT. Figure 7A shows that the kinase inhibitors H-7, staurosporine, and Rp-cAMPS had no effect on the agonist-induced phosphorylation of 5-HT_{1A} receptors in Sf9 cells. Those results suggest that PKA and PKC do not play a major role in the agonist-induced phosphorylation of the 5-HT_{1A} receptor in Sf9 cells. The fact that the specific 5-HT_{1A} receptor antagonist (*S*)-UH-301 and the nonspecific antagonist pindolol attenuated the 5-HT-induced phosphorylation of the ~44-kDa band lends further support to its identity as the 5-HT_{1A} receptor.

In marked contrast to the other kinase inhibitors tested, and similar to findings reported above for desensitization,

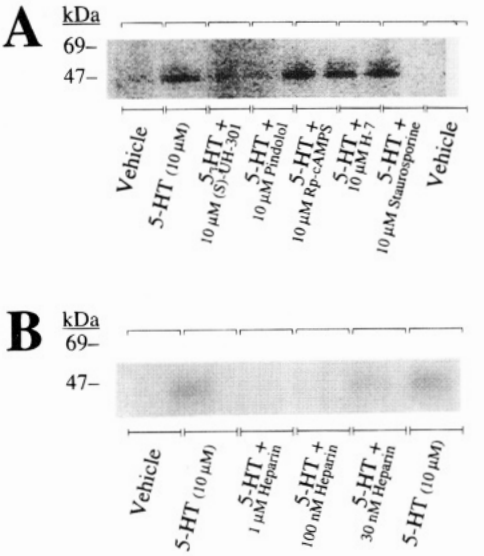

FIGURE 7: Effects of kinase inhibitors on agonist-induced phosphorylation of the human 5-HT_{1A} receptor. Phosphorylation reactions were carried out for 30 min at room temperature in streptolysin-O-permeabilized cells in the presence of vehicle alone or 10 μM 5-HT ± each kinase inhibitor. The gels were exposed to Kodak X-AR film for 28 days at −80 °C (panel B), or were analyzed with a PhosphorImager after a 3-day exposure (panel A). The experiments shown are representative of three (panel A) and two (panel B) experiments. The image of the gel in panel A was printed on a laser printer at 600 × 600 dpi.

heparin at 100 nM and 1 μM markedly inhibited 5-HT-induced phosphorylation of the 5-HT_{1A} receptor in streptolysin-O-permeabilized cells (Figure 7B). Although heparin has many nonspecific effects on cellular kinases, phosphatases, and other important proteins, the effectiveness of 100 nM heparin is consistent with the action of a heparin-sensitive receptor kinase (Lohse et al., 1990; Richardson & Hosey, 1992).

DISCUSSION

Insect cell models, particularly the Sf9 cell, have been used for heterologous expression of a number of GCRs, including β-adrenergic (Parker et al., 1991; Reiländer et al., 1991; Mouillac et al., 1992; Pei et al., 1994), muscarinic (Parker et al., 1991; Richardson & Hosey, 1992; Vasudevan et al., 1992), α_2-adrenergic (Okcr-Bloom et al., 1993; Pei et al., 1994; Marjamäki et al., 1994), D_1 and D_4 dopaminergic (Mills et al., 1993; Ng et al., 1994; Chabert et al., 1994), *N*-formyl peptide (Quehenberger et al., 1992), substance P (Kwatra et al., 1993), and 5-HT_1 (Ng et al., 1993; Mulheron et al., 1994; Parker et al., 1994) receptors. In aggregate, those studies have stressed two common themes. The first is the ability of mammalian receptors to couple to endogenous G proteins and effector−second messenger systems. The second is that the insect cells seem to possess the proper machinery for folding, processing, and inserting receptors into the plasma membrane. Several receptors have also been shown to undergo potentially important posttranslational modifications including glycosylation (Parker et al., 1991; Reiländer et al., 1991), phosphorylation, and palmitoylation (Hosey & Richardson, 1992; Ng et al., 1993, 1994). The current studies add to the previous work by demonstrating that the 5-HT_{1A} receptor appears to be a substrate for phosphorylation by an endogenous, agonist-activated, heparin-sensitive kinase in Sf9 cells and that the phosphorylation of the receptor is associated with a marked decrease in its

ability to propagate a subsequent second messenger signal, that being the inhibition of adenylyl cyclase.

