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Inverse Agonism and Constitutive Activity as Functional Correlates of Serotonin h5-HT_{1B} Receptor/G-Protein Stoichiometry

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ABSTRACT

This study evaluated the influence of receptor/G-protein (R:G) stoichiometry on constitutive activity and the efficacy of agonists, partial agonists, and inverse agonists at human (h) 5-hydroxytryphamine 1B (5-HT_{1B}) receptors. Two Chinese hamster ovary cell lines were used; they expressed 8.5 versus 0.4 pmol h5-HT_{1B} receptors/mg (determined by [^{3}H]GR125,743 saturation analysis) and 3.0 versus 1.5 pmol receptor-activated G-proteins/mg [determined by guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) isotopic dilution], respectively. Thus, they displayed R:G ratios of ~3.0 (RGhigh) and ~0.3 (RGlow), respectively. In competition-binding experiments, the agonists, 5-HT and sumatriptan, displayed fewer high-affinity (HA)-binding sites and the partial agonists, BMS181,101 and L775,606, displayed decreased affinity in RGhigh versus RGlow membranes. In contrast, the inverse agonists, SB224,289 and, to a lesser extent, methiothepin, showed increased affinity. In G-protein activation experiments, both basal and 5-HT-activated [^{35}S]GTPγS binding were higher in RGhigh than in RGlow membranes. Constitutive activity (determined by inhibition of basal [^{35}S]GTPγS binding with GTPγS in the absence of receptor ligands) was more pronounced in RGhigh versus RGlow membranes, as revealed by the >5-fold greater proportion of HA sites. Correspondingly, the negative efficacy of inverse agonists was strikingly augmented, inasmuch as they suppressed approximately two-thirds of HA [^{35}S]GTPγS binding in RGhigh membranes, but only approximately one-third in RGlow membranes. Furthermore, the efficacy of partial agonists was greater at RGhigh versus RGlow membranes, as estimated by their ability to enhance [^{35}S]GTPγS binding. In conclusion, an increase in R:G ratios at h5-HT_{1B} receptors was associated with an increase in relative efficacy of partial agonists and, most notably, an increase in both constitutive G-protein activation and negative efficacy of inverse agonists.

In addition to characterization of the pharmacological profiles of cloned, G-protein-coupled receptors, studies of recombinant cell lines have enabled the exploration of cellular parameters that influence diverse signal transduction pathways. For example, in NIH-3T3 fibroblasts, agonist efficacies increased with augmentation of h5-HT_{1A} receptor expression levels (Varrault et al., 1992), a finding corroborated by studies in other cell lines (Newman-Tancredi et al., 1997; Schoeffter et al., 1997). Conversely, irreversible receptor inactivation by the alkylating agent, *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, reduced agonist efficacies at heterologously expressed h5-HT_{1B}, h5-HT_{1D}, and hD_3 receptors (Adham et al., 1993; Zgombick et al., 1996; Newman-Tancredi et al., 1999b). Other factors are also important in determining agonist efficacies at G-protein-coupled receptors. Thus, Fargin et al. (1989) reported that the extent of the 5-HT-induced decrease in forskolin-stimulated cAMP levels in four HeLa cell lines did not correlate with h5-HT_{1A} receptor expression levels, suggesting that signal transduction characteristics other than receptor density are of importance. Indeed, the efficacy of partial agonists at μ-opioid receptors expressed in Chinese hamster ovary (CHO) cells is related to the stoichiometric ratio of receptors to G-proteins (Selley et al., 1998). In a similar vein, altering receptor/G-protein (R:G) stoichiometry by G-protein coexpression increased the efficacies of agonists at muscarinic receptors (Burnstein et al., 1995) and constitutive activation of *alpha*-2A adrenoceptors (Pauwels et al., 2000). Moreover, the dopamine D_4 receptor ligand, L745,870, originally characterized as an antagonist (Patel et al., 1997), was later reported to be an agonist in different cellular expression systems, an observation attributed to differences in R:G stoichiometry (Gazi et al., 1999; P. Schoeffter, personal communication).

The above considerations show that quantification of both

ABBREVIATIONS: 5-HT_{1A}, 5-hydroxytryphamine 1A; CHO, Chinese hamster ovary; R:G, receptor/G-protein; [^{35}S]GTPγS, guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate; LA, low affinity; HA, high affinity; RGlow, CHO-h5-HT_{1B} membranes exhibiting low R:G stoichiometry; RGhigh, CHO-h5-HT_{1B} membranes exhibiting high R:G stoichiometry; E_{max}, maximal efficiency; h, human.

receptor and G-protein expression levels is important for the appropriate interpretation of drug influences on signal transduction mechanisms (Kenakin, 1997b). However, previous studies have focused on the influence of R:G coupling on agonist effects, whereas the influence of R:G stoichiometry on negative efficacy is relatively uncharacterized. Indeed, to our knowledge, only one study (at 5-HT$_{1A}$ receptors), has investigated the influence of R:G ratios on the efficacy of inverse agonists at serotonergic receptors (Newman-Tancredi et al., 1997).

To address these issues, we studied G-protein activation at h5-HT$_{1B}$ receptors stably expressed in CHO cells.[1] 5-HT$_{1B}$ receptors exhibit marked constitutive activity for G-protein activation, and several inverse agonists have been identified at this site (Thomas et al., 1995; Pauwels et al., 1997; Gaster et al., 1998; Selkirk et al., 1998). Furthermore, inhibitory 5-HT$_{1B}$ receptors are located as autoreceptors on serotonergic neuronal terminals and are key targets for the modulation of serotonin release, both in the central nervous system (Engel et al., 1986; Bruinvels et al., 1993; Millan et al., 1999; Sari et al., 1999) and in the dura matter. Consequently, an understanding of the mechanisms of signal transduction by 5-HT$_{1B}$ receptors is relevant to the treatment of affective disorders and to the management of migraine (Hamel, 1996; Millan, 1999).

