MOLECULAR PHARMACOLOGY, 27:600–611

Receptor-Binding Properties *in Vitro* and *in Vivo* of Ritanserin

A Very Potent and Long Acting Serotonin-S_2 Antagonist

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Received October 4, 1984; Accepted March 18, 1985

SUMMARY

In vitro and *in vivo* receptor-binding properties of the new serotonin antagonist, ritanserin, are reported. In *in vitro* binding assays, ritanserin shows high affinity binding to serotonin-S_2 sites in rat frontal cortex tissue: IC_{50} = 0.9 nM without drug preincubation and 0.3 nM with 30-min drug preincubation; IC_{50} values for histamine-H_1, dopamine-D_2, and adrenergic-α_1 and -α_2 sites were 39-, 77-, 107-, and 166-fold higher, and at up to 1 μM, the drug did not bind to serotonin-S_1 sites. In *in vitro* assays, ritanserin dissociated very slowly from serotonin-S_2 ($t_{1/2}$ = 160 min) and histamine-H_1 sites ($t_{1/2}$ = 77 min) and rapidly from dopamine-D_2 sites ($t_{1/2}$ = 11 min). Half-times of dissociation from adrenergic-α_1 and -α_2 sites were 18 and 26 min. The inhibition by ritanserin of [^{3}H]ketanserin binding was found to be partially noncompetitive and the inhibitory potency increased with drug preincubation. Due to the slow dissociation of ritanserin from the serotonin-S_2 sites, the drug cannot be displaced completely by [^{3}H]ketanserin. In contrast, inhibition by ritanserin of [^{3}H]haloperidol binding to dopamine-D_2 sites in rat striatum was fully competitive, in agreement with the rapid dissociation of the drug from the latter sites. In *ex vivo* binding assays using brain areas of rats and guinea pigs treated subcutaneously with ritanserin, occupation of serotonin-S_2 sites was observed at very low dosage (50% occupation at 0.08–0.1 mg/kg) and sites remained occupied during a prolonged time period (>70% occupation up to 48 hr after 2.5 mg/kg ritanserin). Histamine-H_1 receptor sites in guinea pig cerebellum became occupied at dosages 25-fold higher than the dosage producing occupation of frontal cortical serotonin-S_2 sites. Dopamine-D_2 sites in rat striatum and cortical adrenergic-α_1 sites became only slightly occupied (<20%) at higher dosages and the effect was not dose-dependent. Adrenergic-α_2 sites were not occupied up to doses of 160 mg/kg given subcutaneously. *In vivo* binding assays using [^{3}H]spiperone confirmed the occupation of frontal cortical serotonin-S_2 sites following low dosage of ritanserin and a minor occupation of striatal dopamine-D_2 sites. Levels of dopamine and serotonin and their metabolites remained unchanged in brain areas of rats orally treated with ritanserin up to dosages of 40 mg/kg. At 160 mg/kg, there seemed to be a slight reduction in dopamine and serotonin content. These observations indicated that serotonin turnover was not affected by serotonin-S_2 receptor blockade. The study showed that the *in vitro* and *in vivo* interactions of ritanserin with serotonin-S_2 and histamine-H_1 sites were in good agreement. However, the *in vitro* binding to dopamine-D_2 and α-adrenergic sites was not evident under *in vivo* conditions. The discrepancies between *in vitro* and *in vivo* binding data demonstrate that, for evaluating the activity profile of drugs, both approaches of investigation are necessary. Ritanserin appeared to be a relatively selective, extremely potent, long acting, and centrally active serotonin-S_2 antagonist. The drug probably represents a useful tool for investigating the role of serotonin-S_2 sites in brain disorders.

INTRODUCTION

The serotonergic receptor sites, presently known as 5-hydroxytryptamine$_2$ or serotonin-S_2 sites, were first detected in radioligand-binding studies using the neuroleptic [^{3}H]spiperone (1, 2). This ligand was originally proposed as the ligand of choice for studying dopamine receptor sites (3), but it revealed additional high binding affinities for receptor sites showing serotonergic properties, which were predominantly present in frontal cortex tissue. Known serotonin antagonists displayed high

Part of this work was supported by a grant from I.W.O.N.L.

0026-895X/85/060600-12$02.00/0

binding affinities for the sites, and their binding affinities correlated with their serotonin antagonistic potencies in *in vivo* pharmacological tests measuring serotonin-induced behavioral excitation (1, 2) and quipazine-induced discriminative stimulus (4). Binding affinities for serotonin-S_2 sites also corresponded to drug potencies to antagonize serotonin-induced vasoconstriction *in vitro* (1, 2, 5). Serotonin showed micromolar binding affinities for these sites, a fact which is entirely compatible with the concentrations of agonist which are required for eliciting functional effects (6–8).[1] The sites were distinguished from 5-hydroxytryptamine$_1$ or serotonin-S_1 sites, which were labeled at nanomolar concentration by [^{3}H] serotonin, but for which attributed functional roles have been challenged (5, 9–12). *In vitro* receptor-binding studies provided a new means for investigating the activity profile of drugs. It appeared that known serotonin antagonists were poorly selective agents. Cyproheptadine-like compounds and mianserin showed equally potent interactions with histamine-H_1 and serotonin-S_2 sites. Most ergot derivatives were known to display mixed agonist-antagonist properties and in binding studies these drugs differentiated poorly between serotonin-S_2 and serotonin-S_1 sites (13). This poverty in selective drugs hampered investigations of the therapeutic applications of serotonin antagonists. The discovery of ketanserin, which was found to bind primarily and with high affinity to serotonin-S_2 sites, opened new perspectives for investigating the role of serotonin-S_2 receptor sites. The drug was developed as the first selective ^{3}H-ligand for labeling the sites (14), which contributed to the further investigation of the localization of serotonin-S_2 sites in central nervous system (15) and peripheral tissues (16). Their functional role in particular on blood platelets (8) was characterized and evidence was obtained that phosphatidic acid-phosphatidylinositol turnover formed part of the signal-transducing system coupled to the serotonin-S_2 receptor sites (17).[1]

For many years, serotonin has been considered an important neurotransmitter in depression; more recently, the hypothesis according to which serotonin-S_2 receptors would be affected in depressed patients, and mostly after drug treatment, was inferred from an observed down-regulation of the sites in animals chronically treated with certain antidepressants (18). However, the hypothesis has been debated (19). Thus far, no information has been obtained on the effects of the serotonin antagonists on depressive disorders. In view of the findings on receptor alterations and the related hypotheses, a beneficial action of serotonin antagonists in certain types of depressive or mood disorders could be expected. However, in order to investigate this, potent, selective, centrally active, and long acting serotonin antagonists are required. Ritanserin (R 55 667), a new benzhydrilene piperidine derivative (molecular structure and perspective drawing in Fig. 1), has been investigated for such properties.

We now report on the *in vitro* and *in vivo* receptor-binding profile of ritanserin. We shall discuss the need and the relevance of thorough investigation of *in vitro* binding properties, involving binding affinity, rate of drug receptor dissociation and investigation of the type of inhibition caused by the compound on radioligand binding. The importance of concomitant studies of *in vitro* and *in vivo* binding for evaluating drug properties will be illustrated.

[1] D. De Chaffoy de Courcelles, F. De Clerck, J. E. Leysen, H. Van Belle, and P. A. J. Janssen, *J. Biol. Chem.* in press (1985).

MATERIALS AND METHODS

Female (150 g) and male (200 ± 10 g) Wistar rats were used for *in vitro* and *in vivo* binding experiments, respectively. Female Pirbright guinea pigs (250 g) were used for histamine receptor-binding assays. For *in vitro* binding experiments, no differences were found between tissue of young female and male animals; for economical reasons female animals were used in the *in vitro* experiments. Animals were sacrificed by decapitation and brain areas were rapidly dissected. Fresh tissues were immediately homogenized in ice-cold buffer, for total particulate membrane preparations (3), or in sucrose (0.25 M) for removal of the nuclear fraction, followed by preparation of a mitochondrial + microsomal (M+L+P) membrane fraction (14). Membrane fractions were prepared and washed by successive centrifugation and immediately used for binding assays.

