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Determination of Subtype Selectivity of *Alpha*-Adrenergic Antagonists

Comparison of Selective and Nonselective Radioligands

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SUMMARY

LAVIN, T. N. B. B. HOFFMAN, AND R. J. LEFKOWITZ. Determination of subtype selectivity of *alpha*-adrenergic antagonists: comparison of selective and nonselective radioligands. *Mol. Pharmacol.* **20**:28–34 (1981).

The $alpha_1/alpha_2$-adrenergic receptor subtype selectivity of twelve adrenergic antagonists was assessed by two different radioligand binding approaches in rabbit uterine membranes which contain both receptor subtypes. In the first approach, a nonsubtype selective antagonist radioligand, [^{3}H]dihydroergocryptine ([^{3}H]DHE), was used to label all of the *alpha*-receptors. Selective competing ligands produced complex competition curves which could be analyzed by nonlinear least-squares curve-fitting methods to yield the K_d values of the competitor for the $alpha_1$- and $alpha_2$-receptors, respectively. The second approach utilized the radioligand antagonists [^{3}H]prazosin and [^{3}H]yohimbine. [^{3}H]Prazosin was found to label $alpha_1$-receptors, whereas [^{3}H]yohimbine labeled $alpha_2$-receptors. Competition experiments performed with subtype selective antagonists with these radioligands produced steep uniphasic competition curves in all cases. Dissociation constants of drugs for the [^{3}H]prazosin and [^{3}H]yohimbine sites correlated highly with the $alpha_1$ and $alpha_2$ components of the complex [^{3}H]DHE competition curves, respectively. Good quantitative agreement between the two sets of data was obtained, indicating the validity of both approaches for the determination of receptor subtype affinities. Nonetheless, use of subtype selective radioligands offered several advantages. When the nonsubtype selective radioligand [^{3}H]DHE was used in this system, 100-fold selectivity of a competitor was required in order to determine reliably $alpha_1$ and $alpha_2$ affinities. In contrast, use of the selective radioligands discriminated much smaller degrees of selectivity without the necessity for sophisticated computer analysis of the data.

INTRODUCTION

Alpha-adrenergic receptors have been classified into two subtypes by classic pharmacological techniques and also by radioligand binding studies (1–4). Originally, these *alpha*-receptor subtypes were defined by their presumed anatomical location on presynaptic and postsynaptic sites (5). The "presynaptic" *alpha*-receptors mediate feedback inhibition of the release of norepinephrine from nerve terminals, whereas the "postsynaptic" receptors mediate such typical responses as smooth muscle contraction (1, 2, 6). Pharmacologically, these *alpha*-receptors can be differentiated by their differing affinities for a variety of drugs (3).

More recently, receptors having pharmacological characteristics of typical presynaptic receptors have been found on postsynaptic sites and in non-neural tissues such as platelets (7). Thus, the original anatomical distinctions have become blurred, and *alpha*-receptor subtypes are now generally defined in pharmacological terms. $Alpha_1$-receptors correspond to classical postsynaptic excitatory *alpha*-receptors; $alpha_2$-receptors are those having pharmacological properties similar to the originally defined presynaptic *alpha*-receptors (3). Prazosin and yohimbine have been among the most useful antagonists for defining the *alpha*-receptor subtypes. In studies performed with intact tissues, yohimbine is generally more potent at $alpha_2$ sites than is prazosin, whereas prazosin is much more potent at $alpha_1$-receptor sites than is yohimbine (8, 9, 10).

Radioligand binding studies have also directly demonstrated the $alpha_1$ selectivity of prazosin and the $alpha_2$ selectivity of yohimbine in membrane preparations (11–14). In such direct binding studies, the antagonist

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[^{3}H]prazosin generally appears to label $alpha_1$ sites, whereas the agonists [^{3}H]clonidine or [^{3}H]epinephrine appear to generally label $alpha_2$ sites (15). Competition curves of antagonists with these radioligands reflect the subtype specificity of the tritiated radioligand. Thus, the subtype specificity of a particular drug can be assessed by comparing its affinity for sites labeled by an $alpha_1$ or $alpha_2$ selective radioligand (15).

In contrast, the antagonist radioligand [^{3}H]DHE[3] binds to both $alpha_1$- and $alpha_2$-receptors with indistinguishable affinity (16, 17). Competition curves of subtype selective antagonists with [^{3}H]DHE can be dissected by computer methods to provide the affinities of the competitor for the two receptor subtypes, and the proportion of each receptor type present in the membranes (7).

