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Agonist-induced GTPγ^{35}S binding mediated by human 5-HT$_{2C}$ receptors expressed in human embryonic kidney 293 cells

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Abstract

The 5-HT$_{2C}$ receptor as heterologously expressed in various mammalian cells mediates inositol 1,4,5-triphosphate (IP_3) signal by activating $G_{q/11}$ subtypes of G proteins, but minimally promotes agonist-induced GTPγ^{35}S binding in membranes due to slow GTP turnover rates of the G proteins. Here we discovered robust (over 200%) agonist-induced GTPγ^{35}S binding mediated by the human receptor expressed in human embryonic kidney (HEK) 293 cells, and investigated its pharmacology. Agonists concentration-dependently increased GTPγ^{35}S binding in isolated membranes, which was competitively blocked by antagonists. Intrinsic efficacies of agonists from GTPγ^{35}S binding were comparable to those from IP_3 measurement. Pertussis toxin treatment largely blocked serotonin-induced GTPγ^{35}S binding, serotonin high affinity sites by 70% without altering the total binding sites, and reduced IP_3 release by 40%. GTPγ^{35}S-bound Gα subunits from serotonin-activated membranes were mainly $G\alpha_i$, judging from immobilization studies with various Gα-specific antibodies. Inhibition of forskolin-stimulated cAMP formation, however, was not observed. Apparently, the 5-HT$_{2C}$ receptor-mediated GTPγ^{35}S binding is a unique phenotype observed in HEK293 cells, reflecting its coupling to pertussis toxin-sensitive G_i subtypes, which contribute to the IP_3 signal, along with pertussis toxin-insensitive $G_{q/11}$ subtypes.

Keywords: Human 5-HT$_{2C}$ receptor; GTPγ^{35}S binding; IP_3 signal; G protein coupling; Intrinsic efficacy

1. Introduction

The 5-HT$_{2C}$ receptor, a G-protein coupled receptor with seven transmembrane segments, is highly expressed in the brain (Conn et al., 1979; Julius et al., 1988; Stam et al., 1994) and has been implicated for a number of diseases such as anxiety, obesity, depression, schizophrenia and affective disorders (Canton et al., 1990; Sanders-Bush and Breeding, 1991; Moreau et al., 1993; Dourish, 1995; Tecott et al., 1995; Cowen et al., 1996; Kennett et al., 1996; Epstein et al., 1997). Recently, the receptor has received much attention as a potential target for anxiolytics and anti-obesity agents (Dourish, 1995; Cowen et al., 1996; Kennett et al., 1996), and its recombinant clones have been often used for pharmacological studies. The human 5-HT$_{2C}$ receptor, since the cloning of its cDNA (Julius et al., 1988; Salzman et al., 1991; Stam et al., 1994; Xie et al., 1996), has been heterologously expressed in various cells, including NIH3T3 fibroblast, Chinese hamster ovary, African green monkey SV40 transformed, human embryonic kidney (HEK)293, SH-SY5Y cells, insect Sf9 cells and Xenopus oocytes (Barker et al., 1994; Chen et al., 1994; Akiyoshi et al., 1995; Tohda et al., 1995; Hartman and Northup, 1996; Kennett et al., 1996; Newton and Elliott, 1997). Pharmacological characterization of the cloned receptor have shown that its activation elevates intracellular inositol 1,4,5-triphosphate (IP_3), via $G_{q/11}$ subtypes of G proteins and phospholipase C pathway, and subsequently the Ca^{2+} level (Conn et al., 1979; Julius et al., 1988; Stam et al., 1994; Kaufman et al., 1995). Direct coupling of the receptor with G_q (squid retinal) has been shown in reconstituted receptors from the Sf9 cell expression system (Hartman and Northup, 1996), and also its coupling with G_o and G_{i1} in the Xenopus oocytes expression system, using antisense probes (Chen et al., 1994).

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For G protein-coupled receptors, an early and common signaling step is the agonist-induced binding of GTP to Gα subunits, and this step, using slowly hydrolyzable GTPγ^{35}S, has been frequently used for investigations of receptor-ligand interactions. Interestingly, agonist-induced GTPγ^{35}S binding has been reported to be low or non-existent for the 5-HT$_{2C}$ receptor expressed in NIH3T3 cells (Burns et al., 1997), and this has been attributed to low intrinsic GTP-turnover rate for the receptors coupled to the G_q family of G proteins. Our preliminary study with the human 5-HT$_{2C}$ receptor expressed in HEK293 cells revealed, however, robust enhancements of GTPγ^{35}S binding by serotonin and several agonists, and led us to investigate its pharmacology.

2. Materials and methods

The cDNA for the human 5-HT$_{2C}$ receptor was cloned into the PCRscript vector via a blunt end ligation. The directionality of the insert was determined with polymerase chain reaction (PCR) using primers annealing to the vector and the insert. Then the cDNA insert was transferred to a mammalian expression vector, PCI-Neo™ from Promega, and the recombinant vector was used to transfect HEK293 cells using Ca^{2+} phosphate precipitation techniques. Stably transfected cells were selected in the presence of G418 (400 μg/ ml). Membranes from HEK293 cells expressing the 5-HT$_{2C}$ receptor were prepared by standard procedures including cell homogenization and differential centrifugations as described elsewhere (Pregenzer et al., 1993).

