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5-HT_{2A} receptor-stimulated phosphoinositide hydrolysis in the stimulus effects of hallucinogens

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Abstract

The role of 5-HT_{2A}-mediated stimulation of phosphoinositide hydrolysis in the discriminative effects of hallucinogens was investigated in PC12 cells stably expressing the rat 5-HT_{2A} receptor (PC12-5-HT_{2A} cells). The hallucinogenic compounds, D-lysergic acid diethylamide (LSD), (−)2,5-dimethoxy-4-methylamphetamine (DOM), psilocybin, *N*,*N*-dimethyltryptamine (DMT), 5-methoxy-*N*,*N*-dimethyltryptamine (MDMT) and *N*,*N*-diethyltryptamine (DET), all caused a concentration-dependent increase in the generation of [^{3}H]inositol phosphates. The nonhallucinogenic compounds, 6-fluoro-*N*,*N*-diethyltryptamine (6-F-DET), lisuride and quipazine, also displayed significant efficacy in stimulating phosphoinositide hydrolysis, while 2-bromo-lysergic acid diethylamide (BOL), which is not a hallucinogen, did not alter inositol phosphate generation. The β-carbolines, harmaline and harmane, also did not alter phosphoinositide hydrolysis. Comparison of these results with previous drug discrimination studies indicated the apparent lack of correlation between the degree of substitution in LSD- and DOM-trained animals and efficacy in stimulating phosphoinositide hydrolysis. The present study indicates that 5-HT_{2A}-mediated stimulation of phosphoinositide hydrolysis does not appear to be the sole critical signaling mechanism involved in the discriminative effects of hallucinogens.

Keywords: 5-HT_{2A} receptor; Drug discrimination; Hallucinogens; Phosphoinositide hydrolysis; Arachidonic acid release

1. Introduction

The serotonergic receptor system appears to play a prominent role in mediating the discriminative effects of both indoleamine [e.g., D-lysergic acid diethylamide (LSD)] and phenethylamine [e.g., (−)2,5-dimethoxy-4-methylamphetamine (DOM)] hallucinogens. The initial finding that serotonergic antagonists block the stimulus effects of the phenethylamine hallucinogen mescaline (Winter, 1975; Browne and Ho, 1975) was later expanded to include other hallucinogens including LSD, DOM and *N*,*N*-dimethyltryptamine (DMT) (Kuhn et al., 1997; Winter, 1978; Glennon et al., 1983). Of the seven different families of serotonin receptors, activation of the 5-HT_2 receptor family appears to be the primary site of action of the indoleamine/phenethylamine hallucinogens. Affinity at the 5-HT_2 receptor for a series of phenethylamines strongly correlates with both the potency of these compounds to substitute for DOM as a discriminative cue (Glennon et al., 1984), as well as their potency as hallucinogens (Titeler et al., 1988; Sadzot et al., 1989). Based upon antagonist correlation analysis, Fiorella et al. (1995a) concluded that the 5-HT_{2A} receptor rather than the 5-HT_{2C} receptor mediates the stimulus effects of LSD. Similarly, the discriminative stimulus effects of the phenethylamine DOI are blocked by MDL 100,907, a selective 5-HT_{2A} antagonists, but not by the selective 5-HT_{2C} antagonist SB 200,646 (Schreiber et al., 1994). In addition, behavioral tolerance to the stimulus effects of DOI is associated with a down-regulation of the 5-HT_{2A} and not the 5-HT_{2C} receptor (Smith et al., 1999).

Although the data indicate that the discriminative stimulus effects of indoleamine and phenethylamine hallucinogens involve activation of the 5-HT_{2A} receptor, the resulting biochemical changes responsible for the interceptive state induced by these hallucinogens are unknown. The 5-HT_{2A} receptor is coupled to stimulation of phospholipase C through an activation of the $G_{q/11}$ GTP-binding protein.

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This stimulation results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) with the subsequent generation of inositol 1,4,5-trisphosphate (IP_3), which regulates the release of calcium from internal stores, and *sn*(1,2) diacylglycerol (DAG), which is involved in the activation of protein kinase C. The objective of the present study was to determine whether 5-HT_{2A} receptor-mediated stimulation of phosphoinositide hydrolysis is a necessary component of the discriminative state produced by hallucinogens. To address this question, generation of inositol phosphates was measured in the presence of various indoleamine, phenethylamine and β-carboline hallucinogens, as well as some nonhallucinogenic congeners. For these studies, PC12 cells expressing the rat 5-HT_{2A} receptor cDNA were used to preclude the confounding stimulation of phospholipase C by other members of the 5-HT_2 receptor family.