In vitro binding assays. Assay conditions for measuring serotonin-S_2, serotonin-S_1, dopamine-D_2, adrenergic-α_1, adrenergic-α_2, and histamine-H_1 receptor binding *in vitro* and *ex vivo* are summarized in Table 1. Possible modifications are indicated in the legends to tables and figures. A rapid filtration technique using Whatman GF/B glass fiber filters and a 40-well filtration manifold (Multividor, Janssen Scientific Instruments Division) was used to harvest and rinse labeled membranes. Radioactivity was estimated in a Packard Tri-Carb 4530 liquid scintillation spectrometer; data were expressed in disintegrations per min by a built-in calculation device using an external standard method and referring to a standard quench curve.

Specific binding was calculated as the difference in radioactivity on the filters in assays in the absence and the presence of the "blank" compound.

Dissociation rate of unlabeled drug-receptor complexes in vitro. A new technique using tissue adsorbed to glass fiber filters, previously described in detail (20), was applied. A series of 2-ml samples of the tissue preparation (dilution, 100 v/w) was incubated in the presence of the unlabeled drug at a concentration of 10 × IC_{50} value (IC_{50}, concentration causing 50% inhibition of specific binding of the labeled ligand) in conditions as indicated in Table 1 to saturate receptor sites with the unlabeled drug. Then the samples were filtered under suction over Whatman GF/B glass fiber filters positioned on a filtration manifold, through which the membrane preparations saturated with the unlabeled drug were adsorbed to the filters. The vacuum was released and, to allow dissociation of the drug from the receptor sites, a 5-ml sample of warmed buffer (25°) was applied on the filter. Without suction, the buffer dripped slowly through the filter. Every 5 min, the remaining buffer was sucked off, the vacuum again was released, and a fresh 5-ml buffer sample was applied on the filter. In that way, filters were rinsed from zero to 18 times for 5 min with 5 ml of warmed buffer. After the rinsing session, free receptor sites in the tissue adsorbed to the filter were estimated by incubating the filter kept on the manifold with a small sample (0.4 ml) of a ^{3}H-ligand solution in buffer. A volume of 0.4 ml is sufficient to wet the filter completely, and without suction, it is retained on the filter by capillary forces. After 5-min incubation, the vacuum was applied and the filter was rinsed with 2 × 5 ml of cold buffer. The time of dissociation of the unlabeled drug was taken as the time of rinsing plus the 5-min incubation period with the ^{3}H-ligand. Hence, assayed dissociation time points were from 5 (no rinsing) to 95 min (18 times rinsed).

Simultaneously, an identical procedure was carried out using control tissue samples (preincubated in the absence of unlabeled drug) for assaying total binding by incubation of the ^{3}H-ligand on the filter and for determining nonspecific binding in the presence of blank compound.

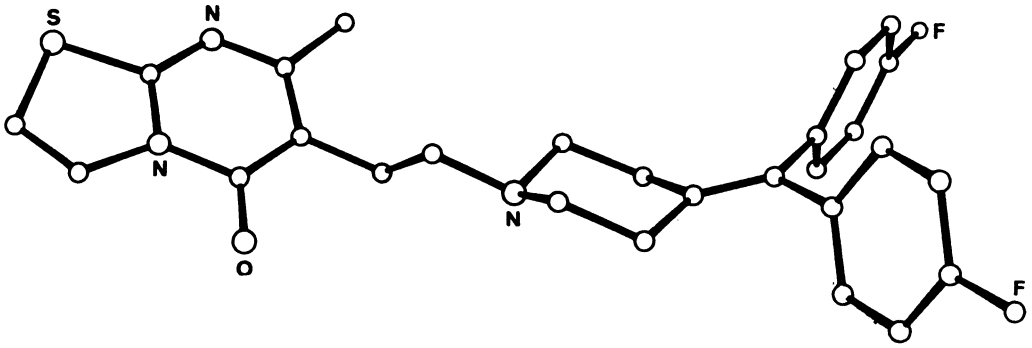

FIG. 1. *Perspective drawing of the molecular structure of ritanserin, 6-[2-[4-[bis(4-fluorophenyl)methylene]-1-piperidinyl]ethyl]-7-methyl-5H-thiazolo[3,2-a]pyrimidinin-5-one, based on X-ray data of pirenperone and cyproheptadine and quantum chemical calculation*

The X-ray crystal structure determination of ritanserin is in progress.[2] The perspective drawing was kindly provided by Dr. J. P. Tollenaere *et al.*

TABLE 1

Binding assay conditions for serotonin-S_2, serotonin-S_1, dopamine-D_2, adrenergic-α_1 and -α_2, and histamine-H_1 receptors, applied in vitro and ex vivo experiments

Conditions for S_2 *ex vivo* binding experiments are indicated in parentheses when they differed from the usual *in vitro* binding conditions.

	S_2	S_1	D_2	α_1	α_2	H_1
Species	Rat	Rat	Rat	Rat	Rat	Guinea pig
Brain region	Frontal cortex	Hippocampus	Striatum	Total cortex	Total cortex	Cerebellum
Membrane preparation	M+L+P (total particulate)	Total particulate	Total particulate	Total particulate	Total particulate	Total particulate
Number of washings	2	2	2	2 (1)	2 (1)	2
Tissue dilution	1:400 (1:200)	1:80	1:80 (1:200)	1:80	1:80	1:100 (1:50)
Buffer	Tris-HCl, 50 mM	Tris-HCl, 50 mM +4 mM $CaCl_2$	Tris-salt[a]	Tris-HCl, 50 mM	Tris-HCl, 50 mM	Na-K phosphate, 50 mM
pH	7.7	7.6	7.6	7.7	7.7	7.4
^{3}H-Ligand concentration	Ketanserin, 1 nM (2 nM)	Serotonin, 3 nM	Haloperidol, 2 nM	WB-4101, 0.5 nM	Clonidine, 3 nM	Pyrilamine, 4 nM
Blank						
Compound and concentration	Methysergide, 2 μM	LSD,[b] 2 μM	(+)Butaclamol, 2 μM	Noradrenaline, 100 μM	Noradrenaline, 2 μM	Astemizole, 2 μM
Incubation						
Volume	4.4 ml (2.2 ml)	2.2 ml	2.2 ml	2.2 ml	2.2 ml	1.1 ml
Temperature	37°	37°	37°	25°	25°	25°
Time	15 min	10 min	10 min	20 min	30 min	30 min

[a] Tris-salt buffer: 50 mM Tris, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 μM pargyline, 0.1% ascorbic acid.
[b] Lysergic acid diethylamide.

^{3}H-Ligands and buffers were as indicated in Table 1, except for estimating serotonin-S_2 sites where 2 nM [^{3}H]spiperone in Tris-salt buffer was used. The ^{3}H-ligand must have the property of fast association with the receptor sites adsorbed to the filter (maximal after 5 min) and must show minimal adsorption to the filter material itself. For serotonin-S_2 receptor measurements in tissue adsorbed to filters, [^{3}H]spiperone was found to be superior to [^{3}H]ketanserin, and Tris-salt buffer reduced the adsorption of [^{3}H]spiperone to the filter material. For the other receptor systems, the ^{3}H-ligands and buffers used in the conventional test tube assays gave satisfactory results. This dissociation method and the binding properties of tissue adsorbed to the filter have been evaluated for the various receptor systems in the same way as described for the dopamine receptor (20). It was found that tissue preparations adsorbed to filters retained the same binding properties as the tissues in aqueous suspension.[3]

Ex vivo binding assays. Animals were treated subcutaneously with ritanserin. Ninety minutes later (for dose response) or after various time intervals, the animals were sacrificed; brain tissue was dissected and immediately processed for *in vitro* ^{3}H-ligand binding as described in Table 1.

In vivo binding assays. In vivo binding was performed using the postlabeling technique, i.e., the unlabeled drug is given to the animal before the ^{3}H-ligand (21). Ritanserin was administered subcutaneously to rats, followed 1 hr later by intravenous injection of 0.5 $\mu g \cdot kg^{-1}$ [^{3}H] spiperone (corresponding to approximately 6.5 μCi/rat). Rats were sacrificed 1 hr after the last injection. Brain areas were immediately dissected and samples corresponding to 15–30 mg of tissue were placed in counting vials. Radioactivity was extracted in 10 ml of Instagel by vigorous shaking and then counted in a liquid scintillation spectrometer. For *ex vivo* and *in vivo* binding assays, the subcutaneous route of administration was used for comparison with data from behavioral studies, where this route is routinely used for all drugs.

