Functional Selectivity of Hallucinogenic Phenethylamine and Phenylisopropylamine Derivatives at Human 5-Hydroxytryptamine (5-HT)_{2A} and 5-HT_{2C} Receptors

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ABSTRACT

2,5-Dimethoxy-4-substituted phenylisopropylamines and phenethylamines are 5-hydroxytryptamine (serotonin) (5-HT)_{2A/2C} agonists. The former are partial to full agonists, whereas the latter are partial to weak agonists. However, most data come from studies analyzing phospholipase C (PLC)-mediated responses, although additional effectors [e.g., phospholipase A2 (PLA2)] are associated with these receptors. We compared two homologous series of phenylisopropylamines and phenethylamines measuring both PLA2 and PLC responses in Chinese hamster ovary-K1 cells expressing human 5-HT_{2A} or 5-HT_{2C} receptors. In addition, we assayed both groups of compounds as head shake inducers in rats. At the 5-HT_{2C} receptor, most compounds were partial agonists for both pathways. Relative efficacy of some phenylisopropylamines was higher for both responses compared with their phenethylamine counterparts, whereas for others, no differences were found. At the 5-HT_{2A} receptor, most compounds behaved as partial agonists, but unlike findings at 5-HT_{2C} receptors, all phenylisopropylamines were more efficacious than their phenethylamine counterparts. 2,5-Dimethoxyphenylisopropylamine activated only the PLC pathway at both receptor subtypes, 2,5-dimethoxyphenethylamine was selective for PLC at the 5-HT_{2C} receptor, and 2,5-dimethoxy-4-nitrophenethylamine was PLA2-specific at the 5-HT_{2A} receptor. For both receptors, the rank order of efficacy of compounds differed depending upon which response was measured. The phenylisopropylamines were strong head shake inducers, whereas their phenethylamine congeners were not, in agreement with in vitro results and the involvement of 5-HT_{2A} receptors in the head shake response. Our results support the concept of functional selectivity and indicate that subtle changes in ligand structure can result in significant differences in the cellular signaling profile.

5-Hydroxytryptamine (serotonin) (5-HT)_{2A} and 5-HT_{2C} receptors are highly homologous G protein-coupled receptors that are primarily distributed in the brain and exert their actions through pertussis toxin-insensitive G_{q/11} and G_{12/13} proteins (Berg et al., 1998; Grotewiel and Sanders-Bush, 1999; Chang et al., 2000). 5-HT_{2A} receptors mediate the effects of many hallucinogens, and they are targets of a number of commonly prescribed drugs, including antipsychotics, antidepressants, and anxiolytics (Meltzer et al., 1989; Kroeze et al., 2002). Conversely, numerous studies have implicated 5-HT_{2C} receptors in conditions, such as anxiety, obesity, and affective disorders (Kennett et al., 1997; Egan et al., 1998; Niswender et al., 2001). Elucidation of the different receptor-mediated signaling mechanisms may provide important insight into the therapeutic modes of action of drugs that target 5-HT_{2A} and 5-HT_{2C} receptors and has implications for the rational design of novel drugs (Gray and Roth, 2002; Kroeze et al., 2002).

Classically, drugs have been characterized by affinity, which describes the tenacity with which they bind to a