Binding of radioactive ligands was measured in membranes expressing recombinant receptors, using filtration techniques as described elsewhere (Pregenzer et al., 1997). Briefly, [^{3}H]serotonin or [^{3}H]mesulergine binding was measured in medium that contained (mM) 100 NaCl, 2 $MgCl_2$, 1 EDTA, 20 HEPES/Tris (pH 7.4), the radioactive ligand at varying concentrations (0.1 to 20 nM for typical binding profiles), and 5 to 8 μg membrane protein, in a total volume of 500 μl. Reaction mixtures were incubated at 23°C for 60 min, and filtered over Whatman GF/B filters under vacuum, which were then washed three times with 4 ml of an ice cold 50 mM Tris/HCl buffer (pH 7.4). Non-specific binding was estimated in the presence of excess unlabeled clozapine (10 μM). Ligand stock solutions were prepared in 0.1% ascorbic acid. Displacement of [^{3}H]mesulergine at 2 nM by test compounds at various concentrations (competition assay) was carried out in the same manner.

GTPγ^{35}S binding was measured following the procedure reported earlier (Chabert et al., 1994) in medium that contained (mM) 25 HEPES, 100 NaCl, 1 EDTA, 3 $MgCl_2$, 0.5 dithiothreitol, 0.003% digitonin, 2 nM GTPγ^{35}S (3–5 $\times 10^5$ cpm /assay), and 10 μg membrane protein in a volume of 120 μl. Membranes were preincubated with 100 μM 5′-adenylylimidodiphosphate for 30 min at room temperature, subsequently with 10 μM GDP for 10 min on ice. Test ligands were included at 10 μM, unless indicated otherwise. Reaction mixtures were incubated for 45 min at 30°C, and were filtered over a Whatman GF/B filter under vacuum. Filters were washed three times with 4 ml of an ice-cold buffer that contained (mM) 100 NaCl, 20 Tris/HCl, pH 8.0, 25 $MgCl_2$. Agonist-induced GTPγ^{35}S binding was obtained by subtracting that observed without agonists. Binding data were analyzed using a nonlinear regression method (Sigma Plot), and presented as mean ± S.E.M. from three experiments.

The agonist-induced IP_3 release in intact cells was measured using the inositol-1,4,5-trisphophate [^{3}H]radioreceptor assay kit from DuPont NEN™. Briefly, cells were grown in a 24-well plate to about 80% confluency, and were treated with serotonin or test ligands at indicated concentrations for 45 sec (initially a time course from 0 to 1200 sec). Each reaction was stopped with trichloroacetic acid (20% final concentration), which were then extracted with 1,1,2-trichloro-1,2,2-trifluoroethane and trioctyl amine. An aliquot (300 μl) was analyzed for IP_3 using [^{3}H]IP_3/IP_3 receptor preparations from calf cerebellum, following the protocols provided by the vender. For each experiment, a dose–response profile for IP_3 was constructed by adding known amounts of exogenous IP_3 to trichloroacetic acid extracts of untreated cells. Cellular changes in cAMP were measured, using a FlashPlate assay kit from NEN™ Life Science Products. Briefly, cells were grown in a 96-well plate to about 80% confluency, and then treated with forskolin or in combination with serotonin or test ligands for 30 min in the presence of 3-isobutyl-1-methylxanthine, a phosphodiesterase inhibitor. cAMP in cell lysates was measured using the competition between [^{125}I]cAMP and non-radioactive cAMP for a fixed number of antibody binding sites in microplates coated with solid scintillants.

GTPγ^{35}S-bound α subunits of G-proteins were identified following the method reported previously (Okamoto et al., 1992) with the following modifications. Briefly, membranes (~10 μg membrane proteins) were incubated in the presence of GTPγ^{35}S (4 nM) and serotonin or other test ligands (10 μM) under the conditions identical to those for GTPγ^{35}S binding as described above. Treated membranes were solubilized with an equal volume of a buffer that contained (mM) 100 Tris/HCl, pH 8.0, 10 $MgCl_2$, 100 NaCl and 0.6% 3-[(3-cholanidepropyl)dimethylammonio]-1-propane-sulfonate (CHAPS, an ionic detergent) for 30 min on ice, and were diluted to a final CHAPS concentration to 0.125%. Aliquots (300 μl) of the mixtures were transferred to individual wells which had been coated, successively with goat anti-rabbit (or mouse) antibodies (1:100 dilution), bovine serum albumin (5 mg/ml), and one of the affinity-purified antibodies for various Gα isoforms (1:200 dilution). The specific antibodies used here include those for $G\alpha_i$ (against the C-terminal sequence, 345–354), $G\alpha_s$ (against the C-terminal sequence,

385–394), $G\alpha_{q/11}$ (against their common C-terminal sequence, QLNLKEYNLV), and $G\alpha_{13}$ (against the sequence, 367–377) from Calbiochem. Also the mouse monoclonal antibody raised against the bovine G_o protein was obtained from Chemicon. Solubilized membranes were added to individual wells and the reaction was carried out at room temperature for 1 h. Then, unbound GTPγ^{35}S was removed, and each well was counted for the bound radioactivity using a standard scintillation cocktail and a β-counter. Ligand-dependent association with GTPγ^{35}S was computed by subtracting that observed without agonist activation.

To estimate the level of three major isoforms of Gα proteins ($G\alpha_i$, $G\alpha_{q/11}$ and $G\alpha_o$) in HEK 293 cells, cell membranes (10 μg protein) were resolved with sodium dodecyl sulfate (SDS) polyacrylamide gel (12%) electrophoresis, were transferred to polyvinylidene difluoride membranes, and probed with antibodies specific for individual Gα subunits. Detection was performed using the ECL Plus™ Western blotting detection system from Amersham Pharmacia Biotech.

