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Species Variations in Transmembrane Region V of the 5-Hydroxytryptamine Type 2A Receptor Alter the Structure-Activity Relationship of Certain Ergolines and Tryptamines

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SUMMARY

Previous work has suggested that species differences in the structure-activity relationship for ergolines and tryptamines at the 5-hydroxytryptamine (5-HT)$_{2A}$ (formerly known as the 5-HT$_2$) receptor are related to aliphatic substitution at the N1-position on the indole nucleus. The present work has confirmed these findings by examining the rat and human cloned 5-HT$_{2A}$ receptors. As previously found, N1-substitution of ergolines or tryptamines had no effect or increased affinity for the rat 5-HT$_{2A}$ receptor but decreased affinity for the human receptor. Also, the N1-unsubstituted analogues had higher affinity for the human 5-HT$_{2A}$ receptor, whereas the N1-alkyl analogues had a higher affinity for the rat receptor. By mutating the rat 5-HT$_{2A}$ receptor, the importance of the Ala/Ser^{242} species variation in amino acid sequence was examined in relation to this structure-activity relationship. Three mutations of the rat 5-HT$_{2A}$ receptor were made, i.e., A242S, A242V, and A242T. All three mutations resulted in functional (able to stimulate inositol phosphate hydrolysis) 5-HT$_{2A}$ receptors with high affinity for [^{3}H]ketanserin and 1-(2,5-dimethoxy-4-[^{125}I]iodophenyl)isopropylamine. The A242S mutation resulted in a pharmacological profile that was almost identical to that of the human 5-HT$_{2A}$ receptor but differed significantly from that of the wild-type rat receptor. This strongly suggests that the Ala/Ser^{242} species variation accounts for the differences in the structure-activity relationship. The A242V and A242T mutations resulted in differing but profound effects on affinity for the different ergolines and tryptamines. The results are discussed in terms of the importance of position 242 in the binding of these ligands to 5-HT$_{2A}$ receptors. In addition, arguments are presented that suggest that a hydrogen-bonding interaction occurs between the human 5-HT$_{2A}$ receptor at Ser^{242} and the N1-hydrogen of N1-unsubstituted ergolines and tryptamines and may serve as an important contact point in the receptor.

A number of compounds have shown differences in their affinity for the 5-HT$_{2A}$ receptor, depending upon the species examined. For instance, mesulergine (see Fig. 1) and methysergide have been found to displace [^{3}H]ketanserin, a 5-HT$_{2A}$ antagonist, with substantially lower affinity in pig or human cortex homogenates than in rat homogenates. Similarly, [^{3}H] mesulergine effectively labels the rat 5-HT$_{2A}$ receptor but not the pig or human receptor (1). Recent work has suggested that these species differences are related to substitution at the N1-position of ergolines, such as in mesulergine. Nelson *et al.* (2) found that N1-unsubstituted ergolines such as ergonovine, LY86057, LY193525, LY254239, and LY225297 displaced [^{3}H] ketanserin from the pig, squirrel monkey, and human 5-HT$_{2A}$ receptors with higher affinity than that for the rat receptor. In contrast, N1-substituted analogues (mesulergine, LY108742, LY53857, amesergide, and sergolexol) showed higher affinity for the rat antagonist-labeled 5-HT$_{2A}$ receptor than for the pig, squirrel monkey, or human receptors.

That work (2) went on to show that this was true of other indole-containing 5-HT$_{2A}$ ligands, namely the tryptamines. Tryptamine, 5-HT, and 5-MeOT all displaced [^{3}H]ketanserin from the human receptor with a higher affinity than that seen in rat cortical homogenates. As with the ergolines, the N1-isopropyl analogues of 5-HT and 5-MeOT showed significantly higher affinity for the rat versus human antagonist-labeled 5-HT$_{2A}$ receptor. Interestingly, when the SAR is closely examined by comparing compounds that differ only in their substitution at the N1-position, the SAR is seen to change dramatically between species. Specifically, N1-substitution of ergolines and tryptamines resulted in either an increase in affinity (seen with all of the ergolines and tryptamine) or no change in affinity (seen with 5-MeOT) of [^{3}H]ketanserin-labeled 5-HT$_{2A}$ receptors in the rat. In contrast, N1-substitution produced a clear decrease in affinity for both the ergolines and the tryptamines with the human 5-HT$_{2A}$ receptor. When the 5-HT$_{2A}$ agonist [^{125}I]DOI was used to label the rat and squirrel monkey 5-HT$_{2A}$ receptors, results similar to those seen with [^{3}H]ketanserin-labeled receptors were found with the ergoline 5-HT$_{2A}$ antagonists and the tryptamine agonists (3).

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); DOI, 1-(2,5-dimethoxy-4-iodophenyl)isopropylamine; 5-MeOT, 5-methoxytryptamine; SAR, structure-activity relationship; TM, transmembrane region; PI, phosphatidylinositol; ANOVA, analysis of variance.

The identification of such a well defined SAR that is dramatically altered between species provides a unique opportunity to attempt to identify which differences in the rat and human 5-HT_{2A} receptors account for these results. By using molecular biology techniques, it has been found that there are only three TM changes between the rat and human 5-HT_{2A} receptors (4–11). Given that the TMs are thought to form a pocket within which the ligands bind (for review, see Ref. 12), it may be possible to identify which amino acid change results in the observed changes in the SAR.

The pharmacological data suggest that a specific type of amino acid variation with species would account for these effects. Given the SAR data, the most likely changes in amino acid sequence in the human receptor, compared with the rat receptor, are a potential increase in the size of the amino acid and/or the addition of a hydrogen-bonding acceptor to the human 5-HT_{2A} receptor. Of the three TM changes found, only one meets these criteria. The amino acid substitution within TM V (Ala^{242} in the rat and Ser^{242} in the human) results in both an increase in size and the addition of a hydrogen-bonding acceptor. It is interesting to note that alignment of several of the monoamine G protein-coupled receptors indicates that this position is a serine in all of the catecholamine receptors and it has been implicated in the binding of dopamine and norepinephrine to their receptors (13, 14). In fact, with several of the catechol receptors the serine that corresponds to position 242 (in the 5-HT_{2A} receptor) has been implicated in the binding of the catechol hydroxy groups (15–17). In addition, a recent report (18) has suggested that the species differences in affinity for psilocin (4-hydroxy-*N*,*N*-dimethyltryptamine) are due to this amino acid substitution. Within TM V the change from an alanine in the rat 5-HT_{2A} receptor to a serine in the human 5-HT_{2A} receptor may easily explain all of the pharmacological differences between species seen for these ergolines and tryptamines. Specifically, the N1-unsubstituted analogue could form a hydrogen bond between the N1-hydrogen and the oxygen of the Ser^{242} in the human receptor, thereby increasing its affinity, but could not form a hydrogen bond with the rat receptor (Ala^{242}). Similarly, with an N1-alkyl analogue an unfavorable steric interaction may occur between the human receptor Ser^{242} and the N1-alkyl group, decreasing affinity, compared with the rat receptor.

