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[^{3}H]WB4101—CAUTION ABOUT ITS ROLE AS AN ALPHA-ADRENERGIC SUBTYPE SELECTIVE RADIOLIGAND*

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Abstract—Quantification of alpha-adrenergic subtypes, using radioligand binding studies, depends on the availability of subtype selective drugs. [^{3}H]WB4101 has been proposed as an alpha$_1$ selective radioligand [D. C. U'Prichard and S. H. Snyder, *Life Sci.* **24**, 79 (1979)]. While confirming that WB4101 is alpha$_1$ selective in calf cerebral cortex, we have found, however, that both unlabeled and tritiated WB4101 bind with indistinguishable affinity to the alpha$_1$ and alpha$_2$ receptors in rabbit uterus. This conclusion is based on three sets of observations: (1) [^{3}H]WB4101 and [^{3}H]dihydroergocryptine bind with high uniform affinity to the same number of sites in rabbit uterus in which only ~30 per cent of the alpha receptors are alpha$_1$; (2) computer modeling of WB4101 competition curves with [^{3}H]dihydroergocryptine indicates that WB4101 had indistinguishable affinity for the alpha$_1$ and alpha$_2$ receptors in rabbit uterus; and (3) competition curves of the alpha$_1$ selective antagonist prazosin with [^{3}H]WB4101 are biphasic, indicating that [^{3}H]WB4101 was bound to both alpha$_1$ and alpha$_2$ receptors. Cautious testing of the alpha-adrenergic subtype selectivity of ^{3}H-labeled ligands, such as [^{3}H]WB4101, needs to be undertaken before any can be utilized to selectively label alpha-adrenergic subtypes.

Since the initial demarcation between alpha- and beta-adrenergic receptors in 1948 [1], it has become clear that there exist subtypes of both beta [2] and alpha [3, 4] receptors. There has not been uniformity in the terminology used to define the alpha-adrenergic receptor subtypes. Initially these were called 'post-synaptic' and 'pre-synaptic' alpha receptors. However, the more general terminology of Berthelson and Pettinger [5] wherein the two classes of alpha receptors are recognized by their different affinities for a variety of drugs and are termed 'alpha$_1$' and 'alpha$_2$' receptors, respectively, seems preferable. These subtypes have also been defined by radioligand binding assays [6–9].

Two general approaches have been utilized to quantitate the alpha-adrenergic subtypes in a tissue by radioligand binding. Firstly, a non-subtype selective radioligand such as [^{3}H]dihydroergocryptine ([^{3}H]DHE) has been used to construct competition curves with the highly alpha$_1$ selective antagonist prazosin; computer modeling of the resultant competition curve can provide estimates of the proportions of alpha$_1$ and alpha$_2$ receptors [8]. Second, 'selective' radioligands can be utilized to label one or the other subtype of alpha-adrenergic receptor by virtue of having very high affinity for one adrenergic subtype and low affinity for the other. Several compounds have been proposed to fulfill this purpose. It has been suggested that [^{3}H]WB4101 binds exclusively alpha$_1$ receptors [6, 10, 11], as does [^{3}H]prazosin [12].

[^{3}H]WB4101 has been used extensively in radioligand binding studies in mammalian brain [6, 13–15] and more recently it has been utilized to label alpha receptors in a wide variety of peripheral tissues [11]. We have demonstrated recently that rabbit uterus contains a mixture of alpha$_1$ and alpha$_2$ receptors, with the alpha$_2$ sites predominating [8, 9]. In the course of examining the alpha receptor subtype specificity of various drugs in this system, we discovered to our surprise that WB4101 has indistinguishable affinities for the alpha$_1$ and alpha$_2$ receptors in this tissue. This was confirmed with both [^{3}H]WB4101 in direct binding studies and with unlabeled WB4101 in competition with the non-selective alpha antagonist [^{3}H]DHE. These findings have important implications for the use of [^{3}H]WB4101 in radioligand binding studies.