3. Results

3.1. Ligand binding

We examined binding of standard serotonergic ligands to the human 5-HT_{2C} receptor in HEK293 cell membranes (Fig. 1 and Table 1). The binding data for [^{3}H]mesulergine at various concentrations fit well to the binding model for a single class of sites, and yielded a dissociation constant (K_d) of 3.0 ± 0.2 nM and maximal binding (B_{max}) of 62 ± 2 pmol/mg protein (Fig. 1A). It should be noted that membranes from untransfected HEK293 cells show no detectable specific binding for [^{3}H]mesulergine or [^{3}H]serotonin. Competition experiments against [^{3}H]mesulergine binding were carried out with several antagonists or partial agonists (Table 1). Metergoline, methiothepin, clozapine and D-lysergic acid diethylamide tartrate (LSD) blocked [^{3}H]mesulergine binding with the inhibition constant (K_i) of 3.1 ± 0.7, 1.9 ± 0.3, 37.9 ± 1.6, and 20 ± 2 nM, respectively. For agonists, competition experiments with [^{3}H]mesulergine also fit to the binding model for a single class of sites. For example, serotonin monophasically displaced [^{3}H]mesulergine binding with a K_i value of 142 ± 4 nM (Fig. 1B), seemingly representing its low affinity sites, probably because a predominant population of cloned receptors may assume the phenotype of G protein-uncoupled receptors, as reported for other overexpressed systems (Butkerait et al., 1995; also see below). Similar studies with several agonists also showed high K_i values, e.g., 85 ± 6, 298 ± 21, 181 ± 9 nM for *R*(−)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane hydrochloride (DOI), 1-(3-chlorophenyl)piperazine dihydrochloride (mCPP) and Organon 37689 ((3*S*)-3-[(5-methoxy-2,3-dihydro-1*H*-inden-4-yl)oxy]pyrrolidine hydrochloride), respectively, which may also represent their low affinity sites. To study the high affinity site for serotonin, we examined [^{3}H]serotonin binding at the concentration range of 0.2 to 8 nM, but its binding curve showed no sign of saturation even at the serotonin concentration of 8 nM (Fig. 1C), due to substantial contributions by its low affinity sites even at such low serotonin concentrations. We computed the portion contributed by low affinity sites, using the equation of (B_{max} * [serotonin]/([serotonin] + 142)) where B_{max} is the total binding site from [^{3}H]mesulergine binding, [serotonin] is its concentration at nM, and the number of 142 (nM) represents the K_i for the serotonin low affinity site. After correction for the low affinity site contributions, the remaining, specific [^{3}H]serotonin

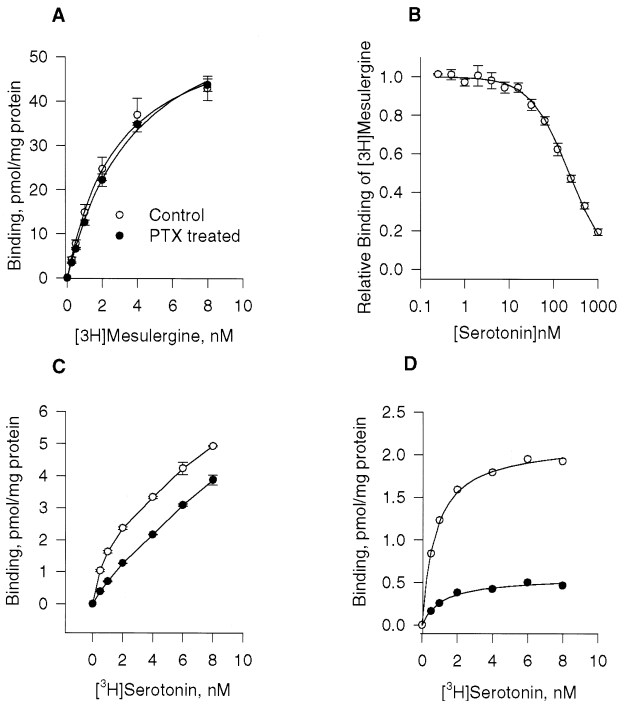

Fig. 1. Plots showing [^{3}H]mesulergine and [^{3}H]serotonin binding to 5-HT_{2C} receptors in membranes from HEK293 cells with or without pertussis toxin treatment. Membranes from cells treated with pertussis toxin (closed circles) or not treated (open circles; Control) were used for ligand binding at 23°C for 1 h, using filtration techniques, as described in Materials and methods. Solid lines in all the plots except for Plot C represent the data fit to the binding model for a single class of binding sites. Non-specific binding was estimated in the presence of clozapine at 10 μM. (A) Binding curves for [^{3}H]mesulergine (an antagonist), representing the total binding sites, were not affected by pertussis toxin treatment. (B) [^{3}H]Mesulergine binding was monophasically displaced by serotonin in control membranes expressing 5-HT_{2C} receptors, with a K_i of 142 ± 4 nM (solid line), representing the low affinity sites for serotonin. (C) Binding profiles for [^{3}H]serotonin at concentrations from 0.2 to 8 nM failed to show saturation, due to the contributions from its low affinity sites. (D) [^{3}H]Serotonin binding in panel C were corrected for the low affinity site contributions (see text), and then analyzed to yield a K_d of 0.8 ± 0.1 nM and B_{max} of 2.1 ± 0.1 pmol/mg protein for control membranes. Note that the pertussis toxin treatment reduced the serotonin high affinity sites by 70% (0.57 ± 0.05 pmol/mg protein) with little effect on its affinity (0.9 ± 0.1 nM).

