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Serotonin 5-Hydroxytryptamine$_{2A}$ Receptor-Coupled Phospholipase C and Phospholipase A_2 Signaling Pathways Have Different Receptor Reserves

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ABSTRACT

NIH3T3 cells stably expressing the rat 5-hydroxytryptamine$_{2A}$ (5-HT$_{2A}$) receptor (5500 fmol/mg) were used to explore further the capacity of structurally distinct ligands to elicit differential signaling through the phospholipase C (PLC) or phospholipase A_2 (PLA$_2$) signal transduction pathways. Initial experiments were designed to verify that 5-HT$_{2A}$ receptor-mediated PLA$_2$ activation in NIH3T3 cells is independent from, and not a subsequent result of, 5-HT$_{2A}$ receptor-mediated PLC activation. In addition, we also explored the extent of receptor reserve for the endogenous ligand, 5-HT, for both PLC and PLA$_2$ activation. Finally, we employed structurally diverse ligands from the tryptamine, phenethylamine, and ergoline families of 5-HT$_{2A}$ receptor agonists to test the hypothesis of agonist-directed trafficking of 5-HT$_{2A}$ receptor-mediated PLC and PLA$_2$ activation. To measure agonist-induced pathway activation, we determined the potency and intrinsic activity of each compound to activate either the PLA$_2$ pathway or the PLC pathway. The results showed that a larger receptor reserve exists for 5-HT-induced PLA$_2$ activation than for 5-HT-induced PLC activation. Furthermore, the data support the hypothesis of agonist-directed trafficking in NIH3T3–5HT$_{2A}$ cells because structurally distinct ligands were able to induce preferential activation of the PLC or PLA$_2$ signaling pathway. From these data we conclude that structurally distinct ligands can differentially regulate 5-HT$_{2A}$ receptor signal transduction.

G protein-coupled receptors (GPCRs) function to transduce an external chemical stimulus into an intracellular biochemical response. Upon agonist binding to the receptor, the receptor undergoes a conformational change that promotes GDP release from the Gα subunit; high levels of intracellular GTP bind to Gα and promote the dissociation of this complex (Downes and Gautam, 1999). Activated Gα_{GTP} and G$\beta\gamma$ are then free to regulate downstream effectors (Gilman, 1987; Clapham and Neer, 1997).

Although models have been proposed to clarify GPCR activation, the mechanism of this process is not completely understood. Crystal structures of Gα, Gβ, and Gγ subunits have led to insight into the function of these proteins in GPCR activation. Very little is known, however, about the receptor, including the nature of the change in receptor conformation upon ligand binding and whether only one or perhaps an infinite range of active conformations are sufficient to explain all the empirical data (Colquhoun, 1998). Indeed, several studies have demonstrated the ability of a single GPCR, when acted upon by different agonists, to activate preferentially two independent signaling pathways (Offermanns et al., 1994; Robb et al., 1994; Berg et al., 1998; Pommier et al., 1999), suggesting that a receptor is able to exist in different active conformations. It is not known, however, whether the receptor is able to achieve a limitless number of active conformations (R^{n*}), each determined by the agonist that binds, or whether the receptor is limited in the number of active conformations, (R^* and R^{**}), thereby requiring the agonist to recognize a restricted subset of receptor states.

For example, Kenakin (1995) formulated the concept of agonist-directed trafficking of a receptor stimulus to explain the ability of structurally diverse agonists to activate differentially GPCR-mediated signaling. According to this model, each agonist is able to promote its own specific active recep-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; 5-HT, 5-hydroxtryptamine, serotonin; PLA$_2$, phospholipase A_2; PLC, phospholipase C; AA, arachidonic acid; IP, a mixture of inositol monophosphate, inositol bisphosphate, and inositol triphosphate; RHC-80267, 1,6-*bis*-(cyclohexyloximinocarbonylamino)hexane; *d*-LSD, *d*-lysergic acid diethylamide; DAG, diacylglycerol; PBZ, phenoxybenzamine; DOB, 1-(4-bromo-2,5-dimethoxyphenyl)-2-aminopropane.

tor state, leading theoretically to a limitless number of receptor conformations, R^{n*}. In contrast, Leff et al. (1997) proposed a three-state model, where the receptor might exist in three states, an inactive (R) and two active conformations (R^*, R^{**}), thereby still accounting for multiple receptor-effector coupling but limiting the number of active conformations. That is, a particular agonist will stabilize either R^* or R^{**}, but not R^{n*}, thereby limiting the complexity of the extended ternary complex model.

Besides taking into consideration the number of receptor states when exploring receptor-effector coupling, the concept of receptor reserve must also be taken into account. Stephenson (1956), based on studies conducted by Furchgott (1955) and Nickerson (1956), expanded the original receptor occupancy theory proposed by Clark (1926) and Ariëns (1954) to include receptor reserve by postulating that a pharmacological response need not be proportional to receptor occupancy. Specifically, different drugs may be able to induce a response with varying efficiencies, and thus could mediate equal responses by occupying different percentages of the receptor pool.

The aims of this study were twofold: 1) to demonstrate that 5-HT_{2A} receptor-mediated PLA_2 and PLC activation are independently coupled to the receptor in NIH3T3–5HT_{2A} cells and 2) to examine the capacity of 5-HT_{2A} receptor agonists to activate preferentially the PLC or PLA_2 signaling pathways. In particular, we were interested in the role of receptor reserve on differential regulation of 5-HT_{2A} receptor-mediated PLA_2 activation and PLC activation. Using 5-HT, the endogenous ligand, studies were conducted to determine the percentage of the total receptor pool that was required to elicit PLC and PLA_2 activation following exposure to phenoxybenzamine, an irreversible GPCR antagonist.

Following the demonstration that a larger receptor reserve existed for 5-HT-induced PLA_2 activation than for 5-HT-induced PLC activation, we then wished to explore the ability of structurally diverse 5-HT_{2A} receptor agonists from the phenethylamine, ergoline, and tryptamine classes of ligands to regulate differentially receptor-mediated AA release and IP accumulation. The data are consistent with the hypothesis of agonist-directed trafficking because many of the ligands were able to display preferential activation of the PLC or PLA_2 signaling pathways. In addition, the results of the present study demonstrate that a larger receptor reserve may exist for many of these agonists at 5-HT_{2A} receptor-mediated AA release because an increase in potency was observed for PLA_2 activation when compared with PLC activation. Interestingly, a similar trend does not exist when the intrinsic activity of these compounds is examined, therefore supporting our decision to measure both potency and intrinsic activity independently. Taken together, these data are consistent with the concept of agonist-directed trafficking but do not fit the predictions of the three-state model because we have demonstrated pathway-specific differences in receptor reserve.

