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Photolabeling of Benzodiazepine Receptors Spares [^{3}H]Propyl β-Carboline Binding

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HIRSCH, J. D. *Photolabeling of benzodiazepine receptors spares [^{3}H]propyl β-carboline binding.* PHARMAC. BIOCHEM. BEHAV. **16**(2) 245–248, 1982.—When the benzodiazepine receptor in mouse cerebellar, striatal, and hippocampal membranes was photoaffinity labeled with nonradioactive flunitrazepam, specific [^{3}H]diazepam binding determined with either unlabeled diazepam or ethyl β-carboline-3-carboxylate (βCCE) as displacer declined ≥80%. In contrast, specific propyl β-carboline-3-carboxylate (PrCC) binding in these regions determined with βCCE as displacer was basically unaltered after photolabeling. Photolabeling lowered specific [^{3}H]PrCC binding with diazepam as displacer to an intermediate extent in the three regions. In cerebellum photolabeling had little effect on either the K_D or B_{max} for specific [^{3}H]PrCC binding determined with βCCE as displacer but significantly lowered the B_{max} for specific [^{3}H]PrCC binding determined with diazepam as displacer. These results do not support the idea that [^{3}H]PrCC and [^{3}H]diazepam have a common recognition site on the benzodiazepine receptor. Instead, they suggest that the benzodiazepine receptor is a multicomponent complex.

Benzodiazepine receptors   Photoaffinity labeling   Propyl-β-carboline-3-carboxylate   Ligand binding
Sparing from inactivation   Multicomponent receptor   Diazepam

IN 1980 Braestrup *et al.* reported the isolation of ethyl β-carboline-3-carboxylic acid (βCCE) from human urine and rat brain [1]. This compound has high affinity for the brain benzodiazepine receptor [1, 10, 12, 13, 16] and we have shown that it antagonizes the anticonvulsant effects of diazepam, lowers the convulsive dose$_{50}$ for pentylenetetrazole, and displaces [^{3}H] flunitrazepam from the benzodiazepine receptor in vivo [6,16]. The former results were recently confirmed and extended [3, 12, 13, 15]. In 1981, Nielsen *et al.* [11] reported on [^{3}H]propyl β-carboline-3-carboxylate ([^{3}H]PrCC) as a ligand for in vitro studies of the benzodiazepine receptor. Although they observed subtle differences between the binding of [^{3}H]PrCC and [^{3}H]benzodiazepines, it was suggested that both types of ligands interacted at a common site on the benzodiazepine receptor. I have studied the differences between [^{3}H]PrCC binding and that of [^{3}H]diazepam in more detail. Photoaffinity labeling of the benzodiazepine receptor with nonradioactive flunitrazepam destroyed ≥80% of the βCCE and diazepam-displaceable [^{3}H]diazepam binding in mouse cerebellar, striatal, and hippocampal membranes. However, almost all of the βCCE-displaceable [^{3}H]PrCC binding in these regions was spared after photolabeling. Diazepam also retained its ability to displace [^{3}H]PrCC after the photolabeling step, but its effect was diminished more than that of βCCE. It is difficult to reconcile these results with a single recognition site for both ligands on the benzodiazepine receptor. Several alternative models for a multicomponent receptor are proposed to explain this data.

METHOD

The [^{3}H]PrCC used in these experiments was prepared at New England Nuclear (Boston, MA) by catalytic tritiation of the unlabeled propenyl derivative provided by G.D. Searle & Co. The material had a specific activity of 48.3 Ci/mmol and after four weeks of storage in 100% ethanol at −20°C, it was 99.1% pure. This was determined by thin-layer chromatography on silica gel G plates developed with toluene-methanol-ammonium hydroxide (70:28:2, v/v). The fluorescent radioactive and standard spots were visualized with long-wave UV light. For use, [^{3}H]PrCC was diluted with 50 mM Tris-HCl buffer (pH 7.4, 4°C).

Membrane fractions used in these studies were prepared from mouse brain regions as previously described [5,11]. The buffer used for all experiments was 50 mM Tris-HCl (pH 7.4, 4°C). For the experiments shown in Table 1, aliquots (0.1 ml) of membranes (150–250 μg protein) were incubated in triplicate or quadruplicate with 0.52 nM [^{3}H]PrCC in a total volume of 0.2 ml for 60 min at 4°C. For saturation experiments (Fig. 1, Table 2) [^{3}H]PrCC was present at 0.31–6.2 nM. When used, [^{3}H]diazepam (84 Ci/mmol, New England Nuclear, Boston, MA) was present at 1.3 nM. With both [^{3}H]ligands, 10 μM diazepam and 10 μM βCCE were used separately to determine nonspecific binding. These displacer concentrations displaced both ligands maximally. However, as also indicated previously [11], benzodiazepines displaced slightly less [^{3}H]PrCC than did β-carbolines. The reverse was true when [^{3}H]diazepam was the ligand. In whole brain membranes, specific [^{3}H]diazepam binding determined with either displacer represented 93–96% of the total binding. Specific [^{3}H]PrCC binding determined with either displacer represented 78–81% of total binding. Following incubation all reactions were terminated by vacuum filtration through Whatman GF/C filters and washing with ice-cold Tris-HCl buffer.