Table 1
Comparison of test ligands for their affinity (K_i against [^{3}H]mesulergine) to the human 5-HT$_{2C}$ receptor and for their intrinsic efficacy as measured with GTPγ^{35}S binding or IP$_3$ release in HEK293 cells expressing the human 5-HT$_{2C}$ receptor. Competition binding experiments against [^{3}H]mesulergine (2 nM) were carried with test ligands at various concentrations in membranes form HEK 293 cells expressing the human 5-HT$_{2C}$ receptor. Half maximal inhibitory concentrations were obtained from three dose–response profiles and converted to K_i values using Cheng–Prusoff equation. GTPγ^{35}S binding was also measured in isolated membranes in the presence or absence of test ligands at 10 μM and the net increase was normalized to the maximal effect of serotonin at 10 μM. The IP$_3$ release was measured in intact cells with 40 s incubation (the peak level) and the net changes were normalized to that observed with serotonin (10 μM) in the same experiments. These values represent the mean ± S.E.M.

Compounds	Affinity K_i, nM	Maximal response	
		GTPγ^{35}S	IP$_3$
Mesulergine	3.0 ± 0.2	−12	−5
Serotonin	142 ± 4	100	100
DOI	85 ± 6	72 ± 6	110 ± 5
mCPP	298 ± 21	72 ± 7	115 ± 10
Org37684	181 ± 9	94 ± 5	112 ± 13
LSD	20 ± 2	21 ± 4	44 ± 12
Metergoline	3.1 ± 0.7	−2 ± 1	−7 ± 3
Methiothepin	1.9 ± 0.3	−3 ± 1	0.7 ± 0.5
Clozapine	37.9 ± 1.6	−5 ± 1	−2.3 ± 2

binding data yielded the K_d of 0.8 ± 0.1 nM and B_{max} of 2.1 ± 0.1 pmol/mg protein (Fig. 1D). This reveals that the high affinity sites for serotonin, the phenotype of G protein-coupled receptors, amount to only 3% of the total binding sites as measured with [^{3}H]mesulergine binding, and is consistent with the view that an overwhelming population of receptors assume low affinity states, the phenotype of G protein-uncoupled ones. This also accounts for the monophasic displacement of [^{3}H]mesulergine by serotonin, despite the marked difference in affinities between its low and high affinity sites (177-fold).

3.2. GTPγ^{35}S binding

Agonist-induced GTPγ^{35}S binding represents an early step for G protein activation by G protein-coupled receptors, namely displacement of GDP from G protein α subunits by GTPγ^{35}S, and has been frequently utilized to characterize ligand interactions. In membranes from HEK293 cells expressing the 5-HT$_{2C}$ receptor, serotonin concentration dependently enhanced GTPγ^{35}S binding with a half maximal concentration (EC$_{50}$) of 21 ± 1.7 nM and maximal increase of 724 fmol/mg protein (Fig. 2), but in membranes from native HEK293 cells, it produced no appreciable effects (data not shown). The EC$_{50}$ value for serotonin (21 ± 1.7 nM) is greater than the K_d value for its high affinity site (0.8 ± 0.1 nM), but less than that for its low affinity site (142 ± 4 nM), implying the involvement of receptors in both states, probably through random and dynamic interactions of G proteins with receptors. Typically, the maximal increase of GTPγ^{35}S binding by serotonin (10 μM) for the 5-HT$_{2C}$ receptor (724 fmol/mg protein) was over 200% above the level without serotonin (about 250 fmol/mg proteins), and exceptionally high as compared to those observed with other serotonin receptors, e.g., 140 fmol/mg protein for the gorilla 5-HT$_{1D}$ receptor, as expressed in the same cell line (unpublished data) using the same vector. Metergoline, a known antagonist for the 5-HT$_{2C}$ receptor, produced no appreciable effect on GTPγ^{35}S binding by itself, but concentration dependently shifted the serotonin dose–response profile to the right; increasing the serotonin EC$_{50}$ value from 21 to 35, 189 and 553 nM in the presence of the drug at 5, 25 and 50 nM, respectively (Fig. 2). The data were fit to the equation for competitive interactions, $-\log[\text{serotonin EC}_{50}] = -\log([\text{metergoline}] + K_d) - \log C$ (Lew and Angus, 1995), where K_d is the dissociation constant for metergoline and C is the ratio of $K_d/\text{EC}_{50-\text{control}}$. Upon non-linear regression fitting (Fig. 2), we obtained the K_d of 2.5 nM for metergoline which is close to the value of 3.1 nM as measured from competitive, equilibrium binding experiments using [^{3}H]mesulergine, and the C value of 0.13 close to the expected one. Several antagonists, ketanserin, clozapine, methiothepin and mesulergine, reduced basal GTPγ^{35}S binding by 3% to 5% as normalized to the maximal serotonin action (Fig. 2 and Table 1). These reductions probably represent their inverse agonistic actions, which were considerably less than those reported earlier with basal IP$_3$ accumulations in other cell lines,

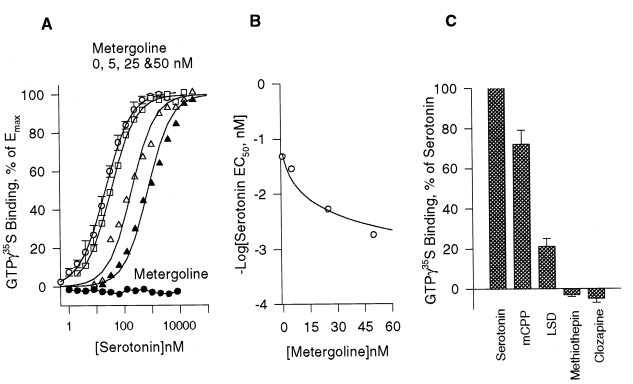

Fig. 2. Serotonin-induced GTPγ^{35}S binding in membranes of HEK293 cells expressing the human 5-HT$_{2C}$ receptor. (A) GTPγ^{35}S binding was measured as a function of serotonin concentrations in the presence of metergoline (antagonist) at 0, 5, 25 and 50 nM. Amounts of serotonin-dependent GTPγ^{35}S binding, as obtained by subtracting that observed without the agonist, were normalized to the level observed with serotonin at a saturating concentration (10 μM). Metergoline by itself showed no appreciable effects on GTPγ^{35}S binding, but shifted serotonin dose–response profiles to the right. (B) Metergoline-induced shifts of the EC$_{50}$ value for serotonin were plotted as a function of metergoline concentrations, and the solid line represents a non-linear regression fit to the equation of competitive interaction, $-\log[\text{Serotonin EC}_{50}] = -\log([\text{metergoline}] + K_d) - \log C$ (C = constant)(Lew and Angus, 1995). (C) Several standard serotonergic ligands at 10 μM were tested for their effects on GTPγ^{35}S binding, and ligand-induced changes were normalized to the maximal effect of serotonin at 10 μM.