Materials and Methods

Materials

myo-[2-^{3}H(N)]Inositol was obtained from PerkinElmer Life Sciences (Boston, MA). [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid was obtained from Amersham Biosciences Inc. (Piscataway, NJ). Melittin, phenoxybenzamine, and bovine serum albumin were all purchased from Sigma-Aldrich (St. Louis, MO). RHC-80267 was obtained from Calbiochem (San Diego, CA). 5-HT, ketanserin, and AA were all purchased from Sigma/RBI (Natick, MA). ET-18-OCH_3 was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). Dialyzed fetal bovine serum was purchased from Hyclone Laboratories (Hogan, UT); all other cell culture reagents were purchased from Invitrogen (Carlsbad, CA). All of the agonist ligands were synthesized in our laboratory, except for *d*-LSD (Nation Institute on Drug Abuse, Bethesda, MD), tryptamine (Sigma-Aldrich), quipazine (a gift from Bayer Corp., Emeryville, CA), and lisuride (a gift from Schering, AG, Berlin, Germany).

Methods

Cell Culture. NIH3T3 fibroblasts stably expressing the 5-HT_{2A} receptor (Julius et al., 1990) were maintained in Dulbecco's modified Eagle's medium, supplemented with 10% dialyzed fetal bovine serum, 2 mM L-glutamine, 50 units/l penicillin, 50 μg/l streptomycin, and 300 mg/ml G-418, and grown at 37°C in a 5% CO_2 environment. Cells were passaged when they reached 95% confluence and discarded after 30 passages.

Radioligand Binding Assays. Cells were grown in 150-mm tissue culture dishes until 90% confluent. Five hours before harvesting of cells, the medium was aspirated, and the cells were rinsed once with phosphate-buffered saline and left to incubate in serum-free Opti-MEM, unsupplemented. After this incubation, the cells were pelleted by centrifugation and stored in a −80°C freezer until needed.

For saturation binding assays, 0.313 to 10.0 nM [^{3}H]ketanserin or 0.125 to 5.0 nM [^{125}I]2,5-dimethoxy-4-iodoamphetamine was used. Nonspecific binding was defined in the presence of 10 μM cinanserin. All drugs and radioligands were diluted in "assay binding buffer" (50 mM Tris, 0.5 mM EDTA, 10 mM $MgCl_2$; pH = 7.4). The assay commenced upon the addition of 25 mg of cellular homogenate to each well of a 96-well plate already containing assay binding buffer, radioligand, and cinanserin, if appropriate. The incubation was carried out at 25°C for 60 min and terminated by rapid filtration using a prechilled Packard 96-well harvester. The filters were rinsed once using chilled wash buffer (10 mM Tris, 150 mM NaCl). Radioactivity was determined using a TopCount (PerkinElmer Life Sciences) scintillation counter.

Competition binding experiments were carried out in a similar manner with slight modifications. Previously harvested cells were resuspended in assay buffer, and 50 mg of protein were added to each well already containing assay binding buffer, [^{125}I]2,5-dimethoxy-4-iodoamphetamine (0.20 nM) or [^{3}H[ketanserin (1.0 nM), and test compound dilutions (10 pM–10 μM). The reaction was carried out at 25°C for 60 min and terminated by rapid filtration as described above. Prism software (GraphPad Software Inc., San Diego, CA) was used to analyze the saturation and competition binding curves.

Phosphoinositide Hydrolysis Assays. Accumulation of total IP was determined using a modified version of a previously published protocol (Berg et al., 1994). Cells were seeded to a final density of 1×10^5 cells/well in 48-well plates. Eighteen hours before beginning the assay, cells were washed once with phosphate-buffered saline, and the medium was replaced with serum- and inositol-free CMRL-1066 media (Connaught Laboratories, Swiftwater, PA), supplemented with 1.0 μCi/ml *myo*-[2-^{3}H(N)]inositol. To start the assay, the cells were pretreated for 15 min at 37°C with 10 μM pargyline, 10 mM LiCl, and any inhibitors, if appropriate. Following this incubation, 5-HT_{2A} receptors were then stimulated with agonists for 30 min at 37°C. The assay was terminated by aspiration of the medium and the addition of 10 mM formic acid; the 48-well plates were then left to sit overnight at 4°C. The [^{3}H]phosphoinositides were separated by placing the termination reaction onto a Dowex-1 ion exchange column (Berridge, 1983). The columns were then washed first with equilibrium buffer (10 mM *myo*-inositol, 3 M ammonium formate) and second with wash buffer (5 mM sodium tetraborate, 10 mM ammonium formate). The [^{3}H]phosphoinositides were then eluted with elution buffer (1.0 M ammonium formate and 0.10 M formic acid) into scintillation vials. Scintillation cocktail was added and the ra-

dioactivity was quantified using a liquid scintillation counter (Beckman Coulter, Fullerton, CA).

PLA$_2$ Assays. The quantity of released AA was determined using a modified version of the procedure of Berg et al. (1998). Cells were seeded into 24-well plates at a density of 2×10^5 cells/well. Cells were labeled with 0.5 μCi/ml [^{3}H]AA in serum-free medium for 4 h prior to assay at 37°C. After this incubation, the cells were washed three times with Dulbecco's modified Eagle's medium supplemented with 0.5% fatty acid-free bovine serum albumin and 2% dialyzed fetal bovine serum. Between each wash the 24-well plate was placed in a 37°C water bath for 5 min. Enzyme inhibitors or antagonists were present during each 5-min incubation (i.e., for 15 min total). The assay was initiated by the addition of 5-HT (10 μM) or other agonist, followed by incubation for 30 min at 37°C. After this final incubation, an aliquot of the cell medium was removed and added to scintillation vials and quantified using liquid scintillation counting.

Results

5-HT$_{2A}$ Receptor Activation Stimulates AA Release and IP Accumulation in NIH3T3 Cells. Initial studies were designed to establish that 5-HT can stimulate both PLA$_2$-AA release and PLC-IP accumulation in NIH3T3 cells that heterologously express the 5-HT$_{2A}$ receptor (5500 fmol/mg). Time course assays revealed that 30 min, which was used for all subsequent experiments, was an optimal 5-HT incubation time to produce a robust stimulation of PLA$_2$ and PLC (results not shown). In NIH3T3–5HT$_{2A}$ cells, PLA$_2$-AA release was stimulated in a dose-dependent manner, to a maximal 5.1-fold over basal levels (Fig. 1A). 5-HT$_{2A}$ receptor-mediated PLC-IP accumulation was maximally increased 19-fold over basal (Fig. 1B). To ensure that 5-HT-coupled PLA$_2$ and PLC activation was 5-HT$_{2A}$ receptor-mediated, ketanserin, a selective 5-HT$_{2A}$ antagonist (Van Nueten et al., 1981) was employed. Exposure of NIH3T3–5HT$_{2A}$ cells to ketanserin (100 μM) prior to stimulation with 5-HT (10 μM) resulted in complete inhibition of both PLA$_2$-AA release and PLC-IP accumulation (Fig. 1, inset graphs). Prior work by Julius et al. (1990), from whom we obtained the NIH3T3–5-HT$_{2A}$ cell line, had shown both a lack of [^{125}I]LSD-specific binding as well as a lack of 5-HT response in parental NIH3T3 cells.