2. Materials and methods

2.1. Material

[^{3}H]*Myo*-inositol was purchased from American Radiochemical (St. Louis, MO) and [^{3}H]ketanserin was obtained from DuPont/NEN (Boston, MA). DOM, LSD, psilocybin, DMT, *N*,*N*-diethyltryptamine (DET) and 2-bromo-lysergic acid diethylamide (BOL) were generously provided by the National Institute on Drug Abuse (Rockville, MD). Quipazine, lisuride, and 5-methoxy-*N*,*N*-dimethyltryptamine (MDMT) were purchased from RBI (Natick, MA). Harmane and harmaline were purchased from Sigma (St. Louis, MO). 6-Fluoro-*N*,*N*-diethyltryptamine (6-F-DET) was provided by Upjohn (Kalamazoo, MI). All drugs were dissolved in water. The rat 5-HT_{2A} cDNA was a generous gift from Dr. David J. Julius (University of California-San Francisco). The plasmid pTR-UF5 was a generous gift from Dr. Thomas Black (Roswell Park Cancer Institute, Buffalo, NY). XL1-Blue cells were obtained from Stratagene (La Jolla, CA). Lipofectamine Plus was purchased from Gibco BRL (Grand Island, NY), and the restriction enzymes were purchased from Promega (Madison, WI). Fetal bovine serum was from HyClone Laboratories (Logan, UT), and the heat-inactivated horse serum was from JRH Bioscience (Lenexa, KS). All other reagents were from common commercial suppliers.

2.2. Cell culture

PC12 cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, 4 mM glutamine and supplemented with 5% heat-inactivated horse serum and 10% fetal calf serum.

2.3. Transfection

A 1.8-kb cDNA fragment encoding the rat 5-HT_{2A} receptor (Julius et al., 1990) was removed from the Bluescript KS_{II} vector (Stratagene) using the restriction enzyme, *Eco*R1, and the ends of the fragment filled using Klenow DNA polymerase. The gel-purified cDNA fragment was ligated into the *Not*I site of pTR-UF5, which had been gel-purified, blunt-ended and dephosphorylated to produce pTR-5-HT_{2A}. Competent XL1-Blue cells were transformed with the vector pTR-5-HT_{2A} using heat shock (2 min at 42 °C), and the transformed cells were selected using agar plates containing ampicillin. Plasmids containing the insert in the proper orientation were determined by restriction digest analysis using *Kpn*I, and the results confirmed by direct DNA sequencing using the core sequencing facility of the Center for Advanced Molecular Biology and Immunology (CAMBI) at SUNY-Buffalo. After purification using the High Purity Plasmid Purification System (Gibco BRL) as per manufacturer's instructions, plasmids were linearized using *Sca*I, and PC12 cells were transfected using Lipofectamine Plus according to the manufacturer's instructions. Cells were allowed to recover for 48 h, and cells expressing genectin (G418) resistance were selected by maintaining cultures in serum-containing DMEM media supplemented with 500 μg/ml of genectin. A total of 17 clones were subsequently isolated, and selection pressure was maintained during the propagation of these cells by the inclusion of 200 μg/ml of genectin in the complete media. Clones were screened for 5-HT_{2A} receptor expression by measuring the ability of 100 μM 5-HT to stimulate phosphoinositide hydrolysis; the clone expressing the highest level of 5-HT-stimulated inositol phosphate generation (PC12-5-HT_{2A}) was used in the present study.

2.4. Phosphoinositide hydrolysis

Phosphoinositide hydrolysis was determined by prelabeling the phosphatidylinositol pool with [^{3}H]*myo*-inositol and subsequently measuring the generation of [^{3}H]inositol phosphates using a previously described method (Kim et al., 1993). Briefly, after an overnight incubation at 37 °C in inositol-free DMEM containing 10% fetal calf serum, 5% heat-inactivated horse serum and 2 μCi/ml [^{3}H]*myo*-inositol, cells were incubated for 15 min at 37 °C in Krebs buffer (125 mM NaCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1 mM $CaCl_2$, 6 mM glucose, 25 mM HEPES, pH 7.4) supplemented with 10 mM LiCl to inhibit inositol monophosphatase and 1 mM *myo*-inositol. Assays were subsequently carried out by incubating 10^6 cells for 5 min at 37 °C in Krebs buffer containing 10 mM LiCl, 1 mM *myo*-inositol and appropriate drugs. Reactions were terminated by the addition of ice-cold perchloric acid (final concentration of 4%), and the samples were sonicated on ice for 2 min. Samples were then centrifuged at $1000 \times g$ for 15 min at 4 °C, and the supernatant was adjusted to pH 8–9 with a solution of 0.5 M KOH, 9 mM $Na_2B_4O_7$ and 1.9 mM EDTA. [^{3}H]Inositol phosphates were separated by anion-exchange chromatography using Dowex AG1-X8 resin. Maximal stimulation of phosphoinositide hydrolysis (V_{max})

and the concentration of drug resulting in half-maximal activation (EC_{50}) were determined by using nonlinear regression analysis (SigmaPlot, Jandel) to fit the general logistic equation to the data from the concentration–response relationships.

