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Conformational Changes in Benzodiazepine Receptors Induced by the Antagonist Ro 15-1788

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SUMMARY

The binding kinetics of [^{3}H]Ro 15-1788, a selectice benzodiazepine receptor antagonist, to synaptosomal membranes of rat cerebral cortices was studied. [^{3}H]Ro 15-1788 binds with high affinity (dissociation constant, 0.53 nM) to a single class of binding sites (maximal binding capacity, 1.97 pmoles/mg of protein). Equilibrium binding was not affected by γ-aminobutyric acid (GABA), NaCl, pentobarbital, or pretreatment of the membranes at 37°. Association at 0° was identical whether measured in the absence or presence of GABA or bicuculline methiodide or after preincubation of the membranes at 37°. The association rate under pseudo-first order conditions was curvilinear and consisted of a fast component and a slow component. Dissociation at 0° with 1×10^{-5} M clonazepam was also curvilinear and could best be fitted by two linear exponential components. The dissociation rate was not altered by GABA, NaCl, pentobarbital, or pretreatment of membranes at 37°. The dissociation rate was similar for 0.1, 1, and 10 nM [^{3}H]Ro 15-1788. The ratio of slow to fast dissociation component for 10 nM [^{3}H]Ro 15-1788 was larger than that for 0.1 and 1 nM [^{3}H]Ro 15-1788. In contrast, the dissociation rate for 20 nM [^{3}H]flunitrazepam ([^{3}H]FNP) was much greater than that for 2 nM [^{3}H]FNP. Using ligand concentrations occupying the same fraction of receptors, the ratio of slow to fast dissociation components was invariably greater for [^{3}H]Ro 15-1788 than that for [^{3}H]FNP. The rate of dissociation for [^{3}H]Ro 15-1788 was faster under pre-equilibrium conditions than under equilibrium conditions. These results, discussed in terms of the cyclic model of interaction between receptors and benzodiazepines, suggest that [^{3}H]Ro 15-1788 is a powerful ligand in inducing conformational changes in the initial, more labile, binary complex. They also suggest that different conformational states deduced from studies of *in vitro* binding kinetics may not correspond to the distinct pharmacological actions of benzodiazepines. It is speculated that intrinsic activities of benzodiazepines probably are determined by the step beyond the complex formation and conformational changes suggested to occur by these studies of binding kinetics.

INTRODUCTION

Several recent studies have suggested that Ro 15-1788, an imidazodiazepine, is a selective brain benzodiazepine receptor antagonist. Ro 15-1788 selectively antagonized the central effects of benzodiazepines [e.g., anticonflict, sedative, anticonvulsant, and muscle-relaxant activities in animals (1)], and antagonized the sedation, incoordination, and muscle relaxation caused by Ro 11-3128 (3-methylclonazepam), a benzodiazepine agonist, in man (2). Ro 15-1788 alone in relatively high doses did not exert any effect in humans or animals (1, 2). Biochemical studies indicated that [^{3}H]Ro 15-1788 binds with high affinity (dissociation constant around 1 nM) to the same set of receptors that interact with [^{3}H]clonazepam; [^{3}H]Ro 15-1788 binding was displaced by benzodiazepines with the same rank order of potency as that found for displacing [^{3}H]diazepam, and its binding site localization was the same as that of [^{3}H]FNP[2] (3, 4). Furthermore, electrophysiological studies indicated that Ro 15-1788 specifically antagonized the effects of benzodiazepines on a variety of spinal cord and brain activities (5). Recently Ro 15-1788 has been shown to precipitate withdrawal symptoms in chronically treated animals (6–9).

GABA, which is known to increase the binding of benzodiazepine agonist (10), did not affect the binding of [^{3}H]Ro 15-1788 to the central benzodiazepine receptors (4). Studies of the temperature dependence of the binding of [^{3}H]clonazepam and [^{3}H]Ro 15-1788 suggested that benzodiazepine agonists such as clonazepam interacted

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[2] The abbreviations used are: [^{3}H]FNP, [^{3}H]flunitrazepam; GABA, γ-aminobutyric acid.