Herein, using two cell membrane preparations, we characterized the effects of a variation in h5-HT$_{1B}$ R:G stoichiometry on several parameters. First, receptor expression levels and receptor-activated G-proteins were quantified by saturation-binding experiments, permitting the calculation of R:G ratios. Second, the competition-binding affinities of chemically diverse 5-HT$_{1B}$ receptor ligands, including several recently described selective compounds, were compared in these cell membranes displaying high versus low R:G ratios. Third, ligand potencies and efficacies were compared by binding of the hydrolysis-resistant GTP analog radioligand, guanosine-5′-*O*-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) (Lorenzen et al., 1993). This technique affords a measure of the activation of the first step of the intracellular transduction cascade (Birnbaumer and Birnbaumer, 1995; Gudermann et al., 1997). Fourth, the effect of contrasting R:G ratios on constitutive 5-HT$_{1B}$ receptor-mediated G-protein activation was investigated with a novel procedure using [^{35}S]GTPγS versus GTPγS homologous inhibition curves. Such binding isotherms allow the detection of high affinity (HA) and low affinity (LA) binding components (Breivogel et al., 1998; Selley et al., 1998), and can be used to directly quantify the amount of agonist-independent constitutive G-protein activation without requiring the use of inverse agonists (Audinot et al., 1999, 2001).

Materials and Methods

Binding with [^{3}H]GR125,743 at CHO-h5-HT$_{1B}$ Membranes. For competition- and saturation-binding experiments, CHO-h5-HT$_{1B}$ cell membranes (15 μg) were incubated for 60 min at 22°C in buffer A (50 mM Tris-HCl, pH 7.7, 4 mM $CaCl_2$, 0.1% ascorbic acid) with [^{3}H]GR125,743 (1 nM; 70 Ci/mmol; Amersham, Les Ulis, France; Doménech et al., 1997) and competing ligands. 5-HT (10 μM) was used to define nonspecific binding. Incubations were terminated by rapid filtration through GF/B filters pretreated with polyethylenimine (0.1%, v/v). Data were analyzed by nonlinear regression using the program Prism (Graphpad Software Inc., San Diego, CA), to yield K_D (dissociation of the radioligand) and B_{max} (maximal binding density) values for saturation experiments, and IC_{50} values for competition experiments. K_i values were calculated according to the equation: $K_i = IC_{50}/(1 + L/K_D)$, where L is the concentration of radioligand.

Effects of Receptor Ligands on [^{35}S]GTPγS Binding at CHO-h5-HT$_{1B}$ Membranes. Receptor-linked G-protein activation at h5-HT$_{1B}$ receptors was determined by measuring stimulation of [^{35}S]GTPγS (1000 Ci/mmol; New England Nuclear, Paris, France) binding as described previously (Newman-Tancredi et al., 1999a). Briefly, membranes (15 μg of protein/well) were incubated (30 min at 22°C) with ligands in a final volume of 250 μl of buffer B [20 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid, pH 7.4, 3 μM GDP, 3 mM $MgCl_2$, 100 mM NaCl, and 0.1 nM [^{35}S]GTPγS]. Nonspecific binding was defined with GTPγS (10 μM).

Inhibition of [^{35}S]GTPγS Binding by Unlabeled GTPγS at CHO-h5-HT$_{1B}$ Membranes. Isotopic dilution experiments were carried out in buffer B and incubations lasted 30 min at 22°C. Binding of radiolabeled [^{35}S]GTPγS was inhibited with GTPγS and the resulting isotherms were best fitted by a two-site nonlinear regression analysis, giving IC_{50} values for HA and LA binding components. HA binding observed under basal conditions (i.e., not agonist induced) reflects endogenous G-protein activation, providing a direct measure of constitutive activity of G-protein-coupled receptors (Audinot et al., 1999, 2001), whereas LA binding likely reflects endogenous GDP/GTP turnover of CHO cell membrane G-protein Gα-subunits. Binding data from these experiments expressed in femtomoles per milligram of protein were normalized to account for the concentration of [^{35}S]GTPγS present in the assay. Hence, units are denoted fmol/mg/nM [^{35}S]GTPγS.

Isotopic dilution [^{35}S]GTPγS versus GTPγS-binding experiments were also used to calculate the total amount of ligand ([^{35}S]GTPγS and GTPγS) bound to G-protein (=$BOUND_{TOT}$), by a modification of the procedure previously described (Newman-Tancredi et al., 1997). The formula applied herein was as follows:

$$BOUND_{TOT} = [^{35}S]GTP\gamma S_{BOUND\text{-}HA} \times GTP\gamma S_{TOT}/[^{35}S]GTP\gamma S_{CONC} \quad (1)$$

where [^{35}S]GTPγS_{CONC} is [^{35}S]GTPγS concentration (~0.1 nM) and GTPγS_{TOT} is [^{35}S]GTPγS_{CONC} plus GTPγS concentration. The value of [^{35}S]GTP$\gamma S_{BOUND\text{-}HA}$ herein was calculated by subtracting LA binding sites ([^{35}S]GTP$\gamma S_{BOUND\text{-}LA}$) from the observed [^{35}S]GTPγS binding ([^{35}S]GTPγS_{BOUND}).

Thus, the present isotopic dilution methodology provided a measure of the G-proteins labeled not only under the influence of agonist (10 μM 5-HT), as in our previous study (Newman-Tancredi et al., 1997) but also in its absence, yielding estimates of the number and affinity of G-proteins endogenously activated in CHO-h5-HT$_{1B}$ cell membranes.