Determination of biogenic amine and amine metabolite levels in brain tissue. Rats starved overnight were orally treated with ritanserin. Two hours later (for dose response) or after different time periods, rats were killed by decapitation. Brain areas were immediately dissected and frozen in liquid nitrogen. The samples were lyophilized for 24 hr and

[2] C. J. De Ranter *et al.*, unpublished data.
[3] Manuscript in preparation.

stored at −80°. For this type of study, oral treatment was used since this has been a routine procedure in our laboratories for all previous investigations with neuroleptics and antiemetics.

The dry tissue samples (between 10 and 40 mg) were weighed and homogenized with an Ultra-Turrax for 2 × 10 sec in 1 ml of ice-cold 0.4 N $HClO_4$. After being kept in ice for a few minutes, the material was centrifuged (Eppendorf centrifuge, 1 min) and 10- (striatum) or 40-μl (other brain areas) sample of the supernatant was injected directly in the high performance liquid chromatography apparatus.

The instrument used was a Varian HPLC model 5060, equipped with a microcomputer-controlled reciprocating single piston pump system. The electrochemical detector was a BAS model 4B from Bioanalytical Systems equipped with a glassy carbon electrode. The detector potential was set at +0.75 V and 5 namp versus the reference electrode Ag/AgCl. Elution time was less than 30 min. All chromatograms were monitored on a microcomputer data system (Varian, Vista 401). The raw data were stored on floppy disks for calculation. Narrow bore stainless steel columns (25 cm; 4 mm i.d.) filled with spherical LiChrospher RP-18 particles of 5 μm and a pore size of 100 Å were used throughout this study. The efficiency ranged from 70,000 to 100,000 theoretical plates per meter. The optimum flow rate was 0.9 ml/min. The columns were obtained from Merck AG as Hibar LiChrospher 100-CH-18. The elution solvent consisted of an aqueous mixture of 0.2 M sodium formate, 0.5 mM EDTA, 5 mM heptanesulfonic acid (brought to pH 3.75 with formic acid), and acetonitrile/methanol (60:40, v/v). To 1 liter of the buffer solution, 115 ml of the organic solvent mixture was added and degassed by ultrasonification.

The system was calibrated several times each day with a known amount of different catecholamines and metabolites. More details will be published in a separate paper.[4]

Drugs. Labeled drugs were: [^{3}H]ketanserin, specific activity of 15.0 Ci/mmol, and [^{3}H]haloperidol, specific activity of 22.0 Ci/mmol from Janssen Life Sciences Products, Beerse, Belgium; [^{3}H]spiperone, specific activity of 22.9 Ci/mmol, [^{3}H]WB-4101, specific activity of 20.7 Ci/mmol, [^{3}H]pyrilamine, specific activity of 24.1 Ci/mmol, and [^{3}H]clonidine, specific activity of 67.6 Ci/mmol from New England Nuclear, Dreieich, W. Germany. ^{3}H-Ligands were regularly checked for radioactive purity using thin layer chromatography as indicated by the manufacturers; it was required to be >99%.

Ritanserin (R 55 667) was from Janssen Pharmaceutica (Beerse, Belgium). Unlabeled drugs were obtained from the companies of origin. For *in vitro* assays, stock solutions in 100% ethanol and dilutions in 10% ethanol in bidistilled water were freshly made before each experiment. Norepinephrine was from Janssen Chimica. For administration to animals, drugs were dissolved in saline.

RESULTS

In vitro receptor binding. The potency of ritanserin to bind to receptor-binding sites *in vitro* was assayed in radioligand-binding models for serotonin-S_2, serotonin-S_1, histamine-H_1, dopamine-D_2, adrenergic-α_1, and adrenergic-α_2 receptor sites. The inhibition curves of ritanserin compared with those of ketanserin are shown in Fig. 2 and IC_{50} values are presented in Table 2. The highest potency of ritanserin concerned binding to serotonin-S_2 receptor sites (IC_{50} = 0.9 nM). Under usual incubation conditions, ritanserin was approximately twice as potent as ketanserin, but when ritanserin was preincubated with the tissue preparation for 30 min, its inhibitory potency was enhanced 3-fold, whereas preincubation of unlabeled ketanserin with the tissue preparation did not alter its inhibitory potency (results not shown). Ritanserin revealed markedly lower binding affinity for various other receptor sites. The difference in

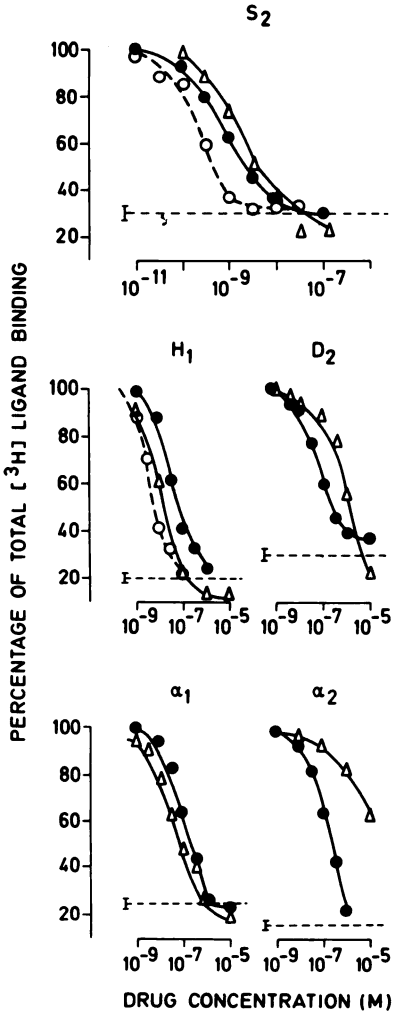

FIG. 2. *Inhibition by ritanserin (●) and ketanserin (Δ) of ^{3}H-ligand binding to receptor binding sites in vitro*

Incubation conditions were as described in Table 1. For binding to serotonin-S_2 and histamine-H_1 receptor sites, additional experiments were performed using preincubation of ritanserin with the tissue preparations during the 30 min before addition of the ^{3}H-ligand (O- - -O). Points are mean values of two to five independently performed assays in duplicate. Standard deviations were less than 5%. Horizontal discontinuous lines indicate the level of the nonspecific binding. The means of the IC_{50} values derived from the separate curves are presented in Table 2.

IC_{50} values was 39-fold for binding to histamine-H_1 sites, 77-fold for binding to dopamine-D_2 sites, 107-fold for binding to α_1-adrenergic sites, and 166-fold for binding to α_2-adrenergic sites. Ritanserin was somewhat less potent than ketanserin for binding to histamine-H_1 and adrenergic-α_1 receptor sites, but it was apparently more potent than ketanserin for binding to dopamine-D_2 and adrenergic-α_2 receptor sites. However, as observed for inhibition of serotonin-S_2 receptor binding, preincubation of ritanserin (30 min) with the cerebellar tissue also increased its potency to inhibit [^{3}H]pyrilamine binding to histamine-H_1 sites (IC_{50} = 5 nM). Neither ritanserin or ketanserin bound to serotonin-S_1 sites tested up to concentrations of 10,000 nM.

In order to gain more insight in the receptor-binding properties of ritanserin, the time of dissociation of the

[4] J. Wynants *et al.*, manuscript in preparation.

TABLE 2

Inhibitory potency in in vitro radioligand receptor binding and physicochemical properties of ritanserin and ketanserin

Physicochemical data were provided by Dr. J. Peeters, Department of Analytical Chemistry, Janssen Pharmaceutica, Beerse, Belgium. Values are mean ± standard error; n = numbers in parentheses.