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with the receptor, forming an initial binary complex which then isomerized to a more stable complex; benzodiazepine antagonists such as Ro 15-1788 were unable to induce further conformational changes after initial binary complex formation (4). We have previously shown from studies of [^{3}H]FNP binding kinetics that benzodiazepine receptors in rat cerebral cortex exist as a homogeneous population with two interconvertible conformations (11). From those results a cyclic model of interactions between benzodiazepines and the receptors was proposed. We have further shown that some benzodiazepines (e.g., FNP) are more effective than others (e.g., diazepam) in inducing conformational changes in the binary complexes formed between ligand and the low-affinity conformation (12). In this study, we report the binding kinetics of [^{3}H] Ro 15-1788 because of its unique characteristics. The results indicate that Ro 15-1788 is a powerful ligand in inducing conformational changes. The results also suggest that the states of the ligand-receptor complexes as revealed from kinetic studies may not reflect the intrinsic activities of benzodiazepine agonists or antagonists.

MATERIALS AND METHODS

Preparation of synaptosomal membranes. Cerebral cortices from male Sprague-Dawley rats (250–350 g) were dissected according to the method of Glowinski and Iversen (13) and frozen at −20° until use, usually within 1 week. Crude synaptosomal membranes (P_2) were prepared and washed three times with 50 mM Tris-HCl buffer (pH 7.5 at 0°) as previously described (14). The whole procedure and the binding experiments were carried out, and the buffer solution was maintained at 0° ± 0.5°. Protein concentration was determined by the method of Lowry *et al.* (15), using bovine serum albumin as a standard.

Equilibrium binding assay. A standard binding technique was used. Synaptosomal membranes (0.2 mg of protein) in 0.5 ml of 50 mM Tris-HCl buffer were incubated with ^{3}H-ligands at 0° for 60 min. Five concentrations (0.5, 1, 2, 4, and 8 nM) of [^{3}H]FNP and [^{3}H]Ro 15-1788 were used. At the end of the incubation period, 5 ml of buffer were added to the incubation mixture, and the entire contents were filtered immediately through a Whatman GF/B glass-fiber filter (which was wetted just before sample filtration) positioned over an Amicon VFM1 filtration manifold under reduced pressure (achieved by a Millipore ⅙ horsepower vacuum pump). The filters were rinsed twice with 5-ml aliquots of buffer. The entire filtration was completed within 10 sec. The filters were removed after suction for an additional 30 sec, dried, and counted as before (14). Nonspecific binding, measured separately in the presence of 1×10^{-6} M clonazepam, was about 7% and 2% of the total binding for 8 nM [^{3}H]FNP and 8 nM [^{3}H]Ro 15-1788, respectively. Specific binding was the difference between total and nonspecific binding. Least-squares regression analysis of the Scatchard plot was used to estimate maximal binding capacity (B_{max}) and the dissociation constant (K_D).

Association kinetics. The procedures described previously were followed (11, 12). Association at 0° was started by adding 1 nM [^{3}H]Ro 15-1788 to the membrane suspension (0.1 mg of protein per milliliter). At various time intervals after the addition of [^{3}H]Ro 15-1788, aliquots of 0.5 ml were removed and filtered to determine total binding. The filters were washed twice with 5-ml aliquots of buffer. Nonspecific binding was measured in the presence of 1×10^{-6} M clonazepam, and reached the steady-state level 1 min after the addition of [^{3}H]Ro 15-1788; it was determined separately at the end of the experiment. Total binding represented less than 5.5% of the added ligand activity. Therefore, the association can be considered to be under a pseudo-first order condition; i.e., the ligand concentration remained unchanged throughout the experiment. Results were plotted according to the integrated rate equation (16), assuming a simple bimolecular reaction as described elsewhere (11, 12).

Dissociation kinetics. Previously described procedures were followed (11, 12). Synaptic membranes (0.5 mg of protein per milliliter) were incubated with ^{3}H-ligand at 0° for 60 min. Aliquots of 0.5 ml were removed to determine the steady-state binding. Nonspecific binding in the presence of 1×10^{-6} M clonazepam was determined separately. Dissociation was started by adding excess clonazepam (1×10^{-5} M) to the equilibrated membrane suspension. At various intervals after the addition of excess clonazepam, aliquots of 0.5 ml were removed to determine [^{3}H]Ro 15-1788 or [^{3}H]FNP remaining bound, as described in the previous section. The dissociation rate constants were estimated by nonlinear regression analysis using a Biomedical Computer program, p-series, developed by the University of California, Los Angeles.

