European Journal of Pharmacology 594 (2008) 32–38

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Agonist-directed trafficking of signalling at serotonin 5-HT_{2A}, 5-HT_{2B} and 5-$HT_{2C\text{-}VSV}$ receptors mediated Gq/11 activation and calcium mobilisation in CHO cells

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ARTICLE INFO

Article history:
Received 22 April 2008
Received in revised form 4 July 2008
Accepted 22 July 2008
Available online 30 July 2008

Keywords:
5-HT_2 receptor
Gq protein
Calcium release
Functional selectivity
Hallucinogen

ABSTRACT

Several examples of agonist-directed trafficking of receptor signalling at 5-HT_{2A} and 5-HT_{2C} receptors have been reported that involve independent downstream transduction pathways. We now report the functional selectivity of a series of chemically diverse agonists at human (h)5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ by examining two related responses, the upstream activation of Gq/11 proteins in comparison with its associated cascade of calcium mobilisation. At the h5-HT_{2A} receptor, *d*-lysergic acid diethylamide (LSD) and the antiparkinsonian agents lisuride, bromocriptine and pergolide exhibit a higher potency for Gq/11 activation than calcium release in contrast with all the other tested ligands such as 5-HT, mCPP and BW723C86, that show an opposite preference of signalling pathway. Comparable observations are made at h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors, suggesting a similar mechanism of functional selectivity for the three serotonin receptors. Interestingly, the non-hallucinogenic compound lisuride behaves as a partial agonist for both Gq/11 activation and calcium release at the three 5-HT_2 receptors, in contrast with DOI, LSD, pergolide and bromocriptine, which are known to provoke hallucinations, and behave as more efficacious agonists. Hence, a functional selectivity for Gq/11 activation together with a threshold of efficacy at h5-HT_{2A} (and possibly h5-HT_{2B} and/or h5-$HT_{2C\text{-}VSV}$) may contribute to hallucinogenic liability. Thus, our results extend the notion of agonist-directed trafficking of receptor signalling to all the 5-HT_2-receptor family and indicate that measures of Gq/11 activation versus calcium release may be useful to identify more effective therapeutic drugs with limited side effects.

1. Introduction

The 5-HT_2 receptor family is involved in multiple physiological functions including smooth muscle contraction, platelet aggregation, control of endocrine secretion, feeding behaviour, mood, pain, sleep, thermoregulation, learning and memory (Buhot, 1997; Roth et al., 1998). It has also been implicated in several central nervous system disorders such as anxiety, depression, migraine, schizophrenia and mania (Akiyoshi et al., 1996; Baxter et al., 1995; Bickerdike, 2003). The 5-HT_2 receptor family comprises the 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors which have distinct regional distributions within the brain (Pompeiano et al., 1994; Clemmett et al., 1999), and it is therefore reasonable to hypothesise that each subtype may be capable of mediating specific physiological functions.

The three 5-HT_2 receptors show high homology in their primary and secondary structure (Humphrey et al., 1993; Baxter et al., 1995) and also in their pharmacology (Porter et al., 1999; Nelson et al., 1999; Jerman et al., 2001; Cussac et al., 2002b). The 5-HT_2 receptors are mainly coupled via heterotrimeric GTP binding protein of the Gq/11 type to phospholipase C (PLC) (Gerhardt and Heerikhuizen, 1997; Berg et al., 1998, 2001; Cussac et al., 2002a). Activation of PLC elevates cytosolic levels of inositol phosphates, and subsequently increases levels of intracellular calcium, two major parameters exploited for characterization of drug efficacy at 5-HT_2 receptor subtypes (Porter et al., 1999; Cussac et al., 2002b). Furthermore, previous studies have shown that both 5-HT_{2A} and 5-HT_{2C} receptors can couple independently to PLC and phospholipase A_2 (PLA_2) and that the relative efficacy of the agonists at these receptors can differ depending upon whether phosphatidylinositol hydrolysis or arachidonic acid release is measured (Berg et al., 1998, 2001; Kurrasch-Orbaugh et al., 2003; Moya et al., 2007). This concept of agonist-directed trafficking of receptor stimulus or functional selectivity (Kenakin, 1995, Urban et al., 2006) has also been extended to 5-HT_{2C} receptors coupled to PLC versus extracellular signal-regulated kinase 1/2 phosphorylation where the rank order of relative efficacy of DOI and quipazine was reversed (Werry et al., 2005). Although the precise nature of G proteins involved in these transduction pathways remains to be elucidated, 5-HT_{2C} receptors are known to interact with other G

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doi:10.1016/j.ejphar.2008.07.040

proteins than Gq/11, notably Gi3 and G13, depending also on the nature of 5-HT_{2C} receptor editing (Alberts et al., 1999; Cussac et al., 2002a; Chang et al., 2000; McGrew et al., 2002). Furthermore, a previous report has shown that agonist efficacy at 5-$HT_{2C\text{-}VSV}$ receptors coupled to Gq/11 and Gi3 activation was both ligand- and receptor reserve-dependent (Cussac et al., 2002a) similar to 5-HT_{2A} receptor coupling to PLC and PLA_2 (Kurrasch-Orbaugh et al., 2003).