Additional support for this hypothesis comes from a mutational study described by Kao *et al.* (19). Previous work illustrated that [^{3}H]mesulergine had a lower affinity for the human receptor than the rat 5-HT_{2A} receptor (1). Kao *et al.* (19) found that mutating only the Ser^{242} of the human 5-HT_{2A} receptor to an alanine resulted in an affinity for [^{3}H]mesulergine that closely resembled that of the rat 5-HT_{2A} receptor. The affinity for [^{3}H]ketanserin was unaffected by this mutation. Interestingly, mesulergine is an N1-methylergoline that fits well with the previously described SAR differences between species. However, no N1-unsubstituted ergolines that have previously been found to show species differences were examined with this

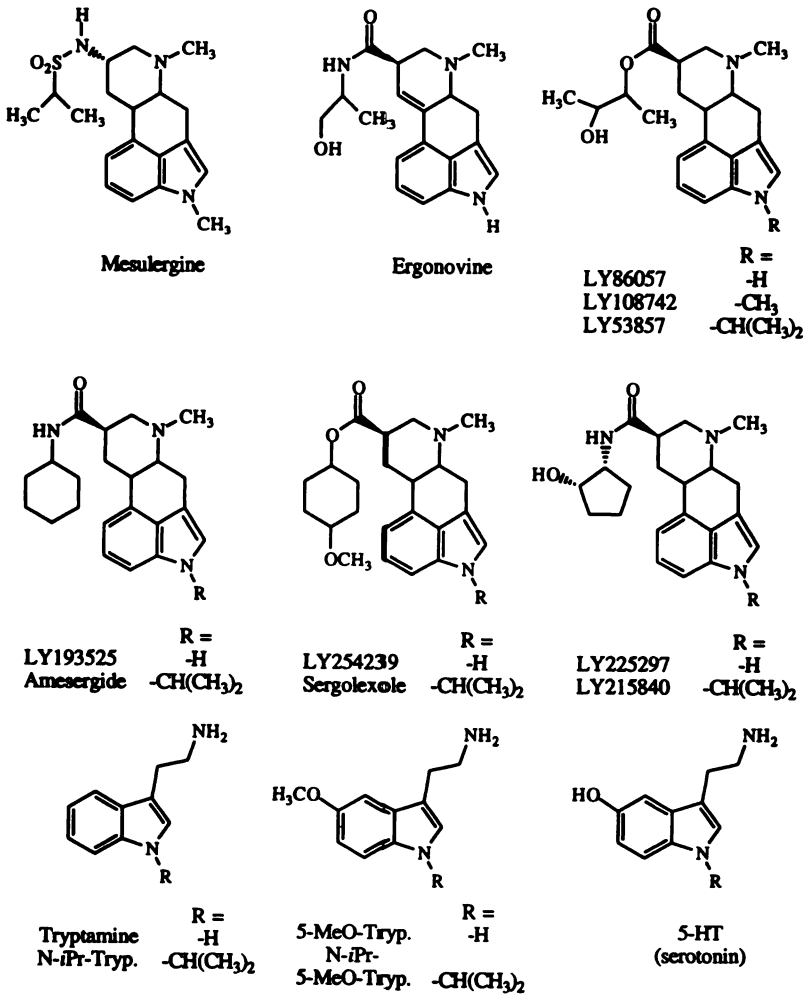

Fig. 1. Structures of the N1-substituted and -unsubstituted ergolines and tryptamines. *N-iPr-Tryp.*, *N*-isopropyltryptamine; *5-MeO-Tryp.*, 5-MeOT; *N-iPr-5-MeO-Tryp.*, *N*-isopropyl-5-MeOT.

mutant of the human 5-HT$_{2A}$ receptor. Furthermore, the effect of the agonist-labeled 5-HT$_{2A}$ receptor was not examined, nor was the affinity for N1-substituted and -unsubstituted tryptamines assessed. Therefore, the present study was undertaken to examine more closely the importance of position 242 in the 5-HT$_{2A}$ receptor, in relationship to the species variability in affinity for certain indole-containing ligands. In addition, attempts were made to characterize the type of interaction involved by systematically altering both the substitution on the ligand and the amino acid substitutions of the receptor at position 242.

Experimental Procedures

Materials. [^{125}I]DOI, [^{3}H]ketanserin, and *myo*-[^{3}H]inositol were obtained from New England Nuclear (Boston, MA), at specific activities of 2200, 61, and 24.6 Ci/mmol, respectively. Ergonovine maleate, 5-HT (serotonin) creatine sulfate, and pargyline HCl were purchased from Sigma Chemical Co. (St. Louis, MO). Ketanserin HCl, spiperone HCl, DOI HCl, 5-MeOT HCl, tryptamine HCl, prazosin HCl, and mianserin HCl were purchased from Research Biochemicals Inc. (Natrick, MA). Mesulergine HCl was kindly donated by Sandoz (Basel, Switzerland). All other compounds were synthesized at Lilly Research Laboratories.

Mutagenesis. A cDNA coding for the rat 5-HT$_{2A}$ receptor (6) was kindly provided by Dr. L. Yu (Indiana University-Purdue University of Indianapolis, Indianapolis, IN). A cDNA encoding the human 5-HT$_{2A}$ receptor (4, 19) was kindly provided by Dr. R. L. Wienshank and co-workers (Synaptic Pharmaceutical Corp., Paramus, NJ). Mutations of the rat 5-HT$_{2A}$ receptor were accomplished utilizing the Altered Sites mutagenesis kit (Promega, Madison, WI), as described in the protocol provided. All mutations were confirmed by sequencing. The mutated rat receptors, as well as the human 5-HT$_{2A}$ receptor, were subcloned into the same expression vector (phd) (20) used to express the wild-type rat 5-HT$_{2A}$ receptor. The vectors containing the cDNA fragments were then stably transfected into the AV12 cell line (CRL 9595) using $CaPO_4$ precipitation (Strategene, La Jolla, CA) and 1 μM methyltrexate for selection. After screening for expression of recombinant proteins, a single-cell population was expanded and adapted to growth in suspension.

[^{3}H]Ketanserin and [^{125}I]DOI binding assays. Cells were harvested by centrifugation at 1000 × *g* for 10 min at 4°. The cells were then gently resuspended in 50 mM Tris·HCl, pH 7.4, frozen in a methanol/dry ice bath, and stored at −70° for up to 3 months. On the day of the experiment aliquots were thawed and centrifuged at 19,800 × *g* for 10 min at 4°. The pellet was washed with Tris buffer and recentrifuged as before. The final pellet was resuspended and homogenized with a Tissuemizer (Tekmar, Cincinnati, OH) (2 × 30 sec), in 67 mM Tris·HCl, pH 7.6, for [^{3}H]ketanserin binding or in 67 mM Tris·HCl, pH 7.4, 0.67 mM EDTA, 13 mM $MgCl_2$, for [^{125}I]DOI binding. Saturation experiments with [^{3}H]ketanserin were carried out with 0.1–10 nM radioligand, with specific binding being defined as that displaceable by 3 μM mianserin. Displacement experiments were carried out with 0.5 nM [^{3}H]ketanserin or 0.2 nM [^{125}I]DOI. Test drugs were dissolved and diluted in water before addition to assay tubes. The final buffer (0.8-ml final volume) composition was 50 mM Tris·HCl, pH 7.6, 100 nM prazosin, for [^{3}H]ketanserin binding, or 50 mM Tris·HCl, pH 7.4, 0.5 mM EDTA, 10 mM $MgCl_2$, 0.12% ascorbate, 10 μM pargyline, for [^{125}I]DOI binding. Incubations at 37° were commenced with the addition of homogenates to the assay tubes and were allowed to equilibrate for 15 or 20 min in [^{3}H]ketanserin or [^{125}I]DOI assays, respectively. Termination of incubations was by filtration through GF/B or GF/C filters (presoaked with 0.5% polyethylenimine), using a Brandel cell harvestor (Brandel, Gaithersburg, MD). Assay tubes and filters were immediately washed three times with ice-cold 50 mM Tris·HCl, pH 7.4. The amount of protein per assay tube was approximately 100 μg, as determined in a modified Bradford protein assay (21).

