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Characterization of Peripheral-Type Benzodiazepine Binding Sites in Brain Using [^{3}H]Ro 5-4864

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SUMMARY

The binding of [^{3}H]Ro 5-4864 to the peripheral-type benzodiazepine binding site in brain is characterized. The binding is saturable, high-affinity (K_D = 1.6 nM), and reversible. The comparison of [^{3}H]Ro 5-4864 and [^{3}H]diazepam binding sites reveals major differences which include the following. There are about one-fourth as many peripheral-type binding sites than central sites in brain. Peripheral sites are present in many extranervous tissues and have a brain regional distribution distinct from that of the central-type receptor. The [^{3}H]Ro 5-4864 binding site also is apparently highly localized in the nuclear membrane in contrast to the central-type receptor, which is synaptosomal. γ-Aminobutyric acid has no effect on [^{3}H]Ro 5-4864 binding, again in contrast to its marked effect on [^{3}H]diazepam binding. Various putative benzodiazepine receptor ligands, such as purines, β-carbolines, and kynurenamines, are also inactive as inhibitors of [^{3}H]Ro 5-4864 binding. Blocking the benzodiazepine receptor by photoaffinity labeling decreases [^{3}H]diazepam binding by more than 80% and has no effect on [^{3}H]Ro 5-4864 binding. These results indicate that the peripheral-type benzodiazepine binding site in brain is a separate entity whose physiological function is probably distinct from that of the central-type benzodiazepine receptor.

INTRODUCTION

A key insight concerning the mechanism of benzodiazepine action has been the finding that a saturable high-affinity binding site specific for this class of minor tranquilizer exists in brain (1–3). The relevance of these receptor sites to the mechanism of benzodiazepine action is supported by the observation that the clinical potency of the benzodiazepines as anticonvulsants and anxiolytics is quite closely correlated to their efficacy as inhibitors of [^{3}H]diazepam binding *in vitro* (2).

The benzodiazepine receptor has been extensively studied in brain with major efforts directed toward the pharmacological characterization of the receptor (4); its modulation by, and interaction with, the major inhibitory neurotransmitter, GABA[3] (5–11); and the identification of putative endogenous ligands (12–15). Shortly after the description of brain benzodiazepine receptors it was shown that [^{3}H]diazepam also labeled benzodiazepine binding sites in kidney (16, 17). The kidney binding site displayed a pharmacology distinct from that of the brain site in that clonazepam was a very weak inhibitor of binding and the clinically inactive benzodiazepine Ro 5-4864 was a very potent inhibitor. This is the exact opposite of what is observed in brain membranes. The brain benzodiazepine receptor has been termed the "central-type" and the kidney site the "peripheral-type" benzodiazepine binding site. The peripheral-type benzodiazepine receptor has also been shown to predominate in various cultured neural cell lines (18).

It has been difficult to assess whether the peripheral-type benzodiazepine receptor is present in brain, since a specific ligand for this site was lacking. The recent availability of [^{3}H]Ro 5-4864 has now made it possible to study the peripheral-type receptor in brain. The availability of this ligand makes it feasible to address questions such as whether these two receptor types are in fact distinct molecules and to determine their respective properties. A preliminary report describing [^{3}H]Ro 5-4864 binding to rat cerebral cortical membranes has appeared (19). In the present report [^{3}H]Ro 5-4864 binding in brain is characterized. The results indicate that the peripheral-type benzodiazepine binding site is distinct from the central-type in its subcellular localization, brain regional distribution, its tissue distribution, and its ability to be modulated by GABA and a series of putative endogenous ligands.

METHODS

Membrane preparation. Male Sprague-Dawley rats (100–125 g) were killed by decapitation and their brains

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[3] The abbreviation used is: GABA, γ-aminobutyric acid.

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were rapidly removed. Each forebrain was homogenized in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4) using a Brinkmann Polytron (Setting 5, 10 sec). The resulting homogenate was centrifuged at 30,000 × *g* for 20 min. The pellet was resuspended in 50 volumes of buffer (Polytron), and the wash step was repeated two additional times (three washes total). The resulting membrane suspension was frozen in 10-ml aliquots at −20°. Prior to use the membranes were thawed and washed one additional time. Peripheral tissue membranes were prepared in a similar manner. Membrane suspensions were stable for several weeks at −20°. In some experiments (brain regional study) the tissue was frozen at −20° for several days before the membranes were prepared. Protein determinations were performed according to the procedure of Lowry *et al.* (20).

