Differential Effects of 5-Methyl-1-[[2-[(2-methyl-3-pyridyl)oxyl]-5-pyridyl]carbamoyl]-6-trifluoromethylindone (SB 243213) on 5-Hydroxytryptamine_2C_ Receptor-Mediated Responses

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ABSTRACT

5-Methyl-1-[[2-[(2-methyl-3-pyridyl)oxyl]-5-pyridyl]carbamoyl]-6-trifluoromethylindone (SB 243213) is a selective, high-affinity 5-hydroxytryptamine (serotonin)_2C_ receptor ligand that has been previously characterized as a competitive 5-HT_2C_ receptor antagonist that has a long duration of activity in vivo. It is active in two preclinical models of anxiety and has an improved anxiolytic profile compared with benzodiazepines. In this study, we further characterized the pharmacological properties of SB 243213 by measuring its effects on each of multiple responses coupled to the 5-HT_2C_ receptor. In Chinese hamster ovary cells, SB 243213 was an inverse agonist for the phospholipase A_2 response, for guanosine 5’-O-(3-thio)triphosphate binding, for reduction of constitutive desensitization, and for enhancement of dopamine release in the rat nucleus accumbens, with relative efficacies of 0.6, 1, 1, and 0.6, respectively. However, for the phospholipase C (PLC) signaling cascade, SB 243213 behaved as an antagonist. Although SB 243213 was previously characterized as a competitive antagonist for the PLC response, the magnitude of the dextral shift of the 5-HT concentration-response curve was time-dependent, and the maximal PLC response to 5-HT was decreased, probably as a result of the slow dissociation rate of SB 243213 (initial dissociation rate was 3.2 times slower than SB206553, a prototypical 5-HT_2C_ receptor inverse agonist). Taken together, these data show that the pharmacological characteristics of SB 243213 at the 5-HT_2C_ receptor differ depending upon the response measured, and they support the hypothesis that different drugs, acting at the same receptor subtype, can differentially regulate multiple cellular signaling systems.

5-Hydroxytryptamine (serotonin) (5-HT)_2C_ receptors are members of the 7-transmembrane spanning (7-TMS or heptahedral) receptor superfamily, frequently referred to as G protein-coupled receptors. The 5-HT_2C_ receptor is involved in the regulation of a variety of physiological functions and behaviors, such as feeding behavior, temperature regulation, and affective state. Therapeutically, 5-HT_2C_ receptors are targets for medications used to treat conditions such as schizophrenia, anxiety, depression, Parkinson’s disease, and obesity. 5-HT_2C_ receptors are also major sites of action of hallucinogenic ligands (Roth et al., 1998; Roth and Shapiro, 2001; Jones and Blackburn, 2002; Leysen, 2004).

Like many, if not all, 7-TMS receptors, 5-HT_2C_ receptors couple to multiple cellular effector systems. Perhaps the best-studied effector system is the phospholipase C (PLC) pathway. Stimulation of PLC by 5-HT_2C_ receptors leads to the production of inositol phosphates (IP) and diacylglycerol. Another effector pathway that couples to 5-HT_2C_ receptors, but

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine, serotonin; 7-TMS, 7-transmembrane spanning; PLC, phospholipase C; IP, inositol phosphate; PLA_2_, phospholipase A_2_; AA, arachidonic acid; DOI, (±)-2,5-dimethoxy-4-iodoamphetamine hydrobromide; CHO, Chinese hamster ovary; SB 243213, 5-methyl-1-[[2-[(2-methyl-3-pyridyl)oxyl]-5-pyridyl]carbamoyl]-6-trifluoromethylindone; SB206553, 5-methyl-1-(3-pyridylcarbamoyl)-1,2,5-tetrahydropyrrolo[2,3-f]indole; Ro 60-0175, S-2-(6-chloro-5-fluorindol-1-yl)-1-methyl ethylamine; SB 242084, 6-chloro-5-methyl-1-(6-[[2-methylpiridin-3-yloxy]pyridin-3-yl carbamoyl] indoline; DA, dopamine; NAc, nucleus accumbens; GTP-γ-S, guanosine 5’-O-(3-thio)triphosphate; ANOVA, analysis of variance; PLSD, protected least significant difference; i.p., intraperitoneal.
that is somewhat less well studied, is the phospholipase A\textsubscript{2} (PLA\textsubscript{2}) signaling cascade, which leads to the liberation of arachidonic acid (AA) and the subsequent production of a myriad of AA metabolites (Leysen, 2004).