PLA$_2$-AA Release Occurs Independently of PLC-IP Accumulation. Inhibitors selective for various steps along the PLA$_2$ or PLC signaling pathways were employed to determine the roles of PLC and PLA$_2$ in 5-HT-induced AA release and IP accumulation. Because the possibility existed that 5-HT$_{2A}$ receptor-mediated AA release could be subsequent to PLC pathway activation, either by cleavage of arachidonyl chains located on DAG or by PKC-coupled PLA$_2$ activation, studies were conducted to determine the extent of cross-talk between 5-HT-induced IP accumulation and 5-HT-induced AA release.

The first inhibitor employed was mepacrine, a PLA$_2$ inhibitor that does not discriminate between the three isoforms of PLA$_2$, namely, secretory PLA$_2$, cytoplasmic PLA$_2$, and Ca^{2+}-independent PLA$_2$ (Mukherjee et al., 1994). Pretreatment with mepacrine (100 μM) abolished 5-HT$_{2A}$ receptor-mediated AA release but resulted in a slight potentiation of PLC-IP accumulation (140% 5-HT response; Fig. 2). Furthermore, this enhancement of the PLC signaling pathway was also observed at a concentration of mepacrine (30 μM) that resulted only in partial inhibition of agonist-induced AA release (57% decrease; Table 1).

To confirm that AA was not produced as the result of cleavage from DAG by DAG lipase, RHC-80267 was employed. RHC-80267 is a direct inhibitor of DAG lipase (Sutherland and Amin, 1982), which functions to cleave the arachidonyl chains of DAG. Preincubation with RHC-80267 (10 μM) had no effect on AA release (>90% 5-HT response), although there was a slight decrease in PLC-IP accumulation (Table 1).

These results, however, do not confirm the ability of the receptor to activate PLA$_2$ directly, or indicate whether agonist-induced PLA$_2$ activation is subsequent to stimulation of the PLC signaling pathway. Therefore, the effects of PLC inhibition on AA release were explored using an inositol-specific PLC inhibitor, ET-18 (Powis et al., 1992). Pretreatment of NIH3T3–5HT$_{2A}$ cells with ET-18 (50 μM) abolished 5-HT-induced IP accumulation (Fig. 2B), although there was a slight inhibition of 5-HT-induced AA release (Fig. 2A). A lower concentration of ET-18 (10 μM), however, resulted in a significant decrease in PLC-IP accumulation (>70% inhibition; Table 1), and in this case there was no effect on PLA$_2$-AA release (Table 1). Thus, inhibition of PLC appears to have no effect on 5-HT-induced AA release.

Even though these data indicate that there is no cross-talk between the PLC and PLA$_2$ signaling pathways, one additional inhibitor that targets a PLC-coupled signaling molecule was employed. In particular, PKC is downstream of PLC

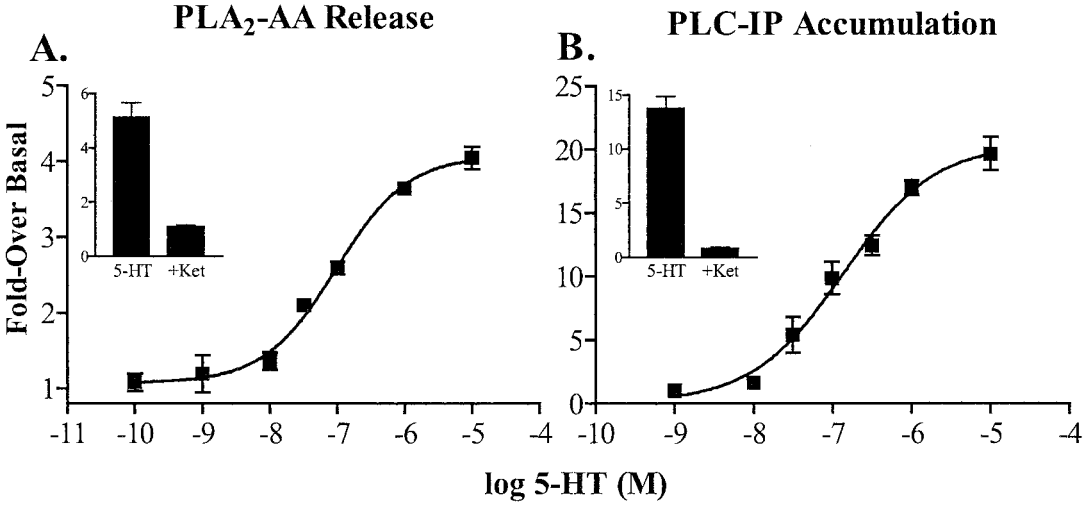

Fig. 1. 5-HT-induced PLA$_2$-AA release (A) and PLC-IP accumulation (B) in NIH3T3 cells. Cells were labeled as described under *Materials and Methods.* The dose-response curves represent 30-min incubations with varying concentrations of 5-HT (0.10 nM–10 μM). The inset graphs illustrate the effect of ketanserin. After preincubation with ketanserin (100 μM, 15 min), cells were exposed to 5-HT (10 μM) for 30 min. Data are the mean ± S.E.M. of three separate experiments.

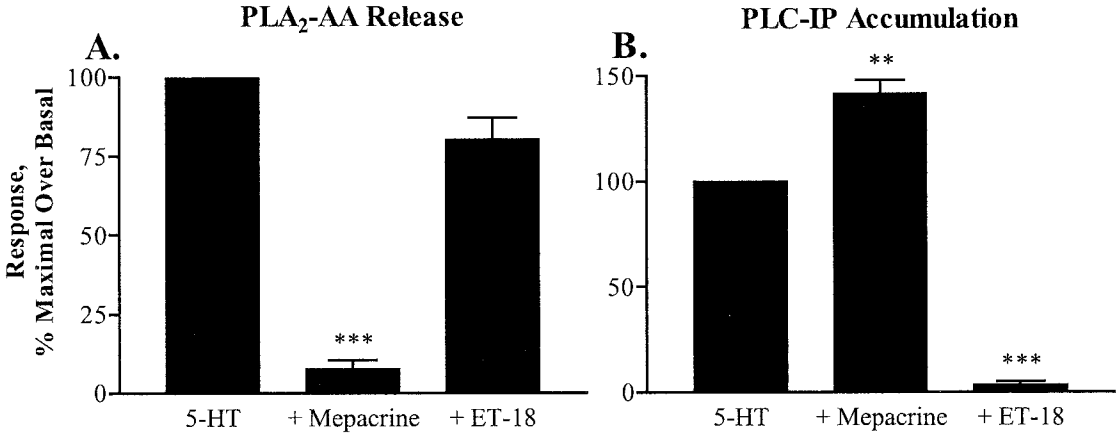

Fig. 2. The effect of mepacrine (PLA_2 inhibitor) or ET-18 (PLC inhibitor) on PLA_2-AA release (A) and PLC-IP accumulation (B). NIH3T3 cells stably expressing the r5-HT_{2A} receptor were preincubated for 15 min with vehicle (control), mepacrine (100 μM), or ET-18 (50 μM). The effect of these inhibitors in the presence of 5-HT (10 μM) for 30 min is shown. The data represent the mean ± S.E.M. of four separate experiments. ***, $p < 0.002$; **, $p < 0.02$ compared with a hypothetical value of 100 (one-sample t test).

