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Rapid Agonist-Induced Internalization of the 5-Hydroxytryptamine$_{2A}$ Receptor Occurs via the Endosome Pathway *In Vitro*

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SUMMARY

The mechanism by which agonists induce 5-hydroxytryptamine$_{2A}$ (5-HT$_{2A}$) receptor internalization was investigated in a clonal cell line stably transfected with the 5-HT$_{2A}$ receptor cDNA. Confocal laser microscopy of immunolabeled 5-HT$_{2A}$ receptors in control (untreated) cells demonstrated that most of the immunoreactivity was associated with the cell surface. After quipazine administration, a significant increase in intracellular immunofluorescence was measured. Time course studies demonstrated rapid agonist-dependent internalization of 5-HT$_{2A}$ receptors, with significant internalization occurring as early as 5 min after agonist administration at 37°. In GF-62 cells, agonist-induced internalization was blocked by preincubation with the 5-HT$_{2A}$ receptor antagonist ketanserin. Internalization was also temperature sensitive because agonist-induced internalization did not occur at 4°. Dual-label experiments disclosed that 5-HT$_{2A}$ and transferrin receptors were internalized via the same endocytotic vesicles. These results suggest that 5-HT$_{2A}$ receptors and transferrin receptors are internalized via the endosomal pathway in GF-62 cells. Although 5-HT$_{2A}$ receptors were internalized, down-regulation, or loss of radioligand binding sites, did not occur. Our results demonstrate that agonists rapidly induce 5-HT$_{2A}$ receptor internalization via the endosomal pathway and that internalization can be dissociated from down-regulation.

The mechanisms by which G protein-coupled 5-HT (serotonin) receptors are regulated remain incompletely understood. Although the phenomena of 5-HT receptor desensitization and down-regulation are well described (1–3), relatively little attention has been focused on the molecular, biochemical, and cellular processes by which agonist-induced alterations in receptor activity might occur. In this study, we use a cell line stably transfected with the 5-HT$_{2A}$ receptor as a convenient model system.

In addition to serving as a model 5-HT receptor, the 5-HT$_{2A}$ receptor is of importance for other reasons. For example, the 5-HT$_{2A}$ receptor has been proposed to mediate the effects of certain atypical antipsychotic drugs (e.g., clozapine, risperidone, olanzepine; see Ref. 4), hallucinogens (e.g., lysergic acid diethylamide; see Ref. 5), some antidepressants (e.g., mianserin; see Ref. 6), and other drugs (6, 7). Also, the 5-HT$_{2A}$ receptor is involved in a number of physiological processes, including vascular and uterine smooth muscle contraction, platelet aggregation, perception, and emotion (7). Finally, disregulation of 5-HT$_{2A}$ receptors has been suggested to occur in a variety of pathological states, including depression, suicide, schizophrenia, and other mental and neurological illnesses (8, 9).

The 5-HT$_{2A}$ receptor is regulated by a wide variety of 5-HT receptor agonists and antagonists. Acute or chronic administration of agonists [e.g., lysergic acid diethylamide and (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane] causes an apparent down-regulation of 5-HT$_{2A}$ receptors *in vivo* (1). *In vitro*, agonists also desensitize 5-HT$_{2A}$ receptors (2, 3). *In vivo*, antagonists also, paradoxically, down-regulate 5-HT$_{2A}$ receptors without altering 5-HT$_{2A}$ receptor mRNA levels (10, 11).

Recently, we investigated the mechanisms responsible for agonist-induced desensitization of the 5-HT$_{2A}$ receptor in a stably transfected cell line (12). In that study, we identified three phases of receptor desensitization: (i) an early protein kinase C-independent process that occurred 10–120 min after agonist exposure, (ii) an intermediate protein kinase C-dependent process that occurred 2–6 hr after agonist administration, and (iii) a late phase that occurred after 24 hr of agonist exposure. Because the initial phase of desensitization was protein kinase C independent, we hypothesized that this early phase of desensitization could be accompanied by receptor sequestration.

In the current study, we tested the hypothesis that brief

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; GPCR, G protein-coupled receptor; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; IP, inositol phosphate; PI, phosphoinositide; BODIPY, 4,4-difluoro-4-bora-3a,4a-diaza-3-alkyl,5-alkyl,7-alkyl-5-indacene.

exposure to agonists may cause receptor internalization. To test this hypothesis, we modified a dual-label, fluorescence confocal microscopic technique by adding image analysis procedures to quantify the extent of internalization. For these experiments, a BODIPY-transferrin derivative was used to label endocytotic vesicles and a rhodamine-labeled secondary antibody was used to visualize 5-HT$_{2A}$ receptors. With these procedures, we were able to simultaneously measure the internalization of 5-HT$_{2A}$ and transferrin receptors. The results indicate that agonists cause a rapid, temperature-dependent internalization of the 5-HT$_{2A}$ receptor, apparently via the endosomal pathway.

Experimental Procedures

Materials. Tissue culture reagents were purchased from GIBCO-BRL (Gaithersburg, MD); [^{3}H]ketanserin (80.9 Ci/mmol) was purchased from New England Nuclear (Boston, MA); quipazine maleate was purchased from Miles Laboratories (Naperville, IL); ketanserin tartrate was purchased from Janssen Pharmaceutica (New Brunswick, NJ); tetramethyl rhodamine isothiocyanate-labeled goat anti-rabbit antibody was purchased from Accurate Scientific (Westbury, NY); BODIPY-transferrin was purchased from Molecular Probes (Eugene, OR); and unlabeled transferrin and ovalbumin was purchased from Sigma Chemical (St. Louis, MO). The antibody directed against the 5-HT$_{2A}$ receptor has been described previously (12, 13).