2.5. [^{3}H]Ketanserin binding

Binding of the antagonist [^{3}H]ketanserin to the 5-HT_{2A} receptor was measured using a modification of a previously described method (Fiorella et al., 1995a). Briefly, cells were homogenized in 50 mM Tris–HCl (pH 7.4) using a Dounce tissue grinder, and the cell lysate was centrifuged at 40,000 × *g* for 15 min at 4 °C. The resulting pellet was resuspended in the Tris buffer and again centrifuged at 40,000 × *g* for 15 min. This procedure was repeated, and the final pellet was resuspended in the Tris buffer. Binding assays were carried out at 30 °C for 30 min in a final volume of 1 ml consisting of 50 mM Tris–HCl (pH 7.4), [^{3}H]ketanserin (six concentrations: 0.25–8 nM) and 100 nM prazosin to prevent binding to α-adrenergic receptors. Reactions were initiated by the addition of tissue (approximately 240 μg protein) and were terminated with a Brandel cell harvester using Whatman GF/B filters that were presoaked in 0.1% polyethyleneimine to minimize radioligand binding to the filters. Preliminary studies verified the above conditions resulted in equilibrium binding and that less than 4% of the radioligand was bound to the tissue. Filters were washed twice with 3 ml of cold 50 mM Tris–HCl (pH 7.4) and incubated overnight in scintillation cocktail before measuring the amount of bound radioactivity by liquid scintillation spectrophotometry. Specific binding was defined as the difference between the amount of radioactivity bound in the absence and presence of 100 μM mianserin. Receptor density and affinity were calculated by nonlinear regression using the EBDA/LIGAND program (Elsevier BIOSOFT).

2.6. [^{3}H]Arachidonic acid release

PC12-5-HT_{2A} cells were labeled using a modified N2 serum-free defined media (1:1 mixture of DMEM/F12 supplemented with 10 μg/ml transferrin, 5 μg/ml insulin, 20 nM progesterone, 10 μM putrescine, 20 nM selenium and 10 mM HEPES, pH 7.4) containing 0.5 μCi/ml [^{3}H]arachidonic acid. Because PC12 cells require serum for attachment (Rabin, 1990), cells were plated (10^6 cells/well) onto collagen-coated 24-well plates in complete serum-containing media the previous day. After a 24-h incubation with [^{3}H]arachidonic acid, cells were rinsed and incubated for 5 min at 37 °C in Krebs buffer containing 0.2% fatty acid free BSA. This procedure was repeated, and [^{3}H]arachidonic acid release was measured by incubating the cells for 10 min at 37 °C in the Krebs buffer containing 0.2% fatty acid free BSA and appropriate drugs. At the end of this incubation, an aliquot of the media was centrifuged at 16,000 × *g* for 1 min to remove any detached cells. The amount of radioactivity in the resulting supernatant, as well as in the cells remaining attached to the plates, was counted by liquid scintillation spectrophotometry. Data were expressed as the percentage of total incorporated radioactivity (i.e., amount of radioactivity in the cells plus in the medium) released into the medium.

Protein content was measured using the colorimetric Bio-Rad protein dye binding procedure with bovine serum albumin (fraction V) as a standard.

2.7. Statistical analysis

Results are presented as means ± S.E.M. Student's *t* test was used to compare the stimulation of [^{3}H]inositol phosphate production by 10 and 100 μM 5-HT. Data for the stimulation of [^{2}H]arachidonic acid release were analyzed by repeated-measures analysis of variance followed by Dunnett's test for multiple comparisons. An alpha of .05 was used.

3. Results

The binding of [^{3}H]ketanserin to PC12-5-HT_{2A} cells was best fitted by a one-site model (Hill coefficient 1.003 ± 0.03) with a B_{max} of 326 ± 70.6 fmol/mg and pK_D of 8.89 ± 0.072 (n = 3; Fig. 1). This affinity is comparable to values observed for the binding of [^{3}H]ketanserin in the rat cerebral cortex (Conn and Sanders-Bush, 1986; Johnson et al., 1993; Helsley et al., 1998a), as well as in other clonal cells expressing the cDNA for the 5-HT_{2A} receptor (Pritchett et al., 1988; Teitler et al., 1990; Berg et al., 1994b).

Stable expression of the cDNA for the 5-HT_{2A} receptor did not appear to alter the phospholipase C system as the increase in the generation of [^{3}H]inositol phosphates by 100 nM bradykinin in the PC12-5-HT_{2A}

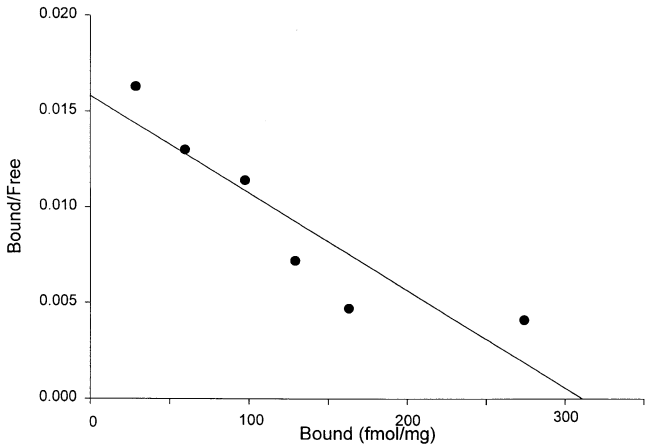

Fig. 1. Scatchard plot of [^{3}H]ketanserin binding in PC12-5-HT_{2A} cells. Equilibrium saturation experiments using six concentrations of [^{3}H]ketanserin (0.25–8 nM) were analyzed by nonlinear regression analysis of the untransformed data. Data are representative of results from three separate experiments and are plotted by the method of Scatchard.

