CHARACTERISTICS OF [3H]E-643-BINDING TO ALPHA ADRENOCEPTORS

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Abstract—Radiolabeled E-643, a newly developed antihypertensive compound, bound specifically to a preparation obtained from rat brain with a maximum of 85 f moles of binding sites per mg protein and a dissociation constant of 0.59 nM. Prazosin markedly inhibited the binding, while yohimbine and clonidine were only weak inhibitors. Other characteristics of the binding of [3H]E-643 to the brain and its specific binding to preparations of peripheral rat organs were also studied. The present findings suggest that [3H]E-643 is useful for labeling α₁-adrenoceptors.

Pharmacological studies showed that a newly developed antihypertensive compound, E-643[4-amino-2-(4-butanoyl-hexahydro-1H-1, 4-diazepin-1-yl)-6, 7-dimethoxy quinazoline hydrochloride] may be a specific and competitive inhibitor of norepinephrine (NE) at α-adrenoceptors (1). Its highly selective action on postsynaptic α-adrenoceptors, but not presynaptic α-adrenoceptors, was also demonstrated by comparison of its effects with those of many kinds of α-blockers on pre- or postsynaptic α-adrenoceptors (2).

Studies on the direct binding of radiolabeled ligand to neurotransmitter receptors provided further evidence for the interactions between receptors and ligands. Radiolabeled agonists (3, 4) and antagonists (4, 5) labeled α-adrenoceptors. Recent studies revealed that [3H]WB 4101 (6–9) or [3H]prazosin (10–13) specifically binds to α₁-adrenoceptors, while [3H]clonidine (7) or [3H]yohimbine (14) binds to α₂-adrenoceptors. This paper reports on the specific binding of [3H]E-643 to α₁-adrenoceptors and the characteristics of this binding.

MATERIALS AND METHODS

Animals: Male Wistar rats weighing 200–300 g were used. They were given the standard laboratory diet (Oriental Yeast Co. Ltd, Tokyo, Japan) and water ad libitum.

Radiolabeled E-643 and other compounds: E-643 hydrochloride and its tritiated derivative were gifts from the Eisai Co. Ltd. (Tokyo, Japan). Clonidine hydrochloride was obtained from Boehringer (Ingelheim, West Germany). Phentolamine hydrochloride and prazosin hydrochloride were gifts from the Takeda Pharmaceutical Co. (Osaka, Japan) and Taito-Pfizer pharmaceutical Co. (Tokyo, Japan), respectively. Tritiated E-643 (specific radioactivity, 50 Ci/m mol) was synthesized in the Chemical Synthetic Laboratories of Eisai Co. Ltd. The purity of the labeled E-643 was almost 100 % as judged by thin layer chromatography on silica gel (Merck, Darmstadt, West Germany) in methanol: tetrahydrofuran: chloroform: ammonium hydroxide: ether (0.5: 13: 15: 0.2: 10 by vol.). Radiolabeled E-643 moved with
authentic E-643 as a single band with an Rf value of 0.40.

Binding of radiolabeled E-643: Tissues isolated from rats were homogenized with a 50-fold volume of 50 mM Tris-HCl buffer (pH 7.4) in a polytron (10-ST, Kinematica, Switzerland) set at an index of 9 for 20 sec. The homogenate was centrifuged for 10 min at 15,000 r.p.m., and the precipitate was washed twice with the same buffer by centrifugation. Then the tissue suspension was passed through nylon bolting cloth (150 mesh) and suspended in a 100-fold volume of 50 mM Tris-HCI buffer (pH 7.4) as described by Greenberg et al. (4). The incubation mixture for the binding assay consisted of tissue suspension (500 μg of protein) and various concentrations of [3H] E-643 with or without other agents at the final concentrations shown in a final volume of 1 ml. Incubation was started by adding [3H]E-643 and carried out for 15 min at 37°C, unless otherwise indicated. The specific binding of the ligand was expressed as the difference between the radioactivities bound in the presence and absence of 100 μM phentolamine. After incubation, the mixture was filtered through a Whatman GF/B glass fiber filter, and the filter was washed three times with 5 ml of 50 mM Tris-HCl buffer (pH 7.4). The radioactivity remaining on the filter was counted in vials containing 5 ml of Insta-Gel in a scintillation spectrometer (Aloka, model LSC 900) with about 41% efficiency.

Other methods: Maximal binding sites and the dissociation constant (Kd) of [3H]E-643 were obtained by Scatchard analysis (15) and the method of least squares. Protein was determined by the method of Lowry et al. (16).

