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Novel Actions of Inverse Agonists on 5-HT_{2C} Receptor Systems

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ABSTRACT

In cell systems where ligand-independent receptor activity is optimized (such as when receptors are overexpressed or mutated), acute treatment with inverse agonists reduces basal effector activity whereas prolonged exposure leads to sensitization of receptor systems and receptor up-regulation. Few studies, however, have reported effects of inverse agonists in systems where nonmutated receptors are expressed at relatively low density. Here, we investigated the effects of inverse agonists at human serotonin $(5\text{-HT})_{2C}$ receptors expressed stably in Chinese hamster ovary cells (≈250 fmol/mg protein). In these cells, there is no receptor reserve for 5-HT and 5-HT_{2C} inverse agonists did not reduce basal inositol phosphate (IP) accumulation nor arachidonic acid (AA) release but behaved as simple competitive antagonists, suggesting that these receptors are not overexpressed. Prolonged treatment (24 h) with inverse agonists enhanced selectively 5-HT_{2C}-mediated IP accumulation but not AA release. The enhancing effect occurred within 4 h of treatment, reversed within 3 to 4 h (after 24-h treatment), and could be blocked with neutral antagonists or weak positive agonists. The enhanced responsiveness was not due to receptor up-regulation but may involve changes in the expression of the G protein, $G_{\alpha q/11}$ and possibly $G_{\alpha 12}$ and $G_{\alpha 13}$. Interestingly, 24-h exposure to inverse agonists acting at 5-HT_{2C} receptors also selectively enhanced IP accumulation, but not AA release, elicited by activation of endogenous purinergic receptors. These data suggest that actions of inverse agonists may be mediated through effects on receptor systems that are not direct targets for these drugs.

Data accumulated over the last few years demonstrate that a percentage of a G protein-coupled receptor population elicits cellular responses in the absence of any ligand (for reviews see Schütz and Freissmuth, 1992; Milligan et al., 1995; Kenakin, 1996; Milligan and Bond, 1997). This capacity to signal in the absence of a ligand has been called ligand-independent or constitutive receptor activity. The experimental evidence for the existence of ligand-independent receptor activity is based largely on results from heterologous expression systems where receptors are expressed at high densities or mutated such that they more efficiently activate effector pathways. In both situations, "basal" effector activity is enhanced and the magnitude of the increase in basal activity is correlated with the level of receptor expression (Samama et al., 1993; MacEwan and Milligan, 1996a). Furthermore, certain ligands (inverse agonists) have the capacity to decrease this basal effector activity (for reviews see Schütz and Freissmuth, 1992; Milligan et al., 1995; Kenakin, 1996; Milligan and Bond, 1997). Initially it was believed that the action of these ligands to decrease basal effector activity was due to competition between the ligand (acting as an antagonist) and an endogenous receptor agonist present in the system. However, the discovery of ligands that bind to the receptor but do not either increase or decrease effector activity (i.e., "true" or "neutral" antagonists with intrinsic efficacy close to zero), suggested that ligands capable of decreasing effector activity have "negative" intrinsic efficacy and actively produce responses opposite to that of agonists (thus the term "inverse" agonist). Further evidence that inverse agonists actively promote decreases in effector activity from interaction with a receptor came from experiments in which the effects of inverse agonists, as well as positive agonists, could be blocked by the neutral antagonists. Thus, the criterion currently used to conclude that a receptor system is constitutively active includes demonstration of effects of inverse agonists which can be blocked in a competitive fashion by neutral antagonists.

These findings lead to the proposal that receptors exist in

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ABBREVIATIONS: 5-HT, serotonin; 5-MXG, 5-methoxygramine; AA, arachidonic acid; BOL, bromo-lysergic acid diethylamide; CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; HBSS, Hanks' balanced salt solution; IP, inositol phosphate; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; 5-HT_{2C}, serotonin type 2C; BSA, bovine serum albumin; PVDF, polyvinylidene fluoride; PBS-T, PBS containing 0.01% Tween-20.

an equilibrium between a variety of conformational states, some of which are spontaneously "active" (i.e., that can interact with effector mechanisms in the absence of ligand). In the simplest model (the two-state model of agonist action; Costa et al., 1992; Samama et al., 1993; Leff, 1995), receptors are proposed to exist in equilibrium between two conformations, an active form (R^*) and an inactive form (R). Basal effector activity is defined, in part, by the absolute level of R^*, which will increase along with increasing receptor density. Agonists act by preferentially binding to and enriching the active conformation, thereby increasing effector activity, whereas inverse agonists bind preferentially to the inactive (R) conformational state, leading to a reduction in "basal" effector activity. Neutral antagonists bind equally well to both R and R^* and thus do not alter the equilibrium between the two states and do not alter effector activity, but because they occupy the receptor, they can block the effects of both agonists and inverse agonists. Typically, inverse agonist effects are most easily detectable in systems where there is a large degree of constitutive receptor activity and consequently high basal effector activity, such as when receptors are overexpressed or are mutated. In systems where basal effector activity is low, inverse agonists usually have no observable effect on basal responses and tend to behave as simple antagonists (i.e., they can block the response to agonists without themselves producing an effect). As a consequence of the frequent use of systems in which constitutive receptor activity is artificially high, there has been considerable debate as to whether inverse agonists have physiological and therapeutically relevant effects or whether they represent merely a pharmacological curiosity (Black and Shankley, 1995; Milligan et al., 1995).

However, evidence is beginning to emerge that suggests that inverse agonists have novel actions that extend beyond simply reducing basal effector activity. There are now several studies in systems with artificially enhanced constitutive receptor activity demonstrating that prolonged (e.g., 24 h) treatment with inverse agonists can lead to increased receptor density (Pei et al., 1994; MacEwan and Milligan, 1996a, b; Smit et al., 1996; Gether et al., 1997; Lee et al., 1997) and enhanced responsiveness (Pei et al., 1994; Lee et al., 1997). Importantly, receptor up-regulation generally does not occur when neutral antagonists are used (MacEwan and Milligan, 1996a, b) and neutral antagonists have been shown to block the effect of inverse agonists (MacEwan and Milligan, 1996b). It is possible that constitutively active receptor systems, just as ligand-dependent receptor activity, activate cellular effector pathways responsible for desensitization and down-regulation. Prolonged treatment with an inverse agonist, by reducing constitutive receptor activity, would permit the system to resensitize and up-regulate receptors.[1]

Here we studied the effects of inverse agonists in cells that express human serotonin type 2C ($5\text{-}HT_{2C}$) receptors at relatively low levels, 250 fmol/mg protein. $5\text{-}HT_{2C}$ receptors couple to at least two distinct cellular signal transduction pathways, phospholipase C (PLC)-mediated inositol phosphate (IP) accumulation and phospholipase A_2 (PLA_2)-mediated arachidonic acid (AA) release (Felder et al., 1990; Berg et al., 1994b, 1996, 1998; Kaufman et al., 1995). In this system, inverse agonists did not reduce basal effector activity but behaved as simple competitive antagonists. However, prolonged treatment with inverse agonists enhanced the responsiveness of the $5\text{-}HT_{2C}$ receptor system to activation by agonists in an effector pathway-dependent manner. The enhanced responsiveness did not involve receptor up-regulation, but may be due to changes in the effector system coupled to the receptor (e.g., G proteins, PLC). Moreover, responses to activation of another receptor system on the cell that is not a direct target for $5\text{-}HT_{2C}$ inverse agonists were enhanced also. These data suggest that inverse agonists may have actions in physiologically relevant systems to regulate heterologous receptor-mediated signal transduction.

