MOLECULAR PHARMACOLOGY, 21:618–628



# Heterogeneity of Brain Benzodiazepine Receptors Demonstrated by [$^3$H]Propyl β-Carboline-3-carboxylate Binding

JAMES D. HIRSCH, RONALD L. KOCHMAN, AND PAUL R. SUMNER

*Department of Biological Research, G. D. Searle & Company, Chicago, Illinois 60680*

Received October 7, 1981; Accepted December 17, 1981

SUMMARY

[$^3$H]Propyl β-carboline-3-carboxylate (PrCC) was used as a ligand for *in vitro* binding studies of the mouse brain benzodiazepine receptor. Initial experiments showed that specific [$^3$H]PrCC binding was saturable in whole brain and cerebellar membranes, regionally variable in the brain, and inhibited by a wide variety of β-carbolines, benzodiazepines, and other drugs with affinities similar to those obtained with [$^3$H]diazepam as the ligand. However, in cerebellar membranes, the $B_{max}$ for specific [$^3$H]PrCC binding (570 fmoles/mg of protein) represented about 80% of the total number of sites labeled by [$^3$H]diazepam. Further studies revealed other differences between specific [$^3$H]PrCC and [$^3$H]diazepam binding. Nickel ion, which markedly stimulated [$^3$H]diazepam binding (+80%), had a strong inhibitory effect on specific [$^3$H]PrCC binding (−70%). γ-Aminobutyric acid (GABA) stimulated [$^3$H]diazepam binding in a concentration-dependent manner (up to 80%), but had a smaller and non-concentration-dependent stimulatory effect on [$^3$H]PrCC binding (49%). Similarly, pentobarbital stimulated specific [$^3$H]diazepam binding (40%) in a picrotoxinin-sensitive fashion, but had no effect at all on [$^3$H]PrCC binding. Also, avermectin $B_{1a}$ treatment strongly stimulated [$^3$H]diazepam binding (76%), but had only a small effect (+32%) on [$^3$H]PrCC binding. In brain membranes not depleted of GABA, specific [$^3$H]diazepam binding was stimulated by exposure to heat (60°), whereas there was no change in [$^3$H]PrCC binding. These results were shown to be due to the differential protective effect of GABA on binding of the two $^3$H-ligands. Although they are different in terms of binding regulation and other properties, studies of kainic acid-induced lesions in rat cerebellum revealed that specific [$^3$H]PrCC and [$^3$H]diazepam binding declined similarly in the lesioned tissue. Photoaffinity labeling of the benzodiazepine receptor with unlabeled flunitrazepam (20 nM, ≤ 4-hr short-wave UV) in membranes prepared from various brain regions and whole brain revealed that specific [$^3$H]diazepam binding determined with either unlabeled diazepam or ethyl β-carboline-3-carboxylate as displacers declined extensively (≥80%). In sharp contrast, specific [$^3$H]PrCC binding determined with unlabeled ethyl β-carboline-3-carboxylate as displacer was basically unaffected by photolabeling (−10% to 20%). Specific [$^3$H]PrCC binding determined with unlabeled diazepam declined to an intermediate extent in the membranes (≤50%), but never as far as [$^3$H]diazepam binding. In cerebellar membranes, photolabeling had little effect (~20%) on either the $K_D$ or $B_{max}$ for specific [$^3$H]PrCC binding determined with ethyl β-carboline-3-carboxylate as displacer, but significantly lowered (68%) the $B_{max}$ for [$^3$H]PrCC binding determined with diazepam as displacer. Similar results could be obtained after UV irradiation times as short as 5 min. The ability of GABA to stimulate [$^3$H]PrCC binding (34%) was preserved after photolabeling, but chloride channel manipulation with avermectin $B_{1a}$ failed to stimulate binding after irradiation. These data suggest that the benzodiazepine receptor is heterogeneous and that one of its subsets has specificity for β-carbolines. Several models are proposed for the heterogeneous benzodiazepine receptor that are consistent with this hypothesis.

## INTRODUCTION

Although the relationship, if any, between β-CCE[1] and the proposed endogenous ligand(s) for the brain benzodiazepine receptor is not known, it is clear that β-CCE is a potent inhibitor of [$^3$H]benzodiazepine receptor binding *in vitro* (1–4). Also, this β-carboline displaces [$^3$H]flunitrazepam from the benzodiazepine receptor *in vivo* (5),

[1] The abbreviations used are: β-CCE, ethyl β-carboline-3-carboxylate; PrCC, propyl β-carboline-3-carboxylate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; GABA, γ-aminobutyric acid.

0026-895X/82/030618-11$02.00/0

acts as a proconvulsant, and antagonizes a number of diazepam's pharmacological and physiological properties (2, 3, 6–9). These results suggest that β-CCE works through binding to the benzodiazepine receptor.

Further evidence for this suggestion was provided by Nielsen *et al.* (10), who used the [$^3$H]propyl ester derivative of β-carboline-3-carboxylic acid as a ligand for *in vitro* studies of the benzodiazepine receptor. Although subtle differences in binding characteristics were found, these workers concluded that [$^3$H]PrCC and [$^3$H]benzodiazepines bound specifically to a common recognition site on the benzodiazepine receptor. However, the possibility has been raised that a second binding site (a "β-carboline site") or subsets of binding sites exist at the receptor level (3, 4, 10–13).

Our preliminary studies with [$^3$H]PrCC showed that its binding was similar to that of [$^3$H]diazepam (see below). However, additional experiments with [$^3$H]PrCC suggested that several of its binding characteristics were considerably different from those of [$^3$H]diazepam. Furthermore, data obtained after photoaffinity labeling of the benzodiazepine receptor revealed that [$^3$H]PrCC binding was not destroyed by this technique whereas [$^3$H]diazepam binding was. Although alternative explanations are possible, our data support the suggestion that [$^3$H]PrCC may bind to a "β-carboline site" which is associated with or part of the benzodiazepine receptor in mouse brain membranes.

## MATERIALS AND METHODS

*Membrane preparation.* Male CD-1 HAM/ICR mice (Charles River Breeding Laboratories, Portage, Mich.) weighing 25–30 g were killed by cervical dislocation and were decapitated. The brains were rapidly removed, rinsed free of blood, and homogenized in 50 volumes of ice-cold 50 mM Tris-HCl buffer (pH 7.4, 4°) at Setting 6.5 (full scale = 10) with a Brinkmann (Westbury, N. Y.) Polytron PCU-2-110 for 25 sec. A crude membrane pellet was recovered by centrifugation at 36,000 × *g* in a Sorvall RC2-B refrigerated centrifuge, rehomogenized in 50 volumes of buffer as described above, recentrifuged, and resuspended for use in 50 volumes of Tris-HCl buffer (pH 7.4, 4°). Variations in this procedure are indicated where appropriate. Fresh and frozen tissue (−80°) were used in these experiments interchangeably. A new preparation of membranes was made for each experiment.

*Binding assays.* Aliquots of membranes were incubated in an ice-water bath in triplicate or quadruplicate for 60 min with [$^3$H]PrCC (final concentration 0.52 nM or as indicated) or [$^3$H]diazepam (final concentration 1.3 nM or as indicated). The assay volume varied from 0.2 to 2 ml depending upon the needs of the experiment, but the volume relationships between the ingredients and the final protein concentration per milliliter remained constant regardless of the assay size. One-half of the final assay volume consisted of membranes. Nonspecific binding was usually determined in the presence of 10 μM diazepam and 10 μM β-CCE assayed separately. Following incubation, assays were terminated by rapid vacuum filtration through Whatman GF/C filters which were washed three times with ice-cold Tris-HCl buffer. The filters were dried in an oven for 30 min at 55°, and counted in 10 ml of PCS (Amersham Corporation, Arlington Heights, Ill.) in a liquid scintillation spectrometer (Searle Analytic, Elk Grove Village, Ill.).

*Photoaffinity labeling.* Photoaffinity labeling of the benzodiazepine receptor was performed with minor modifications as described previously (14). Briefly stated, unlabeled flunitrazepam (final concentration 20 nM) in ethanol was added to an aliquot of membranes prepared as described above. Following a 20-min incubation at 4° in the dark, the mixture was incubated on ice in small glass dishes for various times 6–7 cm below an Ultraviolet Products Chromato-Vue lamp (Model CC-20, San Gabriel, Calif.) emitting short-wave UV light. Care was taken to keep the depth of the membranes in the dishes at 2–3 mm. When large volumes of membranes were needed, multiple dishes were used.

Control membranes were treated identically but were not irradiated, or in some experiments were irradiated without flunitrazepam. In either case the appropriate volume of ethanol vehicle was added to control membranes. After irradiation, all membranes were recovered by centrifugation and washed twice in their original volume of drug-free buffer. Since there were no differences in $^3$H-ligand binding characteristics between non-irradiated flunitrazepam-treated membranes and irradiated no-flunitrazepam membranes (see also ref. 15), the control data reported here were obtained with the former.

