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h5-HT$_{1B}$ receptor-mediated constitutive Gα_{i3}-protein activation in stably transfected Chinese hamster ovary cells: an antibody capture assay reveals protean efficacy of 5-HT

[1,2]Adrian Newman-Tancredi, *,[1]Didier Cussac, [1]Laetitia Marini, [1]Manuelle Touzard & [1]Mark J. Millan

[1]Department of Psychopharmacology, Institut de Recherches Servier, 125, Chemin de Ronde, Croissy-sur-Seine, Paris 78290, France

1 Serotonin 5-HT$_{1B}$ receptors couple to G-proteins of the Gi/o family. However, their activation of specific G-protein subtypes is poorly characterised. Using an innovative antibody capture/guanosine-5′-0-(3-[^{35}S]thio)-triphosphate ([^{35}S]GTPγS) binding strategy, we characterised Gα_{i3} subunit activation by h5-HT$_{1B}$ receptors stably expressed in Chinese hamster ovary (CHO) cells.

2 The agonists, 5-HT, alniditan and BMS181,101, stimulated Gα_{i3}, whereas methiothepin and SB224,289 behaved as inverse agonists. The selective 5-HT$_{1B}$ receptor ligand, S18127, modestly stimulated Gα_{i3} and reversed the actions of both 5-HT and methiothepin. S18127 (1 μM) also produced parallel, dextral shifts of the 5-HT and methiothepin isotherms.

3 Isotopic dilution experiments ([^{35}S]GTPγS *versus* GTPγS) revealed high-affinity [^{35}S]GTPγS binding to Gα_{i3} subunits in the absence of receptor ligands indicating constitutive activity. High-affinity [^{35}S]GTPγS binding was increased 2.8-fold by 5-HT with an increase in the affinity of GTPγS for Gα_{i3} subunits. In contrast, methiothepin halved the number of high-affinity binding sites and decreased their affinity.

4 h5-HT$_{1B}$ receptor-mediated Gα_{i3} subunit activation was dependent on the concentration of NaCl. At 300 mM, 5-HT stimulated [^{35}S]GTPγS binding, basal Gα_{i3} activation was low and methiothepin was inactive. In contrast, at 10 mM NaCl, basal activity was enhanced and the inverse agonist activity of methiothepin was accentuated. Under these conditions, 5-HT *decreased* Gα_{i3} activation.

5 In conclusion, at h5-HT$_{1B}$ receptors expressed in CHO cells: (i) inverse agonist induced inhibition of Gα_{i3}, and its reversal by S18127, reveals constitutive activation of this Gα subunit; (ii) constitutive Gα_{i3} activation can be quantified by isotopic dilution [^{35}S]GTPγS binding and (iii) decreasing NaCl concentrations enhances Gα_{i3} activation and leads to protean agonist properties of 5-HT: that is a switch *to inhibition* of Gα_{i3}.
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Abbreviations: [^{35}S]GTPγS, guanosine-5′-O-(3-[^{35}S]thio)-triphosphate; CHO cells, Chinese hamster ovary cells; SPA, scintillation proximity assay

Introduction

5-HT$_{1B}$ receptors are involved in the control of mood, motor function and cognition, and 5-HT$_{1B}$ autoreceptors on serotonergic terminals modulate serotonin release (Engel *et al*., 1986; Barnes & Sharp, 1999; Meneses, 1999; Millan *et al*., 1999; Sari *et al*., 1999). Consequently, an understanding of the mechanisms of signal transduction at 5-HT$_{1B}$ receptors is relevant to the etiology and treatment of affective, neurological and cardiovascular disorders and to the management of migraine.

5-HT$_{1B}$ receptors couple inhibition of adenylyl cyclase (Schoeffter & Hoyer, 1989; Pauwels & Palmier, 1994), a response which is abolished by pre-treatment with *Bordetella pertussis* toxin which ADP-ribosylates Gα subunits of the Gi/o family, indicating coupling of 5-HT$_{1B}$ receptors to these G-proteins. Several studies have investigated the influence of serotonergic ligands on G-protein activation by 5-HT$_{1B}$ receptors (Thomas *et al*., 1995; Pauwels *et al*., 1997; Gaster *et al*., 1998; Selkirk *et al*., 1998; Newman-Tancredi *et al*., 2000). These studies demonstrated robust activation of G-proteins by agonists, and also revealed that 5-HT$_{1B}$ receptors exhibit marked constitutive activity for G-protein activation. Correspondingly, several inverse agonists have been identified at 5-HT$_{1B}$ receptors, including methiothepin and the selective 5-HT$_{1B}$ ligand, SB224,289 (Gaster *et al*., 1998; Selkirk *et al*., 1998; Audinot *et al*., 2001a, b). However, these studies were carried out using techniques which measure "overall" G-protein activation without distinguishing the precise G-protein subtype(s) involved. 5-HT$_{1B}$ receptors couple, in fact, to several Gα subtypes, including members of the Gi/o family and Gα_{15} subunits (Bae *et al*., 1997; Clawges *et al*., 1997;

*Author for correspondence; E-mail: didier.cussac@fr.netgrs.com
[2]Current address; Neurobiology 2 Division, Institut de Recherche Pierre Fabre, 17, avenue Jean Moulin, 81106 Castres, France.

