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Thermodynamic Changes Associated with Benzodiazepine and Alkyl β-Carboline-3-carboxylate Binding to Rat Brain Homogenates

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SUMMARY

The temperature dependence of binding to the benzodiazepine receptor for several benzodiazepines and their antagonists was investigated. For all compounds, binding to crude membranes from a whole rat brain homogenate was an entropy-driven process between 0° and 30°. With the exceptions of desmethylmedazepam and triazolam, binding affinities for all compounds decreased as incubation temperature increased. In another series of experiments the binding of [^{3}H]diazepam to a thrice-washed P_2 homogenate was also entropy-driven. However, the driving force for [^{3}H]diazepam binding was shifted at around 20° from entropic to enthalpic by the presence of 10 μM γ-aminobutyric acid or 5 mM nickel chloride in the incubation media. This change could be antagonized by ethyl and propyl β-carboline-3-carboxylate. Neither γ-aminobutyric acid nor nickel chloride induced a change from the entropic driving force for [^{3}H]propyl β-carboline-3-carboxylate binding to the same membranes. γ-Aminobutyric acid (10 μM) also did not significantly affect the K_D or B_{max} for [^{3}H]propyl β-carboline-3-carboxylate binding. However, in contrast to its enhancement of [^{3}H]diazepam binding, nickel chloride (0.1 mM) inhibited [^{3}H]propyl β-carboline-3-carboxylate binding, raising the K_D and B_{max} approximately 5-fold and 3-fold, respectively. The results of these studies indicate that in washed membranes the thermodynamic parameters for diazepam binding to its receptor can be modulated by agents such as nickel and γ-aminobutyric acid, and that these transitions can be reversed by the benzodiazepine antagonists ethyl and propyl β-carboline-3-carboxylate.

INTRODUCTION

The approach and binding of a molecule to a receptor are governed by certain attractions and repulsions between the two. These interactions, whether bonded or nonbonded in nature, produce a change in free energy. Binding of the molecule to the receptor may initiate a series of events that could include transduction of a signal to an effector, or a conformational change of the receptor resulting in changed translational and rotational freedom of the molecule-receptor complex. Application of the laws of thermodynamics together with equilibrium receptor binding studies can be useful in evaluating the relationship between a drug molecule and receptor. This approach has been fruitful in understanding *beta*-adrenergic (1), cholinergic (2), glucocorticoid (3), insulin (4), and glutamate (5) receptor binding.

We were interested in investigating the potential utility of the thermodynamics of binding to distinguish compounds that bind to the benzodiazepine receptor *in vitro* but antagonize the effects of the benzodiazepines *in vivo*, such as β-CCE[1] (6). Therefore we determined the thermodynamics of receptor binding for several benzodiazepines; the triazolobenzodiazepines alprazolam and triazolam; zopiclone, a non-benzodiazepine which inhibits [^{3}H]DZ binding *in vitro* and *in vivo* and has central effects like the benzodiazepines (7); and β-CCE and β-PrCC, which bind to the benzodiazepine receptor with high affinity (8, 9).

Since the benzodiazepine receptor has been linked to the GABA receptor (10) and is influenced by divalent cations such as nickel (11), we decided to investigate also the effects that GABA and $NiCl_2$ have on binding thermodynamics.

MATERIALS AND METHODS

Tissue preparation. Male CD/CR rats (Charles River Breeding Laboratories, Wilmington, Mass.), 8–12 weeks old, were decapitated. The whole brain plus attached cervical spinal cord was homogenized in 71 volumes of 50 mM sodium-potassium phosphate buffer (pH 7.4, 0°) containing 0.2 M NaCl, using a Potter-Elvehjem glass homogenizer equipped with a motor-driven Teflon pestle, and centrifuged at 1,000 × *g* for 10 min to obtain the S_1 supernatant. For some experiments, brains were homogenized with the Potter-Elvehjem in 40 volumes of 50 mM

[1] The abbreviations used are: β-CCE, ethyl β-carboline-3-carboxylate; DZ, diazepam; β-PrCC, propyl β-carboline-3-carboxylate; GABA, γ-aminobutyric acid.