Membranes and Compounds. CHO-h5-HT$_{1B}$ cell membranes expressing both high and low levels of h5-HT$_{1B}$ receptors were purchased from Euroscreen (Brussels, Belgium; Euroscreen only now commercializes the membranes from cells expressing the higher level of h5-HT$_{1B}$ receptors). 5-HT creatinine sulfate was purchased from Sigma (Saint Quentin Fallavier, France), methiothepin maleate was from Tocris Cookson (Southampton, England). Sumatriptan (GR43,175) was from Glaxo (Greenford, UK). SB224,289 (1′-methyl-5-[[2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl]-2,3,6,7-tetrahydrospiro-[furo-[2,3*f*]-indole-3,4′-piperidine]-oxalate) was synthesized by Jean-Louis Peglion [Institut de Recherches Servier, Croissy-sur-Seine (Paris), France]. BMS 181,101 (5-fluoro-3-{3-[4-(5-methoxy-pyrimidin-4-yl)-piperazin-1-yl]-propyl}-1*H*-indole) dihydrochloride, GR 125,743 (*N*-[4-methoxy-3-(4-methylpiperazin-1-yl)phenyl]-3-methyl-4-(4-pyridyl)benzamide) hydrochloride, and L775,606 (1-[2-(3-fluorophenyl)ethyl]-4-[3-[5-(1,2,4-triazol-4-yl)-1H-indol-3-yl]-propyl]-piperazine) dicitrate were syn-

[1] Nomenclature of h5-HT$_{1B}$ receptors is according to Hartig et al. (1996).

thesized by Gilbert Lavielle [Institut de Recherches Servier, Croissy-sur-Seine (Paris), France]. Compounds were dissolved in water or in dimethyl sulfoxide and diluted in the appropriate assay buffer to the required experimental concentrations.

Results

[^{3}H]GR125,743 and [^{35}S]GTPγS Saturation Binding to Cell Membranes from Two CHO Cell Lines Expressing h5-HT$_{1B}$ Receptors. Saturation binding with [^{3}H]GR125,743 was carried out in CHO cell membranes expressing a high level (RGhigh membranes) and a low level (RGlow membranes) of h5-HT$_{1B}$ receptors. RGhigh membranes expressed 20 times more h5-HT$_{1B}$ receptors (B_{max} = 8.54 pmol/mg) than RGlow cell membranes (B_{max} = 0.43 pmol/mg) (Table 1 and Fig. 1).

The number of activated G-proteins was calculated from the HA binding sites detected in biphasic [^{35}S]GTPγS versus GTPγS isotopic dilution "saturation"-binding isotherms. This analysis takes account of those G-proteins that are constitutively activated in CHO-h5-HT$_{1B}$ cells and not just those that are activated by the presence of 5-HT. In RGlow cell membranes, G-protein B_{max} values were about 1.5 pmol/mg, both in the presence and in the absence of 5-HT (10 μM). In contrast, the apparent K_D value was reduced by 3-fold in the presence of 5-HT (Table 1 and Fig. 1). In RGhigh cell membranes, the B_{max} values were similarly insensitive to the presence of 5-HT (10 μM) (B_{max} = 2.8–3.0 pmol/mg). In contrast, 5-HT decreased the apparent K_D value by 4-fold (Table 1 and Fig. 1). These data yielded R:G ratios (B_{max} [^{3}H]GR125,743: B_{max} [^{35}S]GTPγS) of ~0.3 for RGlow and of ~3 for RGhigh cell membranes.

Competition for [^{3}H]GR125,743 Binding to RGlow and RGhigh Cell Membranes. Competition-binding isotherms for [^{3}H]GR125,743 binding were carried out at both RGlow and RGhigh cell membranes with six chemically diverse serotonergic ligands (Table 2). Marked differences in the affinities (p$K_{i/H/L}$ values) of certain ligands were observed between the two sets of membranes. 5-HT displayed biphasic isotherms with modest pseudo-Hill coefficients (n_H = 0.73) in both RGlow and RGhigh membranes, but the proportion of HA sites was significantly reduced in the latter. Sumatriptan yielded monophasic isotherms in RGlow membranes but biphasic ones in RGhigh membranes, with a significant reduction in n_H value. For BMS181,101 and L775,606, pK_i values were about 0.5 unit higher at RGlow than at RGhigh cell membranes. In contrast, SB224,289 exhibited a pK_i value that was higher at RGhigh membranes. The other inverse agonist, methiothepin, showed a similar tendency, although it did not reach statistical significance (Table 2).

Concentration-Response Effect of 5-HT Receptor Ligands on [^{35}S]GTPγS Binding to RGlow and RGhigh Cell Membranes. The influence of 5-HT$_{1B}$ ligands on G-protein activation was investigated (Table 3 and Fig. 2). The amount of [^{35}S]GTPγS binding was markedly higher in RGhigh membranes than in RGlow membranes both in absolute and in relative terms. Thus, basal [^{35}S]GTPγS binding was 1222 fmol/mg/nM in RGhigh, but only 477 fmol/mg/nM in RGlow membranes (see Table 4, "Total" column). 5-HT-stimulated [^{35}S]GTPγS binding was 2937 fmol/mg/nM in RGhigh membranes (2.4-fold increase) compared with 650 fmol/mg/nM in RGlow membranes (1.4-fold increase) (Table 4; "Total" column).

As concerns other 5-HT$_{1B}$ receptor ligands, the following points should be noted. First, the full agonists (5-HT and sumatriptan) exhibited similar potency in both sets of membranes. Second, the partial agonists (BMS181,101 and, L775,606) exhibited increased efficacy (relative to 5-HT) in RGhigh cell membranes, with E_{max} (maximal efficacy) values 1.3- to 1.4-fold greater than RGlow cell membranes. Third, the inverse agonists, SB224,289 and methiothepin, which exhibited only slight negative efficacy in RGlow membranes, displayed markedly (3-fold) greater negative efficacy in RGhigh cell membranes (Table 3 and Fig. 2).