The exact nature of the kinase(s) (or phosphatases) involved in the phosphorylation process is not entirely clear. The human 5-HT$_{1A}$ receptor contains a number of consensus sequences for phosphorylation by classical second messenger kinases. Those kinases specifically include PKA, PKC, and cGMP-dependent kinase (PKG), for which the receptor contains three, four, and six potential sites, respectively.[3] No consensus recognition motifs for Ca^{2+}- and calmodulin-dependent protein kinase type II (CAMK II) are present within the sequence of the 5-HT$_{1A}$ receptor. The current work does not support a major role for PKA or PKC in the agonist-induced phosphorylation of the 5-HT$_{1A}$ receptor in Sf9 cells. Our studies show that Rp-cAMPs, a specific inhibitor of protein kinase A, had no effect on agonist-induced phosphorylation of the 5-HT$_{1A}$ receptor. In any case, we would not expect PKA to lead to agonist-induced phosphorylation of the 5-HT$_{1A}$ receptor because we found no evidence that the receptor increases intracellular cAMP content, a prerequisite for PKA activation. Similarly, staurosporine and H-7, two broad-spectrum protein kinase inhibitors, had no effect on agonist-induced phosphorylation of the 5-HT$_{1A}$ receptor. Both H-7 and staurosporine have inhibitory effects on protein kinase A ($IC_{50} \approx 3$ μM and 7 nM, respectively), protein kinase C ($\approx$700 pM and 6 μM), and cGMP-dependent protein kinase ($\approx$9 nM and 6 μM) (Hidaka et al., 1984; Tamoaki et al., 1986; Bouli & Davis, 1990; Schächtale et al., 1991; Tischler et al., 1991).

One caution here is that little is known about serine–threonine kinases endogenous to Sf-9 cells. Indirect evidence for the presence of PKA in Sf9 cells has been presented (Kartner et al., 1991), but the very presence of PKC in Sf9 cells has been debated (Schaap & Parker, 1990; Burns et al., 1990). However, others have shown low levels of PKC activity and [^{3}H]PDBu binding activity in Sf9 cells (Liyanage et al., 1992; Kazanietz et al., 1993). In that light, the negative findings regarding the lack of PKC interaction with the 5-HT$_{1A}$ receptor in Sf9 cells should not be generalized to other systems. The paucity of knowledge regarding endogenous kinases in Sf9 cells should lead to a careful interpretation of all of the negative data obtained using kinase inhibitors.

The previous cautions notwithstanding, the seeming lack of involvement of PKA and PKC in the agonist-stimulated phosphorylation of the 5-HT$_{1A}$ receptor is most likely explained by interactions of members of the G protein-coupled receptor kinase (GRK) family (Inglese et al., 1993). Although specific peptide recognition motifs for those kinases have not been fully worked out, GRK1 and GRK2 (also known as rhodopsin kinase and β-ARK1) have been shown to phosphorylate serine and threonine residues in close proximity to acidic residues (*i.e.*, glutamic acid or aspartic acid) (Palczewski et al., 1989; Onorato et al., 1991). The human 5-HT$_{1A}$ receptor contains 17 intracellular serine and threonine residues, most of which are in close proximity to acidic residues. Our studies show that the 5-HT$_{1A}$ receptor is phosphorylated on both serine and threonine residues after agonist stimulation. Moreover, the phosphorylation is sensitive to heparin at modest concentrations (100 nM and 1 μM). Thus, it is possible that the 5-HT$_{1A}$ receptor serves as a substrate for a GRK native to the Sf9 cell, although that possibility remains speculative at this time.