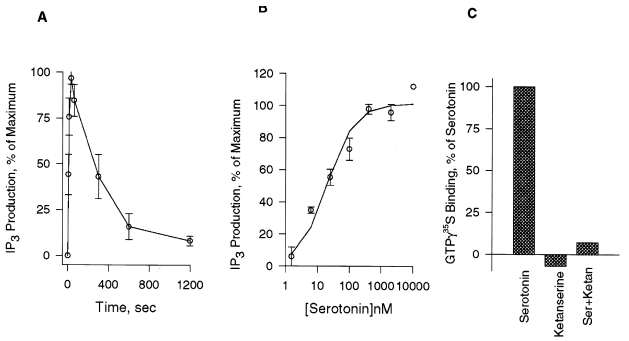

Fig. 3. Characterization of serotonin-induced IP_3 release in HEK293 cells heterologously expressing the human 5-HT_{2C} receptors. IP_3 release was measured using a FlashPlate assay kit (NEN™) at room temperature, as described in Materials and methods. (A) IP_3 release was measured upon treatment of cells with 10 μM serotonin for indicated durations (from 10 to 1200 s). A transient increase of IP_3 release was observed with serotonin treatment, peaking in 40 s, and returning to the basal level in 20 min. (B) Cells were treated with serotonin at various concentrations for 40 s, and the peak response were plotted as a function of serotonin concentrations. The values were normalized to that observed with 10 μM serotonin. (C) Serotonin (100 nM)-induced IP_3 release was abolished by ketanserin (10 μM), an antagonist, which by itself showed no appreciable effect on IP_3 release.

such as NIH3T3 and Sf-9 cells (Barker et al., 1994; Hartman and Northup, 1996). Recently, negligible inverse agonistic actions were reported with the receptor as expressed in HEK293 cells (Herrick-Davis et al., 1998). No further attention to the subject was paid in this study. Several known agonists, mCPP, DOI and Org37684 at 10 μM (a saturating concentration) enhanced GTPγ^{35}S binding by 72%, 72% and 94% of the maximal serotonin action. LSD increased GTPγ^{35}S binding only by 21 ± 4% of the serotonin response.

3.3. IP_3 and cAMP levels

We measured serotonin-induced changes in the intracellular IP_3 level in intact cells, using the radioreceptor competition assay using [^{3}H]IP_3 and calf cerebellum IP_3 receptors (DuPont NEN). Upon serotonin activation, a transient rise of IP_3 was observed, peaking at the incubation time of 30 to 45 s, with a maximum level of 25 pmol/10^6 cells (Fig. 3). Serotonin concentration dependently enhanced the peak IP_3 level (40 s points) with an EC_{50} value of 19.7 ± 5.2 nM. Ketanserin, an antagonist, at 10 μM abolished serotonin(100 nM)-induced enhancement of IP_3, but by itself produced no detectable change in IP_3 level (Fig. 3). Other known antagonists, clozapine, metergoline and methiothepin also showed no appreciable effects on IP_3 release while standard agonists, DOI, mCPP and Org37684 enhanced the transient IP_3 peak as high as serotonin (Table 1). Also LSD produced an IP_3 peak, amounting to 44 ± 12% of that observed with serotonin, confirming its partial agonistic functionality.

We monitored the effect of serotonin on intracellular cAMP level. Forskolin concentration-dependently stimulated cAMP in HEK293 cells expressing the 5-HT_{2C} receptor with an EC_{50} value of 2 ± 0.4 μM. The forskolin actions at submaximal concentrations (0.2, 0.5 and 1.5 μM) were not affected by serotonin at the concentration range from 4 to 2000 nM (data not shown). Also serotonin by itself showed no effect on cAMP production. It appears that adenylyl cyclases in HEK293 cells were not influenced by activation of the 5-HT_{2C} receptor.

3.4. Pertussis toxin treatment

Pertussis toxin blocks G_o- and G_i-mediated signals by ADP-ribosylation of their cysteine residue at the carboxyl terminus. Pertussis toxin treatment for 24h did not alter the maximal binding sites as measured with [^{3}H]mesulergine in the treated cell membranes, but decreased the high affinity serotonin sites (Fig. 1). Analysis of [^{3}H]mesulergine binding data yielded a K_d of 3.2 ± 0.5 nM and B_{max} of 65 ± 3 nM pmol/mg protein, which are not appreciably different from those observed with the untreated cells. Analysis of [^{3}H]serotonin binding data, after correction for the low affinity site contributions as described above, however, yielded B_{max} of 0.57 ± 0.05 pmol/mg protein. This represents a reduction of high affinity sites for serotonin by nearly 70% after the pertussis toxin treatment, with little changes in its affinity (0.9 ± 0.1 nM). At the same time, serotonin-induced GTPγ^{35}S binding greatly decreased with the pertussis toxin treatment (Fig. 4). Serotonin at 10 μM increased GTPγ^{35}S binding by less than