Cell culture. A nonclonal NIH-3T3 cell line stably expressing the rat 5-HT$_{2A}$ receptor was used to isolate a clonal cell line (GF-62) that expresses a 5–7 pmol/mg concentration of the 5-HT$_{2A}$ receptor (12). Cells were grown as detailed previously (12–14). At least 18 hr before agonist exposure, medium was replaced with DMEM with 10% dialyzed fetal calf serum (to remove 5-HT from the medium).

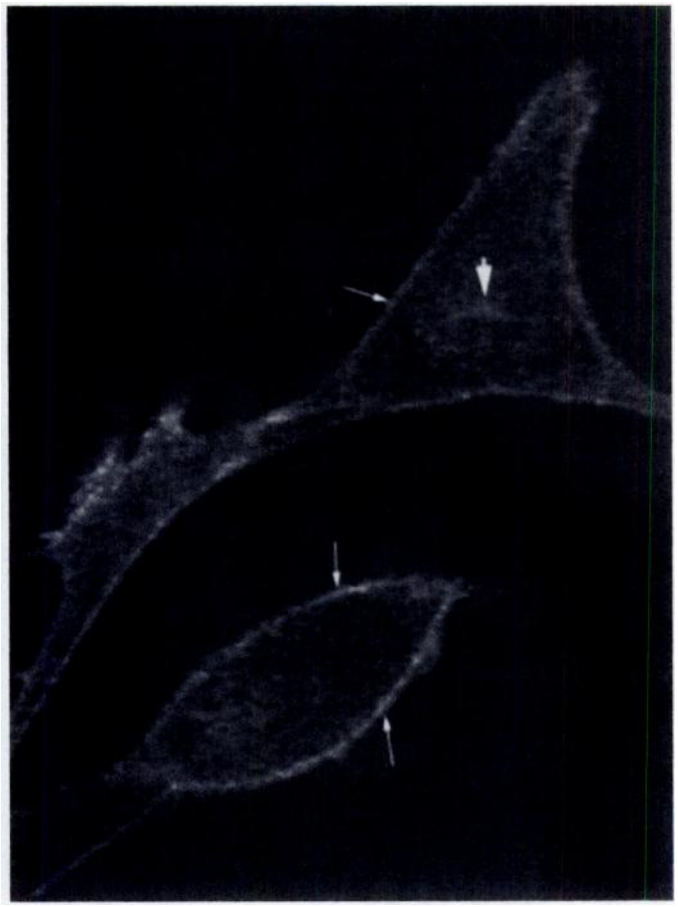

Fig. 1. Distribution of 5-HT$_{2A}$ receptors in quiescent GF-62 cells. Shown is a typical confocal microscopic section from control (untreated) GF-62 cells. *Small arrows*, surface immunofluorescence; *large arrowhead*, intracellular immunofluorescence.

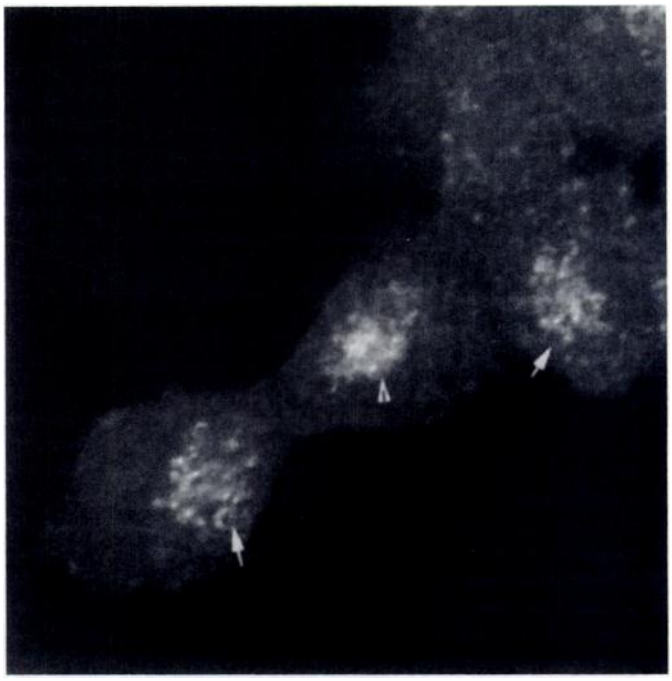

Fig. 2. Quipazine induces 5-HT$_{2A}$ receptor sequestration. Shown is a typical confocal microscopic section from cells that had been exposed to 10 μM quipazine for 30 min at 37°. *Arrowheads*, intracellular accumulation of 5-HT$_{2A}$ receptor immunofluorescence.

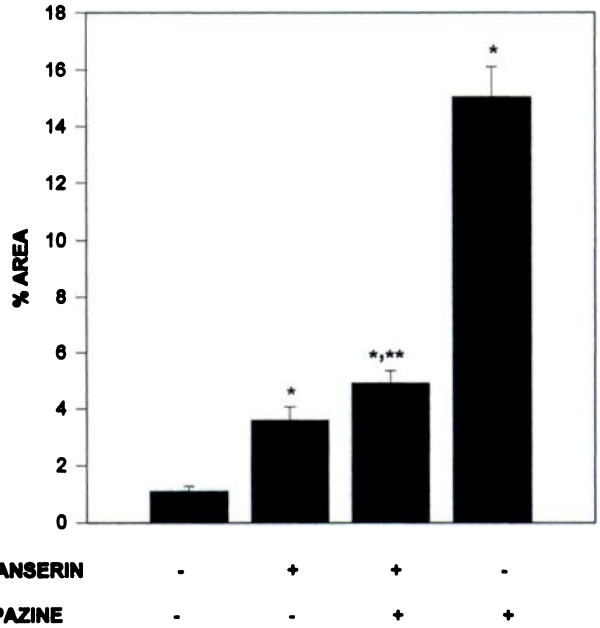

Fig. 3. Ketanserin blocks the ability of quipazine to sequester 5-HT$_{2A}$ receptors. Cells were exposed to vehicle (PBS), 10 μM quipazine, or the combination of 10 μM quipazine plus 10 μM ketanserin for 30 min at 37° and then prepared for confocal microscopy as described in Experimental Procedures. Then, 3–10 0.3-μm confocal sections were made from representative cells, and the percentage of intracellular area that was immunofluorescent (a measure of sequestration) was calculated as described in Experimental Procedures. Data are expressed as mean ± standard error. *, $p < 0.01$ versus control; **, $p < 0.01$ versus quipazine alone.

