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MOLECULAR SIZE OF [^{3}H]WB-4101-BINDING SITES IN RAT CORTEX AS DETERMINED BY RADIATION INACTIVATION

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Abstract—The molecular weight of the [^{3}H]WB-4101-binding sites in rat cerebral cortex was estimated by the irradiation-inactivation technique. The molecular weight was found to be dependent on the assay concentrations of the radioligand in the binding assay. Assays with a [^{3}H]WB-4101 concentration of 0.25 nM showed a molecular weight of 62,100 daltons and 5.1 nM showed 50,800 daltons. Scatchard transformation of the [^{3}H]WB-4101-binding data shows two binding sites (high-affinity: K_D = 0.09 nM, B_{max} = 9.1 pmoles/g; low-affinity: K_D = 20 nM, B_{max} = 80 pmoles/g). It is suggested that the two binding sites exist at two distinct molecules and in that case the observed molecular weights of 62,100 and 50,800 daltons are not true values because the determinations are carried out on a mixture of the two molecule populations. The distribution of the two binding sites was calculated for the two radioligand concentrations, 0.25 nM and 5.1 nM; and on this background the "true" molecular weights of the two [^{3}H]WB-4101-binding sites were estimated to be 68,300 daltons for the high-affinity molecule and 41,400 daltons for the low-affinity molecule. Competition studies with a variety of adrenergic agonists and antagonists against [^{3}H]WB-4101 supported the hypothesis that only the high-affinity binding site is an alpha-1-adrenoceptor.

[^{3}H]WB-4101 labels two binding sites in human [1, 2], calf [3], rat [4], and mouse brain [5]. These results indicate that there may exist either two binding sites at the same molecule or two distinct [^{3}H]-WB-4101-binding molecules. The high-affinity binding site is found to be at the alpha$_1$-adrenoceptor, whereas it is questioned if the low-affinity site deals with an adrenoceptor [4, 6].

Solubilized alpha-1-adrenoceptors from rat-liver plasma membranes labeled with [^{3}H]phenoxybenzamine showed a molecular weight of 96,000 daltons on gel filtration and sucrose gradient centrifugation [7] or 80,000, 58,000, 44,800, 28,000 and 17,900 daltons on gel electrophoresis [8, 9].

Radiation inactivation has been used as a method for target-size analysis of macromolecules [10, 11]. Target-size analysis of the alpha$_1$-adrenoceptor in rat cerebral cortex homogenate showed a molecular weight of 85,000 and 71,500 daltons using, respectively, [^{125}I]HEAT and [^{3}H]prazosin as a ligand and a temperature-correction factor of 2.6 [12].

In the present study we have determined the molecular weight of the [^{3}H]WB-4101-binding sites at high and low affinity using radiation inactivation on non-homogenized rat cortex, and we found differences in the respective molecular weights.

MATERIALS AND METHODS

Animals. Male Wistar rats with a weight of *ca.* 200 g were used.

Drugs. [^{3}H]WB-4101 [(2:6-dimethyoxyphenoxyethyl)aminomethyl-1-4-benzodioxane] with a sp. act. of 25.7 Ci/mmole was obtained from New England Nuclear, Boston, MA. (±)Adrenaline, (±)noradrenaline, dopamine, serotonine, pargyline and ascorbic acid was from Sigma Chemical Co., St. Louis, MO, prazosin from Pfizer, Sandwich, U.K., and phentolamine from Ciba-Geigy. Clonidine was a gift from Lundbeck (Denmark).

Radiation inactivation. Frozen whole-rat cerebral cortex was exposed to high-energy electrons using the 10 MeV linear accelerator at Risø, Denmark. The doses of radiation were determined using calibrated thermo-dosimeters (water). During radiation the samples were cooled at *ca.* −10°. Radiation was delivered in doses of 0.5–2 Mrad and in between the doses the samples were cooled to −15° for at least 2 min to ensure that they remained completely frozen during the radiation process.

Tissue preparation. After radiation the cortex were kept at −20° for 1–8 days. The brain tissue was homogenized in 20 vol. of ice-cold 50 mM Tris–HCl buffer, pH 7.5, and centrifuged twice at 30,000 *g* for 10 min with an intermediate rehomogenization in fresh cold buffer between the spins. The pellets were resuspended in Tris–HCl buffer to a final concentration of 5 mg original wet tissue/ml. To all assays with catecholamines 10^{-5} M pargyline and 0.1% ascorbic acid were added to the Tris–HCl buffer.

