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[^{3}H]Ketanserin (R 41 468), a Selective ^{3}H-Ligand for Serotonin$_2$ Receptor Binding Sites

Binding Properties, Brain Distribution, and Functional Role

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SUMMARY

In rat prefrontal cortex tissue (mitochondrial plus microsomal membrane preparation), 70% of [^{3}H]ketanserin (2 nM) binding is to serotonin$_2$ (S_2) receptor binding sites, defined by inhibition by 1 μM methysergide; less than 10% of the labeling is to *alpha*$_1$-adrenergic receptor sites; and the remainder is nonspecific binding. High-affinity binding to histamine H_1 receptor binding sites is not detectable, and interference with other receptor binding sites (S_1, dopaminergic, *alpha*$_2$- or *beta*-adrenergic, cholinergic, muscarinic, γ-aminobutyric acid or opiate) is unlikely. Analysis of the properties of specific [^{3}H]ketanserin binding in rat prefrontal cortex tissue reveals linear Scatchard plots and first-order dissociation kinetics for assays in various buffer. In Tris-HCl buffer a K_D value of 0.42 nM, a B_{max} of 30.9 fmoles/mg of tissue, and a half-life of dissociation of 3.5 min are found. The presence of physiological concentrations of electrolytes and/or 0.1% ascorbic acid and 1 μM pargyline reduces the binding affinity up to 3-fold, reduces the B_{max} value by 30%, and enhances the rates of association and dissociation. Inhibition isotherms and Hill plots of chemically different serotonin antagonists are steep and indicative of competition for a single type of binding site. Serotonin and some of its agonists produce more shallow inhibition curves. Effects of buffer additives on binding parameters and shapes of inhibition curves can probably be ascribed to surface phenomena. In both rats and guinea pigs, the highest density of specific [^{3}H]ketanserin binding to S_2 receptor binding sites is found in frontal cortical areas, but a high amount of the sites is also detectable in dopaminergic brain areas. The K_D values for specific [^{3}H]ketanserin binding are similar in various cortical and dopaminergic brain areas with the exception of the nucleus accumbens, where it tends to be somewhat higher. Binding affinities of 65 compounds of various pharmacological classes are reported: purported potent serotonin antagonists of different chemical structure show that nanomolar K_i values for specific [^{3}H]ketanserin binding sites and serotonin-like compounds are at least 100 times more potent inhibitors of specific [^{3}H]ketanserin binding than other neurotransmitters and their agonists. A strong resemblance between specific binding sites for [^{3}H]ketanserin and for [^{3}H]spiperone in rat prefrontal cortex is demonstrated, but subtle differences point to a more purely serotonergic nature of the [^{3}H]ketanserin sites. It is determined to what extent *in vitro* binding affinities relate to potencies of drugs in pharmacological tests reflecting serotonergic and antiserotonergic activity (*in vivo*, the tryptamine test and the mescaline test; on isolated tissue, the caudal artery test and the rat fundus test). The relationship so found suggests a role of S_2 receptor binding sites in behavioral excitation. S_2 receptor binding sites are also likely to mediate serotonin-induced vasoconstriction. The [^{3}H]ketanserin binding assay appears to offer a purer model for the vascular serotonin than the [^{3}H]spiperone binding assay. Otherwise, S_2 receptor binding sites do not seem to be involved in serotonin-induced contraction of the rat fundus. It is concluded that because of its marked selectivity and advantageous binding properties, [^{3}H]ketanserin is the most suitable of the ^{3}H-ligands available so far for investigaton of S_2 receptor binding sites.

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INTRODUCTION

Two distinct types of serotonin receptor binding sites have been detected in *in vitro* binding studies: one is specifically labeled with high affinity by [^{3}H]serotonin (1) in various tissues (see references in ref. 2) and is called 5-HT$_1$,[2] or S$_1$ receptor (3, 4); the other is labeled with high affinity by [^{3}H]spiperone in frontal cortex tissue (5, 6) and is designated 5-HT$_2$, or S$_2$ receptor (3, 4). In this study the sites are described as S$_1$ and S$_2$ receptor binding sites, respectively.

Besides [^{3}H]serotonin and [^{3}H]spiperone, various other ^{3}H-ligands have been used to study the serotonin receptor binding sites. However, all of them have the disadvantage of labeling more than one type of binding sites with high affinity. [^{3}H]LSD (3, 7, 8) and [^{3}H]metergoline (9) were found to label both S$_1$ and S$_2$ receptor binding sites and probably also dopamine receptor binding sites; [^{3}H]spiperone labels S$_2$ and dopamine receptor binding sites (6); [^{3}H]mianserin labels S$_2$ and histamine$_1$ (H$_2$) receptor binding sites (10); and [^{3}H]methiothepin shows predominantly nonspecific binding to lipid components of membranes (11). Hence [^{3}H]serotonin appears to be the only selective ligand labeling S$_1$ receptor binding sites. However, the physiological role of the S$_1$ receptor binding sites is not yet clear (2, 4), although a relationship between these sites and serotonin-sensitive adenylate cyclase or alternatively a relationship with neuronal inhibition by serotonin has been proposed (4). On the other hand, S$_2$ receptor binding sites were shown to mediate the antagonism of various serotonergic effects measured *in vivo*, such as tryptamine-induced clonic seizures (5, 6) and 5-hydroxytryptophan-induced head twitches (4), or *in vitro*, such as serotonin-induced contractions in isolated rat caudal arteries (2). Unfortunately, the lack of selectivity of the ^{3}H-ligands used for labeling the S$_2$ receptor binding sites has been a major drawback for an extensive investigation of the regional distribution, the cellular and subcellular localization, and the regulation of these receptor sites (12, 13).

Recently we described the receptor binding profile of ketanserin (R 41 468) (14), a serotonin antagonist of a new chemical series of quinazoline derivatives. Unlike known serotonin antagonists, ketanserin displays primarily a high binding affinity for S$_2$ receptor binding sites (K_i = 2.1 nM); it is inactive at S$_1$ receptor binding sites and shows only moderate binding affinity for histamine H$_1$ and *alpha*$_1$-adrenergic receptor binding sites (K_i = 10 nM); the substance binds very weakly to dopamine receptor binding sites (K_i = 220 nM) and is inactive in other known neurotransmitter receptor binding assays. In pharmacological studies *in vivo* and *in vitro*, ketanserin was found to be a particularly potent antagonist of serotonin-induced vasoconstriction, devoid of any agonist activity (15, 16). In clinical trials the drug revealed promising antihypertensive properties (17, 18) and lacked the adverse reactions of clinically used antiserotonergic agents such as psychotic symptoms, hallucinations, or drowsiness.

The particular receptor profile and its interesting pharmacological and clinical properties were compelling reasons for using ketanserin as a labeled ligand in the investigation of serotonin receptors in binding studies. This study shows that [^{3}H]ketanserin labels selectively the S$_2$ receptor binding sites. The specificity, the regional distribution in rat and guinea pig brain, the binding properties, and the identity and role of the [^{3}H]ketanserin labeled sites were investigated in detail using rat prefrontal cortex tissue.

MATERIALS AND METHODS

Tissue preparation. Female Wistar rats (150 g) were killed by decapitation. The brains were immediately removed from the skull and brain areas for regional distribution studies and were dissected according to the method of Glowinski and Iversen (19). The prefrontal cortex was obtained after careful removal of the bulbus olfactorium by a vertical cut with a scalpel just in front of the edge of the corpus callosum. Procedures similar to those used for rats were used to dissect guinea pig brains (female Pirbrights, 300 g). The brain tissue was immediately placed in ice-cold 0.25 M sucrose (1:10, w/v) and homogenized with the use of a Potter Elvejhem conical glass tube and motor-driven Teflon pestle (four strokes at 120 rpm). Nuclei and cell debris were removed by centrifugation at 1,086 × *g* for 10 min. The pellet was rehomogenized in 0.25 M sucrose (1:5, w/v) as above and centrifuged. Combined supernatants were diluted in Tris buffer (see below) to 1:40, w/v. The suspension was centrifuged at 35,000 × *g* for 10 min. The pellet was washed once by resuspension with a pipette in the same volume of Tris buffer and centrifuged at 35,000 × *g* for 10 min. The final pellet contained washed membranes of a total mitochondrial plus microsomal subcellular fraction (M + L + P). It was suspended in Tris buffer of the indicated composition, using a motor-driven Teflon pestle and conical glass tube as above. The final concentration of the membrane suspension used for binding assays corresponded to 2.5 mg (original wet weight) of tissue per milliliter. The average protein content in washed (M + L + P) membranes of rat prefrontal cortex was 30 ± 1 mg of protein per gram (wet weight) of tissue (n = 11) measured with the Bio-Rad protein assay kit referring to γ-globulin calibration curves (Bio-Rad Laboratories, München, Germany). It is noted that the values were about 50% higher when the method of Lowry *et al.* (20) was used, referring to bovine serum albumin calibration curves. With the method of Lowry *et al.* the protein content (milligrams of protein per gram of tissue) in the first 16 areas in Table 3 was found to vary between 45 ± 3 (prefrontal cortex) and 35 ± 7 (thalamus) in the rat and between 38 ± 4 (frontoparietal cortex) and 26 ± 4 (medulla oblongata) in the guinea pig. The protein content in the last four areas appeared to be somewhat lower.

