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Characterization of α_1-Adrenergic Receptor Subtypes in Rat Brain: A Reevaluation of [^{3}H]WB4104 and [^{3}H]Prazosin Binding

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SUMMARY

[^{3}H]Prazosin and [^{3}H]WB4101 [2-(2,6-dimethoxyphenoxyethyl)aminomethyl-1,4 benzodioxane] have both been proposed to label α_1-adrenergic receptors in the rat central nervous system. As many discrepancies between the binding of these two ligands have arisen, we conducted these studies in order to reevaluate their binding characteristics and resolve the similarities and differences in the pharmacological characteristics of their respective binding sites. [^{3}H]Prazosin binding is characterized by a monophasic saturation isotherm. Prazosin, indoramine, and dihydroergocryptine competitions with [^{3}H]prazosin are steep and monophasic, and model best to a single binding site. In contrast, phentolamine and WB4101 competition curves are shallow in rat cortex, exhibiting Hill coefficients significantly less than 1.0, and model to two binding sites of approximately equal proportions. The higher and lower affinity components are defined as α_{1A} and α_{1B}, respectively. [^{3}H]WB4101 also labels two binding sites in rat cortex and hippocampus with picomolar and nanomolar affinity, respectively. However, the nanomolar binding site is serotonergic and not adrenergic. The picomolar site (K_D = 150 pM) has characteristics of an α_1-receptor binding site: prazosin, WB4101, and phentolamine affinities for this [^{3}H]WB4101 binding site correlate with their affinities for the highest affinity component (α_{1A}) of [^{3}H]prazosin binding. In addition, the B_{max} of this [^{3}H]WB4101-labeled site is equal to one-half of the total [^{3}H]prazosin B_{max}. Agonist competitions with [^{3}H]prazosin binding are multiphasic with pseudo-Hill slopes less than 1.0 and with a rank order of affinity of epinephrine > norepinephrine > phenylephrine. When binding to the α_{1A} component is blocked by a 30 nM phentolamine mask, the same rank order of agonist affinities is preserved. Although the affinities of epinephrine and norepinephrine at the two subtypes are identical, phenylephrine is weaker at the α_{1B} site. The ratio of the potency of phentolamine versus prazosin is about 4 at the α_{1A} component but about 80 at the α_{1B} binding site. We discuss these data in relation to the reported potencies of these antagonists in blocking α_1-receptor-mediated responses which may correlate with our designation of α_{1A} or α_{1B} binding sites.

α-Adrenergic receptors have been classified into two subtypes by classical pharmacological techniques (1–3). In early studies, α-adrenergic receptor subtypes were distinguished by their anatomical localization and pharmacological specificity. Presynaptic α-adrenergic receptors (designated α_2) were hypothesized to inhibit the release of NE from nerve terminals (3), whereas postsynaptic receptors (designated α_1) were hypothesized to mediate smooth muscle contraction and many other postsynaptic effects (for review, see Ref. 1). This classification scheme has fallen into disuse, however, since receptors with pharmacological characteristics of the α_2 subtype have been localized postsynaptically (4, 5) and on platelets (6, 7) which have no innervation. Subsequently, in radioligand binding studies, α_1-adrenergic receptors have been identified by the antagonists [^{3}H]DHE, [^{3}H]WB4101, and [^{3}H]prazosin, and by the agonists [^{3}H]NE and [^{3}H]EPI. These studies have provided a deluge of information regarding the pharmacology of these binding sites but have left several important questions regarding the classification of the α_1-adrenergic receptors unanswered.

Suggestive pharmacological evidence for heterogeneity of α_1-adrenergic receptors has been reported by several groups (8–10). The presence of subtypes of α_1-adrenergic receptors could explain the observations that labeling of α_1-adrenergic receptor sites in the same tissue preparation with several structurally different ligands in parallel rarely results in the identical B_{max} values. For example, in rat liver, there is not good agreement on the total number of α_1 binding sites labeled by [^{3}H]DHE, [^{3}H]prazosin, and the agonist [^{3}H]EPI or [^{3}H]NE (11–15). These discrepancies have led to the suggestion of subtypes of α_1-adrenergic receptors in this tissue (11, 13, 14).

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ABBREVIATIONS: DHE, dihydroergocryptine; WB4101, 2-(2,6-dimethoxyphenoxyethyl)-aminomethyl-1,4 benzodioxane; NE, norepinephrine; EPI, epinephrine; IND, indoramine.

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The specificity of WB4101 for α_1-adrenergic receptors has been challenged in recent years (16, 17) as well as the conclusion from early studies that [^{3}H]WB4101 bound to a single site (4). [^{3}H]WB4101 does exhibit dual labeling of α_1- and α_2-adrenergic receptors in some peripheral tissues. This has been demonstrated in uterus in competition studies showing that WB4101 has indistinguishable affinity for α_1- and α_2-adernergic receptor-binding sites labeled by [^{3}H]DHE (18). In contrast, the selectivity of WB4101 for α_1-adrenergic receptors has been verified in other peripheral tissues *in vivo* (19, 20). In the central nervous system, [^{3}H]WB4101 binding is characterized by biphasic dissociations, nonlinear Scatchard plots, and multiphasic competitions by α-adrenergic antagonists (21–25). These studies have resolved [^{3}H]WB4101 binding into two sites, one with picomolar affinity and one with nanomolar affinity. Conversely, [^{3}H]prazosin has been considered a highly selective ligand for characterizing α_1-adrenergic receptors. However, the characteristics of its binding site do not obviously correlate with those of either of the [^{3}H]WB4101 binding sites.

The purpose of the present paper is to 1) reexamine the characteristics of [^{3}H]prazosin and [^{3}H]WB4101 binding to α_1-adrenergic receptors in the rat brain and to provide a possible explanation for the relationship between the pharmacological properties of these two ligands; and 2) determine the adrenergic characteristics of the nanomolar [^{3}H]WB4101 binding sites.

Materials and Methods

Membrane preparation. Tissue was obtained from 180–200-g Sprague-Dawley-derived rats (Simonsen Laboratories, Gilroy, CA; Charles River Breeding Laboratories), dissected, and frozen at −70° up to 1 month before assay. At the time of assay, tissue was homogenized in 50 mM Tris-HCl buffer, pH 7.7, at 25°, using 10 mg of tissue/ml in a Tekmar homogenizer. The homogenate was centrifuged twice for 10 min at 48,000 × *g* with intermediate resuspension in fresh buffer. The final pellet was suspended in buffer to give an assay tissue concentration of 3–5 mg wet weight of tissue/ml.

Radioligand binding. The assay buffer was identical to that used for tissue preparation. Ascorbic acid (0.01%) was included in assays involving catecholamines to prevent oxidation. This addition did not alter the binding properties of either ligand. Assays were conducted in duplicate or triplicate using glass test tubes containing radioligand and unlabeled drugs to yield a final assay volume of 2 ml. The assay tubes were incubated for 30 min at 25° and then the binding reaction was terminated by rapid filtration over glass fiber filters (Whatman GF/B) under vacuum. The filters were rapidly rinsed twice with 4 ml of ice-cold saline or Tris buffer. Radioactivity trapped on the filters was counted by liquid scintillation spectroscopy at an efficiency of 37–45%. Nonspecific binding of the radioligands was measured in the presence of 10^{-5} M phentolamine unless indicated otherwise. Specific binding was 80–90% of total binding for [^{3}H]prazosin and 50–70% of the total binding for [^{3}H]WB4101.

