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Mechanisms of Ligand-Induced Desensitization of the 5-Hydroxytryptamine$_{2A}$ Receptor

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ABSTRACT

We have examined the cellular processes underlying the desensitization of the 5-hydroxytryptamine (5-HT)$_{2A}$ receptor induced by agonist or antagonist exposure. Treatment of C6 glioma cells with either 5-HT or the 5-HT$_{2A}$ receptor antagonist ketanserin resulted in an attenuation in 5-HT$_{2A}$ receptor function, specifically the accumulation of inositol phosphates stimulated by the partial agonist quipazine. 5-HT-induced desensitization of the 5-HT$_{2A}$ receptor involved receptor internalization through a clathrin- and dynamin-dependent process because it was prevented by concanavalin A, monodansylcadaverine, and by expression of the dominant negative mutants β-arrestin (319–418) and dynamin K44A. Although short-term (i.e., 10 min) 5-HT and ketanserin exposure resulted in the same degree of desensitization, ketanserin-induced desensitization was not prevented by these agents and did not involve receptor internalization. In contrast, prolonged ketanserin exposure (i.e., 2 h) resulted in 5-HT$_{2A}$ receptor internalization through a clathrin- and dynamin-dependent process, as was observed after agonist treatment. Inhibitors of protein kinase C or calcium-calmodulin kinase II did not attenuate or prevent 5-HT-induced desensitization of the receptor. 5-HT$_{2A}$ receptor desensitization induced by 5-HT and prolonged ketanserin treatment, but not by short-term ketanserin treatment, was prevented by the expression of the dominant negative mutant of G protein-coupled receptor kinase (GRK)2, GRK2-K220R, and by an anti-GRK2/3 antibody. Our data indicate a dual mechanism of early and late desensitization by the antagonist ketanserin. Short-term ketanserin treatment reduced the specific binding of the agonist radioligand [^{125}I](±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane ([^{125}I]DOI) and the ability of 5′-guanylylimidodiphosphate to attenuate this binding, suggesting that at the early stage of antagonist-induced desensitization the capacity of the 5-HT$_{2A}$ receptor to couple to G protein is impaired.

Desensitization of G protein-coupled receptors occurs during agonist exposure, often in a matter of minutes. Mechanisms underlying the desensitization of many G protein-coupled receptors have been elucidated in part by using the β-adrenergic receptor as a prototype (Bunemann and Hosey, 1999). In this "classical" multistep process, the agonist-occupied state of the receptor is phosphorylated by a second messenger-dependent kinase (e.g., protein kinase A) and/or a G protein-coupled receptor kinase (GRK). The binding of an adapter protein, arrestin, leads to uncoupling of the receptor from the G protein and receptor sequestration through clathrin-coated vesicles. The internalization of the receptor is dependent on dynamin, a GTPase that is responsible for pinching off the endocytotic vesicle. Internalized receptors may be returned to the cell surface, or degraded in lysosomes.

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The 5-hydroxytryptamine (5-HT)$_{2A}$ receptor has been implicated in the mechanism of action of many psychoactive drugs such as hallucinogens, atypical neuroleptics, and antidepressants. The regulation of the 5-HT$_{2A}$ receptor, however, does not appear to follow the pattern established for many other G protein-coupled receptors. Repeated administration of agonists (Buckholtz et al., 1988; Anji et al., 2000) as well as antagonists (Blackshear and Sanders-Bush, 1982; Gandolfi et al., 1985; Hensler and Truett, 1998) results in the desensitization and down-regulation of central 5-HT$_{2A}$ receptors.

Given the interest in the role of the 5-HT$_{2A}$ receptor in the action of many psychoactive drugs and the apparent anomalous regulation of 5-HT$_{2A}$ receptors by antagonists, including atypical neuroleptics and many antidepressant drugs (Eison et al., 1991; Kuoppamaki et al., 1995), a thorough understanding of the mechanisms that regulate 5-HT$_{2A}$ receptor function is desirable. In the current study, we have examined the effect of both agonist and antagonist exposure on 5-HT$_{2A}$ receptor function in C6 glioma cells. C6 glioma cells endogenously express the 5-HT$_{2A}$ receptor, which is

ABBREVIATIONS: GRK, G protein-coupled receptor kinase; 5-HT, 5-hydroxytryptamine (serotonin); IP, inositol phosphates; PI, phosphoinositol; PBS, phosphate-buffered saline; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; ELISA, enzyme-linked immunosorbent assay; TBS, Tris-buffered saline; con A, concanavalin A; MDC, monodansylcadaverine; PKC, protein kinase C; GppNHp, 5′-guanylylimidodiphosphate.

coupled to the stimulation of phospholipase C (Ananth et al., 1987; Ding et al., 1993). Furthermore, 5-HT$_{2A}$ receptors are down-regulated in C6 glioma cells by antagonist treatment (Toth and Shenk, 1994), as has been observed in vivo (Blackshear and Sanders-Bush, 1982; Anji et al., 2000), making these cells an appropriate model system. In the current study, treatment with either serotonin (5-hydroxytryptamine) or the 5-HT$_{2A}$ receptor antagonist ketanserin resulted in an attenuation in 5-HT$_{2A}$ receptor function, specifically the accumulation of inositol phosphates (IP) stimulated by the partial agonist quipazine. Desensitization, a decrease in response, can occur as a result of receptor uncoupling from G protein, internalization (sequestration of the receptor away from the cell surface), or down-regulation (loss of total receptor number). We have examined the cellular processes underlying the desensitization of the 5-HT$_{2A}$ receptor and report data that indicate a dual mechanism of early and late desensitization by the antagonist ketanserin. As was observed after agonist treatment, prolonged antagonist exposure resulted in the internalization of the 5-HT$_{2A}$ receptor through a clathrin- and dynamin-dependent process, which appears to involve a GRK. Although short-term 5-HT and ketanserin exposure resulted in the same degree of desensitization, the desensitization induced by short-term ketanserin treatment appears to be due to receptor uncoupling from G protein but does not involve receptor internalization.

Experimental Procedures

Cell Culture. C6 glioma cells (ATCC CCL107) were grown in Dulbecco's modified Eagle's medium supplemented with 15% horse serum (Summit Biotechnology, Ft. Collins, CO) and 2.5% fetal bovine serum (Atlanta Biologicals, Norcross, GA) in a humidified atmosphere containing 5% CO_2. Sera were heat-inactivated and charcoal-treated in our laboratory to remove monoamines.

Phosphoinositol Hydrolysis. Three days before the assay cells were plated onto 24-well plates at a density of 100×10^4 cells/plate. At least 22 h before the assay, cells were labeled with [^{3}H]myoinositol (PerkinElmer Life Sciences, Boston, MA) (1 μCi/ml). PI hydrolysis assays were performed as described by Berg et al. (1994). Cells were washed with Hanks' balanced salt solution containing 20 mM $LiCl_2$ and 20 mM HEPES, pH 7.4. After a 15-min preincubation in Hanks' balanced salt solution, the stimulation of PI hydrolysis was initiated by addition of quipazine. Reactions were stopped after 20 min by the addition of ice-cold 10 mM formic acid. The accumulation of total [^{3}H]IP (inositol monophosphate, inositol bisphosphate, and inositol triphosphate) was determined by ion exchange chromatography.

Cell Homogenates. To harvest cells, culture plates were washed with phosphate-buffered saline (PBS). Lysis buffer (5 mM HEPES, 5 mM EDTA) was added and plates set for 10 min at 4°C. Cells were harvested and centrifuged at 20,000*g* for 15 min. The pellet was resuspended in 50 mM Tris buffer, flash frozen, and stored at −80°C. The homogenates were thawed on the day of the binding assay and centrifuged. Pellets were resuspended in 50 mM Tris, incubated at 37°C for 10 min, and centrifuged. The resultant pellet was washed once and resuspended in 50 mM Tris. Protein concentrations were determined by the method of Bradford (Bio-Rad, Hercules, CA).