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Tris-HCl (pH 7.4, 0°), and the S_1 supernatant was pelleted three times at 30,000 × *g* for 10 min, with intermediate resuspensions in 40 volumes of the Tris buffer. The final pellet was resuspended in the original 40 volumes of buffer.

Binding assays. Sample tubes containing approximately 0.23–0.47 mg of homogenate protein, six concentrations of each test compound, and one of the buffers were preincubated at 0°, 10°, 20°, or 30° for 15 min; in some cases tubes also contained 5 mM $NiCl_2$ or 10 μM GABA. The binding reaction was initiated by adding [^{3}H]DZ or [^{3}H]PrCC to all samples, resulting in a final volume of 2 ml, and continuing the incubation at the respective temperature for an additional 30 min. The reaction was terminated by vacuum filtration through Whatman GF/C filter papers, and radioactivity was determined as previously described (12). Unlabeled DZ was used to determine nonspecific binding for [^{3}H]DZ, and unlabeled β-PrCC was used for [^{3}H]PrCC binding studies. Additional details of the binding assays are given in the legends to the tables and figures.

Buffers were prepared to be pH 7.4 at the respective temperature used for the binding assay. Homogenate protein concentration was determined by a biuret method (13), with correction for Tris buffer interference.

Stability of ligands. [^{3}H]DZ is not metabolized or degraded during incubation at 30° with rat brain membranes for up to 40 min (14).

To establish the stability of [^{3}H]PrCC, thrice-washed membranes were prepared as described above and incubated with 0.4 and 12.8 nM [^{3}H]PrCC in 50 mM Tris-HCl (total volume 2 ml) at 30° for 30 min. The reaction was terminated by the addition of 30 ml of ethyl acetate. Controls received 30 ml of ethyl acetate before the addition of the [^{3}H]PrCC to represent zero time. The pH of each sample was adjusted by the addition of one drop of 6 N NaOH; the sample was shaken vigorously and allowed to settle for 1 hr. One-milliliter aliquots from each sample were evaporated under nitrogen and reconstituted in 100 μl of methanol. These samples were analyzed by thin-layer chromatography on glass plates coated with 250 μm of silica gel GF. The plates were developed in a dichloromethane:methanol (9:1, v/v) solvent system, then air-dried. Three-centimeter sections were scraped from the plates, dissolved in scintillation fluid, and counted as described under Materials and Methods. Radiochromatograms indicated single peaks with identical R_F values and amplitudes for controls and incubated samples at both concentrations of [^{3}H]PrCC. Thus, [^{3}H]PrCC was not metabolized or degraded by a whole rat brain homogenate under incubation conditions identical with the binding assays described above.

Binding and thermodynamic calculations. Apparent equilibrium dissociation constants (K_D) and maximal binding capacities (B_{max}) for [^{3}H]DZ and [^{3}H]PrCC at each temperature were calculated from Woolf plot analyses of the saturation isotherms (15). The concentration of test compound that inhibited 50% of [^{3}H]DZ specific binding (IC_{50}) was determined by log-logit analysis, and was converted to a corresponding K_I value (16).

The thermodynamic binding parameters were calculated by using a classical thermodynamic formalism (17). The change in the standard Gibbs energy ($\Delta G°$) was calculated using Eq. 1:

$$\Delta G° = -RT \ln K_A \quad (1)$$

where R is the gas constant (1.99 cal/mole Kelvin),[2] T is the temperature in Kelvin units, and K_A is the apparent equilibrium association constant ($1/K_D$ or $1/K_I$) for the compound at T. For these experiments, $\Delta G°$ was calculated at T = 273 K (0°) unless otherwise indicated. To determine the average enthalpy change ($\Delta H°$) for binding, application of the van't Hoff equation to the temperature variation of the equilibrium constant data was used. A van't Hoff plot of ln K_A versus $1/T$ yields a slope for each compound which is equal to $-\Delta H°/R$. The entropy change ($\Delta S°$) can then be calculated directly from Eq. 2:

$$\Delta S° = (\Delta H° - \Delta G°)/T \quad (2)$$

Materials. [*methyl*-^{3}H]DZ (87.6 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, Mass.). [*propyl*-^{3}H]β-Carboline-3-carboxylate (48.3 Ci/mmole) was prepared by New England Nuclear Corporation by catalytic tritiation of the propenyl derivative provided by G. D. Searle & Company. Nickel chloride hexahydrate and γ-amino-*n*-butyric acid were purchased commercially. Chlordiazepoxide, clonazepam, diazepam, and flunitrazepam were donated by Hoffmann-La Roche, and zopiclone was donated by Rhône-Poulenc. All other compounds were synthesized by members of the Medicinal Chemistry Department of G. D. Searle & Company.

RESULTS

Thermodynamic changes in the crude membranes. Analysis of the binding saturation isotherms for [^{3}H]DZ and [^{3}H]PrCC in crude membranes (S_1) indicated that the apparent K_D for [^{3}H]DZ increased approximately 5-fold as the incubation temperature was raised to 30°, without any significant change in B_{max} (Table 1), which agrees with previous reports (18, 19). The apparent K_D for [^{3}H]PrCC also increased, whereas the B_{max} decreased 28% ($p < 0.01$) as the incubation temperature was raised. Williams *et al.* (20) recently reported increased K_D values for [^{3}H]β-CCE with increases in temperature. Both [^{3}H]DZ and [^{3}H]PrCC had similar enthalpy and entropy changes, and the binding for both was entropy-driven.

The reciprocals of the K_I values obtained for the displacement of 4 nM [^{3}H]DZ by various compounds at each temperature were used in Eq. 1 to calculate the Gibbs energy change and in van't Hoff plots of ln K_A versus $1/T$; representative van't Hoff plots are shown in Fig. 1. The affinities for two compounds increased with increasing incubation temperature. The K_I for triazolam, for example, decreased from 0.67 nM at 0° to 0.13 nM at 30°; desmethylmedazepam had a slightly lower K_I at 30° than at 0°. The enhanced binding of the two compounds at higher temperatures is reflected in their positive $\Delta H°$ values (Table 2), indicating that their binding to the receptor is an endothermic process; for all other compounds, binding was exothermic (negative enthalpy

[2] One calorie = 4.184 J.

TABLE 1

Effect of temperature on [^{3}H]DZ and [^{3}H]PrCC binding and binding thermodynamics

[^{3}H]DZ (1–32 nM) in the absence and presence of 3.2 μM unlabeled DZ, or 0.2–6.4 nM [^{3}H]PrCC in the absence and presence of 640 nM β-PrCC, was incubated in triplicate with 0.4 mg of protein from a 1000 × *g* supernatant of whole rat brain homogenate and 50 mM (Na_2HPO_4-KH_2PO_4) + 0.2 M NaCl buffer (pH 7.4) in a total volume of 2 ml for 30 min at 0°, 10°, 20°, and 30°. Values in parentheses represent 95% confidence limits. The changes in standard Gibbs energy (Δ*G*°), enthalpy (Δ*H*°), and entrophy (Δ*S*°) were calculated as described in the text.

Temperature	[^{3}H]DZ		[^{3}H]PrCC	
	K_D	B_{max}	K_D	B_{max}
	nM	*fmoles/mg protein*	*nM*	*fmoles/mg protein*
0°	2.0	521(494–552)	0.3	493(461–531)
10	3.4	493(428–580)	0.5	449(354–614)
20	4.9	511(487–538)	1.0	415(290–727)
30	9.4	482(356–746)	1.3	353(320–394)[a]
Δ*G*° (kcal/mole)		−10.9		−11.9
Δ*H*° (kcal/mole)		−8.2		−8.3
Δ*S*° (cal/mole Kelvin)		9.8		13.3

[a] Significantly different from B_{max} at 0° ($p < 0.01$, two-tailed Student's *t*-test).

changes). Whether binding was characterized by a gain or release of energy, the driving force for binding in all cases was entropic, with Δ*S*° values ranging from 5.0 to 72.9 cal/mole Kelvin.