One of the important implications of agonist-directed trafficking of 5-HT_2 receptor stimulus is that drugs may have a richer pharmacology than previously thought. By understanding the molecular mechanisms of ligand-selective pathway activation, it may be possible to enhance 5-HT_2 receptor-mediated therapeutic action without eliciting associated side effects such as hallucinations (see Berg and Clarke, 2006 for review). 5-HT_{2A} receptors are thought to be the primary target of hallucinogen compounds (González-Maeso et al., 2003, 2007, see Nichols 2004 for review), although other receptors may play a role (Nichols, 2004; González-Maeso et al., 2008). Interestingly, functional selectivity was observed for a series of phenethylamine and phenylisopropylamine compounds at 5-HT_{2A} and 5-HT_{2C} receptor-mediated PLC and PLA_2 activation (Kurrasch-Orbaugh et al., 2003; Moya et al., 2007). In the present study, we confirm and extend these previous observations by reporting a differential agonist action of a series of ligands. Particularly, we show that *d*-lysergic acid diethylamide (LSD) and ergot derivative antiparkinsonian compounds elicit agonist-directed trafficking of the 5-HT_{2A}, 5-HT_{2B} and 5-$HT_{2C\text{-}VSV}$ receptors coupled to Gq/11 activation versus calcium mobilisation.

2. Methods

2.1. Cell lines and membrane preparations

CHO cells stably expressing human (h)5-HT_{2A} (4 pmol/mg), h5-HT_{2B} (5 pmol/mg) and the edited h5-HT_{2C} receptors (VSV isoform, 20 pmol/mg) were obtained from Euroscreen (Perkin-Elmer, Shelton, USA) and grown in adherent culture in 225 cm^2 flasks with UltraCHO medium (BioWhittaker Europe, Verviers, Belgium) containing dialyzed fetal calf serum (1%) and geneticin (1.25 mg/ml). At confluence, cells were washed twice with PBS and harvested in a buffer containing 20 mM HEPES and 150 mM NaCl, pH 7.4. The suspension was then centrifuged at 500 g for 10 min at 4 °C. Cells were homogenized using a Polytron homogenizer in the same buffer and crude membrane preparation was obtained by centrifugation twice at 20,000 g for 20 min at 4 °C with a step of washing. The pellet was resuspended in 20 mM HEPES and 150 mM NaCl, pH 7.4, and aliquots were stored at −80 °C until assay.

2.2. Competition binding assays

Binding affinity at h5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors was determined on membrane preparations. Briefly, all incubations were performed for 2 h at room temperature in 20 mM HEPES buffer containing 120 mM NaCl, 5 mM KCl, 1 mM EDTA and 5 mM $MgCl_2$ in the presence of 0.5 nM of [^{3}H]ketanserine (Perkin-Elmer, Life and Analytical Sciences, Courtaboeuf, France) for h5-HT_{2A} receptors and 2 nM of [^{3}H]mesulergine (GE Healthcare, Little Chalfont, UK) for h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors. Compounds were tested in half log concentration ranges from 10^{-11} M to 10^{-5} M and non-specific binding was defined with 10 µM 5-HT. Data are expressed as mean ± S.E.M. of at least three independent determinations performed in duplicate.

2.3. Scintillation proximity assay

Specific activation of Gq/11 proteins by the 5-HT_2 receptor family was determined using Scintillation Proximity Assays (SPA) essentially as previously described (Cussac et al., 2002a). CHO-h5-HT_{2A}, CHO-h5-HT_{2B} and CHO-h5-$HT_{2C\text{-}VSV}$ cell membranes (≈30 µg) were preincubated on 96-well plates with agonists and/or antagonists for 30 min at room temperature in a buffer containing 20 mM HEPES, pH 7.4, 50 mM $MgCl_2$, 150 mM NaCl and 0.1 µM GDP. Reaction was started with 0.2 nM [^{35}S]GTPγS (≈1000 Ci/mmol, GE Healthcare, Little Chalfont, UK) in a final volume of 200 µl for 60 min at room temperature. Reaction was stopped by solubilizing cell membranes by addition of 20 µl of Nonidet P40 (0.27% final concentration) and gentle agitation for 30 min. Rabbit anti-Gq/11 polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were added (1.74 µg/ml final dilution) and the microwell plates were incubated for a further 1 h to allow antibody-Gα complexes to form. At the end of the incubation period, SPA beads coated with anti-rabbit secondary antibody (GE Healthcare, Little Chalfont, UK) were added at a dilution indicated by the manufacturer, and the plates were incubated for 1 h under agitation. The plates were then centrifuged (10 min at 1200 g), and radioactivity was detected on a TopCount microplate scintillation counter. Agonist efficacy is expressed relative to that of a saturating concentration of 5-HT in each experiment. Data are expressed as mean ± S.E.M. of at least three independent determinations performed in duplicate.