Standard procedure for [^{3}H]ketanserin binding assays. Incubation mixtures contained in 10-ml plastic tubes were composed of 4 ml of a freshly prepared (M + L + P) membrane suspension in buffer (see below), 0.2

[2] The abbreviations used are: 5-HT$_1$, 5-hydroxytryptamine$_1$; 5-HT$_2$, 5-hydroxytryptamine$_2$; S$_1$, serotonin$_1$; S$_2$, serotonin$_2$; LSD, lysergic acid diethylamide; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; HPLC, high-pressure liquid chromatography; BRY, wetting agent Brij-35 (Merck, Darmstadt, Germany).

ml of 10% ethanol or unlabeled drug solution in 10% ethanol, and 0.2 ml of [^{3}H]ketanserin in 10% ethanol. The mixture was incubated for 15 min at 37° and rapidly filtered under reduced pressure through GF/B glass-fiber filter discs (2.5-cm diameter). Filters were rapidly rinsed three times with 5 ml of ice-cold Tris buffer. Filtration and rinsing were accomplished within 5 sec using a filtration manifold with 40 wells (Janssen Pharmaceutica, Scientific Instruments Division, Beerse, Belgium). Filters were transferred to plastic counting vials, and 6 ml of Instagel (Packard) was added, followed by vigorous shaking for 10 min. The vials were stored for 12 hr at 4° in the dark. Thereafter radioactivity was counted in an Intertechnique liquid scintillation spectrometer equipped with a built-in computer. Data were expressed in disintegrations per minute, using an external standard, and were calibrated against quench curves set up under experimental conditions. Specific [^{3}H]ketanserin binding was obtained by subtracting nonspecific binding, determined in assays containing unlabeled methysergide in 1000-fold excess over the [^{3}H]ketanserin concentration.

As a rule, all incubations were carried out in duplicate and filtered in separate sessions. Experiments were repeated two to four times independently, using fresh tissue preparations and drug solutions.

Mathematical analysis. Equilibrium dissociation constants (K_D values and maximal number of binding sites (B_{max} values) were obtained from Scatchard plots (21). K_i values were calculated from IC_{50} values (drug concentration inhibiting 50% of specific binding) according to the equation of Cheng and Prusoff (22). Linear regression lines were calculated according to the method of least squares. Correlation coefficients (r_s) were calculated using the nonparametric Spearman rank (23).

Buffers. The following buffers were used: Tris buffer, 50 mM Tris-HCl (pH 7.7); Tris-SALT buffer, 50 mM Tris-HCl (pH 7.7), 120 mM NaCl, 5 mM KCl, 2 mM $MgCl_2$, and 1 mM $CaCl_2$. When indicated, 0.1% ascorbic acid and 1 μM pargyline were added to these buffers.

[^{3}H]ketanserin. For most experiments in this study, a [^{3}H]ketanserin preparation (^{3}H-position as indicated in Fig. 1) of 15.5 Ci/mmole with a radiochemical purity > 99% was used. [^{3}H]Ketanserin was prepared and purified on HPLC by Knaeps and co-workers (24) (Department of Drug Metabolism and Pharmacokinetics, Janssen Pharmaceutica, Beerse, Belgium). The compound was stable during storage in 100% ethanol at −15° for at least 6 months. Binding data were highly reproducible with this preparation. Values for radioactivity trapped on the filters by filtration of 4.4 ml of the [^{3}H]ketanserin solution in Tris buffer were 1600 dpm and 2800 dpm at 1 nM and 2 nM, respectively. Besides the above-mentioned [^{3}H]ketanserin preparation, more highly labeled preparations of 87.5 Ci/mmole and 22.5 Ci/mmole prepared by New England Nuclear Corporation (Boston, Mass.) were tested. Preparations with a radiochemical purity of 95–96% yielded fluctuating values for total and nonspecific binding, varying by 25–30% between independent experiments. After purification on HPLC to 99% purity, more reproducible binding was obtained. The purified preparation of 22.5 Ci/mmole was used for ligand saturation binding experiments in the regional distribution study. With the more highly labeled [^{3}H]ketanserin, the radioactivity adsorbed on the filters was higher. This could be reduced by one-half by presoaking the filters in a 0.025% BRY solution.

Drugs. Drugs were kindly donated by the manufacturers of origin. Just prior to use in the assays, the drugs were dissolved in 100% ethanol and diluted further in 10% ethanol. Dilutions were made using plastic disposable pipettes and tubes.

RESULTS

Specificity and levels of [^{3}H]ketanserin binding. Figure 2 shows the inhibition curves of methysergide, prazosin, pyrilamine, and unlabeled ketanserin for [^{3}H]ketanserin binding to rat prefrontal cortex membranes. Methysergide binds at submicromolar concentrations only to S_1 and S_2 receptor binding sites (14), prazosin shows a K_i = 0.6 nM for *alpha*$_1$-adrenergic receptor binding sites, and pyrilamine shows a K_i = 2 nM for H_1 receptor binding sites. Prazosin and pyrilamine show K_i

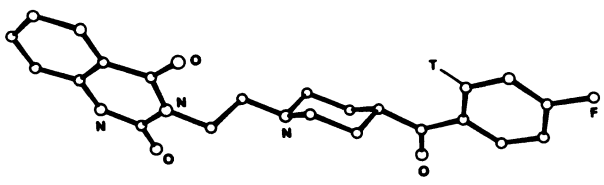

FIG. 1. *Perspective drawing of the molecular structure of ketanserin with indication of the labeled position (T)*

The perspective drawing, based on X-ray crystallographic data (from Dr. C. S. De Ranter, K. U. Leuven) was provided by Dr. J. P. Tollenaere and H. Moereels.

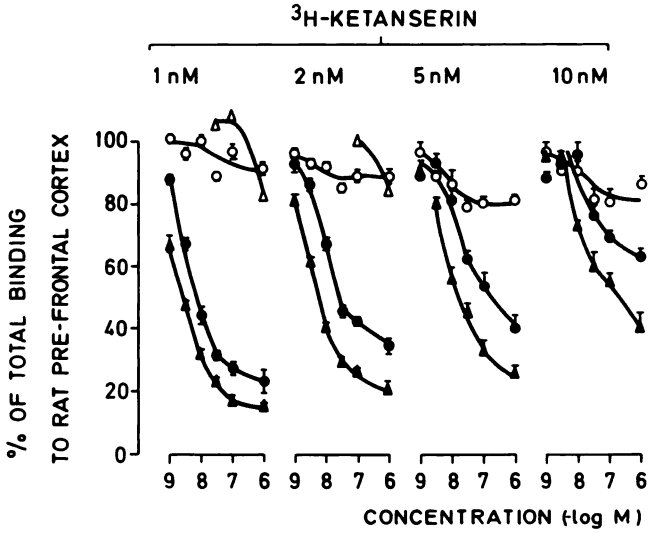

FIG. 2. *Inhibition curves of ketanserin (▲), methysergide (●), prazosin (○), and pyrilamine (△) for [^{3}H]ketanserin binding to rat prefrontal cortex*

[^{3}H]Ketanserin concentrations and corresponding values of total binding were as follows: 1 nM, 27.6 ± 2.4 fmoles/mg of tissue; 2 nM, 36.7 ± 2.5 fmoles/mg of tissue; 5 nM, 53.2 ± 3.7 fmoles/mg of tissue; 10 nM, 67.6 ± 1.7 fmoles/mg of tissue. All data are mean values ± standard error of the mean obtained in four independent experiments. Experiments were performed in a volume of 2.2 ml of Tris buffer containing (M + L + P) membranes corresponding to 10 mg of tissue (original wet weight).