Wurch & Pauwels, 2000) but not to $G\alpha_t$ subunits (Bae *et al*., 1997). Reconstitution of 5-HT$_{1B}$ receptors expressed in Sf9 cells with different purified $G\alpha_i$ subunits increased the affinity of agonists indicating coupling to several G-protein subtypes (Clawges *et al*., 1997). Compared with $G\alpha_{i2}$ and $G\alpha_o$, the $G\alpha_{i3}$ subtype induced the greatest increase in agonist affinity, suggesting preferential coupling of 5-HT$_{1B}$ receptors to this G-protein subtype.

In view of the above considerations, the present study employed a recently developed antibody-capture technique coupled to SPA detection (De Lapp *et al*., 1999; Cussac *et al*., 2002; Newman-Tancredi *et al*., 2002a) to characterise $G\alpha_{i3}$ subunit activation by recombinant human 5-HT$_{1B}$ receptors stably expressed in CHO cells (Newman-Tancredi *et al*., 2000; Audinot *et al*., 2001a, b). This cell line constitutes a useful model to investigate 5-HT$_{1B}$ receptor coupling since they express high levels of $G\alpha_{i3}$ subunits in comparison to only low levels of $G\alpha_o$, while $G\alpha_{i1}$ is undetectable (Gerhardt & Neubig, 1991; Law *et al*., 1993; Gettys *et al*., 1994).

Herein, we investigated the influence of several parameters on $G\alpha_{i3}$ activation. First, we compared ligand potencies and efficacies for $G\alpha_{i3}$ activation with those determined previously in identical membrane preparations (Newman-Tancredi *et al*., 2000) employing conventional [^{35}S]GTPγS binding, which does not distinguish between G-protein subtypes (Lazareno *et al*., 1993; Lorenzen *et al*., 1993). Second, constitutive 5-HT$_{1B}$ receptor-mediated $G\alpha_{i3}$ protein activation was quantified employing [^{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 (Newman-Tancredi *et al*., 2000; Audinot *et al*., 2001b; Rouleau *et al*., 2002). Third, detection of constitutive activity may be dependent on the experimental conditions employed and particularly on the presence of Na^+ ions. Thus, NaCl influences receptor conformation by favouring a shift from a G-protein coupled to an uncoupled state, thereby reducing the affinity of agonists (Pert & Snyder, 1974; Horstman *et al*., 1990). Further, NaCl reduces basal G-protein activation in membranes of cells expressing G-protein coupled receptors (Costa *et al*., 1990; see de Ligt *et al*., 2000 for review). However, few studies have examined the influence of NaCl on specific G-protein subtypes (Wenzel-Seifert *et al*., 1998; Seifert, 2001). Thus, we describe here its influence on the constitutive activity of h5-HT$_{1B}$ receptor-activated $G\alpha_{i3}$ subunits, and upon the actions of 5-HT and methiothepin.

A preliminary communication of these data was presented in abstract form (Newman-Tancredi *et al*., 2002b).

Methods

[^{35}S]GTPγS binding by antibody capture and scintillation proximity assay detection

CHO-h5-HT$_{1B}$ cell membranes expressing 8.5 pmol mg^{-1} of h5-HT$_{1B}$ receptors (Newman-Tancredi *et al*., 2000) were purchased from Euroscreen (Brussels, Belgium). To specifically detect [^{35}S]GTPγS binding to h5-HT$_{1B}$ receptor-mediated $G\alpha_{i3}$ G-protein subunits, an antibody-capture strategy was adopted, coupled to detection by SPA. Procedures were similar to those described by De Lapp *et al*. (1999) and are detailed in Newman-Tancredi *et al*. (2002a) and Cussac *et al*. (2002). Briefly, CHO-h5-HT$_{1B}$ cell membranes were incubated on 96-well plates with agonists and/or antagonists and [^{35}S]GTPγS (0.2 nM) for 1 h at 22°C in a buffer containing: 20 mM HEPES (pH 7.4), 3 μM GDP, 3 mM $MgCl_2$ and 100 mM NaCl unless otherwise indicated. The reaction was stopped by solubilising cell membranes with detergent (NP40 0.3% v/v final) and gently agitating for 30 min. Mouse anti-$G\alpha_{i1/3}$ monoclonal antibodies (Biomol, San Diego, CA) were then added (0.1 μg of IgG per well) and plates incubated for a further 2 h to allow antibody-Gα complexes to form. Since CHO cells do not express $G\alpha_{i1}$, the assay detects activation of $G\alpha_{i3}$ (see Gettys *et al*., 1994; Newman-Tancredi *et al*., 2002a and Introduction). The specificity of the anti-$G\alpha_{i1/3}$ antibody itself was verified by Western blots against a range of purified Gα subunits indicating an absence of cross-reactivity with $G\alpha_{i2}$, $G\alpha_o$, $G\alpha_S$, $G\alpha_q$, $G\alpha_{13}$ (Cussac *et al*., 2002). At the end of the incubation period, SPA beads coated with anti-mouse 2nd antibody (Amersham, Les Ulis, France), were added at the manufacturer's recommended concentrations and incubated with gentle agitation overnight before radioactivity counting. All incubation steps were carried out at room temperature. Non-specific binding was defined with 10 μM GTPγS and, unless indicated otherwise, subtracted from observed [^{35}S]-GTPγS binding prior to analysis.