In the present study, we have directly compared these two different approaches for determining the *alpha* subtype selectivity of widely used *alpha*-adrenergic antagonists in a single model tissue, the rabbit uterus. The subtype selective radioligands employed are both antagonists: [^{3}H]prazosin ($alpha_1$) and [^{3}H]yohimbine ($alpha_2$). The nonsubtype selective radioligand is the antagonist [^{3}H]DHE. The results obtained from computer modeling of complex [^{3}H]DHE competition curves obtained with a variety of *alpha*-antagonists were compared with the results obtained for the same drugs in competition with the two selective radioligands. In this way, the selectivity of a variety of antagonist compounds was examined and the validity and relative merits of the two experimental approaches could be compared.

METHODS

Pharmacological agents. [^{3}H]Yohimbine (specific activity 84 Ci/mmole) and [^{3}H]DHE (31 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). [^{3}H]Prazosin (33 Ci/mmole) was obtained from Amersham Corporation (Arlington Heights, Ill.).

Other compounds were obtained as follows: imiprimine HCl and phentolamine HCl, Ciba-Giegy (Summit, N.J.); clozapine, Roussel UCLAF Corporation (Romainville, France); chlorpromazine HCl, Smith, Kline and French Laboratories (Philadelphia, Pa.); haloperidol, McNeill Laboratories (Fort Washington, Pa.); indoramine, Wyeth Laboratories (Philadelphia, Pa.); mianserin, Organon (Oss, The Netherlands) labetalol, Allen and Hansbury (London, United Kingdom); piperoxan, May and Baker (Essex, United Kingdom); prazosin, Pfizer, Inc. (New York, N.Y.); rauwolscine (*alpha*-yohimbine), New England Nuclear Corporation; thymoximine, William R. Warner and Co. (Hampshire, United Kingdom); yohimbine HCl and (−)-epinephrine bitartrate, Sigma, Chemical Company (St. Louis, Mo.).

Binding assay. Rabbit uteri, mature type II [from Pel-Freeze Biologicals (Rogers, Ark.)], were frozen on Dry Ice, and membranes were prepared as described previously, except for the following changes (18). Homogenization of minced tissue was performed with a Polytron PT-10 homogenizer at maximal speed in three 5-sec bursts with a 20-sec pause between bursts. The final membrane suspension was frozen with liquid nitrogen in 50 mM Tris-HCl and 10 mM $MgCl_2$, pH 7.5, at 25° and stored at −80°. Comparable results were obtained with fresh and frozen membrane preparations. The binding assay was performed as previously described, except that filter washing in [^{3}H]prazosin and [^{3}H]yohimbine experiments was performed with cold buffer (4°) (18). All incubations lasted 20 min at 25°, at which time equilibrium was reached for all concentrations of radioligands employed in the study. Specific binding was defined with 10^{-5} M phentolamine or 10^{-4} M (−)-epinephrine which gave comparable results. When present at concentrations approximating their K_d values, specific binding was as follows: [^{3}H]prazosin, 60–80%; [^{3}H]yohimbine, 40–60%; and [^{3}H]DHE, 50–70%. Saturation curves were constructed from data obtained with all three radioligands in the same membrane preparation. Concentrations of radioligands used in competition experiments were as follows: [^{3}H]DHE, 3–5 nM; [^{3}H]prazosin, 1–2 nM; and [^{3}H]yohimbine, 8–12 nM.

Data analysis. Saturation and competition data were analyzed by a weighted nonlinear least-squares curve-fitting procedure (19, 20) which models data according to the law of mass action (21). Models for binding of the radioligand and competitor (when present) to one or two independent classes of sites were tested, and a two-site model was accepted as appropriate only when it significantly improved the fit of the data ($p < 0.05$). Testing for statistical difference between models was performed by comparing the residual variance of the fits to the data according to the "extra sum of squares" principle using an F-ratio test (22).

When [^{3}H]DHE competition curves fit best to a two-site model, an $alpha_1$ or $alpha_2$ designation for the two receptor subtypes and their respective dissociation constants was determined in the following manner. In each experiment, the fraction of $alpha_1$ and $alpha_2$ sites in the membrane preparation was determined by measuring the amount of [^{3}H]DHE binding which was blocked by 100 nM prazosin. We have previously demonstrated (23) in rabbit uterine membranes that the amount of [^{3}H]DHE binding blocked by 100 nM prazosin represents the $alpha_1$ sites. In the current experiments, this was generally ~20% of the total *alpha*-receptor population labeled by [^{3}H]-DHE. Knowledge of the properties of $alpha_1$ and $alpha_2$ sites in a particular membrane preparation made it possible to assign $alpha_1$ and $alpha_2$ designations to the individual affinities obtained from a biphasic [^{3}H]DHE competition curve; e.g., the component representing ~20% of the sites would be assigned as $alpha_1$ and that representing ~80% would be assigned as $alpha_2$. The various $alpha_1$ and $alpha_2$ dissociation constants of competitors are summarized in Table 1. When a two-site model did not significantly improve the fit of the data, only a single dissociation constant is reported in Table 1. This presumably represents a hybrid of $alpha_1$ and $alpha_2$ affinities which are too similar to be resolved by the computer analysis (see below for further discussion).