Thermodynamic changes in washed membranes and modulation by GABA and $NiCl_2$. Binding to membranes that had been washed three times was similar to that obtained using crude membranes, except that affinities were slightly lower (compare Tables 3 and 4 with Tables 1 and 2). In all cases, affinity decreased as incubation temperature was raised from 0° to 30°. The thermodynamic changes associated with binding were also similar to those obtained using crude membranes.

The addition of GABA or $NiCl_2$ to the incubation media, however, produced dramatic effects in some cases. As previously reported (10, 11) the apparent affinity of the receptor for [^{3}H]DZ was enhanced by 10 μM GABA (21%) and by 5 mM $NiCl_2$ (40%) at 0°, without significantly changing the B_{max} for [^{3}H]DZ (Table 3). However, as Fig. 2 illustrates, there is a break in the van't Hoff plots for [^{3}H]DZ at about 20° when GABA or $NiCl_2$ is present. Similar plots were obtained for unlabeled DZ. At 30°, the enhancement of [^{3}H]DZ binding by GABA and $NiCl_2$ is significantly reduced. Between 20° and 30°, the driving force for binding changes from entropic to enthalpic and the unfavorable loss in entropy (−18 to −32 cal/mole Kelvin) is compensated for by a decrease in enthalpy (−5 to −9 kcal/mole), permitting the binding reaction to occur. The enthalpy and entropy changes for unlabeled diazepam (not shown) are similar to those for [^{3}H]DZ.

GABA had no significant effect on [^{3}H]PrCC binding between 0° and 30° (Fig. 3), in agreement with other reports for [^{3}H]PrCC (21) and [^{3}H]β-CCE (22). Nickel

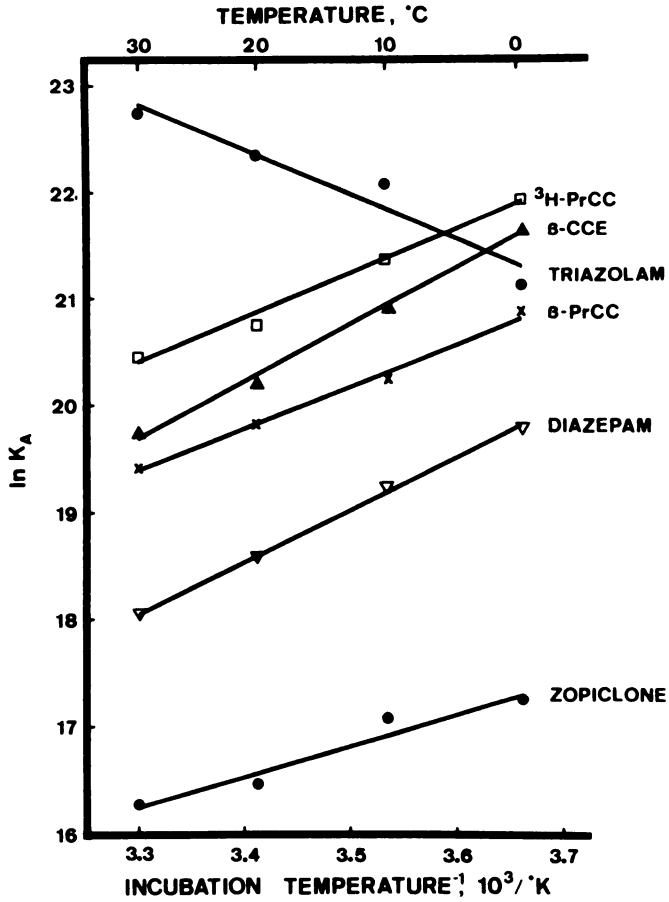

FIG. 1. *Representative van't Hoff plots for the effect of temperature on equilibrium association constants*

Six concentrations of each compound were incubated with 4 nM [^{3}H]DZ in the absence and presence of 4 μM diazepam at 0°, 10°, 20°, and 30° as described under Materials and Methods. [^{3}H]PrCC (0.2–6.4 nM) was incubated in the absence and presence of 640 nM β-PrCC. Equilibrium association constants (K_A) were calculated as described under Materials and Methods.