TABLE 1

The effect of various inhibitors of 5-HT_{2A} receptor-mediated PLC and PLA_2 activation

The effect of PLA_2 inhibition (mepacrine), PLC inhibition (ET-18), DAG lipase inhibition (RHC-80267), and PKC inhibition (staurosporine) on PLA_2-AA release and PLC-IP accumulation. NIH3T3–5HT_{2A} cells were preincubated with vehicle (control) or inhibitor for 15 min prior to the addition of 5-HT (10 μM) for 30 min. The data represent the mean (S.E.M.) of at least three independent experiments.

Inhibitor	Concentration	PLA_2-AA Release	PLC-IP Accumulation
	μM	*% max over basal*	
Control		100	100
Mepacrine	30	43(8.4)*	120(3.8)
ET-18	10	100(8.2)	28(5.3)***
RHC-80267	10	92(9.9)	81(8.1)
Staurosporine	0.1	95(11)	110(7.1)

* $P < 0.05$, *** $P < 0.001$ compared with a hypothetical value of 100 (one-sample t test).

activation and is potentially capable of mediating AA release by functioning as a kinase, either to phosphorylate PLA_2 itself or other intermediary proteins, such as mitogen-activated protein kinases, which then phosphorylate PLA_2. Staurosporine is a microbial alkaloid produced by *Streptomyces* that interacts with the catalytic domain of PKC to inhibit its activity (Kanashiro and Khalil, 1998). Pretreatment with this compound (100 nM) had no effect on PLA_2-AA release (>95% control), whereas a slight, but nonsignificant potentiation of PLC-IP accumulation was observed (Table 1).

The Effect of 5-HT_{2A} Receptor Inactivation on 5-HT-Induced AA Release and IP Accumulation. Having demonstrated that the 5-HT_{2A} receptor can independently activate the PLC and PLA_2 signaling pathways, additional studies were conducted to explore the existence and extent of receptor reserve for 5-HT-induced PLC-IP accumulation or PLA_2-AA release in NIH3T3–5HT_{2A} cells. We employed phenoxybenzamine (PBZ), an alkylating agent that covalently modifies GPCRs (Hoffman and Lefkowitz, 1996), to inactivate irreversibly the 5-HT_{2A} receptor. Saturation isotherm studies were conducted to determine the percentage of the 5-HT_{2A} receptor pool that was inactivated at varying doses of PBZ, and functional studies were carried out to determine the effect of receptor inactivation on agonist-induced PLA_2-AA release and PLC-IP accumulation.

First, NIH3T3–5HT_{2A} cells were treated with increasing concentrations (10 nM–100 μM) of PBZ to determine the magnitude of 5-HT_{2A} receptor inactivation, as determined by saturation isotherm assays conducted using the radiolabeled antagonist, [^{3}H]ketanserin. The data show that pretreatment with PBZ inactivates 5-HT_{2A} receptors without altering the affinity of the remaining receptors because the K_d values were virtually unchanged, whereas the B_{max} values were decreased in comparison to control cells (Table 2). Specifically, 5-HT_{2A} receptor expression was reduced from 5500 fmol/mg in control cells to 2700 fmol/mg (Table 2) following

TABLE 2

The effect of PBZ on 5-HT_{2A} receptor inactivation, AA release, and IP accumulation

NIH3T3–5HT_{2A} cells were preincubated with PBZ (10 nM–100 μM) for 15 min. For saturation isotherm studies, the cells were harvested and saturation assays were conducted using [^{3}H]ketanserin (0.125 nM–10 nM). For functional studies, following the preincubation with PBZ, cells were incubated with 5-HT (10 μM) for 30 min. Different concentrations of PBZ were used for the two signaling pathways to generate the dose-response curves presented in Fig. 3. The data represent the mean (S.E.M.) of three independent experiments.

PBZ Concentration	[^{3}H]Ketanserin Saturation Isotherm			5-HT (10 μM)	
	K_d	B_{max}	% Inactivated	PLA_2-AA Release	PLC-IP Accumulation
μM	*nM*	*fmol/mg*		*% max over basal*	
0	1.2 (0.30)	5500 (101)		100	100
0.01	0.75 (0.05)	2700 (130)	51 (0.60)	N.D.[a]	65 (5.5)
0.10	N.D.	N.D.	N.D.	N.D.	35
1.0	3.2 (2.7)	390 (96)	93 (1.6)	58 (5.6)	0.90 (0.30)
10.0	N.D.	N.D.	N.D.	25	N.D.
100	N.D.	110 (60)[b]	97 (1.1)[b]	−2.0 (3.7)	N.D.

[a] N.D. = concentration not tested.
[b] Expression of the 5-HT_{2A} receptor following pretreatment with 100 μM PBZ as determined by a single 10 nM concentration of [^{3}H]ketanserin.

15 min of pretreatment with PBZ (10 nM). Furthermore, a higher concentration of PBZ (1.0 μM) resulted in 93% receptor inactivation (390 fmol/mg; Table 2). When the highest concentration (100 μM) of PBZ was used, however, the amount of [^{3}H]ketanserin bound at the lower concentrations was not significantly above background noise. To circumvent this problem, a single concentration of [^{3}H]ketanserin was employed (10 nM) that enabled us to estimate the percentage of 5-HT$_{2A}$ receptors inactivated, but not the K_d value, after treatment with the highest concentration of PBZ. Following exposure to PBZ (100 μM), >97% of the 5-HT$_{2A}$ receptors were inactivated, when compared with control cells (Table 2).

Subsequent experiments were conducted to determine the effect of receptor inactivation on 5-HT-mediated PLA$_2$-AA and PLC-IP accumulation. At a concentration of PBZ (1.0 μM) that produced 93% receptor inactivation, 5-HT-mediated PLC-IP accumulation was reduced to near-basal levels, whereas PLA$_2$-AA release was only partially decreased (42% decrease; Fig. 3; Table 2). When the concentration of PBZ was increased to 10 μM, PLA$_2$-AA release was inhibited only by ca. 70% (Fig. 3). It was not until the concentration of PBZ was raised to 100 μM that 5-HT$_{2A}$-stimulated PLA$_2$-AA release was finally reduced to basal levels (Fig. 3).

The capacity of 5-HT$_{2A}$ Ligands to Show Agonist-Directed Trafficking. Having shown that the 5-HT$_{2A}$ receptor couples to the PLC and PLA$_2$ signaling pathways with what appears to be different efficiencies, subsequent experiments were performed to explore the capacity of 5-HT$_{2A}$ ligands to route agonist-directed trafficking. That is, can structurally distinct ligands preferentially activate PLA$_2$-AA release instead of PLC-IP accumulation, or vice versa? To explore this hypothesis, functional studies were conducted to include two properties of a drug necessary to characterize a physiological response: potency and intrinsic activity. Even though the measurements of agonist-induced [^{3}H]IP accumulation and [^{3}H]AA release were determined from two separate assay plates, the assays were conducted side-by-side in cells seeded from the same cell population and, most importantly, without any experimental interventions that would eliminate one signaling pathway, consistent with the designation of an intact system as proposed by Leff et al. (1997). This point is important because under these conditions, all receptor equilibria will be functioning such that enrichment of one signaling pathway by stabilizing one receptor state has consequences for the other signaling pathway, even though [^{3}H]AA release and [^{3}H]IP accumulation are not being measured simultaneously from the same cell.