For some experiments, ligand masks have been employed to experimentally isolate a single binding site when a ^{3}H-ligand labels two binding sites. This technique has one very important advantage in that it allows the investigator to ascertain which portion of a biphasic or "two-site" competition curve reflects competition from the respective binding sites labeled by the ^{3}H-ligand. Without this information, the LIGAND analysis (see below: Data Analysis) could assign the high affinity phase of a competition curve to either site labeled by the ^{3}H-ligand and would obtain the same residual variance for the fit. In the case of [^{3}H]WB4101, prazosin (30 nM) has been used to block the highest affinity [^{3}H]WB4101 binding site so that the nanomolar affinity [^{3}H]WB4101 binding site may be studied. The affinity of the mask for each binding site labeled by the ^{3}H-ligand must be taken into account in the analysis of data obtained with the mask. Thus, the mask must label all or nearly all of one of the binding sites at the ^{3}H-ligand concentration used. Analysis of the remaining site under study must account for the possible effect of the mask at that site. For the case of [^{3}H]WB4101, the prazosin mask (30 nM) occupies nearly all of the α_1-adrenergic receptor sites labeled by [^{3}H]WB4101, but it exhibits no significant effect on the remaining sites since its affinity for those sites is so low (see Results). A phentolamine mask (30 nM) of one of the two sites labeled by [^{3}H]prazosin has also been used. In this case, we have determined that occupancy of one site is essentially complete; however, the mask will clearly occupy some of the remaining binding sites. These calculations are given under Results. When the prazosin mask was used in [^{3}H]WB4101 saturation experiments, a "sliding concentration" of mask was used to provide greater than 95% occupancy of the α_1-adrenergic receptor sites at each ^{3}H-ligand concentration.

NE uptake and preparation of synaptosomes. Fresh tissue was collected and homogenized in cold 0.32 M sucrose, pH 7.2, with a Teflon to glass homogenizer at 10 mg/ml. This suspension was centrifuged at 900 × *g* for 10 min and the resulting supernatant was centrifuged again at 12,500 × *g* for 15 min. The final pellet was then suspended in Krebs-Henseleit buffer, pH 7.4, at 25°, containing 1.1 mM ascorbic acid. Synaptosomal uptake was initiated by adding [^{3}H]NE to a final concentraton of 10^{-7} M to duplicate or triplicate tubes that were prewarmed to 37° for 4 min. The synaptosomes were then incubated for 5 min and the process was terminated by immersing the tubes in cold water (0–4°) and adding 2.5 ml of excess cold NE (10^{-4} M). The synaptosomes were then filtered under vacuum over Whatman GF/B filters and washed twice with 4 ml of cold saline. The radioactivity trapped in the filters was counted as described above. Nonspecific and low affinity uptake was estimated in the presence of 5×10^{-7}M desmethylimipramine. Specific NE uptake consisted of 75–80% of the total signal. For each sample tested, an aliquot was reserved for protein determination by the method of Lowry *et al.* (26).

Data analysis. Radioligand binding data were analyzed with the assistance of a VAX computer using the program LIGAND, an interactive nonlinear regression program based on the law of mass action (27). Saturation curves were analyzed according to a model for mass action binding of the ligand(s) to one or two binding sites. The residual variance between the actual data and each successive model was compared by statistical analysis. A model for two binding sites was utilized only when it fit the data significantly better than the model for a single binding site. Competition curves were analyzed similarly except that the affinities of the labeled ligand were set constant to those determined by saturation analyses. This allowed for a more precise determination of the affinities of the competitors at each of the one or more putative binding sites.

Materials. [^{3}H]Prazosin (20 Ci/mmol), [^{3}H]WB4101 (25 Ci/mmol), and [^{3}H]NE (44.7 Ci/mmol) were obtained from New England Nuclear. (−)EPI (−)NE, phenylephrine, dopamine, clonidine, L-isoproterenol, and ascorbic acid were purchased from Sigma Chemical Co. WB4101 was purchased from Amersham. IND, piperoxane, and verapamil were kindly provided by Dr. Paul Insel. Additional WB4101 was kindly provided by Dr. Henry Yamamura. The remaining drugs were gifts from the indicated pharmaceutical companies: prazosin, Pfizer Pharmaceuticals; phentolamine, Ciba-Geigy; DHE, Sandoz; isomers of NE and EPI, Sterling-Winthrop.

Results

Characterization of [^{3}H]WB4101 Binding: Validation of Blank Drug

Saturation isotherms for [^{3}H]WB4101 can vary depending upon the blank drug used to determine nonspecific binding. As we have reported previously under different assay conditions (28), [^{3}H]WB4101 saturation curves are biphasic when 10^{-5} M phentolamine is employed to define specific binding (Fig. 1A). Phentolamine competition with [^{3}H]WB4101 is multiphasic

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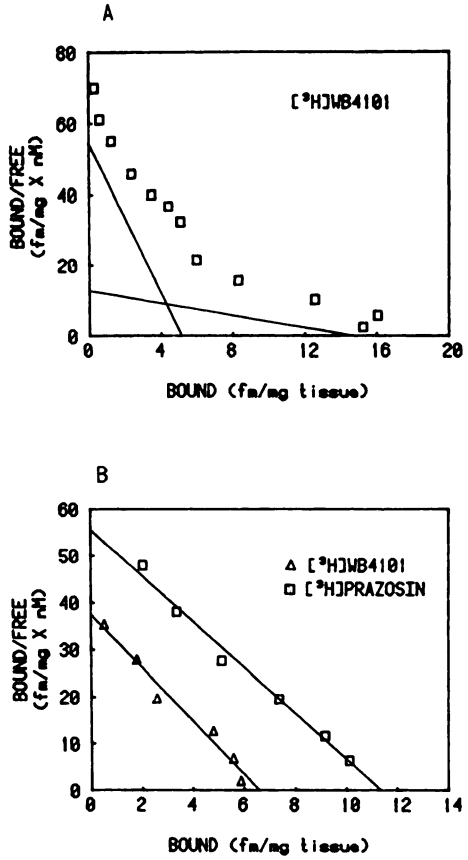

Fig. 1. A. Scatchard plot of [³H]WB4101 saturation assay in rat hippocampal membranes using 10 μM phentolamine to define specific binding. Nonlinear regression analysis revealed that the data best fit to a model for two binding sites represented by the *solid lines*. The B_{max} and K_d values of the highest affinity site in this experiment are 5.46 pmol/g of tissue and 0.10 nM, respectively. The lower affinity site has a B_{max} of 14.55 pmol/g of tissue and K_d = 1.07 nM. The concentrations of [³H]WB4101 ranged from 0.01 to 6.0 nM. B. [³H]WB4101 (△) saturation in rat hippocampal membranes using 30 nM prazosin to define specific binding. The data modeled best to a single site with B_{max} = 6.5 pmol/g of tissue and K_d = 0.17 nM. The concentration of [³H]WB4101 ranged from 0.01 to 3.0 nM. [³H]Prazosin (□) saturation using 10 μM phentolamine to define specific binding is also shown. The B_{max} and K_d values are 11.35 pmol/g of tissue and 0.20 nM, respectively. The concentrations of [³H]prazosin were from 0.05 to 2.0 nM.