Data analysis

Concentration – response isotherms for the effect of drugs on $G\alpha_{i3}$ subunit activation were analysed by nonlinear regression using the program "Prism" (Graphpad Software Inc., San Diego, CA, (U.S.A.)). Isotherms were fitted to a four-parameter, logistic equation to derive values of pseudo-Hill coefficient (nH), potency (pEC$_{50}$) and maximal stimulation (E_{MAX}). The latter was defined as the amount of specific [^{35}S]GTPγS binding expressed as a percentage of basal (agonist-independent) binding (= 100%). Antagonist potency (pK_B values) for dextral shift of agonist-induced stimulation or inverse agonist-induced inhibition isotherms of $G\alpha_{i3}$ activation was calculated by $K_B = [\text{Antagonist}]/\{([EC_{50}']/[EC_{50}])-1\}$, where [Antagonist] = antagonist concentration, [EC$_{50}$] = concentration of agonist/inverse agonist producing half-maximal effect and [EC$_{50}'$] = concentration of agonist producing half-maximal effect in the presence of antagonist. K_B values for inhibition of 5-HT and methiothepin (10 and 100 nM, respectively)-stimulated [^{35}S]GTPγS binding were calculated by $K_B = IC_{50}/\{[(2+(\text{Agonist}/EC_{50})^{nH})^{nH-1}]-1\}$, where IC$_{50}$ is the inhibitory concentration$_{50}$ of the antagonist, Agonist the 5-HT or methiothepin concentration; EC$_{50}$ the effective concentration$_{50}$ of 5-HT/methiothepin alone and nH the Hill coefficient of the 5-HT/methiothepin stimulation isotherm.

Inhibition of [^{35}S]GTPγS binding by unlabelled GTPγS at CHO-h5-HT$_{1B}$ membranes

Isotopic dilution experiments were carried out as described by Newman-Tancredi *et al*. (2000). Binding of radiolabelled [^{35}S]GTPγS to $G\alpha_{i3}$ subunits was inhibited with GTPγS and the resulting isotherms were best fitted by a two-site nonlinear regression analysis, giving inhibitory concentration (IC$_{50}$)

values for HA and LA binding components, respectively. HA binding observed under basal conditions (i.e., not agonist-induced) reflects receptor-dependent G-protein activation and provides a direct measure of constitutive activity (Newman-Tancredi *et al*., 2000; Audinot *et al*., 2001b), whereas LA binding reflects endogenous GDP/GTP turnover of CHO cell membrane Gα subunits. Binding data from these experiments expressed in fmol mg^{-1} of protein were normalised to account for the concentration of [^{35}S]GTPγS present in the assay. Hence, units are denoted fmol of [^{35}S]GTPγS bound mg^{-1} of protein nM^{-1} [^{35}S]GTPγS in the incubation.

Compounds

5-HT creatinine sulphate was purchased from Sigma (Saint Quentin Fallavier, France) and methiothepin maleate from Tocris Cookson (Southampton, England). 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-[2,3f]-indole-3,4'-piperidine]-oxalate) and S18127 (N-[1-(2,3-dihydro[1,4] dioxin-5-yl)piperid-4-yl] indan-2-yl-amine) dichlorohydrate were synthesised by Jean-Louis Peglion, Servier. BMS 181,101 (5-fluoro-3-{3-[4-(5-methoxy-pyrimidin-4-yl)-piperazin-1-yl]-propyl}-1H-indole) di-hydrochloride and alniditan were synthesised by Gilbert Lavielle, Servier. Compounds were dissolved in water at 1 mM or in dimethylsulphoxide at 10 mM and diluted in the appropriate assay buffer to the required experimental concentrations.

Results

Specific Gα$_{i3}$ G-protein subunit activation

The antibody capture/SPA technique detected h5-HT$_{1B}$ receptor-mediated Gα$_{i3}$ subunit activation. 5-HT and methiothepin, up to micromolar concentrations, did not modify Gα$_{i3}$ activation from basal levels in membranes of control (untransfected) CHO cells (not shown) demonstrating that h5-HT$_{1B}$ receptors mediate actions described below. Further, when experiments were carried out in the absence of anti-Gα$_{i3}$ antibodies, or in the presence of another IgG (monoclonal anti-Extracellular Regulated Kinase; pERK, Nanotools, Germany), no stimulation of [^{35}S]GTPγS binding was detected (Newman-Tancredi *et al*., 2002a; and data not shown).

Influence of agonists, partial agonists and inverse agonists

In CHO-h5-HT$_{1B}$ cell membranes, 5-HT yielded sigmoidal [^{35}S]GTPγS binding isotherms with a pEC$_{50}$ value of 8.97±0.20 (Figure 1; Table 1). BMS181,101 and alniditan also efficaciously stimulated [^{35}S]GTPγS binding, whereas the selective 5-HT$_{1B}$ receptor ligand, S18127, only slightly increased Gα$_{i3}$ activation suggesting weak partial agonist properties. Methiothepin and the selective 5-HT$_{1B}$ receptor ligand, SB224,289, exhibited inverse agonist properties in inhibiting basal Gα$_{i3}$ activation (Figure 1; Table 1). 5-HT (10 nM)-stimulated Gα$_{i3}$ activation was concentration-dependently reversed by S18127 (Figure 2) with a pK_B of 7.91±0.35. Further, S18127 (1 μM) shifted the 5-HT isotherm to the right with a pK_B value of 8.01±0.15. S18127 also reversed methiothepin (100 nM)-inhibited Gα$_{i3}$ activation, with a pK_B of 6.80±0.11. Similarly, S18127 (1 μM) shifted the methiothepin inhibition isotherm to the right with a pK_B of 6.86±0.17 (Figure 2).