Receptor binding assays. The [^{3}H]diazepam binding assay was performed as previously described (11). Briefly stated, each assay contained 0.1–0.3 mg of membrane protein, and [^{3}H]diazepam (87 Ci/mmole; New England Nuclear Corporation, Boston, Mass.). The assays were performed in 50 mM Tris-HCl buffer (pH 7.4) with a final volume of 0.5 ml. Incubations were carried out at 0° (ice-water bath) for 30 min, and each assay was terminated by vacuum filtration on Whatman GF-B filters followed by four 3-ml washes with ice-cold buffer. The filters were air-dried and subjected to liquid scintillation counting in 10 ml of Aquasol (New England Nuclear Corporation). In all cases, 3 μM unlabeled diazepam (courtesy of Dr. William Scott, Roche, Nutley, N. J.) was used to determine nonspecific binding, which routinely represented between 4% and 7% of total binding.

The [^{3}H]Ro 5-4864 binding assay was performed in a manner similar to the [^{3}H]diazepam assay. Incubation media contained between 0.2 and 0.4 mg of protein and [^{3}H]Ro 5-4864 (74 Ci/mmole; New England Nuclear Corporation) in a volume of 0.5 ml (Tris-HCl buffer, pH 7.4). Incubations were carried out for 30 min at 0° and were terminated in a manner identical with that for the [^{3}H] diazepam binding assay. Nonspecific binding was determined with either 3 μM unlabeled diazepam or Ro 5-4864 (courtesy of Dr. William Scott). In both instances the nonspecific binding was about 20–25% of the total binding at 1 nM [^{3}H]Ro 5-4864.

Subcellular fractionation of brain was performed according to the method of Whittaker (21). Harvested subcellular fractions were washed twice in 50 mM Tris-HCl buffer (pH 7.4) to remove sucrose and lyse the organelles. Brains were dissected according to the procedure of Glowinski and Iversen (22), and the membrane fractions were prepared as described above.

Photoaffinity labeling experiments were performed by irradiating four-times-washed membranes (prepared as described above) with a UV light source (Mineralight, short-wave UV) in the presence of 20 nM unlabeled flunitrazepam (Roche) at 0° for 5 min. Control membranes were incubated with 20 nM flunitrazepam in the absence of UV light. Both membrane preparations were washed four times in 50 volumes of buffer (10-min incubations between centrifugations) to remove unbound flunitrazepam. The membranes were then frozen at −20° for 16 hr and then washed two additional times before assay.

Statistical analysis. The coefficient of interassay variance was 2.7% for the [^{3}H]diazepam binding assay and 4.5% for the [^{3}H]Ro 5-4864 assay. Data were statistically evaluated by using the paired *t*-test analysis (two-tailed). All direct comparisons between [^{3}H]diazepam and [^{3}H] Ro 5-4864 binding were made from data generated on the same day using the same membrane preparation.

RESULTS

[^{3}H]Ro 5-4864 binding was found to be optimal at 0°. Specific binding decreased at 23° by about 25% and by about 50% at 37°. [^{3}H]Ro 5-4864 binding is therefore significantly less sensitive to temperature than is [^{3}H]

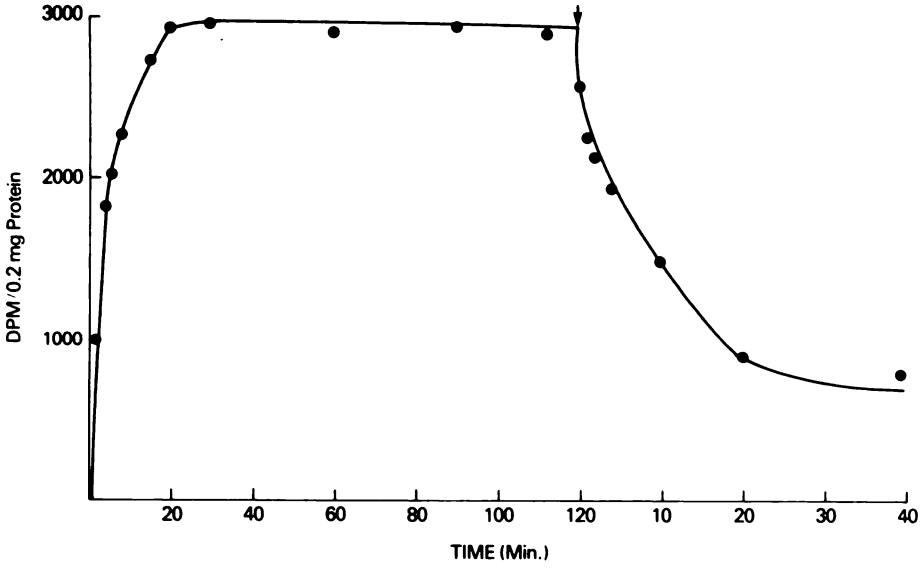

FIG. 1. *Reversibility of [^{3}H]Ro 5-4864 binding*

Rat forebrain membranes were incubated with [^{3}H]Ro 5-4864 (1 nM) at 0°. Aliquots (0.2 mg of protein) were filtered and washed at the indicated times. Unlabeled Ro 5-4864 (5 μM) was added at the point indicated by the *arrow*. This experiment was repeated twice with similar results.