Immunofluorescence. For immunocytochemical detection of $5HT_{2A}$ receptors, cells were grown onto poly(lysine)-coated glass coverslips in 24-well dishes. For time course experiments, cells were exposed to a saturating concentration (10 μM; see Ref. 12) of the 5-HT_{2A} receptor agonist quipazine for various time periods. Medium was then removed, and the monolayers were washed two times with ice-cold PBS. The cells were fixed for 30 min with 4% paraformaldehyde in PBS. Fixative was removed, and cells were washed two times with PBS and then gently permeabilized with 0.3% Triton X-100 in PBS on ice for 20 min. Cells were then incubated with blocking solution (5% nonfat dry milk in PBS) for 1 hr and then incubated for 2 hr with a 1:2000 dilution of primary antibody dissolved in blocking solution (12, 13). After two PBS washes, cells were incubated for 1 hr with a 1:200 dilution of secondary antibody dissolved in blocking solution in the dark. Cells were then washed three times with PBS, dipped into distilled H_2O, and mounted onto glass microscope slides with fluorescent mounting medium (Kirkegaard and Perry Laboratories, Gaithersburg, MD), and the coverslips were sealed with fingernail polish.

For detection of the transferrin receptor, BODIPY-transferrin was used as recently described (15). In brief, cells were grown as above and then incubated at 4° with BODIPY-transferrin (100 μg/ml) in Ham's F-10 solution containing 10 mg/ml ovalbumin (to inhibit nonspecific transferrin binding) and 10 μM quipazine (to saturate 5-HT_{2A} receptors with agonist). After being washed with Ham's F-10 solution, the coverslips were placed into prewarmed 37° medium containing unlabeled transferrin (10 mg/ml) to "chase" BODIPY-transferrin and 10 μM quipazine to induce 5-HT_{2A} receptor internalization. Then, at various times, cells were cooled to 4° on ice, rinsed with PBS, and fixed and prepared for 5-HT_{2A} receptor immunofluorescence as above.

Confocal microscopy. Cells were visualized using a Ziess (Oberkochen, Germany) confocal laser microscope. For these studies, 0.3-μm optical sections were taken of 20–60 representative cells at each time point of interest. Ten sections corresponding to the middle of each cell were taken for quantitative analysis.

Binding assay. Saturation binding assays were performed with [^{3}H]ketanserin in total volumes of 0.5 ml at 25° for 2 hr with 5–20 μg of membrane protein as described previously (16) in 50 mM Tris-Cl buffer (pH 7.4). Then, 1 μM unlabeled ketanserin was used to measure nonspecific binding; specific binding represented 90–95% of total binding for the experiments reported here. Membranes were harvested with a Brandel SM-24 cell harvester followed by three ice-cold washes onto polyethyleneimine-pretreated (0.1%) glass fiber