In addition to the classical signaling mechanisms described above, 5-HT\textsubscript{2C} receptors couple to several other signaling systems. In addition to the PLC and PLA\textsubscript{2} pathways, 5-HT\textsubscript{2C} receptors also activate desensitization mechanisms, such as G protein-coupled receptor kinase (Westphal et al., 1995; Saucier et al., 1998; Backstrom et al., 2000; Berg et al., 2001b; Porter et al., 2001; Schlag et al., 2004). Furthermore, it has been demonstrated that 5-HT\textsubscript{2C} receptors can couple to phospholipase D via G\textsubscript{13} (McGrew et al., 2002), to pertussis toxin-sensitive G proteins (e.g., G\textsubscript{i/o}) (Lucaites et al., 1996), and to PDZ domain-containing proteins (Backstrom et al., 2000). Consequently, the net cellular effect of activation of 5-HT\textsubscript{2C} receptors is a coalescence brought about by the concurrent activation of several effector pathways within cells. Recently, evidence has begun to accumulate from studies with 7-TMS receptor systems that indicates that ligand pharmacology may be more complex than that specified by traditional receptor theory. For example, rather than being a system-independent constant for a particular receptor, it seems that ligand intrinsic efficacy is a variable that changes with cell phenotype and physiological state and is dependent upon cellular signaling machinery (i.e., differs with the response measured) (Clarke and Bond, 1998). This newly discovered ligand behavior has been given many names by different groups: agonist-directed trafficking of receptor stimulus, functional selectivity, stimulus trafficking, and biased agonism to name a few (see references within Kenakin, 1995; Mailman and Gay, 2004; Clarke, 2005).

5-HT\textsubscript{2C} receptors seem to be capable of subserving agonist-directed trafficking of receptor stimulus. Werry et al. (2005) found that the relative efficacy of DOI and quipazine was reversed when measured for PLC-IP and calcium responses versus phosphorylation of extracellular signal-regulated kinase1/2 via the 5-HT\textsubscript{2C} receptor expressed in CHO cells. In NIH-3T3 cells expressing the 5-HT\textsubscript{2C} receptor, it was reported that although lysergic acid diethylamide is equally efficacious as 5-HT in stimulating PLC-IP, it does not elicit increases in intracellular calcium nor does it promote phosphorylation of the 5-HT\textsubscript{2C} receptor (Backstrom et al., 1999).

Studies from our group have also shown different ligand pharmacological properties depending upon the response measured from activation of 5-HT\textsubscript{2C} receptors (Berg et al., 1998, 2001a; Stout et al., 2002; De Deurwaerdere et al., 2004). One of the important implications of agonist-directed trafficking of receptor stimulus is that drugs may have a richer pharmacology than previously thought. Ligands may have more selectivity than that afforded by differential affinity for different receptor subtypes. By developing drugs that are not only receptor subtype-selective but also signal pathway-selective, it may be possible to enhance therapeutic selectivity.

SB 243213 is a selective, high-affinity 5-HT\textsubscript{2C} receptor ligand. SB 243213 has been previously characterized as a competitive 5-HT\textsubscript{2C} receptor antagonist that has a long duration of activity in vivo, is active in two preclinical models of anxiety, and has an improved anxiolytic profile compared with benzodiazepines (Wood et al., 2001; Blackburn et al., 2002). In this study, we further characterized the pharmacological properties of SB 243213 by measuring its relative efficacy on each of multiple responses coupled to the 5-HT\textsubscript{2C} receptor with respect to effects elicited by the 5-HT\textsubscript{2C} inverse agonists SB206553 (Kennett et al., 1996) and clozapine (Herrick-Davis et al., 2000). In addition, the pharmacological profile of SB 243213 was also studied in vivo by assessing its influence on the effects induced by the inverse agonist SB206553 and the agonist Ro 60-0175 (Martin et al., 1998) on the release of DA measured in the rat nucleus accumbens (NAc) (De Deurwaerdere et al., 2004).

Materials and Methods

Materials

The following materials were purchased from commercial sources:

- \textit{myo}-[\textit{3}H]inositol, \textit{[3}H\textit{]arachidonic acid, and \textit{[35}S\textit{]GTP-S were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). 5-HT HCl, DOI, SB206553 HCl, and 8-chloro-11-(4-methyl-1-piperazinyl)-5H-dibenzo[b,e]1,4-diazepine (clozapine) were purchased from Sigma/RBI (Natick, MA). Ro 60-0175 HCl was kindly donated by Dr. P. Weber (F. Hoffman-La Roche, Basel, Switzerland). SB 242084 and SB 243213 were generously provided by Dr. M. Wood (Psychiatry CEDD, GlaxoSmithKline, Harlow, UK). Fetal bovine serum was from Gemini Bioproducts (Calabasas, CA). All other tissue culture reagents were purchased from Invitrogen (Carlsbad, CA). All other drugs and chemicals (reagent grade) were purchased from Sigma-Aldrich (St. Louis, MO, and Illkirch, France) and VWR (Strasbourg, France).