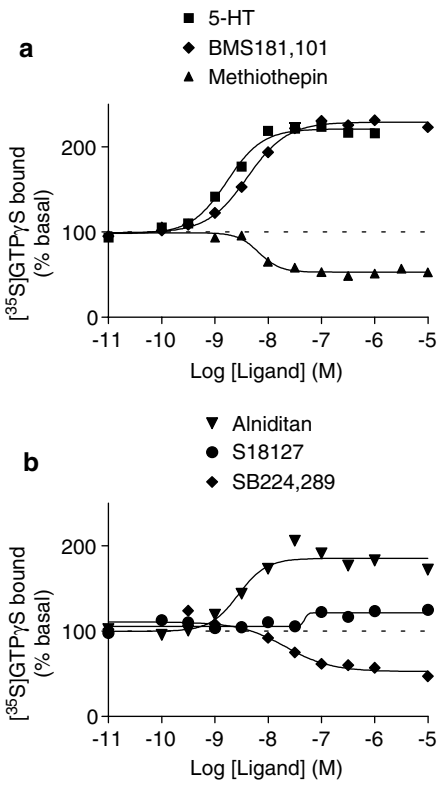

Figure 1 Action of agonists and inverse agonists on [^{35}S]GTPγS binding to Gα$_{i3}$ subunits in CHO-h5-HT$_{1B}$ cell membranes. Panel a: concentration – response isotherms of 5-HT, BMS181,101 and methiothepin. Panel b: concentration – response isotherm of alniditan, S18127 and SB224,287. Data points are means of duplicate determinations from representative experiments repeated on at least three independent occasions. Data from these experiments are summarised in Table 1.

Isotopic dilution "saturation binding"

Under basal conditions (absence of receptor ligand), inhibition of [^{35}S]GTPγS binding by GTPγS produced biphasic isotherms (two-site fit statistically superior to single-site fit; $P<0.05$). This is consistent with spontaneous induction of G-protein activation, that is, constitutive activity (Figure 3). 5-HT (1 μM) increased the number of HA sites by 2.8-fold and increased the pIC$_{50}$ of the HA component from 8.32±0.09 under basal conditions to 9.34±0.04 with 5-HT (Table 2). Conversely, the number of HA sites was reduced by the inverse agonist, methiothepin (1 μM), which also decreased the pIC$_{50}$ value to 7.81±0.07 (Table 2).

In contrast to HA sites, the number of LA binding sites observed in [^{35}S]GTPγS *versus* GTPγS binding isotherms was *not* affected by the presence of receptor ligands. However, an increase in the affinity of the low potency component was observed (pIC$_{50}$ increased from 5.45 under basal conditions to 5.90 with 5-HT), possibly suggesting the presence of additional coupling states of the receptor to Gα$_{i3}$ subunits (cf. Rouleau *et al*., 2002, who reported a third affinity component for H$_3$ receptors).

Table 1 Stimulation of $G\alpha_{i3}$ G protein subunits at h5-HT$_{1B}$ receptors

	$G\alpha_{i3}$ activation		"Total" G–proteins		Affinity
Ligand	*pEC_{50}*	*E_{MAX} (%)*	*pEC_{50}*	*E_{MAX} (%)*	*pk_i*
5-HT	8.97±0.20	100	8.06	100	8.99
BMS181,101	8.67±0.33	109±2	7.90	85	7.56
Alniditan	8.84±0.23	88±12	8.15	87	7.72[a]
S18127	7.17±0.55	30±5	—	0	7.43
SB224,289	7.68±0.04[b]	−44±1	7.79[b]	−40	8.56
Methiothepin	8.15±0.05[b]	−48±6	7.83[b]	−42	8.51

Activation of $G\alpha_{i3}$ subunits in CHO cells stably expressing h5-HT$_{1B}$ receptors was determined employing an antibody-capture/SPA detection technique. Data are expressed as means±s.e.m.'s of three or more determinations performed in duplicate. 5-HT stimulated $G\alpha_{i3}$ subunit activation by 1.81±0.16-fold above specific basal binding (see Fig. 1). E_{MAX} values in the table are expressed as a percentage of the stimulation induced by a maximally effective concentration of 5-HT (1 μM). For comparative purposes, the table shows previously determined potencies and efficacies for stimulation of 'total' G-proteins in identical membrane preparations. Ligand affinities (pK_i) determined by competition binding with [^{3}H]GR125,743 are also shown. (Data are from Newman–Tancredi *et al*., 2000; Audinot *et al*., 2001a.) [a]Unpub. obs. [b]pIC_{50} for inverse agonists.