	Mean IC_{50} value	
	Ritanserin	Ketanserin
	nM	
Serotonin-S_2	0.9 ± 0.1 (5)	1.7 ± 0.5 (4)
	0.3[a] (2)	
Histamine-H_1	35 ± 5 (4)	16 ± 5 (3)
	5[a] (2)	
Dopamine-D_2	70 ± 30 (4)	620 ± 50 (4)
Adrenergic-α_1	97 ± 13 (4)	31 ± 2 (4)
Adrenergic-α_2	150 ± 25 (4)	>1000 (3)
Serotonin-S_1	>1000 (3)	>1000 (3)
Octanol-water partition coefficient (P)		
Un-ionized compound, log P	4.2	3.3
At pH 7.5, log P_{app}	3.42	3.0
Acid dissociation constant (K_a)		
pK_a	8.2	7.5
μ (dipole moment)	3.4	4.9

[a] Value from assays with 30-min preincubation of ritanserin.

drug from the various receptor sites was measured. The new technique, in which tissue preloaded with drug is adsorbed to the filter and dissociation of the drug from the receptor sites is achieved by slowly rinsing the filter with buffer, was found to be applicable for all the various receptor systems in the same way. Control values of specific ^{3}H-ligand binding to tissue adsorbed to filters are reported in the legend to Fig. 3. First order reaction plots [ln (specifically bound drug) versus time] of the dissociation of ritanserin and ketanserin from serotonin-S_2, histamine-H_1, adrenergic-α_1 and -α_2, and dopamine-D_2 sites are presented in Fig. 3. Analysis of up to 50% dissociation of the drug-receptor complex revealed rectilinear plots (calculated by the method of least squares, correlation coefficient, $r > 0.9$); derived dissociation $t_{1/2}$ values are indicated on the graphs. Ritanserin dissociated most slowly from serotonin-S_2 sites ($t_{1/2}$ = 160 min) and rapidly from dopamine-D_2 sites ($t_{1/2}$ = 11 min). Dissociation from histamine-H_1 sites was also slow ($t_{1/2}$ = 77 min) and ritanserin dissociated with intermediate velocity from adrenergic-α_1 and -α_2 sites ($t_{1/2}$ = 18 and 26 min). In contrast to ritanserin, ketanserin dissociated rapidly from all the various receptor sites ($t_{1/2}$ = 4–11 min).

Because of the marked difference in the dissociation rate of ritanserin from serotonin-S_2 and dopamine-D_2 sites, and because of the observation of a decrease in IC_{50} value for binding to serotonin-S_2 sites following preincubation of the drug, the type of inhibition by ritanserin for inhibiting [^{3}H]ketanserin binding to serotonin-S_2 sites and for inhibiting [^{3}H]haloperidol binding to dopamine-D_2 sites was investigated. Specific [^{3}H]ketanserin binding with increasing [^{3}H]ketanserin concentration was assayed in the absence of drug and in the presence of 3×10^{-10} and 1×10^{-9} M ritanserin. Experiments were performed in the usual incubation conditions, i.e., without drug preincubation, and with 15-min

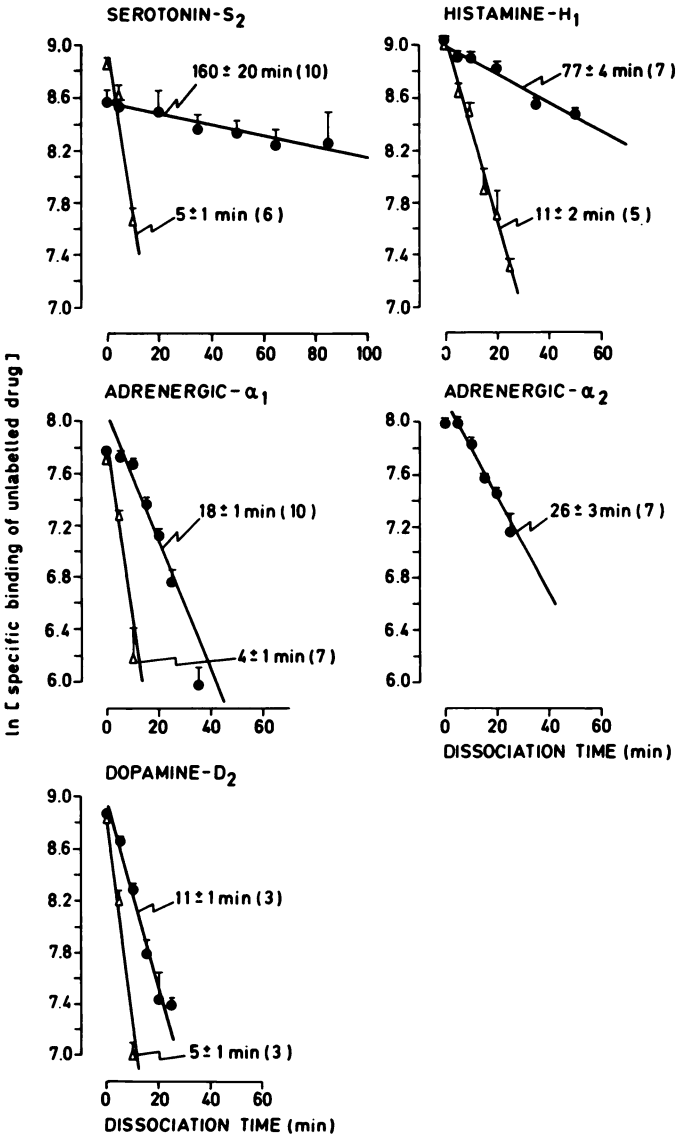

FIG. 3. *First order reaction plots of the dissociation of ritanserin (●) and ketanserin (Δ) from receptor-binding sites in vitro*

The dissociation of the unlabeled drugs from the receptor sites was measured using the tissue adsorbed by the filter technique described under Materials and Methods. Specific binding of unlabeled drug is estimated as total ^{3}H-ligand binding in control tissue adsorbed to the filter minus ^{3}H-ligand binding after a particular period of dissociation. Points are mean values of independently performed experiments. Half-times of dissociation were derived from the slopes of the lines in individual experiments (calculation by the method of least squares, correlation coefficient of the lines, $r > 0.9$). Values on the graphs indicate the mean half-times of dissociation (± standard error) and the number of independent determinations. 100% specific binding of the ^{3}H-ligands incubated on the filters with control tissue (20 mg original wet weight per filter) were for [^{3}H]spiperone (2 nM) binding to serotonin-S_2 sites in rat frontal cortex 6250 ± 380 dpm, for [^{3}H]pyrilamine (4 nM) binding to histamine-H_1 sites in guinea pig cerebellum 8320 ± 430 dpm, for [^{3}H]haloperidol (2 nM) binding to dopamine-D_2 sites in rat striatum 7260 ± 440 dpm, for [^{3}H]WB 4101 (1 nM) binding to adrenergic-α_1 sites in rat cortex 2270 ± 120 dpm, and for [^{3}H]clonidine (3 nM) binding to adrenergic-α_2 sites in rat cortex 8050 ± 330 dpm.

preincubation of ritanserin and the tissue preparations. Results analyzed in Scatchard plots are presented in Fig. 4. Mean values of apparent K_D and B_{max} values are given in the legend to Fig. 4. In assays without preincubation, the inhibition by ritanserin was apparently competitive; 3×10^{-10} M ritanserin did not significantly affect the binding although in each pair of experiments the K_D values were slightly increased compared to controls. In the presence of 1×10^{-9} M ritanserin, the K_D value of [^{3}H]ketanserin was more than 4-fold increased, but the B_{max} value was not significantly changed. When ritanserin was preincubated with the tissue, the inhibition became much more pronounced and was no longer competitive. Preincubation with 3×10^{-10} M ritanserin resulted in an increase in the K_D value of [^{3}H]ketanserin and in a reduction of the B_{max} value. It would appear that in the latter case the plot of mean points from three independent experiments performed in duplicate are not necessarily fitted by a straight line. However, Scatchard plots of individual experiments, calculated according to the method of least squares, revealed highly significant correlation coefficients ($r > 0.95$). Therefore, the means of derived apparent K_D and B_{max} values are probably a close estimate of these values. After preincubation with 1×10^{-9} M ritanserin, the [^{3}H]ketanserin binding was reduced to 13 ± 8% ($n = 4$) (at 0.1 nM [^{3}H]ketanserin) to 19 ± 2% ($n = 4$) (at 3 nM [^{3}H]ketanserin) of control binding. Because of the very low binding, Scatchard plots were scattered and line calculation was no longer significant. It appeared that the binding potency of ritanserin for serotonin-S_2 sites increased with drug preincubation and [^{3}H]ketanserin was unable to displace entirely ritanserin from the serotonin-S_2 receptor sites.