diazepam binding, which is suppressed 3-fold at 23° and 6-fold at 37° relative to 0° (data not shown).

The reversibility of [^{3}H]Ro 5-4864 binding is shown in Fig. 1. The half-time of association was 6 min, and equilibrium was attained at 20 min. The half-time of dissociation was 11 min, and binding was completely reversible. The K_D calculated from the rate constants was 0.9 nM. Figure 2A illustrates the saturation isotherm for [^{3}H]Ro 5-4864 binding to rat forebrain membranes. Scatchard analysis of these data (Fig. 2B) indicated an apparently homogeneous population of binding sites with a K_D of 1.3 nM and a B_{max} of 275 fmoles/mg of protein. The Scatchard plot shown is representative of eight determinations which resulted in the following kinetic parameters: $K_D = 1.61 \pm 0.2$ nM, $B_{max} = 245 \pm 16$ fmoles/mg of protein. The K_D is in good agreement with the value determined using the rate-constant data. Analysis of [^{3}H]diazepam binding on the same membrane preparations yielded the following: $K_D = 3.8 \pm 0.3$ nM, $B_{max} = 930 \pm 33$ fmoles/mg of protein. There are about one-fourth as many [^{3}H]Ro 5-4864 binding sites in forebrain as there are [^{3}H]diazepam binding sites. The exact percentage of peripheral versus central sites is difficult to determine since [^{3}H]diazepam also binds to peripheral