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ABBREVIATIONS:
5-HT, 5-hydroxytryptamine (serotonin); DOI, 2,5-dimethoxy-4-iodophenylisopropylamine; DOM, 2,5-dimethoxy-4-methylphenylisopropylamine; DOB, 2,5-dimethoxy-4-bromophenylisopropylamine; PLC, phospholipase C; PLA2, phospholipase A2; AA, arachidonic acid; 2C-I, 2,5-dimethoxy-4-iodophenethylamine; 2C-B, 2,5-dimethoxy-4-bromophenethylamine; 2C-N, 2,5-dimethoxy-4-nitrophenethylamine; 2C-D, 2,5-dimethoxy-4-methylphenethylamine; DON, 2,5-dimethoxy-4-nitrophenylisopropylamine; TMA, 2,4,5-trimethoxyphenylisopropylamine; 2,5-DMA, 2,5-dimethoxyphenylisopropylamine; 2C-H, 2,5-dimethoxyphenethylamine; CHO, Chinese hamster ovary; IP, inositol phosphate; PIA, phenylisopropylamine; PEA, phenethylamine.
ceptor, and intrinsic efficacy, which describes their ability to alter receptor behavior toward the intracellular environment. At a given receptor, both affinity and intrinsic efficacy were thought to be exclusive properties of a drug that were independent of the response mechanism. Because of their system independence, these drug properties were thought to be of great predictive value for drug discovery. This classical view has been challenged by evidence indicating that intrinsic efficacy can vary across different cell types and is dependent upon the cellular signaling machinery (for reviews, see Berg and Clarke, 2006; Urban et al., 2007). To account for response-dependent, agonist intrinsic efficacy, Kenakin (1995, 2003) developed the concept of agonist-directed trafficking of receptor stimulus (also known as “functional selectivity”), which states that agonists promote/stabilize unique agonist-specific receptor conformational states that can differentially activate effectors. Agonist functional selectivity has been supported by evidence obtained with neurotensin NTS1 receptors (Skrzypelski et al., 2003), dopamine receptors (Meller et al., 1992; Gay et al., 2004), opioid receptors (Piñeyro and Archer-Lahlou, 2007), cannabinoid receptors (Shoemaker et al., 2005), chemokine receptors (Fitzsimons et al., 2006), and 5-HT2A and 5-HT2C receptors (Berg et al., 1998; Kurrasch-Orbaugh et al., 2003), among others (Urban et al., 2007).

Phenylisopropylamines, such as 2,5-dimethoxy-4-iodophenylylisopropylamine (DOI), 2,5-dimethoxy-4-methylphenylisopropylamine (DOM), and 2,5-dimethoxy-4-bromophenylylisopropylamine (DOB), are hallucinogenic amphetamine derivatives. These compounds are 5-HT2A/2C agonists that have extensively been used as radioligands and classic agonists in vitro and in vivo (Gerhardt and van Heerikhuizen, 1997; Nelson et al., 1999; Nichols, 2004). However, most of the in vitro studies with these drugs evaluated their phospholipase C (PLC)-mediated effects, despite several reports showing additional effector routes associated with these receptors, particularly phospholipase A, (PLA,)-mediated arachidonic acid (AA) production (Felder et al., 1990; Gerhardt and van Heerikhuizen, 1997; Berg et al., 1998; Kurrasch-Orbaugh et al., 2003). In this context, several studies showed response-dependent efficacies for a series of 5-HT2A or 5-HT2C agonists (Berg et al., 1998; Stout et al., 2002; Kurrasch-Orbaugh et al., 2003) that support the functional selectivity concept.

Another group of hallucinogenic amphetamine analogs, the 4-substituted 2,5-dimethoxyphenethylamines, have been less explored than their α-methyl-substituted homologs, the phenylisopropylamines, for activity at 5-HT2A/2C receptors, presumably because of their lower in vivo potency. Among the best known are 2,5-dimethoxy-4-iodophenethylamine (2C-I), 2,5-dimethoxy-4-bromophenethylamine (2C-B), and 2,5-dimethoxy-4-methylphenethylamine (2C-D), the α-demethylated analogs of DOI, DOB, and DOM, respectively. The data available for these phenethylamines show that their affinities for 5-HT2A receptors are similar to those of their phenylisopropylamine counterparts, whereas different functional models have shown that their efficacies are lower than those of their α-methylated analogs (Glennon et al., 1992; Nichols et al., 1994; Acuña-Castillo et al., 2002; Parrish et al., 2005). Again, with few exceptions, most in vitro studies on these compounds examined only PLC-mediated responses.

As a result, we chose to compare two homologous series of hallucinogenic phenylisopropylamine and phenethylamine derivatives (Fig. 1), by testing their ability to activate PLC and PLA signaling via 5-HT2A and 5-HT2C receptors. We used a well characterized cell system that allowed us to test PLA and PLC responses simultaneously from the same cells (Berg et al., 1998). To test whether in vitro efficacies of phenylisopropylamines and phenethylamines at the 5-HT2A receptor might be associated with in vivo effects, we also counted head shakes induced by both series of compounds in rats, a behavior mediated by 5-HT2A Receptor activation (Yap and Taylor, 1983; Gewirtz and Marek, 2000).