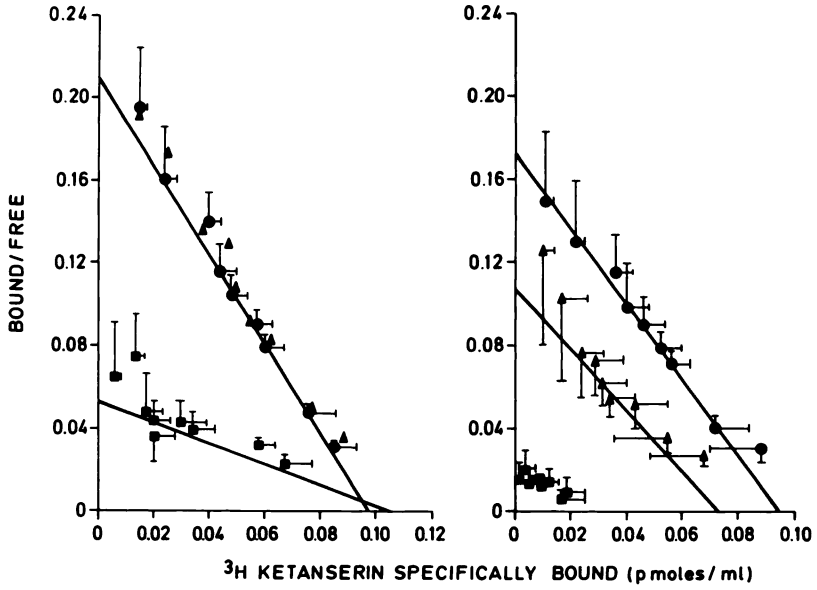

FIG. 4. *Scatchard plots of specific [^{3}H]ketanserin binding to rat frontal cortex tissue preparations in the absence (●) and presence of ritanserin* 3×10^{-10} (▲) and 1×10^{-9} M (■) without preincubation (left) and after 15 min preincubation of ritanserin (right). Incubations were run for 15 min at 37°, using 2 ml of M+L+P membrane fraction of rat frontal cortex tissue, in a dilution of 200 (v/w). [^{3}H]Ketanserin was used at concentrations of 0.1, 0.2, 0.4, 0.5, 0.6, 0.8, 1.0, 2.0, and 3.0 nM. Specific binding was defined by inhibition with 1000-fold excess methysergide. Free [^{3}H]ketanserin was given by the difference between totally added and totally bound [^{3}H]ketanserin. In conformance with the law of mass action, all values are expressed as concentrations present in the incubation mixture (i.e., nanomolar or picomoles per ml). Points represent mean values obtained in three independent experiments performed in duplicate. Separate experiments were analyzed in Scatchard plots and lines were calculated by the method of least squares. The apparent K_D value of [^{3}H]ketanserin was obtained from the reciprocal value of the calculated slope of the line. The apparent B_{max} value is expressed in femtomoles per mg wet weight of tissue, given by the abscissa intercept times 1000 divided by the tissue concentration in milligrams per ml.

	K_D	B_{max}	Correlation coefficient of the lines
	nM	*fmol/mg tissue*	
No preincubation			
Control	0.46 ± 0.06 (5)	21.4 ± 1.8	0.97 ± 0.01
+3 × 10^{-10} M ritanserin	0.52 ± 0.06 (3)	22.2 ± 4.5	0.967 ± 0.001
+1 × 10^{-9} M ritanserin	2.0 ± 0.04 (4)	23.6 ± 3.1	0.81 ± 0.06
Preincubation			
Control	0.55 ± 0.06 (4)	20.8 ± 2.4	0.97 ± 0.02
+3 × 10^{-10} M ritanserin	0.69 ± 0.1 (3)	16.3 ± 2.4	0.96 ± 0.01
+1 × 10^{-9} M ritanserin	Scattered plots because of very low binding (<20% of control binding over the entire [^{3}H]ketanserin concentration range)		

Values are means ± standard error; n = number of experiments in parentheses.

Concentration binding curves of [^{3}H]haloperidol to rat striatal membrane preparations were investigated in the absence of inhibitor and in the presence of 30, 100, and 300 nM ritanserin, respectively. Scatchard analysis of the binding data yielded straight lines, intersecting on the abscissa at the level of the B_{max} value (data not shown) and were hence indicative of a competitive type of binding between ritanserin and [^{3}H]haloperidol. To evaluate further whether the binding of both drugs was fully competitive, the data were analyzed in double reciprocal plots (1/specifically bound versus 1/free [^{3}H]haloperidol concentration) followed by a slope replot (22) (data shown in Fig. 5). Double reciprocal plots revealed that the K_D value of [^{3}H]haloperidol increased with increasing ritanserin concentration (see legend to Fig. 5) B_{max} values, given by the reciprocal value of the intersection point of the lines with the ordinate, remained unchanged (B_{max} = 43 ± 5 fmol/mg of striatal tissue), which again pointed to a competitive type of inhibition. Furthermore, the fully competitive nature of the inhibition by ritanserin of [^{3}H]haloperidol binding was corroborated by the rectilinear slope replots (plotting the slopes of the lines in the double reciprocal plot against the ritanserin concentration) (22). The K_i value of ritanserin, given by the intersection point of the line in the slope replot with the abscissa, was found to be 37 nM. This is in good agreement with the K_i value which can be calculated from the IC_{50} value (see Table 2) according to $K_i = IC_{50}/[1 + C/K_D]$ which yields 30 nM.

Ex vivo receptor binding. Rats were treated subcutaneously with increasing dosages of ritanserin (0.04 to 160 mg/kg); 90 min later, they were sacrificed and brain tissues were assayed for serotonin-S_2, dopamine-D_2, adrenergic-α_1, and adrenergic-α_2 receptor binding under *in vitro* assay conditions as described in Table 1. Results are presented in Fig. 6. It was found that binding to serotonin-S_2 receptor sites in frontal cortex tissue was markedly inhibited in rats treated with ritanserin. After 0.04 mg/kg ritanserin, 30% inhibition of serotonin-S_2 receptor labeling was already observed. The inhibition increased further with increasing dosage of ritanserin given to the rats and was maximal (>90% inhibition) at 2.5 mg/kg. In contrast, only a slight occupation (<20%) of α_1-adrenergic sites after 10, 40, and 160 mg/kg and of dopamine-D_2 sites after 40 and 160 mg/kg ritanserin was apparent, and the effect was not dose-dependent. α_2-Adrenergic sites were not occupied at up to 160 mg/kg ritanserin.

Serotonin-S_2 receptor occupancy in rat frontal cortex was assayed as a function of time after subcutaneous administration of 0.63 and 2.5 mg/kg of ritanserin. Fig. 7 shows that, in rats treated with 0.63 mg/kg, serotonin-S_2 receptor sites were occupied by more than 80% during 6 hr; then a slow decline in receptor occupation was