[^{3}H]Ketanserin Binding. Binding experiments were performed using a single saturating concentration of [^{3}H]ketanserin (PerkinElmer Life Sciences) (~12 nM) in the presence of 100 nM prazosin and 100 nM pyrilamine to prevent the binding of [^{3}H]ketanserin to α_1-adrenergic and H_1 histamine receptors, respectively. Nonspecific binding was defined by 10 μM methysergide. Binding was initiated by the addition of homogenate (100 μg of protein/tube). Assay tubes were covered and incubated for 60 min at 37°C. Binding reactions were terminated by the addition of 5 ml of ice-cold buffer (50 mM Tris, pH 7.4, at 4°C). Membranes were collected on glass fiber filters (no. 25; Schleicher & Schuell, Keene, NH) presoaked in 0.3% polyethylenimine. Filters were washed three times with ice-cold buffer.

[^{125}I]DOI Binding. Homogenates were thawed on the day of the binding assay and centrifuged. Final pellets were resuspended in assay buffer (50 mM Tris, 0.5 mM Na_2EDTA, 10 mM $MgSO_4$), pH 7.4, at 37°C. Binding experiments were performed using the K_d concentration of [^{125}I]DOI (PerkinElmer Life Sciences) (~0.7 nM). Nonspecific binding was defined by 1 μM ketanserin. Binding was initiated by the addition of homogenate (100 μg of protein/tube). Assay tubes were covered and incubated for 20 min at 37°C. Binding reactions were terminated by the addition of 5 ml of ice-cold buffer (50 mM Tris, pH 7.4, at 4°C). Membranes were collected on glass fiber filters (no. 25; Schleicher & Schuell) presoaked in 0.3% polyethylenimine. Filters were washed three times with ice-cold buffer.

Transfections. Cells were transiently transfected with the eukaryotic expression vector pcDNA3 (Invitrogen, Carlsbad, CA) or vector containing the cDNA for β-arrestin (319–418), dynamin K44A, or GRK2-K220R. Twenty-four hours before transfection, cells were plated onto 24-well plates at a density of 100×10^4 cells/plate. Cells were transfected with 6 μg of DNA per plate by activated-dendrimer by using SuperFect according to manufacturer's recommendations (QIAGEN, Valencia, CA). PI hydrolysis assays were performed 48 h after transfection.

Cells were transfected with pcDNA3 (Invitrogen) containing RatSR5-HT$_{2A}$ cDNA. Twenty-four hours before transfection, cells were plated onto 10-cm plates at a density of 5×10^5 cells/plate. Cells were transfected with 5 μg of DNA per plate with LipofectAMINE PLUS (Invitrogen) according to the manufacturer's recommendations. Stably transfected cells were selected by growth in media containing G418 sulfate (Geneticin). Transfected colonies were isolated and cultured for several passages in the presence of G418 (700 μg/ml). The expression and functional status of 5-HT$_{2A}$ receptors in several colonies were measured by the binding of [^{3}H]ketanserin and by PI hydrolysis assay, respectively. For measurement of cell surface 5-HT$_{2A}$ receptors by whole-cell ELISA, a clone expressing 300 fmol/mg of protein of the 5-HT$_{2A}$ receptor was chosen (C62A09).

Whole-Cell ELISA. C62A09 cells were plated onto poly-L-lysine (1 mg/ml)-coated six-well plates at a density of 100×10^4 cells/plate. Three days after plating, cells were fixed with 4% paraformaldehyde in PBS at 4°C for 10 min, washed (1× 3 ml of PBS, followed by 1 × 3 ml of Tris-buffered saline; TBS), and incubated with SuperBlock in TBS (Pierce Chemical, Rockford, IL) overnight at 4°C. Cells were then incubated with monoclonal anti-5-HT2AR (BD PharMingen, San Diego, CA) (5 μg/ml in 1:10 SuperBlock:TBS) for 1 h at room temperature, followed by 2 × 5-min washes with TBS. Antigen-antibody complexes were incubated with a biotinylated, affinity-purified horse anti-mouse IgG (Pierce Chemical) (10 μg/ml in 1:10 SuperBlock:TBS) for 30 min at room temperature, followed by 2 × 5-min washes with TBS. The biotinylated complexes were revealed by incubation with Ultra-Sensitive ABC peroxidase reagent (Pierce Chemical) for 30 min at room temperature in TBS, followed by 2 × 5-min washes with TBS. Cell surface peroxidase was detected with SuperSignal ELISA femto maximum sensitivity substrate (Pierce Chemical) by measurement of absorbance at 405 nm. Absorbance values were normalized by cell count. A background control was included in each plate and subtracted from the final absorbance measurements.

Antibody Delivery. Cells were plated onto 24-well plates at a density of 100×10^4 cells/plate. Three days after plating, cells were incubated with 40 μg/plate of monoclonal anti-GRK 2/3 antibody (Upstate Biotechnology, Lake Placid, NY) in the presence of 180 μl of the protein delivery reagent ProVectin (IMGENEX, San Diego, CA) according to manufacturer's instructions. Cells were incubated for 4 h before PI hydrolysis assay. For the 2-h ketanserin treatment, ketanserin is added during the last 2 h of the ProVectin incubation.

Data Analysis. Dose-response data, expressed as a percentage of basal IP accumulation, were fit by nonlinear regression with Kalei-

daGraph software (version 3.0.5; Synergy Software, Reading, PA) to the equation $E = E_{max}/(1 + (EC_{50}/(A)^n))$, where E is the measured response at a given concentration of agonist (A), E_{max} is the maximal response, EC_{50} is the concentration of agonist producing half-maximal response, and n is the slope factor. Statistical tests were performed using Statistica software (version 4.1; StatSoft, Tulsa, OK).

Materials. Quipazine dimaleate, ketanserin tartrate, prazosin HCl, methysergide maleate, and pyrilamine maleate were purchased from Sigma/RBI (Natick, MA). Concanavalin A (con A), staurosporine, bisindolymaleimide, and KN-93 were purchased from Calbiochem (La Jolla, CA). Serotonin creatinine sulfate, monodansylcadaverine, and 5′-guanylylimidodiphosphate were purchased from Sigma (St. Louis, MO). The dominant negative mutants, dynamin K44A, β-arrestin (319–418), and GRK2-K220R were generously provided by Dr. Jeffrey Benovic (Jefferson University, Philadelphia, PA).

Results

As shown in Fig. 1, treatment of C6 glioma cells with either 5-HT or the 5-HT_{2A} receptor antagonist ketanserin resulted in an attenuation in 5-HT_{2A} receptor function, specifically IP accumulation stimulated by the partial agonist quipazine (intrinsic activity with respect to 5-HT, $\alpha = 0.32 \pm 0.03$, $n = 3$). A concentration of 10 μM 5-HT was used in these experiments to allow us to compare our results with those of other investigators (Ferry et al., 1993; Toth and Shenk, 1994) and with those obtained previously in our laboratory (Anji et al., 2001). For the 24-h time point, 5-HT was added every 12 h because the half-life of 5-HT in culture is 12 to 14 h (Ferry et al., 1993). The concentration of ketanserin used was 100 times the K_i or affinity constant (Leysen et al., 1988) to ensure maximal receptor occupancy. Ketanserin alone, at concentrations of 100 nM to 100 μM, did not change IP accumulation from basal (data not shown). A greater attenuation in the maximal effect (E_{max}) of quipazine to stimulate IP accumulation was observed after treatment of cells with 5-HT than after treatment with the antagonist ketanserin (Fig. 1).