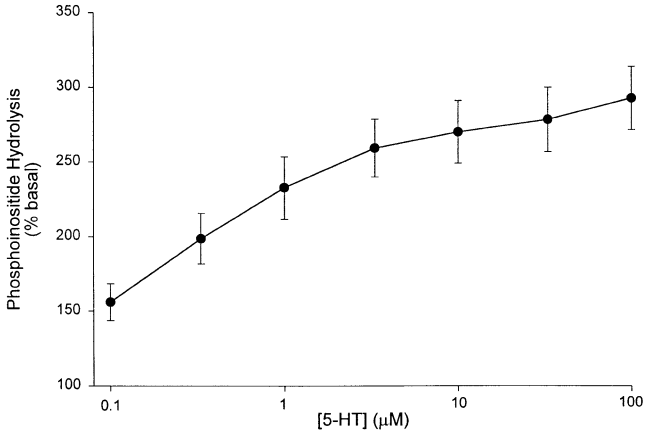

Fig. 2. Stimulation of phosphoinositide hydrolysis by 5-HT in PC12-5-HT_{2A} cells. The effects of various concentrations of 5-HT on the generation of [^{3}H]inositol phosphates were determined after an overnight incubation of the PC12-5-HT_{2A} cells with [^{3}H]*myo*-inositol as described in the Materials and Methods. Data are plotted as means ± S.E.M. of six separate experiments.

cells (75,026 ± 3562 and 120,062 ± 1379 dpm/mg in the absence and presence of bradykinin, respectively) is comparable to that previously observed in naive PC12 cells (Kim et al., 1993). In the PC12-5-HT_{2A} cells, 5-HT caused a concentration-dependent increase in the generation of [^{3}H]inositol phosphates (Fig. 2). The pEC_{50} for 5-HT was 6.45 ± 0.13, and the slope was 0.889 ± 0.099. Separation of the [^{3}H]inositol phosphates into inositol phosphate (InsP), inositol bisphosphate ($InsP_2$) and inositol trisphosphate ($InsP_3$) revealed an increase in all three species by 5-HT (Fig. 3). The above effects in PC12-5-HT_{2A} cells all involved the expression of the cDNA for the rat 5-HT_{2A} receptor as neither specific

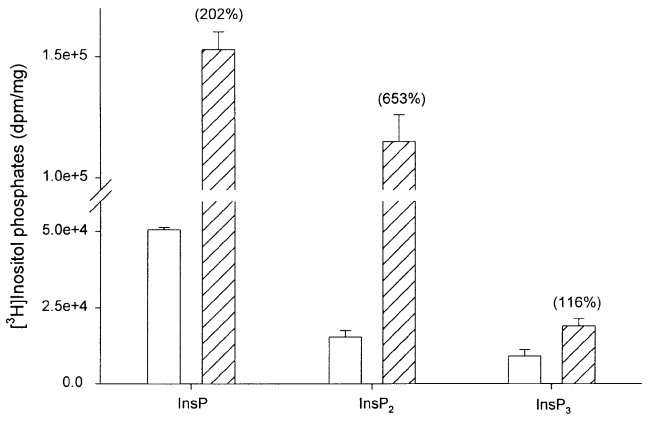

Fig. 3. Stimulation of [^{3}H]inositol phosphate, [^{3}H]inositol bisphosphate, and [^{3}H]inositol trisphosphate by 5-HT. [^{3}H]Inositol phosphates generated by 10 μM 5-HT were separated into inositol phosphate (InsP), inositol bisphosphate ($InsP_2$) and inositol trisphosphate ($InsP_3$) using Dowex resin as described in the Materials and Methods. Control: open bars; 5-HT: striped bars. Data are plotted as the measn ± S.E.M. of two separate experiments carried out in duplicate. Numbers in parentheses represent the percent increase elicited by the addition of 5-HT.

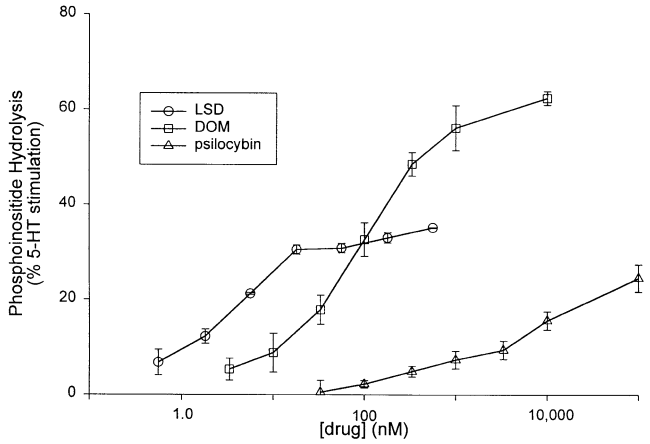

Fig. 4. Stimulation of phosphoinositide hydrolysis by the hallucinogens LSD, DOM and psilocybin. The effects of various concentrations of LSD, DOM and psilocybin on the generation of [^{3}H]inositol phosphates were determined after an overnight incubation of the PC12-5-HT_{2A} cells with [^{3}H]*myo*-inositol as described in the Materials and Methods. Data are plotted as means ± S.E.M. of three to six separate experiments. Abscissa: drug concentration. Ordinate: phosphoinositide hydrolysis expressed as a percentage of the stimulation observed with 10 μM 5-HT in this set of studies.