Inhibition by GTPγS of [^{35}S]GTPγS Binding to RGlow and RGhigh Cell Membranes. Both in the presence and in the absence of receptor ligands, inhibition of [^{35}S]GTPγS binding to both RGhigh and RGlow membranes by GTPγS produced biphasic isotherms (two-site fit was statistically superior to a single-site fit; $P < .05$, F test; Table 4 and Fig. 3). The amount of HA [^{35}S]GTPγS binding (a measure of constitutive activation) was greater in RGhigh cell membranes (683 fmol/mg/nM) than in RGlow cell membranes (126 fmol/mg/nM). Furthermore, a marked difference was observed in the action of 5-HT (10 μM) on HA sites in the two membrane preparations: 5-HT increased the number of HA sites by 3.7-fold in RGhigh membranes but by only 2.8-fold in RGlow membranes (Table 4 and Fig. 3). Conversely, the number of HA sites was reduced by the inverse agonists, SB224,289 (10 μM) and methiothepin (1 μM), but their actions were more profound in RGhigh than in RGlow membranes (Table 4 and Fig. 3). Indeed, methiothepin reduced

TABLE 1

[^{3}H]GR125,743 and [^{35}S]GTPγS saturation binding to cell membranes from two CHO cell lines expressing h5-HT$_{1B}$ receptors

The expression level (B_{max}) of h5-HT$_{1B}$ receptors was determined by saturation-binding experiments with [^{3}H]GR125,743. The number of G-proteins activated by h5-HT$_{1B}$ receptors was determined by [^{35}S]GTPγS isotopic dilution saturation binding as described under *Materials and Methods* in the presence or absence of 5-HT (10 μM). The R:G ratio was calculated by dividing the B_{max} of [^{3}H]GR125,743 by that of [^{35}S]GTPγS. The cell membranes exhibiting a lower B_{max} ratio are denoted "RGlow". The cell membranes exhibiting a higher B_{max} ratio are denoted "RGhigh". Data are means ± S.E. of at least three independent determinations performed in triplicate.

	RGlow		RGhigh	
	B_{max}	K_D	B_{max}	K_D
	fmol/mg	*nM*	*fmol/mg*	*nM*
[^{3}H]GR125,743	426 ± 54	0.46 ± 0.11	8539 ± 95	1.27 ± 0.12
[^{35}S]GTPγS				
Basal	1446 ± 199	11.42 ± 1.45	3025 ± 210	5.12 ± 0.91
(R:G ratio)	(0.29)		(2.82)	
5-HT	1611 ± 90	3.87 ± 0.38[a]	2765 ± 224	1.17 ± 0.15[a]
(R:G ratio)	(0.26)		(3.09)	

[a] $P < .05$, two-tailed unpaired t test versus basal K_D value.

HA sites to 70% of their basal level (=100%) in RGlow membranes but to just 29% of basal levels in RGhigh membranes.

In contrast to HA sites, the LA component of [^{35}S]GTPγS versus GTPγS-binding isotherms was not markedly affected by the presence of receptor ligands. Neither 5-HT nor the inverse agonists, methiothepin and SB224,289, significantly affected the number of LA binding sites in either RGhigh or RGlow membranes.

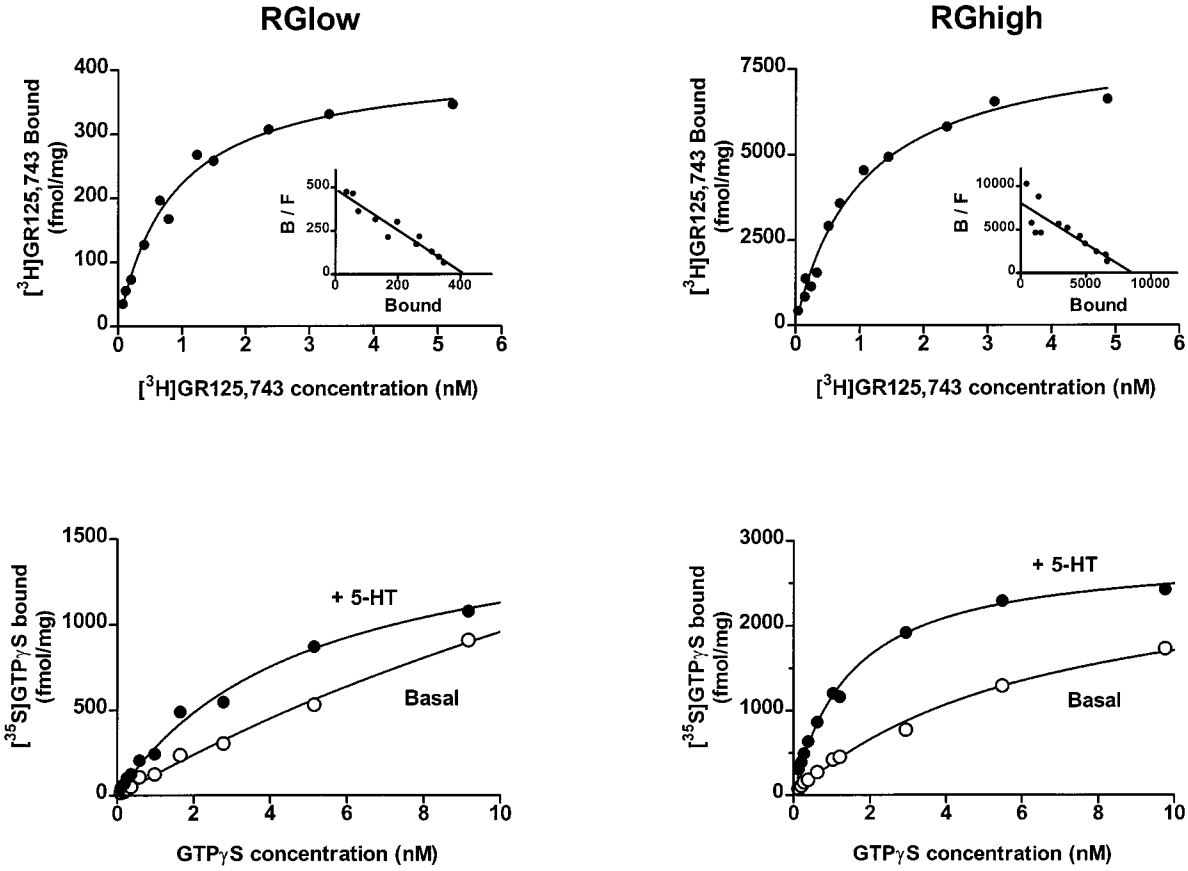

Fig. 1. Saturation binding at RGlow or RGhigh membranes. Top, h5-HT$_{1B}$ receptor saturation binding with [^{3}H]GR125,743 (insets show Scatchard plots of respective saturation isotherms). Bottom, [^{35}S]GTPγS isotopic dilution G-protein saturation binding (as described under *Materials and Methods*) in the presence or absence of 5-HT (10 μM). Left, isotherms from RGlow cell membranes. Right, isotherms from RGhigh cell membranes (note different *y*-axes). Points shown are from representative experiments performed in triplicate and repeated on at least three independent occasions. B, bound radioligand; F, free radioligand.