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TABLE 1

EFFECT OF PHOTOAFFINITY LABELING OF THE BENZODIAZEPINE RECEPTOR ON SPECIFIC BINDING OF [^{3}H]DIAZEPAM AND [^{3}H]PrCC IN MOUSE BRAIN REGIONS

		Percent Decrease in Specific Binding			
		[^{3}H]Diazepam		[^{3}H]PrCC	
		A	B	C	D
Brain Region	displacer:	DZ	βCCE	DZ	βCCE
cerebellum		86 ± 1(4)	90 ± 1(4)	33 ± 1(3)	10 ± 8(3)*
striatum		81 ± 2(4)	86 ± 2(4)	22 ± 8(4)	17 ± 8(4)
hippocampus		83 ± 4(4)	87 ± 4(4)	41 ± 5(4)	14 ± 4(4)†

Preparation of membranes, photolabeling and receptor binding assays were performed as described in Method. The number of separate experiments is shown in parentheses. With [^{3}H]PrCC, the difference between diazepam (DZ) and βCCE as displacers in cerebellum and hippocampus was significant at the $^*p<0.05$ and $^\dagger p<0.01$ levels (2-tailed Student's t-test), respectively. Membranes were irradiated for 4 hr in the presence of 20 nM flunitrazepam.

Photoaffinity labeling (photolabeling) of the benzodiazepine receptor was performed as described by Johnson and Yamamura [7]. After a 20 min preincubation in the dark at 4°, membranes in Tris-HCl (pH 7.4, 4°C) buffer were irradiated for up to four hours on ice with short wave UV light emitted by an Ultraviolet Products Chromato-Vue lamp (Model CC-20, San Gabriel, CA). During the preincubation and irradiation steps, flunitrazepam was present at 20 nM. Following irradiation, membranes were washed twice in drug-free buffer to remove unbound flunitrazepam. Control membranes were treated identically but not irradiated, or they were irradiated without flunitrazepam. Since there were no differences in [^{3}H]ligand binding between the two types of control membranes, the control data reported here were obtained with flunitrazepam-treated-nonirradiated membranes. After photolabeling, total binding of [^{3}H]diazepam decreased markedly (⩾75%); total [^{3}H]PrCC binding decreased only 10 to 15%.

RESULTS

As reported elsewhere [4, 9, 11], I have also observed that [^{3}H]PrCC binding in brain membranes was saturable, of high affinity (K_D=1–2 nM) and regionally variable. In addition, it was inhibited by a large number of benzodiazepines and β-carbolines whose IC_{50}'s were very similar to those obtained with [^{3}H]diazepam as the ligand. Details of these and other experiments designed to reveal differences and similarities between the [^{3}H]ligands will be presented in a subsequent publication (J. D. Hirsch, R. L. Kochman and P. R. Sumner, in press).

In this report, I present evidence which suggests that [^{3}H]PrCC and [^{3}H]diazepam do not bind to a common site on the benzodiazepine receptor. Following photoaffinity labelling of this receptor, specific [^{3}H]diazepam binding determined in the presence of either 10 μM diazepam or 10 μM βCCE declined by ⩾80% in cerebellar, striatal and hippocampal membranes (Table 1, columns A and B). In sharp contrast, photolabeling had almost no effect (−10 to −20%) on the ability of 10 μM βCCE to displace [^{3}H]PrCC from membranes from these regions (Table 1, column D).

It was also important to determine how photolabeling of the benzodiazepine receptor affected the ability of unlabeled diazepam to displace [^{3}H]PrCC. Photolabeling had intermediate effects on displacement of [^{3}H]PrCC by diazepam in cerebellar (−33%) and hippocampal membranes (−41%). In striatal membranes the ability of diazepam to displace [^{3}H]PrCC declined to the same extent as that of βCCE (Table 1, column C). It must be made clear that essentially the same results as shown in Table 1 were obtained at 5, 15, 30 and 60 min of irradiation (not shown).