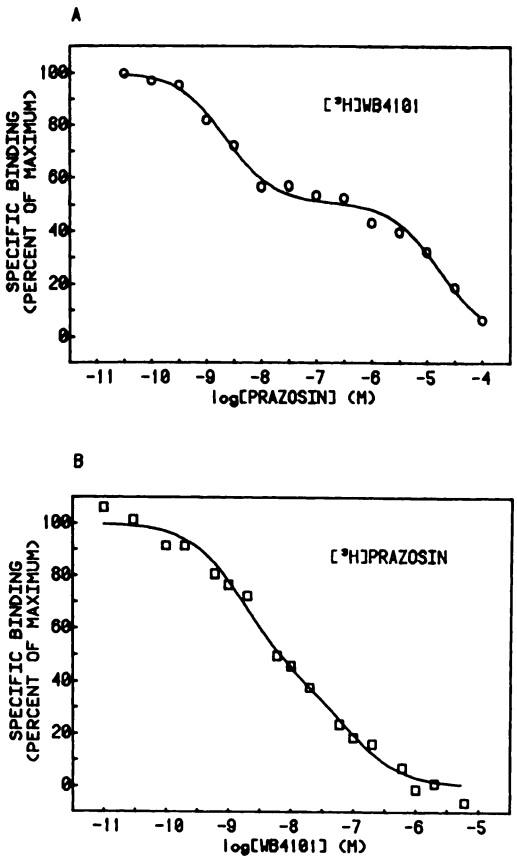

Fig. 2. A. Prazosin/[³H]WB4101 competition in rat hippocampal membranes. Various concentrations of prazosin were incubated in duplicate with 0.98 nM [³H]WB4101. The *points* represent the actual data while the *curve* describes the model for two binding sites which best fit the data. Prazosin had 0.17 nM affinity for the high affinity component which comprised 24% of the specific binding. The low affinity component had 5.2 μM affinity. Nonspecific binding was defined by 1 × 10^{-5} M phentolamine. The same respective affinities were determined in cortical membranes. B. WB4101/[³H]prazosin competition in rat hippocampal membranes using 0.4 nM [³H]prazosin. Data models best to two binding sites with a ratio of 58:42% of specific binding, respectively. The affinities of the two sites described by the computer analysis were 0.40 nM and 18.8 nM. Similar affinities were measured in cortex.

with a pseudo-Hill slope of 0.49 ± 0.04 (n = 7) (shown in Fig. 6). The curve plateaus at 10^{-5} M phentolamine at all relevant concentrations of [³H]WB4101 and remains constant over 1.5 orders of magnitude. Several laboratories have used 1–5 × 10^{-4} M NE to define specific binding (4, 23, 25, 29, 30). Under these conditions [³H]WB4101 binding isotherms are also complex (23, 25, 30). However, the use of NE to determine a baseline is problematic, since NE competition with [³H]WB4101 does not plateau at concentrations as high as 10^{-2} M (data not shown). Prazosin may also be used to define a baseline for [³H]WB4101 binding to α-adrenergic receptors. Prazosin competition with [³H]WB4101 is biphasic, reaching a plateau at approximately 10–30 nM, remaining constant for at least 2 orders of magnitude with further competition in the micromolar concentration range (Fig. 2A). The use of 30 nM prazosin as a baseline for [³H]WB4101 binding yields a monophasic saturation curve (Fig. 1B). Saturation analysis of [³H]WB4101 binding using phentolamine or prazosin baselines reveal an important relationship. In rat hippocampus the B_{max} and K_D values for [³H]WB4101 using the prazosin baseline are 5.96 ± 0.32 pmol/g of tissue and 0.30 ± 0.02 nM, respectively. However, nonlinear regression analysis of the phentolamine-defined saturation curve yields two sites (Fig. 1A). The high affinity site has a B_{max} of 5.56 ± 0.65 pmol/g of tissue and a K_D value of 0.15 ± 0.03 nM, while the second site has a B_{max} of 10.86 ± 0.34 nM and a K_D value of 1.62 ± 0.34 nM (n = 5). Thus, it appears that the higher affinity [³H]WB4101 binding site may be identical to the binding site identified using a 30 nM prazosin blank (Fig. 1). This is further substantiated by nonlinear regression analysis of prazosin competition with [³H]WB4101. Such competition curves are shallow (Fig. 2A), with pseudo-Hill coefficients of 0.26 ± 0.03 (n = 11). Prazosin competes with high affinity for 19 ± 3% of the [³H]WB4101 binding sites with a dissociation constant $K_{(high)}$ of 0.25 ± 0.06 nM for the highest affinity site and with a dissociation constant $K_{(low)}$ of 11.35 ± 2.4 μM for the lower affinity site.

[³H]Prazosin Saturation: Comparison of B_{max} Value with [³H]WB4101

Phentolamine competition with [³H]prazosin plateaus near 10^{-6} M and remains constant to 10^{-4} M at concentrations of

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[^{3}H]prazosin near its K_d. NE and cold prazosin competition curves also plateau at the same level as phentolamine. Phentolamine (1×10^{-5} M) was used to define the baseline of α_1-adrenergic receptor binding of [^{3}H]prazosin to provide consistency for comparisons with [^{3}H]WB4101 binding. Saturation isotherms of [^{3}H]prazosin binding so defined are monophasic. In rat hippocampus the B_{max} is 11.03 ± 0.39 pmole/g of tissue and the K_d = 0.22 ± 0.01 nM (Fig. 1B). This B_{max} value is approximately twice the capacity of the [^{3}H]WB4101 binding sites which have high affinity for prazosin. Thus, the high affinity [^{3}H]WB4101 binding site, defined either by LIGAND analysis or by a prazosin baseline, has about half of the total capacity of the [^{3}H]prazosin binding sites. The affinity of prazosin for the higher affinity [^{3}H]WB4101 binding site is nearly identical to prazosin's affinity as a [^{3}H]ligand labeling α_1-adrenergic receptors directly (0.25 nM versus 0.22 nM). Furthermore, WB4101 has subnanomolar affinity for about half of [^{3}H]prazosin binding sites as determined in competition experiments (Fig. 2B), and this affinity is close to the affinity of [^{3}H]WB4101 at its picomolar affinity binding site (620 pM versus 150 pM). Table 1 shows the relationship between the capacity of [^{3}H]prazosin binding sites and the high affinity component of [^{3}H]WB4101 binding ascertained through LIGAND analysis in several tissues. Whereas the relationship between the two parameters is exactly 2 to 1 in the hippocampus, the ratios in cortex and cerebellum are 2.7 and 2.5, respectively.