binding of [^{3}H]ketanserin nor 5-HT stimulation of phosphoinositide hydrolysis was observed in untransfected PC12 cells or in cells transfected with the plasmid lacking the cDNA insert. In untransfected cells, production of [^{3}H]inositol phosphates was 60,450 ± 5065 and 60,639 ± 3539 dpm/mg in the absence and presence of 10 μM 5-HT (n = 4), respectively, while [^{3}H]ketanserin binding was 1329 and 1373 dpm in the absence and presence of mianserin, respectively. Furthermore, the stimulation of phosphoinositide hydrolysis by 5-HT was

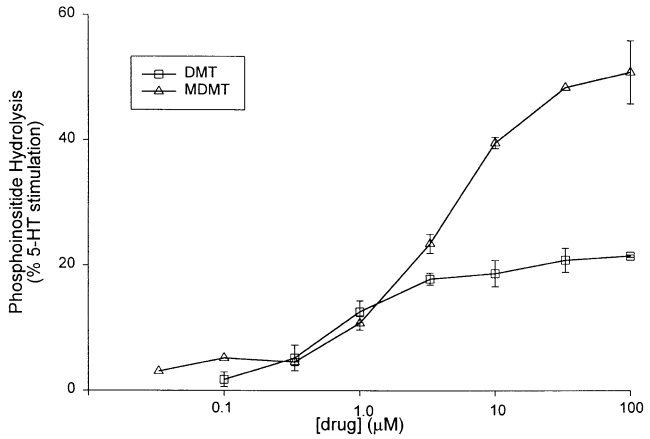

Fig. 5. Stimulation of phosphoinositide hydrolysis by the tryptamines DMT and MDMT. The effects of various concentrations of DMT and MDMT on the generation of [^{3}H]inositol phosphates were determined in PC12-5-HT_{2A} cells. Data are plotted as means ± S.E.M. of three to six separate experiments. Abscissa: drug concentration. Ordinate: phosphoinositide hydrolysis expressed as a percentage of the stimulation observed with 10 μM 5-HT in this set of studies.

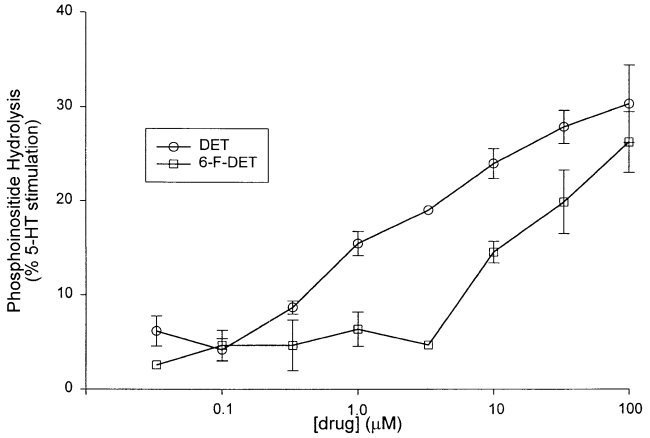

Fig. 6. Stimulation of phosphoinositide hydrolysis by DET and its nonhallucinogenic congener 6-F-DET. The effects of various concentrations of DET and 6-F-DET on the generation of [^{3}H]inositol phosphates were determined in PC12-5-HT$_{2A}$ cells. Data are plotted as means ± S.E.M. of four separate experiments. Abscissa: drug concentration. Ordinate: phosphoinositide hydrolysis expressed as a percentage of the stimulation observed with 10 μM 5-HT in this set of studies.

completely abolished by the addition of the 5-HT$_2$ antagonist ketanserin. Generation of [^{3}H]inositol phosphates was increased by 186 ± 2.0% in the presence of 1 μM 5-HT, while the inclusion of 5-HT plus 10 μM ketanserin caused a 4 ± 1.8% increase (n = 4).

The concentration–response relationships for the stimulation of phosphoinositide hydrolysis by various indolealkylamines and phenalkylamines are shown in Figs. 4–7. Data are expressed as a percentage of the stimulation observed with 10 μM 5-HT. Based upon the observed EC$_{50}$ value for 5-HT, this concentration results in 97% of the maximal response. Furthermore, no statistically significant difference was observed between the increase in generation of [^{3}H]inositol phosphates with 10 μM 5-HT and that with 100 μM 5-HT. A comparison of the in vitro efficacy of the various compounds with the results from

Table 1
Comparison of in vitro (phosphoinositide hydrolysis) and in vivo (LSD or DOM appropriate responding) efficacy of indoleamine, phenethylamine and β-carboline hallucinogens