Similar to the phosphorylation results, several lines of experimentation showed a rapid uncoupling of the 5-HT$_{1A}$ receptor from G proteins and the inhibition of cAMP accumulation after agonist exposure (Figures 1 and 2). Brief agonist pretreatment resulted in uncoupling of the receptor from endogenous G proteins as evidenced by (1) a loss of high-affinity binding sites and (2) a loss of the ability of the receptor to increase covalent labeling of AA-GTP to 40–41-kDa $G_{o\alpha}$-like G proteins in Sf9 cells (Figure 1). Importantly, such pretreatment also significantly attenuated the ability of the receptor to inhibit cAMP accumulation in a time- and concentration-dependent manner (Figure 2). Because the cAMP assays were performed in the presence of the phosphodiesterase inhibitor IBMX, the changes in cAMP levels represent activity of adenylyl cyclases. Similar to the results of the phosphorylation studies, PKA and PKC inhibitors had little effect on desensitization, while heparin markedly attenuated the same. The most probable explanation for this observation again resides in the likelihood of an endogenous insect cell GRK that recognizes and interacts with the 5-HT$_{1A}$ receptor.

Our results must be compared to those of Harrington et al., who also showed a profound agonist-induced desensitization of 5-HT$_{1A}$ receptors in HeLa cells (Harrington et al., 1994). In contrast to the current work, desensitization was blocked in their studies by inhibition of PKC. Together, the two studies suggest that there may be cellular specificity to pathways of agonist-induced short-term desensitization of GCR. In addition, we have not yet been able to show any agonist-induced desensitization of 5-HT$_{1A}$ receptors expressed in CHO cells.[4] In support of the potential for cell-specific pathways of short-term desensitization, downregulation (long-term mechanism of desensitization) of the β_3-adrenergic receptor was recently shown to be cell-specific (Nantel et al., 1994). Moreover, Shih and Malbon (1994) used antisense constructs to elegantly demonstrate cell-specific pathways of short-term, kinase-mediated desensitization of β_2-adrenergic receptors. The curious feature of the current work is that a mammalian receptor would more easily demonstrate interaction with a putative insect cell GRK than with GRKs in mammalian cells. We have no ready explanation for this phenomenon.

A final note of caution needs to be emphasized. Our studies show a close correlation between the processes of phosphorylation and desensitization of the 5-HT$_{1A}$ receptor in insect cells. However, they stop short of a definitive demonstration of a causal relationship between the two processes, although it should be clear that we hypothesize such. Future studies using mutant receptors and reconstitution systems will be necessary to definitively demonstrate a cause–effect relationship between phosphorylation and desensitization.

[3] When examining the 5-HT$_{1A}$ receptor for potential kinase recognition sites, we used the following consensus sequences (Kemp & Pearson, 1991): **R**XS/T*, **RR**XS/T*, **R**XXS/T*, or **KR**XXS/T* for PKA; S/T*X**K/R**, **K/R**XXS/T*(X**K/R**), or **K/R**XS/T*(X**K/R**) for PKC, and **R/K**XS/T*, **R/K**XXS/T*, **R/KR/K**XS/T*, **R/K**XXXS/T*, or S/T*X**K/R** for PKG. Boldface residues are thought to be critical components of the recognition site. Residues in parentheses may be important in some, but not all, settings. Asterisks denote putative phosphorylated residues. Slashes (/) denote that either residue is acceptable in that location.

[4] John R. Raymond, unpublished observation.

In conclusion, the current studies show that the human 5-HT_{1A} receptor, when expressed in Sf9 insect cells, undergoes rapid phosphorylation on serine and threonine residues, and uncoupling from G proteins and adenylyl cyclase after brief pretreatment with agonist. Those effects are mediated through pathways that are mainly insensitive to classical inhibitors of PKC and are sensitive to intracellular heparin. The major question remaining to be addressed is, What is the identity of the putative agonist-activated kinase which interacts with the 5-HT_{1A} receptor in insect cells?

ACKNOWLEDGMENT

We thank Dr. Bob Lefkowitz of the Howard Hughes Medical Institute at Duke University Medical Center for providing the baculovirus construct bearing the DNA of the β_2-adrenergic receptor, Grace Irons from the Lefkowitz laboratory for providing advice and reagents for insect cell culture, and Dr. John Kebabian of Research Biochemicals International for providing (S)-UH-301.