[^{3}H]Prazosin Binding Characterization

Competition with adrenergic antagonists. An explanation for the greater total number of [^{3}H]prazosin binding sites compared to the picomolar affinity [^{3}H]WB4101 binding sites becomes apparent when studying antagonist competitions with [^{3}H]prazosin. Fig. 3 shows competition curves for the antagonists WB4101, phentolamine, DHE, and IND. It is apparent that DHE and IND compete in a monophasic manner with 90% inhibition within 2 orders of magnitude (pseudo-Hill slopes equal 1.0). However, phentolamine and WB4101 competition curves are shallow, with pseudo-Hill slopes less than 1.0. The data from their curves fit best to a model describing two binding sites in approximately equal proportions. Table 2 shows the parameters derived from the computer analyses of the competition curves of several adrenergic compounds for [^{3}H]prazosin binding sites. Phentolamine and WB4101 each bound with different affinities to subtypes of the [^{3}H]prazosin binding sites. The two antagonists had higher affinity for the same subtype since their high affinity components of competition for [^{3}H]prazosin binding were not additive. Thus, under the routine conditions for a filtration binding assay, [^{3}H]WB4101 would only be predicted to label the portion of the [^{3}H]prazosin sites for which it has highest affinity; [^{3}H]WB4101 would dissociate too rapidly from the lower affinity binding site for it to be detected. The two subtypes of [^{3}H]prazosin binding defined in this assay have very different rank orders of antagonist affinities: half of the sites exhibit the pharmacological profile of WB4101 > prazosin > phentolamine > IND > DHE. This site we will term α_{1A}. In contrast, the remaining subtype demonstrates the rank order of prazosin > IND > DHE > WB4101 > phentolamine which we will term α_{1B}.

TABLE 1

Relationship between the binding capacity of [^{3}H]prazosin and the high affinity [^{3}H]WB4101 binding sites measured simultaneously in tissue from several brain regions

Saturation assays were performed using both ligands with the same tissue homogenate. Data were analyzed by nonlinear regression using LIGAND (see Materials and Methods). [^{3}H]WB4101 binding was resolved into two components as discussed above. The binding capacity (pmol/g of tissue) of the prazosin-sensitive or highest affinity component measured in each tissue is shown here.

Region (*n*)	[^{3}H]Prazosin	High affinity [^{3}H]WB4101
	pmol/g of tissue	
Cortex (5)	14.49 ± 0.38	5.36 ± 0.74
Hippocampus (5)	11.03 ± 0.39	5.56 ± 0.65
Cerebellum (5)	7.72 ± 0.11	3.06 ± 0.18

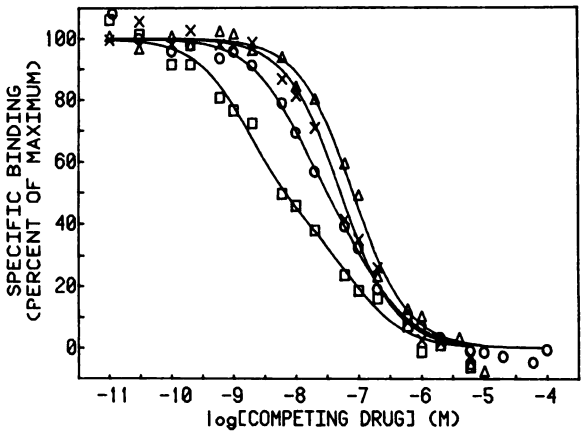

Fig. 3. Computer-fitted curves for the competition of antagonists with 0.4 nM [^{3}H]prazosin in rat cortical membranes. Increasing concentrations of WB4101 (□), phentolamine (○), IND (△), or DHE (*X*) were added to duplicate test tubes. Each *curve* represents a single independent experiment, repeated six times.

Competition with agonists. Several investigators have reported that agonist competitions for [^{3}H]prazosin binding sites result in shallow competition curves with pseudo-Hill slopes < 1.0 (31–34). Fig. 4 shows representative competition curves for (−)NE, (−)EPI and L-phenylephrine. Table 2 gives the parameters derived from nonlinear regression analysis for the two affinity components detected by agonists. It should be noted that the ratio of the high affinity site or state may not be equivalent to the proportions distinguished by antagonists and that the percentage of sites having high affinity, $R_{(high)}$ varies among agonists, the higher affinity compounds having the larger percentage of $R_{(high)}$.

We wished to address the question of how the agonists' affinity states were related to the "subtypes" of [^{3}H]prazosin sites distinguished by WB4101 and phentolamine. Therefore, we conducted competition curves identical to those shown in Fig. 4, except that phentolamine or WB4101 was included at a concentration sufficient to occupy (mask) the higher affinity α_{1A}-subtype of the [^{3}H]prazosin binding sites. Thus, the resulting curves would reflect agonist competition at the [^{3}H]prazosin binding sites having lower affinity for WB4101 or phentolamine, designated α_{1B}. The results for these curves using such a 30 nM phentolamine mask are shown in Fig. 5 and in Table 3. Remarkably, under these conditions, the agonist rank order of affinity is preserved with (−)EPI > (−)NE > L-phenylephrine. If both subtypes of the [^{3}H]prazosin binding sites have identical affinities for these agonsits, then it should be possible to predict exactly how much the agonist curves would be shifted by the presence of the phentolamine mask as it also binds, to some extent, to the α_{1B} site. We have calculated (Table 4) the theoretical values for the shift in the $K_{(high)}$ agonist affinity at

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TABLE 2

Nonlinear regression analysis of [³H]prazosin competition with adrenergic compounds in rat cortex

Competition experiments were performed in duplicate using 21 concentrations of inhibitor ranging from 1×10^{-4} M to 1×10^{-11} M. Values are the means ± SE of 4–6 separate experiments. A two-site fit of the data was accepted only if it resulted in a significant improvement in the agreement between the data and the model parameters.

Drug	Hill coefficient	K_{high}	K_{low}	$\%R_H$[a]
		nM	μM	
WB4101	0.61 ± 0.06	0.62 ± 0.12	0.023 ± 0.004	52[b]
Prazosin	1.0 ± 0.05	0.69 ± 0.16		100
Phentolamine	0.63 ± 0.06	2.44 ± 0.6	0.055 ± 0.011	48[c]
IND	1.02 ± 0.1	11.88 ± 1.0		100
DHE	1.0 ± 0.09	15.76 ± 1.5		100
(−)EPI	0.42 ± 0.01	63.02 ± 7.7	4.11 ± 0.39	44[b]
(−)NE	0.58 ± 0.01	196.2 ± 20.3	9.76 ± 1.4	36[b]
L-Phenylephrine	0.38 ± 0.5	239.5 ± 33.7	9.69 ± 1.2	15[b]

[a] $\%R_H$, the percentage of binding to the highest affinity site.
[b] $p < 0.01$.
[c] $p < 0.02$.