Experimental Procedures

Materials. SB 206553 was generously provided by Dr. T. Blackburn (SmithKline Beecham, Harlow, UK). Initial stock solutions (10^{-3} M) were prepared by dissolving in dimethyl sulfoxide (DMSO). G protein α subunit-specific antiserum was kindly provided by Dr. David Manning (University of Pennsylvania). Bromo-lysergic acid diethylamide (BOL) was obtained from the National Institute on Drug Abuse. The following materials were purchased from commercial sources: *myo*-[^{3}H]inositol and [^{3}H]AA from New England Nuclear (Boston, MA); pertussis toxin, 5-HT HCl, clozapine, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane (DOI), ketanserin, lisuride, mesulergine, and mianserin from Research Biochemicals, Inc. (Natick, MA); hygromycin from Calbiochem Corp. (La Jolla, CA); and 5-methoxygramine from Aldrich (Milwaukee, WI). Fetal bovine serum was purchased from Gemini Bioproducts Inc. (Calabasas, CA). All other tissue culture reagents were purchased from GIBCO BRL (Grand Island, NY). Electrophoresis materials were purchased from Bio-Rad (Hercules, CA). All other drugs and chemicals (reagent grade) were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture. Chinese hamster ovary K1 *(CHO-K1)* cell lines that stably express human $5\text{-}HT_{2C}$ receptors at ≈250 fmol/mg ("low" expressing, CHO-1C19) and 5 to 10 pmol/mg ("high" expressing, CHO-1C7) were used in this study. Cells were maintained in α-minimal essential medium supplemented with 5% fetal bovine serum and 300 μg/ml hygromycin. For all experiments, cells were seeded into 12- or 24-well tissue culture vessels at a density of 4×10^4 cells/cm^2. After a 24-h plating period, cells were washed with Hanks' balanced salt solution (HBSS) and placed into Dulbecco's modified Eagle's medium/F-12 [1:1] with 5 μg/ml insulin, 5 μg/ml transferrin, 30 nM selenium, 20 nM progesterone, and 100 μM putrescine (serum-free media) and grown for an additional 24 h before experimentation. We have previously demonstrated the absence of receptor reserve for 5-HT on both effector pathways (PLC and PLA_2) coupled to the human $5\text{-}HT_{2C}$ receptor in CHO-1C19 cells (Berg et al., 1998).

IP Accumulation and AA Release Measurements. IP accumulation and AA release were measured as described previously (Berg et al., 1994a, 1996, 1998). Unless stated otherwise, measurements of PLC-mediated IP accumulation were made from the same multiwell (simultaneously) as PLA_2-AA release measurements (Berg et al., 1998). Briefly, cells in serum-free medium were labeled with 1 μCi/ml [^{3}H]-*myo*-inositol (25 Ci/mmol) for 24 h and with 0.1 μCi/ml [^{3}H]AA (220 Ci/mmol) for 4 h at 37°C. After the labeling period, cells were washed three times with HBSS containing calcium and magnesium, 20 mM HEPES, and 0.1% fatty acid-free bovine serum

[1] It should be noted however, that Gether et al. (1997) reported that agonists, inverse agonists, and neutral antagonists could up-regulate expression of constitutively active mutants of the β2-adrenergic receptor in Sf9 cells when cells were incubated with ligand during the 48-h infection period. Similar results were reported by Samama et al. (1997) for β2 constitutively active mutants expressed in hearts of transgenic mice. These results were interpreted as due to biochemical stabilization of an inherently unstable, constitutively active receptor. Thus, at least in this treatment paradigm with mutant receptors, up-regulation of receptor density may occur through mechanisms other than by reduction in the activity of effector mechanisms involved in down-regulation.

albumin (BSA; experimental medium). Between washes, the cells were incubated for 5 min in a 37°C water bath (15-min total wash and preincubation time). After the wash procedure, cells were incubated in 0.5 ml of experimental medium containing vehicle (H_2O or 0.01% DMSO) or the indicated drug concentrations. For measurement of basal effector activity, cells were incubated at 37°C for 25 min. For measurement of agonist-mediated stimulation of effector activity, cells were incubated at 37°C for 10 min. After incubation, aliquots (100 μl) of cell media were added directly to scintillation vials for measurement of [^{3}H] content (Berg et al., 1996, 1998). The remaining media were aspirated quickly and 1 ml 10 mM formic acid (4°C) was added to extract the accumulated [^{3}H]-IPs (IP_1, IP_2, and IP_3, collectively referred to as IP; Berg et al., 1994a). For some experiments, data were normalized to protein content, which was measured according to the method of Lowry et al. (1951).

Receptor Binding Studies. 5-HT_{2C} receptor saturation binding experiments were done as described previously (Berg et al., 1994a). Briefly, cells were washed twice with HBSS, scraped, and centrifuged at 500*g* for 5 min. Cell pellets were flash frozen in liquid nitrogen and stored at −135°C until use. All membrane preparation procedures were done at 4°C. Cell pellets were thawed, resuspended in 20 volumes of homogenization buffer (50 mM HEPES, 2.5 mM $MgCl_2$, 2.0 mM EGTA pH 7.4 at 22°C), homogenized twice with a polytron (setting no. 7) for 15 s (separated by 15 s), and centrifuged (39,000*g*; 4°C; 10 min). The resulting membrane pellet was washed three times with homogenization buffer and resuspended in assay buffer (homogenization buffer containing 0.1% ascorbic acid) for use in the binding assay. Aliquots (250 μl) of membrane suspension (≈50 μg protein) were incubated (60 min; 37°C; total volume = 500 μl) with 13 concentrations (0.01–40 nM) of [^{3}H]-mesulergine. Nonspecific binding was determined in the presence of 1 μM mianserin. Samples were filtered through polyethyleneimine-coated Whatman GF/C filters (Whatman Inc., Clifton, NJ) with a Brandel Cell Harvester (Brandel Laboratories, Gaithersburg, MD). The filters were washed twice with 1.5 ml ice-cold buffer and counted with a Beckman LS7500 liquid scintillation counter (Beckman Instruments, Berkeley, CA). Protein was determined with the method of Lowry et al. (1951) using BSA as a standard.