The time course of 5-HT- and ketanserin-induced desensitization of 5-HT_{2A} receptor function in C6 glioma cells is shown in Fig. 2. The attenuation of quipazine-stimulated IP accumulation (E_{max}) after 5-HT exposure was rapid, with a 40% decrease in E_{max} values for quipazine-stimulated IP accumulation occurring by 10 min. Further exposure to 5-HT resulted in a greater attenuation of this response, with a maximal desensitization of quipazine-stimulated IP accumulation by 2 h of 5-HT treatment. A similar time course and extent of desensitization were observed when cells were treated with 100 nM 5-HT (data not shown). Surprisingly, ketanserin treatment also induced a rapid desensitization, with a 40% decrease in E_{max} values for quipazine-stimulated IP accumulation occurring within 10 min (Fig. 2). However, in contrast to what was observed with 5-HT treatment, longer exposure of C6 glioma cells to ketanserin did not result in further desensitization. There was no change in the concentration of quipazine eliciting 50% response (EC_{50}) after treatment with 5-HT or ketanserin at any time point examined (Table 1).

In confirmation of a previous study from our laboratory (Anji et al., 2001), treatment of cells with 5-HT (10 μM) for 2 h did not alter the number of 5-HT_{2A} receptor sites as measured by the binding of a single saturating concentration of [^{3}H]ketanserin (12 nM). Treatment of cells with the antagonist ketanserin (50 nM) for 2 h did not alter the number of 5-HT_{2A} receptor sites [specific bound (fmol/mg of protein): vehicle-treated, 70 ± 4.4 ($n = 9$); 2 h 5-HT-treated, 73 + 7.4 ($n = 3$); and 2 h ketanserin-treated, 66 ± 9.1 ($n = 3$)]. These data indicate that the desensitization of 5-HT_{2A} receptor function after treatment of cells with 5-HT or ketanserin for 2 h is not due to a decrease in 5-HT_{2A} receptor number or expression.

To examine further the cellular processes underlying desensitization of the 5-HT_{2A} receptor, initial experiments were performed in which cells were treated with 5-HT or ketanserin for 10 min, according to time course data shown in Fig. 2. Roth and coworkers (Berry et al., 1996; Willins et al., 1999) have shown that in NIH3T3 cells, stably transfected to express the 5-HT_{2A} receptor, both agonist and antagonist ex-

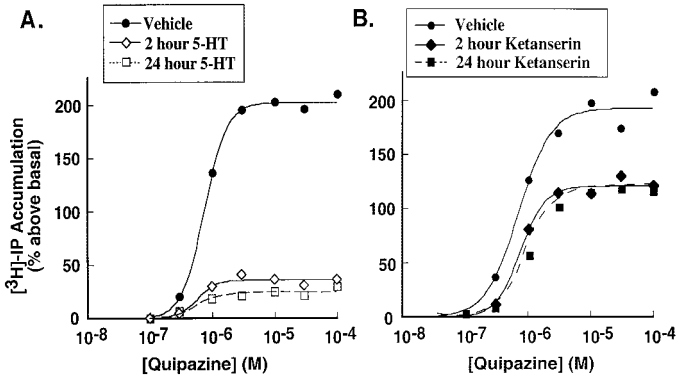

Fig. 1. Desensitization of quipazine-stimulated IP accumulation in C6 glioma cells. Cells were treated for 2 or 24 h with 5-HT (10 μM) (A) or the 5-HT_{2A} receptor antagonist ketanserin (50 nM) (B). Accumulation of [^{3}H]IP was measured in triplicate after a 20-min incubation with quipazine. Data shown are the mean of triplicate determinations of a single representative experiment. Basal [^{3}H]IP accumulation (mean of triplicate determinations) was 209 dpm for vehicle-treated, 196 dpm for 2-h 5-HT-treated, and 231 dpm for 24-h 5-HT-treated cells (A) and 406 dpm for vehicle-treated, 443 dpm for 2-h ketanserin-treated, and 441 dpm for 24-h ketanserin-treated cells (B).

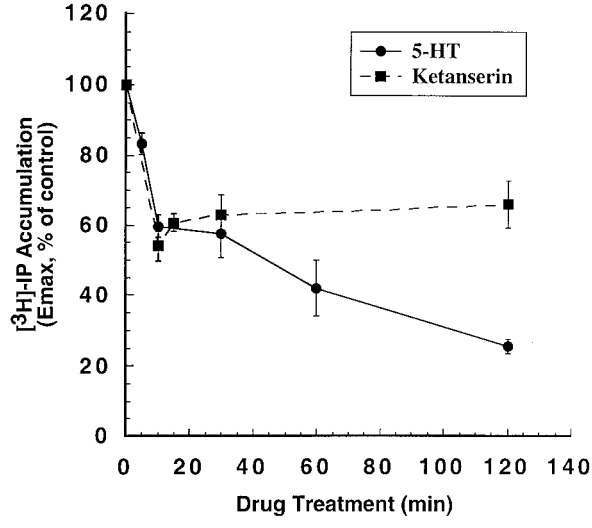

Fig. 2. Time course of 5-HT_{2A} receptor desensitization in C6 glioma cells. Cells were exposed to 5-HT (10 μM) or the antagonist ketanserin (50 nM) for the indicated time periods. Accumulation of [^{3}H]IP was measured in triplicate after a 20-min incubation with quipazine. Quipazine dose-response curves were analyzed as described under *Experimental Procedures*. Data are expressed as E_{max}, percentage of control. Shown are the mean ± S.E.M. of three individual experiments.

TABLE 1

Effect of treatment of C6 glioma cells with 5-HT or ketanserin on the stimulation of IP accumulation by quipazine

Cells were treated with 5-HT (10 μM) and ketanserin (50 nM) for indicated times. Accumulation of [^{3}H]IP was measured in triplicate after a 20-min incubation with quipazine, as described in Fig. 1. Shown are the mean ± S.E.M. EC_{50} values were analyzed by analysis of variance.

Treatment	n	EC_{50}	E_{max}
		μM	*% of control*
Vehicle	6	0.51 ± 0.06	
10-min 5-HT	6	0.48 ± 0.05	61 ± 4.5
2-h 5-HT	6	0.47 ± 0.10	24 ± 8.2
24-h 5-HT	6	0.48 ± 0.20	26 ± 4.4
Vehicle	4	0.54 ± 0.09	
10-min ketanserin	4	0.80 ± 0.18	54 ± 4.2
30-min ketanserin	4	0.83 ± 0.18	63 ± 5.6
2-h ketanserin	4	0.55 ± 0.08	66 ± 6.9

posure results in 5-HT$_{2A}$ receptor internalization. To investigate the role of receptor internalization in either 5-HT- or ketanserin-induced desensitization, experiments were conducted using con A, which inhibits receptor internalization (Waldo et al., 1983; Lohse et al., 1990), or monodansylcadaverine (MDC), which interferes with clathrin-mediated internalization by stabilizing clathrin-coated vesicles (Phonphok and Rosenthal, 1991; Claing et al., 2000). In the current study, both con A and MDC treatment prevented the attenuation of quipazine-stimulated IP accumulation (E_{max}) induced by 10-min exposure to 5-HT, but failed to prevent the desensitization of 5-HT$_{2A}$ receptor function induced by 10-min ketanserin exposure (Fig. 3). The desensitization of 5-HT$_{2A}$ receptor function induced by 2-h ketanserin exposure, however, was blocked by con A and MDC treatment (Fig. 3).