Binding assay. Twenty-five microlitres of [^{3}H]WB-4101 in varying concentrations was added to 2.0 ml of the resuspended brain membranes. The samples were incubated 15 min at 25° followed by at least 30 min at 0°. The samples were filtered through

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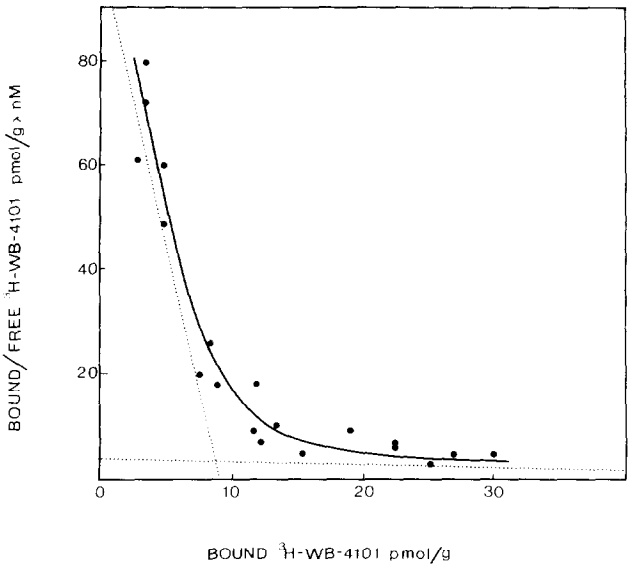

Fig. 1. Scatchard transformation of data from equilibrium binding of [^{3}H]WB-4101 to rat cerebral cortex, frozen at −20° 1 day before preparation. Radioligand concentration was in the range 0.06–10 nM. The stippled lines indicate the best fit to a two-site model, as calculated on an IBM 3033 computer using the program described by Olsen *et al.* [14]. High-affinity site showed K_D = 0.09 nM and B_{max} = 9.1 pmoles/g and low-affinity site K_D = 20 nM and B_{max} = 80 pmoles/g.

Whatman GF/B glass-fiber filters with 3 × 5-ml washes of ice-cold buffer. Radioactivity on the filters were counted in 3 ml Instagel (Packard) using a Nuclear Chicago Mark II scintillation counter. Counting efficiency was in the range 37–41%. Specific binding was calculated as the difference between the total binding of [^{3}H]WB-4101 and the binding in the presence of 1 μM phentolamine. All samples were run in triplicate.

Calculation of molecular weight. This was carried out according to Nielsen *et al.* [13] using a calibration curve of radiation inactivation of seven enzymes with known molecular weight. A mean radiation-inactivation constant K for the enzymes is 730,000 daltons × Mrad in frozen whole cerebral cortex. This value was used to calculate the molecular weight of the binding-site molecules from its inactivation constant, K (Fig. 3) (molecular wt = 730,000 × K). $-K$ is the radiation-inactivation constant determined experimentally from the slope of the logarithmic transcription of the decay curve ($A = A_0 \times e^{-KD}$, where A is residual enzyme activity or receptor binding at the dose D Mrad and A_0 is the activity at $D = 0$). For details in the principles of radiation inactivation technique see, for example, Refs [11, 13, 14].

RESULTS

Data from the binding of [^{3}H]WB-4101 to rat cerebral cortex showed a curved line when transformed to a Scatchard plot (Fig. 1). The best fit to a two-site binding model, as calculated on an IBM 3033 computer [15], resulted in the following dissociation constants and values of maximal receptors: high-affinity site, K_D = 0.09 nM and B_{max} = 9.1 pmoles/g; low-affinity site, K_D = 20 nM and B_{max} = 80 pmoles/g.

In order to examine the pharmacological profile of the [^{3}H]WB-4101 binding, displacement studies were carried out with a variety of agonists and antagonists. The rank order in inhibiting the binding of 0.5 nM [^{3}H]WB-4101 was prazosin > clonidine > (±)adrenaline > (±)noradrenaline > dopamine >