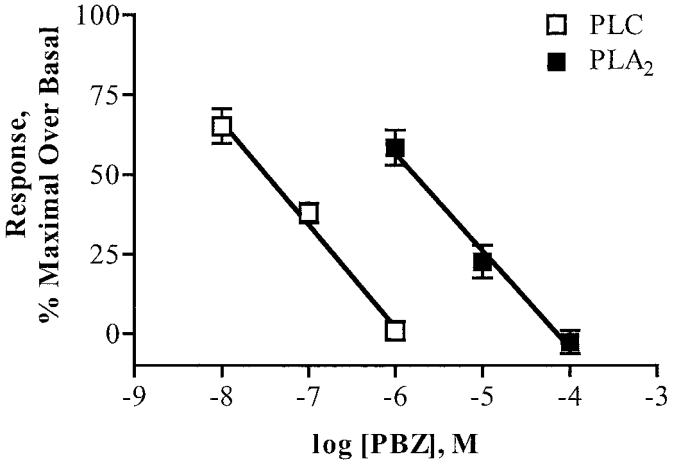

Fig. 3. The effect of phenoxybenzamine pretreatment on 5-HT-induced (□) PLC-IP accumulation and (■) PLA$_2$-AA release. NIH3T3–5HT$_{2A}$ cells were preincubated with PBZ (10 nM–100 μM) for 15 min prior to exposure to 5-HT (10 μM) for 30 min. The data represent the mean ± S.E.M. from three independent experiments.

To determine the effect of distinct structural motifs on ligand-directed pathway activation, we used a series of agonists from the tryptamine, phenethylamine, and ergoline families with known partial agonist activities in naturally expressed 5-HT$_{2A}$ receptors or heterologous cell lines (Sanders-Bush et al., 1988; Zifa and Fillion, 1992; Chambers et al., 2001; D. Kurrasch-Orbaugh and D. E. Nichols, unpublished results; Fig. 4). The capacity of these agonists to elicit both PLA$_2$-AA release and PLC-IP accumulation was determined side-by-side.

Because our receptor reserve studies had demonstrated the existence of a larger reserve at the receptor-coupled PLA$_2$ signaling pathway than at the receptor-coupled PLC signaling pathway, the efficacy of each agonist for activation of the PLC and the PLA$_2$ signaling pathways was determined by measuring both the potency and intrinsic activity at each pathway. If the potency and intrinsic activity of each test compound is taken into account, then four possible scenarios exist for an agonist acting at a single receptor to stimulate two independent signaling pathways. In the first example, the endogenous ligand, 5-HT, had virtually no difference in potency (AA · EC$_{50}$ = 83 ± 7.2 nM; IP · EC$_{50}$ = 120 ± 6.9 nM) for either signaling pathway (Fig. 5A). Serotonin served as the reference compound for all other agonist ligands, so that by definition the maximal 5-HT stimulation of each signaling pathway was considered to be 100%. Thus, the intrinsic activity for both PLC and PLA$_2$ activation was also equal.

In the second illustration, exemplified by tryptamine, no difference in potency for either signaling pathway was observed. Nevertheless, tryptamine functions as a full agonist (intrinsic activity = 91 ± 4.2%) at the PLC signaling pathway, whereas it is only a weak partial agonist for PLA$_2$ activation (intrinsic activity = 41 ± 6.6%; Fig. 5B; Table 3). In contrast, *d*-LSD possesses a slight difference in activation potency (AA · EC$_{50}$ = 20 ± 3.8 nM; IP · EC$_{50}$ = 9.8 ± 3.7 nM) but, interestingly, displays a 2.5-fold increase in intrinsic activity toward PLA$_2$ activation (AA · intrinsic activity = 56 ± 9.4%; IP · intrinsic activity = 22 ± 2.6%; Fig. 5C; Table 3). Finally, in the fourth example, as shown by psilocin, a difference in potency (AA · EC$_{50}$ = 86 ± 3.9 nM; IP · EC$_{50}$ = 2300 ± 289 nM) was observed, but there was no change in intrinsic activity (Fig. 5D; Table 3).

In an attempt to identify trends between structurally similar agonists, additional ligands from the ergoline, phenethylamine, and tryptamine classes of 5-HT$_{2A}$ receptor agonists were employed (Fig. 4). Some interesting patterns can be observed. The first observation is the lack of a generalization between ligand class and differential activation of either PLC or PLA$_2$ (Table 3). Within the ergoline class, for example, *d*-LSD shows increased potency in PLC activation but increased intrinsic activity toward PLA$_2$, whereas lisuride has increased potency and intrinsic activity for PLA$_2$.

Similarly, within the tryptamine class of ligands, psilocin displays higher potency toward PLA$_2$ but has virtually identical intrinsic activity in both pathways. Although 5-methoxy-*N*,*N*-dimethyltryptamine has increased potency for