Materials and Methods

Materials

2,5-Dimethoxyphenethylamine (DOB, DON, DOM, and 2,5-DMA), TMA, 2,5-dimethoxyphenethylamine (2C-B, 2C-N, 2C-D, and 2C-H), and mescaline salts were available from previous studies (Sáez et al., 1994; Acuña-Castillo et al., 2002). The following materials were purchased from commercial sources: [3H]inositol and [3H]arachidonic acid (PerkinElmer Life and Analytical Sciences, Boston, MA), 5-HT HCl (Sigma/RBI, Natick, MA), and fetal bovine serum (Gemini Bioproducts, Calabasas, CA). All other cell culture reagents were purchased from Invitrogen (Carlsbad, CA).

Cell Culture

CHO-1C19 and CHO-FA4 cells are CHO-K1-derived cell lines that stably express human 5-HT2C and 5-HT2A receptors, respectively, at a density of ~200 fmol/mg protein; they have similar maximal responses for inositol phosphate (IP) accumulation and AA release in response to 5-HT, and they have been used and characterized extensively (Berg et al., 1998; López-Giménez et al., 2001; Brea et al., 2003). Cells were maintained in minimum essential medium, α-formulation, supplemented with 5% fetal bovine serum and 300 mg/ml hygromycin. For all experiments, cells were seeded into 24-well tissue culture vessels at a density of 5 × 10^4 cells/cm^2. After a 24-h plating period, cells were washed with Hank’s balanced salt solution and placed into Dulbecco’s modified Eagle’s/Ham’s F-12 media (1:1) with 5 mg/ml insulin, 5 mg/ml transferrin, 30 nM selenium, 20 nM...
progesterone, and 100 μM putrescine (serum-free media). Cells were grown in serum-free media for 24 h before each experiment.

**IP and AA Measurements**

Cells in serum-free medium were labeled with 1 μCi/ml myo-[3H]inositol for 24 h and with 0.1 μCi/ml [3H]arachidonic acid for 4 h at 37°C. Measurements of PLC-mediated IP accumulation and PLA₂-mediated AA release were made from the same multwell, simultaneously, after 10 min of drug exposure, as described previously (Berg et al., 1998; Stout et al., 2002).

**Behavioral Test**

Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with international laws and policies (Guide for the Care and Use of Laboratory Animals, United States National Research Council, 1996). Male Sprague-Dawley rats (180–280 g; ISP, Santiago, Chile) were used. Animals were housed in groups of five to six, under a 12-h light/dark cycle (lights on at 8:00 AM and off at 8:00 PM) at 22 ± 1°C, with free access to food and water. All experiments were done in a climatized, quiet room. Groups of rats (8–10) were injected i.p. with equimolar doses (8.4 mol/kg) of each drug. Immediately after injection, rats were placed in a dark Plexiglas box measuring 50 × 50 × 50 cm, and the number of head shakes, defined as rapid side to side rotations of the head and ears (Bedard and Pycock, 1977), was quantified for 25 min, starting 5 min after drug administration by an experimenter blinded to the drug treatment. Control animals received saline.

**Data Analysis**

**Curve Fitting.** Concentration-response data were fit with nonlinear regression to the model

\[
E = \frac{E_{\text{max}}}{1 + \left(\frac{EC_{50}}{A}\right)^n}
\]

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

**Statistical Analysis.** Means ± S.E.M. were calculated and are presented for each experimental group. In the cell experiments, statistical comparison between responses (IP versus AA) was performed using Student’s paired \(t\) test; for comparisons between drugs, unpaired \(t\) test was used. For behavioral studies, statistical significance was assessed by analysis of variance followed by a Newman-Keuls multiple comparison test. In all cases, the significance level was taken to be \(p < 0.05\).