TABLE 1

Levels of [^{3}H]ketanserin binding (2nM) to rat prefrontal cortex membranes in Tris and Tris-SALT buffer, using various incubation volumes and tissue concentrations

Amount of tissue	Volume of incubation medium	[^{3}H]Ketanserin binding at 2 nM					
		Tris buffer			Tris-SALT buffer		
		TB[a]	TB/added[b]	SB[c]/TB	TB	TB/added	SB/TB
mg	*ml*	*dpm*	%	%	*dpm*	%	%
10	1.1	10 770	13	81	6 050	7.3	79
5	1.1	5 850	7.1	73	3 350	4.1	68
2.5	1.1	3 310	4	63	1 830	2.2	58
20	2.2	19 300	11.4	78	11 220	6.6	80
10	2.2	11 160	6.6	73	6 520	3.9	76
5	2.2	6 380	3.8	58	3 550	2.1	60
20	4.4	21 000	6.3	73	12 490	3.8	77
10[d]	4.4[d]	13 060	3.9	74	6 720	2.0	67
5	4.4	8 100	2.4	49	4 440	1.3	62

[a] TB, totally bound radioactivity.
[b] Added, total amount of radioactivity added to the incubation volume.
[c] SB, specifically bound radioactivity: difference between totally bound and nonspecifically bound in the presence of 1 μM methysergide.
[d] Chosen as standard assay procedure.

values > 1000 nM for other neurotransmitter receptor binding sites (data measured as in ref. 14). [^{3}H]Ketanserin was used at concentrations of 1, 2, 5, and 10 nM; levels of total binding at the various ligand concentrations are given in the legend to Fig. 2. Methysergide inhibits binding at concentrations between 1 and 100 nM in a regular concentration-dependent fashion: maximal inhibition by 1 μM amounts to 75%, 65%, 60%, and 38% of total binding at 1, 2, 5, and 10 nM [^{3}H]ketanserin, respectively. Prazosin at 0.1 and 1 μM inhibits only 10–20% of the total binding. Pyrilamine does not inhibit the binding up to 0.1 μM, but an inhibition of 20% is seen at 1 μM. Unlabeled ketanserin inhibits the [^{3}H]ketanserin binding at nanomolar concentrations, and plateau levels of maximal inhibition are 7–20% below the level of maximal inhibition by 1 μM methysergide. In this report, specific binding of [^{3}H]ketanserin refers to the binding over nonspecific binding measured in the presence of 1000-fold excess methysergide. This appears to represent binding to S_2 receptor binding sites (see Discussion).

To define an optimal tissue to volume ratio for detection of specific [^{3}H]ketanserin binding, the binding of 2 nM [^{3}H]ketanserin was tested in a volume of 1.1, 2.2, and 4.4 ml containing various amounts of rat prefrontal cortex tissue in Tris buffer and in Tris-SALT buffer. Data on total membrane labeling, percentage of totally bound versus totally added radioactivity, and percentage of specific versus total binding are presented in Table 1. In Tris buffer the binding is 70–95% higher than in corresponding assays in the Tris-SALT buffer. Depending on the volume and the tissue content, 2–13% of added radioactivity becomes bound. An incubation volume of 4.4 ml containing a total of 10 mg of tissue (original wet weight) as an (M + L + P) membrane preparation was chosen for further standard binding assays. Under this condition, specific binding varied linearly with the tissue concentration. The large volume and diluted tissue suspension was preferred to avoid substantial loss of freely solubilized compounds because of adsorption by tissue and labware. This precaution is necessary since the danger of reduction in free drug concentration not only pertains to the labeled ligand (where it can be controlled) but also to unlabeled competitors (examples are known where highly lipophilic compounds become adsorbed by more than 90%; ref. 25).

Analysis of binding equilibrium, binding rates, and inhibition of specific [^{3}H]ketanserin binding. [^{3}H]Ketanserin saturation curves (concentration range 0.2–5 nM) for binding to an (M+ L + P) membrane fraction of rat prefrontal cortex were measured (a) in Tris buffer, (b) in Tris buffer containing ascorbic acid plus pargyline, (c) in Tris-SALT buffer, and (d) in Tris-SALT buffer containing ascorbic acid and pargyline. The saturation curve for total and specific binding and the Scatchard plot of specific binding for the experiments performed in Tris buffer, are shown in Fig. 3. It can be seen that the 4 × 10 points obtained in four independent series of experiments can be joined by a straight line in the Scatchard plot. Similarly performed experiments using the other three buffers yielded similar results: high reproducibility between independent experiments and straight Scatchard plots (data not shown). K_D and B_{max} values were calculated from Scatchard plots of separate series of experiments by regression analysis; mean values are shown in Table 2. The highest binding affinity (K_D = 0.42 nM) and highest specific binding site density (B_{max} = 30.9 fmoles/mg of tissue) were found with Tris buffer. The addition of 0.1% ascorbic acid and 1 μM pargyline reduced the binding affinity 1.5-fold and the maximal density in binding sites by 30%. In the presence of SALT with or without ascorbic acid and pargyline, the binding affinity was 3 times lower and the B_{max} value was about 25% lower than in the presence of Tris buffer without additives.

Time curves for reaching equilibrium of specific binding and for dissociation of the [^{3}H]ketanserin binding induced by the addition of 1000-fold excess methysergide at 37° are shown in Fig. 4. For experiments in Tris buffer, [^{3}H]ketanserin was used at 2 nM (saturating concentration of 5 times the K_D value) and at 0.6 nM (subsaturating

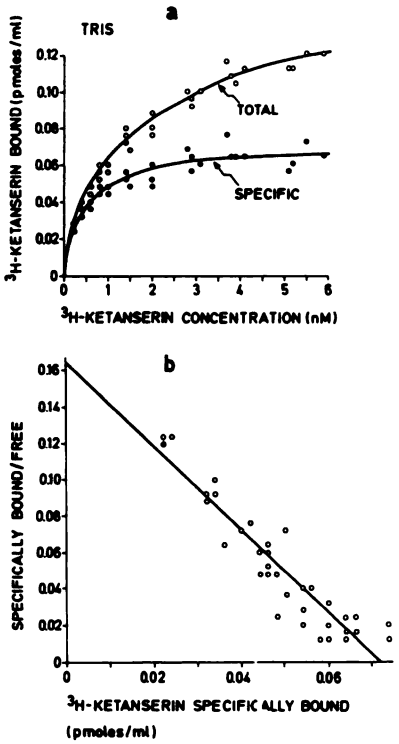

FIG. 3. *Concentration binding curves of total and specific [³H] ketanserin binding to (M + L + P) membranes of rat prefrontal cortex (a) and Scatchard plots of specific binding (b) in Tris buffer, using the standard assay procedure described under Materials and Methods*

Specific binding was obtained by calculating the difference between total binding and binding in the presence of 1000-fold excess methysergide, and the free [³H]ketanserin concentration was obtained by calculating the difference between totally added and totally bound [³H] ketanserin. In the graphs, points obtained in four independent series of experiments are pooled. Mean K_D and B_{max} values derived from Scatchard plots of separate experiments are presented in Table 2.

concentration of 1.4 times the K_D value). Measurements in Tris-SALT buffer were performed at 2 nM [³H]ketanserin (subsaturating concentration of 1.8 times the K_D value). Both association and dissociation were faster in Tris-SALT than in Tris buffer. The half-lives of dissociation derived from linear log [specific binding] versus time plots were 1 and 3.5 min in Tris-SALT and Tris buffer, respectively. Measurements at subsaturating and saturating [³H]ketanserin concentrations yielded the same dissociation rate. Association was too fast to obtain enough reliable points of measurements for calculation of association rates. Full association was reached after 2 min in Tris-SALT buffer and after 3 min in Tris buffer.

Inhibition isotherms and corresponding Hill plots of R 47 465 [3-{2-[4-(4-fluorobenzoyl)-1-piperidinyl]ethyl}-2-methyl-4H-pyrido[1,2-a]pyrimidin-4-one, an LSD antagonist; receptor profile and pharmacological properties in ref. 26], pizotifen, LSD, spiperone, and amitryptiline are shown in Fig. 5. All of these compounds have alleged serotonergic antagonist or mixed agonist-antagonist properties (27) and belong to various pharmacological and chemical classes. For these compounds, inhibition is completed over a concentration range of 2 orders of magnitude with slopes for the Hill plots of 1–1.17. Inhibition isotherms and Hill plots for serotonin agonists and

TABLE 2

Equilibrium binding constants of specific [³H]ketanserin binding at 37° to (M + L + P) membranes of rat prefrontal cortex in Tris buffer with various additives

K_D and B_{max} values are means of values derived from three or four independent series of experiments. Individual values were obtained from Scatchard plots of seperate experiments and calculated by linear regression analysis. Experiments were performed as described in the legend to Fig. 3.