Chemicals. [^{3}H]FNP (86 Ci/mmole) and [^{3}H]Ro 15-1788 (87.5 Ci/mmole) were obtained from Amersham Corporation (Chicago, Ill.) and New England Nuclear Corporation (Boston, Mass.), respectively. GABA and pentobarbital sodium were obtained from Sigma Chemical Company (St. Louis, Mo.). Bicuculline methiodide was obtained from Pierce Chemical Company (Rockford, Ill.). Clonazepam and other benzodiazepines were generously supplied by Hoffmann-La Roche Inc. (Nutley, N. J.). All other chemicals were ACS-grade.

RESULTS

Binding of [^{3}H]Ro 15-1788 or [^{3}H]FNP to cerebral cortices was displaced by active benzodiazepines (e.g., flurazepam, diazepam, FNP, clonazepam, and methyl and ethyl esters of β-carboline-3-carboxylate) but not by inactive benzodiazepines (e.g., Ro 5-3663, a convulsive benzodiazepine, or Ro 5-4864, a ligand for peripheral binding sites). Specific [^{3}H]Ro 15-1788 binding was not affected by GABA, NaCl, pentobarbital, bicuculline methiodide, or pretreatment of membranes at 37° for 2 hr (results not shown). All of these compounds and heat treatment have been shown to affect [^{3}H]FNP and [^{3}H]diazepam binding. As shown in Fig. 1, Scatchard analysis indicated that [^{3}H]Ro 15-1788 binds with high affinity to a single class of binding sites. The B_{max} values (mean ± standard error of the mean of three to five experiments) for [^{3}H] Ro 15-1788 and [^{3}H]FNP were 1.97 ± 0.04 and 2.07 ± 0.08 pmoles/mg of protein, respectively; the K_D values for [^{3}H]Ro 15-1788 and [^{3}H]FNP were 0.53 ± 0.05 and 1.51 ± 0.08 nM, respectively.

The association reaction rate was measured by incubating synaptosomal membrane (0.1 mg of protein per

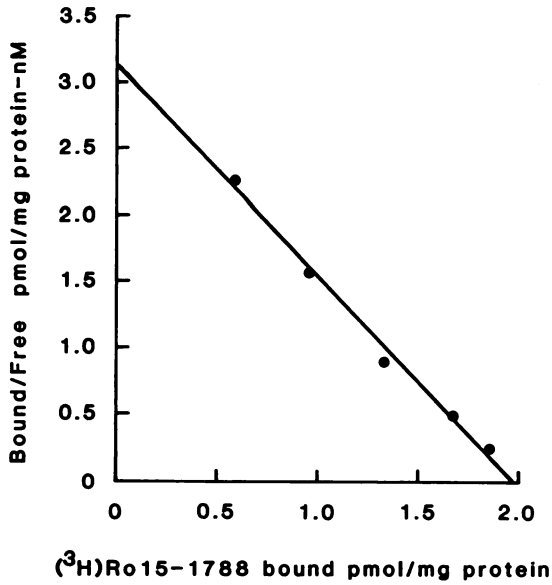

FIG. 1. *Scatchard plot of the saturation binding of [^{3}H]Ro 15-1788*
Triply washed rat cortical synaptosomal membranes were incubated with various concentrations of [^{3}H]Ro 15-1788 at 0° for 60 min. Bound radioactivity was measured by the glass-fiber filtration technique. *Free* is the free [^{3}H]Ro 15-1788 concentration (nanomolar).

milliliter) with 1 nM [^{3}H]Ro 15-1788 at 0°. Binding of [^{3}H]Ro 15-1788 to the receptor sites proceeded without any lag (Fig. 2). Equilibrium was reached after 30 min of incubation at 0°. The pseudo-first order association reaction was curvilinear (Fig. 2, *inset*). The association rate was not altered by GABA, NaCl, pentobarbital, bicuculline methiodide, or pretreatment of membranes at 37° (results not shown).