*Kainic acid injections into rat cerebellum.* Kainic acid lesions were induced in adult rat cerebellum as described by Braestrup *et al.* (16). Adult male Sprague-Dawley rats (Charles River Breeding Laboratories, Wilmington, Mass.) weighing 250–300 g were anesthetized with Nembutal (60 mg/kg i.p.) and fitted into a Kopf stereotaxic apparatus; through craniotomies into the cerebellum the animals received either two injections of 1 μg each (in 2 μl) of kainic acid dissolved in isotonic saline, or saline alone (two injections of 1 μl each). The guide cannula used to position the injection needle was 23-gauge thin-wall (outer diameter = 0.64 mm) and the injection needle was 27-gauge (outer diameter 0.41 mm). An injection rate of 0.5 μl/min was controlled by a motorized pump driving a Hamilton syringe. Injection coordinates were as defined by Braestrup *et al.* (16). After each injection (saline or kainic acid), the needle was left in place for 2 min.

Like Braestrup *et al.* (16), we induced lesions in some of the rats unilaterally in the left ($n$ = 3) or right ($n$ = 3) cerebellar hemisphere, leaving the noninjected side as a control in each rat. In another four rats, we induced lesions in both cerebellar hemispheres with two injections of 1 μg of kainic acid as described above. The controls in this case were four rats which received bilateral injections of equal volumes of isotonic saline.

Twenty-seven days after surgery, the rats were killed by decapitation and the lesioned and control cerebellar hemispheres were removed and frozen on dry ice. Membrane fractions for use in binding assays were prepared in 50 mM Tris-HCl buffer as described above.

*Protein determination.* Protein was determined by the micro-biuret procedure of Itzhaki and Gill (17), using bovine serum albumin as standard. In the kainic acid experiments, protein concentrations were adjusted by dilution with buffer so that all extracts had the same protein concentration per milliliter.

*Materials.* The [$^3$H]PrCC used in these experiments was prepared at New England Nuclear Corporation (Boston, Mass.) by catalytic tritiation of the propenyl derivative provided by G. D. Searle & Company. The labeled material had a specific activity of 48.3 Ci/mmole, and after 4 weeks of storage at −20° in 100% ethanol was 99.1% pure as determined by thin-layer chromatography on silica Gel G plates developed with toluene-methanol-ammonium hydroxide (78:22:2, v/v/v). The fluorescent radioactive and standard spots were located with long wavelength UV light. Several experiments were performed with [$^3$H]PrCC having a specific activity of 102.3 Ci/mmole (New England Nuclear Corporation). [$^3$H] PrCC was diluted for use in 50 mM Tris-HCl buffer (pH 7.4 at 4°). [$^3$H]Diazepam (87.6 Ci/mmole) was purchased from New England Nuclear Corporation.

The drugs used in these studies and their sources are as follows: diazepam, flunitrazepam, clonazepam, tetrabenazine, RO5-3663, and RO3-7355 (Hoffmann-La Roche); avermectin $B_{1a}$, amitriptyline, cyproheptadine, indomethacin, apomorphine, and mecamylamine (Merck); tryptophan, picrotoxinin, tetrodotoxin, and kainic acid (Sigma); pentylenetetrazole, harmaline, taurine, and carbazole (Aldrich); pimozide and fenobam (McNeil); fluoxitine (Lilly); methysergide (Sandoz); bufotenine (Regis); dopamine and DIDS (Calbiochem); cocaine, procaine, and strychnine (Mallinkrodt); piracetam (UCB); meprobamate (Wallace); theophylline (Searle Laboratories); bemegride (Heighart); imipramine (Ciba); propranolol (Ayerst); sotalol and buspirone (Mead-Johnson); loperamide (Janssen); zopiclone (Rhône-Poulenc); fominobene (Karl Thomae); dipyridamole and butazolidin (Geigy); clobazam (Hoechst-Roussel); phenoxybenzamine (Smith Kline & French); clonidine (Boehringer-Ingelheim); butorphanol (Bristol); naltrexone (Endo); pentazocine (Sterling-Winthrop); physostigmine (Schwarz/Mann); ethosuximide (Parke-Davis); trimethadione and pentobarbital (Abbott); ICI 136753 (ICI); baclofen (Ciba-Geigy); D-amphetamine (K and K); oxolinic acid (Warner-Lambert); and nicotinamide (Matheson, Coleman and Bell).

Unlabeled β-CCE, PrCC, all other β-carbolines and the atropine and ouabain used in these studies were synthesized by P. K. Yonan, K. A. Prodan, and other members of the Department of Medicinal Chemistry, G. D. Searle & Company. For routine work, drugs were dissolved in buffer or ethanol and diluted for use in either buffer or 50% (v/v) ethanol, depending on solubility. Final ethanol concentrations ≤10% (v/v) had no effect on specific binding of either radioligand.

## RESULTS

*General binding characteristics.* For saturation binding experiments, 15 concentrations of [$^3$H]PrCC were tested over the range of 0.15–8.0 nM using both 10 μM unlabeled diazepam and 10 μM unlabeled β-CCE for determination of nonspecific binding. Under these conditions, specific [$^3$H]PrCC binding in membranes from whole brain minus cerebellum was saturable with no appreciable difference between the displacers (Fig. 1). In these membranes, specific [$^3$H]PrCC binding determined with either displacer represented 75–80% of the total binding; [$^3$H]diazepam specific binding determined with either displacer was between 93% and 96% of the total binding.

Woolf plot analysis (18) of these saturation data indicated a single apparent $K_D$ for [$^3$H]PrCC of 1.7–2.1 nM and a single $B_{max}$ of 730 fmoles/mg of protein (Fig. 1, *inset*). Scatchard analysis of the data yielded straight lines and the same values for the apparent $K_D$ and $B_{max}$ (data not shown). Hill plots of the data indicated no apparent binding cooperativity with either displacer.

Further analysis of saturation binding was performed in cerebellar membranes using [$^3$H]PrCC, [$^3$H]diazepam, and a combination of the two ligands as discussed by Nielsen *et al.* (10). In these experiments, both β-CCE and diazepam were employed as displacers. When [$^3$H] PrCC was the ligand and nonspecific binding was determined with either 10 μM β-CCE or 10 μM diazepam, the apparent $K_D$ (1.5–2.1 nM) and $B_{max}$ values (570–580 fmoles/mg of protein) obtained were essentially identical (Fig. 2). When [$^3$H]diazepam was the ligand, an apparent $K_D$ of about 4.0 nM was obtained with either displacer. When diazepam displaced [$^3$H]diazepam, the $B_{max}$ (about 730 fmoles/mg of protein) was slightly higher than the $B_{max}$ obtained with β-CCE (690 fmoles/mg of protein) (Fig. 2).

We also performed a [$^3$H]ligand mixture experiment as suggested previously (10). In this case the $B_{max}$ values for [$^3$H]PrCC and [$^3$H]PrCC-[$^3$H]diazepam were the same (Fig. 2). However, as mentioned, higher $B_{max}$ values for [$^3$H]diazepam alone were found consistently in cerebellum when diazepam was displacer. We did not observe that the combined apparent $K_D$ was the mean of the two separate apparent $K_D$ values. Instead, the combined apparent $K_D$ was closer to that observed with [$^3$H]PrCC alone (Fig. 2).

Specific [$^3$H]PrCC binding varied on a brain regional basis (Table 1) and was similar in distribution to [$^3$H] benzodiazepine binding (19). In contrast to a previous study using rat brain membranes (10), we observed no preferential *in vitro* binding of [$^3$H]PrCC in mouse cerebellar membranes over that observed in hippocampal membranes.

Specific binding of [$^3$H]PrCC and [$^3$H]diazepam was inhibited by a variety of benzodiazepines and other drugs in a very similar fashion (Table 2), and the rank orders of potencies for the drugs versus the two ligands were almost identical. Converting these $IC_{50}$ values into $K_i$ values did not change the results significantly (data not shown).

When β-carbolines were used to displace both ligands, some binding specificity differences emerged (Table 3). Methyl-, ethyl-, and propyl-β-carboline-3-carboxylates were approximately equipotent versus both ligands, and all had high affinity (1–2.6 nM). Similarly, norharman was nearly equipotent against the ligands, although its affinity was about 1000-fold lower than its esterified derivatives.