Dissociation constants reported in this paper are the geometric mean (24) of from two to nine independent determinations.

[3] The abbreviation used is: DHE, dihydroergocryptine.

TABLE 1

Dissociation constants of antagonist compounds for alpha-adrenergic receptor subtypes in rabbit uterine membranes as determined by [^{3}H]DHE, [^{3}H]yohimbine, and [^{3}H]prazosin binding

Dissociation constants were determined by computer analysis of ligand binding data as described under Methods. The K_d values are the geometric means derived from two or three experiments each for [^{3}H]yohimbine and [^{3}H]prazosin or "*n*" experiments for [^{3}H]DHE.

Drug	[^{3}H]DHE				[^{3}H]Prazosin	[^{3}H]Yohimbine	
	$K_d alpha_1$	$K_d alpha_2$	(*n*)	$K_d alpha_2$/$K_d alpha_1$	$K_d alpha_1$	$K_d alpha_2$	$K_d alpha_2$/$K_d alpha_1$
	nM	*nM*			*nM*	*nM*	
Prazosin	0.47[1]	7,600[1]		16,000[1]	3.5	13,000	3,800
Indoramine	1.5	5,360	(2)	3,600	10	6,300	630
Chlorpromazine	8	2,700	(3)	340	3.2	1,300	400
Haloperidol	36	4,000	(3)	110	31	2,400	80
Labetalol	64	15,000	(4)	230	41	11,000	270
Yohimbine	3,000[1]	14[1]		.0047[1]	2,100	24	.011
Rauwolscine	7,700	55	(2)	.0071	3,800	17	.0045
Mianserin[2]	310		(9)		250	870	3.5
Clozapine[2]	460		(7)		50	650	13
Piperoxan[2]	1,100		(4)		1,380	280	.20
Thymoxamine[2]	460		(2)		150	390	2.6
Imiprimine[2]	1,900		(9)		120	2,200	18

[1] K_d values from ref. 7.

[2] For these compounds, there was no significant difference between the K_d values at $alpha_1$ and $alpha_2$ sites as determined by computer modeling of [^{3}H]DHE competitioin curves. Occasionally two-site fits could be obtained that did not significantly better fit the data (see Methods). Efforts to utilize the parameter estimates of drug affinity derived from these fits with a *t*-test for paired differences did not suggest significant differences in $alpha_1$ and $alpha_2$ affinities for these drugs.

RESULTS

Saturation curves with [^{3}H]prazosin disclosed a single class of sites with a dissociation constant (K_d) of 0.5 ± 0.15 nM ($n = 4$) and a binding capacity of 19 ± 7 fmoles/mg of protein. The [^{3}H]yohimbine sites were more numerous (72 ± 19 fmoles/mg of protein) and possessed a K_d of 11 ± 5 nM ($n = 9$) (see Fig. 1). [^{3}H]DHE saturation curves performed in the same experiment exhibited 167 ± 35 fmoles/mg of protein with a uniform affinity of 3 ± 1 nM ($n = 4$) (data not shown).

We have previously shown that the fraction of [^{3}H]-DHE sites blocked by 100 nM prazosin is equivalent to the number of $alpha_1$-receptors (23). An average value for all of the membrane preparations in the present experiments was determined to be 23 ± 7%. This is in reasonable agreement with the proportion of [^{3}H]DHE sites labeled with [^{3}H]prazosin = 17 ± 3.6%. Therefore, approximately 20% of [^{3}H]DHE sites are $alpha_1$ and about 80% are $alpha_2$. [^{3}H]Yohimbine appeared to label a somewhat smaller percentage of [^{3}H]DHE sites than would be expected from the proportion of $alpha_2$-receptors in the membranes: 43% obtained versus 80% expected. Nonetheless, its affinity at $alpha_2$ sites determined by direct [^{3}H]yohimbine binding was in close agreement with that obtained from computer modeling of competition curves of [^{3}H]DHE by unlabeled yohimbine (see Table 1).