The rate of dissociation of the complex following the addition of an excess of a nonradioactive ligand (1×10^{-5} M clonazepam) was studied after binding at 0° for 60 min. Figure 3 demonstrates the dissociation after binding with 2nM [^{3}H]FNP or 1 nM [^{3}H]Ro 15-1788. About one-half of the receptor sites were occupied with these ligand concentrations (see Table 1). The dissociation was curvilinear for both ligands. [^{3}H]FNP seemed to dissociate more slowly than [^{3}H]Ro 15-1788 from its respective complex. In contrast, [^{3}H]FNP dissociated at a faster rate than [^{3}H]Ro 15-1788 from its binding sites when nearly all of the receptor sites were occupied (Fig. 4; Table 1). Again, the dissociation was curvilinear for high concentrations of ^{3}H-ligands. The percentage of the complex that remained bound, from four to seven experiments, at various time intervals after initiating the dissociation was averaged and evaluated by nonlinear regression analysis for a two-component model to estimate the dissociation rate constants. The results are summarized in Table 1. The weighted residual sum of the squares, which measures the variability between the fitted regression and the actual data, is small, indicating that the dissociation can best be resolved into two linear exponential components. The analysis also provided the relative percentage of the two complex conformations at equilibrium. The dissociation rate constants were similar for 0.1, 1, and 10 nM [^{3}H]Ro 15-1788. The fraction of the slow exponential component was 64% for 0.1 and 1 nM [^{3}H]Ro 15-1788, and 80% for 10 nM [^{3}H]Ro 15-1788. In contrast, there was a large increase in dissociation rate constants, notably in

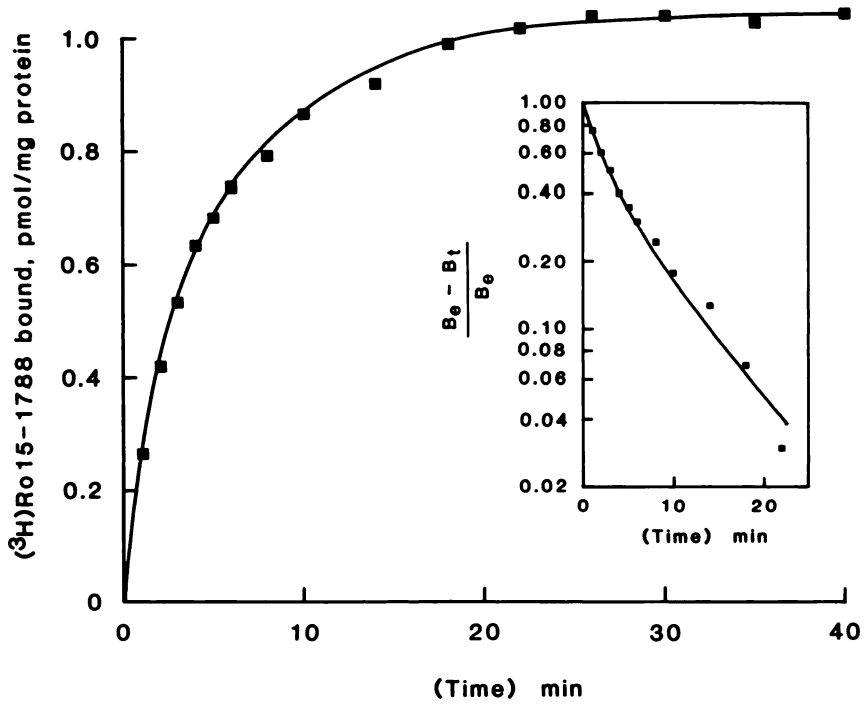

FIG. 2. *Time dependence of [^{3}H]Ro 15-1788 binding*
A homogenate of rat cortical synaptosomal membranes (0.1 mg of protein per milliliter) was incubated with 1.0 nM [^{3}H]Ro 15-1788 at 0° at time zero. Aliquots were removed at the indicated time to determine specifically bound [^{3}H]Ro 15-1788 as described under Materials and Methods. The *inset* shows the rate of [^{3}H]Ro 15-1788 binding according to the integrated rate equation described under Materials and Methods.