2.4. Measurement of intracellular Ca^{2+} responses

CHO cells stably expressing h5-HT_{2A} or h5-HT_{2B} or edited h5-$HT_{2C\text{-}VSV}$ receptors were assayed for Ca^{2+} responses 48 h post-seeding upon 2 h loading with the Ca^{2+} indicator dye Fluo-3 (2 µM) in 20 mM HEPES and 2.5 mM probenicid acid in a 96-well format. Cells were washed 4 times in Hanks' balanced salt solution (HBSS) containing 20 mM HEPES and 2.5 mM probenicid acid; after the final wash, cells were incubated in 100 µl of HBSS. Maximal fluorescent counts (delta of arbitrary fluorescence units (AFU)) were used to determine agonist activity. Fluorescent readings were made every 2 s for a 3 min time period using a fluorometric image plate reader (FLIPR, Molecular Devices, Sunnyvale, CA) as described previously (Pauwels et al., 2000). E_{max} values were defined as the ligand's maximal high-magnitude Ca^{2+} response versus that obtained with 10 µM 5-HT tested in each 96 well plate. Data are expressed as mean ± S.E.M. of at least three independent determinations performed in triplicate.

2.5. Data analysis

Isotherms were analysed by nonlinear regression, using GraphPad Prism v. 4.03 (GraphPad Software Inc., San Diego, CA) to yield E_{max}, EC_{50} and IC_{50} values. Inhibition constants K_i and K_B values were derived from IC_{50} values according to Lazareno and Birdsall (1993): $K_i = IC_{50}/(1+(Ligand/Kd\ Ligand))$ where IC_{50} = Inhibitory Concentration$_{50}$ of the ligand; Ligand = radioligand concentration used in the assay; Kd = Kd of the radioligand and K_B values for inhibition of 5-HT-stimulated Gq/11 activation were: $K_B = IC_{50}/(1+(Agonist/EC_{50}))$ where IC_{50} = Inhibitory Concentration$_{50}$ of the antagonist; Agonist = 5-HT concentration (100 nM for h5-HT_{2A} and h5-HT_{2B} or 10 nM for h5-$HT_{2C\text{-}VSV}$); EC_{50} = Effective Concentration$_{50}$ of 5-HT alone.

2.6. Compounds

5-Hydroxytryptamine (5-HT), bromocriptine, pergolide, 1-2,5-dimethoxy-4-iodophenyl-2-aminopropane (DOI) and 1-(3-chlorophenyl)piperazine (mCPP) were purchased from Sigma-RBI (St Quentin Fallavier, France). Ro600175 (2(S)-1-(6-chloro-5-fluoro-1H-indol-1-yl)-2-propanamine fumarate), Ro600332 (1(S)-methyl-2-(4,4,7-trimethyl-1,4-dihydroindeno[1,2-b]pyrrol-1-yl)ethylamine fumarate) were purchased from Roche (Mannheim, Germany), BW723C86 (1-methyl-2-[5-(2-thienylmethoxy)-1H-indole-3-yl]ethylamine) and 5-carboxytryptamine (5-CT) were purchased from Tocris (Bioblock, Illkirch, France), lisuride was purchased from Schering (Lys-les-Lannoy, France), SB242084 (6-chloro-5-methyl-N-[6-(2-methylpyridin-3-yloxy) pyridin-3-yl]indoline-1-carboxamide), RS127445 (2-amino-4-(4-

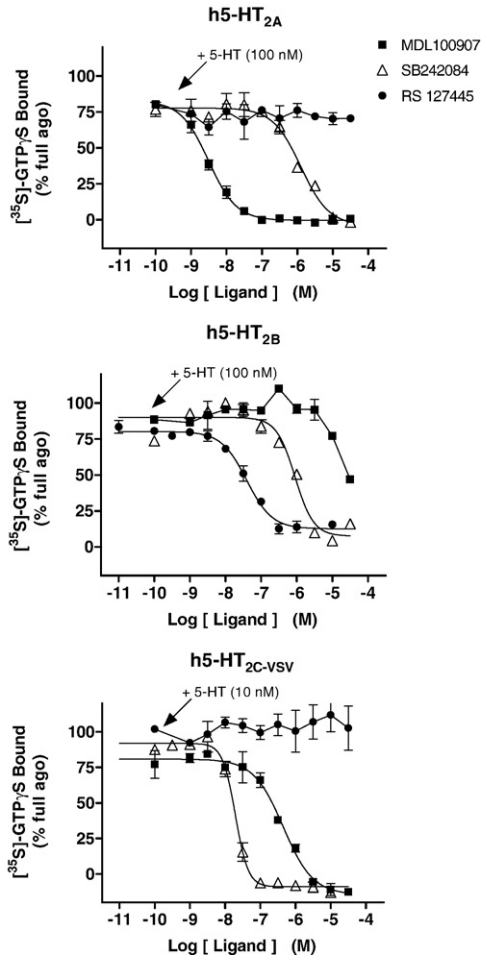

Fig. 1. Antagonism of h5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptor-mediated Gq/11 protein activation. Antagonist concentration–response curves for MDL100907, SB242084 and RS127445 against 5-HT-stimulated [^{35}S]GTPγS binding coupled to a scintillation proximity assay at Gq/11 for h5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors. Points shown are means of duplicate determinations from representative experiments repeated at least three times.

fluoronaphth-1-yl)-6-isopropyl-pyrimidine)-ylcarbamoyl]indoline, MDL100907 (R(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenyl-ethyl)]-4-piperidine-methanol) and *d*-lysergic acid diethylamide (LSD) were synthesized in house.