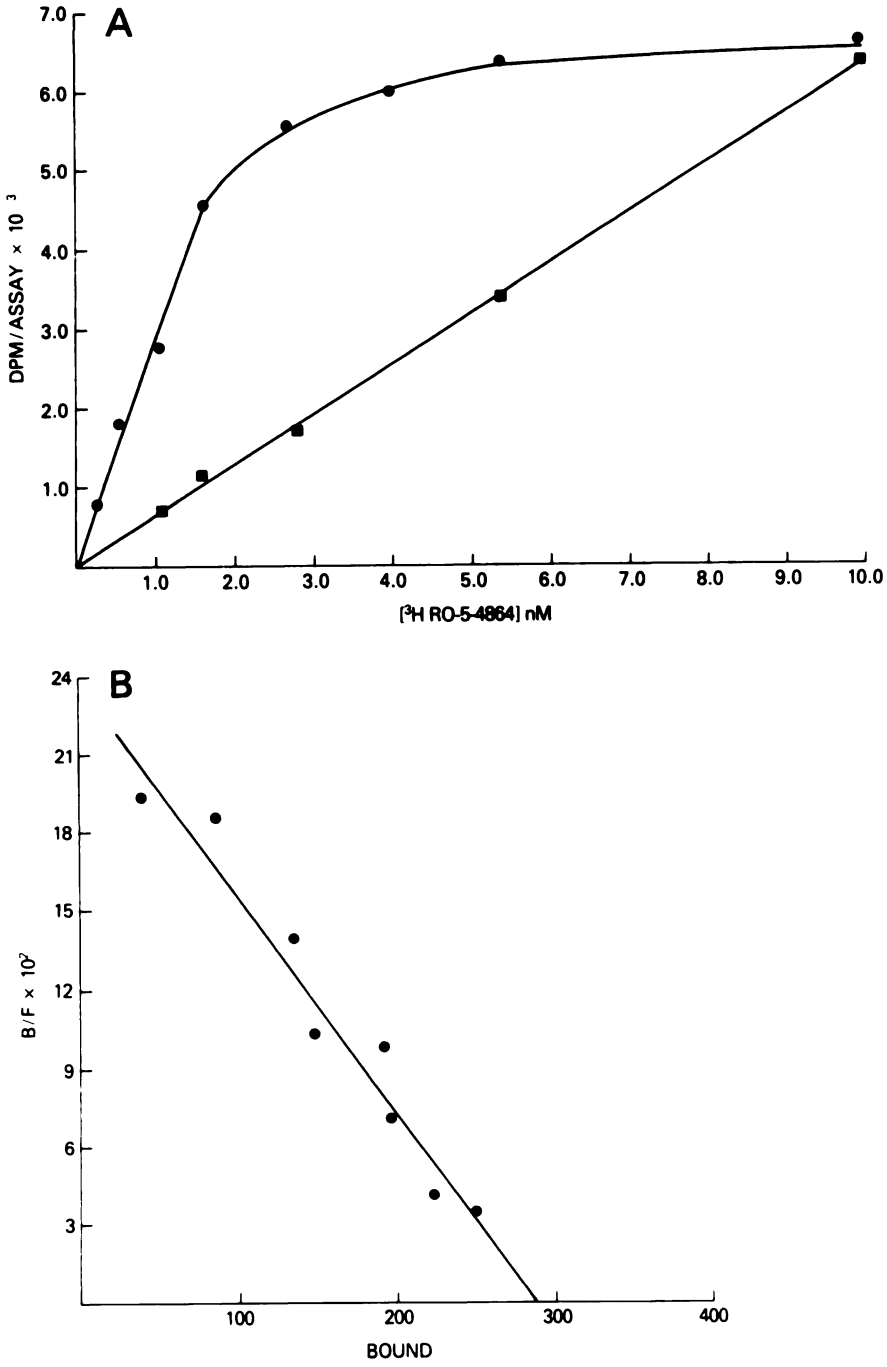

FIG. 2. *Kinetics of [^{3}H]Ro 5-4864 binding to rat forebrain membranes*

Both the saturation isotherm (A) and the Scatchard analysis of those data (B) are shown. This experiment was repeated seven times with similar results.

sites, although the concentration of [^{3}H]diazepam used was too low to label these sites extensively. The affinity of [^{3}H]Ro 5-4864 is also substantially higher than that of [^{3}H]diazepam.

The tissue distribution of [^{3}H]Ro 5-4864 binding in rat is shown in Table 1 and is compared with that of [^{3}H]diazepam. The highest levels of [^{3}H]diazepam binding were found in brain and kidney, with low levels observed in other tissues. [^{3}H]Ro 5-4864 binding, in contrast, was observed in many different tissues, with the level in brain being substantially lower than levels in kidney, lung, testis, and liver.

The brain regional distribution of [^{3}H]Ro 5-4864 binding is shown in Table 2, and the results are compared with those for [^{3}H]diazepam. The two ligands displayed quite distinct regional distribution profiles, with [^{3}H]diazepam binding being highest in cerebral cortex, as previously reported (1–3), and [^{3}H]Ro 5-4864 being highest in the pons. [^{3}H]Ro 5-4864 binding also varied less from one brain region to the other in comparison with that of [^{3}H]diazepam.

The subcellular distribution of [^{3}H]Ro 5-4864 binding sites is graphically illustrated in Fig. 3. In marked contrast to [^{3}H]diazepam, the highest density of binding sites was in the nuclear fraction. The density of [^{3}H]Ro 5-4864 binding sites in the nuclear fraction was 3-fold higher than that in the synaptosomal fraction, whereas the opposite was observed for [^{3}H]diazepam binding sites. The subcellular localization profile of [^{3}H]diazepam binding sites seen here is similar to that reported previously (23).

In an effort to compare the functional properties of the [^{3}H]Ro 5-4864 binding site with those of the [^{3}H]diazepam binding sites, various agents and the treatments which are known to affect [^{3}H]diazepam binding were assessed for their effects on [^{3}H]Ro 5-4864 binding. The effect of GABA on [^{3}H]Ro 5-4864 binding is shown in Fig. 4. No effect was observed at GABA concentrations as high as 1.5 mM, whereas a robust effect was seen on [^{3}H]diazepam binding. The effect of GABA on [^{3}H]diazepam binding is similar to that previously reported (10, 11). These two ligands therefore differ greatly with regard to the effect of GABA on their binding.

TABLE 1

Tissue distribution of [^{3}H]Ro 5-4864 and [^{3}H]diazepam binding

The indicated tissues were processed as described under Methods, and 0.3 mg of membrane protein was assayed for binding using 1 nM [^{3}H]Ro 5-4864 and 1.25 nM [^{3}H]diazepam. Erythrocyte membranes were prepared from red blood cell pellets of human blood; all other tissues were from rat. The experiment was repeated with very similar results.