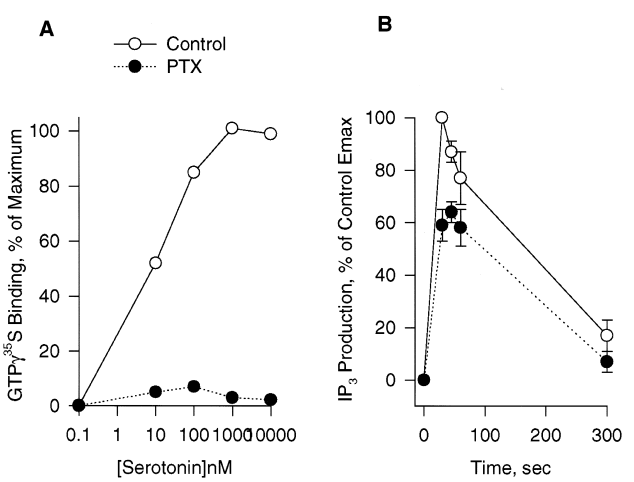

Fig. 4. Effects of pertussis toxin treatment on serotonin-induced GTPγ^{35}S binding and IP_3 release. HEK293 cells expressing the human 5-HT_{2C} receptor were treated with pertussis toxin at 100 ng /ml medium for overnight. (A) GTPγ^{35}S binding profiles as a function of serotonin concentrations in membranes from pertussis toxin-treated or -untreated cells. The pertussis toxin treatment markedly reduced serotonin-dependent GTPγ^{35}S binding. (B) Time courses of serotonin (10 μM)-induced IP_3 release in pertussis toxin-treated or untreated cells. The pertussis toxin treatment reduced the peak level of IP_3 release by only 40%, without alterations in general time course profiles.

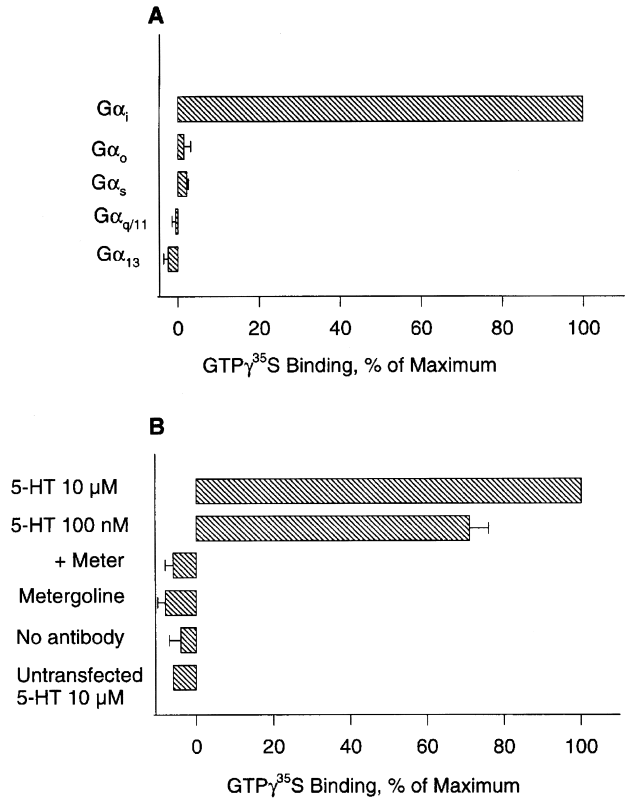

Fig. 5. Primary contribution by G_i subtypes of G proteins to pertussis toxin-sensitive GTPγ^{35}S binding induced by 5-HT_{2C} receptor agonists. Membranes from the HEK293 cells expressing the human 5-HT_{2C} receptor were treated with serotonin at 10 μM in the presence of GTPγ^{35}S at 4 nM, and then solubilized with 0.3% CHAPS. Solubilized membranes were diluted to a final CHAPS concentration of 0.125% and then added to the wells previously coated with various Gα isoform-specific antibodies. (A) After washing, individual wells were counted for ^{35}S radioactivity, and specific binding was calculated by subtracting that observed without serotonin. The highest level of GTPγ^{35}S radioactivity was observed with the antibody specific for $G\alpha_i$, and GTPγ^{35}S levels associated with other Gα isoforms were normalized to that observed with $G\alpha_i$. (B) GTPγ^{35}S levels associated with $G\alpha_i$-specific antibody were measured after treatment with serotonin 10 μM, 100 nM with or without 10 μM metergoline, or metergoline (10 μM) alone, and the values were normalized to that observed with serotonin at 10 μM. Metergoline (10 μM), an antagonist, blocked the serotonin (100 nM)-dependent association of GTPγ^{35}S with the $G\alpha_i$-specific antibody. In the wells not coated with $G\alpha_i$-specific antibodies, we observed no serotonin(10 μM)-induced GTPγ^{35}S association. Also, the serotonin(10 μM)-action was absent in membranes from mock transfected HEK293 cells.

5% of that observed before pertussis toxin treatment. On the other hand, the pertussis toxin treatment reduced the serotonin-induced IP_3 peak only by 40% (Fig. 4).