In contrast to these aromatic β-carbolines, the L- and D-methyl and ethyl tetrahydro-β-carboline-3-carboxylates (derived from L- and D-tryptophan, respectively) showed different absolute potencies, rank orders of potencies, and degrees of stereoselectivity. Versus [$^3$H]

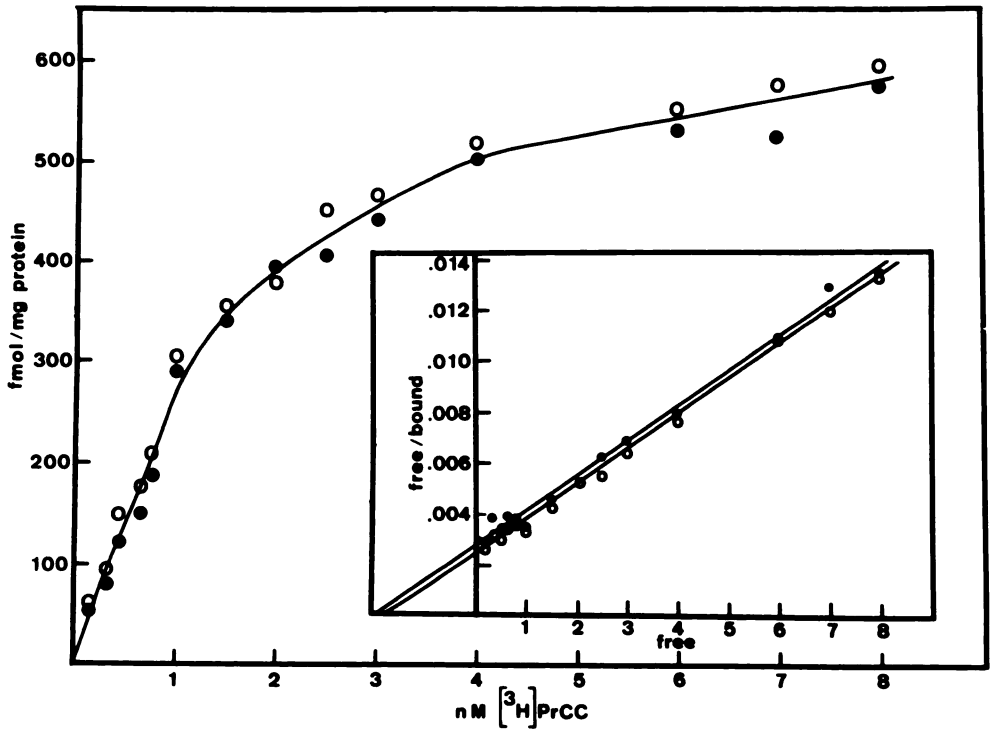


FIG. 1. *Saturable binding of [$^3$H]PrCC in membranes prepared from whole mouse brain minus cerebellum*

Membranes were prepared and binding assays performed as described under Materials and Methods. [$^3$H]PrCC was present at a concentration of 0.15–8.0 nM and each assay contained 400 μg of membrane protein in a final volume of 0.5 ml. *Inset.* Woolf plot of data in the main figure. O——O, Specific [$^3$H]PrCC binding using 10 μM β-CCE as displacer; ●——●, specific [$^3$H]PrCC binding using 10 μM diazepam as displacer. These same symbols are used in the *inset.* Apparent $K_D$ (β-CCE) = 2.1 nM; $B_{max}$ (β-CCE) = 738 fmoles/mg of protein. Apparent $K_D$ (diazepam) = 1.7 nM; $B_{max}$ (diazepam) = 727 fmoles/mg of protein. Data are from a representative experiment conducted in triplicate.

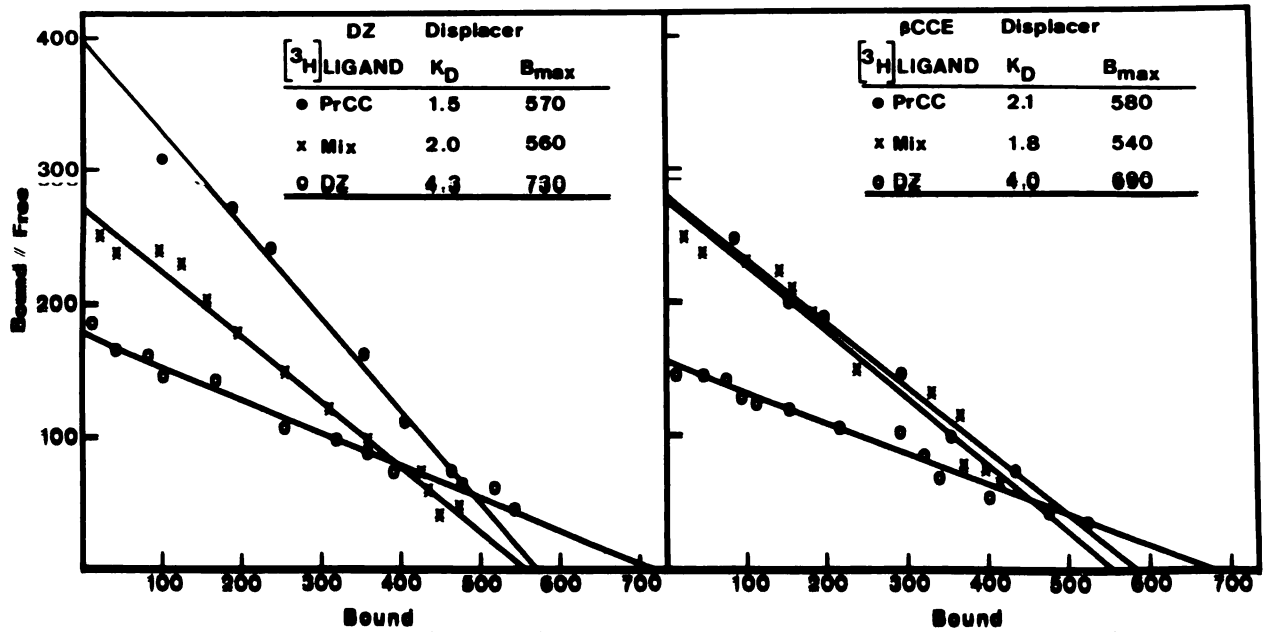


FIG. 2. *Scatchard plots of specific binding of [$^3$H]PrCC, [$^3$H]diazepam (DZ), and the combination of [$^3$H]PrCC and [$^3$H]diazepam (Mix) in cerebellar membranes*

Mouse cerebellar membranes were prepared as described under Materials and Methods. [$^3$H]PrCC (102.3 Ci/mmole) was diluted with unlabeled PrCC so as to give the same specific activity as the [$^3$H]diazepam used (87.6 Ci/mmole). This solution was mixed with a solution of [$^3$H]diazepam so that the concentration of both $^3$H-ligands was the same (92.1 nM). Further dilutions were made with buffer as needed, and the concentration range of the $^3$H-ligand mixture tested was 0.10–9.21 nM. When tested alone, [$^3$H]PrCC was present at a concentration of 0.31–6.21 nM, whereas [$^3$H]diazepam alone was present at a concentration of 0.12–10 nM. Each assay contained 410 μg of membrane protein in a final volume of 0.5 ml. As indicated, nonspecific binding was determined with 10 μM diazepam and 10 μM β-CCE as displacers. The appropriate apparent $K_D$ and $B_{max}$ values are shown. Studies of [$^3$H]PrCC binding alone were conducted three times; [$^3$H]diazepam binding experiments were performed twice. The assay with the $^3$H-ligand mixture was performed once. All experiments were conducted in triplicate or quadruplicate.

TABLE 1

*Regional distribution of specific [$^3$H]PrCC binding in mouse brain*

Mouse brain regions were stored frozen at −80° until assayed. Specific [$^3$H]PrCC (0.52 nM) binding was determined as described under Materials and Methods. Both 10 μM β-CCE and 10 μM diazepam were used for measurement of nonspecific binding with no significant effect on the final results. The number of separate experiments performed is indicated in parentheses, and results are means ± standard error of the mean.

| Region | Specific binding |
|---|---|
| | *fmoles/mg protein* |
| Frontal cortex | 89 ± 1 (4) |
| Hippocampus | 88 ± 6 (6) |
| Cerebellum | 87 ± 7 (6) |
| Hypothalamus | 80 ± 4 (4) |
| Olfactory bulb | 62 ± 5 (4) |
| Striatum | 44 ± 1 (4) |
| Pons-medulla | 39 ± 4 (4) |
| Spinal cord | 26 ± 1 (6) |

PrCC, the D-ethyl β-carboline was 90-fold more potent than the L-ethyl form, whereas the L-methyl form was about 3-fold more potent than its D-methyl isomer. With [$^3$H]diazepam as the ligand, the D-ethyl β-carboline was more potent than the L-ethyl drug, but only by 13-fold. In this case the L- and the D-methyl derivatives were about equipotent versus [$^3$H]diazepam. With the exception of the L-ethyl form, these tetrahydro β-carbolines were 2- to 9-fold more potent against [$^3$H]PrCC than against [$^3$H]diazepam. β-Carboline-3-carboxylic acid displaced both ligands very poorly (Table 3), and tetrahydronorharman was inactive (data not shown). Since preliminary evidence suggested that these tetrahydro β-carbolines were not pure competitive inhibitors of [$^3$H]diazepam binding,[2] $K_i$ values were not calculated for these drugs.