Compound	pEC$_{50}$	Maximum PI hydrolysis (% 5-HT stimulation)	Generalization in LSD/DOM-trained rats[a]
LSD (3)	8.22	32.3 ± 2.45	complete
DOM (3)	7.23	77.4 ± 3.23	complete
Psilocybin (6)	5.39	33.8 ± 5.54	complete
Quipazine (3)	7.30	62.8 ± 5.88	complete
Lisuride (4)	7.08	15.6 ± 2.2	complete
MDMT (4)	6.65	54.0 ± 6.17	intermediate
DMT (3)	6.43	20.4 ± 0.42	intermediate
DET (4)	5.90	31.1 ± 2.51	intermediate
Harmaline (2)	NS	NS	NO/intermediate
Harmane (2)	NS	NS	NO/intermediate
6-F-DET (4)	ND	ND	NO
BOL (2)	NS	NS	

The data from Figs. 4–7 were analyzed by nonlinear regression to determine EC$_{50}$ and V_{max} values. In vitro efficacy is defined as the V_{max} value for the compound relative to the stimulation of phosphoinositide hydrolysis by 10 μM 5-HT and is presented as the mean ± S.E.M. for the number of experiments shown in parenthesis. EC$_{50}$ values are presented as geometric means.

NS: no stimulation (< 10% of the maximum 5-HT-stimulated PI hydrolysis).

ND: maximum stimulation of phosphoinositide hydrolysis could not be determined.

[a] Ability of the various compounds to produce generalization in LSD- or DOM-trained animals is taken from Helsley et al. (1998b), Grella et al. (1998), Nielsen et al. (1982) and Glennon et al. (1983) (see text for details).

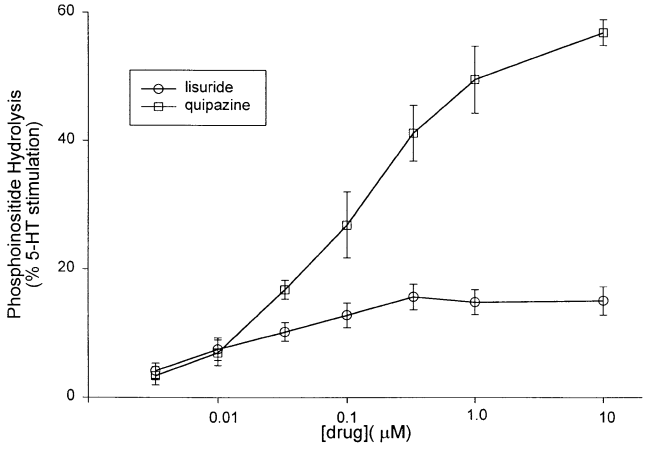

Fig. 7. Stimulation of phosphoinositide hydrolysis by the nonhallucinogens lisuride and quipazine. The effects of various concentrations of lisuride and quipazine on the generation of [^{3}H]inositol phosphates were determined in PC12-5-HT$_{2A}$ cells. Data are plotted as means ± S.E.M. of three to four separate experiments. Abscissa: drug concentration. Ordinate: phosphoinositide hydrolysis expressed as a percentage of the stimulation observed with 10 μM 5-HT in this set of studies.

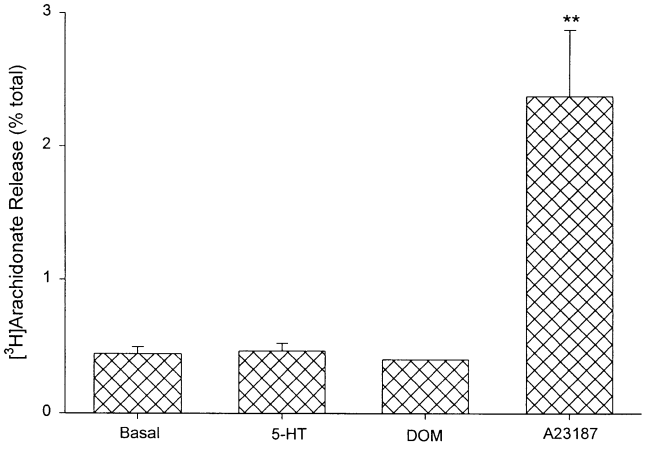

Fig. 8. Stimulation of [^{3}H]arachidonic acid release. The effects of 100 μM 5-HT, 100 μM DOM and 20 μM A23187 on [^{3}H]arachidonic acid release were determined after an overnight incubation of PC12-5-HT$_{2A}$ cells with the radiolabeled fatty acid as described in the Materials and Methods. Data are expressed as the percentage of total incorporated radioactivity (61,057 ± 10,300 dpm/well) that was released into the media and are plotted as the means ± S.E.M. of two to four separate experiments. ** P < .01, Dunnett's test for multiple comparisons compared to basal.

drug discrimination studies using animals trained with either LSD or DOM is shown in Table 1. The hallucinogenic compounds, LSD, DOM, psilocybin, DMT, MDMT and DET, all caused a concentration-dependent stimulation of phosphoinositide hydrolysis. Although concentrations of up to 10 μM BOL, the inactive congener of LSD, did not alter phosphoinositide hydrolysis (Table 1), the nonhallucinogen 6-F-DET increased the generation of inositol phosphates (Fig. 6). Because maximal stimulation of phosphoinositide hydrolysis was not observed with up to 100 μM 6-F-DET, we were unable to calculate pEC_{50} or V_{max} values. Similarly, lisuride and quipazine, which are not hallucinogenic in humans, also caused a concentration-dependent increase in phosphoinositide hydrolysis (Fig. 7). Concentrations of up to 100 μM of the β-carbolines, harmaline and harmane, did not alter the generation of inositol phosphates in the PC12-5-HT_{2A} cells (Table 1).