Competition curves of unlabeled yohimbine and prazosin versus both of the selective radioligands confirmed that [^{3}H]prazosin and [^{3}H]yohimbine were labeling exclusively $alpha_1$ and $alpha_2$ sites, respectively. Figure 2 represents results obtained with unlabeled prazosin which in this preparation was found to be about 4,000-fold more potent at [^{3}H]prazosin sites than at [^{3}H]yohimbine sites. By contrast, results obtained with unlabeled yohimbine (Fig. 3) displayed the reverse relationship: yohimbine was about 100- to 200-fold more potent at [^{3}H]yohimbine sites than at [^{3}H]prazosin sites. Moreover, all four competition curves (in Figs. 2 and 3) modeled best to one site with no improvement in the fit of the data points with a two-site model. Thus, the selectivity of prazosin and yohimbine obtained in this way closely matched the selectivity as determined by analysis of complex biphasic [^{3}H]DHE competition curves (see Table 1).

Competition curves of a variety of *alpha*-adrenergic antagonists were next constructed by using [^{3}H]DHE, [^{3}H]yohimbine, and [^{3}H]prazosin. Figures 4 and 5 illustrate the results obtained with two representative compounds: rauwolscine, an $alpha_2$ selective agent, and labetalol, an $alpha_1$ selective drug. In each case, the $alpha_1$ and $alpha_2$ K_d values of the drug determined by computer modeling of the [^{3}H]DHE competition curve were in good agreement with the $alpha_1$ and $alpha_2$ K_d values of the drug determined at [^{3}H]prazosin ($alpha_1$) and [^{3}H]-yohimbine ($alpha_2$) sites. It can also be appreciated, even by simple visual inspection, that in each case the complex [^{3}H]DHE competition curves representing competition of the ligand for both $alpha_1$ and $alpha_2$ sites is a composite of the simple uniphasic curves obtained in competition with [^{3}H]prazosin and [^{3}H]yohimbine for each drug. Table 1 displays the results of a similar analysis for a series of antagonists. Correlation plots for $alpha_1$ and $alpha_2$ affinities (K_d values) for selective compounds as determined by either of the two methods are shown in Fig. 6. These indicated that the K_d for $alpha_1$ of a drug determined by competition with [^{3}H]DHE correlated highly with the K_d for the [^{3}H]prazosin sites, and the K_d for $alpha_2$ at [^{3}H]DHE sites correlated strongly with the K_d at the [^{3}H]yohimbine sites. There was no correlation

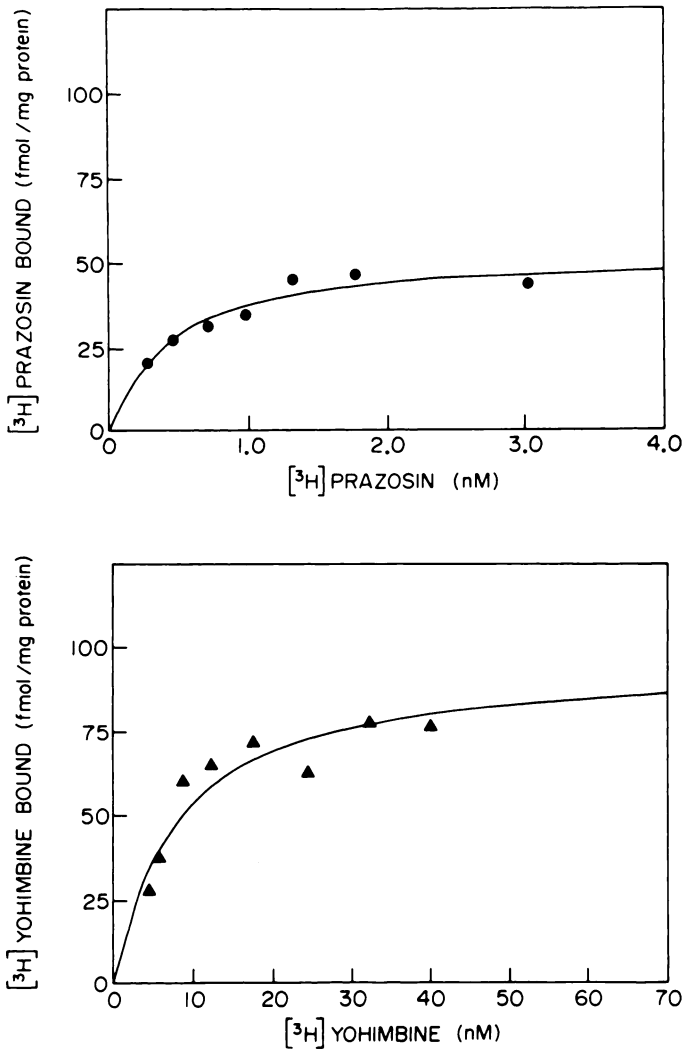

FIG. 1. *Saturation curves of [^{3}H]prazosin (upper panel) and [^{3}H]-yohimbine (lower panel) in rabbit uterine membranes*

In this representative experiment (n = 4), a single membrane preparation was divided and binding with each radioligand was performed. [^{3}H]Prazosin binds with a K_d of 0.3 nM to 50 fmoles/mg of protein sites, while [^{3}H]yohimbine binds with a K_d of 8 nM to 90 fmoles/mg of sites. Each curve represents the best fit model (one site) as determined by computer analysis (see Methods).

of [^{3}H]yohimbine K_d values with *alpha*$_1$ affinities or [^{3}H]-prazosin affinities with *alpha*$_2$ affinities (not shown).