3. Results

3.1. Action of selective antagonists at h5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors

In order to confirm that responses of ligands were due to the presence of the transfected receptor, selective 5-HT_{2A} (MDL100907), 5-HT_{2B} (RS127445) and 5-HT_{2C} (SB242084) receptor antagonists were tested for their ability to antagonize 5-HT-stimulated Gq/11 proteins at each receptor. As expected, MDL100907 potently antagonized 5-HT-stimulated Gq/11 proteins with a pK_B of 9.12±0.02 at h5-HT_{2A} receptor, SB242084 showing only weak antagonist properties (pK_B of 6.56±0.11), and RS127445 being totally inactive (Fig. 1). Similarly, RS127445 antagonized 5-HT action with a pK_B of 7.98±0.14 at the h5-HT_{2B} receptor, while SB242084 was less potent (pK_B of 6.58±0.10), and MDL100907 partially blocked 5-HT action at high concentration (Fig. 1). Finally, for the h5-$HT_{2C\text{-}VSV}$ receptor, SB242084 antagonized 5-HT-stimulated Gq/11 protein activation with a pK_B of 8.33±0.08 whereas RS127445 was inactive and MDL100907 exhibited less potent antagonist properties (pK_B of 6.67±0.12, Fig. 1). Antagonist actions of these compounds were also observed when calcium mobilisation was determined (data not shown).

3.2. Efficacy and potency of ligands at h5-HT_{2A} receptors coupled to Gq/11 activation and calcium release

The endogenous ligand 5-HT as well as 5-CT exhibited a more pronounced potency to induce Ca^{2+} release than that measured for Gq/11 protein activation (20 to 50 fold) whereas mCPP and DOI showed a modest preference for Ca^{2+} release (2 to 3 fold, Fig. 2 and Table 1). BW723C86, Ro600332 and Ro600175 were quasi equipotent in both tests (Table 1). Furthermore, mCPP and, to a lesser extent, BW723C86 behaved as partial agonist at h5-HT_{2A} receptors in both assays in contrast to the full agonist properties of 5-CT, DOI, Ro600332 and Ro600175.

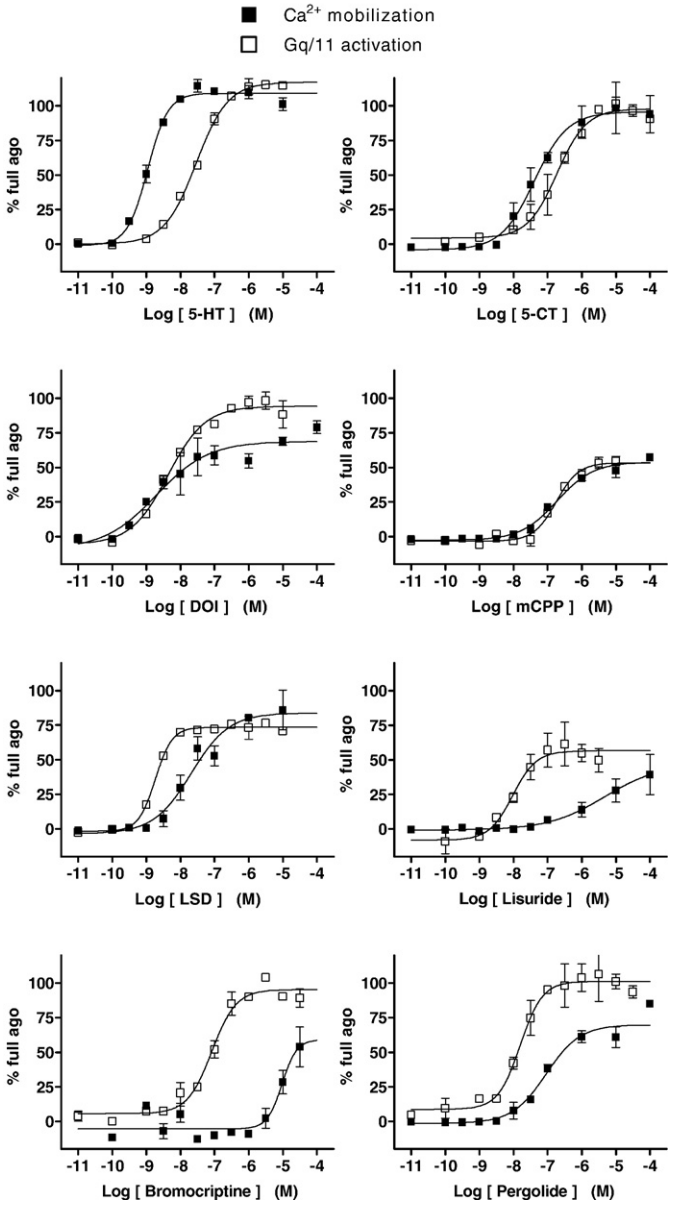

Fig. 2. Agonist concentration–response curves at h5-HT_{2A} receptors coupled to Gq/11 activation (□) and Ca^{2+} release (■). Gq/11 protein activation and Ca^{2+} release are determined in membrane preparation and entire cells respectively, and the results are expressed as a percentage of maximal stimulation obtained with 5-HT at 10 μM (100%). Points are means of duplicate (Gq/11 activation) or triplicate determinations (Ca^{2+} mobilisation) from representative experiments repeated at least three times. E_{max} and pEC_{50} of the series of tested ligands at h5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors are shown in Tables 1 and 2.