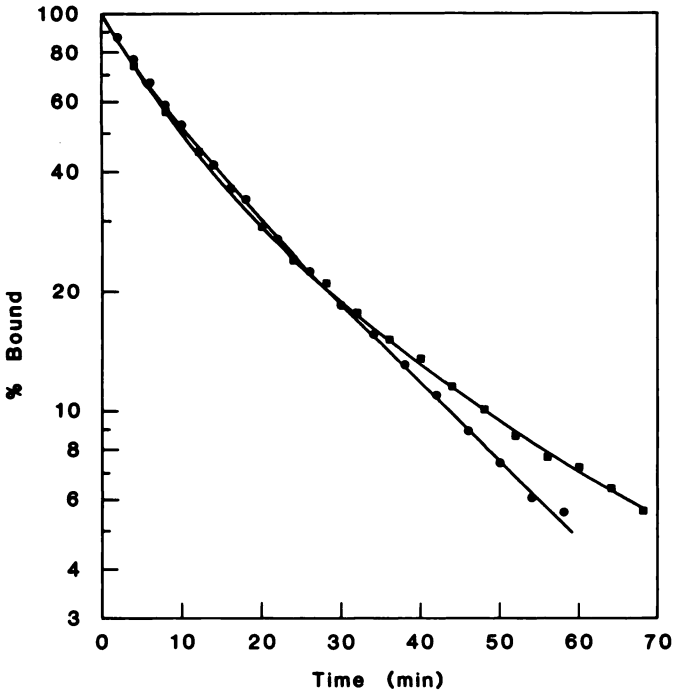

FIG. 3. *Time course of dissociation of [^{3}H]Ro 15-1788 and [^{3}H]FNP complex*

Rat cortical synaptosomal membranes (0.5 mg of protein per milliliter) were incubated with 1 nM [^{3}H]Ro 15-1788 (●) or 2 nM [^{3}H]FNP (■) at 0° for 60 min. Dissociation was started by the addition of 1×10^{-5} M clonazepam at time zero. The percentage of the specifically bound radioactivity after various time intervals was plotted against time. At time zero, there were 0.85 and 0.87 pmoles of bound ligand per milligram of protein for [^{3}H]Ro 15-1788 and [^{3}H]FNP, respectively, which represented about 50% receptor occupation.

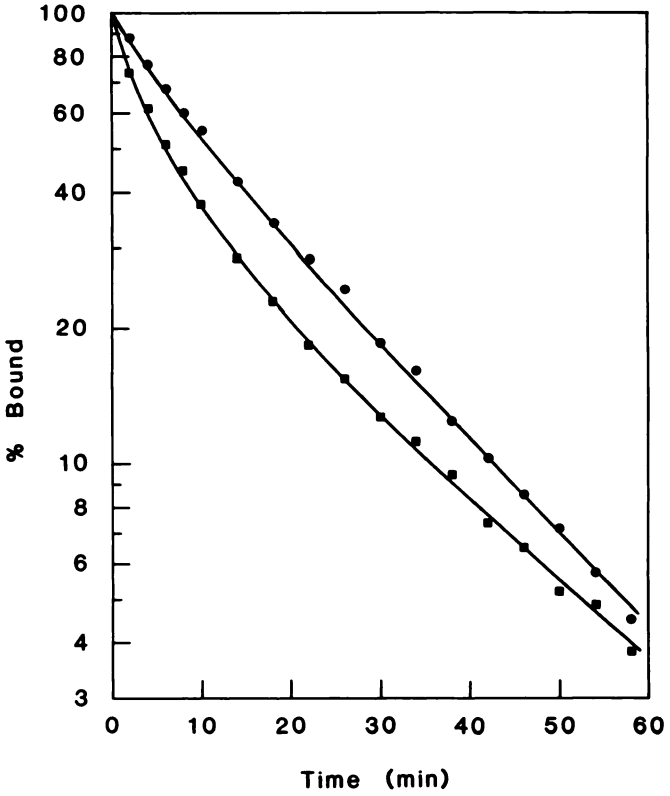

FIG. 4. *Time course of dissociation of [^{3}H]Ro 15-1788 and [^{3}H]FNP complex*