Tissue	Specific binding	
	[^{3}H]Ro 5-4864	[^{3}H]Diazepam
	fmoles/mg protein	
Brain	86	198
Liver	187	24
Testis	288	23
Lung	379	28
Kidney	552	95
Muscle	7	4
Heart	169	40
Erythrocyte	3	7

TABLE 2

Regional distribution of [^{3}H]Ro 5-4864 and [^{3}H]diazepam binding in rat brain

The designated brain areas were dissected from seven rats and the tissue was frozen. Each area from each rat was homogenized in 50 volumes of buffer and washed three times. Each membrane fraction was assayed for [^{3}H]Ro 5-4864 (1 nM) and [^{3}H]diazepam (1.5 nM) binding. Values are means ± standard error of the mean (n = 7) except for hypothalamus, where only two samples (pools of three and four rats) were analyzed.

Brain region	Specific binding	
	[^{3}H]Diazepam	[^{3}H]Ro 5-4864
	pmoles/g tissue	
Cerebral cortex	20.1 ± 1.2	4.3 ± 0.2
Hippocampus	17.5 ± 1.0	7.3 ± 0.4
Caudate	5.0 ± 0.3	4.8 ± 0.3
Cerebellum	7.5 ± 0.7	5.6 ± 0.4
Pons	3.4 ± 0.2	8.5 ± 0.6
Hypothalamus	9.8	4.5

The central-type benzodiazepine receptor can be labeled irreversibly by a photoaffinity process using flunitrazepam and uv light (24, 25). Forebrain membranes were photoaffinity-labeled and tested for both [^{3}H]diazepam and [^{3}H]Ro 5-4864 binding. The results are shown in Fig. 5. Consistent with previous reports (24, 25), [^{3}H]diazepam binding was decreased greatly in the photolabeled membranes. In marked contrast, [^{3}H]Ro 5-4864 binding was unaffected by the photolabeling process.

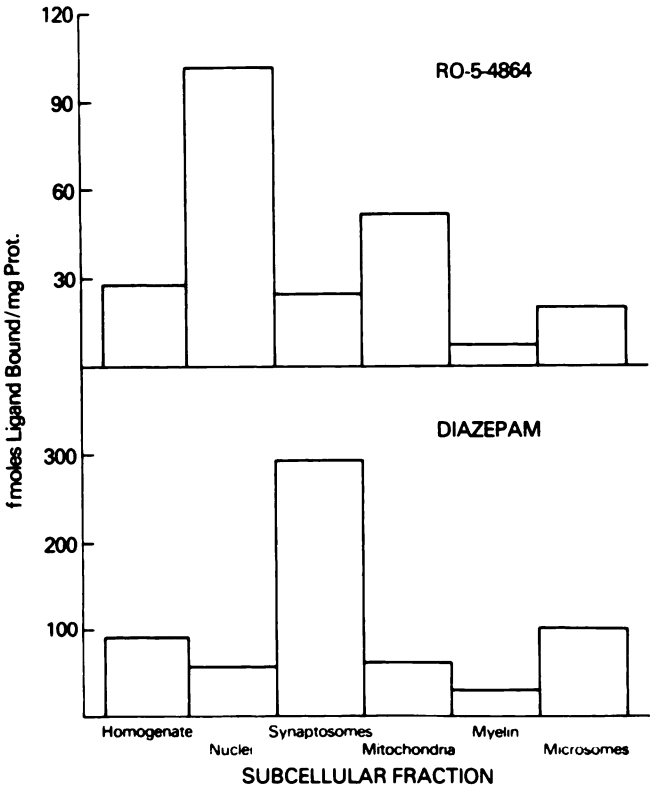

FIG. 3. *Subcellular localization of [^{3}H]Ro 5-4864 and [^{3}H]diazepam binding sites in brain*

Subcellular fractions were assayed for both [^{3}H]Ro 5-4864 (1 nM) and [^{3}H]diazepam (1.5 nM) binding. Each fraction was also assayed for total protein. The recovery of both proteins and binding activity as compared with the crude homogenate was greater than 85%. This experiment was repeated four times with similar results.