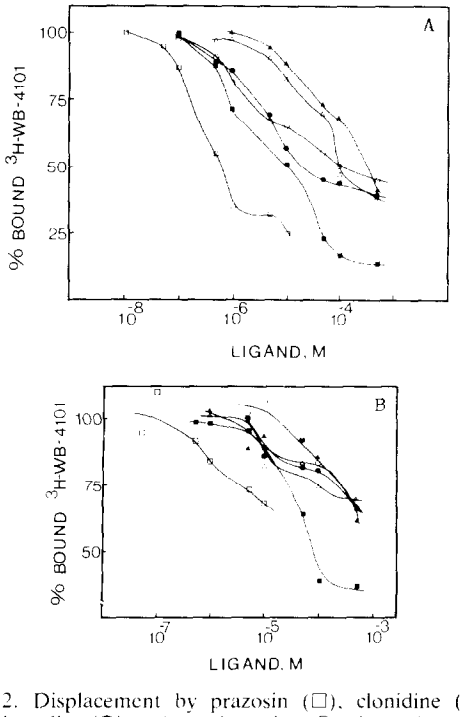

Fig. 2. Displacement by prazosin (□), clonidine (■), (±)adrenaline (●), (±)noradrenaline (○), dopamine (△), and serotonine (▲) of 0.5 nM (A) and 5 nM (B) [^{3}H]WB-4101 bound to membranes from rat cerebral cortex. The values are expressed as percentage of total binding.

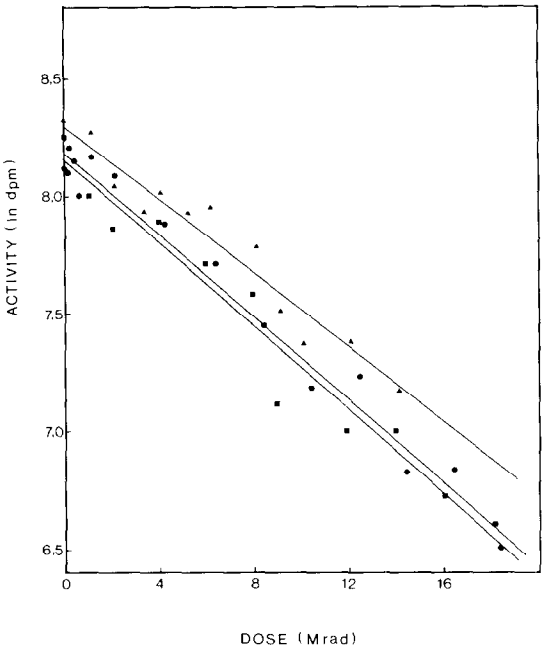

Fig. 3. Irradiation inactivation of [^{3}H]WB-4101 binding to the binding sites in rat cerebral cortex. The data are from 3 experiments with the following radioligand concentrations: 0.22 nM (●, ■) and 0.31 nM (▲). The mean radioligand concentration is 0.25 nM which is mainly in the high-affinity range. Each point is the average of triplicate determinations. The molecular weight is 62,100 ± 3800 daltons (mean ± S.D. of the 3 experiments).

serotonine (Fig. 2A). This order is clearly an alpha$_1$-adrenoceptor profile in agreement with other findings [1, 16–18]. Prazosin, and clonidine to a lesser degree, showed biphasic competition curves indicating two binding sites of [^{3}H]WB-4101. Competition curves of (±)adrenaline, (±)noradrenaline, dopamine and serotonine were monophasic, and only about 60% of the total binding could be displaced. This indicates that these drugs only bind to the high-affinity WB-4101 site.

With a concentration of 5 nM ^{3}H-WB-4101 the rank order was prazosin > clonidine > (±)adrenaline, (±)noradrenaline, dopamine and serotonine (Fig. 2B). The experiment does not give a clear picture of the rank order but shows that the fraction displaced by all 6 drugs decreases with the increasing [^{3}H]WB-4101 concentration. This supports the suggestion that the low-affinity site is not an alpha$_1$-receptor site as reported by others [4].

Radiation inactivation of the binding of [^{3}H]WB-4101 (0.2–0.3 nM) is shown in Fig. 3. At these concentrations the binding is mainly to the high-affinity receptor site. The binding was inactivated as a simple exponential of the radiation dose. The molecular weight was calculated to be 62,100 ± 3800 daltons (mean ± S.D. of 3 values).

Scatchard plots of the binding data for [^{3}H]WB-4101 at varying doses of radiation were carried out. The values of K_D and B_{max}, as shown in Fig. 4, are only approximations because the radioligand concentrations are kept in the range of the high affinity and the contribution from the binding to the low-affinity site is not taken into consideration. The results show that B_{max} decrease with increasing radiation doses without any significant alteration of the K_D values.

To investigate the molecular size at the low-affinity site we made radiation experiments with an assay concentration of 5 nM [^{3}H]WB-4101. The molecular size was found to be 50,800 daltons (the mean of 2 determinations which gave 47,700 and 53,800 daltons). The difference between the molecular weights at, respectively, 0.25 and 5.1 nM [^{3}H]WB-4101 is significant and indicates that there may exist two distinct binding-site populations.