G Protein Immunoblots. The method for Western blot analysis of G protein α subunits was similar to procedures described previously (Berg et al., 1994a). Membranes from cells that had been treated with inverse agonist or vehicle were prepared by homogenization in 45 volumes Tris-$MgCl_2$ buffer (pH = 8.0) in a Teflon/glass homogenizer by hand (20 strokes) on ice. After centrifugation (39,000*g*, 10 min, 4°C), pellets were resuspended in Tris-EDTA buffer (pH = 8.0; final protein concentration ≈ 200 μg/ml). After separation on 10% SDS polyacrylamide mini-gels (0.1–10 μg protein/lane), samples were transferred to Immobilon-P polyvinylidene fluoride (PVDF; 0.45 μm; Millipore Corp., Bedford, MA) membranes using a Trans-blot SD electrophoretic transfer cell (Bio-Rad Laboratories, Hercules, CA). The membranes were incubated overnight at 4°C in PBS containing 0.01% Tween-20 (PBS-T), 5% fetal bovine serum, 5% dry milk, and 1% ovalbumin to block nonspecific sites. After several washes with PBS-T supplemented with 0.01% dry milk and 0.01% ovalbumin (wash buffer), membranes were incubated overnight at 4°C with G protein α subunit-specific polyclonal antibodies (Dr. David Manning, University of Pennsylvania) to the pertussis toxin-insensitive G protein α subunits $\alpha_{q/11}$, α_{12}, α_{13}, or α_{16}, or the pertussis toxin-sensitive G protein α subunit αi_1 diluted 1:100 in wash buffer. After incubation with the primary antibody, membranes were washed three times for 15 min with wash buffer followed by 3 × 15-min washes with PBS-T. Horseradish peroxidase conjugated goat-anti-rabbit IgG second antibody (1:3000 dilution in PBS-T; Amersham; Arlington Heights, IL) was added and the membranes were incubated for 1 h at room temperature. After several washes with PBS-T, blots were made visible by chemiluminescence using the Renaissance detection system (New England Nuclear) according to the manufacturer's instructions. Films were digitized using a COHU model 4912 high-performance CCD camera and a computerized image analysis system and bands were quantified with a Macintosh computer using the public domain National Institutes of Health Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). Integrated optical density values of bands from inverse agonist-treated cells were compared with those of control (vehicle-treated) cells that were run on the same gel and data are expressed as a percentage of the control value. To verify that equivalent protein was loaded onto gels, blots were overexposed to visualize nonspecific bands that did not differ between lanes. In addition, for some experiments, blots previously probed with Gαq/α11 antiserum were chemically stripped according to manufacturer's protocols and re-probed with anti-Gαi_1 antiserum.

Data Analysis. Concentration response data were fit with nonlinear regression to the model:

$$E = \frac{E_{max}}{1 + \left(\frac{EC_{50}}{A}\right)^n} \quad (1)$$

where E is the measured response at a given agonist concentration (A), E_{max} = maximal response, EC_{50} = the concentration of agonist producing half-maximal response, and n = slope index.

Calculation of apparent antagonist dissociation constants (K_B) was determined with the equation:

$$K_B = \frac{[B]}{dr - 1} \quad (2)$$

where B is the concentration of the antagonist used and dr represents the ratio (dose ratio) of concentrations (EC_{50}) that produced equivalent responses in the absence and presence of antagonist.

Data from saturation binding studies were analyzed with nonlinear regression analysis. After fitting nonspecific data to the equation describing a straight line with the origin at 0,0 ($y = mx$) to determine m, total binding data were fit to eq. 3 to provide estimates of B_{max}, K_d, and slope factor (n):

$$B = \frac{B_{max}}{\left(\frac{K_d}{[A]} + 1\right)^n} + m \cdot [A] \quad (3)$$

where m is the slope of the linear regression line for nonspecific binding.

Differences between drug treatment and corresponding vehicle controls were evaluated for statistical significance using Student's paired t test. $p < .05$ was considered significant.

Results

Conventional Demonstration of Inverse Agonist Properties. Fig. 1 shows the effects of a series of 5-HT_{2C} ligands, applied at maximal concentrations (100 × K_B or 100 × K_i values), on basal AA release and IP accumulation in cells overexpressing the 5-HT_{2C} receptor (CHO-1C7 cells; 5–10 pmol/mg protein). There were marked differences in the effects of the ligands measured between effector pathways. SB 206553, clozapine, and mianserin each reduced basal AA release (≈−15 to −20%) and IP accumulation (≈−80%), effects which were blocked by the neutral antagonist 5-methoxygramine (5-MXG). Mesulergine and ketanserin reduced basal IP accumulation (≈−40 to −60%) but did not alter basal AA release. BOL and 5-MXG did not alter either basal IP accumulation or AA release. Lisuride did not alter IP accumulation but weakly stimulated AA release (≈10%). As expected, the agonist 5-HT increased both IP accumulation

(≈60%) and AA release (≈40%). Treatment of cells with pertussis toxin (50 ng/ml, 24 h) did not alter basal nor ligand-induced changes in IP accumulation or AA release (data not shown).

Figure 2 shows that cells that overexpress the 5-HT_{2C} receptor (CHO-1C7) have a high level of basal IP accumulation as compared with cells with low receptor expression (≈250 fmol/mg; CHO-1C19). As expected, the inverse agonist SB 206553 did not reduce basal effector activity in CHO-1C19 cells, however, as shown before (Fig. 1), SB 206553 reduced the high level of basal IP accumulation to a level equivalent to that seen in the low-expressing cells. Interestingly, although basal levels of AA release were not significantly different between the high- and low-expressing cells, SB 206553 reduced basal AA release in the former (−20%).

Although SB 206553 did not alter basal IP accumulation or AA release in the low-expressing line, it did interact with the 5-HT_{2C} receptor in these cells. SB 206553 behaved as a simple competitive antagonist, shifting the concentration-response curve to 5-HT for both responses to the right in a parallel and surmountable fashion (Fig. 3). For AA release, the pEC_{50} for 5-HT was 7.28 ± 0.14 (52 nM) and 5.21 ± 0.15 (6.2 μM) in the absence and presence of SB 206553, respectively. Similarly, for IP accumulation, the pEC_{50} for 5-HT was 7.35 ± 0.07 (45 nM) and 5.27 ± 0.07 (5.4 μM) in the absence and presence of SB 206553, respectively. The apparent dissociation constant (K_B) for SB 206553 was 3 nM for both responses. Note also that the EC_{50} for 5-HT was the same between the two pathways as expected for a drug that acts at a single receptor in the absence of receptor reserve.

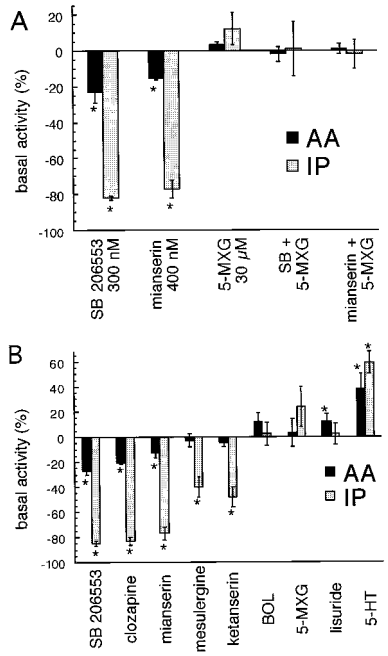

Fig. 1. Effect of 5-HT_{2C} receptor ligands on basal IP accumulation and basal AA release in CHO-1C7 cells. Cells, prelabeled with [^{3}H]AA (4 h) and [^{3}H]-myo-inositol (24 h), were incubated (37°C) with the indicated drugs or vehicle (0.01% DMSO) for 25 min in the presence of 20 mM LiCl and 0.1% BSA. AA release and IP accumulation were measured simultaneously from the same multiwell. Data are expressed as the percent change in basal effector activity. A, basal activity was 3616 dpm ± 195 dpm and 2216 dpm ± 89 dpm for IP accumulation and AA release, respectively. Data represent mean ± S.E.M. of 3 experiments. *$p < .05$ compared with paired vehicle. B, drug concentrations (100 × K_i or K_B values) were as follows: SB 206553, 300 nM; clozapine, 1 μM; mianserin, 400 nM; mesulergine, 100 nM; ketanserin, 1 μM; BOL, 700 nM; 5-MXG, 3 μM; lisuride, 2 μM; *d*-LSD, 1 μM; 5-HT, 3 μM. Basal activity was 3052 dpm ± 313 dpm and 3835 dpm ± 553 dpm for IP and AA, respectively. Data represent mean ± S.E.M. of 4 to 8 experiments. Treatment with pertussis toxin (24 h, 50 ng/ml) did not alter basal activity or ligand-induced changes in basal activity. *$p < .05$ compared with paired vehicle.