GTPγ^{35}S-bound Gα subunits were identified from immobilization studies with various Gα-specific antibodies (Okamoto et al., 1992, see Materials and methods), using membranes activated with serotonin in the presence of GTPγ^{35}S and solubilized with CHAPS (0.3%). The $G\alpha_i$-specific antibody was associated with the highest level of GTPγ^{35}S (Fig. 5), about three times greater than that observed in control wells without serotonin activation; e.g., 19570 ± 490 cpm for the $G\alpha_i$ antibody, and 6035 ± 120 cpm/well for the control wells. The $G\alpha_i$ antibody is known to cross-react with $G\alpha_o$ subtypes, but its cross-reactivity has no consequences in this study, because $G\alpha_o$-specific antibodies showed negligible association with GTPγ^{35}S. Moreover, no appreciable association with GTPγ^{35}S was observed for the other antibodies specific for $G\alpha_s$, $G\alpha_{13}$ or $G\alpha_{q/11}$ (Fig. 5). Relative degrees of GTPγ^{35}S association, as normalized to that observed for $G\alpha_i$ were 2 ± 1% for $G\alpha_o$, 2.2 ± 0.4 for $G\alpha_s$, −0.6 ± 0.8% for $G\alpha_{q/11}$ and −2.4 ± 1.1% for $G\alpha_{13}$. Metergoline (antagonist) at 10 μM blocked the GTPγ^{35}S association

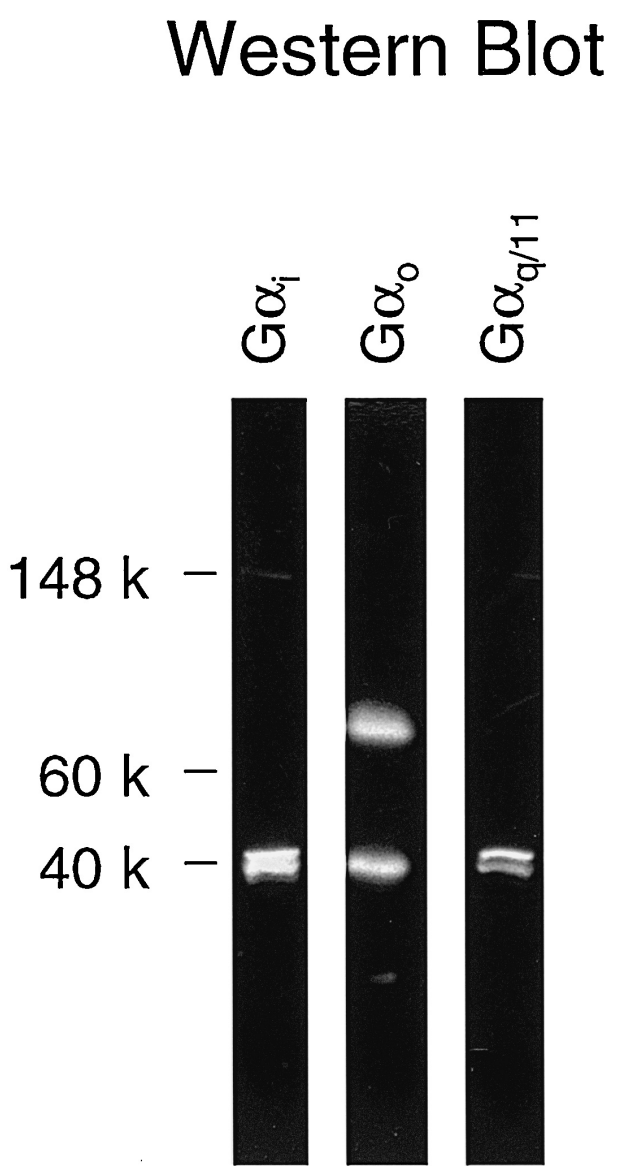

Fig. 6. Western blots for three major Gα isoforms ($G\alpha_i$, $G\alpha_o$ and $G\alpha_{q/11}$) in HEK293 cell membranes. Cell membranes (10 μg protein) were resolved with SDS polyacrylamide gel (12%) electrophoresis, were transferred to polyvinylidene difluoride membranes, and probed with antibodies specific for individual Gα subunits. Detection was performed using the ECL Plus™ Western blotting detection system from Amersham Pharmacia Biotech.

with $G\alpha_i$ induced by serotonin at 100 nM ($70 \pm 5\%$ of that observed with serotonin at 10 μM), but by itself reduced the basal binding to $-8 \pm 3\%$ as normalized to the serotonin response at 10 μM (Fig. 5). $G\alpha_i$ thus appear to be the primary contributor to agonist-induced GTPγ^{35}S binding mediated by 5-HT_{2C} receptors. $G\alpha_i$ appears to be highly abundant in HEK293 cells, judging from Western blots with HEK293 cell membranes, but $G\alpha_o$ and $G\alpha_{q/11}$ isoforms are also reasonably abundant, as detected with antibodies specific individual isoforms (Fig. 6). In the Western blots, the $G\alpha_i$-specific and the $G\alpha_{q/11}$-specific antibodies produced multiple bands centered at the molecular weight of 40 kDa as expected, while the $G\alpha_o$-specific antibody brought out two bands, at 40 and 80 kDa, probably $G\alpha_o$ dimers even under reducing conditions.

4. Discussion

In this study we have shown that serotonin-activation of the human 5-HT_{2C} receptor, as heterologously expressed in HEK293 cells, enhanced GTPγ^{35}S binding via pertussis toxin-sensitive G_i, and IP_3 release via both pertussis toxin-sensitive G_i and -insensitive G proteins (probably $G_{q/11}$). Inhibition of adenylyl cyclases was not observed, despite activation of G_i which are best known as the primary G proteins to inhibit adenylyl cyclases. Binding studies showed that about 3% of the total binding sites are estimated to exist in high affinity states for agonists (G protein-coupled phenotype), as expected from a highly overexpressed system where a dominant receptor population assume low affinity states (G protein-uncoupled phenotype) (Butkerait et al., 1995). Further study with pertussis toxin-treated cells revealed that about 70% of high affinity serotonin sites are coupled to pertussis toxin-sensitive G_i, and the rest to pertussis toxin-insensitive $G_{q/11}$ subtypes. Overall, these observations are in agreement with the 5-HT_{2C} receptor-mediated phosphatidyl inositol pathway in the literature (Conn et al., 1979; Julius et al., 1988; Stam et al., 1994; Kaufman et al., 1995), and at the same time reveals G_i-dependent GTPγ^{35}S binding as a unique phenotype of the receptor in HEK293 cells.