Since these results were obtained at a single concentration of [^{3}H]PrCC, saturation binding experiments were performed in cerebellar membranes to clarify the effects of photolabeling. In control and photolabeled membranes (4 hr irradiated), specific [^{3}H]PrCC binding determined with either 10 μM diazepam or 10 μM βCCE as the displacer was saturable (Fig. 1a and b). This figure also shows that the ability of βCCE to displace [^{3}H]PrCC was spared (Fig. 1b) in cerebellum compared to that of diazepam (Fig. 1a) after photolabeling confirmed the results in Table 1. Results essentially identical to these were obtained after 15 min of irradiation (unpublished observations).

The data in Fig. 1 was analyzed graphically by Woolf plots [8] and the results are shown in Table 2. There was a 68% ($p<0.01$) decrease in the B_{max} for [^{3}H]PrCC after photolabeling when diazepam was used as displacer. There was also a shift in apparent K_D toward higher affinity, but this shift was not statistically significant. In contrast, the ability of βCCE to displace [^{3}H]PrCC was relatively unaffected by photolabeling. In this case there was a nonsignificant decline (−27%) in the B_{max} for [^{3}H]PrCC and a nonsignificant shift in apparent K_D (+33%). Thus, these results confirm the data in Table 1 obtained at a single concentration of [^{3}H]PrCC. In these control cerebellar membranes (n=2 experiments), the B_{max} and apparent K_D for [^{3}H]diazepam determined with either 10 μM unlabeled βCCE or diazepam as displacers were 700±54 fmol/mg protein and 4.1±0.2 nM, respectively.

DISCUSSION

The major finding of this study was that photoaffinity labeling of the benzodiazepine receptor did not lead to equal destruction of specific [^{3}H]PrCC and [^{3}H]diazepam binding

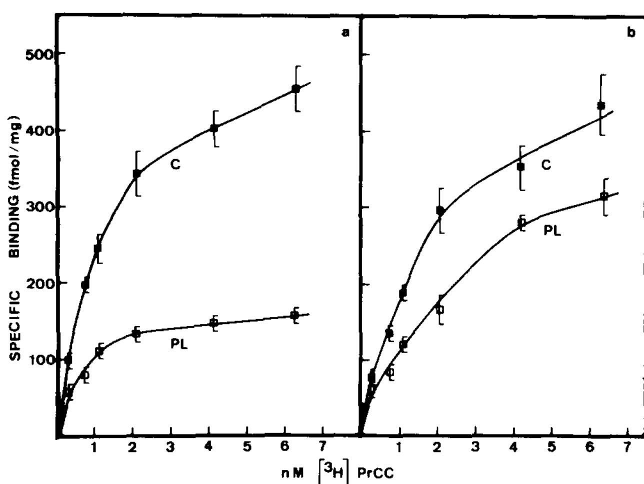

FIG. 1. Saturable specific [^{3}H]PrCC binding in control and photolabeled cerebellar membranes using diazepam and βCCE as displacers. Membranes from frozen mouse cerebella were prepared and photolabeled as described in Method. Binding assays were performed with [^{3}H]PrCC over the concentration range of 0.31–6.2 nM in the presence of either 10 μM diazepam or 10 μM βCCE for determination of nonspecific binding as described in Method. The values are mean±SEM from three separate experiments performed with three different membrane preparations. The K_D and B_{max} values are shown in Table 2. Fig. 1a: [^{3}H]PrCC specific binding using 10 μM diazepam as displacer. Fig. 1b: [^{3}H]PrCC specific binding using 10 μM βCCE as displacer. C=control; PL=photolabeled.

TABLE 2

EFFECT OF PHOTOAFFINITY LABELING OF THE BENZODIAZEPINE RECEPTOR ON SATURABLE BINDING OF [^{3}H]PrCC IN CEREBELLAR MEMBRANES

Displacer	Control	Photolabeled
Diazepam	K_D= 1.0 ± 0.2	K_D= 0.6 ± 0.1
	B_{max}=533 ± 64	B_{max}=171 ± 19*
βCCE	K_D= 1.8 ± 0.3	K_D= 2.4 ± 0.6
	B_{max}=574 ± 86	B_{max}=417 ± 79

Saturation binding data yielding the values shown here are found in Fig. 1 (n=3 separate experiments). These data were derived from Woolf plots constructed using linear regression analysis. The mean correlation coefficient ± SEM for all 12 Woolf plots was 0.98 ±0.01. K_D values are nM and B_{max} values are fmol/mg protein. The decrease in B_{max} (−68%) derived with 10 μM diazepam as displacer was significant at *$p<0.01$ (2-tailed Student's t-test).