DISCUSSION

We have determined the *alpha*-adrenergic receptor subtype selectivity of a number of *alpha*-antagonists by utilizing two distinct experimental approaches. One approach involves the use of a nonsubtype selective antagonist radioligand, [^{3}H]DHE, which labels the entire *alpha*-receptor population of a tissue. The competition curve of a selective drug with this ligand represents a composite of its interactions with *alpha*$_1$- and *alpha*$_2$-receptors. The individual receptor subtype affinities of drugs which showed major selectivity, such as some of the neuroleptics and the *alpha*$_2$-selective ligand rauwolscine (25), could be accurately delineated by this method. A second approach employed radioligands which were

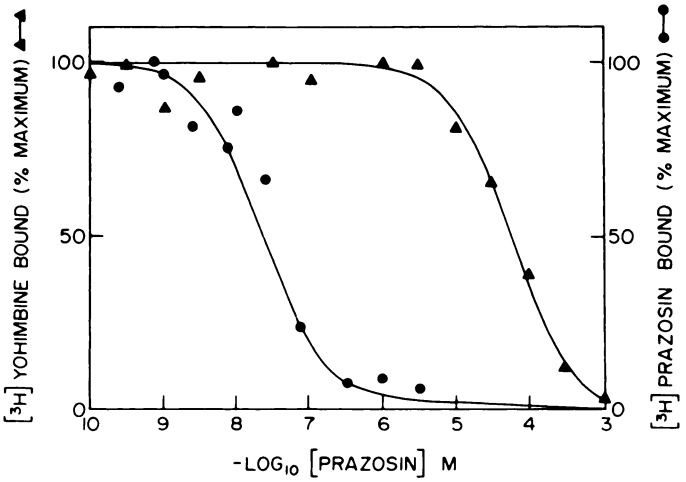

FIG. 2. *Competition binding curves of prazosin with [^{3}H]prazosin or [^{3}H]yohimbine*

Binding was performed simultaneously in aliquots of the same membrane preparation. [^{3}H]Prazosin concentration was 1–2 nM, while [^{3}H]yohimbine concentration was 8–12 nM. Prazosin was approximately 4000-fold more potent at [^{3}H]prazosin sites (K_d = 3 nM) than at [^{3}H]-yohimbine sites (K_d = 14,000 nM). Slope factors were not significantly different from 1. The experiment was replicated three times.

shown to label only the *alpha*$_1$ site, [^{3}H]prazosin, or the *alpha*$_2$ site, [^{3}H]yohimbine. Results obtained with this method were in good agreement with those obtained with [^{3}H]DHE. Moreover, this approach permitted the delineation of affinities of even very weakly selective agents, e.g., piperoxan and imiprimine, which would otherwise have appeared to be "nonselective" by the [^{3}H]DHE technique.

Thus, the use of the individual radioligands [^{3}H]prazosin and [^{3}H]yohimbine offers certain advantages. First, even modest selectivity, on the order of 10-fold, may be

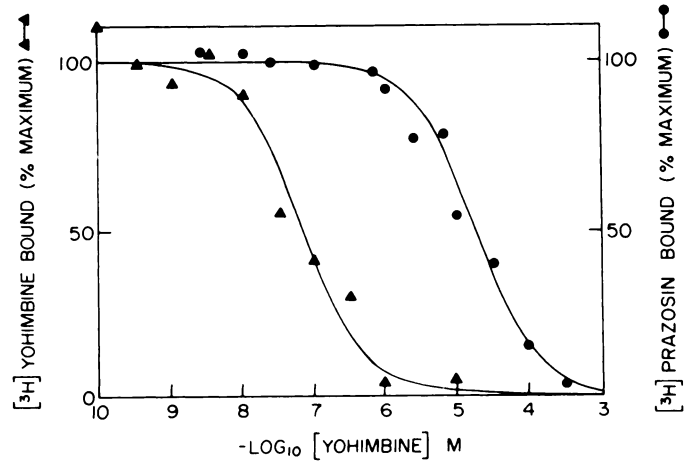

FIG. 3. *Yohimbine competition curves with either [^{3}H]prazosin or [^{3}H]yohimbine*

Concentrations of radioligands were as follows: [^{3}H]prazosin, 1–2 nM; [^{3}H]yohimbine, 8–12 nM. Yohimbine was approximately 100-fold more selective for *alpha*$_2$ sites. The K_d for [^{3}H]yohimbine sites was 18 nM versus the K_d for [^{3}H]prazosin sites of 2000 nM. Both competition curves modeled best to one site with a slope factor not significantly different from 1 and are representative of three such experiments.