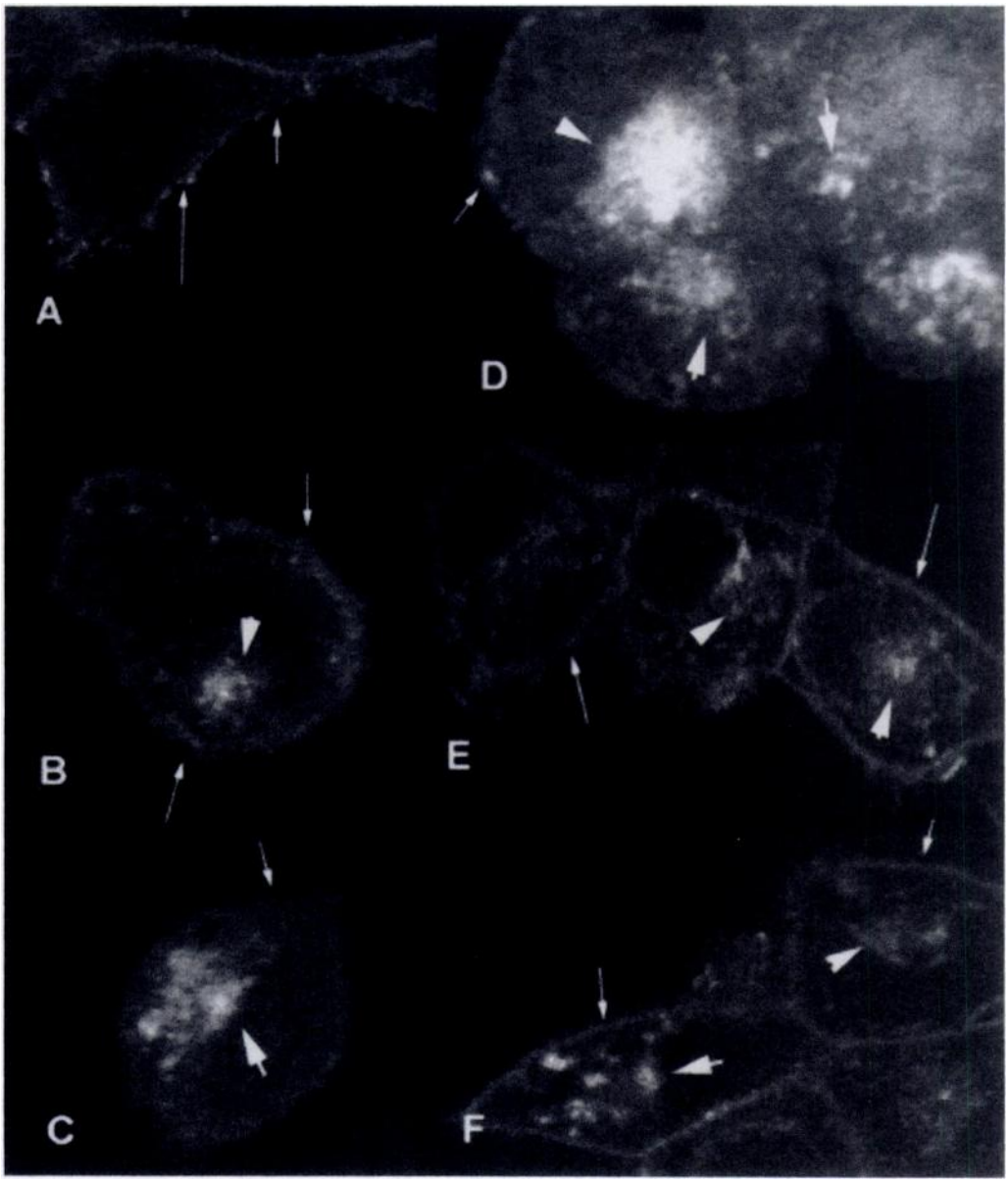

Fig. 4. Time course of quipazine-induced sequestration of 5-HT_{2A} receptors. Cells were exposed to 10 μM quipazine at 37° for various time periods and then fixed and prepared for confocal laser microscopy as described in Experimental Procedures. Representative sections from cells exposed to vehicles (control cells) (A) and cells exposed to quipazine for 5 (B), 15 (C), 30 (D), 60 (E), and 180 (F) min are shown. *Small arrows*, surface immunofluorescence; *large arrowheads*, intracellularly accumulated 5-HT_{2A} receptor immunofluorescence.

filters. Filters were allowed to soak for 18 hr in scintillation fluid before liquid scintillation counting, with efficiency determined by the external standard method.

Semiquantitative image analysis. A computerized image analysis system was used for semiquantitative analysis. In brief, optical sections were digitized and stored as TIF files on a Panasonic rewritable optical drive. Sections were then uploaded onto a Pentium-based computer using Adobe Photoshop (Adobe, Mountain View, CA) and split into red and green channels for independent quantification of 5-HT$_{2A}$ (rhodamine; red) or BODIPY-transferrin (green) fluorescence. Bleedthrough between red and green channels was undetectable. Red and green images were then stored as separate TIF files before quantification using SigmaScan Pro (Jandel Scientific, San Rafael, CA).

For quantification, two measurements were made of each cell: 1) the total cellular area and 2) the total intracellular area that was fluorescent (a measure of internalized receptor). To determine the total cell area, a mouse-driven cursor was used to outline the surface of the cell, and then the total cellular area was calculated in pixels using the image analysis program. To determine the amount of the intracellular area that was filled with internalized receptors, the mouse-driven cursor was used to individually outline each fluorescently labeled intracellular organelle. The area for each fluorescently labeled intracellular organelle was then calculated using the image analysis software and summed for each cell in pixels. Because the intensity of the fluorescent signal in each organelle (e.g., pixel value) is not linearly related to concentration of the fluorescent label and because of the manner in which confocal images are processed before digitizing, no attempt was made to quantify the intensity of fluorescence in each organelle.

The intracellular fluorescent area was then expressed as a percentage of the total cell area. All values for each time point were included in the data set. For a typical data set, 20–50 individual cells (each with 3–10 optical sections) were measured, and the mean percentage of intracellular area (± standard error) was determined. A Student's *t* test was used to detect statistically significant differences between groups. Because the imaging software allows for separate quantification of data stored in different channels, the transferrin receptor internalization image data (stored in green channel) and 5-HT$_{2A}$ receptor internalization image data (stored in red channel) could be measured within the same cell.

Binding data from saturation experiments were analyzed using a weighted, nonlinear least-squares program that determines binding to multiple sites using the law of mass action (LIGAND program) as described previously (17).

PI hydrolysis assays. PI hydrolysis assays were performed as described previously (17) measuring [^{3}H]IP release in cells which were preloaded for 24 hr with 1 μCi/ml [^{3}H]inositol monophosphate (20 Ci/mmol) in inositol-free DMEM.

Results

Subcellular distribution of 5-HT$_{2A}$ receptors. The subcellular location of 5-HT$_{2A}$ receptor on GF-62 cells was determined by immunocytochemical detection of the receptor via a specific antibody (12). Confocal microscopic images of GF-62 cells grown under standard culture conditions are seen in Fig. 1. The rhodamine-labeled 5-HT$_{2A}$ receptors are located primarily on the plasma membrane. However, a small but significant amount of 5-HT$_{2A}$ receptor immunoreactivity is also present intracellularly. This intracellular fluorescence appears in discrete compartments in the perinuclear region.

Agonist effect on receptor internalization. Cultured cells that were exposed to the 5-HT$_{2A}$ agonist quipazine are shown in Fig. 2. After 30 min of exposure to 10 μM quipazine, much of the rhodamine label (representing the 5-HT$_{2A}$ receptor) was found in the cytoplasm in discrete compartments as well as in the perinuclear region. Digitization of these images allowed for quantification of the redistribution of the 5-HT$_{2A}$ receptor immunoreactivity. As can be seen in Fig. 3, quipazine caused a highly significant increase in internalization compared with untreated cells ($p < 0.01$). Similar results were obtained with cells exposed to 10 μM 5-HT (data not shown).