Table 1
Stimulation of [^{35}S]GTPγS binding to Gq/11 proteins and stimulation of Ca^{2+} release by agonists at h5-HT_{2A} receptors

5-HT_{2A}						
	Gq/11		Calcium			
	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	ΔpEC_{50}	pK_i
5-HT	7.53±0.06	108.7±6.4	9.24±0.11	103. 6±6.7	−1.71	7.18±0.04
5-CT	6.21±0.21	100.3±12.4	7.41±0.26	84. 9±3.4	−1.20	6.21±0.07
mCPP	6.79±0.04	54.4±1.3	7.28±0.21	71.6±10.5	−0.49	7.39±0.05
DOI	8.29±0.13	93.6±3.9	8.62±0.25	81.3±6.1	−0.33	8.04±0.05
BW723C86	6.88±0.08	82.3±10.3	7.05±0.08	68.3±14.2	−0.17	6.63±0.06
Ro600332	6.30±0.03	100.7±13.2	6.45±0.16	93.3±4.1	−0.15	6.47±0.11
Ro600175	7.31±0.07	92.5±12.8	7.25±0.14	84.6±8.0	+0.06	6.80±0.08
Pergolide	7.68±0.11	91.9±7.8	7.32±0.14	75.6±5.6	+0.36	8.24±0.06
LSD	8.74±0.04	71.2±2.0	7.47±0.06	84.6±9.5	+1.27	9.49±0.03
Bromocriptine	6.98±0.11	85.1±16.0	4.70±0.20	61.4±12.3	+2.28	8.05±0.07
Lisuride	8.26±0.14	40.7±5.3	5.13±0.34	48.6±8.0	+3.13	9.26±0.06

Data (pEC_{50} and E_{max}) are expressed as mean±S.E.M. of at least three independent experiments performed in duplicate (Gq/11 activation) or triplicate determinations (Ca^{2+} mobilisation). Agonist efficacy is expressed relative to that of a saturating concentration of 5-HT. Ligands were classed following their increasing differences of pEC_{50} between the two assays.

In contrast, other ligands were more potent for Gq/11 protein activation than for Ca^{2+} release (positive values of ΔpEC_{50}, see Table 1). Thus, the hallucinogenic compound LSD showed a 20 fold higher potency to stimulate Gq/11 proteins than that observed to induce Ca^{2+} release (pEC_{50}=8.74±0.04 and 7.47±0.06, respectively), while exhibiting a high efficacy in both assays (about 70–80%; Fig. 2 and Table 1). The antiparkinsonian agents pergolide and bromocriptine also behaved as highly efficacious agonists for Gq/11 protein activation (E_{max} of about 90%), while being slightly less efficacious for Ca^{2+} release (E_{max} of about 60–70%). Furthermore, whereas pergolide showed only a two fold preference for Gq/11 protein activation, bromocriptine was 200 fold more potent in the G protein assay than for Ca^{2+} release (pEC_{50}=6.98± 0.11 versus 4.70±0.20; Fig. 2 and Table 1). The most striking separation was observed with the antiparkinsonian agent lisuride that exhibited a selectivity of more than 1000 fold for Gq/11 activation yet it exhibited partial agonist properties for the two pathways (about 40–50%; Fig. 2 and Table 1).

In control experiments, accessibility of LSD, lisuride, pergolide and bromocriptine to h5-HT_{2A} binding sites was demonstrated by inhibiting [^{3}H]ketanserin binding to intact cells in the same buffer used for calcium assay and with a similar potency as that observed in membrane preparations (data not shown). Furthermore, calcium release in naïve CHO cells via ATP-induced endogenous purinergic receptor activation was not modified in the presence of LSD, lisuride and other compounds, suggesting that they did not interfere on the intracellular mechanisms controlling calcium mobilisation (data not shown). pK_i values determined in competition binding assay (Table 1) correlated well with the potency of the ligands measured for Gq/11 protein activation (coefficient of correlation r=0.87, slope=0.65±0.13) but did not correlate with the potencies determined in calcium release (r=0.24, slope=−0.29±0.38).

3.3. Efficacy and potency of ligands at h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors coupled to Gq/11 activation and calcium release

Results at h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors were similar to those observed at h5-HT_{2A} receptors, although subtle differences appeared. With the exception of mCPP, LSD and the antiparkinsonian agents, all other compounds exhibited full agonist properties at h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors coupled to Gq/11 activation and Ca^{2+} release (Table 2). As for h5-HT_{2A} receptors, mCPP behaved as partial agonist at h5-HT_{2B} sites whereas it behaved as a full agonist with a higher potency at h5-$HT_{2C\text{-}VSV}$ (Table 2), suggesting a preferential action of this compound at this later site. Furthermore, like mCPP, Ro600332 showed a certain selectivity for h5-$HT_{2C\text{-}VSV}$, while BW723C86 and Ro600175 were preferential agonists at h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors as compared with h5-HT_{2A} receptors (Table 2).