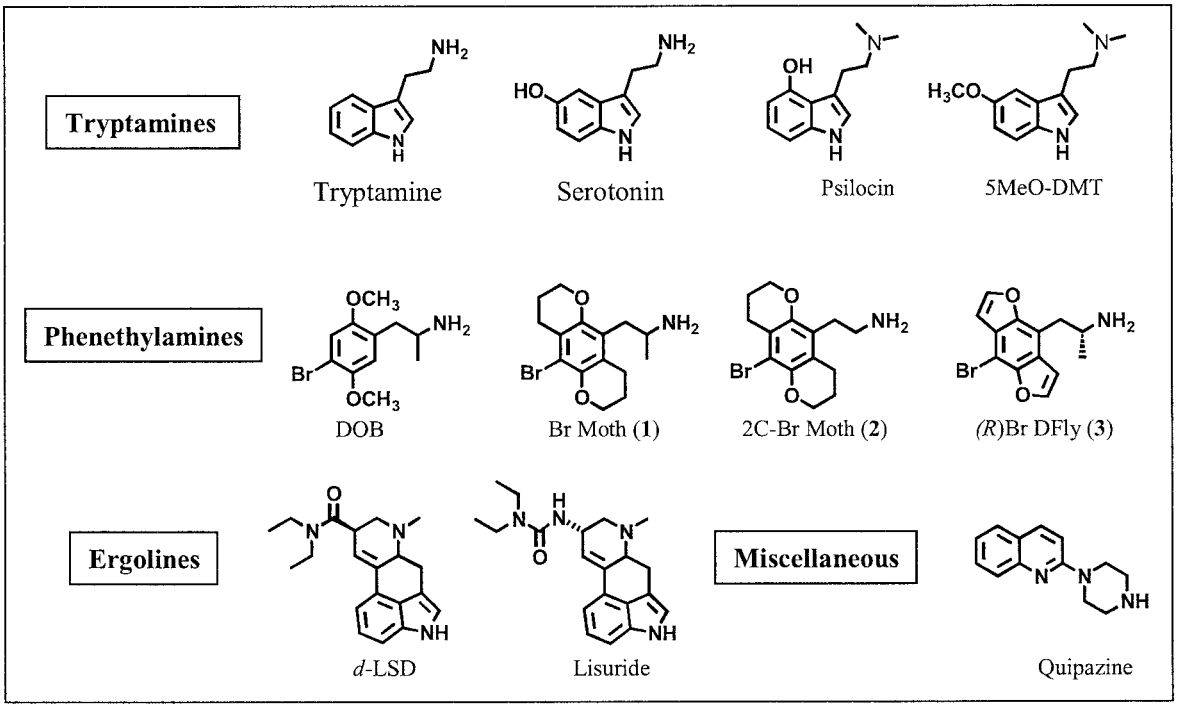

Fig. 4. The chemical structures of 5-HT_{2A} receptor agonists employed in this study. Structurally distinct agonists were selected from the tryptamine, phenethylamine, and ergoline families of 5-HT_{2A} receptor ligands.

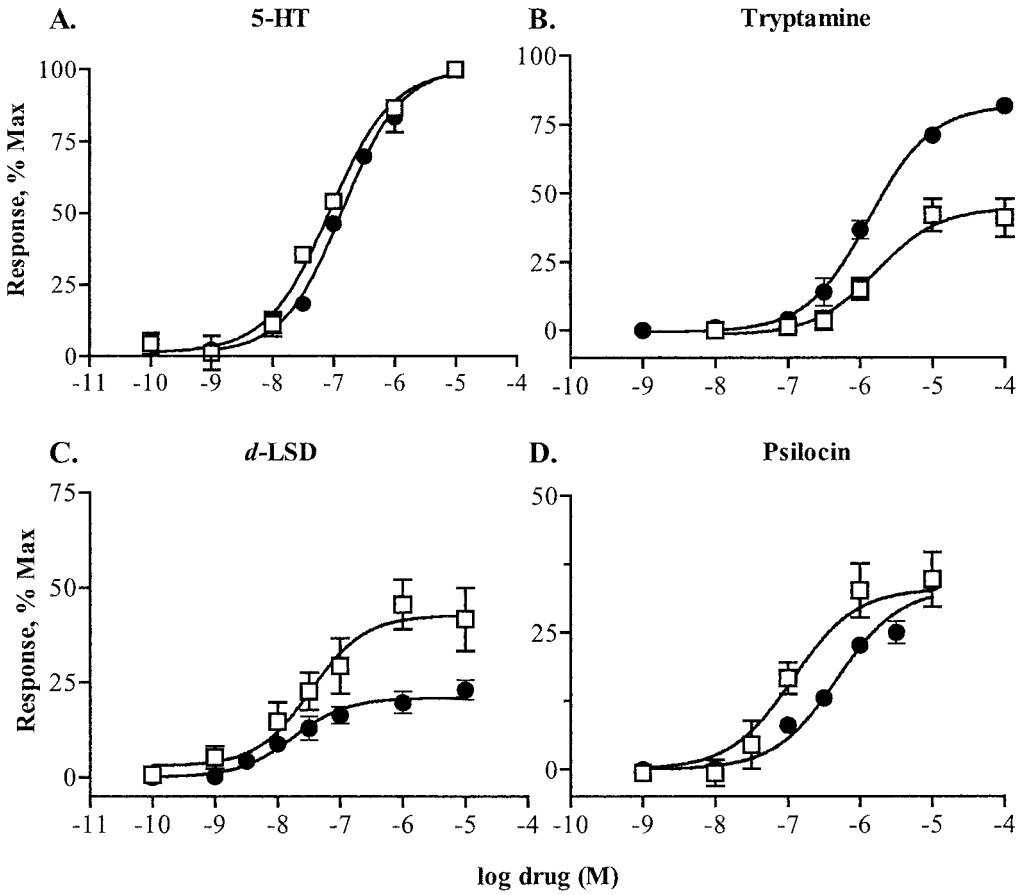

Fig. 5. The demonstration of agonist-directed trafficking by 5-HT_{2A} receptor agonists. NIH3T3–5-HT_{2A} cells were incubated with increasing concentrations of agonists (0.10 nM–10 μM) for 30 min to determine the dose-response effects on PLA_2-AA release (□) and PLC-IP accumulation (●). Basal values were determined by exposure to d_2H_2O for 30 min, and the maximal response was determined by stimulation with 5-HT (10 μM) for 30 min. The data represent the mean ± S.E.M. from three separate experiments.

TABLE 3

The capacity of 5-HT$_{2A}$ receptor agonists to induce agonist-directed trafficking

NIH3T3–5HT$_{2A}$ cells were exposed to increasing concentrations of agonists (0.10 nM–10 μM) for 30 min to determine the potency and intrinsic activity for PLA$_2$-AA release and PLC-IP accumulation. The maximal response was determined by incubation with 5-HT (10 μM) for 30 min. Radioligand competition binding studies were determined as described under *Materials and Methods* using ^{125}I-2,5-dimethoxy-4-iodoamphetamine ([^{125}I]DOI) (0.20 nM). The data represent the mean ± S.E.M. of three to five separate experiments.

Drug	[^{125}I]DOI K_i	PLA$_2$-AA Release		PLC-IP Accumulation		Relative Potency IP · EC$_{50}$/AA · EC$_{50}$
		EC$_{50}$	Intrinsic Activity	EC$_{50}$	Intrinsic Activity	
	nM	*nM*	*% 5-HT*	*nM*	*% 5-HT*	
5-HT	21 (2.8)	83 (7.2)	100	120 (6.9)	100	1.4
d-LSD	3.5 (0.62)	20 (3.8)	56 (9.4)	9.8 (3.7)	22 (2.6)	0.49
Quipazine	31 (1.0)[a]	850 (140)	65 (13)	650 (59)	82 (6.2)	0.76
Tryptamine	49 (10)[a]	1200 (190)	41 (6.6)	1100 (70)	91 (4.2)	0.92
Lisuride	7.2 (0.33)	13 (3.9)	32 (7.9)	41 (15)	13 (3.7)	3.2
1 (Fig. 4)	3.9 (0.95)	35 (8.4)	39 (4.3)	110 (26)	82 (8.1)	3.1
DOB	4.3 (0.28)	15 (4.8)	75 (6.3)	72 (3.6)	79 (6.0)	4.8
3 (Fig. 4)	0.31 (0.04)	0.51 (0.24)	98 (2.2)	2.7 (0.55)	93 (9.9)	5.3
2 (Fig. 4)	1.7 (0.09)	28 (11)	33 (4.6)	250 (47)	45 (3.5)	8.9
5-MeO-DMT[b]	42 (9.9)	190 (31)	70 (10)	2400 (890)	99 (8.5)	13
Psilocin	25 (4.7)	86 (3.9)	42 (5.7)	2300 (290)	46 (2.4)	27

[a] Data taken from Peroutka et al. (1988).
[b] 5-MeO-DMT, 5-methoxy-*N*,*N*-dimethyltryptamine.