The following compounds inhibited [$^3$H]PrCC binding ≤20% at a concentration of 1 μM: *drugs affecting biogenic amines*—tetrabenazine, dextroamphetamine, amitriptyline, cyproheptadine, apomorphine, tryptophan, doxepin, pimozide, fluoxitine, methysergide, bufotenine, dopamine, cocaine, imipramine, propranolol, sotalol, buspirone, phenoxybenzamine, and clonidine; *analgesics, antagonists, and related drugs*—butorphanol, naltrexone, pentazocine, procaine, and loperamide; *cholinergic drugs*—physostigmine, mecamylamine, and atropine; *GABAergic drugs and convulsants*—tetrodotoxin, pentylenetetrazole, harmaline, strychnine, RO5-3663, and baclofen; *miscellaneous drugs*—ouabain, taurine, indomethacin, butazolidin, carbazole, fenobam, ICI 136753, piracetam, bemegride, meprobamate, theophylline, ethosuximide, trimethadione, oxolinic acid, and nicotinamide.

*Regulation of [$^3$H]PrCC binding.* We examined the effect of $Ni^{2+}$ on specific [$^3$H]PrCC binding (Fig. 3). As shown previously (20), $Ni^{2+}$ over the range of $1 \times 10^{-7}$ to $5 \times 10^{-3}$ M stimulated specific [$^3$H]diazepam binding to a maximum of 80%. However, specific [$^3$H]PrCC binding was inhibited up to 70% by this ion over the same concentration range. Identical results were obtained with $Ni^{2+}$ acetate in the usual Tris-HCl buffer, but the ion had no effect on either ligand in 50 mM Tris-citrate buffer

[2] J. D. Hirsch, R. L. Kochman, and P. R. Sumner, unpublished data.

TABLE 2

*Inhibition of [$^3$H]PrCC and [$^3$H]diazepam binding by benzodiazepines and related compounds*

Five to seven concentrations of each drug diluted in 50% ethanol were tested and the results were plotted on log-logit paper. The $IC_{50}$ is the concentration of drug resulting in 50% inhibition of $^3$H-ligand binding. [$^3$H]PrCC and [$^3$H]diazepam were present at concentrations of 0.52 and 1.3 nM, respectively. In these experiments nonspecific binding was determined in the presence of 10 μM diazepam. Each value is the mean of two to five experiments conducted in triplicate or quadruplicate as described under Materials and Methods.

| Compound | $IC_{50}$ | |
|---|---|---|
| | [$^3$H]PrCC | [$^3$H]Diazepam |
| | *nM* | *nM* |
| Clonazepam | 0.6 | 0.7 |
| Flunitrazepam | 1.5 | 2.5 |
| Diazepam | 9.2 | 8.0 |
| Zopiclone | 40 | 78 |
| RO3-7355 | 43 | 64 |
| Clobazam | 236 | 189 |
| Dipyridamole | 740 | 990 |
| Fominobene | 1400 | 1690 |

(data not shown). In Tris-HCl buffer, $Ni^{2+}$ stimulated [$^3$H]diazepam binding by increasing total binding with no effect on nonspecific binding; it inhibited [$^3$H]PrCC binding by increasing nonspecific binding to a much greater extent than total binding.

The effect of $Ni^{2+}$ on ligand binding was explored further by testing how the ion affected the $IC_{50}$ values for unlabeled drugs against [$^3$H]diazepam. Nickel increased the affinity of diazepam by 2- to 3-fold, whereas the binding of all β-carbolines tested was markedly inhibited by the ion. The largest inhibition (13-fold) was observed with the tetrahydro-D-ethyl-β-carboline derivative ($-Ni^{2+}$: 193 nM; $+Ni^{2+}$: 2580 nM). In these experi-

TABLE 3

*Inhibition of [$^3$H]PrCC and [$^3$H]diazepam binding by β-carbolines*

Five to seven concentrations of each drug diluted in 50% ethanol were tested and the results were plotted on log-logit paper. The $IC_{50}$ is the concentration of drug resulting in 50% inhibition of $^3$H-ligand binding. [$^3$H]PrCC and [$^3$H]diazepam were present at concentrations of 0.52 and 1.3 nM, respectively. In these experiments, nonspecific binding was determined in the presence of 10 μM diazepam. Each value is the mean of two to five experiments conducted in triplicate or quadruplicate as described under Materials and Methods. β-CCM, Methyl β-carboline-3-carboxylate; β-C, β-carboline; β-CC, β-carboline-3-carboxylic acid. All of the tetrahydro compounds were substituted as indicated at position 3.

| Compound | $IC_{50}$ | |
|---|---|---|
| | [$^3$H[PrCC | [$^3$H]Diazepam |
| | *nM* | *nM* |
| β-CCE | 1.2 | 2.0 |
| β-CCM | 1.2 | 2.6 |
| PrCC | 2.5 | 1.5 |
| Tetrahydro-D-ethyl β-C | 89 | 190 |
| Tetrahydro-L-methyl β-C | 1,300 | 11,000 |
| Norharman | 2,300 | 1,800 |
| Tetrahydro-D-methyl β-C | 4,400 | 16,000 |
| Tetrahydro-L-ethyl β-C | 8,000 | 2,400 |
| β-CC | 32,000 | 17,000 |

ments, $IC_{50}$ values in the absence of $Ni^{2+}$ were as indicated in Tables 2 and 3.

Two other cations, $Mn^{2+}$ and $Co^{2+}$, had effects similar to those of $Ni^{2+}$ on ligand binding but their stimulation of [$^3$H]diazepam binding was milder ($Mn^{2+}$: 9 to 32% from 0.1 to 100 mM; $Co^{2+}$: 9 to 27% from 0.001 to 1.0 mM). Also, there was less inhibition of specific [$^3$H]PrCC binding ($Mn^{2+}$: 27 to 34% from 0.1 to 100 mM; $Co^{2+}$: 21 to 44% from 0.001 to 1.0 mM) than observed with $Ni^{2+}$.

Other ions tested, including NaCl, KCl, NaI, KI, $CuCl_2$, $HgCl_2$, $FeCl_2$, $MgCl_2$, and $CaCl_2$, had no consistent and/or concentration-dependent effects on [$^3$H]PrCC binding and could not be used to discriminate between the ligands.

Along with ions, GABA can also regulate ligand binding to the benzodiazepine receptor (21, 22). In the membranes routinely used for these experiments (high-GABA), 10 μM GABA moderately stimulated both [$^3$H]diazepam and [$^3$H]PrCC binding (21 to 26%, Table 4). However, when these membranes were washed extensively to remove residual GABA (low-GABA), the amino acid had a much larger concentration-dependent stimulatory effect (68 to 78%) on [$^3$H]diazepam binding. GABA also stimulated [$^3$H]PrCC binding in low-GABA membranes, but the effect was smaller and not concentration-dependent (about 45%).

Drugs interacting with the chloride channel can also alter [$^3$H]diazepam binding (23, 24). One of these drugs, avermectin $B_{1a}$, increased specific [$^3$H]diazepam binding by 54% in high-GABA membranes, but increased [$^3$H]-PrCC binding by only 28%. In low-GABA membranes, the same concentration of avermectin (10 μM) had a greater stimulatory effect on [$^3$H]diazepam binding (76%), but had no additional effect on [$^3$H]PrCC binding (32%) (Table 4).

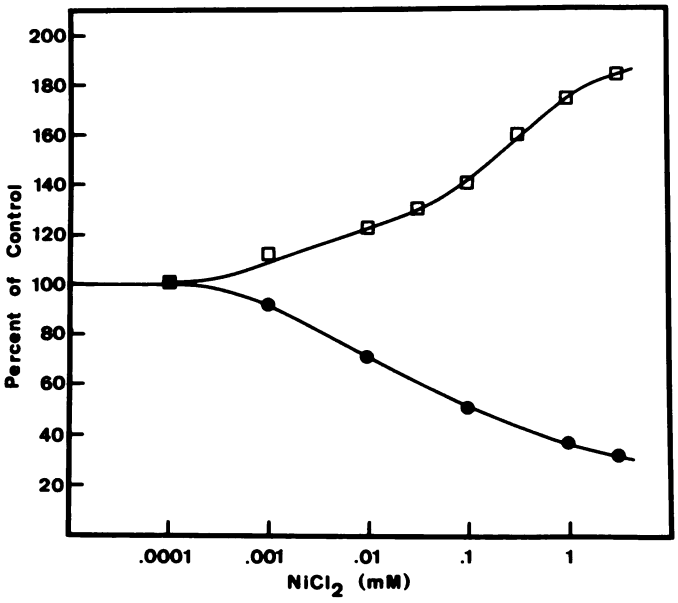


FIG. 3. *Effect of $Ni^{2+}$ ion on specific [$^3$H]PrCC and [$^3$H]diazepam binding*

Whole mouse brain membranes were prepared and binding assays were performed in the presence of the indicated concentrations of $NiCl_2$ as described under Materials and Methods. [$^3$H]PrCC and [$^3$H]diazepam were present at concentrations of 0.52 and 1.3 nM, respectively. Nonspecific binding was determined in the presence of 10 μM diazepam. □—□, [$^3$H]diazepam binding; ●—●, [$^3$H]PrCC binding.