Internalization of the 5-HT$_{2A}$ receptor after 10-min 5-HT or 2-h ketanserin exposure was confirmed using a whole-cell ELISA assay. Because the modest level of endogenous receptor expression made detection of cell surface receptors difficult, cells were stably transfected to increase the number of 5-HT$_{2A}$ receptors. Preliminary experiments demonstrated that in clone C62A09, expressing the 5-HT$_{2A}$ receptor at a density of 300 fmol/mg of protein, quipazine stimulated IP accumulation 694 ± 39% above basal (E_{max}), with an EC_{50} value of 0.423 ± 0.11 (n = 3). Treatment of C62A09 cells with 5-HT or ketanserin resulted in desensitization of this response as was observed for the wild-type C6 cells (data not

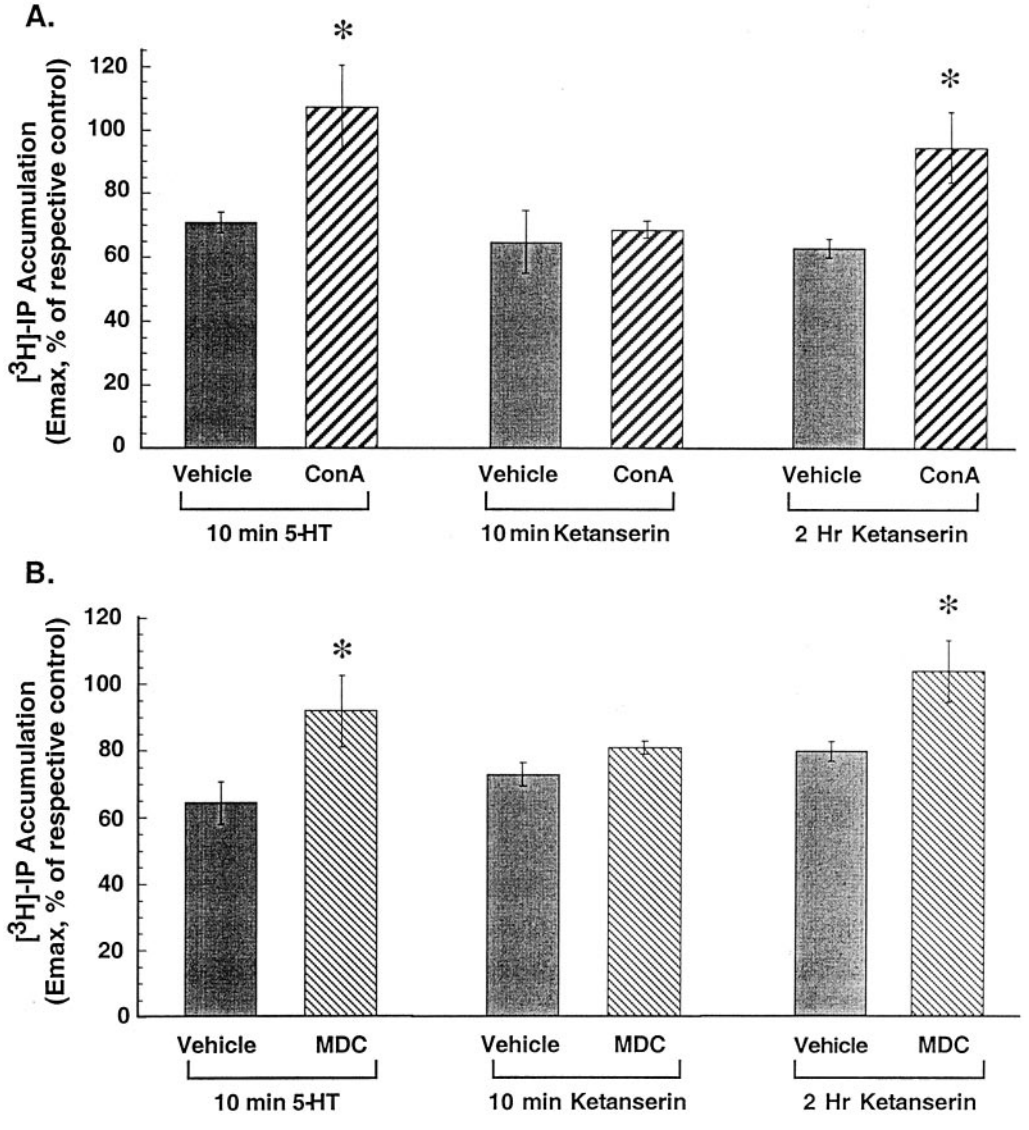

Fig. 3. Effect of con A or MDC pretreatment on 5-HT-induced and ketanserin-induced desensitization of 5-HT$_{2A}$ receptor function. A, cells were pretreated with 0.25 mg/ml con A for 20 min before the addition of 10 μM 5-HT or 50 nM ketanserin for 10 min, or cells were treated with 50 nM ketanserin for 2 h followed by the addition of 0.25 mg/ml con A during the last 30 min. B, cells were pretreated with 100 μM MDC for 30 min before the addition of 10 μM 5-HT or 50 nM ketanserin. Accumulation of [^{3}H]IP was measured in triplicate after a 20-min incubation with quipazine. Quipazine dose-response curves were analyzed as described under *Experimental Procedures*. Treatment of cells with con A or MDC alone did not significantly alter the E_{max} (vehicle, 127 ± 22% above basal; con A-treated, 133 ± 28% above basal; n = 3) (vehicle, 247 ± 58% above basal; MDC-treated, 189 ± 38% above basal; n = 4) or EC_{50} (vehicle, 0.410 ± 0.1 μM; con A-treated, 0.361 ± 0.1 μM; n = 3) (vehicle, 0.391 ± 0.04 μM; MDC-treated, 0.565 ± 0.04 μM; n = 4) of quipazine to stimulate IP accumulation. Data plotted are expressed as E_{max}, percentage of respective control, and each data point represents the mean ± S.E.M. of three to four individual experiments. Data were analyzed by Student's t test; *, $p < 0.05$.

shown). As shown in Fig. 4, exposure of C62A09 cells to 5-HT for 10 min or to ketanserin for 2 h resulted in a decrease in absorbance as measured by whole-cell ELISA, indicating a decrease in cell surface receptor expression after these treatments. The decrease in absorbance induced by 10-min 5-HT or 2-h ketanserin exposure was blocked by MDC (100 μM). Treatment of cells with ketanserin for 10 min, however, did not result in a significant change in absorbance (Fig. 4). Taken together, these data indicate that receptor internalization plays a role in 5-HT_{2A} receptor desensitization induced by 5-HT treatment or prolonged (2-h) exposure to the antagonist ketanserin. Receptor internalization, however, does *not* appear to be involved initially in the desensitization of the 5-HT_{2A} receptor induced by ketanserin.