METHODS

Radioligand binding assay. For the experiments using rabbit uterus, membrane preparation was done as described previously [16]. In assays involving both [^{3}H]DHE and [^{3}H]WB4101, incubations were for 18 min at 25° as described previously for [^{3}H]DHE [16]. For the competition curves involving prazosin, [^{3}H]DHE was at a concentration of ~5 nM and [^{3}H]WB4101 at ~4 nM. Specific binding was 60–70 per cent of total binding for [^{3}H]DHE and 40–60 per cent for [^{3}H]WB4101.

The experiments involving calf brains (supplied by Pel Freeze Biologicals, Rogers, AR) were done using the membrane preparation and assay conditions described by U'Prichard and Snyder [15]. Frozen grey matter from calf frontal cortex was homogenized in 20 vol. of ice-cold buffer (50 mM

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Tris–HCl, pH 7.7 at 23°) with a Brinkmann polytron. The homogenate was centrifuged at 50,000 *g* for 10 min. The pellet was resuspended in buffer and centrifuged again at 50,000 *g* for 10 min. The final pellet was suspended in 46 vol. of ice-cold buffer and used immediately. Binding assays were as described [15], wherein competition curves of [^{3}H]DHE (at a concentration of ~0.5 nM) with unlabeled prazosin and WB4101 were constructed by incubating ~20 mg of cortical membranes in a total volume of 2.0 ml at 25° for 60 min. Bound [^{3}H]DHE was separated from free [^{3}H]DHE by vacuum filtration with a wash of 15 ml of ice-cold buffer.

Materials. [^{3}H]DHE (39 Ci/mmole) and [^{3}H]WB4101 (25 Ci/mmole) were obtained from the New England Nuclear Corp. (Boston, MA). Prazosin (Pfizer, New York, NY) and unlabeled WB4101 (Ward Blenkinsop, Bracknell, Berkshire U.K.) were gifts. Other chemicals were obtained from commercial sources.

Data analysis. All experiments were performed in duplicate and replicated from three to seven times, as indicated in the text. Computer modeling was done as described previously [8, 17]. Briefly, using a PDP 11/45 computer, data were analyzed by a nonlinear least squares curve fitter [18] using a generalized model for ligand–receptor interactions [19]. Data were first fit to a model involving a single class of binding sites; then the data were fit to a model involving two binding sites, and the improvement (if any) in the goodness of fit was statistically tested [20]. A two-site fit was accepted if the fit was improved significantly ($P < 0.05$). Slope factors were determined by a four-parameter logistic equation [20].

The proportions of alpha$_1$ and alpha$_2$ receptors in the various uterine membrane preparations were determined by constructing paired [^{3}H]DHE saturation curves in the presence and absence of a fixed concentration of prazosin (10^{-7} M). We have demonstrated [9] that, at a concentration of 10^{-7} M, prazosin binds essentially all the alpha$_1$ receptors and none of the alpha$_2$ receptors in uterine membranes. Thus, in the presence of 10^{-7} M prazosin, a [^{3}H]DHE saturation curve provides an estimate of the number of alpha$_2$ receptors and, by subtraction from the number of [^{3}H]DHE sites obtained in the absence of prazosin, the number of alpha$_1$ receptors may be calculated. This technique provides estimates of the alpha receptor subtype proportions equivalent to those measured previously by the analysis of prazosin competition curves with [^{3}H]DHE [8].