TABLE 4

*Effect of GABA and avermectin $B_{1a}$ on specific [$^3$H]diazepam and [$^3$H]PrCC binding*

Whole mouse brain membranes (designated high-GABA) were prepared as described under Materials and Methods. Low-GABA membranes were washed by centrifugation and rehomogenization three more times to remove residual GABA. After preparation, membranes were incubated on ice with 10 μM avermectin $B_{1a}$ (AVM) for 60 min and then assayed for specific [$^3$H]diazepam (1.3 nM) and [$^3$H]PrCC (0.52 nM) binding as described in the text. GABA was added at the indicated concentrations directly to the assay tubes. Nonspecific binding in the presence and absence of GABA and AVM was determined in the presence of 10 μM β-CCE. The number of separate experiments with different membrane preparations is indicated in parentheses. Statistical significance was determined by a two-tailed Student's *t*-test.

| Type of membrane | [$^3$H]Ligand | Treatment | % of Control[a] |
|---|---|---|---|
| High-GABA | [$^3$H]Diazepam | None | 100 ± 3 (4) |
| | | 10 μM GABA | 121 ± 3 (4)* |
| | | 10 μM AVM | 154 ± 6 (4)** |
| | [$^3$H]PrCC | None | 100 ± 1 (4) |
| | | 10 μM GABA | 126 ± 5 (4)* |
| | | 10 μM AVM | 128 ± 4 (4)** |
| Low-GABA | [$^3$H]Diazepam | None | 100 ± 7 (3) |
| | | 10 μM GABA | 168 ± 1 (3)** |
| | | 100 μM GABA | 178 ± 1 (3)**# |
| | | 10 μM AVM | 176 ± 3 (3)** |
| | [$^3$H]PrCC | None | 100 ± 7 (3) |
| | | 10 μM GABA | 140 ± 4 (3)*** |
| | | 100 μM GABA | 149 ± 3 (3)*** |
| | | 10 μM AVM | 132 ± 1 (3)## |

[a] Values are means ± standard error of the mean. * $p < 0.005$; ** $p < 0.001$; *** $p < 0.01$; # significantly different from 10 μM, $p < 0.005$; ## $p < 0.025$.

As also reported previously (23), pentobarbital stimulated [$^3$H]diazepam binding (40%) in a picrotoxinin-sensitive manner, but the barbiturate had no effect on [$^3$H]PrCC binding (Table 5). Picrotoxinin itself slightly decreased [$^3$H]diazepam binding in these high-GABA membranes (14%) but had no effect at all on [$^3$H]PrCC binding. Picrotoxinin sensitivity of [$^3$H]diazepam binding in high-GABA conditions has been reported (25).

We also used the drug DIDS to investigate the chloride channel regulation of [$^3$H]PrCC and [$^3$H]diazepam binding (26). Pretreatment of high-GABA membranes with 100 μM DIDS for 30 min at 30° led to a 16% decrease ($n = 4$ experiments; $p < 0.025$) in specific [$^3$H]diazepam binding measured at 0–4° but had no effect on specific [$^3$H]PrCC binding (data not shown). Also, DIDS preincubation blocked the mild stimulation of specific [$^3$H]-diazepam binding obtained with 100 mM NaCl (12%, $n = 4$ experiments; $p < 0.05$). As mentioned, NaCl had no effect on [$^3$H]PrCC binding.

*Other binding characteristics.* In the high-GABA membranes normally used for our binding studies, the heat sensitivity of [$^3$H]PrCC and [$^3$H]diazepam binding was different. Heat shock (60° for 0–60 min) resulted in a time-dependent increase in specific [$^3$H]diazepam binding (28% at 60 min) but had no substantial effect on [$^3$H]PrCC binding (−1% at 60 min). However, since GABA in the presence of chloride (and possibly other

TABLE 5

*Effect of pentobarbital and picrotoxinin on specific [$^3$H]diazepam and [$^3$H]PrCC binding*

Specific binding of [$^3$H]diazepam (1.3 nM) and [$^3$H]PrCC (0.52 nM) was determined in whole mouse brain membranes as described in the text. Pentobarbital and/or picrotoxinin dissolved in buffer were added at the indicated concentrations directly to the assay tubes, which were incubated for 60 min in an ice-water bath. Nonspecific binding in the presence and absence of drugs was determined in the presence of 10 μM diazepam. The number of separate experiments with different membrane preparations is indicated in parentheses. Statistical significance was tested by a two-tailed Student's *t*-test.

| $^3$H-Ligand | Addition | % of Control[a] |
|---|---|---|
| [$^3$H]Diazepam | Control | 100 ± 3 (3) |
| | Pentobarbital (500 μM) | 140 ± 2 (3)* |
| | Picrotoxinin (100 μM) | 86 ± 2 (3)** |
| | Pentobarbital (500 μM) + picrotoxinin (100 μM) | 126 ± 2 (3)*** |
| [$^3$H]PrCC | Control | 100 ± 10 (3) |
| | Pentobarbital (500 μM) | 117 ± 2 (3) |
| | Picrotoxinin (100 μM) | 108 ± 8 (3) |
| | Pentobarbital (500 μM) + picrotoxinin (100 μM) | 116 ± 5 (3) |

[a] Values are means ± standard error of the mean. * $p < 0.001$, ** $p < 0.025$; *** significantly higher than control at $p < 0.005$ but significantly lower than pentobarbital alone at $p < 0.01$.

factors) can protect the benzodiazepine receptor from heat inactivation (27), and since GABA had a differential effect on [$^3$H]ligand binding (Table 4), these heat-shock experiments were repeated in five-times-washed low-GABA membranes. In this case, there was a time-dependent decrease in both [$^3$H]PrCC and [$^3$H]diazepam binding with basically identical rates of heat inactivation (data not shown).

We also used kainic acid lesions to try to differentiate [$^3$H]diazepam binding from that of [$^3$H]PrCC. Twenty-seven days after induction of kainic acid lesions in rat cerebellum (see Materials and Methods), specific [$^3$H]-diazepam binding determined with either displacer had declined by 26 to 29% ($p < 0.005$) while specific [$^3$H]-PrCC binding determined with either displacer had declined by 27% ($p < 0.01$). No binding differences between noninjected and saline-injected control cerebella were noted.

To investigate further the [$^3$H]ligand binding differences, photoaffinity labeling of the benzodiazepine receptor with flunitrazepam (14) was used to destroy specific [$^3$H]diazepam binding in various mouse brain regions and in whole brain. The effects of photoaffinity labeling on [$^3$H]diazepam binding in all regions and in whole brain were identical; specific [$^3$H]diazepam binding was destroyed by 79–93% *no matter what unlabeled ligand was used as displacer* (Table 6). However, the effects of photoinactivation on specific [$^3$H]PrCC binding were completely different (Table 7). First, specific [$^3$H]PrCC binding determined with unlabeled β-CCE as displacer, with the exception of spinal cord, was *relatively unaffected* by photolabeling. Binding declined only 14–30% and was due primarily to small decreases in total binding (10–15%) and small increases in nonspecific binding of similar magnitude. Second, with spinal cord as an exception again, specific [$^3$H]PrCC binding determined with diazepam as displacer was partially preserved, declining 21–48%. These decreases in diazepam-displaceable [$^3$H]PrCC binding were larger than those seen when β-CCE was displacer, but were never as large as those seen when [$^3$H]diazepam was used as the ligand (Table 6).