In Vitro Methods

Cell Culture. CHO-1C19 and CHO-1C7 cell lines are CHO-K1 cells which stably express nonedited 5-HT\textsubscript{2C-INI} receptor at densities of 250 fmol/mg protein and 20 pmol/mg protein, respectively (Berg et al., 1994, 1999).

Cells were maintained in α-minimum Eagle's medium supplemented with 5% fetal bovine serum and seeded into 12- or 24-well tissue culture vessels for functional studies or 15-cm dishes for radioligand binding studies at a density of 4 × 10\textsuperscript{4} cells/cm\textsuperscript{2}. Following a 24-h plating period, cells were washed with Hanks' balanced salt solution and placed into Dulbecco's modified Eagle's medium/Ham's F-12:1 (1:1) with 5 μg/ml insulin, 5 μg/ml transferrin, 30 nM selenium, 20 nM progesterone, and 100 μg/ml putrescine (serum-free media) and cultured for an additional 24 h before experimentation. All prolonged ligand pretreatments were done during the serum-free culture period.

Radioligand Binding. CHO-1C7 membranes were prepared from cell pellets by trituration through a 26-gauge needle, followed by centrifugation at 39,000g and sonication at 4°C. Estimates of ligand dissociation were described as Christopoulos et al. (1999). Membranes were incubated with 1 μM SB206553, 100 nM SB 243213, or 0.1% vehicle (dimethyl sulfoxide) for 60 min at 37°C, washed three times with ice-cold wash buffer (50 mM HEPES, 2.5 mM MgCl\textsubscript{2}, and 2 mM EGTA, pH 7.4, at 23°C), and finally resuspended in assay buffer (wash buffer plus 0.1% ascorbic acid) at a protein concentration of 20 to 40 μg/ml. After washing, 2 nM \textit{[3}H\textit{]mesulergine was added to tubes containing 5 to 10 μg of membrane protein and incubated for various periods up to 1 h. Binding was terminated by addition of ice-cold assay buffer and filtration through 0.5% polyethyleneimine-coated GF/C glass fiber filters. This method of assessing the initial association rate of \textit{[3}H\textit{]mesulergine with the 5-HT\textsubscript{2C} receptor previously occupied with ligand provides an indirect measure of the relative rates of ligand dissociation.

IP Accumulation and AA Release Measurements. CHO-1C19 or CHO-1C7 cells were labeled in serum-free medium with 1 μCi/ml \textit{[3}H\textit{]myo-inositol for 24 h and with 0.1 to 0.2 μCi/ml \textit{[3}H\textit{]arachidonic acid in the presence of 1 μM unlabeled AA for 4 h at 37°C before
experiments. Before initiation of the assay, cells were washed three times with 5-min incubation intervals between each wash. For agonist-stimulation experiments, agonists were added for 10 min in the presence of 20 mM LiCl as described previously (Berg et al., 1998, 1999). Antagonists, when present, were added simultaneously with, or 15 min before, agonists. Basal IP accumulation (i.e., ligand-independent 5-HT$_{2C}$ receptor activity) was measured following a 25-min incubation (37°C) with 20 mM LiCl as described previously (Berg et al., 1998, 1999). For homologous sensitization studies (Berg et al., 1999), cells were treated for 24 h with ligand and washed three times with 5-min incubation intervals at 37°C. IP accumulation and AA release were measured from cells stimulated with the 5-HT$_{2C}$ agonist DOI (1 μM) or vehicle for 10 min. For experiments in which both PLC-PI and PLA$_2$-AA pathways were measured, total [3H]IP accumulation and [3H]AA release were measured from the same multiwell (simultaneously) as described previously (Berg et al., 1998, 1999).

[$[^{35}S]$GTPyS Binding. GTPyS binding to Go$_{q11}$ was done in membranes prepared from CHO-1C7 cells as described previously (Evans et al., 2001; De Deurwaerdere et al., 2004). Membranes were prepared by repeated trituration of cells through a 1-ml pipette in ice-cold wash buffer (20 mM HEPES, 3 mM MgCl$_2$, 0.2 mM EGTA, and 100 mM NaCl, pH 7.4, at 23°C). The homogenate was centrifuged (39,000g; 4°C; 10 min), and the pellet was washed twice by resuspension in 40 volumes of the same buffer and then centrifugation. Membranes were resuspended in assay buffer (wash buffer plus 10 μM GDP, 100 mM okadaic acid, and 10 nM cypemethrin) at a protein concentration of 100 μg/ml. Aliquots (100 μl) of the membrane suspension were preincubated with ligand for 20 min, followed by the addition of [$[^{35}S]$GTPyS (0.3 nM, final concentration) for 30 min at 37°C in 96-well Multiscreen filtration plates. Binding was stopped by the addition of ice-cold buffer and rapid filtration, followed by several washes. Nonspecific binding was defined in the presence of 100 μM guanosine 5′-([β,i]-imidodiphosphate)triphosphate. With this method, all [$[^{35}S]$GTPyS binding was blocked by pretreatment of cells with pertussis toxin and therefore represents binding to Go$_{q11}$ and not Go$_{q11}$.2.