Hydrolysis of inositol phosphates. The assay described by Kursar *et al.* (20) was used with minor modifications. Two days before each experiment *myo*-[^{3}H]inositol (2 μCi/ml of medium) was added and the cells were allowed to grow as normal. Cells were then harvested by centrifugation at 300 × *g* for 7 min at 4°. Cells were gently resuspended in protein-free medium with 10 mM $LiCl_2$ and 10 mM *myo*-inositol. The centrifugation was then repeated once and the cells were resuspended at approximately 20 × 10^{6} cells/ml. Test drugs were diluted in water before addition to assay tubes. Curves were generated from concentrations of drugs spanning 7 log units. Assays were commenced with the addition of labeled cells (corresponding to approximately 100 μg of protein) and were allowed to incubate at 37° for 30 min. Incubations were terminated by centrifugation at 3000 × *g* for 10 min. Pellets were resuspended by repeated pipetting after the addition of 150 μl of water. Methanol/acetone (1:1, 650 μl) was added and each tube was sealed and vortexed for 10 min before the samples were loaded onto prepared columns.

Columns (Accell Plus QMA anion exchange cartridge columns; Waters, Marlborough, MA) were prepared by washing with 10 ml of 1 M ammonium formate/0.1 M formic acid, with a vacuum manifold, followed by washes with 2 × 10 ml of water. Samples were then added to 5 ml of water already loaded on the column and the tubes were washed with 0.5 ml of methanol/acetone. Samples were drawn through the columns under vacuum and the columns were washed with 10 ml of 5.0 mM sodium tetraborate. [^{3}H]Inositol phosphates were eluted from the columns into 20-ml scintillation vials with 3.5 ml of 0.1 M ammonium formate, 10 mM formic acid, 50 mM sodium tetraborate. Scintillation cocktail was added and the vials were counted for ^{3}H.

Statistics. Displacement of either [^{125}I]DOI or [^{3}H]ketanserin was examined at 12 different test drug concentrations spanning 5–6 log units. The ability to stimulate inositol phosphate hydroysis was examined with seven concentrations of agonist spanning 7 log units. The IC_{50}, pseudo-Hill coefficient, EC_{50}, and percentage of 5-HT maximum were estimated using a computer-assisted nonlinear least squares analysis method. The K_d value for [^{3}H]ketanserin was determined from the saturation isotherm curve. The K_d value for [^{125}I]DOI was estimated from the IC_{50} value for displacement by DOI from [^{125}I]DOI-labeled sites using a rearrangement of the Cheng and Prusoff (22) equation, giving $K_d = IC_{50} - [L]$. Inhibition constants (K_i) were estimated using the Cheng and Prusoff equation for K_i values. The means ± standard errors for three or four separate experiments, with each point assayed in triplicate, are presented. Values were converted to pK_i (−log K_i) values, to normalize values, before ANOVA followed by Tukey's *post hoc* comparison was carried out. Partial *F* tests were used to determine whether a single- or two-site model more accurately described the displacement curves.

Results

Competition for [^{125}I]DOI-labeled cloned rat and human 5-HT$_{2A}$ receptors. The human 5-HT$_{2A}$ receptor, the rat 5-HT$_{2A}$ receptor, and the mutated rat 5-HT$_{2A}$ receptors were all subcloned into the same expression vector (phd) and stably transfected into the same cell line (AV12). Displacement of the agonist [^{125}I]DOI from the cloned receptors showed results similar to those found utilizing native tissue (3). For instance, N1-substitution of the ergolines resulted in no change (LY193525 versus amesergide and LY254239 versus sergolexol) or a slight increase in affinity (LY86057 versus LY108742 or LY53857 and LY225297 versus LY215840) when [^{125}I]DOI was used to label the 5-HT$_{2A}$ receptor either in rat frontal cortex or in a cell line expressing the cloned rat 5-HT$_{2A}$ receptor (Table 1). It should be noted that this is in contrast to the SAR seen when the antagonist [^{3}H]ketanserin was used to label the 5-HT$_{2A}$ receptor, where N1-substitution with these same ergolines showed a consistent increase in affinity. N1-substitution of the tryptamines resulted in no change in affinity whether

TABLE 1

Affinity for the rat and human 5-HT$_{2A}$ receptor clones

Twelve different concentrations spanning 5–6 log units of test drugs were used to displace 0.2 nM [^{125}I]DOI from human or rat 5-HT$_{2A}$ cloned receptors. Tubes were allowed to equilibrate for 20 min at 37° before filtration through GF/C filters that had been presoaked in 0.5% polyethylenimine. Determinations were run in triplicate and K_i values from three or four experiments were averaged to give the mean ± standard error. Other K_i values for displacement of [^{3}H]ketanserin or [^{125}I]DOI are from the sources listed and were included for comparison. Unless otherwise indicated, the pseudo-Hill coefficients and partial F test indicated a single-site displacement.

	N1-Substitution	K_i						
		Rat frontal cortex		Cloned rat receptor, [^{125}I]DOI	Monkey frontal cortex		Cloned human receptor	
		[^{3}H]Ketanserin[a]	[^{125}I]DOI[b]		[^{3}H]Ketanserin[a]	[^{125}I]DOI[b]	[^{3}H]Ketanserin[a]	[^{125}I]DOI
		nM						
Ergonovine	–H	16.5	9.11	3.41 ± 0.65	1.25	1.71	1.22	
Mesulergine	$-CH_3$	2.87	3.26	2.66 ± 0.17	16.5	33.9	18.5	
LY86057	–H	20.6	14.8	15.4 ± 3.1[c]	1.17	1.79	0.82	0.67 ± 0.12[c,d]
LY108742	$-CH_3$	3.38	3.89	3.78 ± 0.07	20.9			
LY53857	$-CH(CH_3)_2$	2.67	4.07	3.20 ± 0.19	12.2	25.2	7.17	23.2 ± 5.4[d]
LY193525	–H	14.7	11.5	4.78 ± 0.07	0.77	0.92	0.73	0.25 ± 0.06[c,d]
Amesergide	$-CH(CH_3)_2$	2.56	5.47	3.35 ± 0.49	9.73	23.8	16.2	10.3 ± 1.9[d]
LY254239	–H	15.4		3.42 ± 0.16			1.14	0.26 ± 0.08[c,d]
Sergolexol	$-CH(CH_3)_2$	4.05		3.57 ± 0.43			23.1	19.5 ± 6.0[d]
LY225297	–H	24.0		6.85 ± 0.96[c]			1.17	0.41 ± 0.07[c,d]
LY215840	$-CH(CH_3)_2$	2.17		3.33 ± 0.62			11.3	12.9 ± 1.3[d]
Tryptamine	–H	4127	150	79.3 ± 8.8		68.1	1213	25.6 ± 4.9[c,d]
N-Isopropyltryptamine	$-CH(CH_3)_2$	1450	99.8	72.7 ± 6.8		606	2838	252 ± 45[d]
5-MeOT	–H	1430	24.1	7.5 ± 1.1		1.24[e]	381	1.31 ± 0.60[c,d,e]
N-Isopropyl-5-MeOT	$-CH(CH_3)_2$	1347	51.3	38.4 ± 1.9		843	2551	478 ± 97[d]
5-HT	–H	996		7.23 ± 1.06			252	4.94 ± 0.71

[a] Values were taken from Ref. 2 and the IC_{50} values were converted to K_i values by the Cheng and Prusoff equation.
[b] Values were taken from Ref. 3.
[c] Significantly different from N1-alkyl-substituted analogue ($p < 0.05$, ANOVA followed by Tukey's comparison).
[d] Significantly different from rat receptor K_i ($p < 0.05$, ANOVA followed by Tukey's comparison).
[e] Values are K_i for the high affinity component of a two-site displacement model.

displacement of [^{125}I]DOI in rat recombinant cell membranes or rat frontal cortex was assayed.