Rat cortical synaptosomal membranes (0.5 mg of protein per milliliter) were incubated with 10 nM [^{3}H]Ro 15-1788 (●) or 20 nM [^{3}H]FNP (■) at 0° for 60 min. Dissociation was the same as that described in Fig. 3. At time zero, there were 2.06 and 2.25 pmoles of bound ligand per milligram of protein for [^{3}H]Ro 15-1788 and [^{3}H]FNP, respectively, which represented about 100% receptor occupation.

the fast component, when [^{3}H]FNP was increased from 2 to 20 nM. Under conditions allowing about the same number of receptors to be occupied by either ligand (e.g., 1 nM [^{3}H]Ro 15-1788 versus 2 nM [^{3}H]FNP, and 10 nM [^{3}H]Ro 15-1788 versus 20 nM [^{3}H]FNP), the fraction of receptors in the slowly dissociating complexes was always larger for [^{3}H]Ro 15-1788. It should be noted that the rate constants and the fraction of receptors in either the low-affinity or the high-affinity conformation for [^{3}H]FNP, as estimated by the nonlinear regression analysis for a two-component model, were similar to those estimated previously by a curve-peeling process (11).

In order to characterize further the binding kinetics of [^{3}H]Ro 15-1788, the dissociation of the complex was compared under pre-equilibrium and equilibrium conditions. The dissociation with 1×10^{-5} M clonazepam was

TABLE 1

Dissociation rate constants of [^{3}H]Ro 15-1788 and [^{3}H]FNP binding to rat cortical synaptosomal membranes

Dissociation with 1×10^{-5} M clonazepam was initiated after equilibrium binding with synaptosomal membranes (0.5 mg of protein per milliliter) at 0° for 60 min. The percentage, from four to seven experiments, of specifically bound ^{3}H-ligand at time *t* after the dissociation was averaged and evaluated by nonlinear least-squares regression analysis.

Concentration	^{3}H-Ligand bound	Fast exponential		Slow exponential		RSS[a]
		%	Rate constant	%	Rate constant	
	pmoles/mg protein		*min^{-1}*		*min^{-1}*	
[^{3}H]Ro 15-1788						
0.1 nM	0.10	36.3	0.1194	64.3	0.0410	4.8
1 nM	0.83	36.3	0.1210	63.7	0.0429	0.7
10 nM	1.93	19.7	0.1476	80.3	0.0487	5.6
[^{3}H] FNP						
2 nM	0.85	57.4	0.1114	42.4	0.0302	1.3
20 nM	2.05	36.5	0.3160	62.8	0.0548	14.2

[a] The weighted residual sum of the squares, which is a measure of the variability between the fitted regression and the actual data. The RSS values were 6–80 times larger when the data were fitted to a single-component model.