Buffer additive	K_D[a]	B_{max}[a] (mean ± SEM, *n*)
	nM	*fmoles/mg tissue*
None	0.42 ± 0.02 (4)	30.9 ± 1.2 (4)
Ascorbic acid, 0.1% Pargyline, 1 μM	0.62 ± 0.06 (3)	21.6 ± 1.8 (3)
SALT	1.29 ± 0.09 (3)	23.1 ± 1.5 (3)
SALT Ascorbic acid, 0.1% Pargyline, 1 μM	1.13 ± 0.09 (4)	22.3 ± 1.3 (4)

[a] Values are means ± standard error of the mean. Numbers in parentheses are numbers of experiments.

apomorphine are shown in Fig. 6. The inhibition curves range over 2.5–3.5 orders of magnitude and slopes in Hill plots are between 0.67 (for serotonin) and 1 (for tryptamine). All curves in Figs. 5 and 6 at maximal inhibition reach plateau values around the value for nonspecific binding measured with 1 μM methysergide. IC_{50} values of the compounds are given by the intersection point between inhibition isotherm and the middle line between

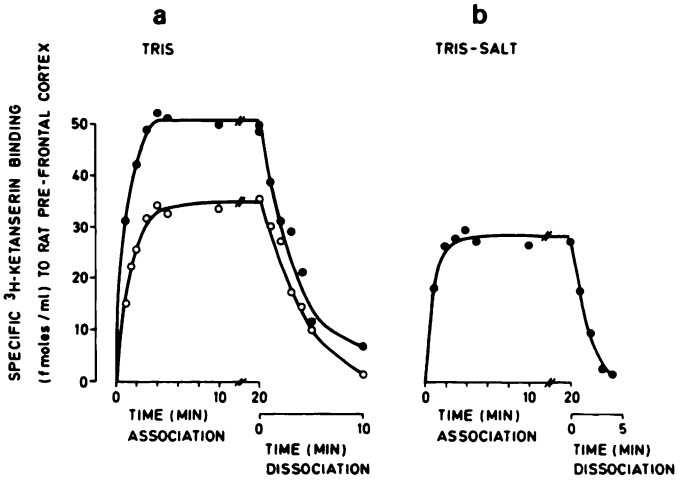

FIG. 4. *Time curves for association and dissociation (induced by adding 1 μM methysergide) of specific [³H]ketanserin binding to (M + L + P) membranes of rat prefrontal cortex in Tris buffer (a) and Tris-SALT buffer (b)*

[³H]Ketanserin was used at 2 nM (●) and at 0.6 nM (○); specific binding was obtained by calculating the difference between total binding and binding in the presence of 1000-fold excess methysergide; the standard assay procedure was used. Association was too fast to allow an accurate determination of the rate constant. For the dissociation, linear log (specific bound) versus time plots revealed a k_{-1} of 0.2 min^{-1} and a $t_{1/2}$ of 3.5 min in Tris-buffer (similar values were obtained when using 0.6 nM and 2 nM [³H]ketanserin) and a k_{-1} of 0.7 min^{-1} and a $t_{1/2}$ of 1 min in Tris-SALT buffer.

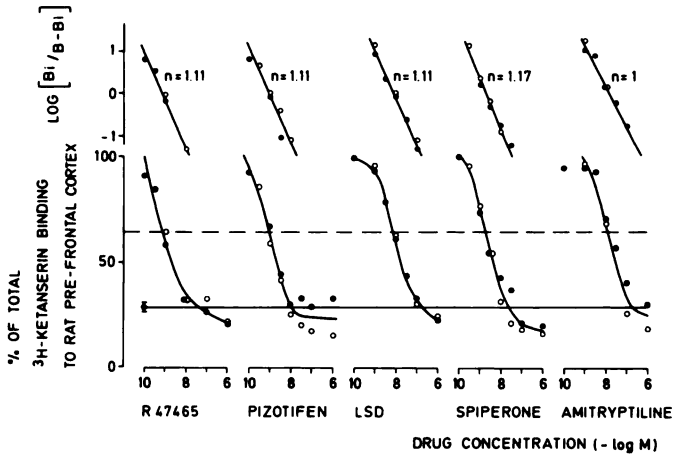

FIG. 5. *Inhibition of [³H]ketanserin binding (1 nM) to rat prefrontal cortex membranes in Tris buffer by alleged serotonin antagonists or mixed agonist-antagonists*

The standard assay procedure was used. Total binding per assay was 9230 ± 400 DPM (n = 8); nonspecific binding in the presence of 1 μM methysergide was 2650 ± 250 dpm. *Upper graphs* show Hill plots (21) of specific binding: B_i = specific binding in the presence of inhibitor; B = specific binding in the absence of inhibitor. The slopes, n, of the lines are indicated. *Lower graphs* show inhibition isotherms fitted by hand: ● and ○ indicate data from independent experiments; ——, the level of nonspecific binding; -·-·-·-, middle line between total and nonspecific binding (the intersection point between this line and an inhibition isotherm gives the IC_{50} value of the drug.

total and nonspecific binding. Values for total and nonspecific binding are reported in the legend to Fig. 5.

Similar K_D and B_{max} values for the different [³H]ketanserin preparations and the same K_i values for unlabeled drugs were obtained with radiochemical preparations of [³H]ketanserin of 87.5 Ci/mmole (New England Nuclear Corporation) of a purity >99%. Presoaking the filters in 0.025% BRY, but rinsing with the usual Tris buffer, did not influence these values.

Regional distribution of specific [³H]ketanserin binding in rats and guinea pigs. [³H]Ketanserin binding at a concentration of 2 nM was assayed in Tris buffer in 18 brain areas, spinal cord, and pituitary of rats and guinea

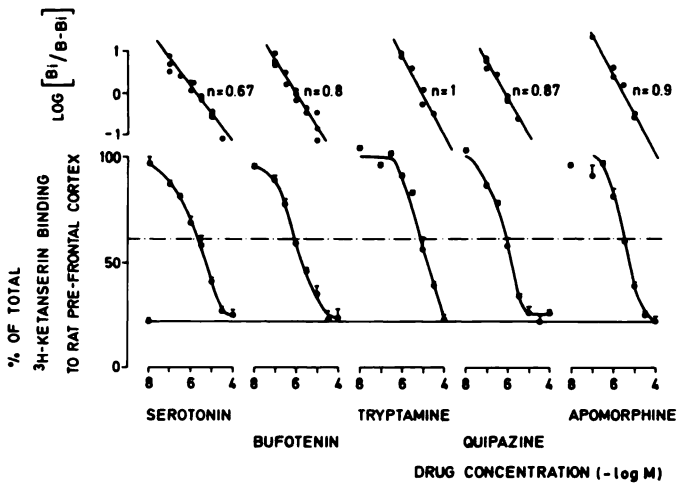

FIG. 6. *Inhibition by serotonin agonists and apomorphine investigated in a manner similar to that described in the legend to Fig. 5.*

Points in the inhibition isotherms indicate mean values obtained in three independent experiments.

pigs. The various areas with specific binding inhibited by methysergide are listed in Table 3. In both species, a substantial amount of specific binding is found in frontal and temporal cortex areas and in mesolimbic and extrapyramidal dopaminergic areas. A number of the remaining areas showed low specific binding (2–3 pmoles/g of tissue). In rats, specific binding was virtually not detectable in occipital cortex, hypothalamus, cerebellum, bulbus olfactorium, and spinal cord, whereas these areas in guinea pigs revealed low specific binding. Virtually no specific binding was found in pituitary of either species. Specific binding at 2 nM [³H]ketanserin was not detectable in guinea pig heart, lung, kidney, or stomach (membrane preparations were assayed using 40 mg of tissue (original wet weight) per 4.4 ml of incubation volume). In guinea pig brain areas and peripheral organs, [³H]ketanserin binding at 2 nM was assayed for inhibition by 1 μM prazosin and 1 μM pyrilamine to trace possible labeling of *alpha*$_1$-adrenergic and H_1 histamine receptor sites. Prazosin inhibited only 3–1 pmoles of [³H]ketanserin binding per g of tissue. Most inhibition occurred in areas with the greatest total binding. Pyrilamine at 1 μM caused a somewhat greater inhibition (nearly one-half the amount of specific binding inhibited by methysergide). However, this appeared to be due to the high concentration of pyrilamine, causing interference with S_2 receptor binding sites, no inhibition of binding was observed at submicromolar concentrations of pyrilamine. K_D and B_{max} values of specific [³H]ketanserin binding were determined in the seven brain areas of rats and guinea pigs showing high specific binding (≥7 pmoles/g of tissue) at 2 nM [³H]ketanserin. All areas were always measured on the same day; the experimental procedure was the same as for experiments shown in Fig. 3, except that another [³H]ketanserin preparation (see Materials and Methods) was used. The data presented in Table 3 reveal that in both species the highest binding site density occurs in the prefrontal cortex and in the frontoparietal cortex. In the nucleus accumbens, the B_{max} value is nearly as high as in the above-mentioned cortical areas, but the K_D value measured in the nucleus accumbens is about 2 times greater than in the other areas. In the tuberculum olfactorium, the temporal cortex, striatum, and occipitoparietal cortex, the number of binding sites represents 64%–33% of the number of sites in the richest cortical area. Some minor differences in the distribution of the specific binding sites between rats and guinea pigs are observed. In some areas, B_{max} values in picomoles per gram of tissue are greater in rats than in guinea pigs; however, it is noted that guinea pig membrane preparations have a lower protein content than those of the rat. Except for the nucleus accumbens, K_D values are about 0.5–0.7 nM; the standard errors of the mean K_D values of two determinations are rather low, yet two determinations are not sufficient to allow speculations on small differences in binding affinity. The K_D and B_{max} values in rat prefrontal cortex found in this series of experiments are not included in the values reported in Table 2, but it is obvious that they correspond completely.