Data Analysis. For analysis of concentration-response curves, data were fit with nonlinear regression to the following model:

$$R = R_0 + \frac{R_m - R_0}{1 + \left(\frac{[A]}{EC_{50}}\right)^d}$$

where $R$ is the measured response at a given ligand concentration [A], $R_0$ is the response in the absence of ligand, $R_m$ is response at maximal ligand concentration, EC$_{50}$ is the concentration of ligand that produces a half-maximal response, and $n$ is the slope factor. Maximal response was calculated as $R_m - R_0$. For analysis of initial association rate, data points for the first 15 min of [$[^{3}H]$mesulergine binding were fit to the following equation:

$$B = B_{max}(1 - e^{-kt})$$

where $B$ is the measured amount of [$[^{3}H]$mesulergine bound at time $t$, $B_{max}$ is the maximal amount of [$[^{3}H]$mesulergine bound at $t = \infty$, and $k$ is the apparent rate constant.

For signal transduction and binding assays, Student’s $t$ test (paired) was used for statistical comparisons. Asterisks denote $p$ values $<0.05 (**), <0.01 (***)$, or $<0.001 (****)$.

In Vivo Methods

Animals. Sprague-Dawley rats (Iffa Credo, Lyon, France) weighing 330 to 380 g were used. Animals were kept at constant room temperature (21 ± 2°C) and relative humidity (60%) with a 12-h light/dark cycle (dark from 8:00 PM) and had free access to water and food. All animals use procedures conformed to International European Ethical Standards (86/609-EEC) and the French National Committee (décret 87/848) for the care and use of laboratory animals. All efforts were made to minimize animal suffering and to reduce the number of animals used.

In Vivo Microdialysis. Surgery and perfusion procedures were performed as described previously, with minor modifications (De Deurwaerdere et al., 2004). In brief, rats were anesthetized with a mixture of halothane and nitrous oxide-oxygen [2%; 2:1 (v/v)]. After tracheotomy for artificial ventilation, the animals were placed in a stereotaxic frame, and their rectal temperature was monitored and maintained at 37.3 ± 0.1°C with a heating pad. One microdialysis probe, 2 mm in length, (CMA/11, 240-μm outer diameter, Cuprophan; Carnegie Medicin, Phymep, Paris, France) was implanted in the right NAc (coordinates from interaural point: AP, 11; L, 1.3; and V, 2) according to the atlas of Paxinos and Watson (1998). The probe was perfused at a constant flow rate of 2 μl/min by means of a microperfusion pump (CMA 111; Carnegie Medicin, Phymep) with artificial cerebrospinal fluid containing 154.1 mM Cl$^-$, 147 mM Na$^+$, 2.7 mM K$^+$, 1 mM Mg$^{2+}$, and 1.2 mM Ca$^{2+}$, adjusted to pH 7.4 with 2 mM sodium phosphate buffer. Dialysates (30 μl) were collected on ice every 15 min. The in vitro response of the probe was about 10% for DA. At the end of each experiment, the brain was removed and fixed in 0.9% NaCl/10% paraformaldehyde solution. The location of the probe was determined histologically on serial coronal sections (60 μm) stained with cresyl violet, and only data obtained from rats with correctly implanted probes were included in the results.

Chromatographic Analysis. Dialysate samples were immediately analyzed by reverse-phase high-performance liquid chromatography coupled with electrochemical detection, as described previously (Bonhomme et al., 1995). The mobile phase (containing 70 mM NaH$_2$PO$_4$, 0.1 mM Na$_2$EDTA, 0.7 mM triethylamine, and 0.1 mM octylsulfonlic acid plus 10% methanol, adjusted to pH 4.8 with orthophosphoric acid) was delivered at 1 ml/min flow rate (Shimadzu system LC-10AD-VP; Shimadzu, Croyssy Beaubourg, France) through a Hypersyl column (C18; 4.6 × 150 mm, particle size 5 μm; Touzard and Matignon, Paris, France). Detection of DA was carried out with a coulometric detector (Coulomech II; ESA, Paris, France) coupled to a dual-electrode analytical cell (model 5014; ESA). The potential of the electrodes was set at −175 and −175 mV. Output signals were recorded on a computer (Shimadzu system class VP-4). Under these conditions, the sensitivity for DA was 0.5 pg/30 μl with a signal-to-noise ratio of 3:1.