Receptor internalization is blocked by ketanserin and is temperature sensitive. To determine whether the quipazine-induced internalization of 5-HT$_{2A}$ receptors was mediated by receptor activation, two types of experiments were performed. In the first types of experiments, cells were exposed to 10 μM quipazine and 10 μM ketanserin, a 5-HT$_{2A}$ receptor antagonist, concomitantly for 30 min, after which intracellular fluorescence was measured. As is seen in Fig. 3, incubation with ketanserin alone caused a very small, but statistically significant, increase in internalization ($p < 0.01$). Preincubation with ketanserin blocked the ability of quipazine to induce receptor internalization (Fig. 3). In the second types of experiment, cells were incubated on ice for 30 min in the presence and absence of agonist. Cells exposed to 10 μM quipazine on ice for 30 min did not differ in intracellular fluorescence compared with vehicle-treated cells on ice (data not shown.)

Because ketanserin induced a small but significant increase in internalization, we investigated the possibility that ketanserin might function as a partial agonist at 5-HT$_{2A}$ receptors.

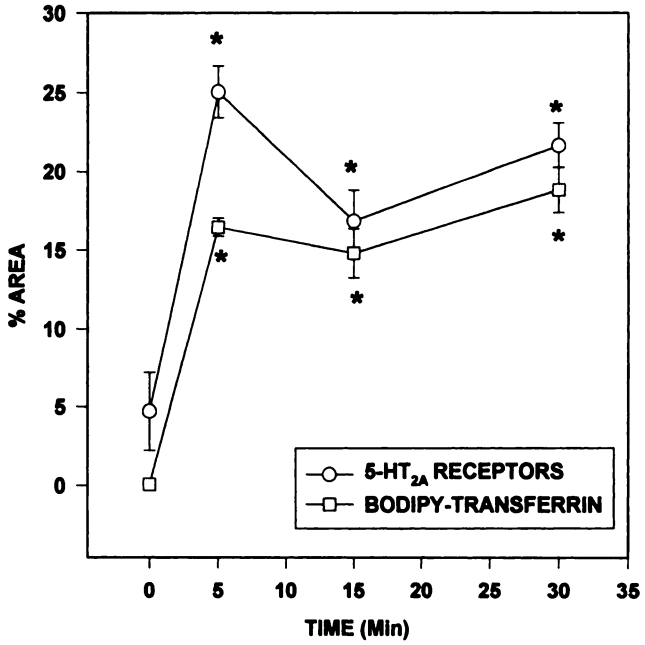

Fig. 5. Quipazine and transferrin induce sequestration with similar kinetics. Cells were prelabeled at 4° with BODIPY-transferrin and simultaneously exposed to 10 μM quipazine to saturate 5-HT$_{2A}$ receptors as described in Experimental Procedures. Cells were then warmed to 37° and chased with an excess of unlabeled transferrin, fixed at various time periods, and prepared for 5-HT$_{2A}$ receptor immunofluorescence. Then, 0.3-μm dual excitation/emission confocal laser fluorescent images were collected and subjected to image analysis as described in Experimental Procedures. Data represent mean ± standard error of the percentage of intracellular immunofluorescence for 5-HT$_{2A}$ receptor immunofluorescence and BODIPY-transferrin fluorescence. *, $p < 0.01$ versus control (0 min).