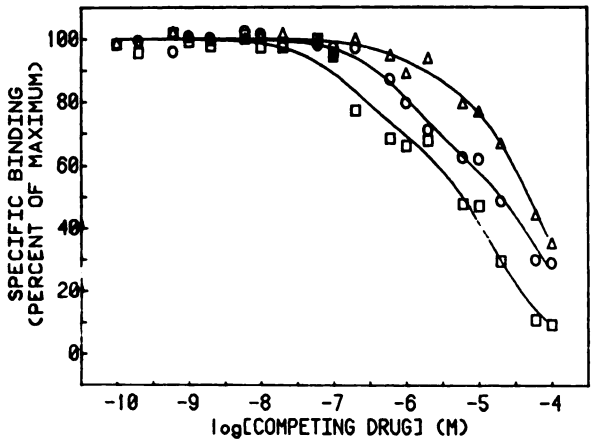

Fig. 4. Computer-fitted curves for the competition of agonists with 0.4 nM [³H]prazosin in rat cortical membranes. EPI (□), NE (○), and phenylephrine (△) curves are shown from individual experiments, repeated 5–6 times.

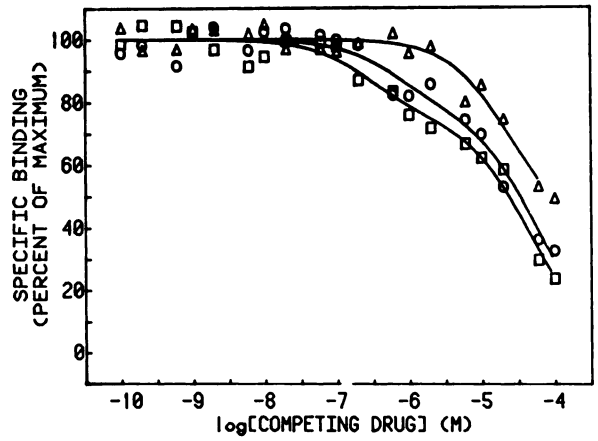

Fig. 5. Agonist competitions with 0.4 nM [³H]prazosin in the presence of a 30 nM phentolamine mask. This concentration occupies the [³H]prazosin binding sites having highest affinity for phentolamine and WB4101 such that agonist interactions at the remaining [³H]prazosin binding sites (the subtype with lower affinity for WB4101 and phentolamine) may be studied. EPI (□), NE (○), and phenylephrine (△) curves are shown from individual experiments, repeated 5–6 times.

the α_{1B}-subtype and show the actual value found. The actual affinity values determined for the $K_{(high)}$ agonist component are close to the theoretical value predicted for NE and EPI, suggesting that both subtypes of the α_1 binding site have the same affinities for these agonists. Furthermore, the percentage of

TABLE 3

Effect of phentolamine mask on agonist/[³H]prazosin competition

Competition experiments were performed as in Table 2, including 30 nM phentolamine which inhibited 50% of total [³H]prazosin binding. Data shown are means ± SE of 5–6 experiments run in duplicate with concentrations of agonist from 1×10^{-10} to 1×10^{-4} M.

Drug	Hill coefficient	K_{high}	K_{low}	R_H/R_L
		μM		
(−)EPI	0.30	0.132 ± 0.026	11.5 ± 1.6	27/73
(−)NE	0.33	0.390 ± 0.090	9.5 ± 0.8	31/69
L-Phenylephrine	0.45	5.32 ± 0.47	*a*	68/32

[a] L-Phenylephrine was too weak for the computer program to make reliable estimates of this value.

TABLE 4

Agonist competition with [³H]prazosin in the presence of a phentolamine mask: Theoretical and experimental values

Theoretical values for the shift in affinity constants of the agonist compounds in the presence of 30 nM phentolamine were calculated using the equation

$$K_{(w)} = \left(1 + \frac{[I]}{K_{(I)}}\right) K_{(w/o)}$$

where $K_{(w)} = K$ (agonist in presence of phentolamine mask), $K_{(w/o)} = K$ (agonist without phentolamine mask), $K_{(I)} = K_{low}$ for phentolamine (given in Table 2), and $[I]$ = [phentolamine mask]. The actual values are determined as described in the text. The predicted values assume that agonists have identical affinity for both subsets of sites distinguished by the antagonists phentolamine and WB4101. It is also assumed that the phentolamine mask occupies all of the binding sites with the highest affinity for the antagonists (α_{1A}).

Drugs	Predicted value		Actual value	
	K_{high} (nM)	$\%R_H$	K_{high} (nM)	$\%R_H$
EPI	95.9	44	132.1 ± 26.1	27
NE	303.2	36	389.5 ± 90.4	31
Phenylephrine	371.3	15	5323.8 ± 472.4	68

$R_{(high)}$ is similar in the presence and absence of the phentolamine mask. However, the high affinity component of phenylephrine binding to [³H]prazosin in the presence of the 30 nM phentolamine mask is radically different from the predicted value. In addition, the percentage of $R_{(high)}$ for phenylephrine is greatly increased when the α_{1B} site is isolated by this technique. Similar results were obtained using a WB4101 mask at a concentration of 15 nM. These data suggest that (−)EPI and (−)NE have the same affinity at both subtypes of the [³H]prazosin binding site but that phenylephrine has higher affinity at the α_{1A} site.

[³H]WB4101 Binding Characterization: Competition with Adrenergic and non-Adrenergic Agents

Fig. 6 shows the competition of several α-adrenergic antagonists with specific [³H]WB4101 binding defined by 10 μM

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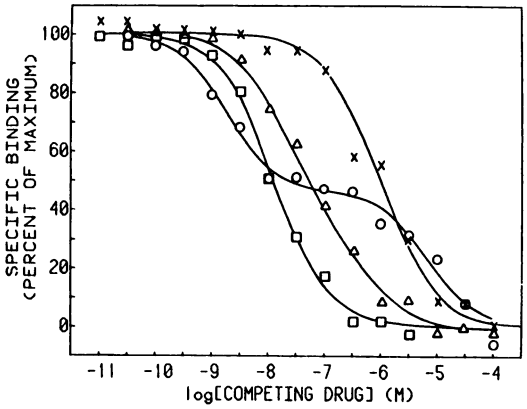

Fig. 6. Antagonist competition with [^{3}H]WB4101 binding sites in rat cortical homogenates. Increasing concentrations of DHE (□), prazosin (○), phentolamine (△), and yohimbine (*X*) were incubated with 1.5 nM [^{3}H]WB4101. The affinities of [^{3}H]WB4101 for the two binding sites determined in saturation studies were used to generate the *curves* in these analyses. Each curve represents an independent experiment. Experiments were repeated 5–8 times.

TABLE 5

IC_{50} values of various adrenergic compounds competing with total [^{3}H]WB4101 specific binding

Competition curves were run in duplicate on membranes of cerebral cortex with 15–19 concentrations of inhibitor. The concentration of [^{3}H]WB4101 was 1.5 nM, labeling both binding sites. Values are mean ± SE of 4–6 separate experiments. Nonspecific binding was defined by 10 μM phentolamine.