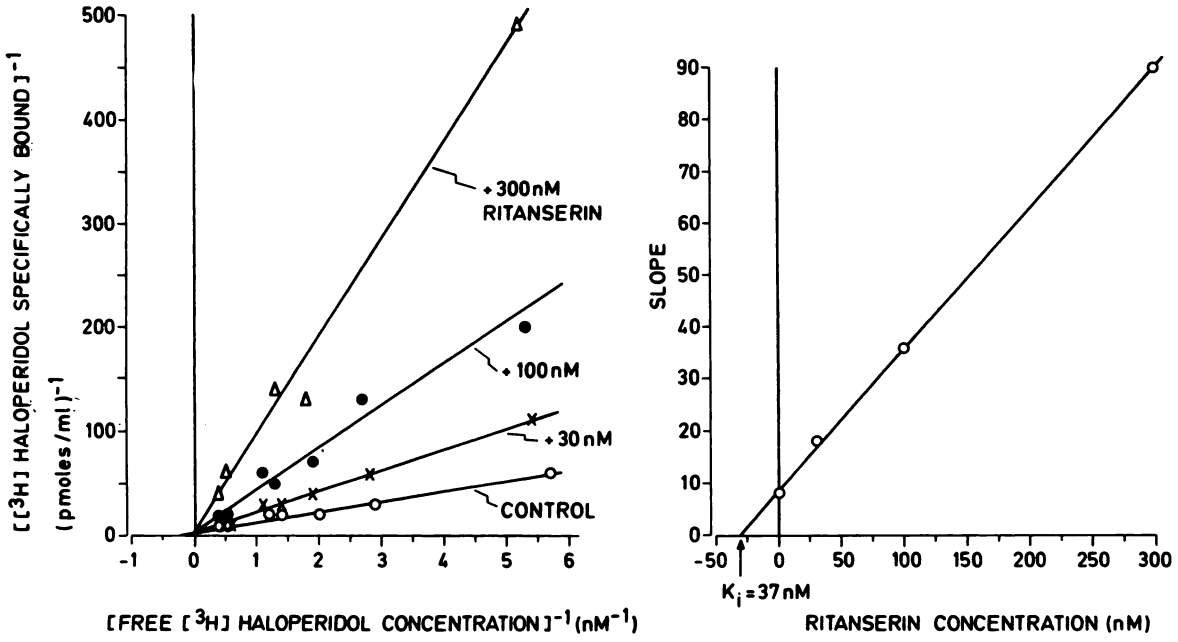

FIG. 5. *[^{3}H]Haloperidol binding to rat striatal tissue*

Left, double reciprocal plot of specific binding in the absence (○) and the presence of 30 (×), 100 (●), and 300 nM (△) ritanserin. Incubations were run for 15 min at 37° using 2 ml of a total particulate membrane fraction of rat striatum in a dilution of 200 (v/w). [^{3}H]Haloperidol was used at concentrations 0.2, 0.4, 0.6, 0.8, 1.0, 2.0, and 3.0 nM. Specific binding was defined by inhibition with 1000-fold excess (+)-butaclamol. Free [^{3}H]haloperidol was given by the difference between totally added and totally bound [^{3}H]haloperidol. All values are expressed as concentrations present in the incubation mixture (see legend to Fig. 4). Points represent mean values of three experiments. Lines were calculated by the method of least squares (correlation coefficients of all lines were >0.98). Apparent K_D values of [^{3}H]haloperidol given by the reciprocal value of the intercept of a line with abscissa were: control, 1.5 nM; +30 nM ritanserin, 3.5 nM; +100 nM ritanserin, 6.6 nM; +300 nM ritanserin, 47 nM. The B_{max} value, 43 ± 5 fmol/mg wet weight of tissue, was obtained from the reciprocal value of the intercept with the ordinate times 1000, divided by the tissue concentration in milligrams per ml. Right, slope replot. The slopes of the lines in the double reciprocal plot were plotted against the ritanserin concentration. The K_i value = 37 nM of ritanserin was obtained from the intersection point of the line with the abscissa (22).

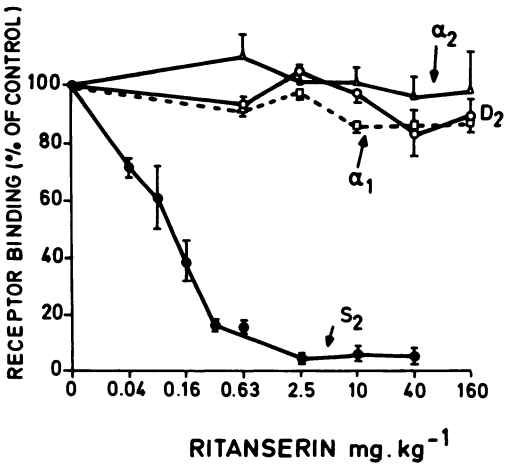

FIG. 6. *Ex vivo binding in rat brain*

Different doses of ritanserin were given subcutaneously to rats; after 90 min, the occupation of various receptor sites was measured *in vitro* using the different binding assays as described in Table 1. Data are means ± standard error of six values obtained from three animals and are expressed as percentage of specific ^{3}H-ligand in control tissue of untreated rats. In control tissue, the following levels of binding were found: serotonin-S_2 sites: total, 18,410 ± 430 dpm; nonspecific, 6,290 ± 170 dpm; dopamine-D_2 sites: total, 22,670 ± 560 dpm; nonspecific 9,020 ± 410 dpm; adrenergic-α_1 sites: total, 11,450 ± 90 dpm; nonspecific, 1,960 ± 40 dpm; adrenergic-α_2 sites: total, 5,510 ± 90 dpm; nonspecific, 1,240 ± 20 dpm.

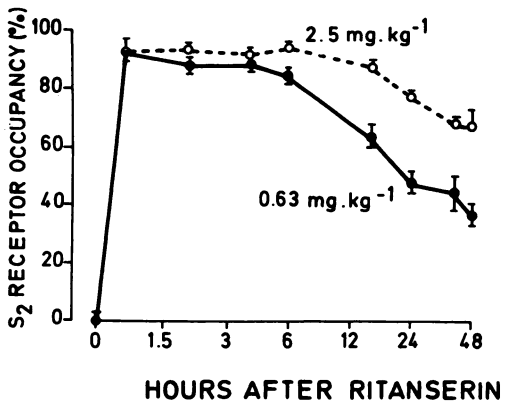

FIG. 7. *Time course of ex vivo binding in rat brain after subcutaneous injection of two doses of ritanserin*

[^{3}H]Ketanserin binding was assayed *in vitro* in frontal cortex tissue preparations. Assay conditions were as described in Table 1. Data are means ± standard error of six values obtained from three animals and are expressed as percentage of receptor sites occupied by ritanserin, calculated according to 100 minus 100 × [specific [^{3}H]ketanserin binding in tissue of treated rats/specific [^{3}H]ketanserin binding in control tissue].

observed, but after 48 hr, 30% of receptors still were occupied. Following 2.5 mg/kg ritanserin, 90% of the receptor sites were occupied during 12 hr, and 48 hr later 70% receptor occupation still was found.

In order to investigate the occupation of histamine-H_1 receptor sites by *ex vivo* binding, guinea pigs were treated subcutaneously with increasing doses of ritanserin and killed 90 min later. Histamine-H_1 receptor binding was measured in the cerebellum and serotonin-S_2 receptor binding was measured in the frontal cortex. Data are shown in Fig. 8. Inhibition of histamine-H_1 receptor binding started at 0.16 mg/kg ritanserin (10% inhibition) and reached 70% inhibition at 10 mg/kg. Inhibition of serotonin-S_2 receptor binding started at lower dosage; as was found in the rat, 30% inhibition of binding was observed after 0.04 mg/kg and a maximal inhibition was reached at 2.5 mg/kg.

In vivo binding. Because of the rapid dissociation *in vitro* of ritanserin from dopamine-D_2 sites, *ex vivo* binding data could be artifactual, due to possible dissociation of the drug from the receptor sites during the tissue preparation. Therefore, occupation of dopamine-D_2 receptor sites was investigated by *in vivo* binding, using intravenous administration of [^{3}H]spiperone. The use of [^{3}H]spiperone allowed concomitant measurement of dopamine-D_2 sites in the striatum and of serotonin-S_2 sites in the frontal cortex (23). Fig. 9 shows that the *in vivo* binding of [^{3}H]spiperone to the serotonin-S_2 sites in the frontal cortex already started to be inhibited at 0.01 mg/kg ritanserin, 50% inhibition occurred around 0.08 mg/kg, and inhibition was maximal at 0.63 mg/kg. There was no inhibition in the cerebellum where the labeling was practically similar to that found in the frontal cortex after maximal inhibition with ritanserin. In the striatum, only a slight inhibition of labeling was seen at dosages between 2.5 and 40 mg/kg. The inhibition remained less than 20% of the labeling in the striatum when the labeling in the cerebellum was subtracted.

Effect on biogenic amines and biogenic amine metabolites. In Table 3, data are presented on the levels of dopamine and its metabolites (3,4-dihydroxyphenylacetic acid and homovanillic acid) and of serotonin and its metabolite (5-hydroxyindoleacetic acid) measured in the striatum and the frontal cortex of rats following oral

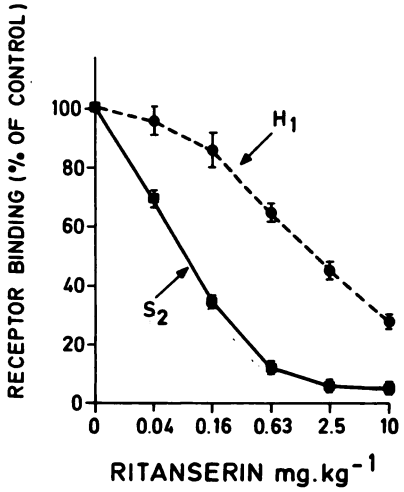

FIG. 8. *Ex vivo binding in guinea pig brain with different doses of ritanserin*

Inhibition of [^{3}H]ketanserin binding to frontal cortex tissue (serotonin-S_2 receptor sites) and [^{3}H]pyrilamine binding to cerebellar tissue (histamine-H_1 receptor sites) of guinea pigs treated subcutaneously with ritanserin 90 min before sacrifice. Data are means of six values obtained in three animals and are expressed as percentage of specific ^{3}H-ligand binding in control tissue of untreated guinea pigs. In control tissues, the following levels of binding were found: serotonin-S_2 sites: total, 11,860 ± 170 dpm; nonspecific, 3,500 ± 90 dpm; histamine-H_1 sites: total, 23,810 ± 630 dpm; nonspecific, 2,970 ± 130 dpm.