It is also clear from examining the values in Table 1 that labeling the cloned human 5-HT$_{2A}$ receptor with [^{125}I]DOI gave results similar to those obtained with monkey cortex. In contrast to the rat receptor, these results were also very similar to those found when [^{3}H]ketanserin was used to label either the cloned human 5-HT$_{2A}$ receptor or the monkey 5-HT$_{2A}$ receptor in cortical homogenates. In all four cases, N1-substitution was found to significantly decrease affinity (i.e., compare LY86057 with LY108742 or LY53857). Interestingly, this was true for both the ergolines and the tryptamines. One other similarity between the monkey cortex and the cloned human receptor should be mentioned, namely, in either homogenate 5-MeOT displaced [^{125}I]DOI with complex competition curves (Fig. 2; Table 2). The reason for the complex competition curves for only 5-MeOT with the cloned human or monkey receptors and not for 5-MeOT with the cloned or native rat receptors, or for 5-HT and the tryptamines in any of these homogenates, is unclear. However, the displacement of [^{125}I]DOI by 5-MeOT had apparently reached equilibrium, because no change in the displacement curves were seen at >4 times the normal incubation period (data not shown).

It is interesting to note that N1-isopropyl substitution may affect not only the potency of tryptamines but also the efficacy at the human 5-HT$_{2A}$ receptor. Using a standard PI accumulation study (Table 3), it can be seen that N1-isopropyl substitution of 5-MeOT not only increased its EC_{50} value by 150-fold but also decreased the percentage of 5-HT maximum to 61% in cells expressing the human 5-HT$_{2A}$ receptor. Therefore, N1-isopropyl substitution not only decreased affinity for the human 5-HT$_{2A}$ receptor and its potency for stimulation of inositol phosphate hydrolysis but also apparently changed a full agonist (5-MeOT) into a partial agonist.

It should also be noted that the N1-substituted and -unsubstituted ergolines and tryptamines showed significant changes in affinity, when [^{125}I]DOI displacement in homogenates from cell lines expressing the cloned rat receptor was compared with that in the human 5-HT$_{2A}$-expressing cell line. All of the N1-alkyl-substituted ergolines and tryptamines showed a higher affinity for the rat versus the human 5-HT$_{2A}$ receptor. In contrast, the N1-unsubstituted ergolines and tryptamines (with the exception of 5-HT) showed significantly higher affinity for the human 5-HT$_{2A}$ receptor. This is in excellent agreement with previous work using either [^{125}I]DOI or [^{3}H]ketanserin to label the rat, monkey, and human 5-HT$_{2A}$ receptors (2, 3).

[^{125}I]DOI binding, [^{3}H]ketanserin binding, and PI turnover with the mutated rat 5-HT$_{2A}$ receptors. All three of the mutants (A242S, A242T, and A242V) were found to retain both the ability to bind radioligands and the ability to stimulate PI hydrolysis. With one exception, the affinity for [^{3}H]ketanserin and [^{125}I]DOI was unaffected by mutation of the rat receptor (Table 4). The one mutation having significant affect on radioligand affinity was the Val^{242} substitution, which caused a slight increase in affinity for [^{3}H]ketanserin. However, the affinity for [^{125}I]DOI was unaffected by this mutation. The ability of DOI to stimulate PI turnover was also unaffected by any of the three mutations. DOI is known to be a partial agonist in native rat tissue (23, 24); however, full agonist activity was seen with the rat, human, A242S, and A242V clones. A value of 81% of 5-HT maximum was seen for DOI with the A242T mutant. This probably relates to the relative amounts of cloned or mutated receptor expressed for any given experiment. For instance, Wainscott *et al.* (25) previously showed that the number of receptors expressed per cell is inversely related to the density of the cells grown in suspension. Furthermore, although identical selection conditions (i.e., 1 μM methyltrex-

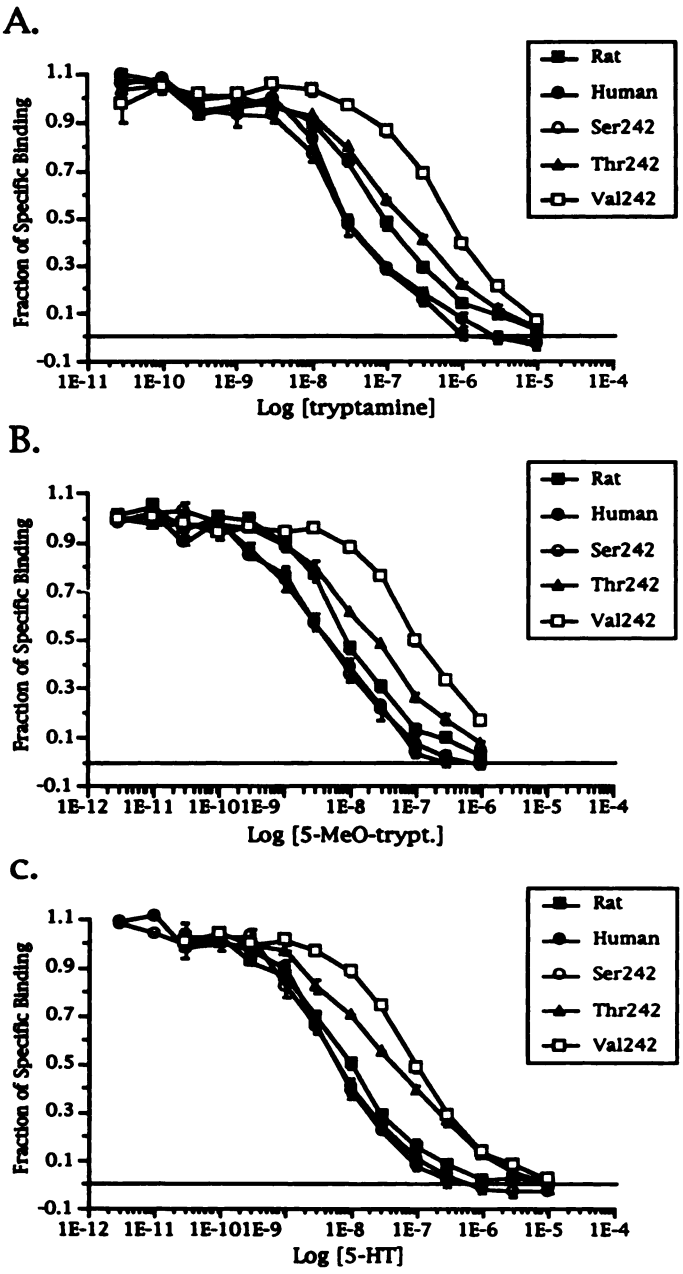

Fig. 2. Displacement curves for the N1-unsubstituted tryptamines from [^{125}I]DOI-labeled sites. Experiments were conducted as described for Table 1. The single-site or two-site K_i values are given in Table 2, as are the proprtions of high affinity sites when a two-site displacement was indicated. Unless otherwise indicated, the pseudo-Hill coefficients and partial *F* test indicated a single-site displacement. The following curves were found to better fit a two-site displacement: tryptamine displacement from the A242T mutant, 5-MeOT displacement from the human, A242S and A242T mutants, and 5-HT displacement from the A242T mutant.

ate) were used to generate all stably transfected cell lines, the actual levels of expressed recombinant proteins are expected to vary for each independent isolate. Consequently, the A242T cell line may inherently express significantly fewer receptors than the other cell lines. Given the fact that a large receptor reserve can make a partial agonist appear to be a full agonist, it would be inappropriate to suggest that the mutation of Ala^{242} to threonine had any effect on the ability of DOI to stimulate PI turnover.

Interestingly the EC_{50} for stimulation of PI turnover with 5-HT and 5-MeOT was similar for all of the cell lines except A242V, where both of these N1-unsubstituted tryptamines were significantly less potent. This agrees well with the results for displacement from a [^{125}I]DOI-labeled receptor (Table 5). It should be noted that the EC_{50} values for 5-HT in all of the cell lines were somewhat lower than might be expected, relative to the potency of 5-MeOT. Although the reason for this is not clear, experiments have shown that this is unaffected by the use of either fluoxetine (a 5-HT uptake inhibitor), pargyline (a monoamine oxidase inhibitor), or ascorbate (an antioxidant), suggesting that it is not related to degradation or uptake of 5-HT (data not shown).