Drug	IC_{50}
	nM
DHE	17.04 ± 1.0
IND	31.2 ± 0.88
Phentolamine	38.3 ± 8.8
Prazosin	173.8 ± 45.3
Yohimbine	591.0 ± 19.6
EPI	3,790 ± 880
NE	4,630 ± 770
Clonidine	4,870 ± 760
Phenylephrine	24,720 ± 730

phentolamine. Table 5 shows the IC_{50} values for various adrenergic agents in competition for [^{3}H]WB4101 binding sites. Prazosin and phentolamine competition curves are shallow, with Hill slopes significantly less than 1.0. Conducting the LIGAND analysis of these curves utilizing the affinities of [^{3}H]WB4101 for its two binding sites as they were determined from saturation analysis (Fig. 1A), we obtained values for the affinities of prazosin and phentolamine for the two binding sites. The affinities of prazosin for the two sites are approximately 0.25 nM and 5 μM. Likewise, phentolamine competes at the two [^{3}H]WB4101 binding sites with K_i values of 1.35 ± 0.33 nM and 163 ± 46 nM (n = 7). DHE competition, however, is steep with pseudo-Hill coefficients greater than or equal to 1.0 and of high affinity (IC_{50} = 17 nM). DHE is not displacing from only one of the [^{3}H]WB4101 binding sites as it inhibits all of the binding defined by the phentolamine baseline. IND competition curves are similar. However, although yohimbine competition with [^{3}H]WB4101 is also monophasic, it is of submicromolar affinity (Tables 5 and 6), demonstrating that [^{3}H]WB4101 is not labeling α_2-adrenergic receptors in cortical tissue.

Fig. 7 illustrates the competition of adrenergic agonists with [^{3}H]WB4101. Table 5 shows the IC_{50} values for agonists which are in reasonable agreement with the values published by other investigators (4, 35, 36) who assumed that [^{3}H]WB4101 labeled

TABLE 6

Affinities of adrenergic drugs and Ca^{2+} channel blockers for [^{3}H] WB4101 in the presence of a prazosin mask (30 nM)

Values are the means ± SE of 3–5 separate experiments.

Drug	K_i
	nM
Antagonists	
WB4101	2.63 ± 0.52
DHE	11.45 ± 1.04
IND	39.13 ± 3.12
Phentolamine	298.0 ± 26.3
Piperoxane	417.1 ± 46.0
Yohimbine	847.0 ± 126
Prazosin	7,220 ± 900
Agonists	
Serotonin	1.54 ± 0.14
Clonidine	1,790 ± 60
L-Phenylephrine	19,230 ± 3,200
(−)EPI	5,380 ± 1,500
(+)EPI	5,130 ± 2,100
(−)NE	26,200 ± 5,200
(+)NE	38,900 ± 7,800
Ca^{2+} channel blockers	
Verapamil	920 ± 140
Nitrendipine	10,080 ± 1,600
Nifedipine	22,430 ± 1,500

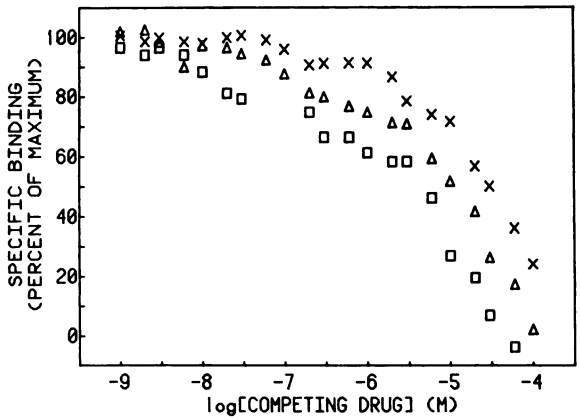

Fig. 7. The competition of epinephrine (□), NE (△), and phenylephrine (*X*) with 1.5 nM [^{3}H]WB4101 in rat cortical membranes. Curves were not fitted by the computer as explained in the text. Data are shown for individual experiments. The IC_{50} values are given in Table 5. Data shown are representative of 5–8 experiments.

a single class of binding sites and therefore determined K_i values using the Cheng and Prusoff (37) relationship between IC_{50} and the inhibition constant (K_i). Analysis of the agonist data in light of the results in this study suggests that agonist competitions should model to at least three sites. Agonists should compete in a biphasic manner with the high affinity [^{3}H]WB4101 binding site, since agonist competition with [^{3}H] prazosin is biphasic even in the presence of a phentolamine mask of one of the [^{3}H]prazosin subtypes. In addition, agonists will compete at the nanomolar affinity [^{3}H]WB4101 binding site. However, a three- or more site analysis of a 21-point competition curve may not be statistically meaningful and, as such, determination of the affinities of agonists was made for the nanomolar [^{3}H]WB4101 binding site by another technique.

Characterization of the Nanomolar Affinity [^{3}H]WB4101 Binding Site

In order to determine the affinities of adrenergic drugs at the nanomolar affinity binding site for [^{3}H]WB4101, we employed

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a prazosin mask (30 nM) to occupy the α_1-adrenergic receptor sites labeled by [^{3}H]WB4101 (Table 6). Whereas DHE, IND and WB4101 have nanomolar affinity at this site, suggesting that it may be adrenergic, prazosin, piperoxane, phentolamine, and yohimbine have, in contrast, much lower affinity than would be expected of an α_1- or α_2-adrenergic receptor. The affinities of prazosin and phentolamine in the presence of the prazosin mask are in good agreement with their lower affinity component for inhibition of [^{3}H]WB4101 derived previously from Fig. 6. (prazosin: 7 μM versus 5 μM; phentolamine: 298 nM versus 163 nM). Furthermore, we can detect no evidence of stereoselectivity between the optical isomers of EPI and NE, suggesting that this site does not label an α-adrenergic receptor at all. It has been suggested that [^{3}H]WB4101 can label Ca^{2+} channel sites (38). However, we found that antagonists for these sites have only micromolar affinity in competition with [^{3}H]WB4101. In contrast, serotonin has nanomolar affinity for the [^{3}H]WB4101 binding sites determined in the presence of 30 nM prazosin (Table 6).

Does [^{3}H]WB4101 Lable an Uptake Site for Norepinephrine?

This question was addressed by measuring the ability of WB4101 to inhibit the uptake of [^{3}H]NE in rat brain synaptosomes. Fig. 8 shows a dose-response curve for the effect of various concentrations of WB4101 in the specific uptake of [^{3}H]NE. The IC_{50} was $1.26 \pm 0.32 \times 10^{-5}$ M. Furthermore, desipramine had micromolar affinity in competition with [^{3}H]WB4101 binding in the presence or absence of a prazosin mask (data not shown), suggesting that the non-α_1 [^{3}H]WB4101 binding site is not related to the NE uptake sites.