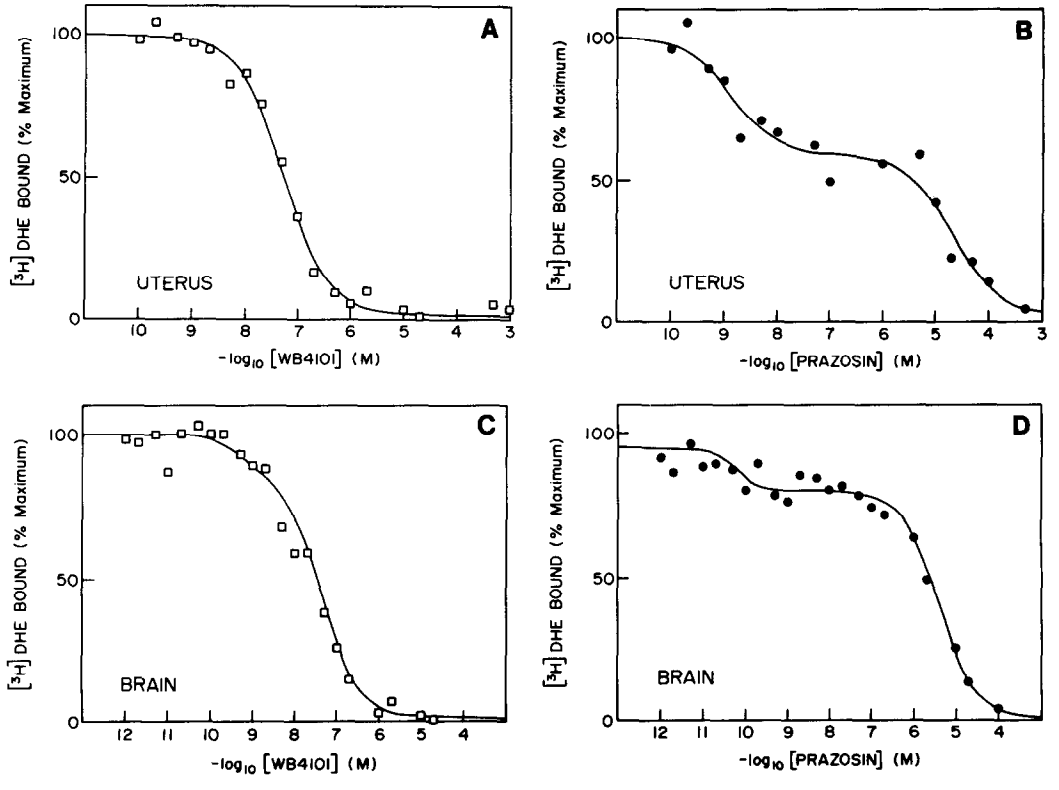

Fig. 1. Competition curves of prazosin and WB4101 with [^{3}H]DHE in rabbit uterine (A and B) and calf brain membranes (C and D). The points are the means of duplicates from a representative experiment, replicated three to seven times, whereas the curves were drawn by the computer modeling technique. The prazosin (D) and WB4101 (C) curves in brain modeled to two classes of binding sites where the proportions of alpha receptors were alpha$_1$ = 20 per cent and alpha$_2$ = 80 per cent. The prazosin curve in rabbit uterus also modeled to two classes of sites (alpha$_1$ = 40 per cent and alpha$_2$ = 60 per cent). The WB4101 curve in uterus was best fit to a single class of binding sites of uniform affinity, suggesting that WB4101 has indistinguishable affinity for alpha$_1$ and alpha$_2$ receptors in uterine membranes.