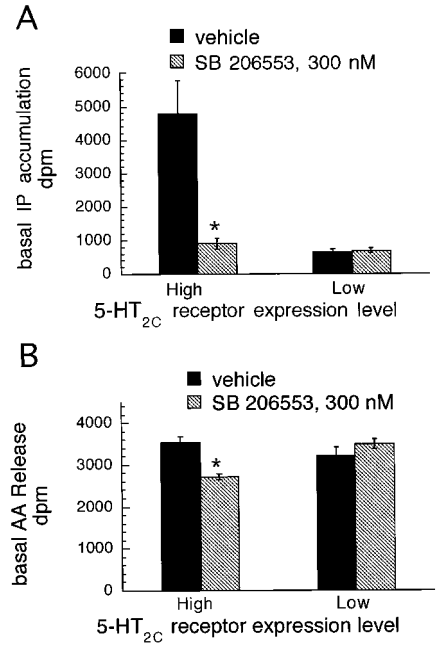

Fig. 2. Comparison of basal effector activity and inverse agonist activity between cells with low (CHO-1C19) or high (CHO-1C7) 5-HT_{2C} receptor expression. Cells, prelabeled with [^{3}H]AA (4 h) and [^{3}H]-myo-inositol (24 h), were incubated (37°C) with the vehicle (0.01% DMSO) or SB 206553 (300 nM) for 25 min in the presence of 20 mM LiCl and 0.1% BSA. Data are expressed as total dpm/well and represent mean ± S.E.M. of four experiments. Normalization of dpm to cell protein content did not change the relative differences in basal activity, indicating that the differences in basal levels between the cell lines was not due to differences in cell densities. *$p < .05$ compared with paired vehicle.

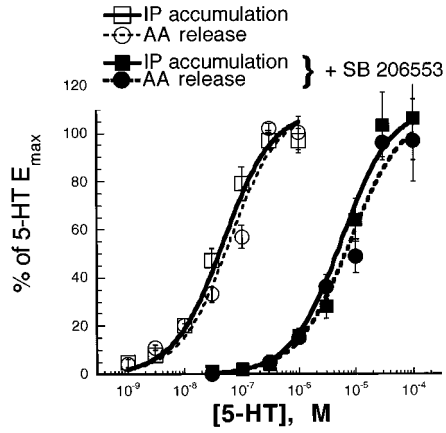

Fig. 3. The effect of acute treatment with SB 206553 in the low-expressing 5-HT_{2C} receptor cell line (CHO-1C19). Cells, prelabeled with [^{3}H]AA (4 h) and [^{3}H]-myo-inositol (24 h), were preincubated (37°C) with the vehicle (0.01% DMSO) or SB 206553 (300 nM) for 15 min. AA release and IP accumulation were measured simultaneously after 10 min of incubation with different concentrations of 5-HT in the presence of 20 mM LiCl and 0.1% BSA. Individual concentration response curves were fit to a logistic equation to determine E_{max}, EC_{50}, and slope parameters. Data are expressed as the percentage of the paired control response and represent the mean ± S.E.M. of five experiments. For these experiments, 5-HT mediated maximal IP accumulation and AA release was 234% ± 28% and 241% ± 26%, respectively.

Mianserin and 5-MXG also behaved as simple competitive antagonists with K_B values of 4 and 200 nM, respectively.

Novel Actions of Inverse Agonists. Although SB 206553 did not alter basal effector activity in the low-expressing CHO-1C19 cells, prolonged treatment with SB 206553 (24 h, 300 nM) selectively enhanced 5-HT_{2C} receptor-mediated IP accumulation (Fig. 4). SB 206553 treatment produced an increase in the maximal IP response to the 5-HT_{2C} agonist DOI with no change in potency. Interestingly, 5-HT_{2C}-mediated AA release was not altered by SB 206553 treatment, indicating that the effect of SB 206553 was effector pathway-dependent. In the high-expressing CHO-1C7 cells, treatment with SB 206553 also increased the maximal IP accumulation response to DOI (81% ± 8% versus 336% ± 26%, vehicle and 300 nM SB 206553, respectively, mean % above basal ± S.E.M., $n = 3$; $p = .005$) with no change in potency [pEC_{50} = 7.16 ± 0.21 (69 nM) versus 7.93 ± 0.30 (117 nM) vehicle and 300 nM SB 206553, respectively; $p = .11$]. Treatment of CHO-1C7 cells with SB 206553 did not alter DOI-mediated AA release (data not shown).

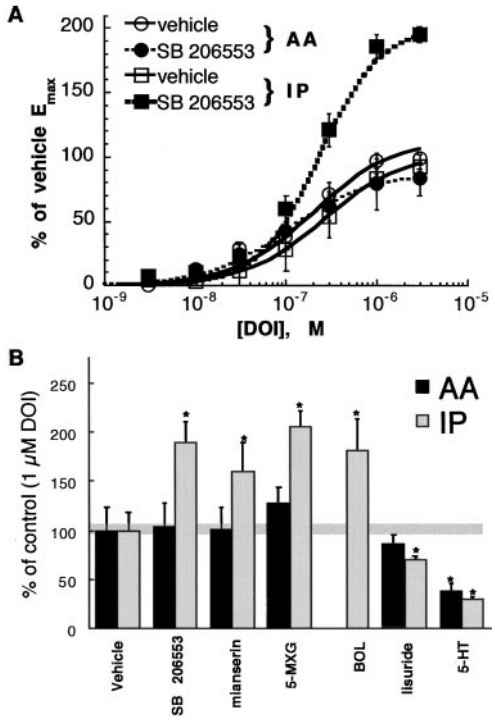

Fig. 4. Effect of 24-h treatment with SB 206553 on 5-HT_{2C} receptor-mediated AA release and IP accumulation in the low-expressing 5-HT_{2C} receptor cell line (CHO-1C19). A, CHO-1C19 cells were incubated for 24 h with vehicle (0.01% DMSO) or SB 206553 (300 nM). After treatment, cells were washed thoroughly and AA release and IP accumulation in response to various concentrations of the 5-HT_{2C} receptor agonist, DOI (10 min, 37°C), were measured. Individual concentration response curves were fit to a logistic equation to determine E_{max}, EC_{50}, and slope parameters. Data are expressed as the percentage of the paired control E_{max} and represent mean ± S.E.M. of five experiments. For IP accumulation the E_{max} and pEC_{50} for DOI was 235% above basal ± 60% versus 474% above basal ± 83% and 6.57 ± 0.12 (267 nM) versus 6.63 ± 0.13 (236 nM), vehicle and SB 206553, respectively. For AA release the E_{max} and pEC_{50} for DOI was 186% above basal ± 22% versus 165% above basal ± 30% and 6.69 ± 0.19 (202 nM) versus 6.97 ± 0.19 (106 nM), respectively. B, CHO-1C19 cells were treated for 24 h with various 5-HT_{2C} ligands at concentrations (100 × K_i or K_B values) as indicated in the legend of Fig. 1. Cells were washed thoroughly and DOI (1 μM)-stimulated AA release and IP accumulation were measured. Each column represents the mean ± S.E.M. of three to six experiments. None of the drugs tested altered basal AA or basal IP accumulation. DOI-mediated AA release was 198% above basal ± 47% and DOI-mediated IP accumulation was 352% above basal ± 63% (mean ± S.E.M., n = 6). *$p < .05$ as compared with paired vehicle control.