Discussion

The purpose of the present study was to reevaluate the characteristics of [^{3}H]WB4101 and [^{3}H]prazosin binding in rat brain and to resolve the similarities and differences in the pharmacological characteristics of their respective binding sites. We suggest that [^{3}H]prazosin labels subtypes of α_1-adrenergic receptor binding sites that are discriminated by the antagonists phentolamine and WB4101, and the agonist phenylephrine. In cortex, we found that the competition of WB4101 and phentolamine for [^{3}H]prazosin binding sites shows deviations from the simple Langmuir isotherm for binding to a single site. Similarly, in frontal cortex (33) and lung (39), others have

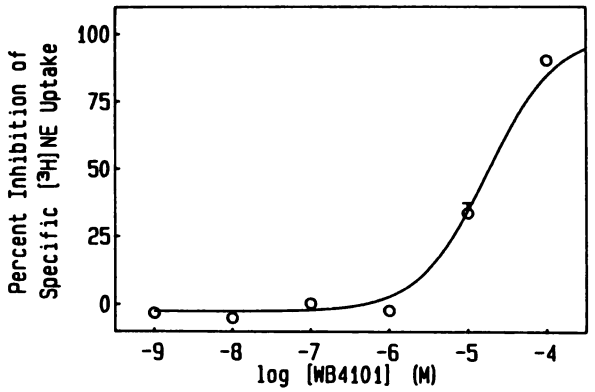

Fig. 8. Dose-response curve for the inhibition of specific [^{3}H]NE uptake by WB4101. Data shown are means ± SE of four separate experiments. *Error bars* are contained within the *circles* in most cases. The IC_{50} for WB4101 inhibition was $1.26 \pm 0.32 \times 10^{-5}$ M.

also reported pseudo-Hill slopes less than 1.0 for one or both of these antagonists. In other tissues, however, phentolamine competition with [^{3}H]prazosin is reported to be monophasic with pseudo-Hill slopes = 1.0 (32, 40, 41). Interestingly, in these cases the affinity of phentolamine in competition with [^{3}H]prazosin correlates well with either the $K_{(high)}$ or $K_{(low)}$ values for phentolamine found in the present study. For example, Battaglia *et al.* (41) report an IC_{50} for phentolamine in pituitary which, when converted to its K_i of 2.58 nM, is comparable to 2.44 nM for its $K_{(high)}$ found in the present study. This suggests that only one subtype (α_{1A}) of the [^{3}H]prazosin binding site is present in pituitary.

In order to establish whether the subtypes identified in our brain radioligand binding assays have any general usefulness in the pharmacological classification of adrenergic receptors, we have examined data in the literature determined in other systems for pharmacological characteristics that may reflect identification of the site with higher affinity for phentolamine (which we have termed α_{1A}) or with lower phentolamine affinity (termed α_{1B}). Since phentolamine has 23 times lower affinity for the α_{1B}- than for the α_{1A}-subtype, but prazosin has equal affinity at both subtypes, the ratio of the affinities or potencies of phentolamine versus prazosin provides a good index for such a classification: prazosin is 3.5-fold more potent than phentolamine at α_{1A} sites defined by our radioligand binding experiments but 80-fold more potent than phentolamine at α_{1B} sites. Table 7 shows the ratio of the potencies of these two drugs in competition with other adrenergic ligands or as antagonists of receptor-mediated responses for which dose-response curves were reported. Studies are listed by the ratio of the potency or affinity of phentolamine versus prazosin in ascending order. Although there is a great deal of variability in these reports for the absolute affinities of the antagonists prazosin and phentolamine (the affinity of prazosin in competition with [^{3}H]prazosin, for example, ranges from picomolar to nanomolar depending upon the tissue and assay conditions), when comparing the ratio of the affinities or potencies of phentolamine to prazosin, a pattern emerged as depicted in Table 7. There is quite a large variation in this ratio among tissues and responses. We propose that subtypes of α_1-receptors may be identified in this manner as suggested by the correlation of these ratios with those obtained from [^{3}H]prazosin binding in this paper. Thus, the ratio of phentolamine versus prazosin at α_{1A} [^{3}H]prazosin binding sites is approximately 4, whereas the same ratio at α_{1B} binding sites is approximately 80. Some ratios, similarly derived from the work of others, fall clearly into a range predicted by our radioligand binding studies. In our cortex data (comparing IC_{50} values), where pseudo-Hill slopes for WB4101 or phentolamine are < 1.0 and both subtypes are present, the ratio is intermediate. Thus, a ratio near 20 may indicate that the tissue contains both subtypes or that the responses are mediated by more than one receptor subtype such as those seen in lung (39) and frontal cortex (33).

Although many of the ratios listed are very close to those defined by our radioligand binding studies, some are clearly aberrant from other studies in the same tissue and are marked with a question mark. For example, although three studies in rat cortex (including the present) provide an intermediate ratio suggesting the presence of both subtypes, Ref. 42 provides a ratio which is more suggestive of pure α_{1B}. Similarly, for rabbit ear artery (42) and rat kidney (43), ratios do not fall clearly

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TABLE 7

Comparison of the pharmacological potency of phentolamine versus prazosin binding in α_1-receptor-mediated responses or [^{3}H]prazosin binding

The ratio of K_i, IC_{50}, or K_D for phentolamine versus prazosin in several tissues is given. Reference source is given in parentheses.

Ratio Phent/Praz	Tissue	Response	Source	Predicted "subtype"
3.1	Submandibular glands	[^{3}H]Prazosin binding	(55)	α_{1A}
3.2	Rat anococcygels muscle	Contraction	(52)	α_{1A}
3.5	Rat cortex	[^{3}H]Prazosin binding, α_{1A} component	(*a*)	α_{1A}
3.7	Rabbit ear artery	Constriction	(53)	α_{1A}
4.7	Rabbit prostate & urethra	Contraction	(54)	α_{1A}
5.1	Renal artery	Renal blood flow	(51)	α_{1A}
6.5	Rabbit aorta	Contraction	(56)	α_{1A}
10	Liver	cAMP-dependent phosphorylase activity	(10)	α_{1A}
11	Rat aorta	Contraction	(57)	α_{1A}
11.5	Intermediate pituitary	[^{3}H]Prazosin binding	(41)	α_{1A}
16	Rabbit ear artery	Constriction	(42)	?
18.6	Rat kidney	Gluconeogenesis	(43)	?
20	Rat submaxillary	[^{3}H]Prazosin binding	(58)	Both
22	Rat cortex	[^{3}H]Prazosin binding	(*a*)[b]	Both
22	Lung	[^{3}H]Prazosin binding	(39)	Both
22	Myometrium	[^{3}H]Prazosin binding	(59)	Both
24	Vas deferens	Contraction	(44)	Both
29	Rat brain	[^{3}H]Prazosin binding	(60)	Both
32	Pithed rat	Diastolic pressure	(57)	Both
35	Frontal cortex	[^{3}H]Prazosin binding	(33)	Both
44	Rat kidney	[^{3}H]Prazosin binding	(51)	?
50	Fat cells	P.I. labeling	(61)	α_{1B}
57	Liver	Ca^{2+}-dependent phosphorylase activity	(10)[c]	α_{1B}
67	Rat cortex	[^{3}H]Prazosin binding	(42)	?
80	Rat cortex	[^{3}H]Prazosin binding, α_{1B} component	(*a*)	α_{1B}
81	Rat heart	[^{3}H]Prazosin binding	(62)	α_{1B}
82	Human lung	[^{3}H]Prazosin binding	(63)	α_{1B}
88	Rat kidney	[^{3}H]Prazosin binding	(32)	α_{1B}
88	BC3H-1 intact cells	[^{3}H]Prazosin binding	(40)	α_{1B}
109	Jejunal epithelial cell	[^{3}H]Prazosin binding	(64)	α_{1B}
145	BC3H-1 cell membranes	[^{3}H]Prazosin binding	(65)	α_{1B}
200	Dog aorta	[^{3}H]Prazosin binding	(66)	α_{1B}
212	Rat liver	[^{3}H]Prazosin binding	(11)	?
218	Dog aorta	[^{3}H]Prazosin binding	(60)	α_{1B}
332	Ca^{2+} mobilization	BC3H-1 cells	(34)	α_{1B}
381	Myocardium	[^{3}H]Prazosin binding	(67)	α_{1B}