Mutation of Ala^{242} to Ser^{242} in the rat 5-HT$_{2A}$ receptor. With the single change of the rat Ala^{242} to a human-like Ser^{242}, a complete alteration in the SAR was found. Whereas the rat receptors showed an increase or no change in affinity with N1-substitution of either ergolines or tryptamines, the A242S mutant showed a significant decrease in affinity with N1-substitution (Tables 5 and 6). Also, the N1-substituted analogues tended to have lower affinity for A242S than the rat 5-HT$_{2A}$ receptor, whereas the N1-unsubstituted ergolines and tryptamines had higher affinity for A242S. Lastly, simply with the change of Ala^{242} to serine, 5-MeOT displacement of [^{125}I]DOI showed a shift in the curve and a change in the shape of the curve such that it fit a two-site model identical to that seen with the human 5-HT$_{2A}$ receptor (Fig. 2B; Table 2) but significantly different from that of the wild-type rat receptor.

Mutation of Ala^{242} to Thr^{242} in the rat 5-HT$_{2A}$ receptor. The effect of mutating the rat 5-HT$_{2A}$ receptor to A242T is substantially more complex, but certain trends in the data are evident. First, it is quite clear that, as with the A242S mutation, N1-substitution of either ergolines and tryptamines resulted in a significant decrease in affinity (Tables 5 and 6). In fact, the N1-substituted ergolines and tryptamines not only showed lower affinity than that for the wild-type rat receptor but also showed lower affinity than that for either the human cloned 5-HT$_{2A}$ receptor or the mutated A242S rat receptor.

The N1-unsubstituted ergolines showed affinities similar to those seen with the A242S mutation. However, the N1-unsubstituted tryptamines (including tryptamine, 5-MeOT, and 5-HT) all gave complex competition curves (Fig. 2) for displacement from [^{125}I]DOI-labeled sites. Because 5-MeOT gave shallow curves with even the cloned human receptor and A242S mutant, where no other compounds did, it would not be prudent to attempt to make any observations based on those data. However, it is interesting to note that, when a two-site analysis was carried out on the displacement curves for tryptamine and 5-HT with the A242T mutant, a high affinity component was seen with an affinity similar to that found with the A242S mutant (Table 2). The low affinity component of these curves had a value similar to that found for displacement of the compounds from the A242V rat mutant.

Mutation of Ala^{242} to Val^{242} in the rat 5-HT$_{2A}$ receptor. The A242V mutant showed some differences in the effect of ergolines and tryptamines. For instance, the N1-isopropyl-ergolines were found to have a similar affinity for either the cloned rat receptor or A242V mutant. However, the N1-isopropyl-tryptamines showed a significant decrease in affinity (to approximately the same extent as seen with the A242S mutation) for the A242V versus the cloned rat receptor. Both the N1-unsubstituted ergolines and tryptamines showed substantial decreases in affinity with the A242V mutation. Interest-

TABLE 2

Displacement of [^{125}I]DOI by N1-Unsubstituted tryptamines and single- and two-site competition curves

Experiments were conducted as described for Table 1. Where two K_i values are given, both the pseudo-Hill coefficient and the partial F test ($p < 0.05$) indicated that a two-site displacement fit the results significantly better than a single-site model. Values are mean ± standard error for three or four experiments. Values are K_i for a single-site displacement or the high and low affinity components of a two-site displacement (two-site displacement was determined by examining the pseudo-Hill coefficients and by comparing the sum of squares for one- and two-site displacement models by Student's t test, $p < 0.05$).

N1-Substitution		K_i[a]				
		Rat receptor	Human receptor	A242S	A242T	A242V
		nM				
Tryptamine	–H	79.3 ± 8.8 (100%)	25.6 ± 4.9 (100%)	24.2 ± 1.2 (100%)	39.7 ± 7.5; 677 ± 179 (57%)	538 ± 34 (100%)
5-MeOT	–H	7.5 ± 1.1 (100%)	1.31 ± 0.60; 12.9 ± 2.0 (55%)	0.88 ± 0.28; 14.0 ± 4.0 (53%)	4.85 ± 1.77; 166 ± 60 (55%)	82.4 ± 4.8 (100%)
5-HT	–H	7.23 ± 1.06 (100%)	4.94 ± 0.71 (100%)	5.13 ± 1.10 (100%)	6.93 ± 0.95; 329 ± 123 (55%)	84.0 ± 18.4 (100%)

[a] Values in parentheses are percentages of high affinity sites.

TABLE 3

Hydrolysis of inositol phosphates with the mutated rat 5-HT$_{2A}$ receptors

Accumulation of [^{3}H]inositol phosphates was measured in triplicate after a 30-min, 37° exposure to seven different concentrations of test drug or water. Values are the mean ± standard error for three to six separate experiments. Maximum values are expressed as a percentage of the maximum amount of stimulation seen after exposure to high concentrations of 5-HT. A concentration of 100 μM 5-HT resulted in a 10-fold increase in [^{3}H]inositol phosphates.

	Cloned rat receptor		Cloned human receptor		A242S		A242T		A242V	
	EC_{50}	Maximum	EC_{50}	Maximum	EC_{50}	Maximum	EC_{50}	Maximum	EC_{50}	Maximum
	nM	% of 5-HT maximum	nM	% of 5-HT maximum	nM	% of 5-HT maximum	nM	% of 5-HT maximum	nM	% of 5-HT maximum
DOI	1.4 ± 0.2	93 ± 2	2.2 ± 0.3	99 ± 13	4.1 ± 0.1	85 ± 6	2.4 ± 0.6	81 ± 3[a]	6.4 ± 3.3	86 ± 3
5-HT	260 ± 57	100	160 ± 63	100	145 ± 17	100	308 ± 90	100	1162 ± 245	100
5-MeOT	18 ± 1	103 ± 6	9.0 ± 0.8	111 ± 17	20 ± 7	89 ± 1	46 ± 15	94 ± 3	615 ± 256[b]	91 ± 3
N-Isopropyl-5-MeOT			1429 ± 129[c]	61 ± 2[a]						

[a] Significantly different from 100% ($p < 0.05$, ANOVA followed by Student's t test).
[b] Significantly different from rat or human receptor EC_{50} ($p < 0.05$, ANOVA followed by Tukey's comparison).
[c] Significantly different from human receptor EC_{50} for 5-MeOT ($p < 0.05$, ANOVA followed by Tukey's comparison).

TABLE 4

Affinity of [^{3}H]ketanserin and [^{125}I]DOI for the mutated rat 5-HT$_{2A}$ receptors

Saturation experimednts were conducted for each of the clonal cell lines, using 0.1–10 nM [^{3}H]ketanserin. The K_d values for [^{3}H]ketanserin were taken from the saturation isotherm curves and are the mean ± standard error of three or four determinations. The K_d values for [^{125}I]DOI were estimated from the displacement curves of unlabeled DOI displacing 0.2 nM [^{125}I]DOI, as described in Experimental Procedures.

	K_d	
	[^{3}H]Ketanserin	[^{125}I]DOI
	nM	
Cloned rat receptor		1.52 ± 0.08
Cloned human receptor	0.29 ± 0.02	0.71 ± 0.05
A242S	0.28 ± 0.01	0.62 ± 0.07
A242T	0.30 ± 0.04	0.70 ± 0.15
A242V	0.12 ± 0.01[a]	1.36 ± 0.26

[a] Significantly different from human, A242S, or A242T receptor K_d ($p < 0.05$, ANOVA followed by Tukey's comparison).

ingly, of the natural and mutated clones examined only the A242V mutation resulted in a significantly different affinity for the N1-methyl analogue LY108742 versus its corresponding N1-isopropyl analogue LY53857.