As in the case of h5-HT_{2A} receptors, LSD and antiparkinsonian agents more potently induced Gq/11 activation than Ca^{2+} release at h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors (Table 2). The only exception concerned the action of pergolide at h5-$HT_{2C\text{-}VSV}$ receptors where the compound showed a higher potency for Ca^{2+} release than for Gq/11 activation. Furthermore, LSD, lisuride and bromocriptine were more efficacious agonists at h5-HT_{2A} and h5-$HT_{2C\text{-}VSV}$ receptors than at h5-HT_{2B} receptors, with bromocriptine being mainly devoid of activity at this latter site (Table 2). In contrast to the other compounds and similarly to DOI, it was also interesting to note that LSD and the antiparkinsonian agents exhibited an overall equilibrated potency at the three h5-HT_2 receptor subtypes or a slight preference for h5-HT_{2A} receptors. pK_i values determined in competition binding assays at h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors (Table 2) correlated only with the potency of ligands measured for Gq/11 protein activation (r=0.84 and 0.74, respectively).

4. Discussion

The main results of this study highlight the differential ability of a series of chemically diverse compounds to activate two signalling pathways in the same cell type via the h5-HT_{2A}, h5-HT_{2B}, and h5-$HT_{2C\text{-}VSV}$ receptors, reinforcing the potential implication of agonist-directed trafficking of signalling at these receptors in the mediation of differential therapeutic or side effects.

Table 2
Stimulation of [^{35}S]GTP$_\gamma$S binding to Gq/11 proteins and stimulation of Ca^{2+} release by agonists at h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors

	5-HT_{2B}						5-$HT_{2C\text{-}VSV}$					
	Gq/11		Calcium				Gq/11		Calcium			
	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	ΔpEC_{50}	pK_i	pEC_{50}	E_{max} (%)	pEC_{50}	E_{max} (%)	ΔpEC_{50}	pK_i
5-HT	8.16±0.11	100.3±2.7	9.47±0.17	109.9±5.1	−1.31	8.38±0.05	8.72±0.05	100±13.4	9.79±0.12	118.3±12.0	−1.07	7.79±0.06
5-CT	7.25±0.17	103.9±8.3	8.64±0.18	94.8±4.2	−1.39	7.33±0.11	6.85±0.10	100.1±3.4	8.18±0.08	104.6±7.4	−1.33	6.35±0.05
mCPP	7.38±0.16	50.8±4.7	7.78±0.15	76.0±4.5	−0.40	8.05±0.08	7.73±0.06	89±5.9	8.55±0.15	112.3±6.6	−0.82	7.59±0.07
DOI	8.01±0.10	85.4±1.0	8.47±0.21	91.0±2.0	−0.46	7.78±0.09	8.13±0.12	96.3±4.8	8.57±0.15	106.7±8.2	−0.44	7.73±0.04
BW723C86	6.90±0.18	105.6±10.4	8.73±0.17	95.2±5.0	−1.83	7.85±0.11	7.44±0.02	116.1±15.8	8.25±0.10	109.3±8.0	−0.81	7.11±0.01
Ro600332	6.29±0.11	94.7±3.6	6.62±0.15	101.5±10.0	−0.33	7.41±0.12	7.85±0.10	104.9±11.9	7.94±0.12	109.1±4.3	−0.09	7.62±0.07
Ro600175	7.98±0.14	97.5±2.5	8.72±0.12	94.5±4.7	−0.74	8.66±0.13	8.60±0.05	104.9±13.1	8.77±0.17	106.1±3.6	−0.17	7.67±0.07
Pergolide	7.48±0.19	88. 9±5.8	7.18±0.15	94.2±8.6	+0.30	8.11±0.04	6.26±0.17	102.9±8.3	6.99±0.13	102.6±7.3	−0.73	6.79±0.03
LSD	8.68±0.12	43.4±3.4	7.40±0.14	86.6±5.2	+1.28	9.22±0.02	7.95±0.13	76.1±9.7	7.08±0.12	104.6±9.4	+0.87	8.52±0.06
Bromocriptine	5.58±0.61	28.2±14.7		na		7.84±0.10	6.30±0.05	102.3±13.0	5.21±0.15	72.4±4.8	+1.09	<5
Lisuride	8.61±0.07	11.6±5.1	6.27±0.09	27.9±4.0	+2.34	9.01±0.02	7.54±0.16	36.4±5.9	6.12±0.11	62.9±9.2	+1.42	8.26±0.02

Data (pEC_{50} and E_{max}) are expressed as mean±S.E.M. of at least three independent experiments performed in duplicate (Gq/11 activation) or triplicate determinations (Ca^{2+} mobilisation). Agonist efficacy is expressed relative to that of a saturating concentration of 5-HT. na=non active.

4.1. Antagonist actions at h5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors

In cell membrane preparations of CHO cells expressing either h5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors, 5-HT-stimulated [^{35}S]GTPγS binding at Gq/11 proteins. Activation of Gq/11 proteins was specifically mediated by these receptors inasmuch as the selective antagonists MDL100907, RS127445 and SB242084 antagonised 5-HT-stimulated [^{35}S]GTPγS binding at Gq/11 proteins at h5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors, respectively. These results exclude a potential contribution of endogenously expressed 5-HT_{1B} receptors in CHO cells to the response induced by 5-HT (Giles et al., 1996).