Pharmacological Treatments. Pharmacological treatments were performed after the stabilization of DA levels in the perfusate. A stable baseline, defined as three consecutive samples in which DA contents varied by less than 10% in the NAc, was generally obtained 135 min after the beginning of the perfusion (stabilization period). The selective 5-HT$_{2C}$ agonist SB 243213 was dissolved in a mixture of 0.9% NaCl containing hydroxypropyl-β-cyclodextrin (8% by weight) plus 25 mM citric acid and was administered intraperitoneally at 1, 3, and 10 mg/kg in a volume of 2 ml/kg. The 5-HT$_{2C}$ agonist Ro 60-0175 was dissolved in 0.9% NaCl and administered intraperitoneally at 3 mg/kg in a volume of 1 ml/kg. The 5-HT$_{2C}$ inverse agonist SB206533 was diluted in a 99:1 (v/v) mixture of aprotic water plus lactic acid and administered intraperitoneally at 5 mg/kg in a volume of 2 ml/kg. SB 243213 was administered intraperitoneally at 1 mg/kg 30 min before Ro 60-0175 and 30 or 60 min before SB206533. The doses and administration times of the different 5-HT compounds used were chosen on the basis of previous studies to keep both selectivity and efficiency toward the targeted sites (Kennett et al., 1996; Gobert et al., 2000; Blackburn et al., 2002; De Deurwaerdere et al., 2004). All drug doses were calculated as the free base. In each experimental group, animals received either drugs or their appropriate vehicle.

Data Analysis. DA content in each sample was expressed as the percentage of the average baseline level calculated from the three fractions preceding any treatment. Data correspond to the mean ±
S.E.M. values of the percentage obtained in each experimental group. Drug overall effect was calculated as the average of DA content from dialysates collected after their administration.

The statistical analysis of the effect of the 5-HT2C ligand SB 243213 on basal DA outflow was assessed by a one-way ANOVA (using group as the main factor) with time as repeated measures, performed for the 10 samples that followed its administration. When significant ($p < 0.05$), the one-way ANOVA was followed by the Fisher’s protected least significance difference test (PLSD) to determine the effect of each dose of SB 243213.

The interaction of SB 243213 (pretreatment) with Ro 60-0175 or SB206553 (treatment) was studied by a two-way ANOVA (pretreatment × treatment) with time as repeated measures, performed for the eight samples that followed the treatment administration. When significant ($p < 0.05$), the ANOVA was followed by the post hoc Fisher’s PLSD test to allow multiple comparison between groups.

**Results**

**Effect of SB 243213 on PLC, PLA2, and [35S]GTPγS Binding.** Figure 1 shows that SB 243213 behaved as a strong inverse agonist for the PLA2-AA and [35S]GTPγS binding responses coupled to the 5-HT2C receptor. In comparison with the full inverse agonist SB206553, SB 243213 was a partial inverse agonist with a relative efficacy of 0.6 for AA release and a pIC50 of 8.77 ± 0.12 (1.7 nM). For [35S]GTPγS binding the relative efficacy of SB 243213 was 1.0 and the pIC50 was 8.89 ± 0.31 (1.3 nM). In contrast to the AA release and basal [35S]GTPγS binding responses, SB 243213 did not alter basal activity of the PLC-IP pathway (Figs. 1 and 2A) at concentrations expected to produce full receptor occupancy (1 µM; 1000 × $K_i$). Thus, SB 243213 seems to be an antagonist for this response. To further define the antagonist property for the PLC-IP response, we tested the ability of SB 243213 to block both inverse agonist and agonist effects on IP accumulation. As shown in Fig. 2, SB 243213 blocked the effects of the inverse agonists, clozapine and SB206553, and of the agonist Ro 60-0175. SB 243213 had no effect in CHO cells that did not express the 5-HT2C receptor.

**Time-Dependent Antagonism by SB 243213.** As shown in Figs. 1 and 2, SB 243213 behaved as an antagonist at the
5-HT$_{2C}$ receptor-PLC-IP pathway. Figure 3 shows the effect of SB 243213 on concentration-response curves for 5-HT-mediated IP accumulation. When 30 nM SB 243213 was coincubated with 5-HT for 15 min, the concentration-response curve to 5-HT was shifted to the right by approximately 13-fold without change in the maximal response. The apparent $p_A^2$ for this shift was 8.62 (2.4 nM). Increasing the incubation time with SB 243213 to 25 min (added 15 min before 5-HT) lead to a greater shift (250-fold) in the 5-HT curve (apparent $p_A^2$ of 9.92; 0.12 nM) and reduced the maximal response by 50%.