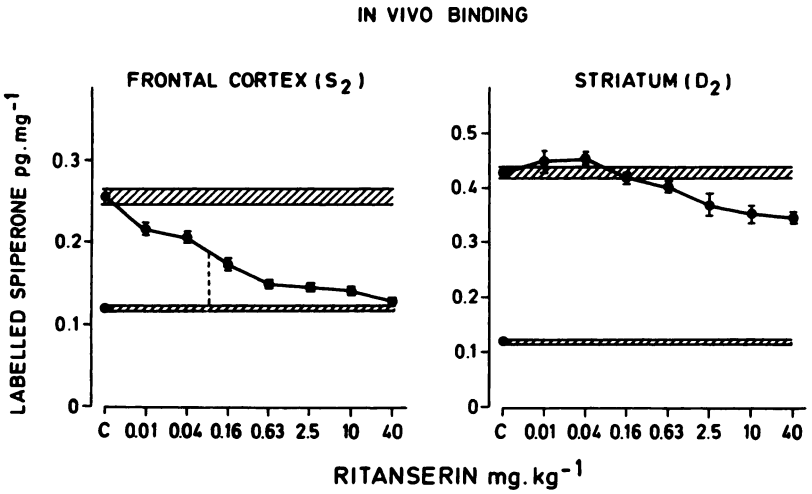

FIG. 9. *In vivo binding: ritanserin dose curve*

Inhibition by ritanserin of [^{3}H]spiperone binding to serotonin-S_2 sites in rat frontal cortex tissue and to dopamine-D_2 sites in rat striatal tissue assayed under *in vivo* binding conditions as described under Materials and Methods. Values are means ± standard error of measurements in three animals. The horizontal upper line represents total labeling in frontal cortex (left) and striatum (right) in saline-treated animals; the lower horizontal line represents labeling in the cerebellum of these animals.

TABLE 3

Determination of biogenic amines and amine metabolite levels in brain areas of rats treated with ritanserin

Time course of biogenic amine or biogenic amine metabolite levels in the striatum of rats treated orally with 0.63 mg/kg of ritanserin did not reveal any change between 2 and 48 hr after drug administration. Values are mean ± standard error. The ratio between wet weight and dry weight of striatal tissue was 4.0 ± 0.1 (mean value ± SD, n = 16). HVA, homovanillic acid; 5HIAA, 5-hydroxyindoleacetic acid; Dopac, 3,4-dihydroxyphenylacetic acid.

Dose	Dopamine	Dopac	HVA	Serotonin	5HIAA
mg/kg					
Control values (pmol/mg dry weight)					
Striatum (n = 6)	210 ± 14	25 ± 2	13 ± 2	9 ± 1	10 ± 1
Frontal cortex (n = 11)				11.1 ± 0.3	3.9 ± 0.1
Ritanserin, at 2 hr			*% control in striatum*[a]		
0.04	95 ± 6	104 ± 7	95 ± 6	96 ± 8	101 ± 6
0.16	102 ± 7	114 ± 14	96 ± 4	92 ± 7	91 ± 8
0.63	93 ± 7	99 ± 14	87 ± 8	90 ± 5	86 ± 7
2.5	95 ± 6	91 ± 6	86 ± 4	92 ± 7	98 ± 6
10	86 ± 7	90 ± 8	87 ± 8	94 ± 5	99 ± 10
40	81 ± 10	107 ± 11	95 ± 4	96 ± 14	89 ± 16
160	77 ± 14	112 ± 9	95 ± 5	61 ± 7	89 ± 15
			% control in frontal cortex[b]		
0.63				82 ± 12	103 ± 12

[a] n = 6.
[b] n = 4.

administration of ritanserin. No obvious effect on any of the biogenic amine or amine metabolite levels was seen over a dose range of 0.04 to 40 mg/kg ritanserin or over a time period between 2 and 48 hr. At the very high dosage of 160 mg/kg, levels of dopamine and of serotonin were apparently somewhat decreased, but the levels of the amine metabolites were not significantly altered.

DISCUSSION

In *in vitro* binding assays, ritanserin was found to bind primarily and with high affinity (subnanomolar concentration) to serotonin-S_2 receptor sites. As with ketanserin, ritanserin shows selectivity for this type of serotonin receptor-binding site since, at up to micromolar concentrations, the compound did not interact with serotonin-S_1 sites. Ritanserin is even more selective than ketanserin in regard to *in vitro* binding to various other neurotransmitter receptor sites.

A new technique was applied for the measurement of the dissociation time of unlabeled drugs from receptor sites. The method shows the advantage of being applicable to various receptor systems in the same way, and the dissociation can be considered as being achieved through infinite dilution of the drug-receptor complex. Evaluation of the method has been fully described for measuring the dissociation of drugs from rat striatal dopamine-D_2 receptor sites (20), and was similarly investigated for the various receptor sites which are con-

sidered in this study.[3] The disadvantage of the method is that the shortest dissociation time point which can be measured is 5 min since the duration of a dissociation period has been taken as the sum of the buffer-rinsing period plus the time of incubation with the ^{3}H-ligand. Therefore, exact rate measurement cannot be done for rapidly dissociating compounds. Using this technique, it was found that ritanserin dissociates very slowly from serotonin-S_2 and histamine-H_1 receptor sites ($t_{1/2}$ = 160 and 77 min), it dissociates with intermediate velocity from adrenergic-α_1 and -α_2 sites, but the compound dissociates rapidly from dopamine-D_2 sites ($t_{1/2}$ = 11 min) (Fig. 3). In contrast, ketanserin dissociates rapidly from all the receptor sites. The reliability of the presently used dissociation method could be verified for the serotonin-S_2 receptor system by comparison of the findings with the drug dissociation rate measured in preliminary studies with [^{3}H]ritanserin (specific activity of 5.6 Ci/mmol) and with those previously measured with [^{3}H]ketanserin using a conventional method (14). After loading a dilute suspension of rat frontal cortical tissue with a low concentration of [^{3}H]ritanserin (5×10^{-11} M), the dissociation induced by addition of excess methysergide (1×10^{-6} M) was similarly slow. The reaction proceeded according to first order kinetics and a dissociation rate constant $k_{-1} = 0.0037$ min^{-1} corresponding to a dissociation $t_{1/2}$ = 187 min in Tris-salt buffer, or $k_{-1} = 0.0033$ min^{-1} corresponding to $t_{1/2}$ = 210 min in Tris-HCl buffer was measured. Otherwise, [^{3}H]ketanserin was previously found to dissociate from serotonin-S_2 sites with a k_{-1} = 0.7 min^{-1} or $t_{1/2}$ = 1 min in Tris-salt buffer and a k_{-1} = 0.2 min^{-1} or a $t_{1/2}$ = 3.5 min in Tris-HCl buffer. Hence, the dissociation times measured with the radioactive ligands using a conventional method were in good agreement with the $t_{1/2}$ values measured with the unlabeled drugs using receptor preparations adsorbed to glass fiber filters. Even marked differences in assay conditions, i.e., low subsaturating concentration of the ^{3}H-ligands and dissociation induced by excess cold drug compared to high saturating concentrations of unlabeled drugs and dissociation induced by rinsing with buffer, seemed to exert little influence on the drug-receptor dissociation rate.

The slow dissociation of ritanserin from serotonin-S_2 receptor sites in particular and also from histamine-H_1 receptor sites poses problems for equilibrium binding studies. Indeed, equilibrium of binding cannot be reached during currently applied relatively short incubation periods. This was reflected by a three to seven times decrease in IC_{50} values for binding to serotonin-S_2 and histamine-H_1 sites, respectively, when the tissue preparations were preincubated with ritanserin for 30 min. The observations prompted a more thorough investigation of the type of inhibition caused by ritanserin on [^{3}H]ketanserin binding to serotonin-S_2 sites in rat frontal cortex tissue. Analysis in Scatchard plots of [^{3}H]ketanserin binding in the absence and the presence of ritanserin revealed a partially noncompetitive inhibition, when ritanserin was preincubated with the tissue; 15-min preincubation with 1×10^{-9} M ritanserin even virtually abolished the [^{3}H]ketanserin binding, rendering calculation of Scatchard plots by the method of least squares no longer statistically significant. The apparent noncompetitive inhibition is probably to be ascribed to the very slow dissociation of ritanserin from the serotonin-S_2 receptor sites. Therefore, the true binding affinity of ritanserin for serotonin-S_2 sites cannot be assessed in inhibition binding experiments. In preliminary experiments with [^{3}H]ritanserin, incubated for 1 hr at 37° with a very dilute suspension of rat frontal cortex tissue, a K_D value = 12 ± 1 pM (n = 3) and a B_{max} = 25 fmol/mg of tissue were found. Hence, the K_D value measured with [^{3}H]ritanserin was 75 to 25 times lower than the IC_{50} values derived from the inhibition experiments.