Figure 4B shows the effects of 24-h treatment with a series of 5-HT_{2C} ligands in the low-expressing cell line. Mianserin, like SB 206553, selectively enhanced DOI-mediated IP accumulation. The effects of SB 206553 and mianserin were insensitive to treatment of cells with pertussis toxin (50 ng/ml; 24 h). Interestingly, 5-MXG, which appeared to be a neutral antagonist in the high-expressing cells, also selectively enhanced 5-HT_{2C}-mediated IP accumulation in the low-expressing cells. Similarly, BOL, which has been reported to be a neutral antagonist at 5-HT_{2C} receptors (Barker and Sanders-Bush, 1993; Barker et al., 1994), and had no effect on reduction of basal effector activity in the high-expressing cell line (Fig. 1), also enhanced DOI-mediated IP accumulation after a 24-h exposure (Fig. 4B). In contrast, 24-h treatment with the agonists, lisuride, and 5-HT resulted in decreased responsiveness of the IP accumulation pathway. Because in this experimental paradigm 5-MXG and BOL produced effects qualitatively similar to those of the inverse agonists SB 206553 and mianserin (and thus appeared not to be neutral antagonists), we could not use these ligands to attempt to block the SB 206553 effect. Lisuride, however, appeared to be a weak agonist in this system (Figs. 1 and 4B) and was effective in blocking the enhanced responsiveness produced by SB 206553. The increase in DOI-mediated IP accumulation was reduced from 179% ± 4% of control in the presence of 300 nM SB 206553 to 88% ± 8% of control in the presence of SB 206553 and 2 μM lisuride (mean ± S.E.M., $n = 3$; $p < .01$).

The effect of SB 206553 to enhance the responsiveness of the 5-HT_{2C} receptor system in CHO-1C19 cells was evident as early as 4 h of treatment (Fig. 5A). DOI-mediated IP accumulation was increased by about 50% after 4 h of treatment with SB 206553, whereas DOI-mediated AA release was not changed. 5-MXG, which enhanced DOI-mediated IP accumulation after 24 h of treatment, did not enhance the IP response after 4 h, but blocked the effect of SB 206553. The enhancing effect of 24 h of treatment with SB 206553 was fully reversible within 3 to 4 h of washout of SB 206553 (Fig. 5B).

Recent studies have demonstrated that prolonged exposure to inverse agonists can cause receptor up-regulation (Pei et al., 1994; MacEwan and Milligan, 1996a, b; Smit et al., 1996; Lee et al., 1997). To determine if receptor up-regulation was the mechanism for the enhanced responsiveness of the 5-HT_{2C} receptor system, we examined the binding characteristics of the 5-HT_{2C} receptor antagonist, [^{3}H]-mesulergine after treatment of CHO-1C19 cells with SB 206553 for 24 h. Neither the B_{max} nor the K_d for [^{3}H]-mesulergine was changed in response to SB 206553 treatment. Values for B_{max} and pK_d were 260 ± 58 versus 233 ± 49 fmol/mg protein and 8.94 ± 0.16 (1.3 nM) versus 8.90 ± 0.13 (1.3 nM) for vehicle versus SB 206553-treated cells respectively ($n = 3$).

Because reductions in levels of G proteins have been reported in response to prolonged exposure to agonists (Mullaney et al., 1993; Shah and Milligan, 1994), we tested the hypothesis that treatment with inverse agonists would increase G protein levels. 5-HT_{2C} receptor-mediated IP accumulation and AA release are insensitive to treatment with pertussis toxin (Berg et al., 1998), indicating that G proteins of the G_q and/or $G_{12/13}$ family likely mediate these responses (Fields and Casey, 1997). Western analysis of G proteins in membranes prepared from CHO-1C19 cells using α subunit-

selective antibodies revealed the expression of $\alpha q/_{11}$, α_{13}, and low levels of α_{12}. As shown in Fig. 6, in the low-expressing cells (CHO-1C19), 24-h treatment with SB 206553 produced an increase in immunoreactivity for $\alpha q/_{11}$(29% ± 9% above controls, mean ± S.E.M.; $n = 3$) that was blocked in the presence of lisuride. SB 206553 treatment also increased levels of α_{12} (28% ± 9%) and resulted in the appearance of a second band under α_{13} suggesting the possibility of altered post-translational processing of α_{13} (data not shown). In comparison with CHO-1C19 cells, 24-h treatment of the high-expressing cell line (CHO-1C7) with SB 206553 produced a greater increase in $\alpha q/_{11}$ immunoreactivity of 90% ± 16% above controls (mean ± S.E.M.; $n = 6$), which is consistent with the larger enhancement of agonist-mediated IP accumulation detected in CHO-1C7 cells versus that of CHO-1C19 cells. Treatment for 24 h with the inverse agonists mianserin and clozapine also produced an increase in $\alpha q/_{11}$ immunoreactivity (53% ±5% and 52 ± 8% above controls, mianserin, and clozapine, respectively, mean ± S.E.M.; $n = 3$) in the high-expressing cell line. In contrast, inverse agonist treatment had no effect on the expression of Gαi1 in either CHO-1C19 or CHO-1C7 cells, which is consistent with the fact that inverse agonist-mediated reduction in basal activity and enhancement of agonist-mediated IP accumulation were insensitive to prior treatment with pertussis toxin (data not shown).

Figure 7 shows that in addition to enhancing the responsiveness of the 5-HT_{2C} receptor system to activation by agonists, 24-h treatment of CHO-1C19 cells with SB 206553 or clozapine enhanced the responsiveness of the endogenously expressed purinergic P_2 receptor to activation by ATP. As with enhanced 5-HT_{2C} receptor responsiveness, ATP-mediated IP accumulation was increased with no change in AA release after inverse agonist treatment. Similar enhancement of ATP-mediated IP accumulation occurred in the high-expressing cell line after SB 206553 treatment (data not shown). In contrast, treatment of nontransfected (parent) CHO-K1 cells with SB 206553 for 24 h had no effect on ATP-mediated IP accumulation, indicating that the effect of SB 206553 treatment on responsiveness of the purinergic receptor system required the presence of 5-HT_{2C} receptors.