**Results**

5-HT₂C Receptor. Figure 2, A to D, shows representative curves obtained for several compounds tested with CHO-1C19 cells expressing 5-HT₂C receptors. The data are expressed as the percentage of the maximal response to 5-HT, concentration-response curves for which were run in each experiment. Thus, the plateau for each curve represents the relative efficacy of the test drug with respect to 5-HT. As can

![Figure 2](https://jpet.aspetjournals.org/doi/10.1124/jpet.105.089889)
be seen, different drugs were able to differentially stimulate the effector pathways tested. The naturally occurring hallucinogen mescaline (Fig. 2A) had similar relative efficacies for both PLA₂-AA and PLC-IP signal transduction pathways. In contrast, DOM (Fig. 2B) and DON (Fig. 2C) preferentially stimulated either the AA or the IP response, respectively. Moreover, the analog lacking a substituent at the 4-position, 2,5-DMA (Fig. 2D), elicited virtually no AA response, whereas it behaved as a strong partial agonist (65% maximal response relative to 5-HT) in the PLC pathway.

Figure 3 summarizes the relative efficacy values obtained for all drugs evaluated at the 5-HT₂C receptor subtype. It can be seen that drug efficacy varied from full to weak agonism, with most of the compounds acting as partial agonists. It is noteworthy that the relative efficacy of some drugs differed substantially depending upon the response measured. In addition, when the relative efficacy values of phenylisopropylamine/phenethylamine pairs with identical substituent patterns were compared, no consistent trend was observed across the series (Fig. 3, B and C). Thus, in some cases, (e.g., DOM/2C-D) the relative efficacy of the phenylisopropylamine (α-methylated) derivative was significantly higher for both responses compared with its phenethylamine (α-demethylated) counterpart, whereas in others (e.g., DOB/2C-B) relative efficacy was similar between responses.

In the case of the phenylisopropylamine derivatives, the rank order of efficacies for IP accumulation (DON > DOM > TMA > 2,5-DMA > DOI > DOB) differed from that for AA release and IP accumulation were measured as described in the legend to Fig. 2. Maximal response for each drug was obtained by fitting data points from concentration-response curves to equation under Materials and Methods. Relative efficacy values for DOI are from (Berg et al., 1998). *, p < 0.05; **, p < 0.01; and ***, p < 0.001 obtained with paired Student’s t test for comparisons between effector pathways.
release (DOM > DOI > DON ≥ DOB > TMA ≥ 2,5-DMA). Interestingly, phenethylamine derivatives showed very similar rank orders of efficacy for both signaling pathways tested (mescaline ≥ 2C-I = 2C-N = 2C-B > 2C-D ≥ 2C-H), and, with the exception of the least efficacious drug (2C-H), they exhibited no preference for either effector route.

The pEC$_{50}$ values for IP accumulation and AA release are summarized in Table 1. Generally, the potencies of phenylisopropylamine derivatives were higher than those of their phenethylamine counterparts. As expected for a cell system lacking receptor reserve, no differences in potency were observed between responses for each compound.

5-HT$_{2A}$ Receptor. Figure 4 shows the relative efficacy for AA release and IP accumulation obtained for all compounds tested at the 5-HT$_{2A}$ receptor. As in the case of the 5-HT$_{2C}$ receptor, some of the drugs evaluated were able to preferentially activate one of the two pathways under study, whereas others did not show this functional selectivity. Remarkably, we found that 2C-N only elicited AA release without activating the PLC-mediated response, whereas 2,5-DMA only induced IP accumulation without activating the PLA$_2$-mediated response.

Even though all of the compounds behaved as partial agonists, all the phenylisopropylamine derivatives were clearly more efficacious than their phenethylamine counterparts in both responses (Fig. 4, B and C), unlike findings at the 5-HT$_{2C}$ receptor (compare DOB/2C-B and TMA/mescaline in both receptors).

Table 1 shows the pEC$_{50}$ values for both AA and IP responses at the 5-HT$_{2A}$ receptor. No statistically significant differences were found between the potencies of phenylisopropylamine/phenethylamine pairs. However, the potency values of the phenethylamines must be viewed with caution, because the low efficacy of these compounds renders the determination of an accurate EC$_{50}$ value difficult.

Figure 5 shows that phenylisopropylamine derivatives induced a robust head shake behavior, which was significantly stronger than that observed with saline or with equimolar doses of the corresponding phenethylamine analogs. Preinjection of 1 mg/kg ketanserin, a 5-HT$_2$ antagonist, abolished the effect of DOI. In contrast, the head shake response was not significantly different in phenethylamine and saline groups. Increasing phenethylamine doses up to 15 mg/kg (about 5-fold higher molar doses) did not increase head shakes significantly, and higher doses resulted in disrupted behavior (data not shown).