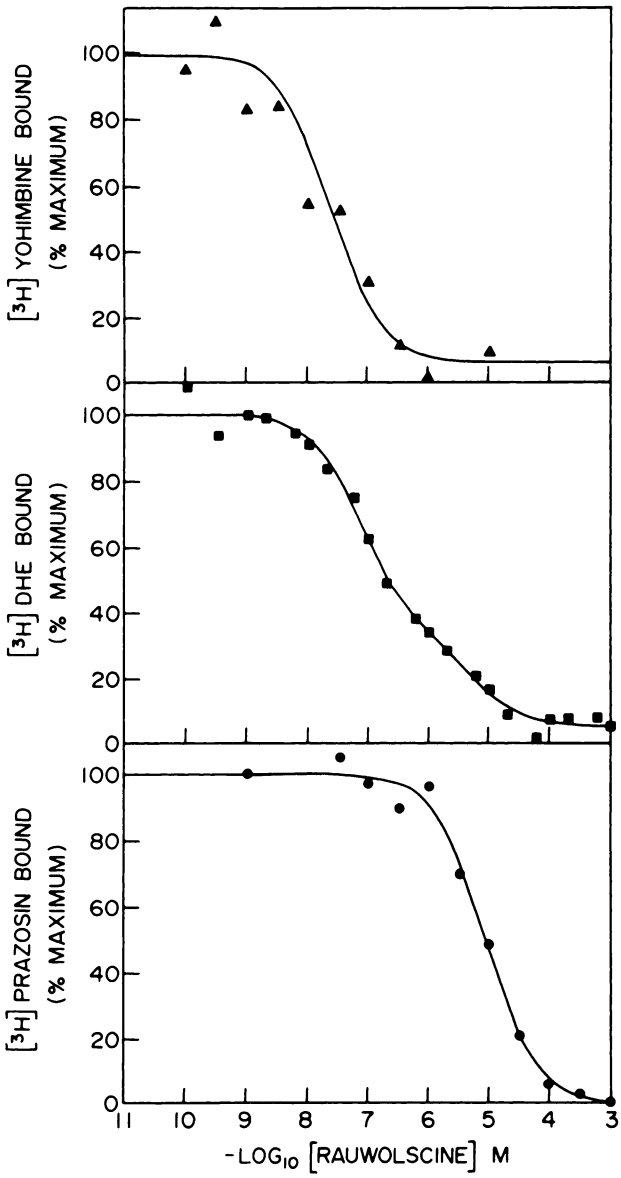

FIG. 4. *Rauwolscine competition curves with [^{3}H]yohimbine (top panel), [^{3}H]DHE (middle panel), and [^{3}H]prazosin (bottom panel)*

Rauwolscine was more potent at [^{3}H]yohimbine sites than at [^{3}H]-prazosin sites. Computer modeling of the [^{3}H]DHE competition curve delineated two sites, with K_d values corresponding to those obtained by the selective radioligands. Dissociation constants are given in Table 1.

detected. It should be noted that in the present system approximately 100-fold selectivity allowed reliable assessment of the *alpha*$_1$ and *alpha*$_2$ K_d values by computer modeling of individual [^{3}H]DHE competition curves. If curves are meaned prior to analysis, selectivity in the range of 30- to 70-fold can be determined in this system (17). It must also be stressed that the sensitivity of the method depends critically on the proportion of receptor subtypes (50:50 mixture is best), the number of data points in an experiment, and the level of nonspecific binding (which is relatively high—30–40% in this system). For example, in a model system of *beta*$_1$- and *beta*$_2$-receptors created by employing a 1:1 mixture of frog and turkey erythrocyte membranes and a radioligand which gives 95% specific binding, [^{3}H]dihydroalprenolol, selectivity of 5- to 7-fold can be reliably determined (24). A second potential advantage of using the individual selective radioligands is that computer modeling procedures are not required and all of the competition curves analyzed are simple and uniphasic.