Because stimulation of 5-HT_{2A} receptors has been reported to increase arachidonic acid release (Felder et al., 1990; Berg et al., 1998), the possible role of phospholipase A_2 was also investigated. Although the ionophore A23187 caused a fivefold increase in the amount of radioactivity released into the media, inclusion of 100 μM 5-HT had no effect (Fig. 8). Similar results were obtained when cells were labeled for 4 h (data not shown) and when 100 μM DOM was added (Fig. 8).

4. Discussion

Hallucinogens can serve as discriminative stimuli in nonverbal animals (see Winter et al., 1999). Although interactions mediating stimulus control may not be completely identical to those responsible for the induction of hallucinations, nonessential interactions can be identified by using a series of compounds for behavioral and biochemical studies. Previous studies have shown that activation of the 5-HT_{2A} receptor is necessary for the stimulus effects of both the indoleamine LSD and the phenethylamine DOM (Schreiber et al., 1994; Fiorella et al., 1995a). The 5-HT_{2A} receptor is typically coupled through $G_{q/11}$ to stimulation of phospholipase C with a resulting increase in the generation of the second messengers inositol 1,4,5-trisphosphate and protein kinase C. The primary question investigated in the present study was whether 5-HT_{2A}-mediated stimulation of phosphoinositide hydrolysis plays an essential role in the stimulus effects of hallucinogenic compounds. This question was investigated by comparing the discriminative stimulus effects of some indoleamines, phenethylamines and β-carbolines with the in vitro ability of these compounds to stimulate phosphoinositide hydrolysis in PC12-5-HT_{2A} cells. Because of the dearth of reports on the potency of these compounds in discriminative stimulus studies, efficacy rather than potency was investigated.

The hallucinogens LSD, DOM and psilocybin show complete cross-generalization and substitution but display more than a twofold difference in in vitro efficacy as indicated by the ability to stimulate phosphoinositide hydrolysis relative to 5-HT. The tryptamines, DET and MDMT, display an in vitro efficacy comparable to or greater than LSD and psilocybin, yet produce only an intermediate level of generalization (Helsley et al., 1998b). The β-carboline, harmaline, has been shown to be hallucinogenic in humans and was reported to display significant substitution in both LSD- and DOM-trained animals (Nielsen et al., 1982; Glennon et al., 1983), although contrary findings have been reported (Helsley et al., 1998b). In addition, cross-generalization occurs with DOM resulting in 76% drug-appropriate responding in harmaline-trained animals (Grella et al., 1998). Harmaline, however, did not increase [^{3}H]inositol phosphate production. The tryptamines, MDMT, DMT, DET and 6-F-DET, all display comparable affinities at the 5-HT_{2A} receptor in rat prefrontal cortex (K_i values were 3.47 ± 0.37, 4.89 ± 0.61, 5.80 ± 0.18 and 1.99 ± 0.57 μM, respectively, $n = 3$; R.A. Rabin and J.C. Winter, unpublished data), and in the present study, these compounds all caused significant stimulation of phosphoinositide hydrolysis. However, whereas partial substitution is observed with DET, DMT and MDMT (Helsley et al., 1998b), 6-F-DET is neither hallucinogenic in humans (Faillace et al., 1967) nor does it show substitution in LSD-trained animals (Helsley et al., 1998b). Thus, because the ability to increase [^{3}H]inositol phosphate production did not correlate with the degree of generalization in LSD- and DOM-trained animals, it would appear that 5-HT_{2A}-mediated stimulation of phosphoinositide hydrolysis may not be the sole critical component in the stimulus effects of the hallucinogens. It is, however, possible that an increase in phosphoinositide hydrolysis above a threshold level might be one of several required intracellular changes involved in the discriminative effects of the hallucinogens.

The validity of the above conclusion is dependent on the compounds stimulating phosphoinositide hydrolysis only through the activation of the 5-HT_{2A} receptors on the PC12-5-HT_{2A} cells. Excluding neurotrophins that act through activation of receptor tyrosine kinases, phosphoinositide hydrolysis in PC12 cells is increased by bradykinin (Kim et al., 1993), purinergic P_2 receptor agonists (Kim et al., 1993) and imidazole I_2 receptor agonists (Scparovic et al., 1996, 1997). We are unable to find any evidence that the compounds used in the present study interact with these receptors. Furthermore, agonist stimulation of phosphoinositide hydrolysis in the PC12 cells was dependent upon the level of 5-HT_{2A} receptor expression (manuscript in preparation), which is not consistent with an involvement of non-5-HT_{2A} receptors. PC12 cells have been reported to express 5-HT_3 receptors, but this occurs only after several days of treatment with nerve growth factor or 8-Br-cAMP (Gordon and Rowland, 1990; Furkawa et al., 1992). As untreated PC12 cells display neither specific binding of [^{3}H]zacopride (Gordon and Rowland, 1990), 5-HT_3 mRNA (Hanna et al., 2000), nor 5-HT-induced electrophysiological