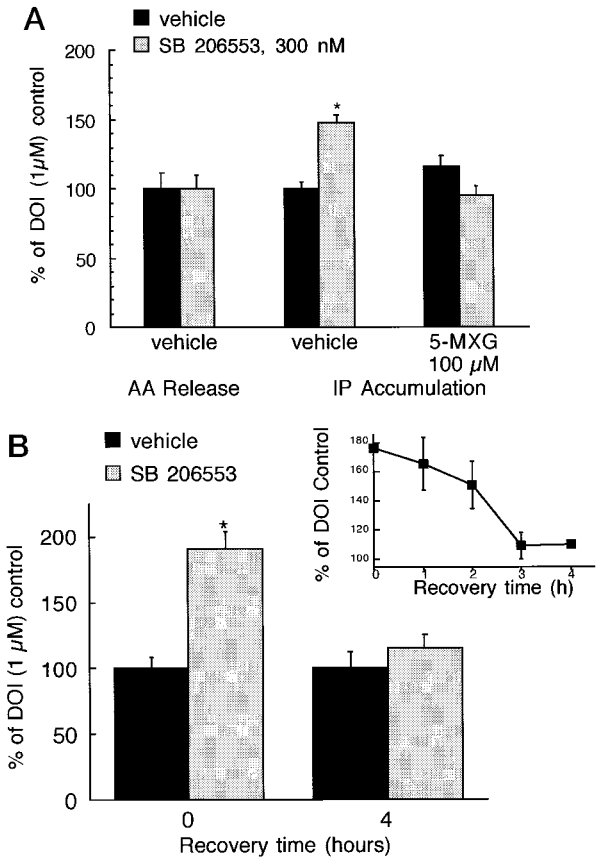

Fig. 5. Time course of the effect of SB 206553 on DOI-mediated AA release and IP accumulation in the low-expressing 5-HT_{2C} receptor cell line. A, effect of SB 206553 on DOI-mediated IP accumulation occurs as early as 4 h of treatment. CHO-1C19 cells were incubated with vehicle (0.01% DMSO) or 300 nM SB 206553 in the absence and presence of the antagonist, 5-MXG, for 4 h. Cells were then washed thoroughly and DOI (1 μM)-stimulated AA release and IP accumulation were measured. Data are expressed as the percentage of control DOI response and represent the mean ± S.E.M. of three to six experiments. Basal AA release was 987 dpm ± 35 and DOI-mediated AA release was 88% ± 11% above basal (mean ± S.E.M., $n = 3$). Basal IP accumulation was 669 dpm ± 31 dpm and DOI-mediated IP accumulation was 194% ± 11% above basal (mean ± S.E.M., $n = 6$). *$p < .05$ as compared with paired vehicle control. Basal activity was unchanged by treatment with SB 206553 or 5-MXG, either alone or together. B, enhancing effect of 24-h treatment with SB 206553 on DOI-stimulated IP accumulation reverses within 3 to 4 h. CHO-1C19 cells were treated for 24 h with 300 nM SB 206553, washed, and further incubated (37°C) for 0 to 4 h (recovery time). Data are expressed as the percentage of control DOI response and represent the mean ± S.E.M. of three experiments. Data shown in figure inset are mean ± S.D. of two experiments. Basal IP accumulation was 541 dpm ± 52 dpm and DOI-stimulated IP accumulation was 243% above basal ± 17%. *$p < .01$ as compared with paired vehicle control.

Discussion

Typically, the inverse agonist properties of a drug are detectable most easily in systems where a large degree of constitutive receptor activity exists, such as when receptors are overexpressed or are mutated (for reviews see Schütz and

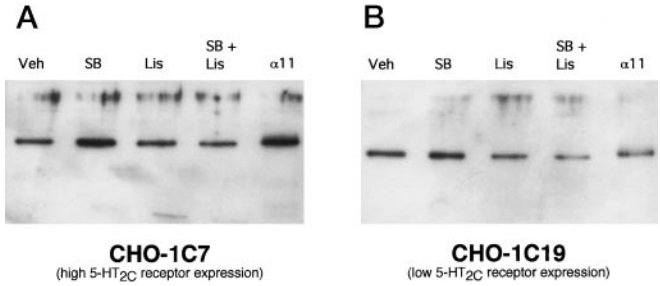

Fig. 6. Western analysis of Gαq/11 protein expression in high-expressing CHO-1C7 (A) and low-expressing CHO-1C19 (B) cells after 24-h treatment with SB 206553. Cells were treated for 24 h with 300 nM SB 206553 or vehicle (0.01% DMSO). Cell membrane proteins (0.2–2 μg protein/lane) were resolved by 10% SDS-polyacrylamide gel electrophoresis, transferred to 0.45 μm PVDF membranes, and probed with antibodies selective for G protein αq/11 subunits. Lane 1 represents vehicle-treated cells, Lane 2 represents cells treated with SB 206553, Lane 3 shows cells treated with lisuride alone, and Lane 4 shows cells treated with SB 206553 and lisuride. Recombinant α11 (Calbiochem) was loaded into lane 5 and used for reference. In this experiment, 2 μg of CHO-1C7 membrane protein was loaded per lane, whereas for CHO-1C19 cells, 0.2 μg of membrane protein was loaded. To verify that equivalent amounts of protein were loaded in each lane, blots were overexposed to visualize nonspecific bands that did not differ between lanes. Additionally, there was no difference in the levels of Gαi1 in PVDF membranes that were chemically stripped and reprobed with antibodies selective for Gαi1. SB-206553 treatment produced an increase in $G\alpha_{q/11}$ immunoreactivity, which was blocked in the presence of lisuride. Data shown are representative of three to six separate experiments.

Freissmuth, 1992; Milligan et al., 1995; Kenakin, 1996; Milligan and Bond, 1997). Accordingly, there has been considerable debate over whether inverse agonists have physiological effects and therapeutic potential in systems where nonmutated receptors are expressed at "normal" levels (Black and Shankley, 1995; Milligan et al., 1995). We report here that 5-HT_{2C} receptor inverse agonists enhance the responsiveness of 5-HT_{2C} and purinergic P_2 receptor systems in a cell line (CHO-1C19) where 5-HT_{2C} receptors are not overexpressed or mutated.

To assess inverse agonist properties of several 5-HT_{2C} receptor ligands, we first used the conventional method of measuring ligand-induced reductions in basal effector activity. Using a cell line (CHO-1C7) that overexpresses the human 5-HT_{2C} receptor at high levels (5–10 pmol/mg protein) and in which basal IP accumulation is high, we demonstrated that 5-HT_{2C} ligands, SB 206553, clozapine, mianserin, mesulergine, and ketanserin behave as inverse agonists. These ligands each reduced basal IP accumulation with rank order of inverse activity of SB 206553 = clozapine = mianserin > mesulergine = ketanserin. The effect of these ligands to reduce basal effector activity was effector pathway-dependent, being much greater for IP accumulation than for AA release. The reduction in basal IP accumulation and AA release produced by SB 206553 and mianserin could be blocked by the neutral antagonist 5-MXG, confirming the inverse agonist nature of these ligands. Although mianserin, mesulergine, clozapine, and ketanserin have been demonstrated previously to be inverse agonists at 5-HT_{2C} receptors (Barker et al., 1994; Labrecque et al., 1995), SB 206553 has only been characterized as a 5-HT_{2C} receptor antagonist (Kennett et al., 1996).