Discussion

Previous experiments have indicated that [^{125}I]DOI can effectively label either the 5-HT$_{2A}$ or the 5-HT$_{2C}$ (formerly termed the 5-HT$_{1C}$) receptor under appropriate conditions (26). Based on the pharmacology of known antagonists (i.e., the ability of ketanserin and spiperone to displace [^{125}I]DOI with a single-site high affinity) it was presumed that [^{125}I]DOI labeled primarily the 5-HT$_{2A}$ receptor in rat frontal cortex under the conditions described (3). A comparison of data from rat frontal cortex and from a clonal cell line expressing the rat 5-HT$_{2A}$ receptor (Table 1) shows very similar results. Because the parental cell line AV12 does not normally express either the 5-HT$_{2C}$ or 5-HT$_{2A}$ receptor, these data support the previous hypothesis that [^{125}I]DOI labels the 5-HT$_{2A}$ receptor and not the 5-HT$_{2C}$ receptor in rat frontal cortex homogenates. Furthermore, the similar K_i values in the rat frontal cortex and the rat 5-HT$_{2A}$ clonal line serve to validate the use of this system to examine the 5-HT$_{2A}$ receptor.

The affinities of compounds for [^{125}I]DOI-labeled human 5-HT$_{2A}$ receptors in a clonal cell line were virtually identical to those obtained in monkey cortex homogenates (3). This is in good agreement with the previous work, where similar results were seen with pig, squirrel monkey, and human 5-HT$_{2A}$ receptors labeled with [^{3}H]ketanserin (2). Furthermore, by comparing the cloned rat and human 5-HT$_{2A}$ receptors in clonal lines developed from the same parental cell line using the same expression strategies, the possibility of any extraneous factors affecting the observed changes in the SAR is minimized. Therefore, it seems very reasonable to suggest that SAR changes seen in different species are due entirely to differences in the species forms of the 5-HT$_{2A}$ receptors.

TABLE 5

Affinity for the [^{125}I]DOI-labeled site in the mutated rat 5-HT$_{2A}$ receptor

Experiments were conducted as described for Table 1.

	N1-Substitution	K_i				
		Rat receptor[a]	Human receptor[a]	A242S	A242T	A242V
				nM		
LY86057	–H	15.4	0.67	0.63 ± 0.08[b,c]	1.09 ± 0.13[b,c]	116 ± 7[b,c]
LY53857	–CH(CH$_3$)$_2$	3.20	23.2	26.0 ± 1.8[c]	63.4 ± 8.0[d]	6.14 ± 0.87
Tryptamine	–H	79.3	25.6	24.2 ± 1.2[b,c]	39.7 ± 7.5[b,e]	538 ± 34[b,c]
N-Isopropyl-tryptamine	–CH(CH$_3$)$_2$	72.7	252	300 ± 35[c]	2636 ± 447[d]	497 ± 84[c]
5-MeOT	–H	7.5	1.31[e]	0.88 ± 0.28[b,c,e]	4.85 ± 1.77[e]	82.4 ± 4.8[b,c]
N-Isopropyl-5-MeOT	–CH(CH$_3$)$_2$	38.4	478	708 ± 82[c]	1712 ± 91[d]	699 ± 71[c]
5-HT	–H	7.23	4.94	5.13 ± 1.10	6.93 ± 0.95[e]	84.0 ± 18.4[b,c]

[a] Values were taken from Table 1.
[b] Significantly different from N1-alkyl-substituted analogue ($p < 0.05$, ANOVA followed by Tukey's comparison).
[c] Significantly different from rat receptor K_i ($p < 0.05$, ANOVA followed by Tukey's comparison).
[d] Significantly different from human, Ser^{242}, or rat receptor K_i ($p < 0.05$, ANOVA followed by Tukey's comparison).
[e] Values are K_i for the high affinity component of a two-site displacement model.

TABLE 6

Affinity for the [^{3}H]ketanserin-labeled site in the mutated rat 5-HT$_{2A}$ receptor

Twelve different concentrations spanning 5–6 log units of test drugs were used to displace 0.3 nM [^{3}H]ketanserin from the mutated rat 5-HT$_{2A}$ receptors. Tubes were allowed to equilibrate for 15 min at 37° before filtration through GF/B filters that had been presoaked in 0.5% polyethylenimine. Determinations were run in triplicate and K_i values from three or four experiments were averaged to give the mean ± standard error. Other K_i values for displacement from [^{3}H]ketanserin are from the sources listed and were included for comparison. Unless otherwise indicated, the pseudo-Hill coefficients and partial *F* test indicated a single-site displacement.

	N1-Substitution	K_i				
		Rat receptor[a]	Human receptor[a]	A242S	A242T	A242V
				nM		
Ergonovine	–H	16.5	1.22	0.57 ± 0.09		
Mesulergine	–CH$_3$	2.87	18.5	11.5 ± 1.5		
LY86057	–H	20.6	0.82	1.17 ± 0.28[c,d]	1.15 ± 0.13[c,d]	166 ± 5[c,d,e]
LY108742	–CH$_3$	3.38	20.9	14.5 ± 1.1	65.6 ± 6.3[d,e]	17.4 ± 1.5[f]
LY53857	–CH(CH$_3$)$_2$	2.67	7.17	10.3 ± 1.2	33.8 ± 3.2[d,e]	3.0 ± 0.4
LY193525	–H	14.7	0.73	0.53 ± 0.05[c,d]	0.63 ± 0.03[c,d]	159 ± 3[c,d,e]
Amersergide	–CH(CH$_3$)$_2$	2.56	16.2	12.2 ± 1.7	35.7 ± 4.1[d,e]	4.34 ± 0.36
LY254239	–H	15.4	1.14	0.66 ± 0.1[c,d]		
Sergolexol	–CH(CH$_3$)$_2$	4.05	23.1	16.1 ± 1.1		

[a] Values were taken from Ref 2 and the IC$_{50}$ values were converted to K_i values by the Cheng and Prusoff equation.
[b] Value is for displacement of LY108742 in monkey cortex.
[c] Significantly different from N1-alkyl-substituted analogue ($p < 0.05$, ANOVA followed by Tukey's comparison).
[d] Significantly different from rat receptor K_i ($p < 0.05$, ANOVA followed by Tukey's comparison).
[e] Significantly different from human and A242S receptor K_i ($p < 0.05$, ANOVA followed by Tukey's comparison).
[f] Significantly different from either LY86057 or LY53857 K_i with A242V ($p < 0.05$, ANOVA followed by Tukey's comparison).

Both the previous (2, 3) and present results can be summarized in a few simple statements. In rats, N1-substitution of the ergolines and tryptamines has no effect or slightly increases affinity for the 5-HT$_{2A}$ receptor. In contrast, N1-alkyl substitution of these same ergolines and tryptamines results in a substantial decrease in affinity for the pig, squirrel monkey, and human 5-HT$_{2A}$ receptors. Therefore, the SAR changes significantly when different species are studied. Interestingly, all of the N1-substituted ergolines and tryptamines were found to have a higher affinity for the rat 5-HT$_{2A}$ receptor, whereas the N1-alkyl-substituted compounds had a higher affinity for the pig, squirrel monkey, and human receptors.

From a comparison of the amino acid sequence of the rat and human 5-HT$_{2A}$ receptors it can be seen that there are only three amino acid differences within the TMs and only one of these three is likely to account for the very specific SAR changes that are limited to differences in N1-substitutions on ergolines and tryptamines. The rat Ala^{242}, compared with the human Ser^{242}, substitution in the 5-HT$_{2A}$ receptor could potentially account for all of the species differences seen with these compounds. Thus, in the present study amino acid Ala^{242} of the rat 5-HT$_{2A}$ receptor was mutated to a serine and the mutant receptor was expressed in the same manner as the cloned rat and human receptors. In contrast to what was found with the wild-type rat receptor, N1-substitution in the A242S mutant significantly decreased affinity for the indole-containing ligands. In other words, mutating the rat receptor Ala^{242} to Ser^{242} gave a pharmacological profile that was virtually identical to that of the human receptor and differed significantly from that of the rat receptor. Neither the affinity for [^{3}H]ketanserin, the affinity for [^{125}I]DOI, nor the ability of agonists to stimulate PI turnover was affected by this mutation.