RESULTS
Saturation of [3H]E-643 binding to a preparation from rat brain

Incubation of the rat brain preparation with [3H]E-643 in a concentration range of 0.5 to 5 nM showed that radiolabeled compound bound to the preparation with high affinity and that much of the total binding was specific. Nonspecific binding was less than 25% of the total binding at 5 nM [3H]E-643 and was negligible at concentrations of less than 1 nM (Fig. 1, A). The specific binding was saturable (Fig. 1, A) and the Scatchard analysis had a single straight line, indicating a single population of binding sites (Fig. 1, B). The values for maximal binding sites and the Kd value calculated from experiment shown in Fig. 1 and four other experiments were 84.6±8.4 f mol/mg protein (mean±S.E.) and 0.59±0.04 nM, respectively. A Hill plot of specific binding of [3H]E-643 had a straight line with a Hill coefficient (nH) of 1.06, indicating no cooperativity among the [3H]E-643 binding sites.

Fig. 1. Binding of [3H]E-643 to a preparation from rat brain. A. The tissue preparation was incubated with various concentrations of [3H]E-643 and the specific binding (○) was determined as described in Methods. Values for total (O) and nonspecific (△) binding are for duplicate determinations in a single experiment. B. Scatchard plot of the specific binding of [3H]E-643. The maximal number of binding sites and Kd value were calculated as 88.4 f moles per mg protein and 0.62 nM, respectively.
Kinetics of [³H]E-643 binding

Specific binding of [³H]E-643 to the rat brain preparation was rapid, reaching equilibrium within 10 min at 37°C (Fig. 2, A). The pseudo-first order rate constant (K₁obs) for association (Fig. 2, B) and the rate constant for dissociation (K₂) (Fig. 3) were determined. A second order rate constant for association was obtained by the equation of Williams et al. (5):

\[ K_1 = \frac{(K_{obs} - K_2)}{[³H]E-643} \]

where \([³H]E-643\) is the concentration of [³H]E-643. The value of K₁ was calculated as 0.294/nM/min. A Kᵦ value of 0.56 nM was obtained kinetically from the ratio of the two constants (K₂/K₁). This Kᵦ value agreed fairly well with that determined by Scatchard analysis of the saturation binding (Fig. 1, B).

Conditions affecting [³H]E-643 binding

Temperature: Total binding of [³H]E-643 at a given concentration to the brain preparation reached equilibrium within 10 min at 25°C or 37°C. Nonspecific binding decreased with an increase in the reaction temperature, resulting in a temperature-dependent increase in specific binding as shown in Table 1.

pH: In the experiment performed with Tris-maleate buffer, total binding of [³H]E-643 showed a broad pH optimum, but nonspecific binding decreased gradually with an increase in pH, and this specific binding was

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Table 1. Effect of temperature on [³H]E-643 binding to the rat brain preparation

| Temperature (°C) | [³H]E-643 bound (fmol/mg protein) | Total | Nonspecific | Specific |
|-----------------|----------------------------------|-------|-------------|---------|
| 15              | 45.81                            | 36.07 | 9.74        |
| 25              | 52.49                            | 8.18  | 44.31       |
| 37              | 67.37                            | 7.68  | 49.69       |

Incubation was carried out with 1.0 nM [³H]E-643 at the temperatures indicated. Values are the mean of duplicate determinations in a single experiment. For further details, see the Methods.
Table 2. Effect of pH on $[^{3}H]$E-643 binding to the rat brain preparation

| pH | Total | Nonspecific | Specific |
|----|-------|-------------|---------|
| 6.0| 41.58 | 9.60        | 31.98   |
| 6.5| 51.32 | 7.33        | 43.99   |
| 7.0| 50.05 | 7.33        | 42.72   |
| 7.5| 44.31 | 5.23        | 39.08   |
| 8.0| 35.78 | 4.72        | 31.06   |

Incubation was carried out with 1.0 nM $[^{3}H]$E-643 by substituting Tris-HCl buffer with 50 mM Tris-maleate buffer adjusted to the pH values indicated. Values are the mean of duplicate determinations in two replicate experiments. For further details, see the Methods.

Table 3. Binding of $[^{3}H]$E-643 to preparations from peripheral organs

| Maximal Binding Sites (fmol/mg protein) | $K_{D}$ (nM) |
|----------------------------------------|--------------|
| Liver                                  | 148±37.4 (3) | 0.48±0.13 (3) |
| Kidney                                 | 102±8.5 (3)  | 0.63±0.12 (3) |
| Brain                                  | 85±8.4 (5)   | 0.59±0.04 (5) |
| Lung                                   | 76 (55, 97)  | 0.56±0.09 (3) |
| Heart                                  | 72 (73, 70)  | 0.54 (0.51, 0.56) |
| Vas deferens                           | 48±5.6 (3)   | 1.08±0.21 (3) |
| Parotid                                | 30±2.2 (6)   | 1.73±0.31 (6) |
| Ileum                                  | 16±5.9 (3)   | 2.28±0.88 (3) |

The maximal binding sites and $K_{D}$ value were determined by Scatchard analysis as shown in Fig. 1. Details of the procedures and experimental conditions were as described in the Methods. Values are the means±standard errors with the number of experiments in parentheses.

maximal at about pH 7.0 (Table 2).