TABLE 2

Competition for [^{3}H]GR125,743 binding to RGlow and RGhigh cell membranes

Affinities at h5-HT$_{1B}$ receptors were determined by competition with [^{3}H]GR125,743 in two preparations of RGlow or RGhigh. Inhibition constants (pK_i) and pseudo-Hill coefficients (n_H) were determined by nonlinear regression. For 5-HT, biphasic isotherms were observed so data are shown for HA (pK_H) and LA (pK_L) binding components. Sumatriptan exhibited biphasic isotherms only in RGhigh membranes. The percentage of high-affinity binding sites for 5-HT and sumatriptan (denoted "% high") is shown in italics. Data are means ± S.E. of at least three independent experiments.

	RGlow		RGhigh	
	pK_i	n_H	pK_i	n_H
		% high		*% high*
Full agonists				
5-HT				
pK_H	9.01 ± 0.24	0.73 ± 0.04	8.99 ± 0.15	0.73 ± 0.03
pK_L	7.64 ± 0.24	*(59 ± 10)*	7.42 ± 0.06	*(31 ± 2[a])*
Sumatriptan				
pK$_H$	7.61 ± 0.04	0.81 ± 0.02	7.94 ± 0.08	0.73 ± 0.03[a]
pK$_L$	N.C.	N.C.	6.54 ± 0.02	*(45 ± 11)*
Partial agonists				
BMS181,101	8.09 ± 0.03	0.98 ± 0.07	7.56 ± 0.13[a]	0.89 ± 0.02
L775,606	6.93 ± 0.10[b]	0.87 ± 0.05[b]	6.31 ± 0.04	1.06 ± 0.15
Inverse agonists				
SB224,289	8.18 ± 0.07	1.09 ± 0.18	8.56 ± 0.09[a]	1.07 ± 0.04
Methiothepin	8.32 ± 0.04	0.87 ± 0.09	8.51 ± 0.10	0.88 ± 0.07

N.C., not computable.
[a] $P < .05$, Student's two-tailed *t* test, compared with pK_i value for RGlow cell membranes.
[b] Mean ± range, $n = 2$.

Discussion

The key findings of the present study are that an augmentation of h5-HT_{1B} R:G stoichiometry is associated with changes in ligand-binding affinities, increased relative efficacies of partial agonists, and, notably, increased constitutive G-protein activation and negative efficacy of inverse agonists at h5-HT_{1B} receptors.

Determination of R:G Stoichiometry. In a comparison of two recombinant CHO cell lines, the first (RGlow) had a low h5-HT_{1B} receptor expression level, whereas the second (RGhigh) expressed 20-fold more receptors (Table 1 and Fig. 1). Interestingly, in [^{35}S]GTPγS saturation-binding experiments, RGhigh membranes exhibited a G-protein B_{max} 2-fold higher than RGlow, resulting in an R:G ratio of ~3.0 (for RGhigh) versus ~0.3 (for RGlow). The present data therefore indicate that, in studies of receptor density on signal transduction, it is advisable to quantify variations in G-protein levels from one recombinant cell line to another. The R:G ratio determination methodology was similar to that used at some other receptors (Lorenzen et al., 1993; Newman-Tancredi et al., 1997; Breivogel et al., 1998; Pauwels et al., 1998, 2000; Selley et al., 1998). However, a distinctive aspect herein was that the density of receptor-activated G-proteins took into account those G-proteins that are endogenously activated, by calculating the amount of HA [^{35}S]GTPγS binding (see under *Materials and Methods*). In systems that exhibit a marked degree of constitutive G-protein activation, this factor may considerably affect estimates of G-protein density and affinity (Audinot et al., 1999, 2001; Pauwels et al., 2000). Herein, the B_{max} of [^{35}S]GTPγS was not altered by the presence or absence of 5-HT (Table 1). Thus, instead of a change in B_{max}, 5-HT induced an increase in the apparent binding affinity of [^{35}S]GTPγS. These data are consistent with the model of agonist action proposed by Breivogel et al. (1998), in which agonists alter G-protein affinity for guanine nucleotides (see also González-Maeso et al., 2000). Thus, in CHO-h5-HT_{1B} cell membranes, the total number of activated G-proteins was unchanged, and the effect of the agonist, 5-HT, is to increase their ability to bind low concentrations of [^{35}S]GTPγS.

Competition Binding at RGhigh and RGlow Membranes. The present data indicate that RGhigh and RGlow membranes differed in [^{3}H]GR125,743 competition-binding experiments (Table 2) while maintaining a pharmacological profile in general accordance with previous reports (Plosker and McTavish, 1994; Doménech et al., 1997; Pauwels et al., 1998; Selkirk et al., 1998; Longmore et al., 2000). It is noteworthy that the proportion of HA sites detected in RGhigh membranes for 5-HT was significantly lower than in RGlow membranes. Furthermore, the binding isotherms for sumatriptan were biphasic in RGhigh membranes but monophasic in RGlow membranes (Table 2), and the pK_i value of the partial agonist, BMS181,101, was significantly reduced in RGhigh membranes. The simplest interpretation of these observations, in the light of the increased R:G stoichiometry of RGhigh membranes, is that the proportion of G-protein-uncoupled receptors is higher. Indeed, agonists display reduced affinity at receptors that are not coupled to G-proteins (Wregget and De Léan, 1984; Kenakin, 1997a). Conversely, the affinity (pK_i values) of the inverse agonist, SB224,289, showed an increase in RGhigh membranes. Methiothepin showed a similar tendency (Table 2). Given that inverse agonists exhibit higher binding affinity at receptors that exist in inactive conformation(s) (Samama et al., 1994; Leff, 1995), this is also in accordance with the interpretation that attributes this change to an increase in the proportion of G-protein-uncoupled receptors.