Table 1. Molecular weights of the [^{3}H]WB-4101-binding sites estimated at varying concentrations

[^{3}H]WB-4101 concentration (nM)	Molecular weight (daltons) means ± S.D.	Number of experiments
0.25	62,100 ± 3800	3
3.0	59,500	1
5.1	50,800 ± 3100	2

Table 2. Distributions of the two[^{3}H]WB-4101 binding sites in frozen rat cerebral cortex at two different concentrations of the radioligand

[^{3}H]WB-4101 (nM) assay concentration	High-affinity binding sites (%)	Low-affinity binding sites (%)
0.25	77	23
5.1	35	65

The calculations are carried out by using the equation described in the text.

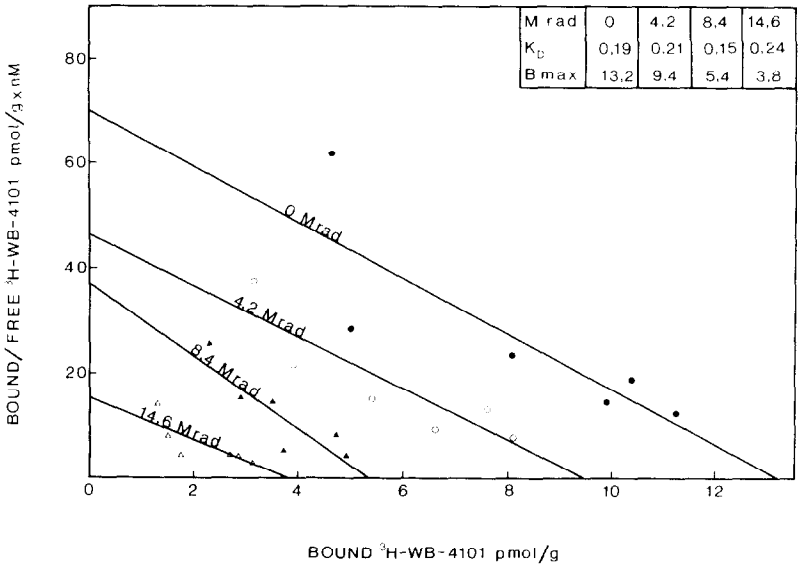

Fig. 4. Scatchard analysis of [^{3}H]WB-4101 binding to rat cerebral cortex exposed to 0 (●), 4.2 (○), 8.4 (▲) and 14.6 (△) Mrad. Six concentrations (0.1–1.5 nM) of the radioligand were used. These estimates are mainly from the high-affinity binding site. The inset shows the approximate K_D values (nM) and B_{max} values (pmoles/g).

The molecular weights of 62,100 and 50,800 daltons cannot be true values because the determinations are carried out on a mixture of two molecular sizes. However, it is possible to find the distribution of the two receptor molecules at any concentration of radioligand by using the following equation:

$$B = [(B_{max_1} \times L)/(K_{D_1} + L)] + [(B_{max_2} \times L)/(K_{D_2} + L)],$$

where B = ligand bound, L = ligand concentration in assay and the numbers 1 and 2 refer to B_{max} and K_D at the high- and low-affinity sites, respectively.

We calculated the distributions of the high- and low-affinity sites at the [^{3}H]WB-4101 concentrations 0.25 and 5.1 nM. The results can be seen in Table 2. The distribution of the two molecular populations and the molecular weights found can be used to put up two equations with two unknowns. By solving the equations we found the "true" molecular weights to be 68,300 daltons for the high-affinity site and 41,400 daltons for the low-affinity site.

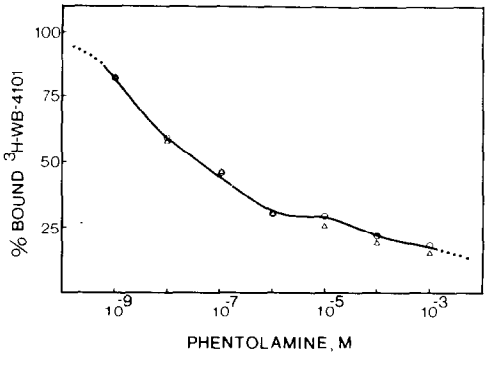

Fig. 5. Displacement by phentolamine of 0.18 nM [^{3}H]WB-4101 bound to membranes from rat cerebral cortex. Non-irradiated frozen cortex (○) and frozen cortex exposed to 5 Mrad (△).