The specific binding of [³H]ketanserin in the striatum was proven to be of serotonergic nature since various serotonergic antagonists such as methysergide, cyproheptadine, and pipamperone inhibited the binding at

TABLE 3

Regional distribution in rat and guinea pig brain of specific [^{3}H]ketanserin binding

Specific binding was taken as the difference between total binding and nonspecific binding in the presence of 1000-fold excess methysergide. Binding assays were carried out in Tris buffer using the standard procedure described under Materials and Methods. Values are means ± standard error of the mean of two independent experiments in triplicate. K_D and B_{max} values were derived from experiments performed in a manner similar to that described in Fig. 3, but using another [^{3}H]ketanserin preparation (see Materials and Methods).

Brain area	Rat				Guinea pig			
	Specific binding at 2 nM		B_{max} (pmoles/g tissue)	K_D (nM)	Specific binding at 2 nM		B_{max} (pmoles/g tissue)	K_D (nM)
	pmoles/g tissue	% total binding			pmoles/g tissue	% total binding		
Prefrontal cortex	23.7 ± 0.5	71 ± 1	33.1 ± 1.2	0.50 ± 0.03	14.6 ± 0.9	70 ± 1	24.3 ± 2.1	0.42 ± 0.04
Frontoparietal cortex	13.7 ± 1.6	59 ± 1	30.1 ± 2.8	0.85 ± 0.03	15.5 ± 0.1	71 ± 1	29.7 ± 0.5	0.52 ± 0.03
Nucleus accumbens	6.9 ± 0.1	27 ± 2	26.0 ± 4.6	1.07 ± 0.08	10.68 ± 0.01	47 ± 2	22.1 ± 2.8	1.15 ± 0.16
Tuberculum olfactorium	10.3 ± 0.7	42 ± 5	21.4 ± 2.2	0.52 ± 0.01	7.8 ± 0.2	49 ± 1	14.1 ± 4.0	0.56 ± 0.12
Temporal cortex	10.7 ± 2.0	51 ± 1	19.4 ± 0.9	0.67 ± 0.13	6.8 ± 1.6	58 ± 7	11.2 ± 1.2	0.73 ± 0.08
Striatum	7.6 ± 0.4	28 ± 4	15.3 ± 1.8	0.64 ± 0.02	7.5 ± 0.2	38 ± 1	9.3 ± 1.3	0.56 ± 0.14
Occipitoparietal cortex	3.8 ± 1.0	24 ± 1	11.3 ± 0.9	0.51 ± 0.12	7.8 ± 3.5	55 ± 14	13.0 ± 2.1	0.45 ± 0.04
Substantia nigra	4.5 ± 0.2	29 ± 1			3.2 ± 0.5	32 ± 4		
Thalamus (frontal part)	3.1 ± 0.1	23 ± 1			3.2 ± 0.2	31 ± 2		
Amygdala	3.0 ± 1.7	21 ± 8			3.8 ± 1.3	44 ± 6		
Hippocampus	2.5 ± 1.3	21 ± 7			2.6 ± 0.7	35 ± 8		
Thalamus (occipital part)	2.3 ± 0.2	16 ± 1			3.1	31		
Medulla oblongata	2.3 ± 0.9	19 ± 5			2.4 ± 0.4	27 ± 3		
Raphe area	2.0 ± 1.3	15 ± 9			3.4 ± 0.5	31 ± 2		
Occipital cortex	1.5 ± 1.9	18 ± 17			5.4 ± 1.5	53 ± 3		
Hypothalamus	0.7 ± 0.4	5 ± 2			5.2 ± 0.6	41 ± 1		
Cerebellum	ND[a]				3.1 ± 1.6	36 ± 11		
Bulbus olfactorius	ND				3.3	49		
Spinal cord	ND				3.3 ± 0.2	37 ± 1		
Pituitary	ND				0.5 ± 0.2	12 ± 4		

[a] ND, Not detectable.

nanomolar concentrations whereas haloperidol and domperidone, which are more selective dopamine antagonists, and prazosin and pyrilamine did not inhibit the binding at concentrations below 100 nM. A peculiar observation is that nonspecific binding in the striatum and nucleus accumbens of both rat and guinea pig is twice as high as in the other brain areas (the values can be calculated from the data in Table 3). [^{3}H]Ketanserin binding in the presence of excess methysergide was not additionally inhibited by dopaminergic, adrenergic, and histamine antagonists and agonists, showing that the high nonspecific binding in the striatum was not due to labeling of other neurotransmitter receptors.

Inhibitory potency of various drugs and relationship to pharmacological activity. For 65 compounds belonging to various pharmacological and chemical classes IC_{50} values for inhibition of specific [^{3}H]ketanserin binding to rat prefrontal cortex membranes were derived from inhibition isotherms (examples given in Figs. 5 and 6). The list of compounds, grouped according to their purported pharmacological activity, is presented in Table 4. Most extensively investigated was the inhibition of specific [^{3}H]ketanserin binding assayed in Tris buffer (yielding lowest K_D and highest B_{max} values); mean log IC_{50} values and derived K_i values are shown in Table 4. For a number of compounds the inhibition was also measured in Tris-SALT buffer (K_i values in Table 4). For comparison, K_i values for inhibition of stereospecific [^{3}H]spiperone binding to rat prefrontal cortex membranes in Tris-SALT buffer [values derived from previously published (6, 14) and original data[3]] are also included in Table 4. For all compounds, the ratio in K_i values for [^{3}H]ketanserin binding measured in Tris and Tris-SALT buffer was 2.4 ± 0.2; this ratio is similar to the ratio in K_D values of [^{3}H]ketanserin in the different buffers. Purported serotonin antagonists of various chemical classes [(a) ketanserin derivatives, (b) tricyclic cyproheptadine-like compounds, (c) ergoline derivatives, (d) a butyrophenone/pipamperone, (e) xylamidine, and (f) cinanserin] are extremely potent inhibitors of [^{3}H]ketanserin binding, showing K_i values between 0.28 and 2.5 nM. Among dopamine antagonists, spiperone is a very potent inhibitor of [^{3}H]ketanserin binding, whereas selective dopamine antagonists such as haloperidol, penfluridol, and domperidone are very weak. For the histamine antagonists, the new long-acting antihistaminic astemizole (25) and its derivative R 43 448 (12) show rather high affinity for the specific [^{3}H]ketanserin sites, whereas pyrilamine has an IC_{50} above 1 μM. Adrenergic antagonists at *alpha*$_1$-, *alpha*$_2$- and *beta*-receptors, with the exception of phenoxybenzamine, are very weak to inactive inhibitors. Among amine reuptake blockers, tricyclic derivatives show a high to moderate inhibitory potency—amitryptiline being the most active—whereas compounds of other chemical classes are virtually inactive.

In a series of various neutrotransmitter agonists, the

[3] J. E. Leysen, unpublished observations.

TABLE 4

Inhibitory potency of various antagonists and agonists of biogenic amines for specific [^{3}H]ketanserin binding (1 nM) in Tris and in Tris-SALT buffer and for stereospecific [^{3}H]spiperone binding (2 nM) in Tris-SALT buffer using rat prefrontal cortex tissue

−Log IC_{50} values (IC_{50}: concentration inhibiting 50% of specific [^{3}H]ketanserin binding) were derived from inhibition isotherms as presented in Figs. 5 and 6. Values for inhibition of [^{3}H]ketanserin binding in Tris buffer are means ± standard error of the mean of two or three independent experiments; values measured in Tris-SALT buffer are from single inhibition isotherm. Values for inhibition of [^{3}H]spiperone binding are calculated from IC_{50} values in refs. 6 and 14 and from unpublished mean IC_{50} values (n = 2 or 3), K_i values were calculated according to the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + C/R_D)$ with C the concentration and K_D the equilibrium dissociation constant of the ^{3}H-ligand (for [^{3}H] ketanserin, K_D values in Table 3; for [^{3}H]spiperone, K_D = 0.97 nM). A Spearman rank correlation coefficient r_s = 0.96, $p < 0.01$, n = 33 between K_i values for specific [^{3}H]ketanserin binding sites in Tris buffer and K_i values for stereospecific [^{3}H]spiperone binding sites in Tris-SALT buffer was found (the extremely high K_i values for the tetralin derivative and dopamine were not taken into account).