4.2. Agonist actions at h5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors

Overall, the rank order of the agonist potencies and efficacies reported herein for Gq/11 activation at h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors was similar to that observed for PLC activation using the same cell lines (Cussac et al., 2002b). In contrast, some discrepancies were observed between our results observed in Ca^{2+} signalling and the results obtained by Porter et al. (1999) who used cell lines with a low level of h5-HT_{2A}, h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptor expression (0.2, 0.6 and 0.2 pmol/mg, respectively). As discussed before (see Cussac et al., 2002b), differences in potency and efficacy may depend on the choice of different expression systems as well as on the level of receptor expression (Lucaites et al., 1996; Burns et al., 1997; Jerman et al., 2001). BW723C86 and Ro600175 showed here an equilibrated action at h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ as previously reported (Cussac et al., 2002b) whereas mCPP and Ro600332 exhibited a slight selectivity for h5-$HT_{2C\text{-}VSV}$ receptors in agreement with previous reports on the actions of these compounds at h5-HT_{2C} receptors *in vitro* and *in vivo* (Kennett and Curzon, 1988; Newton et al., 1996; Martin et al., 1998; Porter et al., 1999; Cussac et al., 2002b).

As concerns calcium release, BW723C86 showed a preferential potency at h5-HT_{2B} in comparison with h5-HT_{2A} and h5-$HT_{2C\text{-}VSV}$ receptors, in accordance with the results of Porter et al. (1999). As mentioned above, the agonist selectivity described here should thus be interpreted in the context of the high expression level of h5-$HT_{2C\text{-}VSV}$ in comparison with that of h5-HT_{2A} and h5-HT_{2B} receptors. However, DOI, LSD and the antiparkinsonian agents lisuride, bromocriptine and pergolide were slightly more potent at 5-HT_{2A} receptors, particularly for Gq/11 activation; although these compounds exhibited a high efficacy at the h5-$HT_{2C\text{-}VSV}$ receptor, no doubt due to marked receptor reserve in the latter expression system (Cussac et al., 2002a,b). A similar profile of action has been found for these compounds in measures of PLC activity at the three 5-HT_2 receptor subtypes (Newman-Tancredi et al., 2002) where bromocriptine and lisuride behaved as antagonists at the 5-HT_{2B} site, in accordance with the very weak efficacy reported herein.

4.3. Differential Gq/11 activation versus calcium mobilisation

Interestingly, the compounds showed differential preference for the activation of specific cellular signalling responses. Indeed, at h5-HT_{2A} receptors, lisuride, bromocriptine, LSD and, to a lesser extent, pergolide preferentially induced activation of Gq/11 proteins while the other agonists were more potent for Ca^{2+} release. Moreover, a similar tendency was observed when h5-HT_{2B} and h5-$HT_{2C\text{-}VSV}$ receptors were considered, with the exception of pergolide at 5-$HT_{2C\text{-}VSV}$ receptors. These observations are unlikely to be accounted for in a simply quantitative manner or by a differential strength of signal dependent on receptor reserve. Indeed, lisuride was more potent than mCPP to induce Gq/11 protein activation at h5-HT_{2A} receptor while the opposite result was observed for induction of Ca^{2+} signalling. Furthermore, both compounds behaved as partial agonists at both pathways, suggesting that a contribution of differential receptor reserve is unlikely. In contrast, these results are highly suggestive of the existence of agonist-specific receptor states that preferentially engage distinct cellular pathways. Evidence for ligand-specific preferential activation of PLC, PLA_2 and phospholipase D by 5-HT_2 receptors have been presented repeatedly *in vitro* (Berg et al., 1998, 2006; Moya et al., 2007) and more recently *in vivo* for 5-HT_{2A} receptor (González-Maeso et al., 2007 and see below). In NIH-3T3 cells, LSD also induced weak 5-HT_{2C} receptor coupling to Ca^{2+} response whereas an efficient activation of PLC pathway was observed (Backstrom et al., 1999), in accordance with the results presented here. Thus, our results confirm and extend this notion of "agonist-specific trafficking" especially with respect to the h5-HT_{2B} receptor, for which agonist-specific receptor states have not been shown before.

4.4. Possible molecular mechanisms involved in differential Gq/11 activation and calcium mobilisation

A particular concern in our context is the nature of the different pathways examined. We assayed activation of Gq/11 proteins and of Ca^{2+} signalling. These pathways are, at least in a classical view, closely linked to each other, such that Ca^{2+} mobilisation can be considered as a simple downstream consequence of Gq/11 activation and subsequent PLC stimulation. Furthermore, this link may explain an amplification of potency observed with a group of compounds. On the other hand, in view of the pharmacological differences between these two processes observed in our assay, it is logical to postulate that Gq/11 signalling is at least not the only determinant for the strength of Ca^{2+} signalling. The agonist-directed-trafficking of signalling reported here seemed to be conserved to a certain degree for all 5-HT_2 receptors, suggesting a common mechanistic basis for these observations.

One obvious hypothesis is that 5-HT_2-family receptors couple to different G proteins in CHO cell lines and that the propensity to activate these different G proteins varies according to the agonist tested. Indeed, 5-HT_{2C} receptors couple to G13 and Gi3, as well as to Gq/11 (Chang et al., 2000; McGrew et al., 2002; Cussac et al., 2002a) and some 5-HT_{2A} intracellular signalling involves pertussis toxin sensitive G proteins (Kurrasch-Orbaugh et al., 2003; González-Maeso et al., 2007, 2008). Although, to our knowledge, 5-HT_{2B} receptors have not been reported to couple to G proteins other than Gq/11, its transduction mechanism remains to be examined in more detail. Thus, the differential signalling reported herein may be the result of the agonist-specific activation of a combination of distinct G proteins.