To testify the hypothesis that there are two molecular populations with two different molecular sizes we carried out another radiation-inactivation experiment. The radioligand concentration was 3 nM and by using the calculated distribution of the receptor types at this concentration we found that the expected molecular weight was 54,000 daltons. We actually found the molecular weight to be 59,500 daltons (Table 1), which is relatively close to the expected value.

In the radiation experiments we observed an increase of the unspecific binding after radiation. This was in the range 10–30% and was independent of the radiation dose. To investigate if there was any bias from similar alterations in specific binding we carried out competition assays with 7 concentrations of phentolamine displacing 0.2 nM [^{3}H]WB-4101 without radiation and with a radiation dose of 5 Mrad. The IC_{50} value was 15 nM at 0 and 5 Mrad (Fig. 5) indicating that the affinity of the high-affinity site for phentolamine was not changed after radiation. This result further substantiates that the affinity of the high-affinity alpha$_1$-adrenoceptor is not changed upon radiation.

DISCUSSION

This work is the first giving evidence to the hypothesis that the two binding sites of [^{3}H]WB-4101 in rat cortex represent two distinct molecules. Hoffman and Lefkowitz [6] were the first to warn about the alpha$_1$-selectivity of the [^{3}H]WB-4101 and this study supports the theory that only the high-affinity binding site is an alpha$_1$-receptor, which is also reported by other investigators [1, 4]. Atlas and Adler [4] suggest that the low-affinity binding site of [^{3}H]WB-4101 is connected to Ca^{2+} channels.

In the present study we have applied the method of radiation-inactivation technique to estimate the functional site of molecules *in situ* in brain tissue. We found significantly different molecular weights dependent on the [^{3}H]WB-4101 concentration used in the binding assay. Assuming the existence of two distinct [^{3}H]WB-4101-binding molecules we estimated the molecular weights of the high-affinity receptor to be 68,300 daltons and the low-affinity [^{3}H]WB-4101-binding molecule to be 41,400 daltons.

Few and variable results have been reported on the molecular weight of the alpha$_1$-adrenoceptor [7–9, 12]. The radiation-inactivation technique has been used by Lübbecke and colleagues [12]. The results were not quite clear but the authors concluded that the alpha$_1$-adrenoceptor size was between 71,500 and 91,500 daltons. Binding assays with [^{3}H]-prazosin gave the lowest molecular weight and [^{125}I]-HEAT the highest. The molecular weight found with prazosin and a correction factor of 2.6 was 71,500 daltons which is close to our results of 68,300 daltons for the high-affinity receptor.

Studies have been carried out to determine the molecular weight of the alpha$_1$-adrenoceptor in rat-liver plasma membranes labeled with [^{3}H]phenoxybenzamine [7–9]. The receptors have been solubilized in Lubrol or sodium dodecylsulphate and molecular weights determined with either gel filtration and sucrose density gradient centrifugation or gel electrophoresis. The molecular weights found were, respectively, 96,000 daltons [7], 80,000 and 58,000 daltons [8] and 44,800, 28,000 and 17,900 daltons [9]. The molecular weight of 58,000 daltons is suggested by the authors to be a proteolytic fragment of the protein of 80,000 daltons, but the 58,000-dalton unit could possibly be an integral part of the alpha$_1$-adrenoceptor. In the study [9] where 3 different molecular weights were found, it was shown that the alpha$_1$-adrenoceptor subunit, which possesses the catecholamine-binding site, is the 44,800 unit because the binding of [^{3}H]phenoxybenzamine could be displaced with phentolamine and epinephrine. This displacement was not found on the 28,000 and 17,800 units. These results are not in agreement with ours, unless some of the subunits found by Guellaen *et al.* [9] are proteolytic fragments of the alpha$_1$-receptor. The addition of the 44,800 and 28,000 subunits thus gives a molecular weight of 72,800 daltons, close to the 68,300 daltons found in this study.

The distribution of the [^{3}H]WB-4101-binding sites at different concentrations have been used to calculate the "true" values of the molecular weights. This gives the results an extra unsafety factor. However, it has been shown by Lyon and Randall [3] that the distribution of the two binding sites at 0.22 nM [^{3}H]WB-4101 in calf neocortical membrane homogenate can be calculated to 74% at high-affinity sites and 26% at low-affinity sites. These results are very close to the values shown in Table 2.

The irradiation-inactivation technique has been a valuable tool in this study, which shows that [^{3}H]-WB-4101 binds with high affinity to a molecule of 68,300 daltons and with low affinity to a unit of 41,400 daltons. In the future the technique can be used to give information about the character of multiple-binding sites of other antagonists or agonists.

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