Agent	[^{3}H]ketanserin			[^{3}H]Spiperone: Tris-Salt, K_i
	Tris		Tris-Salt, K_i	
	−log IC_{50}	K_i		
	M	nM	nM	nM
Serotonin antagonists				
Ketanserin derivatives				
Ketanserin	8.88 ± 0.11	0.39		2.1
R 47 465	9.03 ± 0.11	0.28	0.59	2.0[b]
Tricyclic cyproheptadine compounds				
Pizotifen	9.03 ± 0.11	0.28	0.84	6.5
Clopipazan	8.88 ± 0.04	0.39		
Metitepine	8.88 ± 0.04	0.39		1.9
Cyproheptadine	8.83 ± 0.04	0.44	1.7	6.5
Mianserin	8.33 ± 0.04	1.4		13
Ergoline derivatives				
Metergoline	9.03 ± 0.25	0.28	0.67	0.8
Methysergide	8.50 ± 0.07	0.94	3.0	12
LSD	8.08 ± 0.11	2.5	4.2	8.2
Pipamperone	8.58 ± 0.04	0.78	2.4	5.3
Xylamidine	8.30 ± 0.07	1.5	3.0	18[b]
Cinanserin	8.18 ± 0.04	2.0		41
Dopamine antagonists				
Spiperone	8.75 ± 0.07	0.53	1.9	1.2
Benperidol	8.38 ± 0.32	1.2		6.5
Clozapine	8.05 ± 0.07	2.6		16
Chlorpromazine	7.95 ± 0.07	3.3		20
Fluspirilene	7.98 ± 0.25	3.1		41[b]
Pimozide	7.70 ± 0.01	5.9	8.4	33
Haloperidol	7.13 ± 0.18	22	60	48
Penfluridol	6.70 ± 0.14	59		327[b]
Domperidone	6.58 ± 0.18	78	335	327[b]
Histamine antagonists				
R 43 448	8.88 ± 0.11	0.39	1.1	2.3[b]
Astemizole	7.75 ± 0.07	5.3		33[b]
Ketotifen	7.25 ± 0.07	17	75	231[b]
Pyrilamine	<6			2600[b]
Adrenergic antagonists				
Phenoxybenzamine	7.35 ± 0.07	13	150	111[b]
Phentolamine	6.2	187		
Prazosin	<6			
WB 4101	<6			4110[b]
Yohimbine	5.65	660		
Propranolol	5.7	590		
Alprenolol	5.1	2350		
Amine reuptake blockers				
Amitryptiline	7.85 ± 0.01	4.2	15	21[b]
Chlorimipramine	7.48 ± 0.11	9.8		82[b]
Benztropine	7.23 ± 0.04	17		206[b]
Protryptiline	7.05 ± 0.01	26		
Imipramine	6.90 ± 0.21	37	53	260[b]
Desimipramine	6.58 ± 0.04	78	335	
Nomifensine	5.90 ± 0.99	372		
Citalopram	5.25 ± 0.07	1660		
(+)-Imafen	<5			
(−)-Imafen	<5			

TABLE 4—*Continued*

Agent	[^{3}]Ketanserin			[^{3}]Spiperone: Tris-SALT, K_i
	Tris		Tris-SALT, K_i	
	$-\log IC_{50}$	K_i		
	M	*nM*	*nM*	*nM*
Serotonin agonists				
Bufotenin	6.4 ± 0.4	118		518
Quipazine	6.1 ± 0.2	235	335	
5-Hydroxytryptamine	6.0 ± 0.3	296	335	1,033
5-Methoxytryptamine	5.6 ± 0.4	742	668	
Tryptamine	5.3 ± 0.4	1,482	2,660	5,420
Mescaline	4.7 ± 0.3	5,900		
Dopamine agonists				
Apomorphine	5.5 ± 0.1	935		3,270
2-(*N*,*N*-Dipropyl)amino-5,6-dihydroxytetraline	4.1	23,500		26,000
Dopamine	3.5 ± 0.2	93,500		82,000
Adrenergic agonists				
Isoproterenol	3.9 ± 0.1	37,200		
Epinephrine	3.8 ± 0.1	47,000		
Phenyleprine	3.3 ± 0.1	150,000		
Norepinephrine	<3			
Clonidine	<3			
Miscellaneous[b]				
Acetylcholine	3.4 ± 0.1	120,000		
Glutamic acid	<3			
γ-Aminobutyric acid	<3			

[a] Unpublished data.[3]

[b] Inactive at 1 μM: dexetimide, atropine, diazepam, clonazepam, morphine.

serotonin agonists are by far the most potent inhibitors of specific [^{3}H]ketanserin binding. They exhibit K_i values between 0.1 and 6μM, which are more than 100 times lower than K_i values of dopaminergic and adrenergic agonists. An exception is apomorphine, which is only a 3-times less potent inhibitor of [^{3}H]ketanserin binding than serotonin. Many neurotransmitters such as norepinephrine, histamine, acetylcholine, and amino acids are virtually inactive ($K_i > 1$ mM).

The correlation between K_i values for [^{3}H]ketanserin binding in Tris buffer and K_i values for [^{3}H]spiperone binding is highly significant (Spearman rank correlation coefficient $r_s = 0.96$, $p < 0.01$) on the basis of a series of 33 compounds. However, inspection of the ratio of K_i values for individual compounds reveals some marked exceptions. All compounds have an apparent higher binding affinity for specific [^{3}H]ketanserin binding sites than for stereospecific [^{3}H]spiperone binding sites in rat prefrontal cortex; however, the serotonin antagonists pizotifen, mianserin, and cyproheptadine have a 23- to 15-times lower K_i value for [^{3}H]ketanserin binding than for [^{3}H]spiperone binding, whereas haloperidol, spiperone, metergoline, and LSD have only a 3- to 2-times lower K_i value in the former than in the latter test. Equal K_i values are found for dopamine and the tetraline derivative.

For 19 compounds the correlations between K_i values for [^{3}H]ketanserin binding and the potencies *in vivo* for antagonizing tryptamine-induced clonic seizures (28) and mescaline-induced head-twitches (29) in rats are shown in Fig. 7a and b, respectively. Highly significant correlations are found with a somewhat higher Spearman rank correlation coefficient for comparison with activity in the mescaline test ($r_s = 0.88$) than for comparison with activity in the tryptamine test ($r_s = 0.83$). It is noteworthy that haloperidol and also chlorpromazine are considerably more potent in both *in vivo* tests than would be expected from their K_i values *in vitro*. On the contrary, ketanserin is markedly weakly active in the tryptamine test, whereas it has an activity in the mescaline test corresponding to its K_i value. Correlations regarding the same 19 compounds were also made between K_i values for [^{3}H]spiperone binding and the activities in the *in vivo* tests; comparison with the tryptamine test revealed a somewhat higher correlation coefficient ($r_s = 0.85$, $p < 0.01$) than comparison with the mescaline test ($r_s = 0.78$, $p < 0.01$). Figure 8a shows the correlation between the potencies of the compounds to antagonize serotonin-induced contraction in isolated preparations of rat caudal arteries (15) and the K_i values for [^{3}H]ketanserin binding ($r_s = 0.91$, $p < 0.01$, $n = 25$). The correlation coefficient regarding the same series of compounds between potencies in the caudal artery test and K_i values for [^{3}H]spiperone binding was lower ($r_s = 0.85$, $p < 0.01$) but still significant. Data in Fig. 8b show that no relationship exists between the binding affinities for the [^{3}H]ketanserin-labeled sites and potencies of compounds to antagonize serotonin-induced contractions in isolated strips of the rat fundus (15).

DISCUSSION

Specificity of [^{3}H]ketanserin binding. The receptor binding profile of ketanserin as compared with those of purported serotonin antagonists (14) indicates [^{3}H]ketan-