TABLE 2

Thermodynamic parameters for compounds displacing [^{3}H]DZ binding

[^{3}H]DZ (4 mM) was incubated in triplicate with 0.4 mg of protein from a 1000 × *g* supernatant of whole rat brain homogenate, six concentrations of each test compound, and 50 mM (Na_2HPO_4-KH_2PO_4) + 0.2 M NaCl buffer (pH 7.4) in a total volume of 2 ml for 30 min at 0°, 10°, 20°, and 30°. Nonspecific binding was determined in the presence of 4 μM diazepam.

	Δ*G*°	Δ*H*°	Δ*S*°	K_I at 0°
	kcal/mole	*kcal/mole*	*cal/mole Kelvin*	*nM*
Alprazolam	−11.2	−7.7	12.9	1.1
Chlordiazepoxide	−8.6	−5.3	11.8	144
Clonazepam	−11.9	−7.5	16.3	0.28
Diazepam	−10.7	−8.4	8.4	2.7
Flunitrazepam	−11.3	−8.6	9.9	0.3
Desmethyldiazepam	−6.9	5.6	45.8	3372
Triazolam	−11.5	8.4	72.9	0.67
Zopiclone	−9.4	−6.0	12.4	31.0
β-CCE	−11.8	−10.4	5.0	0.53
β-PrCC	−11.3	−7.9	12.7	0.88

TABLE 3

[^{3}H]DZ binding and thermodynamic parameters in the absence and presence of GABA and $NiCl_2$

For saturation binding studies, 1–32 nM [^{3}H]DZ ± 3.2 μM unlabeled DZ was incubated with 0.23–0.47 mg of protein from whole rat brain homogenate that had been centrifuged three times at 30,000 × *g* (see Materials and Methods) and 50 mM Tris-HCl buffer (pH 7.4) in a final volume of 2 ml for 30 min at 0°, 10°, 20°, 25°, and 30°. Values represent the means ± standard error of the mean for three or four experiments.

Temperature	K_D			B_{max}		
	No addition	+10 μM GABA	+5 mM $NiCl_2$	No addition	+ 10 μM GABA	+ 5 mM $NiCl_2$
	nM			*fmoles/mg protein*		
0°	4.7 ± 0.2	3.7 ± 0.1	2.8 ± 0.3	1174 ± 183	1391 ± 147	1333 ± 210
10	8.7 ± 0.3	5.3 ± 0.6	4.2 ± 0.5	1498 ± 272	1698 ± 322	1522 ± 275
20	11.6 ± 1.8	6.6 ± 0.8	6.4 ± 0.4	1554 ± 408	1577 ± 386	1457 ± 312
25	16.2 ± 2.6	10.5 ± 0.7	11.6 ± 1.6	1085 ± 91	1132 ± 65	1204 ± 86
30	19.6 ± 0.5	14.8 ± 1.2	13.2 ± 1.1	1334 ± 86	1369 ± 190	1218 ± 138

Addition		$\Delta G°$	$\Delta H°$	$\Delta S°$
		kcal/mole	*kcal/mole*	*cal/mole Kelvin*
No addition:	0°–30°	−10.4	−7.6	10.4
+10 μM GABA:	0°–20°	−10.6	−4.7	21.3
	20°–30°	−11.0	−14.0	−10.2
+5 mM $NiCl_2$:	0°–20°	−10.7	−7.3	12.4
	20°–30°	−11.0	−12.7	−5.8