RESULTS

We have demonstrated previously that the selective antagonists prazosin (alpha$_1$) and yohimbine (alpha$_2$) yield complex biphasic competition curves with [^{3}H]DHE in rabbit uterus, a tissue which contains a mixture of alpha$_1$ and alpha$_2$ receptors with the latter predominating [8, 9]. Figure 1 illustrates [^{3}H]DHE competition curves of unlabeled WB4101 and prazosin in uterine membranes, and in brain membranes which also contain a mixture of alpha$_1$ and alpha$_2$ receptors [6, 10]. The prazosin curves in both tissues were biphasic (Fig. 1, panels B and D) and modeled significantly better to a two-site fit in each tissue. The dissociation constants in brain of prazosin at alpha$_1$ ($K_{D\text{alpha}_1}$) and alpha$_2$ ($K_{D\text{alpha}_2}$) receptors were $K_{D\text{alpha}_1} = 8.6 \times 10^{-10}$ M and $K_{D\text{alpha}_2} = 5.3 \times 10^{-6}$ M (N = 3). These K_D values in brain are similar to those reported previously for prazosin in uterus, which has a $K_{D\,\text{alpha}_1} = 4.7 \times 10^{-10}$ M and a $K_{D\text{alpha}_2} = 7.6 \times 10^{-6}$ M [8]. The competition curve of unlabeled WB4101 in brain (Fig. 1, panel C) also modeled best to a two-site fit. The dissociation constants in three such WB4101 curves in brain membranes were: $K_{D\,\text{alpha}_1} = 4.0 \times 10^{-10}$ M and $K_{D\text{alpha}_2} = 2.5 \times 10^{-8}$ M, in reasonable agreement with the values of 3×10^{-10} M and 1.1×10^{-7} M, respectively, reported by U'Prichard *et al.* [6]. These authors, however, determined the K_D of WB4101 at alpha$_1$ receptors by competition with [^{3}H]WB4101 and the K_D of WB4101 at alpha$_2$ receptors by WB4101 competition with [^{3}H]clonidine. Because we find WB4101 to be only ~60-fold alpha$_1$ selective in brain (as compared to the ~10,000-fold alpha$_1$ selectivity of prazosin), the visual effect of WB4101's selectivity is evidenced only by a flattened shoulder at the top of the competition curve (Fig. 1, panel C) in contrast with the frankly biphasic shape of the prazosin curve (Fig. 1, panel D). An additional manifestation of the two-site fit of WB4101 in brain is that the slope factor of the illustrated WB4101 curve in brain is 0.73 which is significantly less than a slope factor of 1.0 ($P < 0.001$) which would be anticipated for a one-site fit.

The situation with WB4101 in uterus is quite different (Fig. 1, panel A). The illustrated WB4101 competition curve modeled to one site with a slope factor of 1.07. In seven of seven experiments, WB4101 competition curves in rabbit uterine membranes each modeled to a single class of binding sites of uniform affinity with a mean K_D of $2.4 \pm 0.5 \times 10^{-8}$ M, strongly suggesting that WB4101 does not discriminate between alpha$_1$ and alpha$_2$ receptors in uterus. We have demonstrated previously that phentolamine also does not discriminate between alpha$_1$ and alpha$_2$ receptors in the uterus [8]. It is of interest to note that the uniform K_D of WB4101 in the rabbit uterus is similar to the K_D of WB4101 at alpha$_2$ sites in brain membranes (2.4×10^{-8} M vs 2.5×10^{-8} M, respectively).

To further test the hypothesis that WB4101 does not discriminate between alpha$_1$ and alpha$_2$ receptors in uterus, saturation curves of [^{3}H]WB4101 and [^{3}H]DHE were performed in the same membrane preparation (Fig. 2, panels A and B). In the illustrated experiment, both saturation curves modeled best to a single class of sites; the [^{3}H]WB4101 sites = 103 fmoles/mg protein and the [^{3}H]DHE sites = 99 fmoles/mg protein. In the illustrated experiment, the proportion of alpha receptor subtypes was alpha$_1$ = 32 per cent and alpha$_2$ = 68 per cent, determined by prazosin as described in Methods. Thus, if [^{3}H]WB4101 were alpha$_1$ selective, it would be expected to label only ~$\frac{1}{3}$ of the [^{3}H]DHE sites; in actuality, it labeled, with uniform affinity, the same number of sites as [^{3}H]DHE. This further confirms the hypothesis that WB4101 does not discriminate between alpha$_1$ and alpha$_2$ receptors in rabbit uterus. In four such experiments, the number of [^{3}H]DHE sites was 140 ± 3 fmoles/mg protein and the number of [^{3}H]WB4101 sites was 153 ± 19 fmoles/mg protein which were not significantly different ($P < 0.9$, paired *t*-test). Moreover,