responses (Furkawa et al., 1992), it seems unlikely that this serotonergic receptor would play a relevant role in the present study. Although specific binding of [^{3}H]ketanserin and stimulation of [^{3}H]inositol phosphate generation were not observed in untransfected PC12 or in cells transfected with the empty vector, Humblot et al. (1997) reported an increased expression of egr-1 in PC12 cells that appeared to involve the 5-HT$_2$ receptor. There are, however, several variants of the PC12 cell line, and while the cells used in the present study were maintained in DMEM, those used by Humblot et al. required RPMI and were serum-starved for 15 h, which in our hands, induces apoptosis (J. Oberdoerster and R.A. Rabin, unpublished data). Nevertheless, the possible presence of endogenous 5-HT$_{2A}$ receptors in PC12 cells would not alter interpretation of the data.

In the present study, the indoleamines and phenethylamines tested appeared to be partial agonists with intrinsic activities ranging from 0.15 to 0.77 with respect to the stimulation of phosphoinositde hydrolysis by 5-HT. Similar results have been reported using brain tissue and cells expressing 5-HT$_{2A}$ receptors (Sanders-Bush et al., 1988; Cory et al., 1987; Newton et al., 1996; Egan et al., 1998). Furthermore, the partial agonist activity of these hallucinogens is consistent with the electrophysiological responses to LSD and DOI in rat piriform cortex (Marek and Aghajanian, 1996).

Lisuride and quipazine have been referred to as ''false positives'' as these compounds have been reported not to be hallucinogenic in humans (Herrmann et al., 1977; De Cecco et al., 1979; Parati et al., 1980) yet display complete generalization in LSD- and DOM-trained animals (Fiorella et al., 1995b). Although a few studies report hallucinations with lisuride (Critchley et al., 1988; Obeso et al., 1988), these occurred with continuous drug infusion in patients with Parkinson's disease, which is associated with psychiatric problems, and these patients also were taking levodopa, which can induce psychosis and hallucinations (Klawans, 1988). In the present study, MDMT and DET caused a greater increase in [^{3}H]inositol phosphate production than lisuride, yet, these tryptamines showed only an intermediate level of generalization. It could be argued that only a minimal increase in phosphoinositide hydrolysis is required for the stimulus effects of the hallucinogens, and because of the potency difference, lisuride, but not the tryptamines, is able to attain this level of inositol phosphate generation in vivo. However, this idea does not appear to be supported by the data with psilocybin; this compound is less potent than the tryptamines but displays full substitution. To the extent that in vivo and in vitro efficacy are correlated, the data would suggest that it is unlikely that the ability of lisuride to substitute for the DOM/LSD stimulus involves only the stimulation of phosphoinositide hydrolysis.

The 5-HT$_{2A}$ receptor has been reported to stimulate phospholipase A$_2$ activity resulting in an increase in arachidonic acid release in mixed neuronal–glial cultures (Felder et al., 1990), C6 glioma cells (Garcia and Kim, 1997), 1C11 cells, which are derived from F9 multipotential embryonal carcinoma cells (Tournois et al., 1998), and in CHO cells tranfected with the cDNA for the human 5-HT$_{2A}$ receptor (Berg et al., 1998). Although activation of phospholipase A$_2$ activity was observed with the ionophore A23187, stimulation of the 5-HT$_{2A}$ receptor by either 5-HT or DOM did not alter arachidonic acid release from the PC12-5-HT$_{2A}$ cells. Similarly, in cerebellar granule cells, activation of the 5-HT$_{2A}$ receptor also did not stimulate phospholipase A$_2$ activity (Chen et al.,1995). The reason for this discrepancy is unclear but may reflect differences in the relative stoichiometry and/or coupling of the various signaling components involved in phospholipase A2 activation by the 5-HT$_{2A}$ receptor.

Currently, it is unclear which signaling pathways coupled to the 5-HT$_{2A}$ receptor are responsible for the stimulus effects of LSD and DOM. The results of the present study suggest that stimulation of phosphoinositide hydrolysis does not appear to be the sole critical mechanism in the stimulus effects of hallucinogens. Although the present data also suggest that arachidonic acid release is not involved in the stimulus effects of the hallucinogens, further studies are required to elucidate definitively the role of phospholipase A$_2$. The 5-HT$_{2A}$ receptor, however, also has been suggested to affect other signaling pathways. For example, this receptor was reported to activate the mitogen-activated protein kinase pathway (Banes et al., 1999) and to induce early response genes (Goppelt-Struebe and Stroebel, 1998). Furthermore, activation of the 5-HT$_{2A}$ receptor also has been reported to amplify cyclic AMP production secondary to activation of protein kinase C- and calcium/calmodulin-dependent pathways (Berg et al., 1994a). Which, if any, of these signaling pathways is involved in the stimulus effects of the hallucinogens is the focus of future investigations.

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