Experiments were performed to investigate the potential involvement of the cellular proteins arrestin, clathrin, and dynamin in the desensitization of the 5-HT_{2A} receptor after 5-HT or ketanserin exposure. Arrestin binding to the receptor promotes the uncoupling of the receptor from G protein and receptor internalization through clathrin-coated vesicles (Goodwin et al., 1996; Krupnick et al., 1997a). To interrupt endogenous clathrin-arrestin interactions, C6 glioma cells were transiently transfected with β-arrestin (319–418), the clathrin binding domain of β-arrestin that acts as a dominant negative mutant (Krupnick et al., 1997b). As shown in Fig. 5A, transient transfection with β-arrestin (319–418) blocked the attenuation of quipazine-stimulated IP accumulation (E_{max}) induced by 10-min exposure to 5-HT or 2-h exposure to ketanserin. β-Arrestin (319–418), however, it did not block the desensitization of 5-HT_{2A} receptor function induced by 10-min ketanserin exposure (Fig. 5A). In separate experiments we used the dynamin dominant negative mutant dynamin K44A, which is void of GTPase activity (van der Bleik et al., 1993; Damke et al., 1994). Through GTP binding and hydrolysis, dynamin pinches off clathrin-coated invaginations to form endocytotic vesicles. Transient transfection with dynamin K44A blocked the attenuation of quipazine-stimulated IP accumulation (E_{max}) induced by treatment with either 5-HT (10 min) or ketanserin (2 h) (Fig. 5B). As expected, the desensitization of 5-HT_{2A} receptor function induced by 10-min exposure to ketanserin was not prevented by dynamin K44A (Fig. 5B). Taken together, these data indicate that the desensitization of 5-HT_{2A} receptor-mediated PI hydrolysis in C6 glioma cells induced by 10-min treatment with 5-HT or 2 h treatment with ketanserin involves receptor internalization through a clathrin- and dynamin-dependent process.

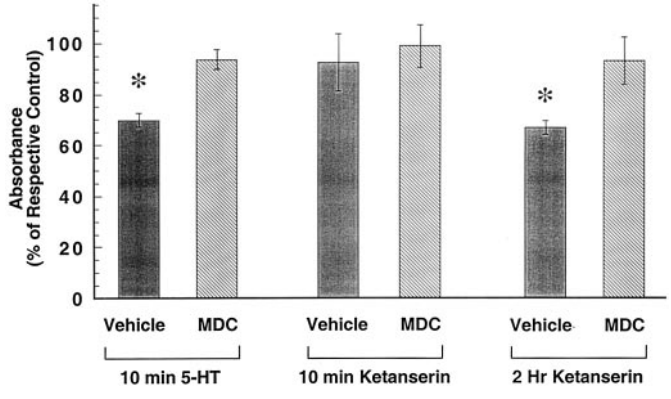

Fig. 4. Cell surface 5-HT_{2A} receptor expression. Cell surface immunoreactivity of the 5-HT_{2A} receptor was measured by ELISA as absorbance at 405 nm. Absorbance values were normalized to cell count. Cells were pretreated with 100 μM MDC for 30 min before the addition of vehicle, 10 μM 5-HT, or 50 nM ketanserin. MDC alone did not alter absorbance. Data plotted are expressed as absorbance, percentage of respective control, and each data point represents the mean ± S.E.M. of four individual experiments. Data were analyzed by Student's t test; *, $p < 0.05$.

The striking difference in the speed of 5-HT- versus ketanserin-induced internalization led us to hypothesize that second messenger-dependent kinases and/or GRKs play a role in agonist, but not antagonist-induced desensitization of the 5-HT_{2A} receptor. To examine whether second messenger-dependent kinases, such as PKC or calcium-calmodulin kinase II, play a role in 5-HT-induced desensitization of the 5-HT_{2A} receptor, cells were pretreated with the broad range serine/threonine kinase inhibitor staurosporine, the selective PKC inhibitor bisindolymaleimide, or the selective calcium-calmodulin kinase II inhibitor KN 93. The concentrations of inhibitors and duration of pretreatment were chosen from our previous studies and from the literature (Zhang et al., 1997; Muraoka et al., 1998; Anji et al., 2001). As shown in Fig. 6, pretreatment of cells with any one of these inhibitors did not block or prevent 5-HT-induced attenuation of quipazine-stimulated IP accumulation (E_{max}). The apparent potentiation of 5-HT-induced desensitization by staurosporine may be due to the nonselective nature of this serine/threonine kinase inhibitor.

To examine whether GRKs play a role in 5-HT_{2A} receptor desensitization, we used the dominant negative mutant of GRK2 GRK2-K220R, which lacks kinase activity (Kong et al., 1994). Classically, phosphorylation of the agonist-occupied state of G protein-coupled receptors by GRKs promotes the binding of receptor to arrestin and receptor internalization (Krupnick and Benovic, 1998). As shown in Fig. 7A, transient transfection with GRK2-K220R blocked the attenuation of quipazine-stimulated IP accumulation (E_{max}) induced by 10-min exposure to 5-HT. Surprisingly, the desensitization of 5-HT_{2A} receptor-mediated PI hydrolysis induced by 2-h ketanserin exposure was also prevented by the expression of GRK2-K220R (Fig. 7A). In separate experiments, we delivered an antibody directed against GRK 2/3 to disrupt GRK activity (Oppermann et al., 1996). Incubation with anti-GRK 2/3 antibody blocked the attenuation of quipazine-stimulated IP accumulation (E_{max}) induced by treatment with either 5-HT (10 min) or ketanserin (2 h) (Fig. 7B). The desensitization of 5-HT_{2A} receptor function induced by 10-min exposure to ketanserin was not prevented by the GRK 2/3 antibody (Fig. 7B). Taken together, these data suggest that GRK plays a role in 5-HT_{2A} receptor desensitization induced by 10-min 5-HT or 2-h ketanserin exposure. GRK, however, appears not to be involved initially in antagonist-induced 5-HT_{2A} receptor desensitization.

We hypothesized that although 5-HT_{2A} receptor desensitization induced by short-term ketanserin exposure is not a result of receptor internalization, the capacity of the 5-HT_{2A} receptor to couple to G protein is impaired at this early stage of antagonist-induced desensitization. The ability of an agonist to promote receptor-G protein coupling can be assessed as the amount of high-affinity agonist binding that is sensitive to guanine nucleotides (Kenakin, 1997). As shown in Fig. 8, GppNHp (10 μM) markedly reduced the specific binding of [^{125}I]DOI (0.7 nM) in homogenates of vehicle-treated cells to 10% of control values. Treatment of cells with ketanserin for