Since these results were obtained at a single concentration of [$^3$H]PrCC, saturation binding experiments

TABLE 6

*Effect of photoaffinity labeling of the benzodiazepine receptor on specific [$^3$H]diazepam binding in mouse brain regions*

Mouse brain membranes were prepared, photoaffinity-labeled with nonradioactive flunitrazepam, and washed as described under Materials and Methods. Specific binding of [$^3$H]diazepam (1.3 nM) was determined in triplicate or quadruplicate. Diazepam at 10 μM and 10 μM β-CCE (displacers) were used separately to determine nonspecific binding. In these experiments, the decreases in specific binding were due almost entirely to large declines (75–90%) in total [$^3$H]diazepam binding with little or no change in nonspecific binding. The number of separate experiments with different membrane preparations is indicated in parentheses.

| Region | % Decrease in specific [$^3$H]diazepam binding (mean ± SEM) by displacer: | |
|---|---|---|
| | Diazepam | β-CCE |
| Hippocampus | 87 ± 1 (4) | 90 ± 1 (4) |
| Cerebellum | 86 ± 1 (3) | 90 ± 1 (3) |
| Striatum | 82 ± 2 (4) | 86 ± 2 (4) |
| Frontal cortex | 89 ± 4 (4) | 88 ± 3 (4) |
| Spinal cord | 83 ± 4 (4) | 87 ± 4 (4) |
| Pons-medulla | 83 (2) | 86 (2) |
| Olfactory bulb | 85 (2) | 93 (2) |
| Hypothalamus | 79 (2) | 84 (2) |
| Whole brain | 87 (2) | 89 (2) |

TABLE 7

*Effect of photoaffinity labeling of the benzodiazepine receptor of specific [$^3$H]PrCC binding in mouse brain regions*

Mouse brain membranes were prepared, photoaffinity-labeled with nonradioactive flunitrazepam, and washed as described under Materials and Methods. Specific binding of [$^3$H]PrCC (0.52 nM) was determined in triplicate or quadruplicate. Diazepam at 10 μM and 10 μM β-CCE (displacers) were used separately to determine nonspecific binding. In these experiments, total [$^3$H]PrCC binding decreased ≤ 20% after photolabeling in all regions tested, and decreases in specific binding resulted from decreases in the amount of [$^3$H]PrCC displaced by the diazepam or β-CCE. The number of separate experiments with different membrane preparations is indicated in parentheses. Control levels of binding were comparable to those indicated in Table 1. Statistical significance was tested by a two-tailed Student's *t*-test.

| Region | % Decrease in specific [$^3$H]PrCC binding (mean ± SEM) by displacer: | |
|---|---|---|
| | Diazepam | β-CCE[a] |
| Hippocampus | 41 ± 5 (4) | 14 ± 4 (4)* |
| Cerebellum | 33 ± 1 (3) | 10 ± 8 (3)** |
| Striatum | 21 ± 8 (4) | 17 ± 8 (4) |
| Frontal cortex | 47 ± 4 (4) | 30 ± 5 (4)** |
| Spinal cord | 74 ± 4 (4) | 48 ± 6 (4)*** |
| Pons-medulla | 37 (2) | 14 (2) |
| Olfactory bulb | 44 (2) | 23 (2) |
| Hypothalamus | 38 (2) | 14 (2) |
| Whole brain | 48 (2) | 21 (2) |

[a] Decrease is less than that seen with diazepam as displacer: * $p < 0.01$; ** $p < 0.05$; *** $p < 0.025$.

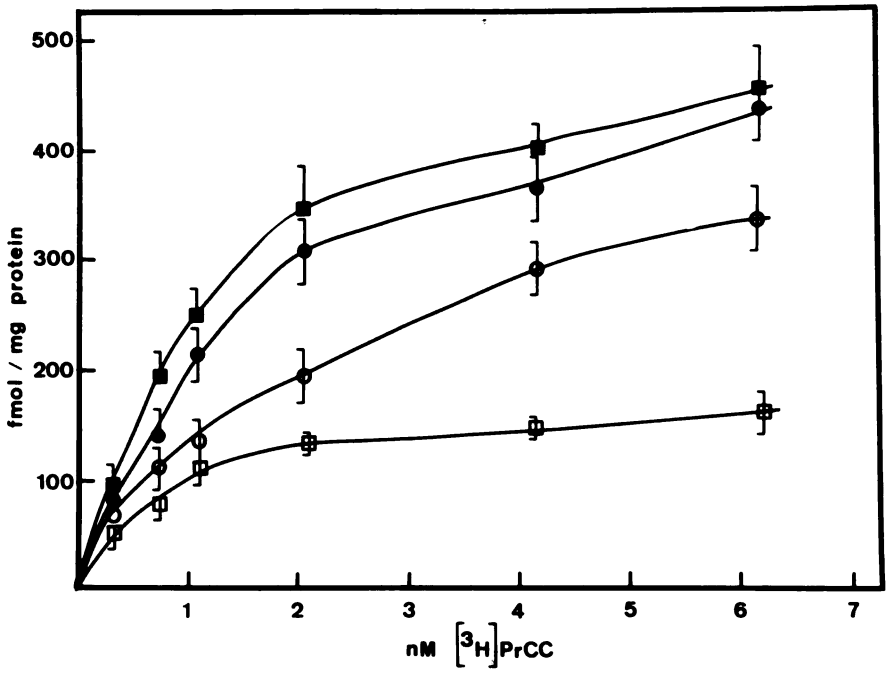


FIG. 4. *Saturable specific binding of [$^3$H]PrCC in control and photolabeled mouse cerebellar membranes using diazepam and β-CCE as displacers*

Membranes from frozen mouse cerebella were prepared and photolabeled for 4 hr as described under Materials and Methods. Binding assays were performed with [$^3$H]PrCC over the concentration range of 0.31–6.2 nM in a final volume of 0.2 ml. Diazepam at 10 μM and 10 μM β-CCE were used to determine nonspecific binding. Data points represent means ± standard error of the mean from three separate experiments performed in quadruplicate with three different membrane preparations. ■——■, [$^3$H]PrCC binding in control membranes determined with diazepam as displacer; □——□, [$^3$H]PrCC binding in photolabeled membranes determined with diazepam as displacer; ●——●, [$^3$H]PrCC binding in control membranes determined with β-CCE as displacer; ○——○, [$^3$H]PrCC binding in photolabeled membranes determined with β-CCE as displacer. The apparent $K_D$ and $B_{max}$ values are shown in Table 8.

tration of [$^3$H]PrCC, saturation binding experiments were performed in cerebellar membranes to clarify the effects of photolabeling. In control and photolabeled membranes, specific [$^3$H]PrCC binding determined with either 10 μM diazepam or 10 μM β-CCE as the displacer was saturable (Fig. 4). After photolabeling, the ability of β-CCE to displace [$^3$H]PrCC was spared compared with that of diazepam, confirming the results in Table 7. However, diazepam-displaceable [$^3$H]PrCC binding did not decline as extensively as diazepam- or β-CCE-displaceable [$^3$H]diazepam binding (see Table 6).

Woolf plots (18) were used to analyze these data, yielding the apparent $K_D$ and $B_{max}$ values in Table 8. There was a significant 68% decrease ($p < 0.01$) in the $B_{max}$ for [$^3$H]PrCC after photolabeling when diazepam was used as the displacer and a nonsignificant shift toward higher binding affinity. In contrast, the ability of β-CCE to displace [$^3$H]PrCC was relatively unaffected by photolabeling. In this case, there was a nonsignificant decline in the $B_{max}$ for [$^3$H]PrCC and a nonsignificant increase in apparent $K_D$. There was no change in Hill coefficient for any ligand-displacer combination after photolabeling ($n_H$ = 0.98–1.11). Thus, these results confirm the cerebellar data in Table 7 obtained at a single concentration of [$^3$H]PrCC. Similar experiments have not yet been performed in other brain regions. However, additional work has shown that the effects of photolabeling as described in cerebellum were essentially the same after 5, 15, 30, and 60 min of UV irradiation as they were after 4 hr (data not shown).

Selected aspects of the regulation of [$^3$H]PrCC binding were examined in photolabeled membranes. As observed in high- and low-GABA membranes (Table 4), β-CCE-displaceable [$^3$H]PrCC binding in photolabeled membranes was moderately stimulated (34 ± 6%, $p < 0.025$; $n$ = 3 experiments) in a non-concentration-dependent way by 10 or 100 μM GABA. It should be noted that the

TABLE 8

*Effect of photoaffinity labeling of the benzodiazepine receptor on saturable binding of [$^3$H]PrCC in cerebellar membranes*

Saturation binding data shown below are depicted graphically in Fig. 4. Mouse cerebellar membranes were prepared and photoaffinity-labeled as described under Materials and Methods. Specific binding of [$^3$H]PrCC was tested in triplicate over the concentration range of 0.31–6.2 nM, and nonspecific binding was determined in the presence of 10 μM diazepam and 10 μM β-CCE (displacers) assayed separately. These data were derived from Woolf plots (18) constructed with linear regression analysis. The mean correlation coefficient (± standard error of the mean) for all 12 Woolf plots was 0.98 ± 0.01. The number of separate experiments with different membrane preparations is shown in parentheses. Statistical significance was tested by a two-tailed Student's *t*-test.

| Displacer | Cerebellar membranes | Mean ± SEM | |
|---|---|---|---|
| | | Apparent $K_D$ | $B_{max}$ |
| | | *nM* | *fmoles/mg protein* |
| Diazepam | Control | 1.0 ± 0.2 (3) | 533 ± 64 (3) |
| | Photolabeled | 0.6 ± 0.1 (3) | 171 ± 19 (3)* |
| β-CCE | Control | 1.8 ± 0.3 (3) | 574 ± 86 (3) |
| | Photolabeled | 2.4 ± 0.6 (3) | 417 ± 79 (3) |

* $p < 0.01$.

photolabeled membranes used were essentially low-GABA membranes because of the extra washes needed to prepare them. However, unlike the results in Table 4 derived with high- and low-GABA membranes, avermectin $B_{1a}$ treatment of photolabeled membranes had no effect on β-CCE-displaceable [$^3$H]PrCC binding (−4 ± 6%, $n = 3$ experiments).