[a] This paper.
[b] Comparison of IC_{50} values.
[c] ED_{50} for phentolamine, estimated from dose response curve.

into either pure α_{1A} or mixed. In rat liver (12), the ratio suggests pure α_{1B}, whereas other studies (11) suggest that both subtypes are present. Whether these differences between studies represent differences in assay conditions or tissue regions used is not clear. Interestingly, contraction of vas deferens (44) gave a ratio comparable to that seen in tissues in which α_{1A} and α_{1B} binding sites are both detectable in radioligand binding studies. In vas deferens, heterogeneity of α_1-receptors mediating contraction has also been suggested by a lack of cross-desensitization by structurally different agonists (45).

Our binding data suggest that [^{3}H]WB4101 selectively labels the α_{1A} subtype. This is reinforced by the observation that the ratio of the affinities of phentolamine and prazosin for the picomolar [^{3}H]WB4101 binding site is 5.40, consistent with this designation of α_{1A}.

Studies using a phentolamine mask of the α_{1A} component of [^{3}H]prazosin binding suggest that the agonists EPI and NE have the same affinity for both subtypes of the [^{3}H]prazosin binding site but that phenylephrine has higher affinity at the α_{1A}-subtype. It would therefore be of interest to compare the affinity of phenylephrine in the pituitary (α_{1A}) with another tissue such as kidney (α_{1B}). Agonist binding was complex both in the presence and absence of the phentolamine mask of [^{3}H]prazosin binding. This may reflect a role of a guanine nucleotide-binding protein at one or both subtypes of [^{3}H]prazosin binding sites. In rat kidney (32), for example, agonist competition curves are shallow and guanine nucleotides have a small effect on agonist affinities. Guanine nucleotide effects have not been tested in a tissue which appears to contain only α_{1A} binding sites. Further studies on potential guanine nucleotide interactions with the subtypes of α_1 binding sites would be of interest. These studies should ideally be conducted in tissues that contain only one of the subtypes, respectively.

In summary, [^{3}H]prazosin labels two binding sites with equal affinity, each with characteristics of α_1-receptors. [^{3}H]WB4101 labels only one of these sites—termed α_{1A}. WB4101 has much lower affinity for the other binding site, termed α_{1B}, which [^{3}H]WB4101 will not label in filtration radioligand binding assays.

A fundamental question regarding the relationship between [^{3}H]prazosin and [^{3}H]WB4101 binding is the characterization of the other binding site which [^{3}H]WB4101 labels with nanomolar affinity. This binding site comprises approximately 65% of the specific binding of this ligand in cortex. As detailed above, this site does *not* correspond to the α_{1B} site. Using 30

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nM prazosin to block the prazosin-sensitive or α_{1A} portion of [^{3}H]WB4101 binding, we have shown that the remaining binding cannot be classified as α-adrenergic. The lack of stereospecificity in the binding of (+)- and (−)-EPI or -NE argues very strongly that this site is not an adrenergic receptor. Adrenergic receptors are known to have considerable stereochemical requirements for the binding of phenylethylamines (46). Furthermore, the affinities of the adrenergic antagonists tested are not in the range expected for α-receptor blockade. We have previously suggested under different assay conditions that [^{3}H]WB4101 binding to this site has characteristics of a serotonin S-1 receptor (47). We have recently confirmed this finding under the same conditions used for binding assays in the present study. Thus, serotonin itself (as shown in Table 6), 8-hydroxy-diproplyaminotetraline, metergoline, and lysergic acid diethylamide all have affinities for this [^{3}H]WB4101 binding site of less than 12 nM in the presence of a prazosin mask (48). This conclusion is in direct conflict with a recent publication by Wang *et al.* (30) which suggested that the nanomolar [^{3}H]WB4101 binding site is an agonist-preferring state of the α_1-receptor. Those authors, however, did not demonstrate that the nanomolar [^{3}H]WB4101 binding site had high affinity for any selective α_1-antagonist, nor did they demonstrate stereoselectivity for agonists. Clearly, the affinity of serotonergic agonists reported here and previously is far greater than the affinity of NE in competition with [^{3}H]WB4101 binding at its nanomolar affinity binding site.

Considering this evidence that [^{3}H]WB4101 can label a serotonin binding site, it may seem perplexing that its pharmacological characteristics have been mistaken as purely adrenergic for so long. The simplest explanation lies in the percentage occupancy of the two binding sites by the concentration of [^{3}H]WB4101 typically used. Given that the affinities of WB4101 for the two sites are approximately 0.2 nM and 2 nM, and that the usual concentration of [^{3}H]WB4101 used for the characterization of its binding site(s) has been 0.5 nM, the fractional occupancy of the α_1 site would be 71% whereas the fractional occupancy of the nanomolar site would be 20%. Thus, when the fractional occupancy of each site is multiplied by the percentage of total [^{3}H]WB4101 binding accounted for by each site (35% versus 65%), the resulting values (0.25 versus 0.13) show that the binding characteristics will predominantly reflect the characteristics of the highest affinity site—the α_1-adrenergic receptors. Only in the presence of a prazosin mask, therefore, is it possible to clearly detect the high affinity of serotonergic agents for [^{3}H]WB4101 binding without resorting to computer analysis of complex competition curves.

This study provides evidence for subtypes of α_1-adrenergic receptor binding sites labeled by [^{3}H]prazosin. Whether these two binding sites represent different proteins or simply different conformations of the recognition site of the same receptor protein is not clear from these data. In the rat liver, α_1-receptors mediating phosphorylase activation show greater potency for phentolamine and WB4101 when this response is dependent upon cAMP production than when the same response is coupled to Ca^{2+} mobilization (10, 49). Thus, it is possible that coupling to a second messenger in the membrane alters the conformation of the recognition site such that the affinities for phentolamine and WB4101 are altered but not the affinity for prazosin. Solubilization studies of α_1-receptors have not yet reached the point of clearly discriminating multiple recognition sites. However, ligand binding sites (identified by photoaffinity probes) of differing molecular weights have been identified, depending upon the tissue investigated (50). Moreover, the fact that some tissues appear to contain only one of the subtypes of [^{3}H]prazosin receptor binding sites would tend to argue that these binding sites may represent discrete receptor proteins.

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