**Discussion**

For decades, pharmacologists have maintained that drugs have two properties, affinity, which describes a drug’s ability to bind to a receptor, and intrinsic efficacy, which describes the ability of a drug to change the behavior of a receptor toward cellular signaling machinery (Kenakin, 2004). Both affinity and intrinsic efficacy were defined as ligand constants independent of response mechanisms and thus were of great predictive value for drug discovery efforts. Recently, clear evidence from many seven-transmembrane-spanning receptor systems has accumulated to indicate that ligands may not obey the tenets of classical receptor theory. In fact, intrinsic efficacy seems to be variable (changes with cell phenotype and physiological state) and dependent upon cellular signaling machinery (response-dependent). Consequently, ligands seem to have multiple intrinsic efficacies (for reviews, see Clarke and Bond, 1998; Kenakin, 2003; Mailman and Gay, 2004; Berg and Clarke, 2006). Previous studies have reported functional selectivity for some drugs at 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors (Berg et al., 1998; Kurrasch-Orbaugh et al., 2003). The results of the present work also support the functional selectivity hypothesis in that some, but not all, compounds differentially stimulated the PLC-IP and the PLA$_2$-AA pathways, measured in the same cells, simultaneously.

In addition, our results show that, even in a closely related series of compounds, subtle structural modifications can have a profound impact on drug efficacy, and importantly, they affect the different signaling pathways in a different (and at this time, unpredictable) manner, as was suggested by Shapiro et al. (2000). The comparison of DOM and 2,5-DMA, acting on the 5-HT$_{2C}$ receptor is a good example: for PLC-IP accumulation response, both compounds are partial agonists, with relative efficacies (with respect to 5-HT) of 85 and 65%, respectively. However, DOM is a full agonist for PLA$_2$-AA release, whereas 2,5-DMA does not elicit any significant response. It should be noted that DOM and 2,5-DMA differ only in the presence or absence of a methyl group at C4. Our results with 2,5-DMA, which selectively induced PLC-IP accumulation in cells expressing 5-HT$_{2C}$ or 5-HT$_{2A}$ receptors, strongly suggest that the presence of a substituent at C4 of phenylisopropylamine congeners (and apparently also of phenethylamines, viz., 2C-H) is critical for activation of the PLA$_2$-mediated response, at least in this model.

Likewise, 2C-N was only able to elicit PLA$_2$-AA release when acting at the 5-HT$_{2A}$ receptor. The inability of 2C-N to activate the PLC-IP pathway, is consistent with our previous observation of the lack of efficacy of this compound in the *Xenopus* oocyte model (Acuña-Castillo et al., 2002), which measures chloride currents produced by calcium transients coupled to PLC activation. The behavior of 2,5-DMA and 2C-N has been called “active state-selective agonism” (Kenakin, 2001), and highlights the possibility of developing these leads to exploit this novel degree of selectivity.

Published data for PLA$_2$-mediated responses to 5-HT$_{2A/C}$ activation induced by phenylisopropylamines and phenethylamines are scanty. Partial-to-full agonism in this pathway

<table>
<thead>
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<th>Drug</th>
<th>5-HT$_{2A}$</th>
<th>5-HT$_{2C}$</th>
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<tr>
<td></td>
<td>AA pEC$_{50}$</td>
<td>IP pEC$_{50}$</td>
</tr>
<tr>
<td>DOI</td>
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<td>6.53</td>
</tr>
<tr>
<td>DOB</td>
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<td>6.18</td>
</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
<td>TMA</td>
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<td>5.15</td>
</tr>
<tr>
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</tr>
<tr>
<td>2C-B</td>
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</tr>
<tr>
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</tr>
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</tr>
<tr>
<td>2C-H</td>
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<td>N.D.</td>
</tr>
<tr>
<td>Mescaline</td>
<td>4.52</td>
<td>4.64</td>
</tr>
</tbody>
</table>

N.D., not determined.
has been shown for DOB and DOI at 5-HT2A and 5-HT2C receptors, respectively (Kenakin, 2001). Our results extend earlier observations on the pharmacology of phenylisopropylamine derivatives, and they also provide for the first time a relative assessment of their $\alpha$-demethylated phenethylamine congeners acting upon the PLA2-mediated signal transduction pathway.