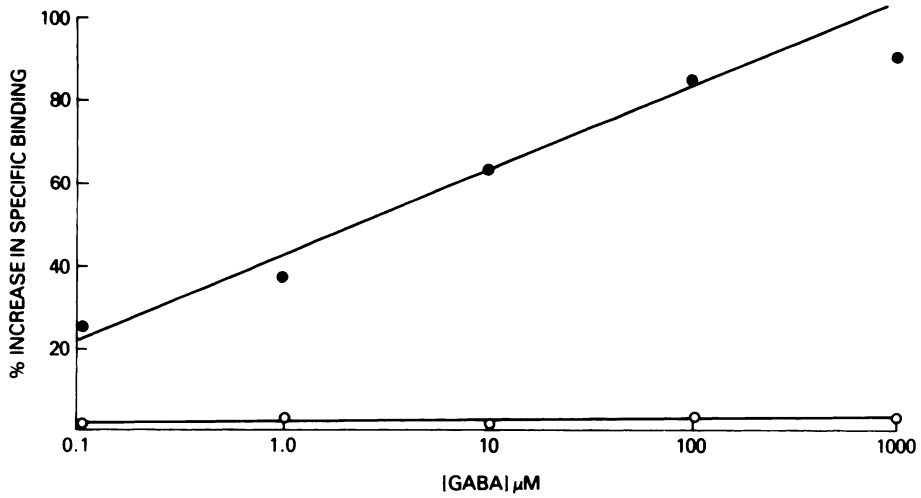

FIG. 4. *Effect of GABA on [^{3}H]Ro 5-4864 and [^{3}H]diazepam binding*
The indicated concentrations of GABA were incorporated into the [^{3}H]Ro 5-4864 (1 nM) and [^{3}H]diazepam (1.5 nM) binding assay. The results are expressed as percentage increase in specific binding relative to controls which did not contain GABA. GABA did not affect the nonspecific binding of either ligand. This experiment was repeated twice with similar results.

Scatchard analysis of the photolabeled membranes with each ligand revealed no change in either binding affinity or number of binding sites for [^{3}H]Ro 5-4864 in the photolabeled membranes (data not shown), whereas the B_{max} for [^{3}H]diazepam binding was greatly decreased with little effect on the K_D (data not shown).

Recent studies have generated a growing list of putative endogenous ligands for the benzodiazepine receptor (12–15). In an effort to determine whether these factors were also effective inhibitors of [^{3}H]Ro 5-4864 binding, their inhibitory potency was compared using the two ligands (Table 3). In almost every case the putative ligands were ineffective as inhibitors of [^{3}H]Ro 5-4864 binding. Various purines, indoles, and β-carbolines, all of which have been implicated as ligand candidates for the benzodiazepine receptor (12–15), were tested. In only one case, that of melatonin, was a higher potency observed for inhibition of [^{3}H]Ro 5-4864 binding. Various benzodiazepines and quinoline derivatives were also tested with the newly described antagonist Ro 15-1788 (26) and the specific anticonflict compound, PK 9084 (27) (supplied by Dr. G. LeFur, Paris, France), being relatively inactive as inhibitors of [^{3}H]Ro 5-4864 binding. As expected, clonazepam was a very poor inhibitor of [^{3}H]Ro 5-4864 binding, as was chlordiazepoxide.

DISCUSSION

The existence of specific benzodiazepine receptors in brain has given rise to the hypothesis that these binding sites represent the primary recognition site in the action of this class of drugs. The anxiolytic effects of the benzodiazepines further implied that characterization of this

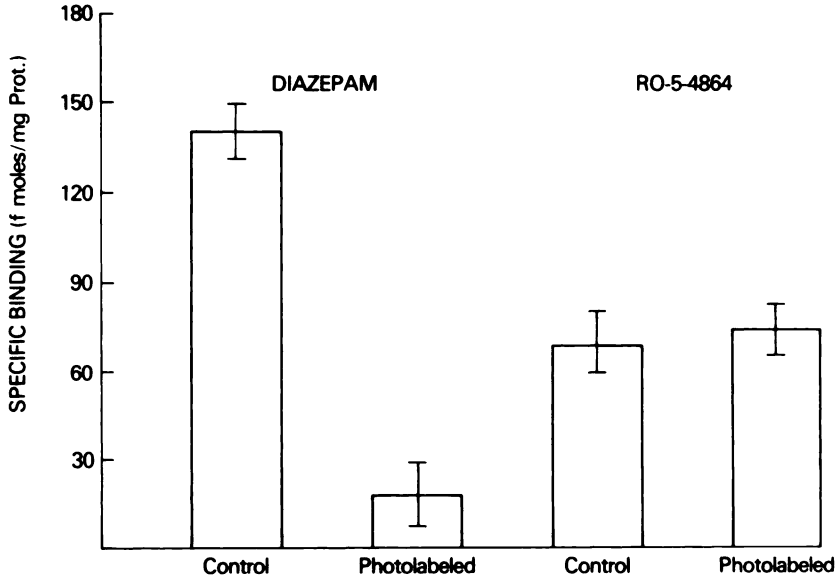

FIG. 5. *Binding of [^{3}H] RO-5-4864 and [^{3}H]diazepam to photoaffinity-labeled rat forebrain membranes*
Rat forebrain membranes were photolabeled with flunitrazepam as described under Methods. The extensively washed, photolabeled membranes were then assayed for both [^{3}H]Ro 5-4864 (1 nM) and [^{3}H]diazepam (1.5 nM) binding. Controls were treated in a manner identical with that for the photolabeled membranes, but they were not irradiated. This experiment was repeated with nearly identical results.