Our results confirm the earlier findings of others that [^{3}H]prazosin appears to label exclusively *alpha*$_1$-receptors (11, 14). We also find that [^{3}H]yohimbine labels exclusively *alpha*$_2$-receptors in this system. Similar findings have recently been obtained in rat liver plasma membranes (26). In the latter system, the sum of [^{3}H]-prazosin and [^{3}H]yohimbine sites equaled the number of [^{3}H]DHE sites, as would be anticipated. By contrast, in the present studies, although the number of [^{3}H]prazosin

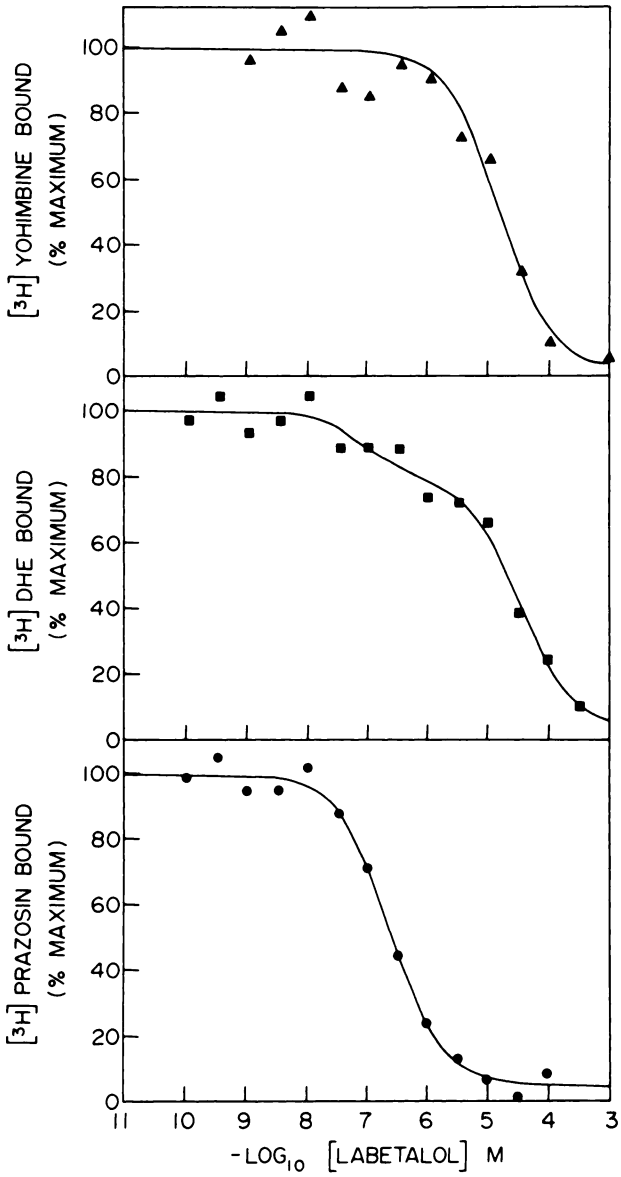

FIG. 5. *Competition curves of labetalol with [^{3}H]yohimbine (top panel), [^{3}H]DHE (middle panel), and [^{3}H]prazosin (lower panel)*

Computer modeling of the biphasic [^{3}H]DHE curve resulted in resolution of both *alpha*$_1$ and *alpha*$_2$ sites (see text for details). Labetolol was more potent at [^{3}H]prazosin sites than at [^{3}H]yohimbine sites, indicating its *alpha*$_1$ selectivity. Dissociation constants given in Table 1.