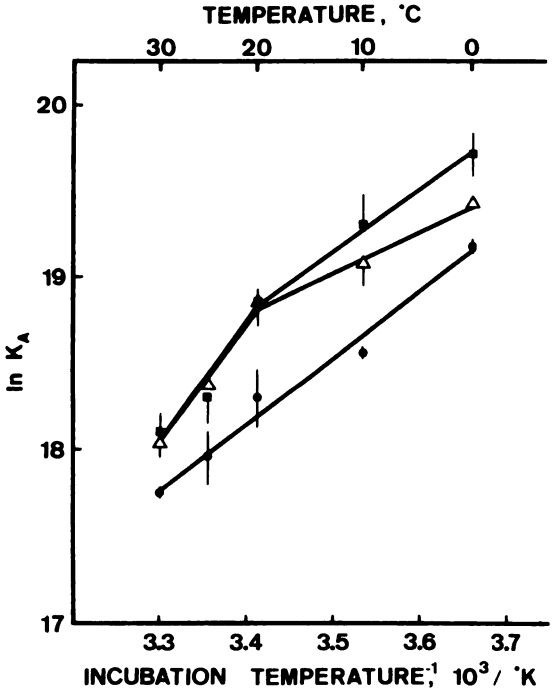

FIG. 2. *van't Hoff plots for the effect of temperature on [^{3}H]DZ binding in the absence and presence of GABA and $NiCl_2$*

[^{3}H]DZ (1–32 nM) was incubated at 0°–30° with thrice-washed whole brain membranes in the absence and presence of 3.2 μM diazepam. K_A values were calculated as described under Materials and Methods. ●, Binding in the absence of added regulatory factors; △, binding in the presence of 10 μM GABA; ■, binding in the presence of 5 mM $NiCl_2$. Points represent the means ± standard error of the mean of three to four different experiments, each performed in triplicate. The standard error of the mean of points with no error bars indicated were less than the size of the symbols.

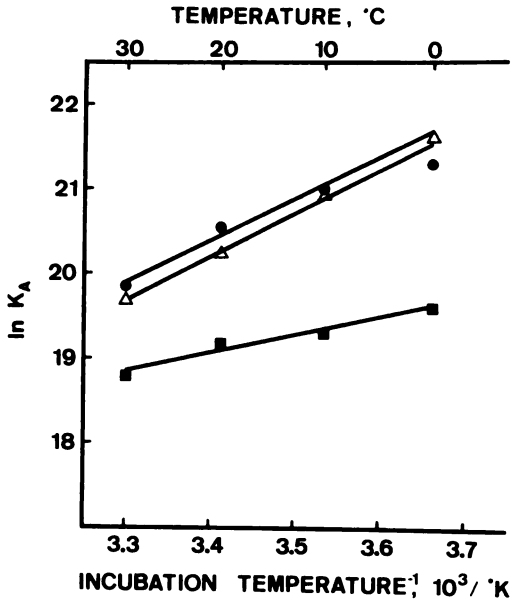

FIG. 3. *van't Hoff plots for the effect of temperature on [^{3}H]PrCC binding in the absence and presence of GABA and $NiCl_2$*

[^{3}H]PrCC (0.4–12.8 nM) was incubated at 0°–30° with thrice-washed whole brain membranes in the absence and presence of 1.28 μM β-PrCC. K_A values were calculated as described under Materials and Methods. ●, Binding in the absence of added regulatory factors; △, binding in the presence of 10 μM GABA; ■, binding in the presence of 0.1 mM $NiCl_2$.

chloride (5 mM) severely disrupted [^{3}H]PrCC binding by increasing nonspecific binding more than 400%. The highest concentration of $NiCl_2$ tested that did not disrupt binding, 0.1 mM, caused a 3- to 5-fold loss in apparent affinity for [^{3}H]PrCC while increasing B_{max} from approximately 1200 fmoles/mg of protein to 3300 fmoles/mg of protein (data not shown). The 5.8 kcal/mole increase in enthalpy in the presence of Ni^{2+} (Table 4) may reflect a weakening of the ligand-receptor complex, resulting in the 4-fold increase in entropy.