TABLE 3

Effect of various putative benzodiazepine receptor ligands on [^{3}H] Ro 5-4864 and [^{3}H]diazepam binding in rat brain

Seven concentrations of each compound were tested for inhibition of [^{3}H]Ro 5-4864 (1 nM) and [^{3}H]diazepam (1.5 nM) binding in rat brain. Each compound was tested at least three times. Values are means ± standard error of the mean.

Inhibitor	IC_{50}	
	[^{3}H]Ro 5-4864	[^{3}H]Diazepam
	μM	
Purines		
Inosine	>10,000	1,100 ± 55
2-Deoxyguanosine	>10,000	200 ± 16
2-Deoxyinosine	>10,000	310 ± 14
Caffeine	>10,000	395 ± 22
Theophylline	9,500	580 ± 29
β-Carbolines		
Harmane	>1,000	200 ± 9
Harmaline	>1,000	125 ± 6
Norharman	>1,000	5 ± 0.4
Indoles		
Melatonin	85 ± 6	520 ± 24
N-Acetyl-5-methoxy kynurenamine	1,700 ± 130	55 ± 8
Benzodiazepines and quinolines		
Diazepam	0.042 ± 0.003	0.011
Chlordiazepoxide	>60	0.900
Clonazepam	28 ± 4	0.001
Ro 5-4864	0.021 ± 0.002	>60
Ro 15-1788	>0.100	0.002
Flunitrazepam	0.035	0.003
PK 9084	>5	0.220

receptor and its naturally occurring endogenous ligands might provide insights concerning the neuronal systems in brain that mediate or have some effect on anxiety. The correlation of clinical potency of the benzodiazepines as anxiolytics and anticonvulsants with their receptor binding potency (2) has provided support for the physiological relevance of the benzodiazepine receptor, suggesting that it is involved in these processes.

A slightly perplexing finding reported in the early studies involving the characterization of the benzodiazepine receptor was the existence of a [^{3}H]diazepam binding site in various peripheral tissues, notably kidney (16, 17). Brain and kidney binding sites were termed "central-type" and "peripheral-type" benzodiazepine binding sites, respectively, since their pharmacological properties were quite distinct. Although the central-type benzodiazepine receptor is thought to be involved in the central effects of these drugs, a role for the peripheral-type receptor has not been suggested.

Both the central- and peripheral-type benzodiazepine receptors bind [^{3}H]diazepam in the nanomolar range, with the former having a significantly higher affinity (16, 17). The distinction between the two sites resides in the relative inability of clonazepam to inhibit [^{3}H]diazepam binding to the peripheral site and the great difference in potency of Ro 5-4864 as an inhibitor of the binding of [^{3}H]diazepam to each site. However, the question of whether the peripheral and central benzodiazepine receptors are different molecules or merely the same receptor associated with different membranes has remained largely unanswered owing to the lack of a specific ligand for the peripheral-type binding site.

The recent availability of [^{3}H]Ro 5-4864, the apparently specific peripheral benzodiazepine binding site ligand, makes possible a variety of studies concerning the relationship of the two major benzodiazepine binding sites. A preliminary report has revealed that [^{3}H]Ro 5-4864 binds with high affinity to brain membranes (19), indicating that some of the peripheral-type site is present in brain. In the present report we have characterized the binding of [^{3}H]Ro 5-4864 in brain and have provided evidence that the two binding sites are quite distinct.

[^{3}H]Ro 5-4864 binds with high affinity (K_D = 1.6 nM) to an apparently homogeneous population of binding sites, with the maximal number of sites being 245 fmoles/mg of protein. The affinity is therefore higher than that of [^{3}H]diazepam whereas the number of binding sites for [^{3}H]Ro 5-4864 is significantly lower than that observed with [^{3}H]diazepam. Our equilibrium parameters are in good agreement with the preliminary data previously reported (19). [^{3}H]Ro 5-4864 binding sites are present in many peripheral non-nervous tissues at levels much higher than those found in brain. In contrast, [^{3}H]diazepam binding is highest in brain (Table 1). The tissue distribution of the peripheral-type site is not consistent with its serving a function specific to nervous tissue.