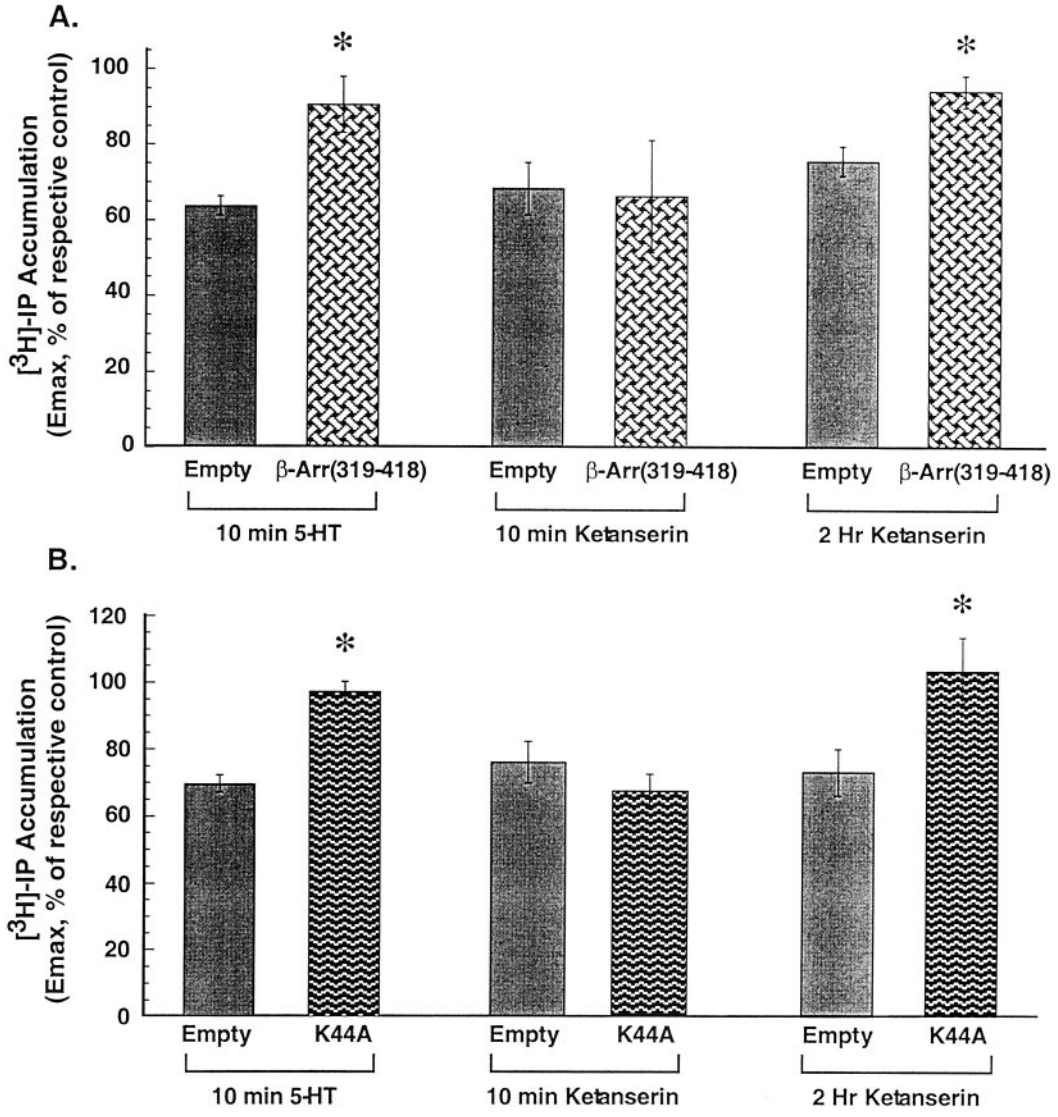

Fig. 5. Effect of the dominant negative mutants β-arrestin (319–418) or dynamin K44A on 5-HT-induced and ketanserin-induced desensitization of 5-HT$_{2A}$ receptor function. Cells were transfected with either the empty vector or β-arrestin (319–418) (A) or dynamin K44A (B) before exposure to 10 μM 5-HT or 50 nM ketanserin. Accumulation of [^{3}H]IP was measured in triplicate after a 20-min incubation with quipazine. Quipazine dose-response curves were analyzed as described under *Experimental Procedures*. Transfection of cells with β-arrestin (319–418) or dynamin K44A alone did not alter the E_{max} (empty vector, 179 ± 23% above basal; β-arrestin (319–418), 197 ± 16% above basal; n = 3) (empty vector, 282 ± 78% above basal; K44A, 240 ± 64% above basal; n = 3) or EC_{50} (empty vector, 0.565 ± 0.1 μM; β-arrestin (319–418), 0.635 ± 0.2 μM; n = 3) (empty vector, 0.548 ± 0.1 μM; K44A, 0.529 ± 0.1 μM; n = 3) of quipazine to stimulate IP accumulation. Data plotted are expressed as E_{max}, percentage of respective control, and each data point represents the mean ± S.E.M. of three individual experiments. Data were analyzed by Student's t test; *, $p < 0.05$.

10 min reduced the amount of GppNHp-sensitive [^{125}I]DOI binding by 92% (Fig. 8, inset). These data suggest that short-term ketanserin treatment reduced the capacity of the 5-HT$_{2A}$ receptor to couple to G protein. As expected, 2-h ketanserin exposure resulted in an 86% reduction in high-affinity agonist binding of the 5-HT$_{2A}$ receptor, consistent with the 5-HT$_{2A}$ receptor being internalized after prolonged antagonist exposure (Fig. 8).

Discussion

We have examined the effect of agonist or antagonist exposure on 5-HT$_{2A}$ receptor function in C6 glioma cells. Treatment with either 5-HT or the antagonist ketanserin attenuated 5-HT$_{2A}$ receptor function, specifically the stimulation of IP accumulation by the partial agonist quipazine. We have confirmed and extended previous studies (Berry et al., 1996; Willins et al., 1998, 1999) by demonstrating that both agonist- and antagonist-induced desensitization of the 5-HT$_{2A}$ receptor appears to involve receptor internalization through a clathrin- and dynamin-dependent process. Moreover, our data indicate a dual mechanism of early and late desensitization by the antagonist ketanserin. As was observed after agonist treatment, prolonged antagonist exposure resulted in the internalization of the 5-HT$_{2A}$ receptor through a clathrin- and dynamin-dependent process that appears to involve a GRK. Although short-term 5-HT and ketanserin exposure resulted in the same degree of desensitization, the desensitization induced by short-term ketanserin treatment appears to be due to reduced capacity of the 5-HT$_{2A}$ receptor to couple to G protein, but does not involve receptor internalization.

In the current study, treatment of C6 glioma cells with 5-HT or ketanserin resulted in a rapid, almost immediate reduction in response (i.e., quipazine-stimulated IP accumulation). For a partial agonist, such as quipazine, response is proportional to receptor occupancy (Kenakin, 1997). Therefore, maximal stimulation of IP accumulation by quipazine (E_{max}) is expected to result from 100% receptor occupancy, and a decrease in functional 5-HT$_{2A}$ receptors would be reflected in a decrease in quipazine's E_{max}. Desensitization, a decrease in response as a result of continuous agonist exposure, can occur as a result of receptor uncoupling from G protein, internalization (sequestration of the receptor away from the cell surface), or down-regulation (loss of total receptor number). The desensitization of 5-HT$_{2A}$ receptor function

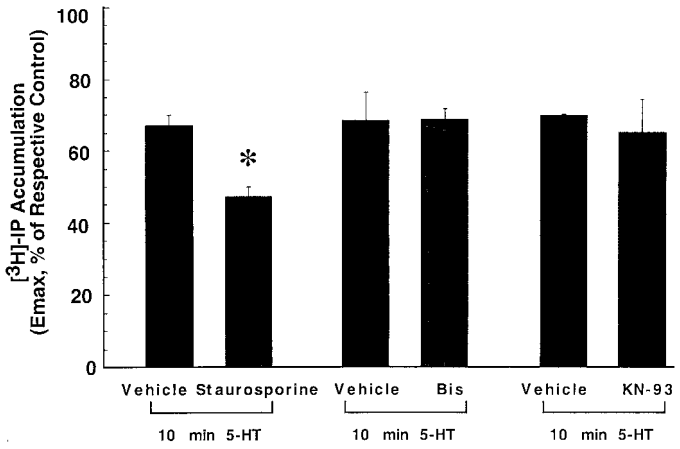

Fig. 6. Inhibitors of second messenger-dependent kinases do not prevent 5-HT-induced desensitization of 5-HT_{2A} receptor function. Cells were pretreated with 300 nM staurosporine for 15 min, 100 nM bisindolylmaleimide for 30 min, or 1 μM KN-93 for 15 min before the addition of 10 μM 5-HT for 10 min. Accumulation of [^{3}H]IP was measured in triplicate after a 20-min incubation with quipazine. Quipazine dose-response curves were analyzed as described under *Experimental Procedures*. Treatment of cells with staurosporine alone did not alter the E_{max} (vehicle, 133 ± 14% above basal; staurosporine-treated, 142 ± 9% above basal; $n = 3$) or EC_{50} (vehicle: 0.461 ± 0.03 μM; staurosporine-treated: 0.311 ± 0.06 μM; $n = 3$) of quipazine to stimulate IP accumulation. Treatment of cells with bisindolylmaleimide alone did not alter the E_{max} (vehicle, 154 ± 24% above basal; bisindolylmaleimide-treated, 140 ± 23% above basal; $n = 4$) or EC_{50} (vehicle: 0.346 ± 0.05 μM; bisindolylmaleimide-treated: 0.370 ± 0.05 μM; $n = 4$) of quipazine to stimulate IP accumulation. Treatment of cells with KN-93 alone did not alter the E_{max} (vehicle, 299 ± 143% above basal; KN-93-treated, 280 ± 141% above basal; $n = 3$) or EC_{50} (vehicle, 0.583 ± 0.06 μM; KN-93-treated, 0.450 ± 0.06 μM; $n = 3$) of quipazine to stimulate IP accumulation. Data plotted are expressed as E_{max}, percentage of respective control, and each data point represents the mean ± S.E.M. of three to four individual experiments. Data were analyzed by Student's t test; *, $p < 0.05$.