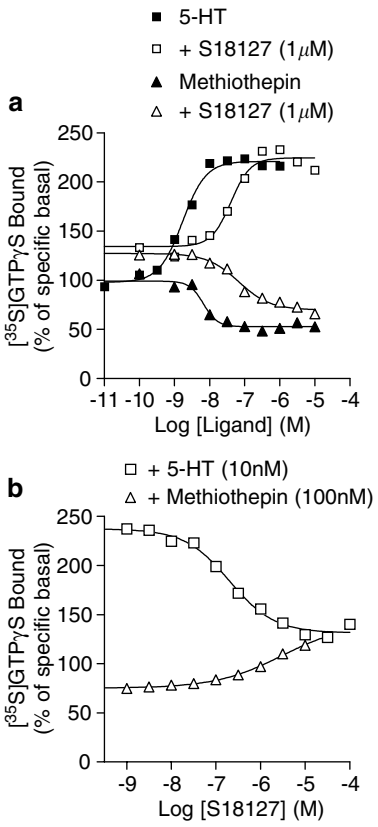

Figure 2 Antagonism by S18127 of 5-HT-stimulated and methiothepin-inhibited [^{35}S]GTPγS binding to $G\alpha_{i3}$ subunits in CHO-h5-HT$_{1B}$ cell membranes. Panel a: concentration – response isotherms of 5-HT and methiothepin alone or in the presence of S18127 (1 μM). Panel b: concentration-dependent reversal by S18127 of 5-HT (10 nM)-induced stimulation or methiothepin (100 nM)-induced inhibition of $G\alpha_{i3}$ activation. Data points are the means of duplicate determinations from representative experiments repeated on at least three independent occasions.

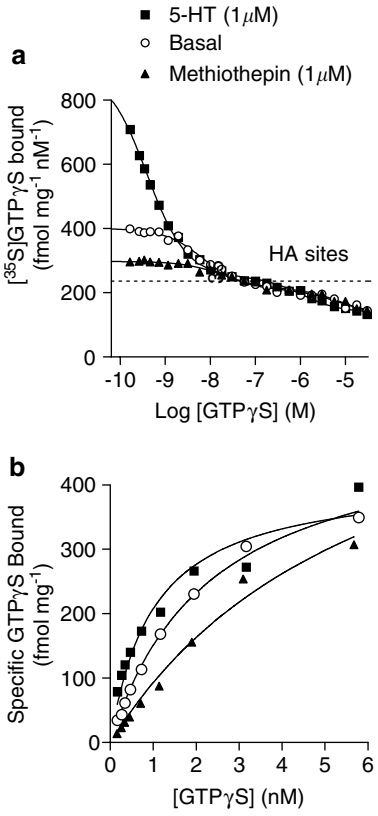

Figure 3 Inhibition, by isotopic dilution with GTPγS, of [^{35}S]GTPγS binding to $G\alpha_{i3}$ subunits in membranes of CHO cells expressing h5-HT$_{1B}$ receptors. Panel a: [^{35}S]-GTPγS isotopic dilution under basal conditions (no receptor ligands) and in the presence of 5-HT (1 μM) or methiothepin (1 μM). The dotted lines indicate the HA binding component of the isotherms. Isotherms determined by nonlinear regression are biphasic (two-site fit statistically superior to a single site fit; $P<0.05$, F-test). Panel b: $G\alpha_{i3}$ subunit saturation binding isotherms derived from [^{35}S]GTPγS isotopic dilution experiments (as described in Materials and Methods). Isotherms are shown under basal conditions and in the presence of 5-HT (1 μM) or methiothepin (1 μM). Points shown are from representative experiments performed in duplicate and repeated on at least three independent occasions. Data from these experiments are summarised in Table 2.

The isotopic dilution experiments ($n=4$) were used to derive K_D (nM) and B_{MAX} values (fmol mg^{-1}) for $G\alpha_{i3}$ subunits as described by Newman-Tancredi *et al*. (2000). B_{MAX} values did not vary significantly: basal conditions: 900±280 fmol mg^{-1}; with 1 μM 5-HT: 510±30 fmol mg^{-1}; with 1 μM methiothepin: 1180±270 fmol mg^{-1}. In contrast, K_D values were significantly modified in the presence of receptor ligands: basal conditions: 3.0±0.6 nM; with 1 μM 5-HT: 1.2±0.2 nM ($P<0.05$, two-tailed t-test *versus* basal conditions); with 1 μM methiothepin: 6.5±1.0 nM ($P<0.05$, two-tailed t-test *versus* basal conditions).

Table 2 Inhibition by GTPγS of [^{35}S]GTPγS binding to Gα_{i3} G protein subunits at h5-HT$_{1B}$ receptors

	HA sites+LA sites (*fmol mg^{-1} nM^{-1}*)	*HA sites* (*fmol mg^{-1} nM^{-1}*)	*pIC$_{50}$ (HA)*	*LA sites* (*fmol mg^{-1} nM^{-1}*)	*pIC$_{50}$ (LA)*
Basal	336 (*1.0*)	244±27 (*1.0*)	8.32±0.09	92±9	5.45±0.10
5-HT	792 (*2.4*)	675±84[a](*2.8*)	9.34±0.04[a]	117±5	5.90±0.07[a]
Methiothepin	236 (*0.7*)	129±22[a](*0.5*)	7.81±0.07[a]	107±12	5.29±0.16

Activation of Gα_{i3} G-protein subunits in CHO cells stably expressing h5-HT$_{1B}$ receptors was determined employing an antibody-capture/SPA detection technique. [^{35}S]GTPγS binding was inhibited with unlabelled GTPγS in the presence or absence of 5-HT (1 μM) or methiothepin (1 μM). Isotherms were analysed by nonlinear regression yielding high-affinity (HA) and low-affinity (LA) specific binding components (expressed as fmol of [^{35}S]GTPγS bound mg^{-1} of protein nM^{-1} [^{35}S]GTPγS present in the experiment). In all cases a two-site fit was statistically superior to a single site fit ($P<0.05$, F-test). Data are means±s.e.m.'s of at least three independent experiments. Data in italics within parentheses are the ratios of values in the presence of ligand to those under basal conditions. [a]$P<0.05$, two-tailed unpaired *t*-test versus respective basal values.