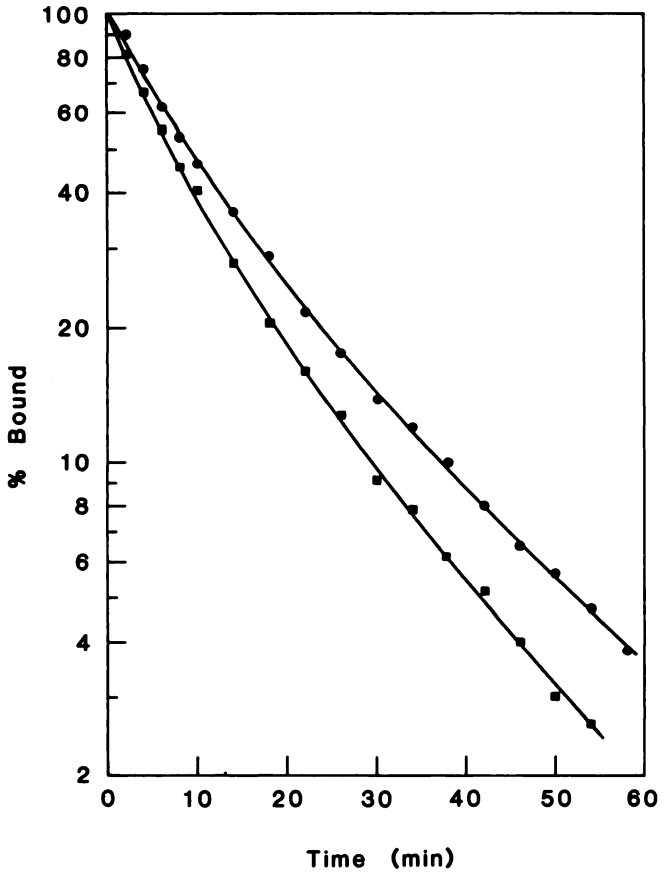

FIG. 5. *Rate of [^{3}H]Ro 15-1788 dissociation before and at steady-state level of binding*

Rat cortical synaptosomal membranes (0.5 mg of protein per milliliter) were incubated with 1.0 nM [^{3}H]Ro 15-1788 at 0°. After 4 (■) and 30 min (●) of incubation, 1×10^{-5} M clonazepam was added to portions of the reaction mixture, and the specific [^{3}H]Ro 15-1788 remaining bound at the indicated time was measured and plotted against time.

started after a 4- or 30-min incubation of the synaptosomal membranes with 1 nM [^{3}H]Ro 15-1788 at 0°. Figure 5 shows that dissociation after a 4-min incubation was faster than that after 30 min. Two distinct components were observed whether dissociation was under pre-equilibrium or equilibrium conditions. The fraction of the slow component was larger under equilibrium conditions.

DISCUSSION

Behavioral, biochemical, and electrophysiological studies have indicated that Ro 15-1788, an imidazodiazepine, is a selective antagonist of benzodiazepine agonists (1–9). We have demonstrated in this study that [^{3}H]Ro 15-1788 binds specifically and to the same set of receptors that interact with [^{3}H]FNP, confirming previous observations (1, 3, 4). It is interesting that the equilibrium binding of [^{3}H]Ro 15-1788 was not affected by several groups of compounds, particularly GABA and chloride ions, known to increase benzodiazepine agonist binding (4). The present study confirmed these findings and also demonstrated that the on- and off-rates of [^{3}H]Ro 15-1788 were not altered by GABA, chloride ions, pentobarbital, or the GABA antagonist, bicuculline methiodide. The unique properties of Ro 15-1788 may prove to be useful in studying the molecular mechanism of benzodiazepine-receptor interaction.

On the basis of the lack of susceptibility of the binding of [^{3}H]Ro 15-1788 to GABA, and the differential effects of temperature on the binding of [^{3}H]Ro 15-1788 and [^{3}H]clonazepam, an isomerization scheme was proposed for the binding of benzodiazepine agonists consisting of the formation of binary complexes, which isomerize to more stable complexes capable of inducing biological responses; antagonists (e.g., Ro 15-1788), on the other hand, would form only the initial binary complexes, which do not isomerize to another conformation and thus are unable to trigger biological responses (4). If the reaction mechanism for antagonists is correct, then a single exponential component for the dissociation of the complex should be observed. However, we found in this report that dissociation of the [^{3}H]Ro 15-1788 complex could best be described by two linear exponential components whether there was only 5% or nearly 100% occupation of the receptors (see Table 1). The dissociation kinetics, therefore, did not support a simple bimolecular reaction between antagonists and the receptors. Consequently, isomerization cannot be used to indicate the presence or absence of intrinsic activity as was previously suggested (4).