in C6 glioma cells after treatment with 5-HT or ketanserin for 2 h was not due to a decrease in 5-HT_{2A} receptor number or expression. Our data indicate that receptor internalization plays a role in the desensitization of 5-HT_{2A} receptor function after short-term (i.e., 10-min) 5-HT treatment and prolonged (i.e., 2-h) ketanserin exposure. Our findings are consistent with those of Roth and coworkers (Berry et al., 1996; Willins et al., 1999). In NIH3T3 cells expressing the 5-HT_{2A} receptor, 30 min of treatment with a variety of antagonists results in 5-HT_{2A} receptor internalization (Berry et al., 1996; Willins et al., 1999); significant internalization of receptors is observed as early as 5 min of agonist exposure (Berry et al., 1996).

The results of the current study indicate that in C6 glioma cells 5-HT_{2A} receptor desensitization induced by short-term 5-HT treatment or prolonged ketanserin exposure involves receptor internalization through a clathrin- and dynamin-dependent process. Immunohistochemical studies have shown the colocalization of clathrin and the 5-HT_{2A} receptor after agonist exposure of NIH3T3 cells stably expressing the 5-HT_{2A} receptor (Willins et al., 1998). For some G protein-coupled receptors, in particular the β_2-adrenergic receptor, the interaction of arrestin with clathrin and with the receptor promotes receptor internalization through clathrin-coated vesicles (Bunemann and Hosey, 1999). Interestingly, agonist- and antagonist-induced internalization of the 5-HT_{2A} receptor in human embryonic kidney 293 cells appears to be through another pathway, one which is arrestin-independent, but dynamin-dependent (Bhatnagar et al., 2001). Similar observations have been made in human embryonic kidney 293 cells for agonist-induced internalization of m1, m2, and m4 muscarinic cholinergic receptors (Lee et al., 1998). Mechanism(s) of regulation of receptor function may be cell-specific, reflecting differences in cellular machinery and different molecular pathways of desensitization.

The carboxy tail of the 5-HT_{2A} receptor contains PKC consensus sites and potential phosphorylation sites for GRKs (Vouret-Craviari et al., 1995). The striking difference in the speed of 5-HT- versus ketanserin-induced internalization led us to hypothesize that second messenger-dependent kinases and/or GRKs play a role in agonist, but not antagonist-induced desensitization of the 5-HT_{2A} receptor. PKC inhibitors have been shown to attenuate agonist-induced desensitization of 5-HT_{2A} receptors in human platelets (Kagaya et al., 1990). However, in NIH3T3 cells stably expressing the 5-HT_{2A} receptor, down-regulation of PKC attenuates only the intermediate phase (i.e., 2–6 h) of agonist-induced desensitization (Roth et al., 1995). In C6 glioma cells, PKC appears not to be involved in the acute phase of agonist-induced desensitization of receptor function (i.e., 10 min of 5-HT exposure; this study), but is involved in regulation of 5-HT_{2A} receptor expression and mRNA levels with chronic agonist exposure (i.e., 2–6 h of 5-HT exposure) (Anji et al., 2001). 5-HT_{2A} receptor desensitization is independent of PKC in hamster fibroblasts (CCL39 cells) (Vouret-Craviari et al., 1995). Thus, cell-specific mechanisms of regulation predominate for this family of receptors.

In the current study, expression of the dominant negative mutant GRK2-K220R or incubation with a monoclonal anti-GRK 2/3 antibody prevented 5-HT_{2A} receptor desensitization resulting from treatment of cells for 10 min with 5-HT or 2 h with ketanserin. Taken together, these data suggest that GRK may be involved in receptor desensitization as a result of short-term 5-HT treatment or prolonged ketanserin exposure. Our data with the antagonist ketanserin are particularly intriguing. We speculate that the slower rate of internalization of the 5-HT_{2A} receptor induced by ketanserin versus 5-HT, and the apparent involvement of GRK in the late but not early stage of ketanserin-induced desensitization, may reflect stabilization of a receptor conformation differing in its capacity to serve as substrate for GRK, to bind arrestin and to undergo endocytosis, or to activate G protein (Clark et al., 1999).

Our data indicate that in C6 glioma cells, 5-HT_{2A} receptor desensitization induced by short-term ketanserin exposure is not a result of receptor internalization. Perhaps the most parsimonious explanation is that the capacity of the 5-HT_{2A} receptor to couple to G protein is reduced at this early stage of antagonist-induced desensitization. Our data with the binding of the agonist radioligand [^{125}I]DOI are consistent with this. A reduced capacity of the 5-HT_{2A} receptor to couple to G protein may be due to regulatory processes (e.g., phosphorylation) at the level of the receptor or distal to the receptor, most notably at the level of the G protein (Lohse, 1993). Desensitization of 5-HT_{2A} receptor function as a result of 10-min exposure to ketanserin is prevented by pretreatment of C6 glioma cells with the PKC inhibitor bisindolylmaleimide (N. R. S. Hanley and J. G. Hensler, unpublished observations). The processes underlying this early effect of ketanserin exposure on 5-HT_{2A} receptor function remain to be elucidated and are currently under investigation. In ad-