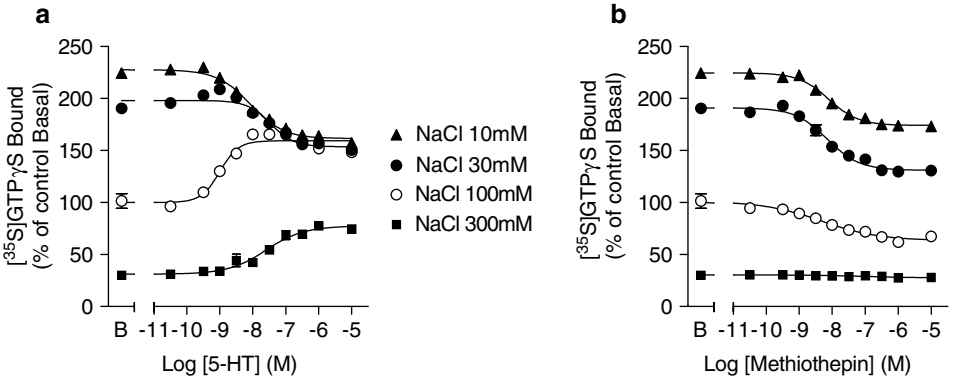

Figure 4 Influence of NaCl concentrations on [^{35}S]GTPμS binding to Gα_{i3} subunits in CHO-h5-HT$_{1B}$ cell membranes. The influence of four concentrations of NaCl on the actions of a full agonist, 5-HT (panel a), and on the actions of the inverse agonist, methiothepin (panel b), are shown. B = basal binding. Data points are the means of duplicate determinations from representative experiments repeated on at least three independent occasions with similar results. Data from these experiments are summarised in Table 3.

Table 3 Influence of NaCl on [^{35}S]GTPγS binding to Gα_{i3} G protein subunits at h5-HT$_{1B}$ receptors

	10 mM NaCl	*30 mM NaCl*	*100 mM NaCl*	*300 mM NaCl*
5-HT				
Bottom	167±5	154±5	100	39±6
Top	237±6	207±4	161±4	84±5
*p*EC$_{50}$	8.49±0.28[a]	7.86±0.27[a]	9.08±0.12	7.44±0.08
nH	−0.98±0.11	−1.36±0.09	+1.53±0.17	+0.83±0.07
Methiothepin				
Top	237±12	204±8	109±9	46±8
Bottom	192±18	153±11	81±9	44±8
*p*EC$_{50}$	8.27±0.16	8.17±0.23	8.32±0.03	n.c.
nH	−0.83±0.13	−1.09±0.10	−0.90±0.20	n.c.

Activation of Gα_{i3} in CHO cells stably expressing h5-HT$_{1B}$ receptors was determined employing an antibody-capture/SPA detection technique. Isotherms were analysed by a 4-parameter logistic equation. "Bottom" refers to the lower plateau (normalised to the binding observed under standard basal conditions, that is, 100 mM NaCl without non-specific subtraction). "Top" is the upper plateau, pEC$_{50}$ is the concentration of ligand inducing a half-maximal effect and nH is the pseudo-Hill coefficient (positive for stimulation of Gα_{i3}, negative for its inhibition). Data are means±s.e.m.'s of at least three independent experiments performed in duplicate. n.c. = not computable. Representative isotherms for these data are shown in Fig. 4. [a]Inhibition of basal binding.

Modulation of Gα_{i3} activation by NaCl concentration

The influence of NaCl on Gα_{i3} activation was examined by investigating the responses to 5-HT and methiothepin in the presence of four different concentrations (10, 30, 100, 300 mM) (Figure 4; Table 3). When the concentration of NaCl was increased to 300 mM, basal Gα_{i3} activation was reduced to just ~40% of basal control values (100 mM NaCl; Table 3). Under these conditions, no constitutive activity was detectable as demonstrated by the absence of inhibition by the inverse agonist, methiothepin. Conversely, at 10 mM NaCl, basal Gα_{i3} activation was high, attaining 237% of control values. Under

these conditions, methiothepin exhibited robust inhibition of Gα_{i3} activation (Table 3). In the case of 5-HT, a striking NaCl-dependent reversal of its actions from stimulation to inhibition of Gα_{i3} activation was observed. Thus, at 300 and 100 mM NaCl, basal binding was low and 5-HT robustly stimulated Gα_{i3} activation. In contrast, at 30 or 10 mM NaCl, no stimulation by 5-HT was observed: indeed, 5-HT concentration-dependently inhibited Gα_{i3} activation. The isotherms of 5-HT in the presence of 10, 30 or 100 mM NaCl converged at a similar level of Gα_{i3} activation ($\sim$160% above basal control values; Figure 4), suggesting stabilisation of a common conformation of a h5-HT$_{1B}$ receptor-Gα_{i3} subunit complex.