We have previously proposed a cyclic model of interaction between benzodiazepine agonists and the two interconvertible conformations of receptors from the following observations (11, 12): (a) The association reaction for [^{3}H]FNP and [^{3}H]diazepam under pseudo-first order conditions showed two linear exponential components. The ratios of these two components varied with [^{3}H]FNP concentration. (b) Dissociation of the [^{3}H]FNP or the [^{3}H]diazepam complex also demonstrated two exponential components. (c) The dissociation rate was decreased by GABA, NaCl, GABA plus NaCl, and NaCl plus pentobarbital, which decreased dissociation of the complex formed between the ligand and the low-affinity receptor conformation (i.e., labile complex) and increased the ratio of high- to low-affinity receptor conformation. (d) Preincubation of the synaptosomal membrane at 37° shifted the receptors to the high-affinity conformation, thereby slowing dissociation. (e) The dissociation rate of the [^{3}H]FNP complex was faster under pre-equilibrium conditions than under equilibrium conditions. (f) At any given receptor occupancy, the ratio of the slow-dissociation component to the fast-dissociation component (representing the complex formed between ligand and the high- and low-affinity conformations, respectively) for [^{3}H]FNP was greater than that for [^{3}H]diazepam. The isomerization step for agonists was substantiated by the thermodynamic measurements of benzodiazepine binding (4, 17). However, these studies did not propose the existence of two interconvertible conformations of benzodiazepine receptors. The finding of two linear exponential components in studies of association and dissociation kinetics does not by itself prove the existence of two distinct types of receptors. On the contrary, the curvilinear association and dissociation kinetics in conjunction with the proposed isomerization of receptor requires a single component in saturation binding studies (18), as

was found in our earlier studies (11, 12) and in studies of the nicotinic acetylcholine receptor system (19).

It is expected that binding of [^{3}H]Ro 15-1788, a selective benzodiazepine antagonist, should conform to the proposed cyclic model. The curvilinear association and dissociation kinetics seemed to support this contention. Conditions that favor the high-affinity conformation or decrease the dissociation of the labile complex should increase the equilibrium binding or decrease the rate of dissociation of [^{3}H]Ro 15-1788. However, results from this and another study (4) clearly indicate that binding of [^{3}H]Ro 15-1788 was not affected by GABA, NaCl, pentobarbital, and some other compounds known to enhance benzodiazepine binding, or by pretreatment of the synaptosomal membrane at 37°. This was not due to the exclusive binding of [^{3}H]Ro 15-1788 to a subset of receptors that are insensitive to these compounds or to heat treatment because Ro 15-1788 binds to the same receptors that interact with benzodiazepine agonists (1–9). Rather, the inability of these treatments to alter the binding of [^{3}H]Ro 15-1788 may reflect the powerful activity of this ligand in inducing isomerization of the labile complex. Such activity would mask the effects of procedures known to enhance the binding of benzodiazepine agonists. Several lines of evidence tend to support this hypothesis. The observation that dissociation of [^{3}H]Ro 15-1788 was faster at pre-equilibrium than at equilibrium was consistent with the existence of an isomerization step rather than binding to two distinct sites, which would demonstrate slower dissociation under pre-equilibrium conditions (11). At concentrations where the same number of receptors was occupied, Ro 15-1788 caused a larger fraction of the receptor-ligand complex to be in the more slowly dissociating conformation than did FNP (Table 1). Furthermore, our earlier studies showed that FNP is more powerful than diazepam in inducing the conversion of the receptor to the higher-affinity state (12). This suggests that Ro 15-1788 is especially efficient in shifting the receptor to the high-affinity conformation. It has been shown in our previous study that GABA exerts very little effect on [^{3}H]FNP dissociation kinetics when a low [^{3}H]FNP concentration is used or after prolonged incubation at 37°, which favors binding to the high-affinity conformation (11). These results, taken together with the curvilinear association and dissociation kinetics for [^{3}H]Ro 15-1788, strongly support the cyclic model proposed from agonist binding kinetics.

As was shown in our previous study (11), prolonged incubation at 37° or preparing the membrane with buffer maintained at 37° favored the high-affinity conformation. Thus, it is reasonable to suggest that most benzodiazepine receptors exist *in vivo* in the high-affinity conformation. If this is indeed the case, then the isomerization observed *in vitro* may not occur *in vivo*. Furthermore, the present results indicate that Ro 15-1788 may be even more powerful than agonists in inducing conformational changes. An alternative mechanism, therefore, must be sought to explain the difference between benzodiazepine agonists and the antagonist Ro 15-1788. It is likely that intrinsic activities of benzodiazepines are determined by the step beyond the complex formation and the conformational changes suggested to occur by these studies of binding kinetics.

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