REFERENCES

Blier, P., & De Montigny, C. (1990) *J. Cardiovasc. Pharmacol. 15* (7), S24.
Boulis, N. M., & Davis, M. (1990) *Brain Res. 525*, 198.
Boyle, W. J., van der Geer, P., & Hunter, T. (1991) *Methods Enzymol. 201*, 110.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.
Burns, D. J., Bloomenthal, J., Lee, M.-H., & Bell, R. H. (1990) *J. Biol. Chem. 265*, 12044.
Chabert, C., Cavegn, C., Bernard, A., & Mills, A. (1994) *J. Neurochem. 63*, 62.
Duclos, B., Marcandier, S., & Cozzone, A. J. (1991) *Methods Enzymol. 201*, 10.
Fargin, A., Raymond, J. R., Lohse, M., Kobilka, B. K., Caron, M. G., & Lefkowitz, R. J. (1988) *Nature 335*, 358.
Gettys, T. W., Fields, T. A., & Raymond, J. R. (1994) *Biochemistry 33*, 4283.
Harrington, M. A., Shaw, K., Zhong, P. Y., & Ciaranello, R. D. (1994) *J. Pharmacol. Expt. Ther. 268*, 1098.
Hartig, P. R., Branchek, T. A., & Weinshank, R. L. (1992) *Trends Pharmacol. Sci. 13*, 152.
Hausdorff, W. P., Caron, M. G., & Lefkowitz, R. J. (1990) *FASEB J. 4*, 2881.
Hen, R. (1992) *Trends Pharmacol. Sci. 13*, 160.
Hidaka, H., Inagaki, M., Kawamoto, S., & Sasaki, Y. (1984) *Biochemistry 23*, 5036.
Huganir, R. L., & Greengard, P. (1990) *Neuron 5*, 555.
Inglese, J., Freedman, N. J., Koch, W. J., & Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 23735.
Kagaya, A., Mikuni, M., Muraoka, S.-i., Saitoh, K., Ogawa, T., Shinno, H., Yamawaki, S., & Takahashi, K. (1993) *J. Neurochem. 61*, 1050.
Kartner, N., Hanrahan, J. W., Jensen, T. J., Naismith, A. L., Sun, S. Z., Ackerley, C. A., Reyes, E. F., Tsui, L. C., Rommens, J. M., Bear, C. E., & Riordan, J. R. (1991) *Cell 64*, 681.
Kazanietz, M. G., Areces, L. B., Bahador, A., Mischak, H., Goodnight, J., Mushinski, J. F., & Blumberg, P. M. (1993) *Mol. Pharmacol. 44*, 298.
Kemp, B. E., & Pearson, R. B. (1990) *Trends Biol. Sci. 15*, 342.
Kwatra, M., Schwinn, D., Schreurs, J., Blank, J. L., Kim, C. M., Benovic, J. L., Krause, J. E., Caron, M. G., & Lefkowitz, R. J. (1993) *J. Biol. Chem. 268*, 9161.
Liu, Y. F., & Albert, P. R. (1991) *J. Biol. Chem. 266*, 23689.
Liyanage, M., Frith, D., Livneh, E., & Stabel, S. (1992) *Biochem. J. 283*, 781.
Lohse, M. J., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1990) *J. Biol. Chem. 265*, 3202.
Maricq, A. V., Peterson, A. S., Brake, A. J., Myers, R. M., & Julius, D. (1991) *Science 254*, 432.
Marjamäki, A., Pohjanoksa, K., Ala-Uotila, S., Sizman, D., Oker-Bloom, C., Kurose, H., & Scheinin, M. (1994) *Eur. J. Pharmacol., Mol. Pharmacol. Sect. 267*, 117.
Mills, A., Allet, B., Bernard, A., Chabert, C., Brandt, E., Cavegn, C., Chollet, A., & Kawashima, E. (1993) *FEBS Lett. 320*, 130.
Mouillac, B., Caron, M., Bonin, H., Dennis, M., & Bouvier, M. (1992) *J. Biol. Chem. 267*, 22249.
Mulheron, J. G., Casañas, S. J., Arthur, J. M., Garnovskaya, M. N., Gettys, T. W., & Raymond, J. R. (1994) *J. Biol. Chem. 269*, 12954.
Nantel, F., Marullo, S., Krief, S., Strosberg, A. D., & Bouvier, M. (1994) *J. Biol. Chem. 269*, 13148.