Within the brain, the distribution of [^{3}H]Ro 5-4864 binding sites is quite distinct from that of [^{3}H]diazepam (Table 2). There is little regional variation in [^{3}H]Ro 5-4864 binding, with the highest areas being the pons and the lowest the cerebral cortex. This is the reverse of the [^{3}H]diazepam binding profile, which is highest in cerebral cortex and lowest in the pons. In addition to having a distinct tissue distribution and brain regional distribution as compared with [^{3}H]diazepam, [^{3}H]Ro 5-4864 also has a unique subcellular distribution (Fig. 3). Although [^{3}H]diazepam binding sites are highly enriched in the synaptosomal fraction, the highest density of [^{3}H]Ro 5-4864 binding sites is found in the nuclear fraction. These results suggest that the [^{3}H]Ro 5-4864 binding site may be localized on nuclear membranes, a site that would not be compatible with these receptors having a role in neuromodulation. The relative impurity of this fraction makes it difficult to state that the peripheral site is on nuclear membranes. However, it is of interest that nuclear binding of [^{3}H]flunitrazepam has been previously reported (28) and that diazepam has marked effects on mitogenesis and differentiation of various cells in culture (29). It is therefore possible that the peripheral-type benzodiazepine binding site may be involved in some of the cellular effects of diazepam. This hypothesis requires further examination.

The major inhibitory neurotransmitter, GABA, markedly stimulates the binding of [^{3}H]diazepam (9, 10) by increasing the affinity of the receptor. This, coupled with the enhancement of various GABA-ergic effects by the benzodiazepines (5–8), indicates that the benzodiazepine and GABA systems are probably coupled. However, GABA is without effect on [^{3}H]Ro 5-4864 binding (Fig. 4), suggesting that the peripheral-type receptor is not coupled to the GABA system. This observation is complicated by the fact that the binding of ^{3}H-labeled β-carboline-3-carboxylate ethyl ester, which is a central-

type ligand, is also not modulated by GABA (30), even though it is thought that the receptors labeled with this ligand are coupled to GABA (31). Modulation by GABA may therefore be a ligand-dependent as well as a receptor-dependent phenomenon.

Another piece of evidence indicating that the peripheral- and central-type benzodiazepine receptors in brain are distinct entities is that brain membranes photolabeled with flunitrazepam did not show any change in [^{3}H]Ro 5-4864 binding whereas [^{3}H]diazepam binding was greatly reduced. These data, taken in conjunction with some of the other findings in this report, indicate that [^{3}H]Ro 5-4864 is interacting with a binding site in brain totally distinct from that interacting with [^{3}H]diazepam.

Of the various putative endogenous ligands for the benzodiazepine receptor, all are virtually inactive as inhibitors of [^{3}H]Ro 5-4864 binding with the exception of melatonin, an observation that is difficult to rationalize at present. The recently described benzodiazepine antagonist Ro 15-1788 is also much less potent as an inhibitor of [^{3}H]Ro 5-4864 binding than of [^{3}H]diazepam binding, as is the pure anticonflict quinoline derivative PK 9084. The pharmacology of [^{3}H]Ro 5-4864 is therefore quite different from that of [^{3}H]diazepam. The lack of any correlation between therapeutic potency and IC_{50} values for inhibition of [^{3}H]Ro 5-4864 indicates that this binding site is probably not involved in the anticonvulsant or anxiolytic effects of the benzodiazepines.

A substantial amount of evidence has been presented indicating that the peripheral-type and central-type benzodiazepine binding sites as they exist in brain are probably separate and distinct molecules. Other possibilities that are much less consistent with the data are that the peripheral- and central-type benzodiazepine binding sites are merely different conformations of the same molecule or that [^{3}H]Ro 5-4864 is binding to a subunit of the same benzodiazepine receptor that is different from the subunit to which [^{3}H]diazepam is binding. Only a limited number of benzodiazepines interact with the peripheral-type site, but [^{3}H]Ro 5-4864 does not appear to interact with the central-type receptor. This ligand therefore represents an apparently specific probe that should be highly useful in the further study of the peripheral-type benzodiazepine binding sites. The physiological relevance of the peripheral-type receptor remains to be established, as does the existence of possible endogenous ligands. The elucidation of endogenous ligands for this site may indeed provide key insights concerning its function, as will the further study of possible nuclear effects of [^{3}H]Ro 5-4864 binding and possible effects of these binding sites on methylation (32).

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