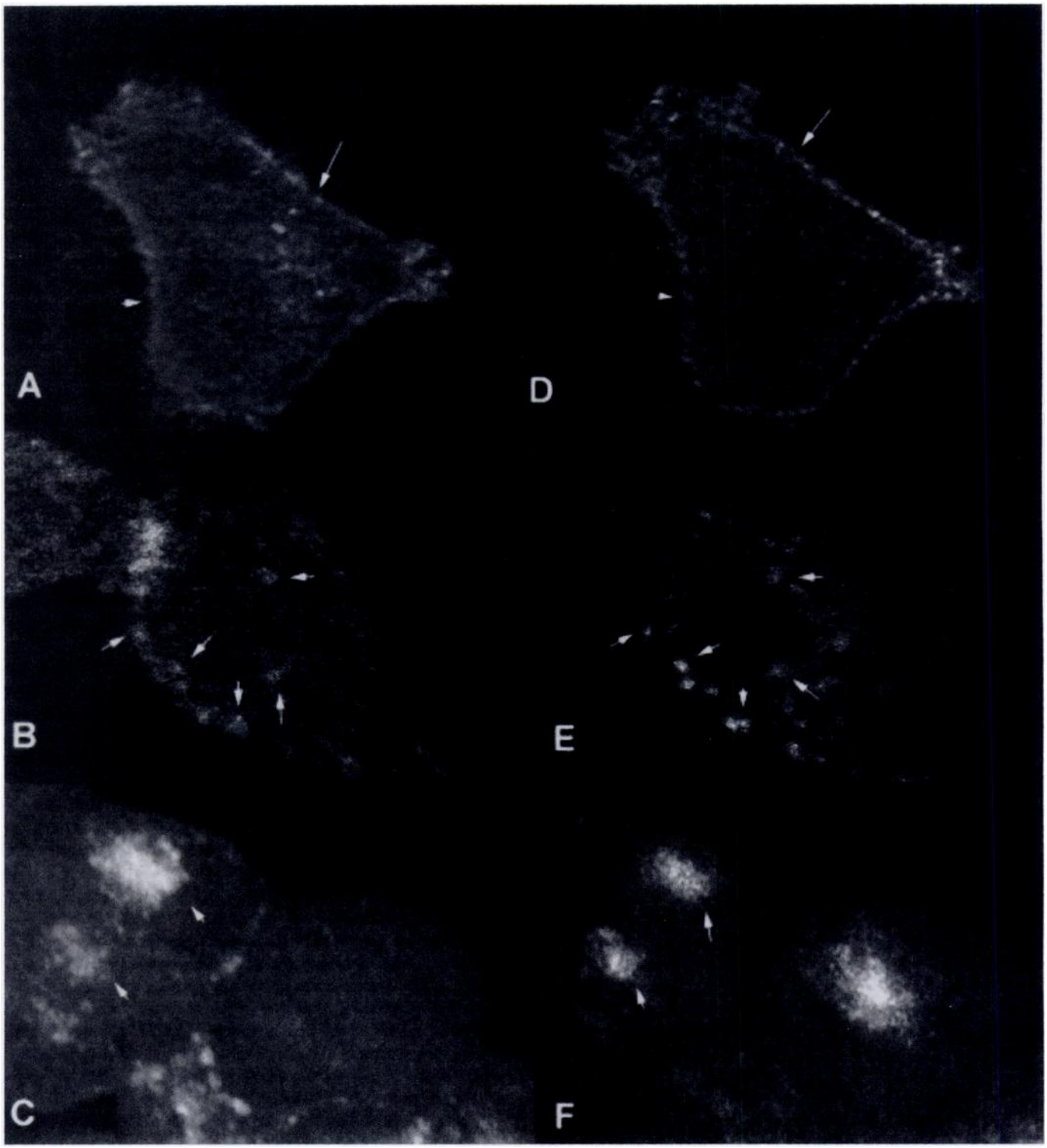

Fig. 6. 5-HT_{2A} and transferrin receptors are sequestered via the same endocytotic vesicles. Cells were prelabeled at 4° with BODIPY-transferrin and simultaneously exposed to 10 μM quipazine to saturate 5-HT_{2A} receptors as described in Experimental Procedures. Cells were then warmed to 37° and chased with an excess of unlabeled transferrin, fixed at various time periods, and prepared for 5-HT_{2A} receptor immunofluorescence. Dual-label confocal fluorescence sections (0.3-μm sections) were made to visualize 5-HT_{2A} receptor immunofluorescence (A–C) and BODIPY-transferrin fluorescence (D–F) of representative cells, which had been incubated for 0 (A and D), 2 (B and E), and 30 (C and F) min at 37°. *Small arrows*, colocalization of transferrin and 5-HT_{2A} receptors on the cell surface (A and D) or intracellular organelles (B, C, E, and F).

For these studies, the ability of graded doses of ketanserin to induce PI hydrolysis (as measured by [^{3}H]IP accumulation) was compared with that of the full agonist 5-HT in GF-62 cells. Basal [^{3}H]IP accumulation was 322 ± 72 cpm, which was not different ($p > 0.05$ versus ketanserin) from that induced by 10 μM ketanserin (388 ± 30 cpm) but was significantly less ($p < 0.01$ versus 5-HT) than that induced by 10 μM 5-HT (5973 ± 604 cpm). These results indicate that the ability of ketanserin to induce a small increase in internalization was not due to a partial agonist activity of ketanserin.

Kinetics of receptor internalization. Confocal sections from a typical time course study of agonist-induced internalization of the 5-HT$_{2A}$ receptor immunoreactivity are shown in Fig. 4. The quipazine-induction of 5-HT$_{2A}$ receptor internalization was rapid, with an increase in intracellular fluorescence seen as early as 5 min after exposure to 10 μM quipazine (Fig. 4B). The quipazine-induced increase in intracellular fluorescence was maintained for ≥3 hr. These results indicate that quipazine induces a rapid increase in intracellular 5-HT$_{2A}$ receptors.

We next quantified the degree of intracellular accumulation of 5-HT$_{2A}$ receptor immunofluorescence by using an image analysis technique. Fig. 5 reveals a time course experiment in which intracellular fluorescence was measured at 0, 5, 15, and 30 min after agonist exposure. For these experiments, total area and percentage of intracellular area that was fluorescent were measured in an average of 31 cells/time point. A large increase in intracellular fluorescence (average, 531% increase) occurred after quipazine exposure. For all time points measured, the increase in intracellular fluorescence was statistically significant ($p < 0.002$ versus control).

Colocalization with the transferrin receptor. Transferrin receptors are known to be internalized via the endosomal pathway when exposed to an agonist (15). To determine whether 5-HT$_{2A}$ receptors are internalized via the same endosomal pathway, cells were incubated at 4° with BODIPY-transferrin and 10 μM quipazine for 30 min and then treated simultaneously with both 10 mg/ml transferrin and 10 μM quipazine for 30 min at 37°. For these experiments, BODIPY transferrin (which fluoresces green) and a rhodamine-labeled secondary antibody to label 5-HT$_{2A}$ receptors (which fluoresces red) were used. Standard dual excitation/emission methods allowed us to examine the internalization of both receptors within one cell.

Fig. 6A clearly demonstrates that before agonist exposure, virtually all of the BODIPY-transferrin was localized on the plasma membrane, whereas the majority of 5-HT$_{2A}$ receptor immunoreactivity was on the plasma membrane. After agonist exposure (2 min at 37°), much of the BODIPY-transferrin was lost from the plasma membrane and was seen in vesicles apparently budding from the plasma membrane (Fig. 6B). By 30 min (Fig. 6D), all of the remaining BODIPY-transferrin was localized in perinuclear vesicles.

Similarly, the 5-HT$_{2A}$ receptor immunofluorescence was found predominantly on the plasma membrane before agonist exposure (Fig. 6D). After 2 min of agonist exposure (Fig. 6E), much of the 5-HT$_{2A}$ receptor immunoreactivity was found in vesicles that seemed to be budding from the plasma membrane. The location of these intracellular vesicles was apparently identical with BODIPY-transferrin (compare Fig. 6, B with E). After 30 min of quipazine exposure (Fig. 6F), the 5-HT$_{2A}$ receptor immunoreactivity was, as seen with BODIPY-transferrin, highly compartmentalized in a cellular region presumably occupied by endosomes.