Effect of SB 243213 on Extracellular DA Levels in the NAc. Absolute basal levels of DA in dialysates collected from the NAc did not differ across the different experimental groups throughout the course of the study and were 5.3+/−0.3 pg/30 μl (mean ± S.E.M., without adjusting for probe recovery; n = 102 animals). Intraperitoneal administration SB 243213 produced a progressive and long-lasting increase in DA extracellular levels in the NAc reaching approximately 30% of basal values at the end of the experiment at the three doses used (one-way ANOVA, $F_{3,33} = 7.1$, $p < 0.001$; Fig. 4A). As reported previously (De Deurwaerdere et al., 2004), administration of Ro 60-0175 (3 mg/kg i.p.) inhibited basal DA outflow by 23%. The effect of Ro 60-0175 was reversed by SB 243213 (1 mg/kg i.p.) administered 30 min before ($p < 0.001$; Fisher’s PLSD test after a two-way ANOVA, $F_{1,23} = 5.6$, $p < 0.05$). SB 243213 alone elicited a significant progressive increase in DA efflux per se, reaching approximately 130% of baseline 105 min after its injection (Fig. 4B).

Figure 5 illustrates the effect of the administration of 1 mg/kg SB 243213 (i.p.) on the increase in DA extracellular levels induced by SB206553 (5 mg/kg i.p.). As reported previously (De Deurwaerdere et al., 2004), SB206553 elicited a significant overall increase in DA outflow, reaching about 50% over basal. SB 243213 (1 mg/kg i.p.) significantly blocked the increase produced by SB206553 when it was administered 30 min before ($p < 0.05$; Fisher’s PLSD test after a two-way ANOVA $F_{1,18} = 12.9$, $p < 0.01$; Fig. 5A) or 60 min before SB206553 ($p < 0.01$; Fisher’s PLSD test after a two-way ANOVA $F_{1,22} = 19.4$, $p < 0.001$; Fig. 5B).

Effect of SB 243213 on Constitutive Desensitization of the 5-HT$_{2C}$ Receptor System. Constitutive receptor ac-
tivity, like agonist-stimulated activity, can activate desensitization mechanisms (Berg et al., 1999; Wilbanks et al., 2002). Consequently, receptor systems can exist in a partial, constitutively desensitized state. Reduction of constitutive receptor activity by prolonged exposure to inverse agonists can lead to sensitization of a receptor system, evidenced by enhanced constitutive receptor activity (i.e., increased basal response) and enhanced responsiveness to agonist. Using this inverse agonist treatment paradigm, we determined whether prolonged treatment with SB 243213 influenced basal or agonist-stimulated IP accumulation. As shown in Fig. 6A, prolonged treatment (24 h) with clozapine or SB 243213 enhanced basal IP accumulation by approximately 90%. Treatment (24 h) with clozapine also enhanced agonist (DOI) stimulation of IP accumulation by about 90% (Fig. 6B). In contrast, agonist stimulation of IP accumulation after prolonged treatment with SB 243213 was reduced by about 60%. Further study of the increased constitutive 5-HT2C receptor activity toward PLC also revealed that the increase in basal activity after prolonged SB 243213 treatment was not sensitive to reduction by acute treatment with the inverse agonist mianserin (Fig. 7).

**Dissociation rate of SB 243213 Binding to the 5-HT2C Receptor.** To determine whether slow dissociation of SB 243213 from the 5-HT2C receptor could be responsible for the time-dependent shift in the 5-HT concentration-response curve (Fig. 3) and the different effects of prolonged SB 243213 treatment with respect to clozapine (Figs. 6B and 7), we measured the association rate of [3H]mesulergine binding after pretreatment of membranes with maximal concentrations of the ligands (with respect to receptor occupancy). The association rate of [3H]mesulergine was markedly slower in membranes pretreated with SB 243213 compared with SB206553 by measuring the association rate constants for [3H]mesulergine binding. The initial association rate constants for [3H]mesulergine binding were 0.423 ± 0.08, 0.249 ± 0.11, and 0.078 ± 0.01 min⁻¹ for vehicle,
SB206553-, and SB 243213-pretreated membranes, respectively.

**Discussion**

The data presented here demonstrate that SB 243213 has different pharmacological properties at the 5-HT$_{2C}$ receptor, depending upon the response measured. For the PLA$_2$-AA and G$_{i}$/H9251 pathways, SB 243213 followed by extensive washing, A, IP was measured for 25 min. Basal IP accumulation was 1971 ± 88 dpm. Data shown represent mean percentage of basal accumulation ± S.E.M. of four experiments. **,** $p < 0.001$ compared with vehicle, Student’s paired t test. B, accumulation of IP with or without the 5-HT$_{2C}$ agonist DOI (1 μM) for 25 min was measured. DOI-stimulated IP accumulation in vehicle-treated cells was 210 ± 12% above basal. Data shown represent mean percentage of DOI-stimulated IP accumulation ± S.E.M. of four experiments. **,** $p < 0.001$; **,** $p < 0.01$ compared with vehicle, Student’s paired t test.