Another possibility is that GPCR-interacting proteins (GIPs) may modulate 5-HT_2 receptor signalling (see Bockaert et al., 2004 for review). Among the various GIPs, proteins containing the PDZ domains can interact with the C-terminal part of three 5-HT_2 receptor subtypes (Manivet et al., 2000; Bécamel et al., 2004; Gavarini et al., 2006). For example, the PDZ-containing protein PSD-95 increases 5-HT_{2A} receptor-mediated inositol phosphate accumulation (Xia et al., 2003) and proteins with PDZ domains are also involved in the desensitisation and resensitization of 5-HT_{2C} receptor signalling (Backstrom et al., 2000; Gavarini et al., 2006). Interestingly, the efficacies of agonists to induce 5-HT_{2C} receptor desensitisation and internalisation have been found to be distinct from those that activate a G protein-dependent pathway (Stout et al., 2002). We can thus speculate that ligands may differentially induce a balance of the active states responsible for signalling via Gq/11 proteins and modulation of desensitisation of the Ca^{2+} signalling pathway via PDZ domain-containing proteins.

4.5. Differential functional signalling at h5-HT_2 receptors and physiological implication

One of the major influences of potent activation of 5-HT_{2A} receptors in man by LSD, DOI and their derivates is induction of hallucinations (Nichols, 2004). In animal models, hallucinogenic behaviour has been related to head-twitch response in rodents. Furthermore, Gq protein KO mice displayed a decreased DOI-induced head-twitch response, supporting a key role for this G protein in hallucinogenic drug effects

(Garcia et al., 2007). We likewise observed that DOI was a potent and efficacious agonist at 5-HT_{2A} receptor-coupled Gq/11 proteins, a property which was shared also by LSD, pergolide and bromocriptine whereas lisuride was less efficacious. Pergolide and, to a lesser degree, bromocriptine have been reported to induce hallucination in Parkinsonian patients (Arnold et al., 2005; Weintraub and Hurtig, 2007). However, lisuride, which is generally considered to be non-hallucinogenic in human (Nichols, 2004), as well as LSD, bromocriptine and pergolide exhibited a high propensity to stimulate Gq/11 proteins in comparison with Ca^{2+} mobilisation. Thus, our results suggest that the differences in hallucinogenic properties between these compounds may not be directly related to a higher efficacy and potency to stimulate Gq/11 proteins versus Ca^{2+} mobilisation, but that a threshold of efficacy at 5-HT_{2A} receptor may be of importance. Interestingly, LSD but not lisuride induced some cortical gene expression via a pertussis toxin sensitive Gi/o protein and the kinase Src, suggesting an agonist-directed trafficking at the 5-HT_{2A} receptor level (González-Maeso et al., 2007). However, Gi/o recruitment by LSD may be rather due to its efficacious activation of 5-HT_{2A} receptors than to specific active states, since lisuride seemed of weaker potency and efficacy than LSD at 5-HT_{2A} receptors whatever the assay conditions (Porter et al., 1999; González-Maeso et al., 2007 and result herein). In line with this hypothesis, it has been shown at 5-$HT_{2C\text{-}VSV}$ receptors that lisuride partially stimulated Gq/11 protein whereas LSD was able to induce both Gq/11 and Gi3 protein activation (Cussac et al., 2002a). Furthermore, stimulation of 5-HT_{2C} receptors may also play a role in hallucinogenic experience (see Nichols, 2004 for review), and the antagonist or weak partial agonist properties of lisuride at this receptor in comparison with the higher efficacy of hallucinogenic agents (Porter et al., 1999; Cussac et al., 2002a and results herein), may account for its absence of hallucinogenic properties.

Concerning 5-HT_{2B} receptors, their role in the CNS is still under evaluation although they appear to play a role in control of mood. However, the hallucinogenic drug 3,4-methylenedioxymethamphetamine (MDMA, ecstasy), behaved as an agonist at 5-HT_{2B} receptors (Setola et al., 2003) and the hyperlocomotor effect of MDMA in mice was blocked by a selective 5-HT_{2B} antagonist (Doly et al., 2007). Interestingly, lisuride and bromocriptine behaved as antagonists (Newman-Tancredi et al., 2002) or very weak partial agonists at 5-HT_{2B} receptors (result herein), a property that may participate in the lack (lisuride) or weak (bromocriptine) hallucinogenic liability of these drugs.

4.6. Summary and conclusion

The present data demonstrate that agonists acting at 5-HT_{2A}, 5-HT_{2B} and 5-$HT_{2C\text{-}VSV}$ receptors can exhibit highly divergent properties for activation of two connected signalling responses: Gq/11 protein activation and calcium mobilisation. The data suggest that high efficacy for 5-HT_{2A} receptor-mediated-Gq/11 activation, possibly associated with 5-HT_{2B} and/or 5-$HT_{2C\text{-}VSV}$ receptor activation, may contribute to the hallucinogenic liability of drugs such as LSD. An important issue would be to explore the differential contribution of Gq/11 protein activation versus Ca^{2+} mobilisation by 5-HT_2-family receptors in view of the pharmacological identification of more effective therapeutic drugs with limited side effects.

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