Ligand Efficacy at RGhigh and RGlow Membranes. RGhigh and RGlow membranes also differed in their functional responses, as determined by [^{35}S]GTPγS binding (Table 3 and Fig. 2). First, the overall degree of stimulation attained above basal binding with 5-HT was markedly higher in RGhigh membranes (2.4-fold) than in RGlow membranes (1.4-fold) (see Table 4; "Total" column), probably reflecting a faster rate of G-protein "cycling" due to the greater availability of h5-HT_{1B} receptors per G-protein (Birnbaumer and Birnbaumer, 1995; Gudermann et al., 1997; Breivogel et al., 1998). Thus, the relative efficacies of the partial agonists, BMS181,101 and L775,606, were also increased in RGhigh membranes versus RGlow membranes. It is important to note that the present data differ from those for 5-HT_{1A} receptors expressed in CHO cells (Newman-Tancredi et al., 1997). Therein, the potency of 5-HT, but not its efficacy, was increased by an augmentation of R:G stoichiometry, whereas

TABLE 3

Concentration-response effects of 5-HT receptor ligands on [^{35}S]GTPγS binding to RGlow and RGhigh cell membranes

The influence of 5-HT_{1B} receptor ligands was compared by [^{35}S]GTPγS binding to two preparations of RGlow and RGhigh cell membranes. Concentration-response isotherms were analyzed by nonlinear regression. pEC_{50} values are shown for agonist effects, whereas pIC_{50} values are shown for inverse agonists (SB224,289 and methiothepin). E_{max} is expressed as a percentage of binding observed with a maximally effective (10 μM) concentration of 5-HT (=100%). Data are means ± S.E. of at least three independent experiments.

	RGlow		RGhigh	
	pEC_{50}/pIC_{50}	E_{max}	pEC_{50}/pIC_{50}	E_{max}
		%		%
Full agonists				
5-HT	8.30 ± 0.16	100	8.06 ± 0.07	100
Sumatriptan	7.25 ± 0.08	95 ± 3	7.09 ± 0.05	98 ± 5
Partial agonists				
BMS181,101	7.82 ± 0.14	68 ± 8	7.90 ± 0.23	85 ± 5[a]
L775,606	6.89 ± 0.22	48 ± 5	6.38 ± 0.03	67 ± 2[a]
Inverse agonists				
SB224,289	7.64 ± 0.10[b]	−12 ± 5[b]	7.79 ± 0.17	−40 ± 6
Methiothepin	7.32 ± 0.17[b]	−14 ± 2[b]	7.83 ± 0.14	−42 ± 4

[a] $P < .05$, Student's two-tailed t test, compared with E_{max} value for RGlow cell membranes.
[b] Mean ± range ($n = 2$).

the reverse was true in the present study. One factor potentially implicated is that the R:G ratio of RGlow membranes herein (~0.3) was substantially less than its counterpart in CHO-h5-HT$_{1A}$ cell membranes (1.4; Newman-Tancredi et al., 1997). Thus, CHO-h5-HT$_{1B}$ RGlow membranes may not have attained a "ceiling" whereby, for example, the number of available G-proteins was limiting, as may have been the case for CHO-h5-HT$_{1A}$ RGlow cell membranes (Newman-Tancredi et al., 1997; Selley et al., 1998). In comparison, when Gq proteins were coexpressed with m_3 receptors, both potency and efficacy were augmented (Burnstein et al., 1995), suggesting that either or both of these parameters may be affected, depending on the R:G stoichiometry and limiting factors in each cellular expression system. These data again highlight the importance of thoroughly characterizing different expression systems at the level of both receptor and coupled G-proteins. Second, inverse agonists displayed increased negative efficacy in RGhigh membranes. Indeed, in RGlow membranes, the inhibitory actions of methiothepin were modest, whereas in RGhigh membranes about 40% of basal [^{35}S]GTPγS binding was inhibited by SB224,289 and methiothepin (Table 3 and Fig. 2). These data are reminiscent of observations at CHO-h5-HT$_{1A}$ cell membranes showing that an increase in R:G ratio augmented the negative efficacy of the inverse agonist, spiperone (Newman-Tancredi et al., 1997). The most likely explanation is that inverse agonists stabilize G-protein-coupled (as well as uncoupled) receptors in inactive conformation(s) (Samama et al., 1994; Leff, 1995; Newman-Tancredi et al., 1997). This would result in a reduction in the pool of G-proteins available for activation by non-inverse agonist-occupied receptors. The present data therefore provide evidence that R:G stoichiometry is an important factor in the detection of inverse agonist actions at h5-HT$_{1B}$ receptor-coupled G-proteins.