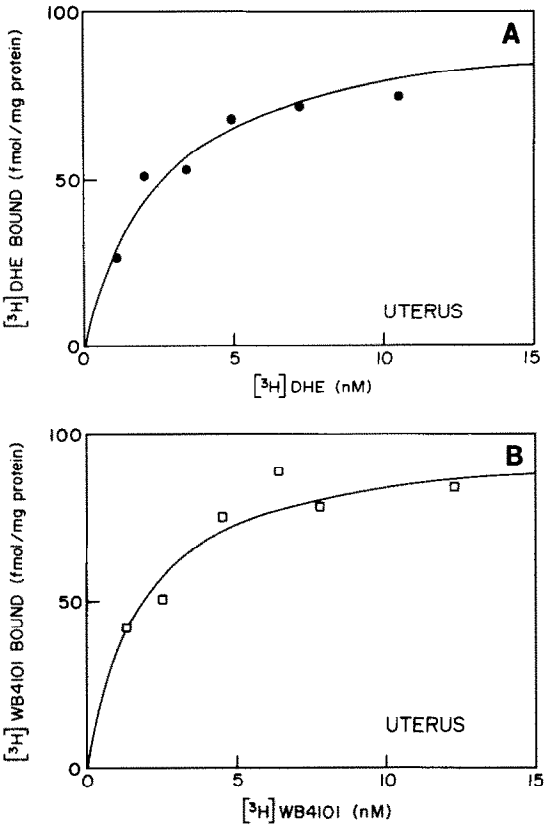

Fig. 2. [^{3}H]DHE and [^{3}H]WB4101 saturation curves in uterine membranes. The points represent the means of duplicates from a representative experiment. Both saturation curves were done with membranes from the same preparation in each experiment. The number of [^{3}H]DHE sites in panel A was 99 fmoles/mg protein. The number of [^{3}H]WB4101 sites in panel B was 103 fmoles/mg protein. The proportion of alpha receptor subtypes in this preparation, determined with prazosin as described in Methods, was alpha$_1$ = 32 per cent and alpha$_2$ = 68 per cent. In four such saturation curves, the K_D of [^{3}H]DHE was $4.9 \pm 1.0 \times 10^{-9}$ M and the K_D of [^{3}H]WB4101 was $4.0 \pm 0.9 \times 10^{-9}$ M. The reason for the 4-fold difference in the affinity of WB4101, determined by direct binding with tritiated WB4101 vs competition with unlabeled WB4101, is not known.

in all four experiments, computer modeling of the [^{3}H]WB4101, as well as the [^{3}H]DHE saturation curves indicated that each ligand bound to the entire alpha receptor population with uniform affinity. The proportions of alpha$_1$ and alpha$_2$ receptors in these four experiments were: alpha$_1$ = 29 ± 3 per cent and alpha$_2$ = 71 ± 3 per cent.

A third test of the hypothesis is that [^{3}H]WB4101 binds to the entire alpha receptor population (alpha$_1$ and alpha$_2$) of rabbit uterus involved the construction of [^{3}H]WB4101 competition curves with prazosin in uterine membranes (Fig. 3). In agreement with the data shown above, the modeling of such curves suggested that [^{3}H]WB4101 binds to the entire alpha

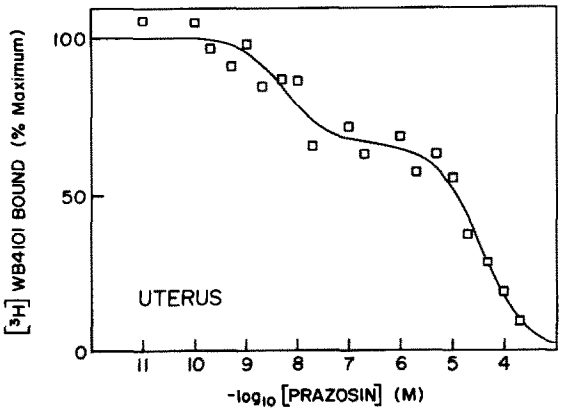

Fig. 3. Competition curve of prazosin and [^{3}H]WB4101 in uterine membranes. The illustrated experiment is representative of four such curves. The biphasic prazosin curve modeled to two sites of binding, indicating that [^{3}H]WB4101 was binding to both alpha$_1$ and alpha$_2$ receptors. The concentration of [^{3}H]WB4101 in the assay was 4 nM.