PLA$_2$ activation, it possesses a higher intrinsic activity for PLC activation (Table 3), similar to what was observed with tryptamine. In contrast, within the phenethylamine class, compounds 1, 2, 3, and DOB each showed increased potency for PLA$_2$ activation, but there was no consistent trend for intrinsic activity, with some ligands displaying higher intrinsic activity for PLC (i.e., compound 1) but others having no difference (i.e., DOB and compound 3).

With the idea that the receptor-G protein complex, in the absence of agonist, might in part dictate preferential activation of either PLC or PLA$_2$ signaling, re-examination of the data in Table 3 reveals an additional trend. With the exception of *d*-LSD, which shows increased potency toward PLC, and quipazine, tryptamine, and 5-HT, which have similar potencies in both pathways, all other agonists tested displayed 3- to 27-fold higher potency for PLA$_2$ activation. When the relative potency of PLA$_2$ activation versus the relative potency of PLC activation is compared, 7 of the 11 agonists tested show increased potency for PLA$_2$ over PLC (Fig. 6). In contrast, there is no apparent trend when the intrinsic activity values are examined; two compounds have increased intrinsic activity for PLA$_2$, four compounds show preferential activation of PLC, and four other ligands have no difference in intrinsic activity.

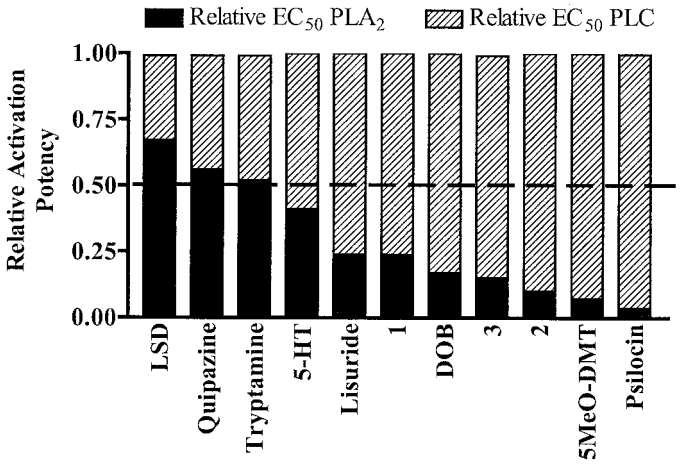

Fig. 6. Preferential activation of the PLA$_2$ pathway by 5-HT$_{2A}$ receptor agonists. The bars represent the relative potency of each agonist for the PLA$_2$ signaling pathways versus the PLC signaling pathway. That is, the smaller the bar area (i.e., lower EC$_{50}$), the greater the potency for activating that signaling pathway. The data represent the AA · EC$_{50}$/(IP · EC$_{50}$ + AA · EC$_{50}$) and vice versa.

Discussion

In the present study, we exploited the ability of the 5-HT$_{2A}$ receptor to activate two intracellular signaling cascades, namely the PLC and PLA$_2$ pathways, to test the hypothesis that the 5-HT$_{2A}$ receptor possesses differing degrees of receptor reserve for these pathways, which affect the capacity of 5-HT$_{2A}$ receptor agonists to activate preferentially the PLC or PLA$_2$ signaling pathways. Initial experiments were conducted to verify that 5-HT$_{2A}$ receptor-mediated PLC and PLA$_2$ activation in NIH3T3 cells was: 1) dose-dependent and 5-HT$_{2A}$ receptor-specific and 2) independently coupled to the receptor. Increasing concentrations of 5-HT led to a dose-dependent increase of both PLA$_2$-AA release and PLC-IP accumulation, which was blocked by pretreatment with ketanserin. Furthermore, by employing inhibitors of various enzymatic steps along the PLC and PLA$_2$ signaling pathways, we were able to show that 5-HT$_{2A}$ receptor-coupled PLA$_2$ activation was independent of, and not subsequent to, receptor-mediated PLC activation in NIH3T3–5HT$_{2A}$ cells. These latter results are consistent with the findings of Berg et al. (1998) who showed that the 5-HT$_{2A}$ receptor stably expressed in Chinese hamster ovary cells can independently activate both the PLC and PLA$_2$ signaling pathways.

Having demonstrated 5-HT$_{2A}$ receptor-mediated PLA$_2$-AA release to be independent of receptor-mediated PLC-IP accumulation in NIH3T3–5HT$_{2A}$ cells, we were then able to study the ability of 5-HT$_{2A}$ receptor ligands to couple preferentially to these signaling pathways. We also wished to explore the possibility that a larger receptor reserve might exist for one signaling pathway over the other. To do so, PBZ was employed to inactivate irreversibly the 5-HT$_{2A}$ receptor. The most striking result from these studies was that a 100-fold larger concentration of PBZ was required to prevent PLA$_2$-AA release than PLC-IP accumulation in response to receptor activation by the endogenous ligand, 5-HT. We conclude from these data that in this system a larger receptor reserve is present for 5-HT$_{2A}$ receptor-coupled PLA$_2$-AA re-

lease than for PLC-IP accumulation. Evidence for this conclusion is based on the fact that 1.0 μM PBZ pretreatment abolished 5-HT-induced IP accumulation, whereas 5-HT-induced AA release was only partially inhibited. This concentration of PBZ inactivated 93% of the receptors, indicating that at least 7% of the receptor population must be available for measurable PLC stimulation to occur. Presumably, fewer receptors are necessary for PLA_2 stimulation because it required inactivation of 97% of the receptors before PLA_2-AA release was abolished. Although a difference of only 4% receptor occupancy may seem a narrow range to conclude that a higher receptor reserve exists for PLA_2-AA release, Nickerson (1956) demonstrated that only 1% occupancy of the histamine receptor population in guinea pig ileum was required to elicit a maximal response, suggesting that occupancy of a very small percentage of the total receptor population may be sufficient to achieve a response.

Similarly, when we used ketanserin to block 5-HT_{2A} receptor-mediated signaling, initial studies showed that 10 μM ketanserin was sufficient to inhibit completely 10 μM 5-HT-mediated PLC activation, whereas only a partial inhibition of 5-HT-mediated PLA_2 activation occurred (61 $\pm$ 5.7% inhibition; data not shown). Based on the K_i values of ketanserin and 5-HT, these concentrations can be estimated to result in about 0.3% receptor occupancy by 5-HT. It was not until a 10-fold larger concentration of ketanserin was utilized (ca. 0.03% receptor occupancy by 5-HT) that 5-HT-induced AA release was blocked completely. These results support our conclusion that a larger receptor reserve exists for 5-HT_{2A} receptor-mediated AA release than for 5-HT_{2A} receptor-mediated IP accumulation.