SB206553-, and SB 243213-pretreated membranes, respectively.

**Fig. 6.** Effect of 24-h treatment with clozapine or SB 243213 on basal (A) and agonist-stimulated (B) 5-HT$_{2C}$ constitutive receptor activity. CHO-1C7 cells were treated for 24 h with vehicle, 1 μM clozapine, or 1 μM SB 243213 followed by extensive washing. A, IP was measured for 25 min. Basal IP accumulation was 1971 ± 88 dpm. Data shown represent mean percentage of basal accumulation ± S.E.M. of four experiments. **,** $p < 0.001$ compared with vehicle, Student’s paired t test. B, accumulation of IP with or without the 5-HT$_{2C}$ agonist DOI (1 μM) for 25 min was measured. DOI-stimulated IP accumulation in vehicle-treated cells was 210 ± 12% above basal. Data shown represent mean percentage of DOI-stimulated IP accumulation ± S.E.M. of four experiments. **,** $p < 0.001$; **,** $p < 0.01$ compared with vehicle, Student’s paired t test.

**Fig. 7.** Mianserin-sensitive 5-HT$_{2C}$ constitutive receptor activity following 24-h treatment with clozapine or SB 243213. CHO-1C7 cells were pretreated with 100 nM SB 243213, 1 μM SB206553, or vehicle (0.1% DMSO) for 60 min at 37°C. After treatment, membranes were washed extensively at 4°C and then incubated for various times with 2 nM [³H]mesulergine at 37°C. Data are normalized to the maximal [³H]mesulergine binding in vehicle-treated membranes and represent mean ± S.E.M. of three experiments.

**Fig. 8.** Association of [³H]mesulergine with the 5-HT$_{2C}$ receptor in CHO-1C7 cell membranes that were pretreated with 100 nM SB 243213, 1 μM SB206553, or vehicle (0.1% DMSO) for 60 min at 37°C. After treatment, membranes were washed extensively at 4°C and then incubated for various times with 2 nM [³H]mesulergine at 37°C. Data are normalized to the maximal [³H]mesulergine binding in vehicle-treated membranes and represent mean ± S.E.M. of three experiments.

the 5-HT$_{2C}$-PLC-IP pathway. At concentrations that should produce full receptor occupancy (1000 × $K_i$), SB 243213 did not alter basal IP accumulation and blocked inverse agonist (clozapine and SB206553)-induced decreases and agonist (5-HT and Ro 60-0175)-stimulated increases in IP accumulation.

In vivo, SB 243213 seemed to behave as a partial inverse agonist for 5-HT$_{2C}$ receptor-mediated regulation of DA release. SB 243213 alone produced a small increase in DA release and antagonized both agonist- (Ro 60-0175) and inverse agonist (SB206553)-induced changes in DA release in the NAc measured with microdialysis. These actions of SB 243213 were similar to those of SB 242084 on DA release in the NAc (De Deurwaerdere et al., 2004); however, the antagonism of the SB206553-induced increase in DA release by SB 243213 had a longer latency than that of SB 242084, requiring approximately 100 min to occur. This is consistent with
the in vitro results that showed increased potency of SB 243213 to antagonize 5-HT-stimulated IP accumulation with incubation time, likely as a result of the slow dissociation of SB 243213 from the receptor (see below). Considering that ligands that activate the 5-HT2C-PLC pathway decrease DA release, ligands that reduce 5-HT2C-PLC signaling increase DA release and ligands with weak efficacy for the PLC response antagonize effects of stronger agonists and inverse agonists, it seems likely that the 5-HT2C-PLC pathway plays a predominant role in regulating DA release in vivo, although extrapolation of results from in vitro cell culture systems to in vivo physiological processes should be done with caution.

Taken together, these data demonstrate that the relative efficacy of SB 243213 differs depending upon the response measured. Such ligand action is not consistent with traditional receptor theory that requires that ligand relative efficacy, as a measure of intrinsic efficacy, be response-independent. Although somewhat sporadic, strong evidence has been accumulating for the 5-HT2C receptor system, and others (for reviews, see Kenakin, 2002; Perez and Karnik, 2005), which suggest that traditional receptor theory requires modification. Previously, we showed that some 5-HT2C agonists preferentially activate the PLC over the PLAr pathway, whereas for other agonists the reverse was true (Berg et al., 1998). In addition, 5-HT2C agonists differ in their relative efficacy to elicit desensitization of the PLC and the PLAr responses (Stout et al., 2002). Werry et al. (2005) reported that 5-HT2C agonists differentially regulate PLC versus extracellular signal-regulated kinase1/2 signaling, and Backstrom et al. (1999) showed that the relative efficacy of LSD differs for PLC versus calcium mobilization and 5-HT2C receptor phosphorylation. Differential relative efficacy toward different cellular signaling cascades coupled to the same receptor suggests that drugs may have more specificity than that afforded by differential affinity for receptor subtypes. Such selectivity may be exploited for improved therapeutic efficacy.