Effect of buffer: No significant difference was observed among the values obtained by using 50 mM Tris-HCl buffer, 50 mM Tris-maleate buffer or 50 mM sodium phosphate buffer at pH 7.4 (data not shown).

Ionic conditions: Under the present experimental conditions, divalent cations did not affect nonspecific binding significantly, but decreased total binding, resulting in a decrease of specific binding. Calcium ion was more effective than magnesium ion and monovalent cations had no significant effect (Fig. 4).

$[^{3}H]$E-643 binding to preparations from peripheral organs

$[^{3}H]$E-643 bound specifically to all the preparations of peripheral organs tested, but the $K_{D}$ values of the preparations varied: binding was high in the kidney and liver as it was in the brain, but low in the parotid, vas deferens and ileum (Table 3).

Displacement of $[^{3}H]$E-643 binding to brain by $\alpha$-agonists, $\alpha$-antagonists and other drugs

The most potent adrenergic agonists were I-epinephrine (Epi) and I-NE, whereas dl-isoproterenol (IPR) was less potent. The $\alpha$-antagonist, phentolamine, was more effective than the $\beta$-antagonists, dl-propranolol and I-alpranolol, indicating competition at $\alpha$-adrenoceptors. Prazosin inhibited the binding more than yohimbine or clonidine (Table 4).
DISCUSSION

Many radiolabeled ligands such as [3H]WB-4101 (4), [3H]clonidine (4) and [3H]Epi (3) have been used to investigate the characteristics of α-adrenoceptors. The present results show that [3H]E-643 is also useful for this purpose. Results on displacement of [3H]E-643 binding by several drugs indicated that [3H]E-643 binds predominantly to α-adrenoceptors; that is, the Kᵢ values of Epi and NE are significantly lower than that of the β-agonist, IPR, and the Kᵢ values of phentolamine and ergotamine are also significantly lower than those of the β-antagonists, propranolol and alprenolol. [3H]E-643 may have little effect on receptors for muscarinic ACh, histamine, serotonin and γ-amino butyric acid. Furthermore, the ratio of specific to total binding of [3H]E-643
binding was higher than those of other ligands: the ratio in the rat brain preparation at a concentration corresponding to the \( K_D \) value of the ligand was 89.6±1.2% (mean ± S.E., n=5) for \[^3H\]E-643 binding, and 58±8%, about 45%, and about 60% for \[^3H\]WB-4101 (unpublished data and ref. 17), \[^3H\]clonidine (17) and \[^3H\]dihydroergocryptine (18), respectively. Alpha-adrenoceptors can be classified into two subtypes on the basis of their pharmacological natures (19-21). Binding studies showed that \[^3H\]WB-4101 (6-9) and \[^3H\]prazosin (10-13) bind specifically to \( \alpha_1 \)-adrenoceptors although \[^3H\]WB-4101 has been suggested to bind both \( \alpha_1 \)-adrenoceptors and \( \alpha_2 \)-adrenoceptors in rabbit uterus (22). In the present study, prazosin, a specific antagonist of \( \alpha_1 \)-adrenoceptors (23, 24), as well as E-643 itself, inhibited \[^3H\]E-643 binding more than yohimbine, a specific antagonist (25, 26) of \( \alpha_2 \)-adrenoceptors and clonidine, a specific agonist (7) of \( \alpha_2 \)-adrenoceptors. There was a thousand-fold difference in their \( K_D \) values. These results strongly support the conclusion from pharmacological studies that E-643 is a potent and highly selective postsynaptic \( \alpha \)-blocker (2). Thus \[^3H\]E-643 and \[^3H\]prazosin seem to be the most suitable ligands available for labeling \( \alpha_1 \)-adrenoceptors.

Large differences were found in the maximal binding sites and \( K_D \) values in different tissue preparations. The correlation between maximal binding sites and \( K_D \) values seems interesting: the greater the number of binding sites, the lower was the \( K_D \) value. The physiological meaning of this correlation seen in all tissues examined except the liver, as shown in Fig. 5, is still unknown. Possibly there are more than two kinds of \( \alpha_1 \)-adrenoceptors with different affinities for E-643, or the affinities for E-643 may differ in different organs.

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