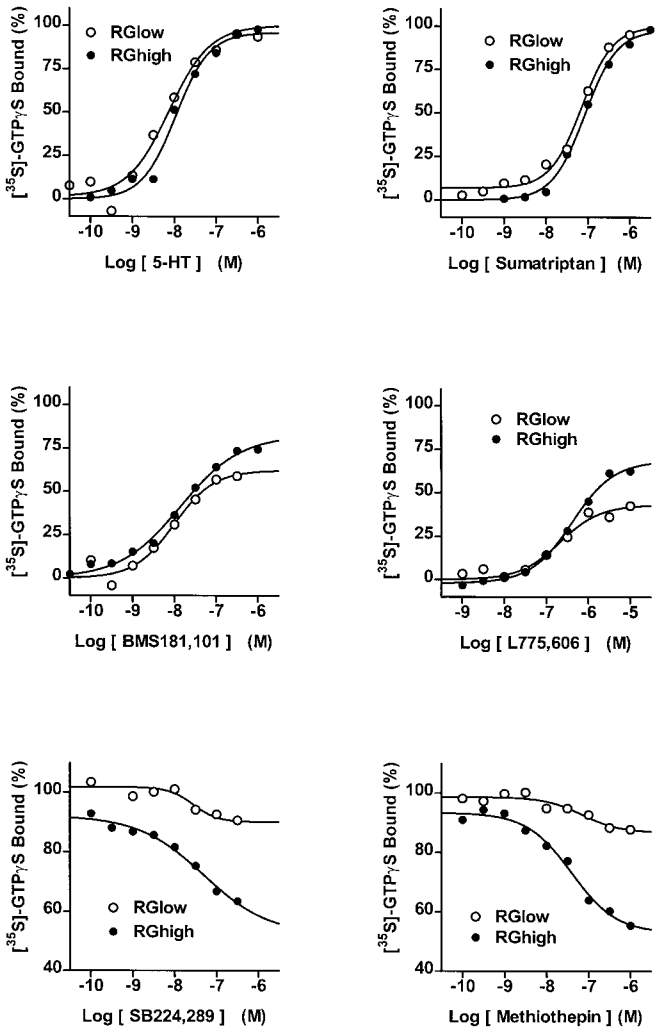

Fig. 2. Concentration-response isotherms of 5-HT$_{1B}$ receptor ligands for stimulation of [^{35}S]GTPγS binding to RGlow (○) or RGhigh (●) membranes. 5-HT and sumatriptan exhibited similar efficacy in both membrane preparations. BMS181,101 and L775,606 were more efficacious in RGhigh than in RGlow membranes. The inverse agonists, methiothepin and SB224,289, exhibited greater negative efficacy in RGhigh versus RGlow membranes. Data for the agonists are expressed as percentages of [^{35}S]GTPγS binding induced by a maximally effective concentration of 5-HT (10 μM). Data for the inverse agonists are expressed as percentages of [^{35}S]GTPγS binding observed under basal conditions (absence of receptor ligand). Points shown are from representative experiments performed in triplicate and repeated on at least three independent occasions.

Constitutive Activity at RGhigh and RGlow Membranes. The degree of constitutive h5-HT$_{1B}$ receptor activation differed markedly between RGhigh and RGlow membranes. As stated in the introduction, the quantitative influence of R:G stoichiometry on constitutive activity is poorly characterized. Herein, constitutive activity was directly quantified by an innovative procedure by which the HA and LA components of homologous inhibition experiments of [^{35}S]GTPγS versus GTPγS (Audinot et al., 1999, 2000) were analyzed. The stimulatory action of 5-HT on [^{35}S]GTPγS binding was due to an action on HA sites, consistent with results at other receptor systems (Breivogel et al., 1998; Pauwels et al., 1998, 2000; Selley et al., 1998), but the key finding of the present study was the increase in the number of HA sites observed in RGhigh cell membranes under *basal* conditions. Indeed, HA binding in RGhigh membranes was 5-fold greater (683 fmol/mg/nM; Table 4 and Fig. 3) than that observed in RGlow membranes (126 fmol/mg/nM). Given that in both cases no agonists were present, the increase is, most likely, attributable to increased R:G stoichiometry (Kenakin, 1997a). Thus, as R:G stoichiometry increases, the augmented availability of receptors per G-protein favors coupling of the latter to receptors in active conformations, yielding a greater amount of HA binding in the absence of agonist (i.e., constitutive activity). In contrast, LA binding increases by only about 2-fold in RGhigh membranes relative to RGlow membranes, a change similar to the increase in the B_{max} derived from [^{35}S]GTPγS saturation binding (Table 1). Indeed, receptor ligands (whether agonists or inverse agonists) have little, if any, influence on LA sites but exert a major influence on HA binding. Indeed, the present study reveals that methiothepin and SB224,289 reduced the number of HA sites, an effect that was more pronounced in RGhigh membranes than in RGlow membranes. These observations suggest that as R:G stoichiometry increases, the number of constitutively active receptors per G-protein increases, thus providing a greater basal activity on which inverse agonists can exert their inhibitory actions. These data are consistent with a two-state receptor activation model (Leff, 1995; Kenakin, 1997a) but, once again, differ from those obtained for CHO-h5-HT$_{1A}$ receptors (Newman-Tancredi et al., 1997). For the latter, basal [^{35}S]GTPγS binding was not altered by the increase in R:G ratio, whereas for h5-HT$_{1B}$ receptors it was (Table 4). As discussed above, these data suggest that h5-

HT_{1B} receptor-mediated [^{35}S]GTPγS binding in the present CHO cell line is not subject to the same limitation or ceiling as h5-HT_{1A} receptors in our previous study (Newman-Tancredi et al., 1997; Selley et al., 1998). The limiting factor could be the G-protein expression level, which was ~1 pmol/mg for CHO-h5-HT_{1A} membranes but ~3 pmol/mg for RGhigh membranes in the present study.

Conclusions

The present study provides evidence that R:G stoichiometry is a key factor in the pharmacological profile of h5-HT_{1B} receptors in CHO cells. Increased R:G ratios are associated with alterations in binding affinity, increased G-protein activation by full agonists, and increased relative efficacy of

TABLE 4

Inhibition by GTPγS of [^{35}S]GTPγS binding to RGlow and RGhigh cell membranes

[^{35}S]GTPγS binding to two preparations of RGlow or RGhigh cell membranes, was inhibited with GTPγS in the presence or absence of 5-HT (10 μM), SB224,289 (10 μM), or methiothepin (1 μM). Isotherms were analyzed by nonlinear regression yielding an HA and an LA component. In all cases, a two-site fit was statistically superior to a single-site fit ($P < .05$, F test). The number of HA and LA sites is expressed as femtomoles per milligram per nanomolar [^{35}S]GTPγS present in the assay. For Total and HA sites, numbers in parentheses indicate the amount of binding as a fraction of that observed under respective basal conditions. Data are means ± S.E. of at least three independent experiments.