We have reported previously that prolonged reduction of constitutive 5-HT2C receptor activity (with inverse agonists) toward desensitization mechanisms results in enhanced responsiveness of the 5-HT2C-PLC signaling pathway (Berg et al., 1999). This enhanced responsiveness occurs as a result of increased expression of Grα111 in the absence of a change in 5-HT2C receptor density. Here, we found that prolonged treatment with SB 243213 or clozapine enhanced constitutive 5-HT2C signaling to the PLC pathway. However, surprisingly and unlike the clozapine effect, the enhanced basal IP response was not sensitive to reduction by acute treatment with the inverse agonist mianserin. Furthermore, prolonged SB 243213 treatment did not enhance 5-HT2C agonist stimulation of IP accumulation, as does clozapine and SB206553 (Berg et al., 1999), but instead reduced it.

One explanation for the opposite effects of prolonged SB 243213 treatment on basal versus agonist-stimulated IP accumulation is perhaps due to response-dependent differences in the pharmacological properties of SB 243213 combined with a slow rate of dissociation from the receptor. As an inverse agonist toward constitutive desensitization, prolonged treatment with SB 243213 would increase basal IP accumulation; however, with a slow rate of dissociation, SB 243213 would remain bound to the receptor after a washout procedure that is effective at removing clozapine and SB206553. Our data suggest that the dissociation of SB 243213 from the 5-HT2C receptor is markedly slower than that of SB206553. Continued occupancy of the receptor by SB 243213 after prolonged treatment would not alter the enhanced basal IP response [SB 243213 is an antagonist (zero efficacy) for this response], but would block the action of agonists (DOI) to stimulate IP accumulation and would prevent inverse agonists (mianserin) from decreasing the enhanced basal response, as we observed here. It should be noted that although slow dissociation of SB 243213 from the 5-HT2C receptor is the most parsimonious explanation for our data, which showed that pretreatment of membranes containing the 5-HT2C receptor with SB 243213, but not SB206553, reduced the rate of association of [3H]mesulergine, there are other interpretations. For example, it is possible that SB 243213, but not SB206553, pretreatment produces a change in the 5-HT2C receptor conformation (e.g., association with an accessory protein, ligand-induced changes in receptor dimerization, phosphorylation, differential receptor distribution into membrane microdomains) that persists after dissociation of SB 243213 such that affinity of [3H]mesulergine is reduced.

Slow dissociation of SB 243213 would also be expected to alter the kinetics of antagonism of the PLC response. We found that the magnitude of the dextral shift of the 5-HT concentration-response curve for IP accumulation was time-dependent. Furthermore, even though SB 243213 was previously characterized as a competitive antagonist (Wood et al., 2001), we found that SB 243213 reduced the maximal 5-HT response. Although insurmountable antagonism is generally associated with uncompetitive or irreversible antagonism, maximal response depression also occurs for competitive, but slowly dissociating, antagonists when used in experiments in which agonists are applied for short periods. In these cases, the agonist cannot bind until the antagonist dissociates from the receptor. This “hemi-equilibrium” can result in pharmacological characteristics that seem to be time-dependent; insurmountability when measured under nonequilibrium (short-term) conditions versus surmountability in equilibrium (long-term) assays (Christopoulos et al., 1999). Consistent with this hypothesis, equilibrium binding assays indicate that SB 243213 binding to the 5-HT2C receptor is reversible and surmountable (Wood et al., 2001); however, indirect measurement of the dissociation rate of SB 243213 suggested that it dissociates very slowly from the receptor. Slow receptor dissociation could explain the long duration of action of SB 243213 seen in some behavioral assays (Wood et al., 2001).

In summary, our data demonstrate that SB 243213 displays different pharmacological properties depending upon the response measured. For 5-HT2C receptor-mediated activation of the PLAr-A2 cascade, [35S]GTPγS binding, reduction of constitutive desensitization of PLC signaling and regulation of DA release in vivo, SB 243213 is an inverse agonist with different relative efficacy depending upon the response. However, for stimulation of PLC, SB 243213 behaves as an antagonist. Compared with SB206553, SB 243213 has a relatively slow dissociation rate from the 5-HT2C receptor, which likely explains the time-dependent increase in antagonist activity (increase potency and reduction of maximal response) of the 5-HT2C receptor-mediated PLC response. These results support the hypothesis that ligands can traffic the receptor stimulus differentially to cellular signaling cas-
cades and suggest that pharmacological labeling of drugs (as agonists, antagonists, and inverse agonists) should be done with reference to the response measured.

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References


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