Discussion

h5-HT$_{1B}$ receptor-mediated Gα_{i3} activation by agonists and inverse agonists

Although, as noted in the Introduction, previous studies have investigated the coupling of 5-HT$_{1B}$ receptors to G-proteins, little is known concerning the activation patterns of specific G-protein subtypes. As shown in Table 1, the pattern of activation of Gα_{i3} generally resembled that described using classical [^{35}S]GTPγS binding filtration assays (Thomas *et al*., 1995; Selkirk *et al*., 1998; Newman-Tancredi *et al*., 2000; Audinot *et al*., 2001a). Thus, BMS181,101 and alniditan exhibited high efficacy for Gα_{i3} activation relative to 5-HT, whereas SB224,289 and methiothepin exhibited inverse agonist properties. However, a detailed comparison of results revealed some interesting differences. First, whereas BMS181,101 behaved as an efficacious but submaximal agonist (E_{MAX} 85%) in conventional [^{35}S]GTPγS binding experiments, its efficacy for Gα_{i3} activation consistently exceeded that of 5-HT. Second, S18127 behaved as a "neutral" antagonist in [^{35}S]GTPγS binding (Audinot *et al*., 2001a) but exhibited modest partial agonist properties for Gα_{i3} activation. Third, potencies of agonists for Gα_{i3} activation were markedly greater than for "total" G-protein activation. Thus, the profile of action of h5-HT$_{1B}$ receptor ligands for Gα_{i3} subunit activation may differ from that of other G-potein subtypes. "Classical" (overall) G-protein activation assays mask these differences by yielding a composite response of multiple G-proteins. Future SPA studies of other G$_i$ subtypes, in particular Gα_{i2} (Clawges *et al*., 1997), coupled to h5-HT$_{1B}$ receptors must address this question directly. In contrast to agonists, negative efficacies of inverse agonists for inhibition of Gα_{i3} activation corresponded closely to those reported by conventional methods. This raises the possibility that a major proportion of constitutive activity in CHO-h5-HT$_{1B}$ cell membranes may be mediated by Gα_{i3} subunits. A further point should be noted: the selective ligand, S18127, reversed both 5-HT-mediated stimulation and methiothepin-mediated inhibition of Gα_{i3} subunit activation (Figure 2), but pK_B values obtained differed (7.9 *versus* 5-HT compared with 6.8 *versus* methiothepin). The lower pK_B values observed for S18127 against methiothepin are consistent with previous data derived from "total" G-protein assays (Audinot *et al*., 2001a) and may be due to methiothepin's proposed capacity to stabilise specific conformations of G-protein-coupled receptors, such as 5-HT$_{1A}$ receptors (McLoughlin & Strange, 2000). Whatever the mechanism involved, blockade by S18127 of the actions of inverse agonists provides compelling evidence for constitutive activation of Gα_{i3} subunits in these CHO-h5-HT$_{1B}$ cell membranes.

Isotopic dilution Gα_{i3} saturation binding

In previous studies, we (Newman-Tancredi *et al*., 2000; Audinot *et al*., 2001b) and others (Rouleau *et al*., 2002) described a methodology to directly quantify the degree of constitutive G-protein activation without the need for inverse agonists. The strategy was based on isotopic dilution of [^{35}S]GTPγS with increasing concentrations of GTPγS. Thus, high-affinity [^{35}S]GTPγS binding sites observed under basal conditions (absence of receptor ligands) provide a measure of constitutive G-protein activation. In our previous study of h5-HT$_{1B}$ receptors, agonists increased the amount of high-affinity [^{35}S]GTPγS binding. Conversely, inverse agonists decreased the number of high-affinity binding sites. Neither agonists nor inverse agonists markedly influenced low-affinity [^{35}S]GTPγS binding sites (Newman-Tancredi *et al*., 2000; Audinot *et al*., 2001b). A similar pattern was observed in the present study of Gα_{i3}. Thus, under basal conditions, [^{35}S]GTPγS binding to Gα_{i3} was biphasic, indicating the presence of constitutive Gα_{i3} activation. As for "total" G-protein activation assays, 5-HT increased HA [^{35}S]GTPγS binding, with a marked increase in the affinity of GTPγS for Gα_{i3} subunits (pIC_{50} increased from 8.32 to 9.34; Table 2). In contrast, methiothepin decreased the number of high affinity sites with a reduction in the affinity of GTPγS (pIC_{50} decreased from 8.32 to 7.81; Table 2). It is interesting that methiothepin reduced the number of high affinity sites by about 50% compared with a 70% decrease in conventional [^{35}S]GTPγS binding experiments (Newman-Tancredi *et al*., 2000). This suggests that methiothepin displays sub-maximal "negative" efficacy at Gα_{i3}. Indeed, just as agonists exhibit "agonist-directed trafficking of receptor signalling" (Kenakin, 1995a), inverse agonists may differentially inhibit signalling of receptors to specific G-proteins (Berg *et al*., 1999). Indeed, the B_{MAX} of Gα_{i3} subunits determined from the isotopic dilution experiments ($\leqslant$1 pmol mg^{-1}), amounted to a third of "total" G-protein activation ($\sim$3 pmol mg^{-1}) in this expression system (Newman-Tancredi *et al*., 2000) indicating that other populations of G-proteins expressed in CHO cells couple to h5-HT$_{1B}$ receptors. It would be interesting to investigate the influence of alterations in receptor/G-protein stoichiometry on activation of Gα_{i3} subunits. Changes in the expression levels of Gα_{i3} may lead to alterations in the equilibrium of coupling of 5-HT$_{1B}$ receptors to other G-protein subtypes. Though Gα_{i2} is likely to be involved (Clawges *et al*., 1997), this remains to be directly demonstrated.