This result alone strongly supports the hypothesis that the species differences seen with ergolines and tryptamines at the 5-HT$_{2A}$ receptor are due to changes in amino acid sequence at position 242 of TM V. This is completely in agreement with the previous report by Kao *et al.* (19), where mesulergine affinity was found to be affected by mutating the human 5-HT$_{2A}$ receptor at Ser^{242} to an alanine. The results of the present study extend this finding to suggest that species variance at

Ala/Ser^{242} is responsible for the species differences with a large number of ergolines and tryptamines. Specifically, it appears very likely that the differences in species pharmacology are due only to substitution at the N1-position of these indole-containing compounds. Furthermore, the present work strongly suggests that the species differences with both the N1-substituted and -unsubstituted indole-containing compounds are due exclusively to this Ala/Ser^{242} species variation.

There is also striking evidence from the present work that the N1-position of some indole-containing ligands interacts directly with amino acid 242 of the 5-HT$_{2A}$ receptors. Only the N1-unsubstituted ergolines and tryptamines (except for 5-HT at the [^{125}I]DOI-labeled site) showed higher affinity for the human receptor and A242S mutant 5-HT$_{2A}$ receptor than for the rat receptor. These same compounds have a potential hydrogen-bond donor at the N1-position that is not present in the N1-alkyl-substituted ligands. The human 5-HT$_{2A}$ receptor and A242S mutant have a potential hydrogen-bond acceptor in the hydroxyl group of Ser^{242} that is not present in the rat. Therefore, a potential hydrogen bond can occur between the N1-hydrogen of the indole and the oxygen of Ser^{242} in the human receptor, but not with Ala^{242} of the rat 5-HT$_{2A}$ receptor (Fig. 3A).

Experimentally, the K_i values for displacement from the rat or human 5-HT$_{2A}$ receptor can be converted to free energy of binding (ΔG°) and therefore the difference in energy of binding between species can be examined. For instance, by converting the K_i values for LY86057 a difference of 2.0 kcal/mmol is obtained (where rat ΔG° is −10.91 and human ΔG° is −12.90 kcal/mmol). Similar changes in ΔG° were seen for all of the N1-unsubstituted ergolines (1.6–2.0 kcal/mmol) and tryptamines (0.7–1.1 kcal/mmol) at either the [^{3}H]ketanserin- or [^{125}I]DOI-labeled sites. A recent article has suggested that hydrogen-bonding energy can range from 0.5 to 5.0 kcal/mmol, depending upon the nature of the hydrogen-bond acceptor and donor groups (27). Therefore, the changes in ΔG° are well within the range of hydrogen-bonding interactions. As illustrated in Fig. 3A, when the indole ring has an N1-alkyl substitution no hydrogen bonding can occur and the potential steric hindrance between the N1-alkyl group and the larger Ser^{242} could actually result in a significant decrease in affinity.

The present results with the two other 5-HT$_{2A}$ mutants, A242T and A242V, also suggest a close proximity between the N1-indole position and amino acid 242 of the receptor. First, it is important to note that, whereas A242T and A242V did show certain anomalies (which are discussed below), there was no indication of a major disruption of the receptor with these mutations. None of the mutants described resulted in any decrease in affinity for either the antagonist [^{3}H]ketanserin or the agonist [^{125}I]DOI. Furthermore, the mutated receptors were

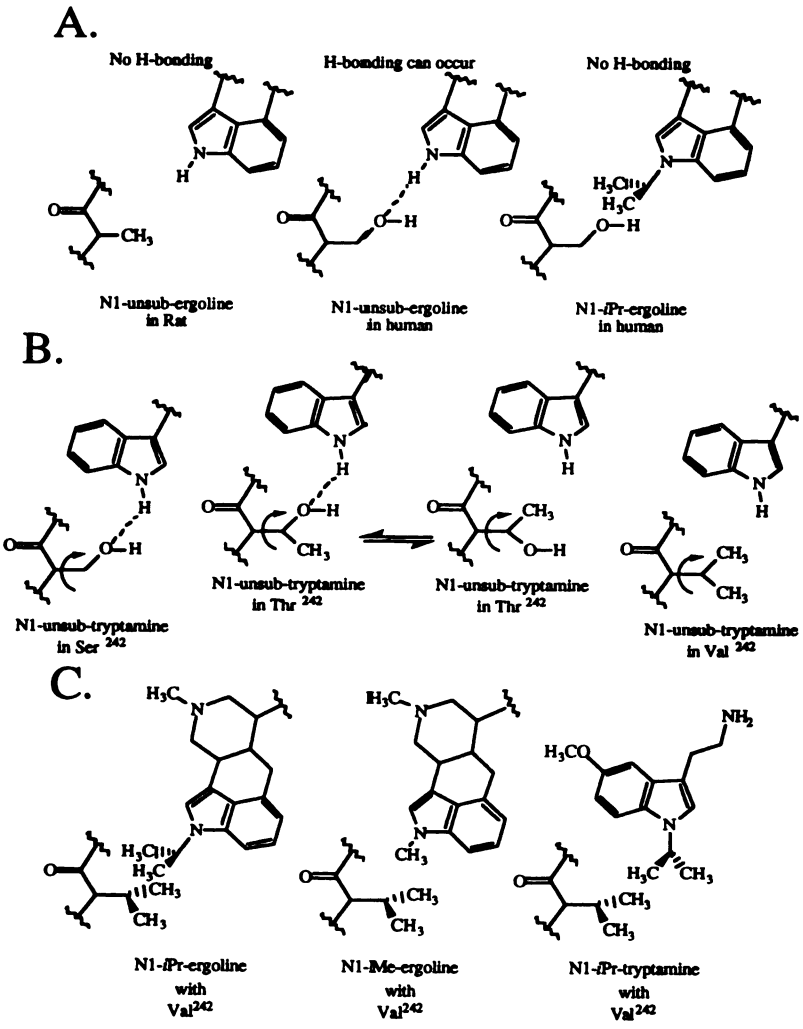

Fig. 3. Graphical representations of the possible interactions of amino acid 242 of the 5-HT$_{2A}$ receptor with the indole-containing compounds. A, Potential interactions of either alanine (as in the rat receptor) or serine (as in the human receptor) with either an N1-substituted or -unsubstituted (*unsub*) indole-containing ligand. As seen, a hydrogen bond can occur only with an N1-unsubstituted analogue and a serine at position 242. B, Potential interactions of serine, threonine, or valine with N1-unsubstituted tryptamines. Depending upon rotation around the indicated carbon-carbon bond, threonine can appear to be like serine or like valine in its interaction with the N1-position of the tryptamines. C, Potential interactions of valine with either N1-isopropryl-ergolines or -tryptamines. It can be seen that, depending upon the approach angle of the ligand, stacking between the isopropyl group of valine and the isopropyl group of the N1-substituent may occur (as illustrated for N1-isopropylergoline). This aliphatic stacking would not occur without an N1-isopropyl substitution (as illustrated for N1-methylergoline). Furthermore, with a different angle of approach (as illustrated for tryptamine) alpiphatic stacking could also not occur. *iPr*, isopropyl; *Me*, methyl.

found to stimulate PI hydrolysis, with DOI being equally potent in all cell lines. Therefore, neither the A242S, A242T, nor A242V mutation appears to result in any major change in the ability of the receptor to express, activate, or trigger a second messenger system.