Quantitative analysis of these time course experiments is shown in Fig. 5. As can be seen, the time course of BODIPY-transferrin internalization was quite similar to that shown for 5-HT$_{2A}$ receptor immunofluorescence. These results strongly imply that 5-HT$_{2A}$ receptors and transferrin receptors are cointernalized via an endosome-mediated process.

Fate of the internalized 5-HT receptor. To determine whether 5-HT$_{2A}$ receptors were down-regulated, saturation binding studies were performed after 0, 30, and 120 min of exposure to 10 μM quipazine. These data (Table 1) demonstrate that agonist exposure and subsequent receptor internalization do not alter the number or affinity of 5-HT$_{2A}$ receptors. This is consistent with the concept that after agonist exposure, 5-HT$_{2A}$ receptors are internalized but not down-regulated. It is possible that these internalized 5-HT$_{2A}$ receptors are post-translationally modified; studies to address this possibility are in progress.

Our prior studies demonstrated that 24 hr of agonist exposure does not down-regulate 5-HT$_{2A}$ receptors and does not alter surface 5-HT$_{2A}$ receptor immunoreactivity (as measured by immunoperoxidase techniques; see Ref. 12). We have now examined 5-HT$_{2A}$ receptor distribution using fluorescence confocal laser microscopy in control cells (Fig. 7A) and cells exposed to quipazine for 24 hr (Fig. 7B). A small, but statistically significant, increase in the amount of intracellular immunoreactivity was noted after 24 hr (Fig. 7). No apparent change in surface fluorescence is seen in a comparison of control (Fig. 7A) and quipazine-treated (Fig. 7B) cells.

Discussion

The major finding of these studies is that serotonergic agonists like quipazine and 5-HT induce a rapid internalization of 5-HT$_{2A}$ receptors via the endosomal pathway in a time- and temperature-dependent fashion. These data are reminiscent of findings obtained for the β_2-adrenergic receptor. In a series of reports, von Zastrow and Kobilka (18, 19) described the agonist-induced internalization of the human β_2-adrenergic receptor stably transfected into a cell line. As in our system, the internalization process is blocked by receptor antagonist, the receptors are internalized via the endosomal pathway, and the process is temperature sensitive.

Because agonist-induced internalization via the endosomal pathway occurs for at least two GPCRs, we suggest that this may be an important cellular pathway for regulating many, but not all, GPCRs. Interestingly, tachykinin receptors are also internalized *in vivo*, although the subcellular organelle responsible remains unidentified (20). In this regard, our prior studies demonstrated enrichment of various GPCRs in endosomes and coated vesicles isolated from nervous tissue (21–23), although it was not clear from these studies whether these organelles were involved in agonist-induced internalization of GPCR.

Alternatively, results of other studies have suggested that novel organelles known as caveoli may be involved in agonist-mediated internalization of GPCRs (24) and other cell surface proteins (25). One recent report (24) investigating

TABLE 1

Quipazine does not down-regulate 5-HT$_{2A}$ receptors

For these experiments, cells were exposed to 10 μM quipazine for various time periods, harvested, and washed to remove unbound quipazine. Then, membranes were prepared for binding assays as described in Experimental Procedures. Data represent mean ± standard error of computer-derived estimates from three separate experiments.

Time	K_d	B_{max}
min	*nM*	*fmol/mg*
0	0.6 ± 0.01	4580 ± 458
30	0.6 ± 0.1	5250 ± 577
60	0.8 ± 0.14	4470 ± 671
180	0.5 ± 0.25	4870 ± 146