in mouse brain membranes (see Table 1). If both [^{3}H] ligands and both classes of displacers bound solely to a common site on the benzodiazepine receptor, then photolabeling that site should result in similar decreases in specific [^{3}H] ligand binding no matter what the ligand or displacer combinations were. Since this was not observed, the present results do not support the suggestion made by Nielsen *et al.* [11] that the two [^{3}H] ligands bind to a common recognition site on the benzodiazepine receptor. These results do support their hypothesis that a "β-carboline" site may exist on this receptor. Interestingly, Ehlert *et al.* [4], using [^{3}H]PrCC as ligand, have demonstrated apparent heterogeneity of the benzodiazepine receptor as well. Additional data is available demonstrating several considerable differences between [^{3}H]PrCC and [^{3}H]diazepam binding (J. D. Hirsch, R. L. Kochman and P. R. Sumner, in press).

If we assume that the benzodiazepine receptor is a multicomponent complex, several models can be proposed to explain the present results and still support the convincing body of evidence that β-carbolines bind to the benzodiazepine receptor. The first is that binding sites for [^{3}H]PrCC and [^{3}H]diazepam overlap on the benzodiazepine receptor. These sites are similar enough pharmacologically that, with few exceptions, unlabeled β-carbolines and benzodiazepines are essentially equipotent at displacing both ligands ([4, 9, 11, 13] and J. D. Hirsch *et al.*, in press). However, these proposed multiple sites are not identical because they are not destroyed equally by photoaffinity labeling.

Another model consistent with the present data and possibly that of Ehlert *et al.* [4] proposes the existence of a β-carboline site and a benzodiazepine site linked by or closely associated with a "hybrid" site with overlapping specificity. Based on the data in Table 1, the benzodiazepine site (which recognizes diazepam and βCCE equally) is destroyed by photolabeling. However, it is suggested that the β-carboline site recognizing βCCE is not destroyed and the β-carboline site recognizing diazepam is only partially inactivated by photoaffinity labeling. An examination of Table 2 tends to support this proposal. Regardless of which displacer was used, the B_{max} for [^{3}H]PrCC in cerebellum was the same (~550 fmol/mg protein). After photolabeling, the number of [^{3}H]PrCC sites binding diazepam decreased to 170 fmol/mg protein. This value may represent the number of "hybrid" sites. It is important to notice that this site did not decline to the extent we would predict (~90% destroyed) if it were the same as the [^{3}H] diazepam site determined with βCCE as displacer (see Table 1). If we add the number of sites labeled by [^{3}H]PrCC determined with either displacer (~550) to the number of proposed overlapping "hybrid" sites (170), we obtain a value of 720 which is essentially identical to the total number of sites labeled by [^{3}H]diazepam in cerebellum (see Results). In this case, the [^{3}H]PrCC sites in cerebellum represent about 80% of the [^{3}H]diazepam sites. This proportion of sites is in line with the results of Müller *et al.* [9].

A third possibility is that photolabeling itself causes restructuring of the benzodiazepine receptor. In this model, one recognition site for both ligands is transformed in vitro into multiple recognition sites having different binding properties. This transformation is incomplete, even after 4 hr of UV, accounting for the partial preservation of diazepam's ability to displace [^{3}H]PrCC after photolabeling (Table 1, column C). A restructuring model cannot, however, explain why the ability of both diazepam and βCCE to displace [^{3}H]diazepam declines identically in each tissue shown (Table 1, columns A and B) as well as others (J. D. Hirsch *et al.*, in press). Also, such a model might predict that the ability of βCCE and diazepam to displace both [^{3}H] ligands would change with irradiation time. As mentioned, this does not occur.

The present data combined with that of Ehlert *et al.* [4] do not suggest a common recognition site for both ligands on the same benzodiazepine receptor entity. In fact, Nielsen *et al.* [11] postulate the presence of a second binding site ("β-carboline site") in their paper and Müller *et al.* [9] suggest that [^{3}H]PrCC labels a subclass of benzodiazepine receptor. Thus, further experimentation is necessary to determine which of the models discussed here (or others that could be formulated) represents the relationship of [^{3}H]PrCC and [^{3}H]diazepam binding to the benzodiazepine receptor. Experiments involving differential solubilization of [^{3}H] diazepam and [^{3}H]PrCC sites or autoradiography with [^{3}H]PrCC after photoaffinity labeling with nonradioactive flunitrazepam [14, 17, 18] may provide support for one of the models presented or suggest a new approach. In view of the fact that βCCE [3, 12, 16] and other β-carbolines [13,15] antagonize a number of actions of benzodiazepines, a detailed resolution of β-carboline interactions with the benzodiazepine receptor is needed.

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