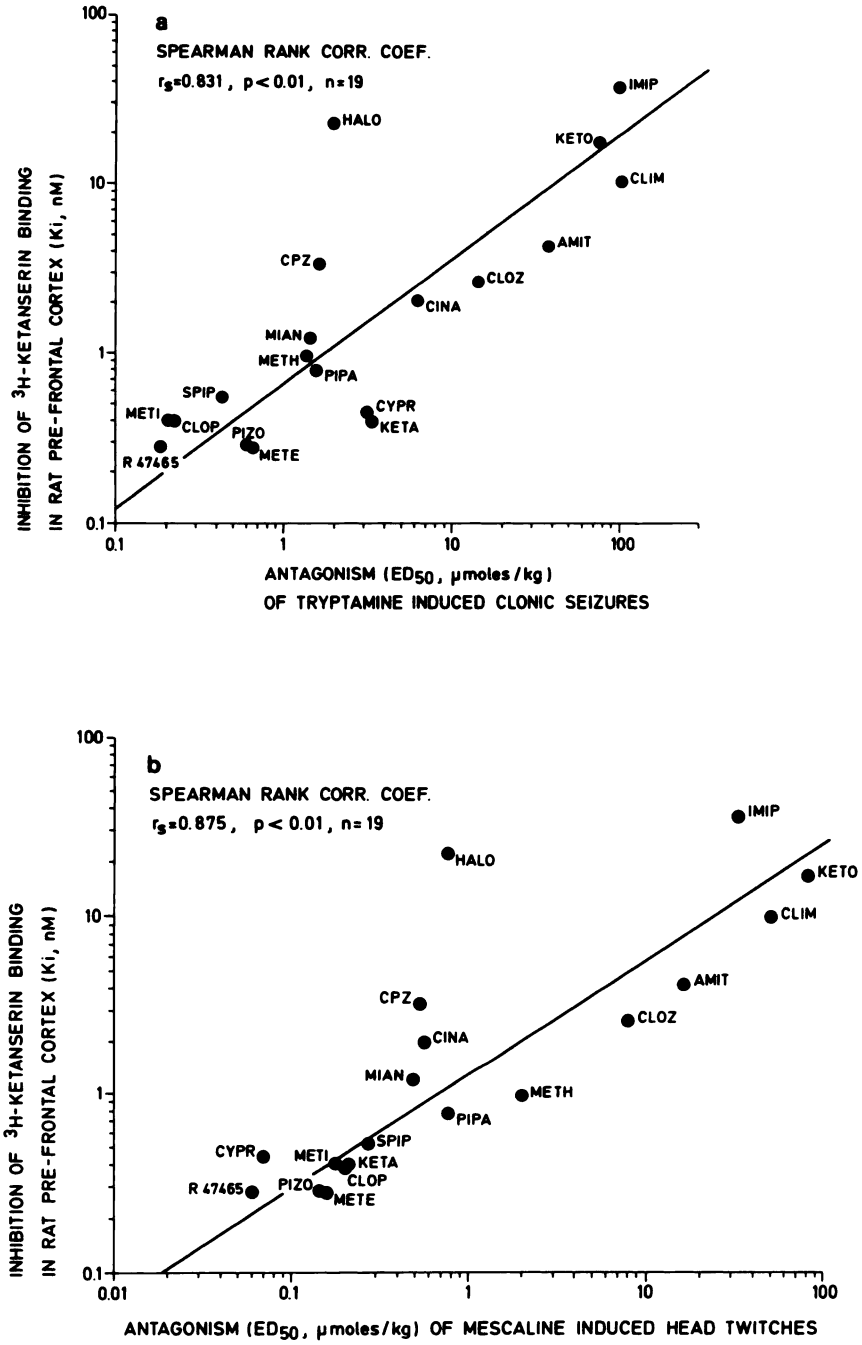

FIG. 7. *Correlation between binding affinities of drugs (K_i values) measured in Tris buffer (see Table 4) for specific [^{3}H]ketanserin binding sites in rat prefrontal cortex and the drug potencies in vivo (ED_{50}) to antagonize tryptamine-induced clonic seizures in rats (a) or to antagonize mescaline-induced head twitches in rats (b)*

ED_{50}, dose of the drugs antagonizing the effect in 50% of the animals. *In vivo* pharmacological data are measured according to published procedures (28, 29). Abbreviations are the first four letters of compounds listed in Table 4, except for chlorpromazine (*CPZ*) and chlorimipramine (*CLIM*).

serin to be the most suitable ligand for selective labeling of S_2 receptor binding sites, which have been reported to occur in large amounts in rat prefrontal cortex (5, 6, 30). It is demonstrated in this study that [^{3}H]ketanserin labels predominantly S_2 receptor binding sites and that binding to *alpha*$_1$-adrenergic or histamine (H_1) receptor sites is negligible up to a concentration of 2 nM [^{3}H] ketanserin (see Fig. 2). The slight inhibition of [^{3}H]ketanserin binding observed at only 1 μM pyrilamine cannot be considered competition for binding to histamine H_1 receptor binding sites, since it is not in agreement with the nanomolar binding affinity of pyrilamine for these sites. Methysergide is selected for defining [^{3}H]ketanserin binding to S_2 receptor binding sites because it is the only compound among known potent serotonin antagonists which lacks interaction with *alpha*$_1$-adrenergic and histamine H_1 receptor binding sites up to a concentration of 1 μM (14). The fact that methysergide does not differentiate greatly between S_1 (K_i = 99 nM) and S_2 receptor binding sites (K_i = 12 nM) is not considered inconvenient

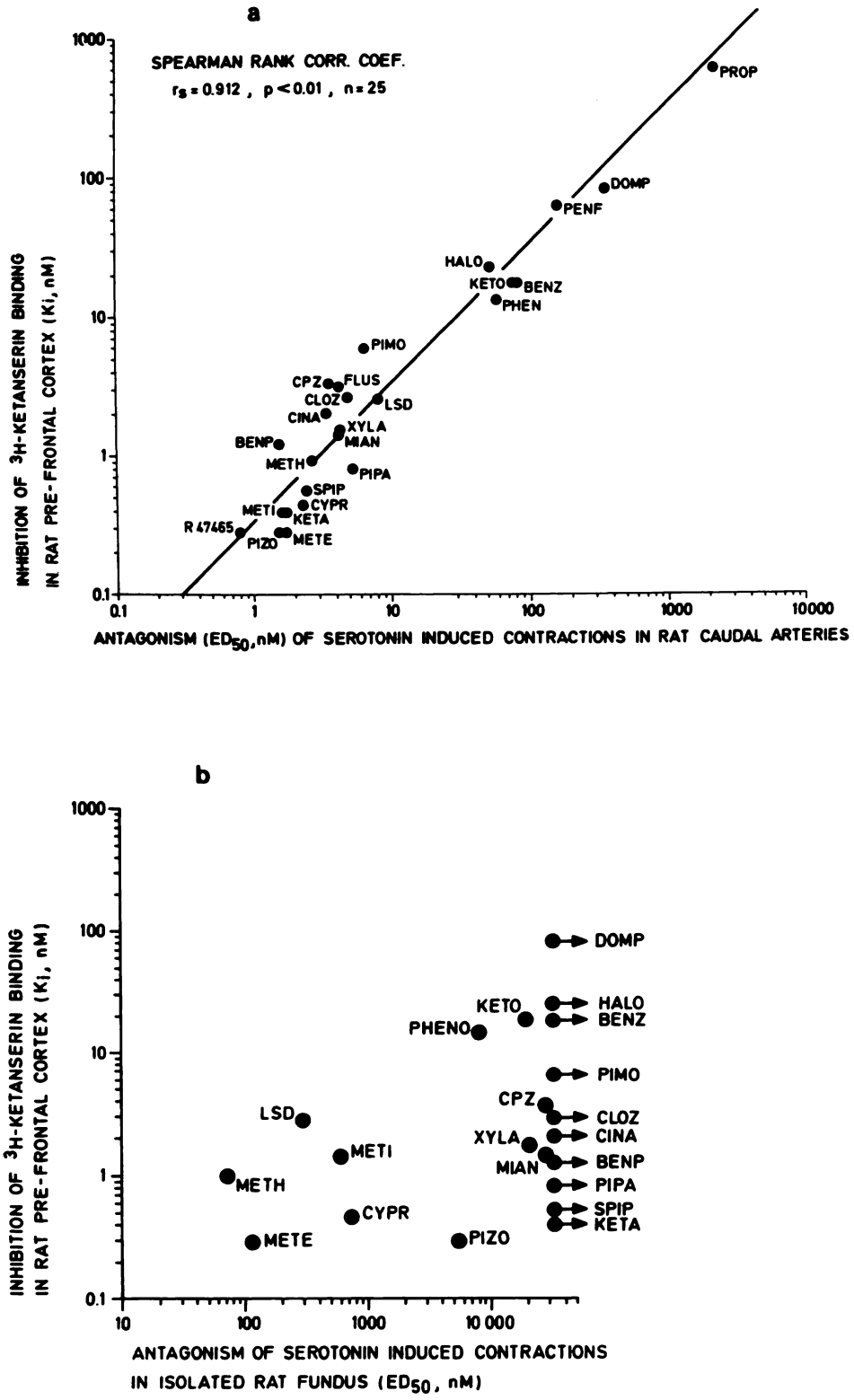

FIG. 8. *Correlation between binding affinities of drugs (K_i values) measured in Tris buffer (see Table 4) for specific [³H]ketanserin binding sites in rat prefrontal cortex and the drug potencies in vitro (ED_{50}) to antagonize serotonin-induced contractions in isolated rat caudal arteries (a) and in rat fundus strips (b)*

ED_{50}, concentration of the compound causing 50% inhibition of the original response. *In vitro* pharmacological data on the isolated organ were measured according to published procedures (15).

since [³H]ketanserin does not at all bind to the S_1 sites (14). Important points in the demonstration of the identity of the [3H]ketanserin-labeled sites are the finding of nanomolar binding affinities for serotonergic antagonists not resembling the chemical structure of ketanserin and the fact that all of the inhibition curves of various types of serotonin antagonists and agonists show a plateau at the same level of specific binding (see Figs. 5 and 6). This

proves that specific binding is accurately defined and that interaction with other receptor sites or with chemical recognition sites of the ketanserin structure is not involved. Further evidence of the specificity of [^{3}H]ketanserin binding to S_2 receptor binding sites is gained from the regional distribution studies. In no brain area is an appreciable labeling of *alpha*$_1$-adrenergic or histamine$_1$ receptor binding sites found using prazosin and pyrilamine as inhibitors, respectively.