Previous data have shown that $\alpha$-methylation of phenethylamines causes an increase in drug efficacy for the 5-HT$_{2A}$/2C PLC-IP response, which could be associated with the higher hallucinogenic potency of phenylisopropylamines compared with the corresponding phenethylamines (Nichols et al., 1994). In a recent report, the Nichols group showed that some phenylisopropylamines have higher intrinsic activities than their phenethylamine analogs regarding the PLC-IP effector route coupled to the 5-HT$_{2A}$ receptor (Parrish et al., 2005). Our present results are in good agreement with this trend (with DOI/2C-I being an exception), and also indicate that $\alpha$-methylation produces differential increases in efficacy, depending upon which signaling pathway coupled to 5-HT$_{2A}$ receptor activation is considered.

In contrast, no consistent effect of $\alpha$-methylation was observed on drug efficacy at the 5-HT$_{2C}$ receptor. Thus, in several cases, similar efficacies were found when phenethylamines were compared with their phenylisopropylamine analogs. These results suggest that the differences in human hallucinogenic potency between these two types of com-

Fig. 4. Relative efficacy for PLA$_2$-AA release and PLC-IP accumulation for the series of tested compounds on 5-HT$_{2A}$ receptors. A, comparison of the relative efficacies of the PIAs and PEAs for the PLA-IP response versus the PLA$_2$-AA response. B and C, effect of $\alpha$-methylation on agonist relative efficacy for the PLC-IP response (B) and the PLA$_2$-AA response (C). AA release and IP accumulation were measured as described in the legend to Fig. 2. Maximal response for each drug was obtained by fitting data points from concentration-response curves to the equation under Materials and Methods. Relative efficacy values for DOI are from (Berg et al., 1998). $*, p < 0.05; **, p < 0.01; and $$$, p < 0.001$ obtained with paired Student's $t$ test for comparisons between effector pathways.
partial 5-HT2A agonists, and our results are in agreement with this view (Nichols, 2004): mescaline, 2C-D, and 2C-I were partial agonists, eliciting both PLA2 and PLC release, whereas their phenethylamine congeners are not, even after administering 5-fold higher doses. This is consistent with the lower intrinsic efficacy of these compounds obtained in vitro in cells expressing 5-HT2A receptors, and it supports the contention that head shake behavior is mediated by 5-HT2A receptor activation (Gewirtz and Marek, 2000). However, these results do not provide evidence for functional selectivity in vivo. Further experiments are necessary to determine which signaling pathway (PLC-IP, PLA2-AA, or both or others) mediates the behavioral response. The identification of active state-selective ligands will be a useful tool to explore these possibilities.

It has been shown that “classical” hallucinogens (i.e., phenylalkylamine, tryptamine, and ergoline derivatives) act as partial 5-HT2A agonists, and our results are in agreement with this view (Nichols, 2004): mescaine, 2C-D, and 2C-I were partial agonists, eliciting both PLA2 and PLC responses. As mentioned above, 2C-N elicited only PLA2-AA release, and 2C-B induced little activation of the 5-HT2A receptor, eliciting weak responses (5–10%) in both AA release and IP accumulation. These results also agree with our previous reports in different models (Sáez et al., 1994; Acuña-Castillo et al., 2002), suggesting that phenylethylamines can be hallucinogenic even if they have low efficacy at 5-HT2A receptors, at least in the two pathways studied here. However, it will be necessary to evaluate the effects of these compounds on other signaling pathways (e.g., phospholipase D) coupled to 5-HT2A receptors. As noted by Nichols (2004), it is possible that the effects in the phospholipase D pathway might be relevant to the mechanism of hallucinogenesis by these compounds.

In conclusion, the results of this work support the hypothesis that ligands can promote differential signaling to effector pathways within cells (functional selectivity). In addition, we found that small changes in the structure of a ligand can result in significant differences in the cellular signaling profile, that are, at present, unpredictable. The capacity for ligands to differentially regulate cellular signaling pathways suggests that drugs have more actions and more specificity than previously thought. Perhaps by exploiting these ligand-specific signaling properties, new drugs with improved therapeutic selectivity and efficacy may be developed.

Acknowledgments

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