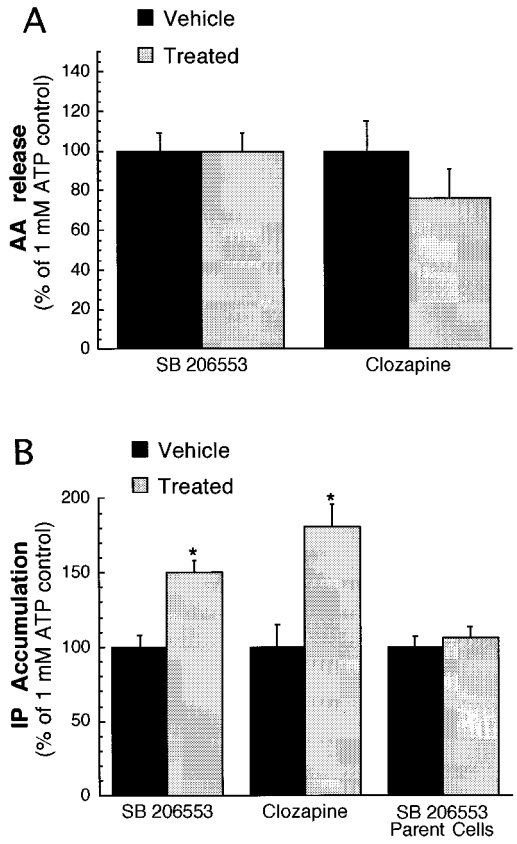

Fig. 7. Effect of 5-HT_{2C} receptor inverse agonists, SB 206553 and clozapine, on P_2-purinergic receptor-mediated AA release and IP accumulation in CHO-1C19 cells. CHO-1C19 cells or parent CHO-K1 cells were incubated with vehicle (0.01% DMSO), SB 206553 (300 nM), or clozapine (1 μM) for 24 h before measurement of ATP (1 mM)-mediated AA release (A) and IP accumulation (B). Data are expressed as the percentage of control ATP response and represent the mean ± S.E.M. of four to seven experiments. Basal AA release was 1482 dpm ± 101 and ATP-mediated AA release was 57% above basal ± 7% (mean ± S.E.M., $n = 4$). In CHO-1C19 cells, basal IP accumulation was 424 dpm ± 31 and ATP-mediated IP accumulation was 222% above basal ± 37% (mean ± S.E.M., $n = 7$). For CHO-K1 parent cells, basal IP accumulation was 540 dpm ± 31 and ATP-mediated IP accumulation was 153% above basal ± 8% (mean ± S.E.M., $n = 6$). *$p < .05$ as compared with paired vehicle control.

The difference in the capacity of inverse agonists to reduce basal IP accumulation versus that of basal AA release suggests that there is a difference in the level of constitutive receptor activity between these two pathways in CHO-1C7 cells. The finding that the increase in basal IP accumulation between CHO-1C7 and CHO-1C19 cells is considerably greater than that for AA release is also consonant with this idea. Other experimental evidence for effector pathway dependence of constitutive receptor activity comes from Perez and colleagues, who report that a mutation of the α_{1B} adrenergic receptor (C128F) results in constitutive activation of PLC but not PLA_2 (Perez et al., 1996). Similarly, mutation of the β2-adrenoceptor (C116F) results in constitutive activity for Na^+/H^+ exchange and not for cAMP production (Zuscik et al., 1998). Pathway-dependent constitutive receptor activity suggests that there are differences in the coupling efficiency between a receptor and its effector pathways and/or there are multiple active conformational states of the receptor, each with its own level of constitutive activity and which couple to an effector pathway. Leff et al. (1997) have recently proposed a three-state model of receptor activation in which receptors can exist in two active conformational states (R* and R**), each of which activates an effector pathway. Within this framework, effector pathway-dependent constitutive activity within the 5-HT_{2C} receptor system would suggest that the equilibrium between R and R^* (for PLC) and R^{**} (for PLA_2) is such that there is considerably more R^* than R^{**}.

As expected, ligands that behaved as inverse agonists in a system artificially optimized to detect inverse agonist properties (overexpressed receptors in CHO-1C7 cells) behaved as simple competitive antagonists in a system that expressed the 5-HT_{2C} receptor at a relatively low level (CHO-1C19 cells). Historically, these data would be interpreted as demonstration that the 5-HT_{2C} receptor system in CHO-1C19 cells has little, if any, constitutive activity. Examples, such as this, where ligands may exhibit inverse agonism in systems with overexpressed receptors, but simple competitive antagonism in systems with receptors expressed at natural levels are common and support the notion that inverse agonists may be simply a pharmacological curiosity (because they behave as simple competitive antagonists in physiologically relevant systems).

Although inverse agonists did not reduce basal effector activity in the low-expressing cell line, they did have actions attributable to their inverse agonist properties. Twenty-four-hour treatment of CHO-1C19 cells with the inverse agonists SB 206553 and mianserin enhanced the responsiveness of the 5-HT_{2C} receptor system to activation by agonist. The effect was selective for the PLC effector pathway and could be blocked with lisuride. Interestingly, although 5-MXG and BOL appeared to be neutral antagonists using the conven-

tional method in the high-expressing 5-HT_{2C} receptor cell line (i.e., they did not reduce basal IP accumulation), 24-h treatment with these ligands enhanced 5-HT_{2C} agonist-induced IP accumulation in the low-expressing cell line in a manner similar to that of the inverse agonists SB 206553 or mianserin. Thus, 5-MXG and BOL may have weak inverse agonist properties that are revealed after prolonged treatment of cells. This suggests that this prolonged treatment method may be a more sensitive measure of a drug's inverse agonist properties than the conventional measure of reduction of basal effector activity.

In some receptor systems with artificially high levels of constitutive receptor activity, prolonged treatment with inverse agonists results in enhanced responsiveness (Pei et al., 1994; Lee et al., 1997) and receptor up-regulation (Pei et al., 1994; MacEwan and Milligan, 1996a, b; Smit et al., 1996; Lee et al., 1997). This has led to the idea that ligand-independent receptor activity, just as ligand-dependent receptor activity, can activate cellular effector pathways responsible for desensitization and down-regulation. Treatment with inverse agonists, by reducing constitutive receptor activity, permits the receptor system to resensitize, which is observed as enhanced responsiveness and/or receptor up-regulation. Because prolonged treatment of CHO-1C19 cells with inverse agonists enhanced the responsiveness of the 5-HT_{2C} receptor system, this suggests that even at low levels of receptor expression, there may exist a level of constitutive receptor activity capable of producing partial desensitization.