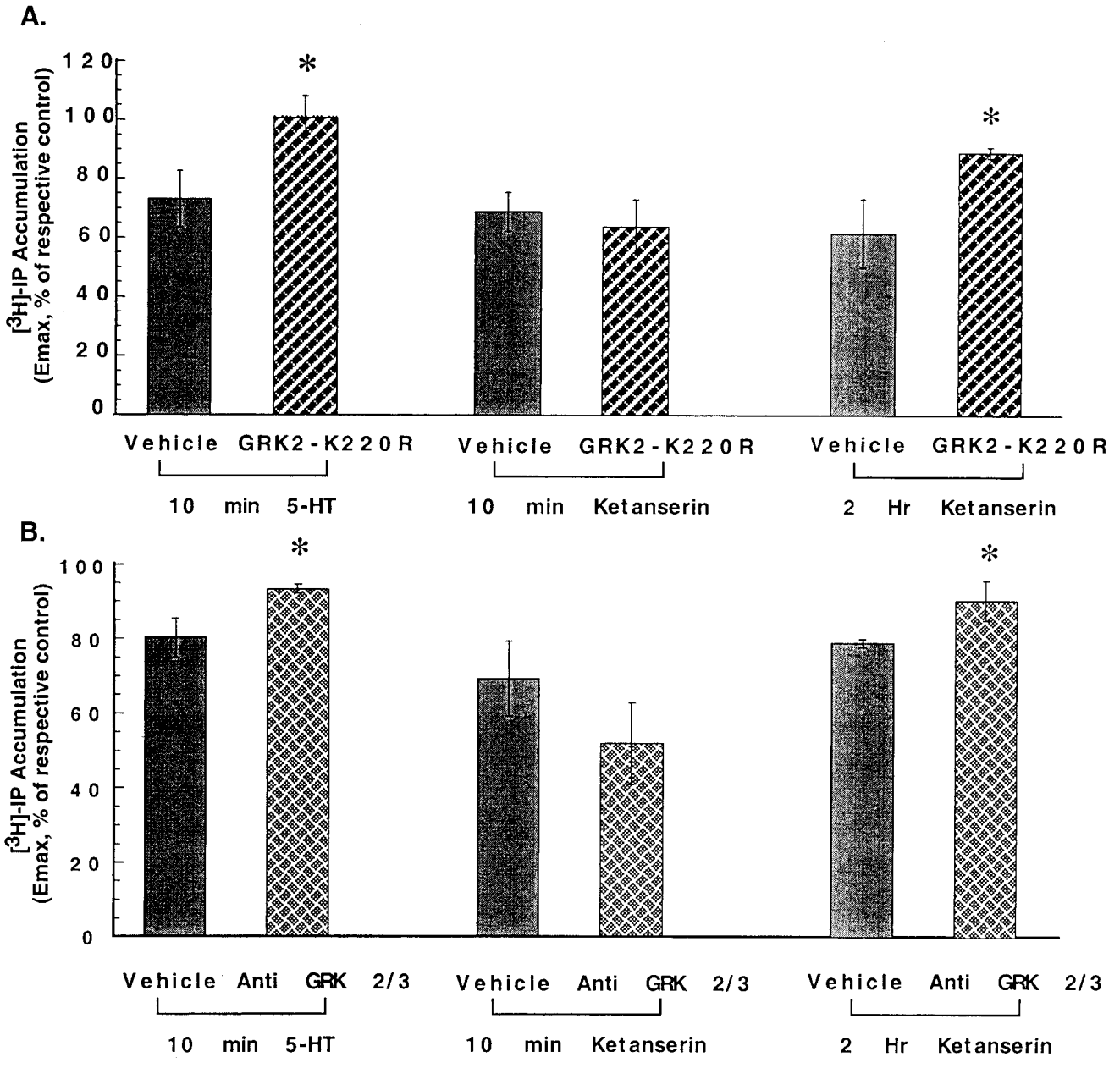

Fig. 7. Effect of the dominant negative mutant GRK2-K220R or anti-GRK 2/3 antibody on 5-HT-induced and ketanserin-induced desensitization of 5-HT$_{2A}$ receptor function. A, cells were transfected with either the empty vector or GRK2-K220R before exposure to 10 μM 5-HT for 10 min, 50 nM ketanserin for 10 min, or 50 nM ketanserin for 2 h. B, cells were incubated with either the vehicle or anti-GRK 2/3 antibody before exposure to 10 μM 5-HT for 10 min, 50 nM ketanserin for 10 min, or 50 nM ketanserin for 2 h. Accumulation of [^{3}H]IP was measured in triplicate after a 20-min incubation with quipazine. Quipazine dose-response curves were analyzed as described under *Experimental Procedures*. Transfection of cells with GRK2-K220R alone did not alter the E_{max} (empty vector, 223 ± 37% above basal; K220R, 219 ± 24% above basal; $n = 3$) or EC_{50} (empty vector, 0.549 ± 0.13 μM; K220R, 0.456 ± 0.02 μM; $n = 3$) of quipazine to stimulate IP accumulation. Incubation with anti-GRK 2/3 antibody alone did not alter E_{max} (vehicle, 178 ± 6.4% above basal; anti-GRK 2/3, 199 ± 13% above basal). Data plotted are expressed as E_{max}, percentage of respective control, and each data point represents the mean ± S.E.M. of three individual experiments. Data were analyzed by Student's t test; *, $p < 0.05$.

dition, it will be of great interest to examine further the nature of ketanserin's interaction with the 5-HT$_{2A}$ receptor. Although ketanserin is an antagonist at many 5-HT$_{2A}$ receptor-mediated responses, ketanserin appears to possess agonist-like properties when it comes to the regulation of 5-HT$_{2A}$ receptor function.

Desensitization and down-regulation of 5-HT$_{2A}$ receptors occur after chronic administration of a variety of antidepressants, as well as atypical neuroleptics, and may be central to the therapeutic action of these drugs. In vivo, clozapine and other atypical antipsychotic drugs, which are antagonists at the 5-HT$_{2A}$ receptor, induce a redistribution of 5-HT$_{2A}$ receptors within neurons of the medial prefrontal cortex (Willins et al., 1999). Although mechanism(s) of regulation of receptor function is determined by cellular machinery and cell-specific molecular pathways, our data from the current study indicate that in C6 glioma cells ketanserin- and 5-HT-induced desensitization of the 5-HT$_{2A}$ receptor is mediated by clathrin- and dynamin-dependent receptor internalization, a process that appears to involve GRK. It will be of great interest

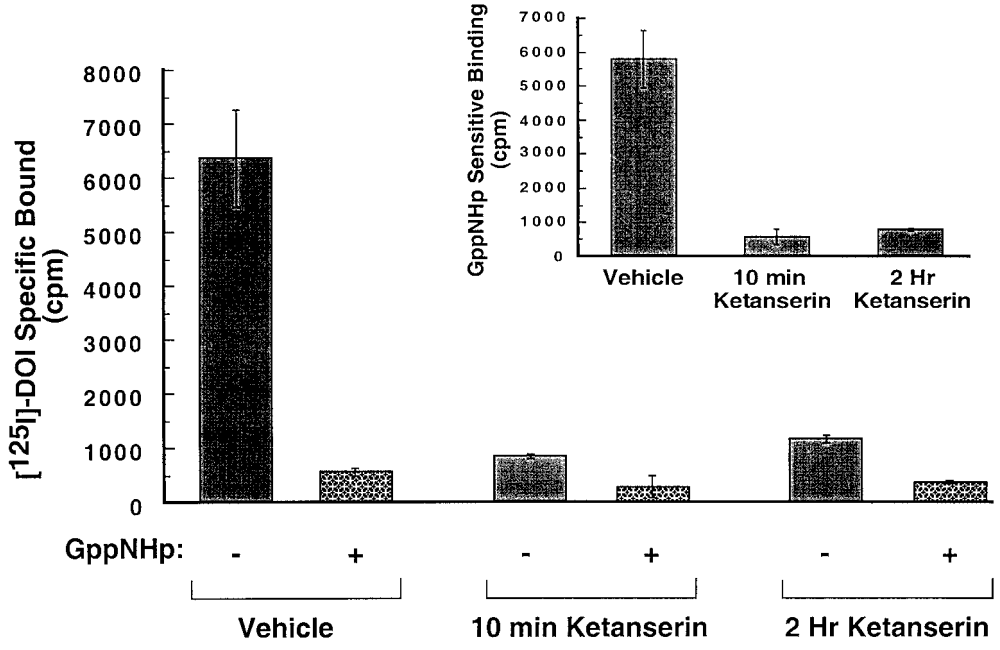

Fig. 8. Effect of ketanserin treatment on the binding of [^{125}I]DOI to 5-HT$_{2A}$ receptor sites. Cells were treated with vehicle, or 50 nM ketanserin for 10 min or 2 h. Binding was performed with a single concentration of [^{125}I]DOI (~0.7 nM) in the presence or absence of GppNHp (10 μM) as described under *Experimental Procedures*. Nonspecific binding was defined by 1 μM ketanserin. Inset, GppNHp-sensitive binding of [^{125}I]DOI was calculated by subtracting specific binding in the presence of GppNHp (10 μM) from specific binding in the absence of GppNHp. Shown are the mean ± S.E.M. of three experiments performed in triplicate.

to examine further the nature of GRK involvement in ligand-induced desensitization of the 5-HT$_{2A}$ receptor by using this model cell system.

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