Ng, G.Y. K., George, S. R., Zatawny, R. L., Caron, M., Dennis, M., Bouvier, M., & O'Dowd, B. F. (1993) *Biochemistry 32*, 11727.
Ng, G. Y. K., Mouillac, B., George, S. R., Caron, M., Bouvier, M., Dennis, M., & O'Dowd, B. F. (1994) *Eur. J. Pharmacol., Mol. Pharmacol. Sect. 267*, 7.
Oker-Bloom, C., Jansson, C., Karp, M., Lindqvist, C., Savola, J.-M., Vlak, J., & Akerman, K. (1993) *Biochim. Biophys. Acta 1176*, 269.
Onorato, J. J., Palczewski, K., Regan, J. W., Caron, M. G., Lefkowitz, R. J., & Benovic, J. L. (1991) *Biochemistry 30*, 5118.
Palczewski, K., Arendt, A., McDowell, J. H., & Hargrave, P. A. (1989) *Biochemistry 28*, 8764.
Parker, E. M., Kameyama, K., Higashijima, T., & Ross, E. M. (1991) *J. Biol. Chem. 266*, 519.
Parker, E. M., Grisel, D. A., Iben, L. G., Nowak, H. P., Mahly, C. D., Yocca, F. D., & Gaughan, G. T. (1994) *Eur. J. Pharmacol., Mol. Pharmacol. Sect. 268*, 43−53.
Pauwels, P. J., Van Gompel, P., & Leysen, J. E. (1990) *Life Sci. 47*, 2009.
Pei, G., Tiberi, M., Caron, M. G., & Lefkowitz, R. J. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3633.
Pleus, R. C., & Bylund, D. B. (1992) *J. Pharmacol. Expt. Ther. 261*, 271.
Quehenberger, O., Prossnitz, E. R., Cochrane, C. G., & Ye, R. D. (1992) *J. Biol. Chem. 267*, 19757.
Raymond, J. R. (1991) *J. Biol. Chem. 266*, 14747.
Raymond, J. R., Fargin, A., Lohse, M. J., Regan, J. W., Senogles, S., Lefkowitz, R. J., & Caron, M. G. (1989) *Mol. Pharmacol. 36*, 15.
Raymond, J. R., Kim, J. L., Beach, R. E., & Tisher, C. C. (1993) *Am. J. Physiol. 264*, F9.
Raymond, J. R., Arthur, J. M., Olsen, C. L., Gettys, T. W., & Mortensen, R. M. (1994) *J. Biol. Chem. 269*, 12954.
Reiländer, H., Boege, F., Vasudevan, S., Maul, G., Hekman, M., Dees, C., Hampe, W., Helmreich, E. J. M., & Michel, H. (1991) *FEBS Lett. 282*, 441.
Richardson, R. M., & Hosey, M. M. (1992) *J. Biol. Chem. 267*, 22249.
Schaap, D., & Parker, P. J. (1990) *J. Biol. Chem. 265*, 7301.
Schächtele, C., Seifert, R., & Osswald, H. (1988) *Biochem. Biophys. Res. Commun. 151*, 542.
Shih, M., & Malbon, C. C. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 12193.
Sibley, D. R., Benovic, J. L., Caron, M. G., & Lefkowitz, R. J. (1987) *Cell 48*, 913.
Sommers, M. D., & Smith, G. E. (1987) *Tex., Agr. Exp. Sta., [Bull.] 1555*, 1.
Sugamori, K. S., Sunahara, R. K., Guan, H.-C., Bulloch, A. G. M., Tensen, C. P., Seeman, P., Niznik, H. B., & Van Tol, H. H. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 11.
Tamaoki, T., Nomoto, H., Takahashi, I., Kato, Y., Morimoto, M., & Tomita, F. (1986) *Biochem. Biophys. Res. Commun. 135*, 397.
Tecott, L. H., & Julius, D. (1993) *Curr. Opin. Neurobiol. 3*, 310.
Tischler, A. S., Ruzicka, L. A., & Perlman, R. L. (1990) *J. Neurochem. 55*, 1159.
Unsworth, C. D., & Molinoff, P. B. (1992) *Mol. Pharmacol. 42*, 464.
Vasudevan, S., Premkumar, L., Stowe, S., Gage, P. W., Reiländer, H., & Chung, S.-H. (1992) *FEBS Lett. 311*, 7.

BI951001K