## DISCUSSION

We observed early in these studies that photoaffinity labeling of the benzodiazepine receptor in mouse brain membranes did not lead to equal destruction of specific [$^3$H]PrCC and [$^3$H]diazepam binding. This suggested that [$^3$H]PrCC might bind to a subtype of the benzodiazepine receptor that was not identical with the [$^3$H]diazepam binding site. Our aim was to investigate this possibility in detail.

As others have reported (4, 10, 12, 13), we have also shown that [$^3$H]PrCC and [$^3$H]benzodiazepines share a number of characteristics as ligands for the benzodiazepine receptor, including their brain regional variability, saturability, and pharmacological specificity. Although these results support the idea that the two ligands bind to the same receptor (10), further work showed that this receptor binding is more complex than was originally thought.

First, $Ni^{2+}$ ion had opposite effects on [$^3$H]PrCC and [$^3$H]diazepam binding, inhibiting the former and stimulating the latter. Nickel also readily distinguished between unlabeled β-carbolines and benzodiazepines when these drugs were used to displace [$^3$H]diazepam. Since $Ni^{2+}$ stimulates [$^3$H]diazepam binding by increasing the affinity of the receptor for the ligand (20), and since we have not been able to relate the $Ni^{2+}$ effect to the chloride channel by blocking it with picrotoxinin or DIDS,[2] we suggest that $Ni^{2+}$ alters ligand binding by a direct effect on the receptor. This opposite effect of $Ni^{2+}$ on [$^3$H]PrCC and [$^3$H]diazepam binding may support the concept that these ligands bind to nonidentical sites on the receptor.

Similar support for nonequivalence of [$^3$H]PrCC and [$^3$H]diazepam binding comes from other regulation studies. As reported elsewhere (4, 10), we have shown that GABA consistently stimulated [$^3$H]PrCC binding less than [$^3$H]diazepam binding. Also, [$^3$H]diazepam binding was stimulated markedly by avermectin $B_{1a}$ (24), whereas [$^3$H]PrCC binding was not. Washing out GABA from the membranes magnified the effect of avermectin on [$^3$H]diazepam binding (24) but had no effect on the small stimulation of [$^3$H]PrCC binding. Moreover, pentobarbital had no effect on [$^3$H]PrCC binding while stimulating [$^3$H]diazepam binding in a picrotoxinin-reversible way. Picrotoxinin itself as well as DIDS inhibited [$^3$H]diazepam binding without affecting [$^3$H]PrCC binding. These results suggest that, in contrast to [$^3$H]diazepam binding, chloride channel regulation of [$^3$H]PrCC binding is minimal.

Our results on receptor regulation supported the hypothesis for non-equivalent [$^3$H]PrCC and [$^3$H]diazepam binding sites within the benzodiazepine receptor-GABA receptor-chloride channel complex, but more definitive evidence was needed. Thus, we tried to demonstrate differential heat sensitivity of [$^3$H]PrCC and [$^3$H]diazepam binding in whole brain membranes. This phenomenon was observed in high-GABA membranes where, upon heat exposure, [$^3$H]diazepam binding increased while [$^3$H]PrCC binding was unaffected. However, removing GABA from the membranes used for binding resulted in identical heat inactivation profiles for specific binding of both ligands. The role of GABA in the differential heat effect was strengthened by the fact that GABA replacement in GABA-depleted membranes partially restored protection from 60° heating for both ligands.[2] Since protection of [$^3$H]benzodiazepine binding from heat inactivation by GABA has been shown (27), as has a stimulation of binding by heating membranes (28), these particular results pointed more to differential binding site regulation (see above) than to completely separate binding entities.

Another approach was to induce kainic acid lesions in rat cerebellum and determine whether one type of ligand binding declined while the other remained unchanged or increased (16). This would imply different cellular loci for the two proposed sites and make it unlikely that they were identical. However, kainic acid-induced lesions resulted in similar moderate declines in both specific [$^3$H]PrCC and [$^3$H]diazepam binding. Our results are qualitatively similar to those published by Braestrup *et al.* (16), who used [$^3$H]flunitrazepam, but are quantitatively smaller. This is probably due to the fact that we did not dissect a block of tissue from around each lesion site as they reported, but processed the entire lesioned cerebellar hemisphere. However, we did observe declines in hemisphere weight after induction of the lesion (23%; $p < 0.05$) as reported previously (16). These results did not resolve the question of separate binding sites.

In contrast, photoaffinity labeling of the benzodiazepine receptor led to apparent specific inactivation of [$^3$H]diazepam binding sites in a number of brain regions. UV irradiation times as short as 5 min resulted in extensive destruction of specific [$^3$H]diazepam binding determined with either diazepam or β-CCE as displacer, but had little or no effect on β-CCE-displaceable [$^3$H]PrCC binding. Diazepam-displaceable [$^3$H]PrCC binding declined partially after photolabeling but never decreased as much as [$^3$H]diazepam binding. We analyzed this effect in detail in cerebellum and found that photolabeling significantly decreased the number of binding sites for [$^3$H]PrCC when diazepam was used to displace it, but had only a small effect on the number of [$^3$H]PrCC sites determined with β-CCE as displacer. The apparent $K_D$ values for [$^3$H]PrCC in cerebellum changed relatively little after photolabeling.

An important point is that, if both $^3$H-ligands and both classes of displacers bound exclusively to a common site in all brain regions, photoaffinity inactivation of that site should have destroyed specific $^3$H-ligand binding equally. This was not observed in any brain region. One possible conclusion is that the two ligands do not bind to a common recognition site on the benzodiazepine receptor as suggested previously (10). Instead we propose that the benzodiazepine receptor is composed of subsets of binding sites and that β-carbolines bind to one of these subsets.

Several models for a heterogeneous receptor can be

suggested that still support the evidence that β-carbolines bind to the benzodiazepine receptor *in vitro* and *in vivo* (1–5, 10, 12, 13). The first is that binding sites for [$^3$H]PrCC and [$^3$H]diazepam overlap on the receptor. These sites are similar enough that, with a few exceptions, unlabeled benzodiazepines and β-carbolines show essentially the same rank order of potencies at displacing both ligands (Tables 2 and 3 and refs. 3, 4, 10, and 13). We suggest that these proposed overlapping sites are not completely identical or equivalent because they are not destroyed equally by photoaffinity labeling and are regulated differently.

In addition, the number of [$^3$H]PrCC binding sites in mouse cerebellum (~550 fmoles/mg of protein) represents only about 80% of the number of binding sites labeled by [$^3$H]diazepam (~700 fmoles/mg of protein). This proportion agrees with the results of Müller *et al.* (12), who have also suggested receptor heterogeneity. Similarly, Braestrup and Nielsen (11, 13) have stated that, in hippocampus, [$^3$H]PrCC labeled only 50–60% of the sites labeled by [$^3$H]flunitrazepam, and used this fact to support their belief that there are multiple binding sites for the two ligands. However, in rat cerebellum, the number of sites labeled by the two ligands is reported to be the same (13), in contrast to the present results in mice. The species difference as well as nonidentical methods of membrane preparation may account for this discrepancy.

Another possibility is that photoaffinity labeling itself causes a restructuring of the benzodiazepine receptor. In this case, one recognition site for both ligands is transformed *in vitro* into multiple recognition sites having different binding properties. First, there are a number of differences between the ligands in normal membranes, particularly in terms of binding regulation, so we do not even need to consider the photolabeling results to postulate receptor heterogeneity. Second, the results with photolabeling indicate that a differential effect on ligand binding can be obtained after as little as 5 min of UV irradiation. In this case, diazepam and β-CCE-displaceable [$^3$H]diazepam binding declined by 70–75% whereas β-CCE-displaceable [$^3$H]PrCC binding was unchanged.[2] Thus, long periods of UV irradiation, which might physically modify receptors artifactually and apparently can change the kinetics of irreversible binding (29, 30) are not necessary to demonstrate binding site heterogeneity by the photolabeling technique.

An analogous explanation for our data is that during photolabeling flunitrazepam becomes covalently linked to a site on the benzodiazepine receptor in such a way (as yet undefined) that it prevents the receptor from recognizing benzodiazepines but does not affect the way β-carbolines bind to the same site. Such a model could account for the similarities in $^3$H-ligand binding observed in normal membranes and explain the results after photolabeling. Further work is necessary to resolve these issues.