In contrast to the findings of the serotonin-S_2 sites, the inhibition by ritanserin of the [^{3}H]haloperidol binding to dopamine-D_2 sites was fully competitive. This appeared from analysis of [^{3}H]haloperidol binding in the presence of ritanserin, in Scatchard plots, and in double reciprocal plots combined with a slope plot (Fig. 5). Preincubation with ritanserin did not affect the inhibition (data not shown). The observation of competitive binding to dopamine-D_2 sites between ritanserin and [^{3}H]haloperidol is in agreement with the rapid dissociation of both drugs from this receptor. The reason why the binding of ritanserin to serotonin-S_2 sites and also to histamine-H_1 sites is virtually irreversible is still poorly understood. The chemical structure of ritanserin does not contain moieties which are prone to bind covalently to functional groups in the receptor sites. The physicochemical properties of ritanserin are not exceptional compared to those of ketanserin, e.g., at pH 7.5 ritanserin is only 2.6-fold more lipophilic than ketanserin (calculated from the octanol-water partition coefficients at pH 7.5), and its dipole moment is lower than that of ketanserin (see Table 2). The slow dissociation seems indeed not to be ascribed to these physicochemical properties, because in that case a similarly slow dissociation from different receptor sites would be expected. Ritanserin probably has the ability to form an energetically stable complex with serotonin-S_2 receptor sites. More information regarding energy changes which occur upon formation of the ritanserin-serotonin-S_2 receptor complex, are expected to be obtained from investigations using [^{3}H]ritanserin.

In receptor-binding studies *ex vivo*, following administration of ritanserin to rats and guinea pigs, only serotonin-S_2 and histamine-H_1 sites were found to be occupied in a dose-dependent manner. An *in vivo* potency ratio of about 25-fold for occupation of the two receptor sites was observed which corresponds approximately to the ratio of the *in vitro* binding affinity for these sites. Serotonin-S_2 sites remained occupied for more than 24 hr after subcutaneous as well as after oral administration of the compound to male or female rats.[5] It was noted that the prolonged serotonin-S_2 receptor occupation did not run parallel with the plasma levels of the drug. After oral administration of 10 mg/kg of ritanserin to male rats, plasma levels of unchanged drug declined rapidly and were below detection level (≤0.01 μg eq/ml) after 24

[5] Unpublished observations.

hr.[6] At that time point, after administration of 2.5 mg/kg ritanserin (orally or subcutaneously), serotonin-S_2 receptors were still 80% occupied. Surprisingly, however, dopamine-D_2 and adrenergic-α_1 sites became only slightly occupied and the occupation was not dose-dependent. Hence, regarding these receptor sites, a discrepancy between the *ex vivo* and *in vitro* binding data apparently exists. Using the *ex vivo* binding technique, the drug can possibly dissociate from the receptor sites during the tissue preparation; this particularly could be the case for the dopamine-D_2 receptor sites, because of the fast *in vitro* dissociation of ritanserin from these sites. The *in vivo* binding experiments, however, using [^{3}H]spiperone, confirmed the minor occupation of dopamine-D_2 sites in the striatum, whereas in the same animal serotonin-S_2 sites in the frontal cortex were virtually completely occupied at 0.63 mg/kg of the drug. Moreover, the dose yielding 50% serotonin-S_2 receptor occupation (0.08 mg/kg) was found to be similar in the *ex vivo* binding experiment in the rat and the guinea pig and in the *in vivo* binding experiments in the rat.

Also the measurements of biogenic amines and amine metabolite levels in various areas of the brain revealed no interaction with dopamine receptor sites. There was no effect on brain levels of dopamine and dopamine metabolites after administration of ritanserin in various dosages (up to 40 mg/kg) or for various time periods. The slight apparent reduction of dopamine content after 160 mg/kg ritanserin should not be considered as a specific effect of the drug. Activation or blockade of dopamine receptor sites seems not to occur since this would result in attenuation or activation, respectively, of the dopamine turnover. Whereas feed-back regulation of dopamine turnover following receptor activation or blockade is a well known and generally observed phenomenon, regulation of serotonin turnover following serotonin receptor blockade or activation is not as well evidenced (24). Previous studies of effects of serotonin antagonists on serotonin turnover were hampered by the poverty in specific drugs; either the drugs had mixed agonist-antagonist properties (e.g., quipazine, methysergide, metergoline) or blockade of serotonin receptor sites was not the primary effect of the drugs (e.g., methiotepin, cyproheptadine, pizotifen, etc.). Our observations would indicate that blockade of serotonin-S_2 receptor sites does not provoke enhanced turnover of serotonin in the forebrain. This could mean that the serotonergic system is regulated differently than the dopamine system, or otherwise that feed-back regulation is not mediated by the serotonin-S_2-type receptor sites.

Finally, the observations in *in vivo* and *ex vivo* receptor-binding experiments with ritanserin are in agreement with the *in vivo* pharmacological activities of the drug in rats (25). Ritanserin antagonized at low subcutaneous dosages tryptamine-induced cyanosis (ED_{50} = 0.021 mg/kg) and tryptamine-induced clonic seizures (ED_{50} = 0.074 mg/kg). The latter dose corresponds to the dose producing 50% occupation of serotonin-S_2 sites in the frontal cortex *ex vivo* and *in vivo*. At 0.51 mg/kg, the drug protected against compound 48/80-induced lethality, which is due to histamine release from mast cells. In contrast, behavior related to activation of dopaminergic or adrenergic receptor sites was poorly or not antagonized by the drug.

It is difficult to explain fully the discrepancy between the *in vitro* receptor-binding properties and the *in vivo* observations with ritanserin. Thus far, no indications were found that ritanserin would be metabolized into compounds with more selective serotonin-S_2 receptor-blocking properties, i.e., lacking the *in vitro* interaction with dopamine-D_2 and α-adrenergic receptor sites. An alternative explanation would be that the dopamine and α-adrenergic receptor sites in the brain are more difficult to reach than serotonergic and histaminergic receptor sites. Serotonin-S_2 and dopamine-D_2 receptors are not localized on the same neurones and the neuronal disposition is certainly not identical in the striatum and the frontal cortex; it is likely that membrane barriers to be crossed markedly differ in both brain regions. It could be that the free concentration of ritanserin in the vicinity of dopaminergic or α-adrenergic receptor sites is never sufficiently high to produce sufficient receptor occupation. Unfortunately, experimental evidence to that will be hard to obtain.

This study has amply demonstrated that ritanserin is an extremely potent and very long acting serotonin-S_2 receptor blocker. However, it also has revealed pitfalls in *in vitro* receptor-binding studies. First, it has been shown that, for drugs which dissociate very slowly from receptor sites, the real binding affinity cannot be assessed from *in vitro* inhibition of radioligand binding. Second, it has appeared that such drugs reveal a pattern of partial noncompetitive inhibition of radioligand binding. It is important to keep this in mind for studies of receptor regulation following long term treatment of animals with the drug. Indeed, noncompetitive inhibition of binding could be confounded with an apparent receptor down-regulation. Third, it is clear that *in vitro* receptor-binding data, although they have been proven useful in several cases, are not sufficient to predict the *in vivo* activities of the drugs; these should always be verified in *in vivo* receptor-binding and pharmacological studies. From the *in vivo* receptor-binding data, it can be concluded that ritanserin is one of the more selective, extremely potent, long acting, and centrally active serotonin antagonists which have been described thus far. The first clinical investigations with ritanserin have revealed therapeutic activity of the drug in dysthymia and anxiety disorder (27–29). In the past, neuroleptic drugs, in particular the selective dopamine antagonists, have largely contributed to a better understanding of the dopaminergic system of the brain. A selective serotonin-S_2 antagonist like ritanserin should facilitate the elucidation of the functional role of serotonin in the brain in normal and pathological situations.

[6] M. Michiels, unpublished observations.

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