The N1-unsubstituted ergolines and tryptamines were found to have a higher affinity for the A242T mutant than for the wild-type rat 5-HT$_{2A}$ receptor. This might be expected, because A242T, like the human and A242S 5-HT$_{2A}$ receptors, has a hydroxyl group available for hydrogen bonding to the N1-hydrogen of the indoles. In contrast, the N1-substituted ergolines and tryptamines were found to have a substantially lower affinity for the A242T receptor than for any of the other 5-HT$_{2A}$ receptors examined (e.g., a 2–9-fold decrease was seen versus the human or A242S receptor). Therefore, it appears that there is a significant negative interaction between a threonine on the receptor and an isopropyl group at the N1-position of indole ligands. Because this decrease is only seen with N1-alkyl-substituted compounds, the most likely explanation is that a negative steric effect is seen in this case, due to the close proximity of the N1-position and amino acid 242 of the 5-HT$_{2A}$ receptor.

With a mutation of Ala^{242} to Val^{242} in the rat 5-HT$_{2A}$ receptor, the N1-isopropyl-ergolines (such as amesergide and LY53857) had a high affinity for the receptor. In contrast, the N1-unsubstituted ergolines and tryptamines showed a much lower affinity (7–12-fold decrease versus the rat receptor and even lower affinity versus the human receptor). Furthermore, the A242V mutation produced the only 5-HT$_{2A}$ receptor for which the N1-methyl-ergoline LY108742 had significantly lower affinity than did its N1-isopropyl homologue LY53857. With the rat receptor LY108742 and LY53857 had higher affinities than did the N1-unsubstituted homologue LY86057, whereas with the human, A242S, and A242V receptors both N1-alkyl-ergolines had lower affinities. One possible explanation for all of these data would be that stacking occurs between the N1-isopropyl group and the isopropyl group of the A242V receptor, stabilizing the binding of N1-isopropyl-ergolines (Fig. 3C). Obviously this would not occur in the case of LY108742, with its N1-methyl substitution. This type of stacking would be very analogous to the leucine zipper proposed for a number of proteins (28). This interpretation is also very consistent with the results for the A242T mutant, where the N1-isopropyl-indole-containing compounds had very low affinities, possibly due to the inability of an isopropanol group (as in threonine) to participate in aliphatic stacking with an N1-isopropyl group.

Taken together, all of the results with these mutants and the effect on the SAR of the ergolines and tryptamines with only changes at position 242 of the 5-HT$_{2A}$ receptor suggest that this part of the receptor is in close proximity to the N1-position of indoles. Furthermore, there is compelling evidence that under certain conditions (i.e., in the human receptor with N1-unsubstituted ergolines and tryptamines) amino acid 242 serves as a contact point, through hydrogen bonding, in the 5-HT$_{2A}$ receptor.

Nevertheless, it merits discussion that certain anomalies were seen with the 5-HT$_{2A}$ receptor mutants that were not naturally occurring, namely A242T and A242V. One of the most interesting observations from the data is that all of the N1-unsubstituted tryptamines (including tryptamine, 5-MeOT, and 5-HT) gave complex competition curves against the agonist [^{125}I]DOI with the A242T mutant 5-HT$_{2A}$ receptor (Fig. 2; Table 2). This was true despite the fact that [^{125}I]DOI appears to label only the agonist high affinity state of these receptors. In the case of 5-MeOT displacement from the A242T mutant, an interpretation of the results is difficult, because 5-MeOT was also found to give complex competition curves with the squirrel monkey (3), human, and A242S mutant (Fig. 2B; Table 2) 5-HT$_{2A}$ receptors. In contrast, tryptamine and 5-HT gave single-site displacement of [^{125}I]DOI in all receptor homogenates, except those of the A242T mutant. Therefore, it may be that 5-MeOT actually displaces [^{125}I]DOI from the A242T mutant with three distinct affinities. Under these conditions it would be impossible to accurately estimate the binding parameters of a three-affinity displacement curve. Therefore, the results obtained with 5-MeOT and the A242V mutant cannot be commented upon and are best left for future studies.

However, the results from a two-site analysis of tryptamine and 5-HT displacement from the A242T homogenates do suggest an interesting relationship. As seen in Table 2, the high affinity component of the displacement curves shows an affinity that is very similar to that seen for the compound in the human and A242S homogenates. In contrast, the low affinity component more closely resembles the affinity of the compounds for the A242V mutant receptor. In both cases approximately 50% of the sites were in the 'high affinity' state and this proportion did not change with increasing incubation times (data not shown).

Taken together, these results suggest one interesting possibility related to carbon-carbon bond rotation around the amino acid backbone and the α-carbon of A242T. As illustrated in Fig. 3B, it is possible that in one conformation of the carbon-carbon bond the hydroxyl group of threonine is exposed to the N1-position of tryptamines, similar to what would be seen with a serine at this position. However, in another rotational conformer the terminal methyl group of threonine is exposed to the N1-position, similar to what might be seen with valine at position 242. It should be cautioned that this cannot be concluded from the present study, and other interpretations of the data may prove to be more relevant in future studies. If this effect of bond rotation is true, however, it would indicate that the tryptamines and ergolines do bind in similar but not identical orientations with respect to the indole nucleus. This follows from the different effects of the A242T mutation on the N1-unsubstituted ergolines, for which a high affinity was seen, and the N1-unsubstituted tryptamines, for which high and low affinities were found.

Additional evidence that these two types of indole-containing ligands do not bind in exactly the same orientation within the receptor can be found by closely examining the A242V mutational effects on the SAR. Here the N1-isopropyl-ergolines and -tryptamines were not affected in the same manner. The N1-isopropyl-ergolines showed equal affinity for the wild-type rat and A242V receptors. In contrast, N1-isopropyl-tryptamines showed a significantly lower affinity with [^{125}I]DOI for the A242V mutant versus the rat receptor (7–18-fold). This is approximately the same magnitude of decrease in affinity as seen when the rat receptor is compared with the human, A242S, or A242T receptors.

It should be noted that the A242V mutant 5-HT$_{2A}$ receptor is not known to be a naturally occurring form of the 5-HT$_{2A}$ receptor and, therefore, caution should be used when the data are interpreted. In fact, A242V was the only mutation that resulted in any change in radioligand affinity (increase in [^{3}H]

ketanserin affinity) and significantly lower affinity for the native ligand 5-HT (increase in K_i versus [^{125}I]DOI and increase in the EC_{50} for inositol phosphate hydrolysis). With that in mind, one interesting possible explanation for these results is that the N1-positions of ergolines and tryptamines approach amino acid 242 of the receptor in different orientations. For instance, the N1-isopropyl-ergolines may approach this area of the receptor in such a way that stacking of the isopropyl side chain occurs, thereby increasing affinity. However, the N1-isopropyl-tryptamines may approach the receptor in such a manner that aliphatic stacking cannot occur, thereby decreasing affinity (Fig. 3C). It is hoped that, as our understanding of exactly how these ligands bind to receptors increases through the use of mutagenesis studies and molecular modeling of G protein-coupled receptors, the reasons for these difficult-to-interpret results will become clear.

Despite these minor anomalies and complicated results, several important conclusions can be drawn with a reasonable amount of confidence. First, there is a clear SAR difference for some ergolines and tryptamines between the rat 5-HT_{2A} receptor and the human 5-HT_{2A} receptor. This SAR difference is clearly due to the Ala/Ser^{242} change between the rat and human receptors. By examining both the SAR of the test compounds and the amino acid substitutions in the mutants examined, it can be suggested that amino acid 242 of TM V is in close proximity to the N1-position of indole-containing compounds. Lastly, there is some strong evidence to suggest that amino acid 242 serves as an important contact point between some ligands and the 5-HT_{2A} receptor.

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