In conclusion, our results suggest that binding of [$^3$H]PrCC to mouse brain membranes is not totally identical with that observed with [$^3$H]diazepam. This may be evidence that β-carbolines bind to a subset of benzodiazepine binding sites that are in or very close to the benzodiazepine receptor-GABA receptor-chloride channel complex. The evidence for this hypothesis includes the observations that GABA can regulate [$^3$H]PrCC binding in an attenuated way before or after photolabeling, heat inactivates [$^3$H]PrCC and [$^3$H]diazepam binding equally under appropriate conditions, kainic acid destroys both binding activities equally, and pharmacological and other studies indicate such a close association. However, the proposed β-carboline recognition site(s) does not appear to be coupled to the chloride channel, its responses to $Ni^{2+}$ ion are entirely different from [$^3$H]diazepam binding, and it is spared under conditions that destroy the bulk of the specific [$^3$H]diazepam binding. On the basis of electrophysiological data, O'Brien *et al.* (3) have recently suggested that a β-carboline-specific site is associated with a benzodiazepine site and the GABA receptor, but not with the chloride channel. Such an arrangement may allow β-carboline derivatives to block the actions of benzodiazepines (2, 3, 6–8) and, in the case of β-CCE, be proconvulsant without actually causing seizures (2, 6, 7, 9). It is not known how this proposed heterogeneity of the benzodiazepine receptor relates to its endogenous ligand(s), but the recently proposed endogenous anxiogenic and anxiolytic substances (3) might interact at β-carboline and benzodiazepine sites, respectively.

## ACKNOWLEDGMENTS

We are very greatful to P. F. East for his help with the kainic acid injections. Our thanks also go to Carole Ryan for excellent secretarial assistance.

## REFERENCES

1. Braestrup, C., M. Nielsen, and C. E. Olsen. Urinary and brain β-carboline-3-carboxylates as potent inhibitors of brain benzodiazepine receptors. *Proc. Natl. Acad. Sci. U. S. A.* **77**:2288–2292 (1980).
2. Tenen, S. S., and J. D. Hirsch. β-Carboline-3-carboxylic acid ethyl ester antagonizes diazepam activity. *Nature (Lond.)* **288**:609–610 (1980).
3. O'Brien, R. A., W. Schlosser, N. M. Spirt, S. Franco, W. D. Horst, P. Polc, and E. P. Bonetti. Antagonism of benzodiazepine receptors by beta carbolines. *Life Sci.* **29**:75–82 (1981).
4. Ehlert, F. J., W. R. Roeske, and H. I. Yamamura. Multiple benzodiazepine receptors and their regulation by γ-aminobutyric acid. *Life Sci.* **29**:235–248 (1981).
5. Hirsch, J. D., and J. L. Lydigsen. Binding of β-carboline-3-carboxylic acid ethyl ester to mouse brain benzodiazepine receptors in vivo. *Eur. J. Pharmacol.* **72**:357–360 (1981).
6. Oakley, N. R., and B. J. Jones. The proconvulsant and diazepam-reversing effects of ethyl-β-carboline-3-carboxylate. *Eur. J. Pharmacol.* **68**:381–382 (1980).
7. Cowen, P. J., A. R. Green, D. J. Nutt, and I. L. Martin. Ethyl β-carboline-3-carboxylate lowers seizure threshold and antagonizes flurazepam-induced sedation in rats. *Nature (Lond.)* **290**:54–55 (1981).
8. Mitchell, R., and I. L. Martin. Ethyl β-carboline-3-carboxylate antagonizes the effect of diazepam on a functional GABA receptor. *Eur. J. Pharmacol.* **68**:513–514 (1980).
9. Cepeda, C., T. Tanaka, R. Besselievre, P. Potier, R. Naquet, and J. Rossier. Proconvulsant effects in baboons of β-carboline, a putative endogenous ligand for benzodiazepine receptors. *Neurosci. Lett.* **24**:53–57 (1981).
10. Nielsen, M., H. Schou, and C. Braestrup. [$^3$H]Propyl β-carboline-3-carboxylate binds specifically to brain benzodiazepine receptors. *J. Neurochem.* **36**:276–285 (1981).
11. Braestrup, C., and M. Nielsen. Multiple benzodiazepine receptors. *Trends Neurosci.* **3**:301–303 (1980).
12. Müller, W. E., H. O. Borbe, K. J. Fehske, and U. Wollert. Benzodiazepine receptor binding in the bovine central nervous system as demonstrated by the specific binding of [$^3$H]propyl β-carboline-3-carboxylate and of [$^3$H]flunitrazepam. *Hoppe-Seyler's Z. Physiol. Chem.* **362**:225 (1981).
13. Braestrup, C., and M. Nielsen. [$^3$H]Propyl β-carboline-3-carboxylate as a selective radioligand for the $BZ_1$ benzodiazepine receptor subclass. *J. Neurochem.* **37**:333–341 (1981).
14. Johnson, R. W., and H. I. Yamamura. Photoaffinity labeling of the benzodiazepine receptor in bovine cerebral cortex. *Life Sci.* **25**:1613–1620 (1979).

15. Mohler, H., M. K. Battersby, and J. G. Richards. Benzodiazepine receptor protein identified and visualized in brain tissue by photoaffinity label. *Proc. Natl. Acad. Sci. U. S. A.* **77**:1666–1670 (1980).
16. Braestrup, C., M. Nielsen, G. Biggio, and R. F. Squires. Neuronal localization of benzodiazepine receptors in cerebellum. *Neurosci. Lett.* **13**:219–224 (1979).
17. Itzhaki, R. F., and D. M. Gill. A micro-biuret method for estimating proteins. *Anal. Biochem.* **9**:401–410 (1964).
18. Keightley, D. D., and N. A. C. Cressie. The Woolf plot is more reliable than the Scatchard plot in analyzing data from hormone receptor assays. *J. Steroid Biochem.* **13**:1317–1323 (1980).
19. Braestrup, C., and R. F. Squires. Specific benzodiazepine receptors in rat brain characterized by high affinity [$^3$H]diazepam binding. *Proc. Natl. Acad. Sci. U. S. A.* **74**:3805–3809 (1977).
20. Mackerer, C. R., and R. L. Kochman. Effect of cations and anions on the binding of [$^3$H]diazepam to rat brain. *Proc. Soc. Exp. Biol. Med.* **158**:393–397 (1978).
21. Tallman, J. F., J. W. Thomas, and D. W. Gallager. GABAergic modulation of benzodiazepine binding site sensitivity. *Nature* (*Lond.*) **274**:383–385 (1978).
22. Karobath, M., and G. Sperk. Stimulation of benzodiazepine receptor binding by γ-aminobutyric acid. *Proc. Natl. Acad. Sci. U. S. A.* **76**:1004–1007 (1979).
23. Leeb-Lundberg, F., A. Snowman, and R. W. Olsen. Barbiturate receptor sites are coupled to benzodiazepine receptors. *Proc. Natl. Acad. Sci. U. S. A.* **77**:7468–7472 (1980).
24. Supavilai, P., and M. Karobath. In vitro modulation by avermectin $B_{1a}$ of the GABA/benzodiazepine receptor complex of rat cerebellum. *J. Neurochem.* **36**:798–803 (1981).
25. Fujimoto, M., and T. Okabayashi. Effect of picrotoxin on benzodiazepine receptors and GABA receptors with reference to the effect of $Cl^-$ ion. *Life Sci.* **28**:895–901 (1981).
26. Rodbard, D., T. Costa, and C. B. Pert. Untitled reply to Candy, J. M. and I. L. Martin. *Nature* (*Lond.*) **280**:173–174 (1979).
27. Squires, R. F. GABA receptors regulate the affinities of anions required for brain specific benzodiazepine binding, in *GABA and Benzodiazepine Receptors* (E. Costa, G. DiChiara, and G. L. Gessa, eds.). Raven Press, New York, 129–138 (1981).
28. Speth, R. C., G. J. Wastek, T. D. Reisine, and H. I. Yamamura. Benzodiazepine receptors: effect of tissue preincubation at 37°C. *Neurosci. Lett.* **13**:243–247 (1979).
29. Sieghart, W., and M. Karobath. Molecular heterogeneity of benzodiazepine receptors. *Nature* (*Lond.*) **286**:285–287 (1980).
30. Thomas, J. W., and J. F. Tallman. Photolabeling of membrane-bound and soluble benzodiazepine binding sites. *Trans. Am. Soc. Neurochem.* **12**:124 (1981).

**Send reprint requests to:** Dr. James D. Hirsch, Department of Biological Research, G. D. Searle & Company, Box 5110, Chicago, Ill. 60680.