	Total (HA + LA)	HA Sites	pIC_{50} (HA)	LA Sites	pIC_{50} (LA)
	fmol/mg/nM	*fmol/mg/nM*		*fmol/mg/nM*	
RGlow					
Basal	477 (1.0)	126 ± 22 (1.0)	8.70 ± 0.22	351 ± 47	6.18 ± 0.06
5-HT	650 (1.4)	351 ± 47[a] (2.8)	8.58 ± 0.06	299 ± 25	6.21 ± 0.06
Methiothepin	410 (0.86)	88 ± 22 (0.70)	7.82 ± 0.15[a]	322 ± 69	5.96 ± 0.03[a]
RGhigh					
Basal	1222 (1.0)	683 ± 76 (1.0)	8.59 ± 0.11	539 ± 52	6.42 ± 0.09
5-HT	2937 (2.4)	2511 ± 369[a] (3.7)	9.11 ± 0.04[a]	426 ± 47	6.61 ± 0.08
SB224,289	787 (0.64)	273 ± 51[a] (0.40)	8.01 ± 0.20[a]	514 ± 49	6.19 ± 0.10
Methiothepin	722 (0.59)	201 ± 24[a] (0.29)	7.92 ± 0.23[a]	521 ± 84	6.18 ± 0.06

[a] $P < .05$, two-tailed unpaired t test versus respective basal values.

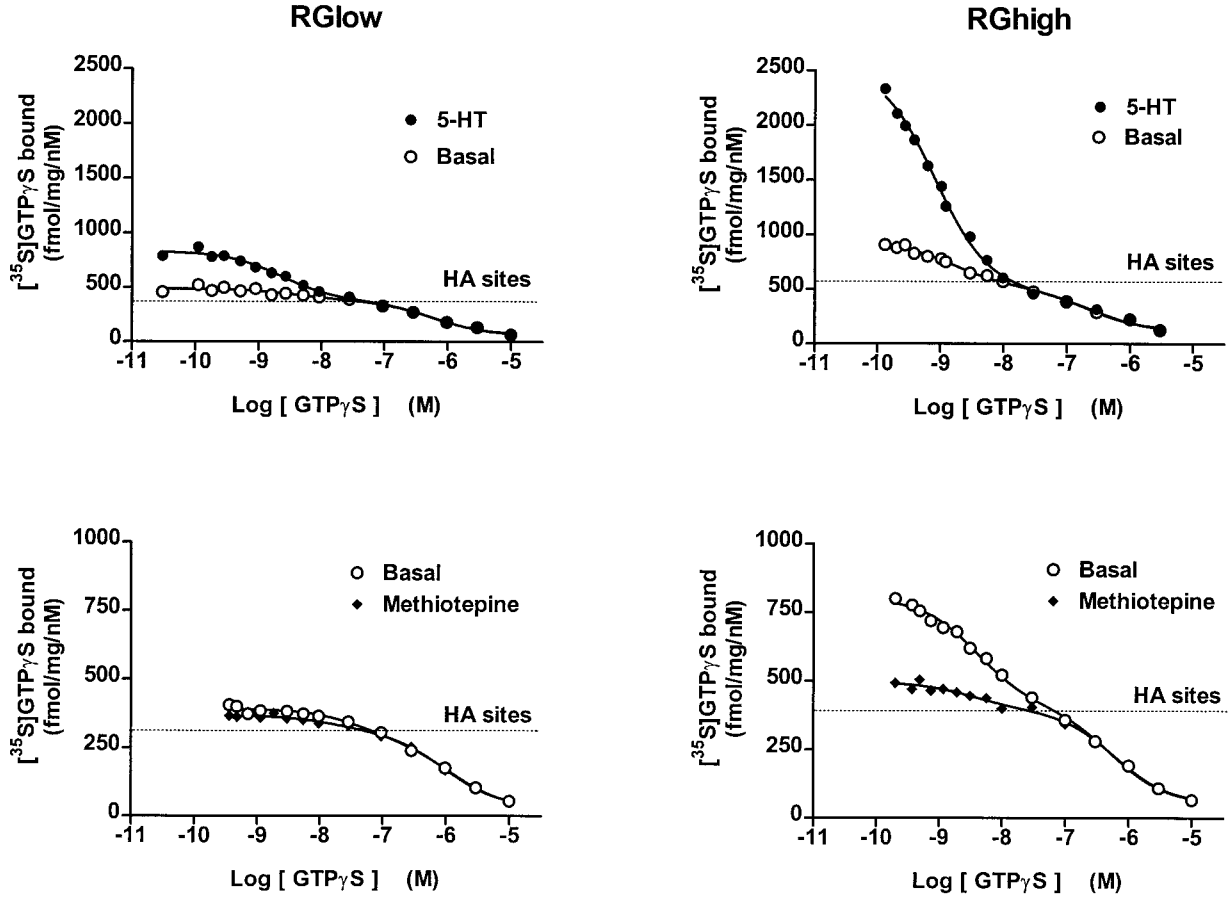

Fig. 3. Inhibition by isotopic dilution with GTPγS of [^{35}S]GTPγS binding to RGlow or RGhigh membranes. Top, [^{35}S]GTPγS isotopic dilution in the presence or absence of 5-HT (10 μM). Bottom, [^{35}S]GTPγS isotopic dilution in the presence or absence of the inverse agonist methiothepin (1 μM). Left, isotherms from RGlow cell membranes. Right, isotherms from RGhigh cell membranes. The dotted lines indicate the HA binding component of the isotherms that were modulated by receptor ligands. Isotherms determined by nonlinear regression are biphasic (two-site fit was statistically superior to a single-site fit; $P < .05$, F test). Points shown are from representative experiments performed in triplicate and repeated on at least three independent occasions.

partial agonists at h5-HT$_{1B}$ receptors. Importantly, the present study reveals that increased R:G ratios are also associated with increased negative efficacy of inverse agonists and increased constitutive G-protein activation of h5-HT$_{1B}$ receptors, effects that may be receptor subtype dependent, because they differ from previously reported data at h5-HT$_{1A}$ receptors. The implications of the present observations for analysis of data obtained from native 5-HT$_{1B}$, or other G-protein-coupled receptors, remains to be more fully ascertained but suggest that determination of R:G stoichiometry is an important parameter to consider when interpreting data pertaining to ligand efficacy and receptor constitutive activity.

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