Many of the ligands we examined were 5-HT_{2A} receptor partial agonists. The observation that some of them preferentially activated the PLA_2 signaling pathway, with some having 10-fold differences in potency, is also consistent with the hypothesis that a larger receptor reserve exists for 5-HT_{2A} receptor-mediated PLA_2 activation than for 5-HT-mediated PLC activation. We tested both hallucinogenic and nonhallucinogenic compounds, because the possibility existed that preferential activation of PLA_2 over PLC, or vice versa, might correlate with the biological activity of these agonists. We did not, however, observe differential activation of either PLC or PLA_2 by the two types of compounds, suggesting that psychotropic versus nonpsychotropic agonists, as a group, do not differ in their ability to activate selectively 5-HT_{2A} receptor-mediated PLA_2 or PLC signaling. Whether or not the generally greater potency of most ligands for activating the PLA_2 pathway is relevant to the action of hallucinogenic drugs remains to be investigated.

Taken together, these results are not compatible with the hypothesis that structurally similar compounds might stabilize a particular conformation of the receptor such that one 5-HT_{2A} receptor-mediated PLC or PLA_2 pathway might be differentially enhanced. Instead, these data suggest either that the 5-HT_{2A} receptor can exist in R^{n*} conformations, one complementary for each ligand that is independent of any structural similarities, or perhaps that intracellular signaling is controlled to a certain extent by receptor-G protein coupling, independent of the agonist. Thus, the ability of the majority of the agonists to preferentially activate PLA_2, as defined by increased potency, leads us to speculate that the 5-HT_{2A} receptor generally couples more efficiently to the PLA_2 signaling pathway, independent of the structural characteristics of the ligand.

Although several findings have been published that provide experimental support for the hypothesis of agonist-directed trafficking (see references in introduction), the study most relevant to the data presented here was that conducted by Berg et al. (1998). Using Chinese hamster ovary-K1 cells stably expressing the 5-HT_{2A} and 5-HT_{2C} receptors, the relative efficacies (in the absence of receptor reserve, intrinsic activity = relative efficacy) of a series of 5-$HT_{2A/2C}$ agonists were measured for PLA_2-AA release and PLC-IP accumulation. Because their ligands were found to have differing relative efficacies for the two signaling pathways without any difference in potencies, their data were consistent not only with the hypothesis of agonist-directed trafficking, but also with the predictions of the three-state receptor model proposed by Leff et al. (1997).

The data presented here are also consistent with the concept of agonist-directed trafficking, because various 5-HT_{2A} receptor agonists possessed the capacity to activate differentially the PLC and PLA_2 signaling pathways. Nevertheless, our data are not consistent with the predictions of a discrete intact three-state system as proposed by Leff et al. (1997) because many 5-HT_{2A} receptor agonists displayed a difference in potency, in addition to the difference in intrinsic activity, for the PLC and PLA_2 signaling pathways. In an attempt to simplify the three-state model to determine how events at the receptor level can explain agonist pharmacology, Leff et al. (1997) ignored postreceptor coupling and the possibility of receptor reserve. The data presented here, however, suggest that postreceptor coupling and/or receptor reserve may play an integral role in the ability of agonists to activate differentially multiple signaling cascades. For example, the 5-HT_{2A} receptor presumably couples to these signaling cascades by activation of intracellular G proteins, although recent studies have demonstrated that GPCR-mediated signal transduction can also be G protein-independent (Hall et al., 1998). Nonetheless, if 5-HT_{2A} receptor-mediated PLC activation is $G\alpha_q$-coupled, whereas 5-HT_{2A} receptor-mediated PLA_2 activation is assumed to be $G\alpha_x$-coupled, then different ternary complexes might dictate the ability of structurally distinct 5-HT_{2A} receptor agonists to activate preferentially the PLC and PLA_2 signaling cascades.

Consistent with this reasoning, a recently published study reported that when NIH3T3 cells stably expressing the α_2-adrenergic receptor were transiently transfected with $G\alpha_{o1}$, the partial agonist activity of clonidine and oxymetazoline was shifted to full agonist (Yang and Lanier, 1999). The authors concluded that although these compounds were equally able to stabilize the α_2-$G\alpha_{i2,3}$ versus α_2-$G\alpha_{o1}$ complex, G protein coupling affected the subsequent step of intracellular pathway activation. Taken together, in light of the data presented here, it can be hypothesized that receptor-G protein coupling, at least in part, drives the existence of agonist-specific receptor states instead of simply being subsequent to agonist binding. If the intracellular G protein partially defines the overall conformation of the ternary complex with respect to agonist-directed trafficking, then postreceptor coupling should be included in agonist-directed effector activation models.

In the presence of receptor reserve, according to conventional interpretations, partial agonists will show increased intrinsic activity because they will be able to bind the spare receptors to produce an increased response. Consequently, partial agonist activity of a ligand within a given system has been utilized as a crude prediction of the existence of receptor

reserve. If this interpretation is correct, then the finding that a larger receptor reserve exists for PLA_2 than for PLC is in direct conflict with the observation that some 5-HT_{2A} receptor ligands possess increased intrinsic activity in the PLC signaling pathway versus the PLA_2 pathway. To explain those data, it can be speculated that some ligands, regardless of their extent of receptor occupancy, never behave as full agonists.

To shed some insight into this idea, several recent papers have examined the ability of weak to full β_2-adrenergic receptor agonists to promote two different steps of the G protein activation/deactivation cycle that would ultimately affect full agonist activity: stabilization of the ternary complex and the steady-state GTPase activity (Seifert et al., 2001; Ghanouni et al., 2001a,b). Their results suggest that, in contrast to full agonists that stabilize the receptor state that promotes GDP release/GTP binding, partial agonists stabilize the ternary complex, thereby resulting in reduced G protein turnover and decreased intrinsic activity. If these findings are substantiated, they would offer a mechanism to explain partial agonist activity whereby intrinsic activity need not be proportional to receptor occupancy. In particular, this potential mechanism of partial agonist activity may help to explain the unexpected data presented here, where many of the ligands employed had partial agonist activity for PLA_2-AA release even in the presence of a large receptor reserve.

In summary, the data presented in this study are consistent with the hypothesis of agonist-directed trafficking, but not the three-state receptor model for agonist action. In particular, irreversibly inactivating the 5-HT_{2A} receptor with 1.0 μM and 100 μM PBZ abolished PLC-IP accumulation and PLA_2-AA release, respectively. The larger PBZ concentration required to block 5-HT-mediated activation of PLA_2 suggests the existence of a larger receptor reserve for the PLA_2 signaling pathway than for the PLC pathway. In addition, and also consistent with the hypothesis of agonist-directed trafficking, we showed that a diverse series of 5-HT_{2A} receptor agonists displayed differential activation of the PLC and PLA_2 signaling pathways. Taken together, these data suggest that the 5-HT_{2A} receptor can differentially regulate the PLA_2 and PLC signaling pathways in NIH3T3–5HT_{2A} cells, and point to the importance of G protein coupling in agonist-directed trafficking. Finally, the differential potencies of the efficacious hallucinogens DOB, 5-methoxy-*N,N*-dimethyltryptamine, and psilocin for activating the PLA_2 pathway (Table 3) also suggest that this signaling pathway may be relevant to the psychopharmacology of these substances.

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