Agonist-induced GTPγ^{35}S binding represents an early step of G protein activation, but has been reported to be non-existent for the 5-HT_{2C} receptor as heterologously expressed in NIH3T3 fibroblasts (Burns et al., 1997). For the receptor in HEK 293 cells, however, as described above, the pertussis toxin-sensitive(G_i subtypes) pathway primarily contributed to agonist-induced GTPγ^{35}S binding, but the pertussis toxin-insensitive pathway only marginally. This minimal association of GTPγ^{35}S with $G\alpha_{q/11}$ subtypes, despite their major involvement (nearly 60%) in the IP_3 pathway of 5-HT_{2C} receptors, could be attributed to the well documented observations that the pertussis toxin-insensitive G_q family of G proteins binds GTP (or its analogs) poorly in isolated systems and also shows low GTP-turnover rates (Pang and Sternweis, 1990; Smrcka et al., 1991). For example, GTP hydrolysis rates by $G\alpha_q$ amount to less than 5% of those observed with $G\alpha_i$ or $G\alpha_o$, and seems to be not compatible with the rate of phospholipase C-β activation by G_q observed in intact cells. This discrepancy is not accounted for at present. On the other hand, one may also wonder if the primary contribution of G_i to agonist-induced GTPγ^{35}S binding reflects their selective interactions with the receptor or their non-selective interactions as possibly dominant G proteins in HEK293 cells. To evaluate the levels of Gα subunits in HEK293 cells, we carried out Western blots, using the antibodies specific for the three major Gα subunits ($G\alpha_i$, $G\alpha_o$ and $G\alpha_{q/11}$). The blots in Fig. 6 show semiquantitatively that the three major Gα subunits were reasonably abundant in HEK293 cell membranes. From intense bands for all the Gα subunits seen with the blots, one may assume that these antibodies are fully capable of interacting with their selective Gα partners, and their immunoreactivities could not be a limiting factor for detecting agonist-induced GTPγ^{35}S binding to Gα subunits, including $G\alpha_q$. Also in the same cell line, the human D3 dopamine receptor which presumably activates G_i/G_o subtypes of G proteins, failed to enhance GTPγ^{35}S binding to $G\alpha_i/G\alpha_o$ subtypes, because of the absence of its target effectors (submitted manuscript). These observations are consistent with the view that G_i subtypes interact with 5-HT_{2C} receptors in a highly receptor-selective manner.

Thus, negligible agonist-induced GTPγ^{35}S binding for the 5-HT_{2C} receptor in NIH3T3 cells could be attributed to its primary coupling to $G_{q/11}$, but no detectable coupling to G_i. Recently, functional receptor-G protein-effector complexes have been proposed to accommodate highly selective couplings of G protein-coupled receptors in intact cells, which could not be accounted for by promiscuous interactions of G proteins with both receptors and effectors, in isolated states (Chidiac, 1998). This proposal implies the existence of cell-line specific mechanisms by which a given receptor harnesses target G proteins and effectors into a functional complex. Such mechanisms would enable 5-HT_{2C} receptors in HEK293 cells to form functional complexes with phospholipase C, $G_{q/11}$ and G_i, in NIH3T3 cells to form similar complexes but without G_i, and in Xenopus oocytes to couple primarily with G_o and G_{i1} (Chen et al., 1994). Moreover, such structural restraints could explain the selective activation of phospholipase C via Gβγ from G_i in HEK293 cells, without inhibition of adenylyl cyclases by $G\alpha_i$, as if such complexes do not include adenylyl cyclases.

Apparently, the agonist-induced GTPγ^{35}S binding represents an easy and useful means to test if 5-HT_{2C} receptors in various cell lines are coupled to pertussis toxin-sensitive G_i subtypes of G proteins. Furthermore, all the agonists we tested here showed the same relative efficacy for stimulating GTPγ^{35}S binding as that for stimulating IP_3

release in HEK293 cells. Thus, these two assays should complement each other for determining intrinsic efficacy of test ligands, and also provide a unique opportunity to see if certain ligands differentially activate pertussis toxin-sensitive or -insensitive pathways.

For G protein coupled-receptors, high and low affinity sites for agonists represent the phenotypes of G protein-coupled and -uncoupled receptors, respectively. For 5-HT_{2C} receptors in HEK293 cells, we found the K_i value of 142 nM for the low affinity site for serotonin, and the K_d of 0.8 nM for its high affinity site, a 177-fold affinity difference. Despite this large affinity difference, our study on the high affinity sites for serotonin was not straightforward, because a predominant receptor population assume low affinity states (see Fig. 1 and text). In this respect, it is noteworthy that most studies in the literature have reported the binding properties of cloned 5-HT_{2C} receptors with [^{3}H]serotonin binding at the concentration range up to 20 nM, without considering its low affinity site contributions, the magnitude of which depends on the total receptor expression level and the K_i value of its low affinity sites, as discussed above. In many studies, the K_d values for [^{3}H]serotonin for cloned 5-HT_{2C} receptors have been reported above 5 nM, and B_{max} values above 5 pmol/mg protein. These values are close to those we would obtain without the correction for the low affinity site contribution. This underscores the need to dissect out high and low affinity site contributions to [^{3}H]serotonin binding.

In summary, we discovered robust agonist-induced GTPγ^{35}S binding mediated by the human 5-HT_{2C} receptor as heterologously expressed in HEK293 cells, representing the activation of G_i subtypes of G proteins by the receptor, and seems to be expressed in cell-type specific manner.

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