Hence, [^{3}H]ketanserin is a far more selective ligand for S_2 receptor binding sites than are [^{3}H]spiperone, which labels dopamine receptor binding sites with higher affinity than S_2 receptor binding sites, and [^{3}H]mianserin, for which considerable interference with histamine H_1 receptor binding sites is reported (10).

Binding equilibrium properties of [^{3}H]ketanserin. Specific [^{3}H]ketanserin binding to S_2 receptor binding sites apparently fits a regular one-site interaction according to mass-action theories: Scatchard plots are linear over a wide concentration range, and the dissociation follows first-order reaction kinetics. Hence, also in this regard [^{3}H]ketanserin can be considered a superior ligand for S_2 receptor binding sites than [^{3}H]spiperone, for which nonlinear Scatchard plots were observed (30). However, [^{3}H]ketanserin-binding parameters are considerably influenced by buffer additives which are currently used in receptor binding assays. The presence of a physiological mixture of electrolytes (SALT) and/or low concentrations of ascorbic acid and pargyline significantly reduces both the binding affinity and the maximal number of receptor sites. SALT apparently increases rates of dissociation and association. It is important to note that for 18 tested compounds (see Table 4) with serotonergic antagonistic properties but listed in different chemical and pharmacological classes, a constant ratio of 2.4 ± 0.2 between binding affinities measured in Tris and Tris-SALT buffer is found. This ratio is similar to the ratio in K_D values of [^{3}H]ketanserin measured in the various buffers. This observation implies that the specific binding measured in both buffers is related to the same pharmacological receptor. At this stage the effect mechanism of the buffer additives is not yet clear. Various possibilities exist, but we believe that surface phenomena which are inherent in the interaction between ligands in solution and membrane micelles (31) probably play an important role in the effects of assay conditions on the "apparent" equilibrium binding parameters. Also, for the interpretation of shallow slopes in Hill plots such as are found here for the inhibition of [^{3}H]ketanserin binding with serotonin, the contribution of surface phenomena must not be neglected.

Regional distribution of S_2 receptor binding sites. In previous attempts to demonstrate S_2 receptor binding sites in brain areas other than the frontal cortex, using [^{3}H]spiperone, many difficulties were encountered. Auxiliary drugs had to be added to the incubation medium to prevent labeling of dopaminergic receptor sites (13), or the binding to serotonergic sites had to be differentiated by using supposedly selective inhibitors (32). Neither method was satisfactory. By virtue of its specificity, [^{3}H]ketanserin proved to be particularly suitable for detailed investigations of the regional distribution of the S_2 receptor binding sites. An attempt was made in making a comparison between two species. Large similarities were found between rats and guinea pigs. The greatest specific binding site density occurred in the frontal cortical areas, but large amounts were also observed in the dopaminergic mesolimbic areas (nucleus accumbens and tuberculum olfactorium) and the extrapyramidal area (the striatum). Also in the dopaminergic areas the specific binding sites were identified as S_2 receptor binding sites, and labeling of dopaminergic or other neurotransmitter receptor sites was not detected. The binding affinity of [^{3}H]ketanserin for the S_2 receptor sites was similarly high in both species in six of the measured brain areas. The observation of an apparently slightly lower binding affinity in the nucleus accumbens needs further confirmation and exploration before any conclusions can be drawn. It is noted that, in the striatum (dissected apart from the nucleus accumbens), the S_2 receptor binding site density is about one-fifth of the dopamine receptor site density measured with [^{3}H]haloperidol. It also appears that the distribution of the S_2 receptor binding sites is distinct from the S_1 receptor binding sites detected with [^{3}H]serotonin. According to previous reports, the latter are most enriched in the hippocampus and striatum (1, 2).

It is not known on which types of membranes the S_2 receptor binding sites are localized, and very little is known about the function of serotonin in the various areas. Innervation of cortical areas, mesolimbic areas, and the striatum by serotonergic neurones has been reported (33), and a marked specific uptake of [^{3}H]serotonin in rat prefrontal cortex synaptosomes points to the presence of serotonergic nerve terminals.[3] Hence the occurrence of serotonergic receptors in these areas is not surprising, yet it is uncertain whether a relationship exists between binding site density and the function of the receptors. In that regard it is to be mentioned that in peripheral tissues, such as in certain arteries, marked functional effects of serotonin are observed, whereas receptor binding sites are as yet not detectable in these tissues using *in vitro* binding techniques (see below).

Identity and role of S_2 receptors. The distinct binding affinities of a wide variety of compounds for the specific [^{3}H]ketanserin binding sites unequivocally demonstrate the serotonergic nature of these sites. Indeed, all compounds with alleged potent serotonin antagonist or mixed-agonist antagonist properties reveal a binding affinity of nanomolar order. Serotonin-like compounds interact with the sites at micromolar concentrations, whereas other neurotransmitters or their agonists are more than 100 times less active. The binding affinities of the serotonin-like agents seem to be related to their pharmacological potencies: bufotenin is the most active; quipazine, a 2-(1-piperazinyl)quinidine with potent serotonin-like activity in peripheral organs (34), reveals a binding affinity comparable to that of serotonin; but mescaline, showing central serotonin-like activity at high dosages (29), is less active. Otherwise, specific dopamine antagonists (haloperidol, penfluridol, domperidone); specific antihistaminics (ketotifen, pyrilamine); antagonists

at *alpha*$_1$-, *alpha*$_2$, and *beta*-adrenergic receptors; specific serotonin reuptake blockers (citalopram) or catecholamine reuptake inhibitors; antimuscarinic agents; benzodiazepines; and opiates are very weak or inactive inhibitors of [^{3}H]ketanserin binding. However, it is again noted that among substances listed in various pharmacological classes, there are compounds which, besides their alleged activity, show an important serotonergic component.

The highly significant correlation between the binding affinities of a large number of compounds for the [^{3}H]ketanserin-labeled sites and the [^{3}H]spiperone-labeled sites in the prefrontal cortex is an indication that both compounds label the same types of S_2 receptor binding sites. Nevertheless, [^{3}H]ketanserin binding involves more selectively serotonergic sites, whereas it is possible that the spiperone binding in the frontal cortex still contains a minor dopaminergic component. This is suggested by the observation that the serotonergic antagonists are relatively more potent in the [^{3}H]ketanserin binding assay than in the [^{3}H]spiperone binding assay, and vice versa for the specific dopamine antagonist.

To investigate the functional role of S_2 receptor binding sites, comparison is made with two *in vivo* and two *in vitro* pharmacological models to measure serotonergic activity. The good relationships between the binding affinities and the drug potencies in the tryptamine and mescaline test point to a role of S_2 receptors in behavioral excitation which is in agreement with previous findings using [^{3}H]spiperone as a ligand (4–6). However, behavioral excitatory effects of serotonin must be distinguished from neuronal excitation by serotonin, since no evidence exists that excitation of behavior reflects neuronal excitation in any simple or direct manner. A relationship between receptor binding and the behavioral effect must therefore not be extended to events on the neuronal level, as was suggested before (4). Despite a strong resemblance, some observations (see results in Fig. 7a and b) point to subtle differences between both *in vivo* pharmacological tests and also between the binding tests with [^{3}H]ketanserin and [^{3}H]spiperone.

The remarkable relationship between binding affinities and drug potencies in the *in vitro* pharmacological test using rat caudal arteries provides evidence that serotonin-induced vasoconstriction is mediated by S_2-type receptor binding sites. The results of this study indicate that the binding assay using [^{3}H]ketanserin is a better model for receptors involved in vasoconstriction by serotonin than is the binding assay using spiperone. However, although the role of S_2 receptor binding sites in vascular tissue is obvious, the sites have not yet been detected by ^{3}H-ligand binding in arterial homogenates. Receptor sites which mediate serotonin-induced contraction in rat fundus tissue, proposed as "D" receptors according to a pharmacological classification (35), are clearly distinct from the S_2 receptor binding sites.

It can be concluded that, because of its marked selectivity and its advantageous binding properties, [^{3}H]ketanserin is the most suitable ^{3}H-ligand thus far used for investigating S_2 receptor binding sites. It opens new perspectives for a detailed investigation of subcellular and cellular localization of the S_2 receptors and for the study of the functional role and regulation of the receptors in various areas. Using this selective agent, a more refined analysis of the link between behavioral, neuronal, and biochemical events becomes possible.

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