Although previous studies have demonstrated that prolonged treatment with inverse agonists can produce receptor up-regulation (Pei et al., 1994; MacEwan and Milligan, 1996a, b; Smit et al., 1996; Lee et al., 1997), the mechanism for the enhanced responsiveness in CHO-1C19 cells did not involve changes in receptor density. We found no change in [^{3}H]-mesulergine affinity or 5-HT_{2C} receptor density in response to inverse agonist treatment. This is consistent with the effector pathway-dependent nature of the inverse agonist effect. Given the lack of receptor reserve for 5-HT in CHO-1C19 cells (Berg et al., 1994b, 1998), we would expect a change in receptor density to be conveyed equally to both effector pathways coupled to the receptor and visualized as changes in the maximal response to a partial agonist such as DOI. Instead, the mechanism may involve, at least in part, changes in the expression of the G proteins, $G_{\alpha q/11}$, $G_{\alpha 12}$, and/or $G_{\alpha 13}$. Because decreased expression of G protein subunits may be a mechanism for agonist-induced desensitization (Lohse, 1993), the most plausible mechanism for inverse agonist-induced enhanced responsiveness of 5-HT_{2C} receptor-mediated IP accumulation in CHO-1C19 cells is the increased expression of $G_{\alpha q/11}$, which are known to couple many receptors to phosphoinositide-specific PLC isoforms (Rhee and Bae, 1997). An increase in the levels of $G_{\alpha q/11}$ could enhance the efficiency of 5-HT_{2C} receptor-G protein coupling, resulting in an increase in agonist-stimulated IP accumulation. $G_{\alpha 12}$ and/or $G_{\alpha 13}$ family G proteins are generally not thought to couple receptors to PLC but instead appear to regulate low molecular weight GTPases in the Ras superfamily such as the Rho-, Ras-, and Rac-dependent signaling pathways (Wadsworth et al., 1997). If these G proteins participate in the enhancement of 5-HT_{2C}-mediated IP accumulation, cross talk regulation of $G_{q/11}$ signaling cascades could be involved (Offermanns and Simon, 1996). Alternatively, the mechanism for the inverse agonist effects shown here could involve changes in the enzyme PLC itself. Inhibition of protein kinase C (PKC) has been shown to enhance receptor-mediated PLC activity (Cockcroft and Thomas, 1992; Rhee and Bae, 1997; Berg et al., 1998), suggesting a negative feedback relationship between PKC and PLC. Although it is possible that 5-HT_{2C} inverse agonists may increase the sensitivity of PLC by reducing PLC-mediated activation of PKC this seems unlikely because we have found that PKC inhibition has no effect on 5-HT_{2C} receptor-mediated IP accumulation (Berg et al., 1998). On the basis of these results, we hypothesize that a fraction of 5-HT_{2C} receptors in CHO-1C19 cells are constitutively active, leading to a level of constitutive, partial desensitization of the receptor system that involves changes in the effector system (e.g., G protein, PLC), as opposed to receptor down-regulation. By reducing constitutive receptor activity, inverse agonists can relieve the constitutive desensitization and allow the system to resensitize, which appears as enhanced responsiveness.

The present results demonstrate that treatment with inverse agonists alters subsequent responsiveness of the 5-HT_{2C} receptor system to activation by agonist. Recently Chidiac et al. (1996) showed that treatment with an agonist could alter subsequent responsiveness of the β_2-adrenergic receptor system to inverse agonists. In Sf9 cells expressing the human β_2-adrenergic receptor, desensitization produced by treatment with isoproterenol resulted in enhanced inhibitory actions of inverse agonists in a manner that was inversely proportional to their intrinsic activity as inverse agonists. These data reinforce the notion that the physiological state of a receptor system or cell at the time of drug application can influence the efficacy of drugs.

For several years it has been recognized that certain "antagonists" at 5-HT_{2C} receptors were capable of producing "atypical" down-regulation of the receptors. In vivo, chronic treatment (72, but not 24 h) of rats with 5-HT_{2C} ligands characterized as antagonists such as mianserin, ritanserin, metergoline, methiothepin, methysergide, and cyproheptadine, results in decreased density of 5-HT_{2C} binding sites in choroid plexus and spinal cord (Sanders-Bush and Breeding, 1988; Pranzatelli et al., 1993). Similar effects on 5-HT_{2C} receptor density were also found after treatment (72, but not 24 h) of primary cultures of rat choroid plexus epithelial cells with mianserin, but not BOL (Barker and Sanders-Bush, 1993; Barker et al., 1994). Although originally thought to be due to inverse agonism of the ligands (Barker et al., 1994), subsequent work showed that the down-regulation of 5-HT_{2C} receptors was not correlated with inverse agonist properties of the ligands (Labrecque et al., 1995). Recent evidence suggests that a ligand can promote receptor conformations that predispose a receptor to down-regulation independently from its ability to activate/inactivate receptor-mediated signaling (Keith et al., 1996; Roettger et al., 1997). The atypical down-regulation of 5-HT_{2C} receptors appears to require treatment of longer than 24 h. It is possible that there may be a time-dependent, biphasic action of some of 5-HT_{2C} ligands, such as mianserin, in which enhanced responsiveness due to inverse agonism is followed by receptor down-regulation.

Although the finding that 5-HT_{2C} inverse agonists enhance 5-HT_{2C} receptor system responsiveness suggests that inverse agonists may have actions in systems where receptors are not overexpressed, they still do not greatly alter the balance of

opinion concerning the debate over physiological/therapeutic relevance versus pharmacological curiosity. This is because enhanced responsiveness would be observed only after the inverse agonist is removed from the system. While present, it would function to occupy and block, if not actively reduce, receptor activation, thereby negating any enhanced responsiveness. Nonetheless, there may be some physiological/therapeutic impact in situations where a patient is treated chronically with a drug that is an inverse agonist (such as clozapine for treatment of schizophrenia or betaxolol for hypertension). Abrupt cessation of treatment might result in supersensitivity to the endogenous agonist and exacerbation of symptoms (Milligan et al., 1995; Milligan and Bond, 1997), suggesting that drug withdrawal should be gradual.

Perhaps the most exciting result of this study is that prolonged exposure to 5-HT_{2C} inverse agonists produced enhanced responsiveness of another receptor system (the purinergic P_2 receptor system) expressed endogenously in CHO cells. Like 5-HT_{2C} receptors, the purinergic P_2 receptor in CHO cells is coupled to both PLC-IP accumulation and PLA_2-AA release (Berg et al., 1994b, 1996). Twenty-four-hour treatment of CHO-1C19 cells with the 5-HT_{2C} inverse agonists SB 206553 or clozapine increased the capacity of ATP to stimulate IP accumulation without altering ATP-mediated AA release. Treatment of parent CHO-K1 cells with SB 206553 for 24 h did not affect ATP-mediated IP accumulation, indicating that the enhancement of purinergic receptor function requires interaction of the inverse agonist with the 5-HT_{2C} receptor. Our working hypothesis is that the action of 5-HT_{2C} inverse agonists to enhance purinergic receptor function is the result of either: 1) a reduction in heterologous desensitization of the purinergic receptor system elicited by the constitutive activity of the 5-HT_{2C} receptor system or 2) changes in components of the signaling system (e.g., G protein, PLC) that are shared by the two receptor systems. Data such as these could mean that cellular actions of inverse agonists may be mediated through changes in receptor systems that are not direct targets for these drugs.

Because the action of the inverse agonist to enhance responsiveness of the purinergic receptor system is through its interaction with the 5-HT_{2C} receptor, the inverse agonist does not have to be removed for the effect to occur. Thus, the therapeutic mechanism of action of drugs previously thought to be simple antagonists, but which are in fact inverse agonists, may not be related solely to their properties as antagonists at their target receptors but rather, indirect actions on other coexpressed receptor systems may be involved as well.

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