receptor population since both subtypes of alpha receptor could be distinguished with the alpha$_1$ selective anatogonsist prazosin. Prazosin had a K_D of $2.4 \pm 0.7 \times 10^{-9}$ M at the higher affinity (alpha$_1$) site and a K_D of $2.3 \pm 0.5 \times 10^{-5}$ M at the lower affinity (alpha$_2$ site) (N = 4). This 9500-fold greater potency of prazosin at alpha$_1$ sites than at alpha$_2$ sites, each labeled with [^{3}H]WB4101, is very similar to the 8800-fold selectivity of prazosin at alpha$_1$ and alpha$_2$ receptors labeled with [^{3}H]DHE [8]. These results further confirm that [^{3}H]WB4101 labels both alpha$_1$ and alpha$_2$ receptors. If only alpha$_1$ receptors were labeled by [^{3}H]WB4101, then the prazosin curve would have been expected to model to a single class of sites of high (alpha$_1$) affinity.

DISCUSSION

We have shown that WB4101 binds with indistinguishable affinity to the alpha$_1$ and alpha$_2$ receptor in rabbit uterine membranes. This conclusion is based on three major observations. First, WB4101 competition curves with [^{3}H]DHE repeatedly model with a single affinity to the entire alpha receptor population of rabbit uterus, a tissue demonstrated to contain a mixture of alpha$_1$ and alpha$_2$ receptors. Second, [^{3}H]WB4101 labels, with uniform affinity, the entire alpha receptor population of the uterus rather than just the alpha$_1$ fraction. Third, prazosin competition curves with [^{3}H]WB4101 modeled to two classes of binding sites, indicating that [^{3}H]WB4101 was binding to both alpha$_1$ and alpha$_2$ receptors. We were able to confirm, however, that WB4101 is alpha$_1$ selective in calf brain membranes as has been reported previously [6, 10].

The explanation for the lack of alpha$_1$ selectivity of WB4101 in rabbit uterus is unknown. The observation that the affinity of WB4101 for the alpha$_1$ and alpha$_2$ receptors in uterus is similar to the affinity of WB4101 for alpha$_2$ receptors in brain suggests that there is some difference between alpha$_1$ receptors in rabbit uterus, where WB4101 has a K_D of 2.4×10^{-8} M, and the alpha$_1$ receptors in calf brain where WB4101 has a K_D of 4.0×10^{-10} M.

WB4101 has not yet been extensively tested in a variety of tissues in physiological experiments to determine its potency at alpha$_1$ and alpha$_2$ receptors. The two such studies which compare pre- and postsynaptic potency, which we are aware of, were both performed in rat vas deferens [21, 22]. In both of these studies, WB4101 was reported to be alpha$_1$ selective. In neither study, however, was pre-synaptic blocking potency assessed directly with measurements of neurotransmitter efflux. Also, in the study of Kapur and Mottram [21], phentolamine was found to be highly alpha$_2$ selective. This selectivity of phentolamine does not hold true in other tissues [8], underscoring the importance of tissue variability in the selectivity of alpha–adrenergic compounds.

The present studies have important implications for the design and interpretation of alpha-adrenergic receptor radioligand binding studies. It is currently assumed that [^{3}H]WB4101 can be widely used to study exclusively the alpha$_1$ subtype of alpha receptors [11]. However, our results indicate that, before [^{3}H]WB4101 could be utilized in tissues other than calf brain to label exclusively alpha$_1$ receptors, it is necessary to check that it is sufficiently alpha$_1$ selective to accomplish this task reliably. As we have seen, it would be inadequate for this purpose in rabbit uterus.

The technique which we favour for quantitating alpha$_1$ and alpha$_2$ receptors involves the computer modeling of competition curves of highly selective alpha receptor antagonists such as prazosin, utilizing [^{3}H]DHE which appears to label the entire alpha receptor population of a tissue nonselectively [8].

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