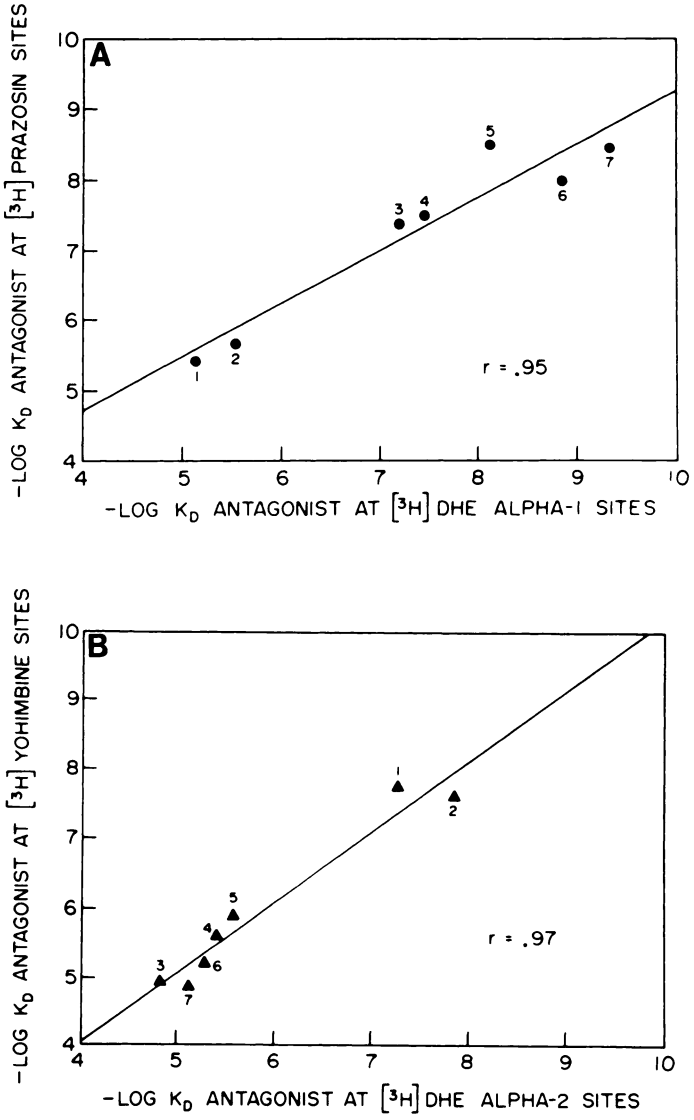

FIG. 6. *Correlation of alpha$_1$ and alpha$_2$ dissociation constants derived from [^{3}H]DHE competition curves with those derived from [^{3}H]prazosin and [^{3}H]yohimbine competition data*

The antagonists are identified as follows: *1*, rauwolscine; *2*, yohimbine; *3*, labetalol; *4*, haloperidol; *5*, chlorpromazine; *6*, indoramine; *7*, prazosin.

A. Dissociation constants at [^{3}H]prazosin sites (*alpha$_1$*) are plotted versus dissociation constants for the *alpha$_1$* component of [^{3}H]DHE binding derived from computer modeling. Linear regression yields the *r* value as shown.

B. Dissociation constants at [^{3}H]yohimbine sites (*alpha$_2$*) are plotted versus dissociation constants for the *alpha$_2$* component of [^{3}H]DHE binding derived from computer modeling. Linear regression yields the *r* value shown.

sites was equivalent to the number of *alpha$_1$* sites determined by prazosin competition with [^{3}H]DHE, the number of [^{3}H]yohimbine sites was somewhat lower than the number of *alpha$_2$* sites determined by analysis of [^{3}H]-DHE binding. Nonetheless, the *alpha$_2$* affinity of yohimbine in competition with [^{3}H]DHE was identical with that determined by direct [^{3}H]yohimbine binding. The reason for this discrepancy in site number is unclear.

Recent physiological data indicate that it is the *alpha$_1$*-receptors which are responsible for mediating the contractile effects of norepinephrine in rabbit uterus.[4] The physiological role of the *alpha$_2$*-receptors detected in these and other studies in rabbit uterus remains a matter of conjecture at this point.

A number of previous attempts to determine *alpha*-adrenergic receptor subtype selectivity by using subtype selective radioligands have utilized the antagonist [^{3}H]WB4101 (2-([2′,6′-dimethoxy]-phenoxyethylamino)-methyl benzodioxan) (*alpha$_1$*) and the agonists [^{3}H]epinephrine, [^{3}H]norpinephrine, and [^{3}H]clonidine (*alpha$_2$*) (4, 15, 27). [^{3}H]Prazosin would appear to be preferable to [^{3}H]WB4101 since it is considerably more *alpha$_1$*-selective. In the rabbit uterus, for example, WB4101 shows very little *alpha$_1$*/*alpha$_2$* selectivity (28). The *alpha$_2$*-antagonist [^{3}H]yohimbine would also appear to have advantages over the tritiated agonists noted above. The binding of these agonists appears at least in part to be related to a high-affinity guanine nucleotide-sensitive form of the *alpha$_2$*-receptor which constitutes a variable proportion of the *alpha$_2$* sites (16, 27). Thus, there are additional complexities in the binding of the agonists which could complicate data interpretation; these difficulties are not present when using an antagonist such as [^{3}H]yohimbine.

The selectivity of the various drugs as determined by our radioligand binding methods compares favorably with the selectivity as determined by more standard pharmacological techniques. For example, indoramine is *alpha$_1$*-selective (29), mianserin is *alpha$_1$*-nonselective (30), and piperoxan is weakly *alpha$_2$*-selective (30, 31). We also confirm previous suggestions (31) that labetalol is an *alpha$_1$*-selective agent.

In summary, we have compared two experimental approaches for determining the *alpha*-adrenergic selectivity of antagonists. Each method appears to be technically valid and to give closely comparable results. However, the use of selective antagonist radioligands has the advantage of finer discrimination and obviates the need for computer modeling of the data. The results obtained and conclusions reached here may be applicable to the determination of receptor subtype selectivity by radioligand binding methods in a variety of systems.

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