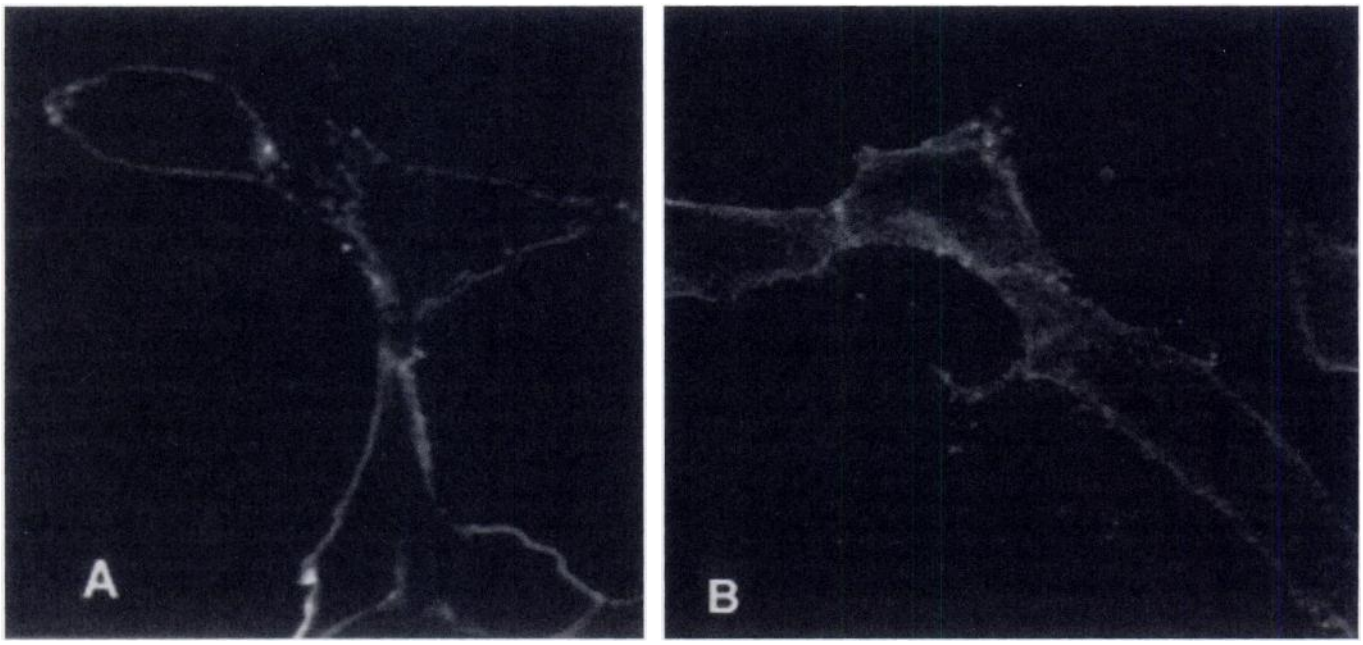

Fig. 7. Effect of 24-hr quipazine exposure on the subcellular distribution of 5-HT_{2A} receptors in GF-62 cells. GF-62 cells were exposed to vehicle (A) or 10 μM quipazine (B) for 24 hr in DMEM containing 10% dialyzed serum and then prepared for confocal laser microscopy. Semiquantitative analysis of images showed a small but significant increase in intracellular immunoreactivity in a comparison of control and quipazine-treated cells (1.6 ± 0.8% versus 5.2 ± 0.63%; p = 0.04; 107 cells).

endothelin receptor internalization suggested that caveoli and the protein known as caveolin were essential for agonist-mediated internalization. This is interesting in view of the fact that the endothelin receptor is, like the 5-HT_{2A} receptor, coupled to phospholipase C. Thus, there is uncertainty regarding whether GPCRs use the coated-pit/endosomal pathway or caveoli. Because the transferrin receptor uses coated vesicles, our findings imply that 5-HT_{2A} receptors use the coated-pit/endosomal pathway. Indeed recent preliminary findings[1] have indicated that caveolin and 5-HT_{2A} receptors have distinctly different surface and subcellular localizations after agonist stimulation.

The precise function of receptor-mediated endocytosis of GPCRs is unclear. Initial studies by von Zastrow and Kobilka (18) implied that sequestration via endosomes was important for agonist-mediated desensitization. Thus, according to this hypothesis, receptors were sequestered away from G proteins and therefore inactivated. More recent studies by several groups of investigators (26–29) have supported the hypothesis that sequestration is instead important for resensitizing receptors. Thus, work by these groups showed that inhibition of sequestration inhibits the resensitization of receptors and that resensitization occurs via dephosphorylation (26–29), perhaps in endosomes. Whether 5-HT_{2A} receptors are also resensitized by endosomal-mediated internalization and recycling is unknown and is the subject of current studies.

One novel feature of the model system we are currently using is that although sequestration and desensitization occur after agonist exposure, 5-HT_{2A} receptors are not down-regulated in GF-62 cells. Classically, down-regulation is defined as a loss of receptor protein. It is clear, however, that internalized receptors may be uncoupled from second messenger production, so that the net result is a functional down-regulation.

In this regard, our prior studies (12) demonstrated that 5-HT_{2A} receptors expressed in GF-62 cells were not down-regulated after 24 hr of agonist exposure as measured by radioligand binding. In those studies, receptor immunoreactivity was measured using the immunoperoxidase technique. No change in apparent surface labeling was seen after 24 hr of agonist exposure. Similar results were obtained in this study, in which receptor distribution was measured via fluorescence confocal laser microscopy, although a small but significant increase in intracellular labeling was noted after 24 hr of incubation. Our prior study using the immunoperoxidase technique (12) also demonstrated a small but statistically significant increase in intracellular immunoreactivity. It is doubtful that the minimal amount of apparent receptor internalization can account for the magnitude of desensitization that is measured after 24 hr of agonist administration. These results indicate that although agonists induce a rapid internalization of receptors, the receptors are either quickly resynthesized or recycled back to the plasma membrane within 24 hr.

Because in GF-62 cells 5-HT_{2A} receptors are sequestered but not down-regulated, we can now distinguish between the processes of internalization and down-regulation for 5-HT_{2A} receptors. Not all cell lines are defective in down-regulation, however. Others have noted that the 5-HT_{2A} receptor is apparently down-regulated in P11 cells (3). Groteweil and Sanders-Bush (30) found that 5-HT_{2A} receptors expressed in various cell lines differ in their abilities to be down-regulated after agonist exposure. Indeed, we have preliminary findings indicating that in transiently transfected COS-7 cells, 5-HT_{2A} receptors are subject to down-regulation.[2]

Prior studies have demonstrated that antagonists like ketanserin and mianserin down-regulate 5-HT_{2A} receptors *in vivo*. Previous studies (10, 11) showed that this antagonist-

[1] N. Khan and B. L. Roth. Manuscript in preparation.

[2] N. Khan and B. L. Roth, unpublished observations.

mediated down-regulation was not accompanied by changes in 5-HT$_{2A}$ receptor mRNA; these findings suggested that 5-HT$_{2A}$ receptors are down-regulated by post-transcriptional means. Our present results show that ketanserin induces a small but significant degree of internalization of 5-HT$_{2A}$ receptors in GF-62 cells. Although not of the magnitude seen with agonist, the results are consistent with the idea that antagonists might also induce sequestration of 5-HT$_{2A}$ receptors. Our PI hydrolysis experiments demonstrate that the internalization induced by ketanserin is not due to partial agonist activity.

In conclusion, we discovered that quipazine induces a rapid internalization of 5-HT$_{2A}$ receptors via the endosomal pathway. We found that this process was time and temperature sensitive and that a specific receptor antagonist (ketanserin) blocked internalization. Our results showed that 5-HT$_{2A}$ receptors were cointernalized with transferrin receptors and imply that agonist-mediated endocytosis is important for regulating the number of cell surface 5-HT$_{2A}$ receptors. We also demonstrated that internalization and down-regulation can be dissociated using native receptor proteins. Whether sequestration is related to 5-HT$_{2A}$ receptor desensitization or resensitization, however, remains unknown and is the subject of current studies.

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