Influence of NaCl on h5-HT$_{1B}$ receptor-mediated Gα_{i3} constitutive activation

Previous studies have demonstrated the importance of NaCl in influencing receptor/G-protein coupling (see Introduction). Indeed, as for "total" G-protein assays at other G-protein coupled receptors (De Ligt *et al*., 2000), NaCl concentrations herein were inversely related to basal Gα_{i3} activation (Figure 4) and the inverse agonist, methiothepin, failed to inhibit [^{35}S]GTPγS binding in the presence of 300 mM NaCl when constitutive activity was entirely suppressed. These data are

reminiscent of studies at 5-HT$_{1A}$ receptors, where inverse agonist properties of certain ligands could only be observed in the absence of NaCl (Cosi & Koek, 2000). However, a striking result was obtained concerning the influence of 5-HT on Gα_{i3} activation. Whereas, under standard conditions (100 mM NaCl), 5-HT exhibited robust stimulation of [^{35}S]GTPγS binding to Gα_{i3} subunits, at low NaCl concentrations (10 mM) it *inhibited* Gα_{i3} activation. Hence, under the latter condition, 5-HT behaved as an inverse agonist, reversing basal [^{35}S]GTPγS binding to Gα_{i3} subunits in a manner similar to that of methiothepin (Figure 4; Table 3). This observation is consistent with the concept of a protean agonist (Kenakin, 1995b), by which ligands can exhibit either agonist or inverse agonist properties depending on ''receptor tone''. When the latter is low (i.e., little constitutive G-protein activation), agonist properties of the ligand (in this case, 5-HT) are revealed. In contrast, when receptor tone is pronounced (i.e., a high level of constitutive activity), the ligand stabilises the receptor in a conformation which is *less* able to activate G-proteins than the free, non-ligand-occupied, receptor. It is noteworthy that, over a wide range of NaCl concentrations (10–100 mM), saturating levels of 5-HT induced a similar degree of Gα_{i3} labelling, either by stimulating or inhibiting [^{35}S]GTPγS binding from basal levels. At an intermediate NaCl concentration (about 40 mM), 5-HT would be expected to have *no* effect on Gα_{i3} activation, thus behaving as a neutral antagonist. It would be interesting to examine other 5-HT$_{1B}$ agonists to determine whether they display similar sensitivity to NaCl.

Previous reports have described protean agonist properties of secretin at constitutively active mutants of secretin receptors (Ganguli *et al*., 1998), and of medetomidine and the dexefaroxan analogue, RX831003, at α_{2A}-adrenoceptors (Jansson *et al*., 1998; Pauwels *et al*., 2002). However, to our knowledge, this is the first demonstration that 5-HT can exhibit protean agonism at non-mutant receptors. The physiological implications of these data are unclear, but Na^+/H^+ exchange in CHO cells is known to be regulated by Gα_{i3} subunits (Garnovskaya *et al*., 1997) and modulation of Na^+ currents is involved in multiple physiological functions (Urenjak & Obrenovitch, 1996; Cantrell & Catterall, 2001). Indeed, whereas plasma levels of sodium are about 120 mM, G-proteins located on the intracellular surface of plasma membranes are exposed to sodium concentrations of 5–10 mM, except under neuronal depolarisation conditions, when extracellular sodium enters the cells. Thus, it may be speculated that the response to 5-HT by 5-HT$_{1B}$ receptor-activated Gα_{i3} subunits may shift from inhibition to stimulation depending on polarisation state. Further, it would be of interest to investigate whether these actions of NaCl are due to an influence upon Gα_{i3} subunits and/or its allosteric regulation of a conserved aspartate residue at the intracellular terminal of the putative second transmembrane segment of the receptor. Mutation of this residue in α_2-adrenoceptors abolishes the ability of Na^+ to modulate receptor–ligand interactions (Horstman *et al*., 1990).

Conclusions

The present data demonstrate that: (i) h5-HT$_{1B}$ receptor activation of Gα_{i3} subunits can be increased by agonists and decreased by inverse agonists. The reversal of the inhibitory actions of methiothepin by the selective ligand, S18127, demonstrates the presence of constitutive activity; (ii) quantification of Gα_{i3} constitutive activity by homologous [^{35}S]GTPγS *versus* GTPγS inhibition isotherms indicates that methiothepin inhibits about half of the high affinity binding sites (i.e. constitutive activity) suggesting that it exhibits submaximal negative efficacy for Gα_{i3} subunit activation and (iii) modulation of NaCl concentrations reveals the protean agonist properties of 5-HT for activation of Gα_{i3} subunits. It would be interesting to investigate whether similar signalling patterns are observed for other Gα subunits and signal transduction responses, such as adenylyl cyclase inhibition.

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