The displacement of 4 nM [^{3}H]DZ binding by β-PrCC and β-CCE was unaffected by 10 μM GABA (Table 4). The K_I values for both compounds were the same in the absence or presence of GABA. Another report (23) indi-

cates a similar effect on the displacement of [^{3}H]flunitrazepam by β-PrCC. Enthalpy and entropy changes were little affected by 10 μM GABA. Nickel chloride, on the other hand, raised the K_I for β-PrCC 7-fold and the K_I for β-CCE 5-fold. Although enthalpy and entropy for β-PrCC were changed little by Ni^{2+}, the gain in enthalpy by β-CCE was compensated for by a 17 cal/mole Kelvin increase in entropy.

Figure 4 is the van't Hoff plot for the displacement of 4 nM [^{3}H]DZ binding by β-PrCC. It is important to note

TABLE 4

Thermodynamic parameters for the binding of β-carbolines in the absence and presence of GABA and $NiCl_2$

For saturation binding studies, 0.4–12.8 nM [^{3}H]PrCC ± 1.28 μM β-PrCC was incubated in triplicate with 0.23–0.47 mg of protein from whole rat brain homogenate that had been centrifuged three times at 30,000 × *g* (see Materials and Methods) and 50 mM Tris HCl buffer (pH 7.4) in a final volume of 2 ml for 30 min at 0°, 10°, 20°, and 30°. For studies of the displacement of [^{3}H]DZ binding, six concentrations of each test compound were incubated in triplicate with 4 nM [^{3}H]DZ and other media components as described above; 4μM DZ was used to determine nonspecific binding.

	ΔG°	ΔH°	ΔS°	K_D or K_I at 0°
	kcal/mole	*kcal/mole*	*cal/mole Kelvin*	*nM*
[^{3}H]PrCC	−11.6	−9.9	6.2	0.6
+ 10 μM GABA	−11.7	−10.6	4.2	0.4
+ 0.1 mM $NiCl_2$	−10.6	−4.1	23.9	3.1
β-PrCC	−11.1	−6.2	17.8	1.4
+ 10 μM GABA	−11.2	−6.8	16.1	1.2
+ 5 mM $NiCl_2$	−10.2	−6.2	14.7	9.9
β-CCE	−11.4	−9.6	6.6	0.7
+ 10 μM GABA	−11.4	−8.9	9.0	0.8
+ 5 mM $NiCl_2$	−10.6	−4.1	23.7	3.3

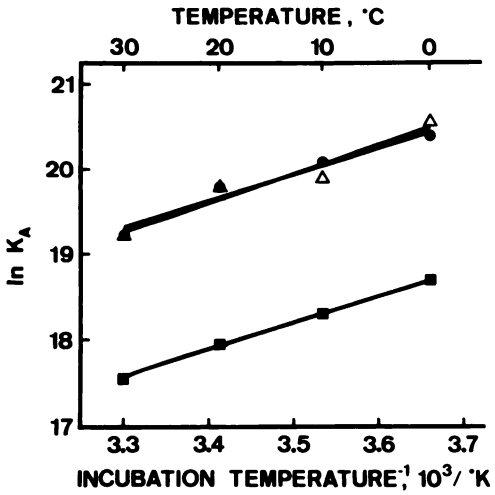

FIG. 4. *van't Hoff plots for the effect of temperature on the displacement of 4 nM [^{3}H]DZ by β-PrCC in the absence and presence of GABA and $NiCl_2$*

β-PrCC (0.3–100 nM) was incubated at 0°–30° with 4 nM [^{3}H]DZ as described in Table 4. K_A values were calculated from K_I values as described in the text. ●, β-PrCC binding in the absence of added regulatory factors; △, binding in the presence of 10 μM GABA; ■, binding in the presence of 5 mM $NiCl_2$. Points represent the means of three experiments, each performed in triplicate.

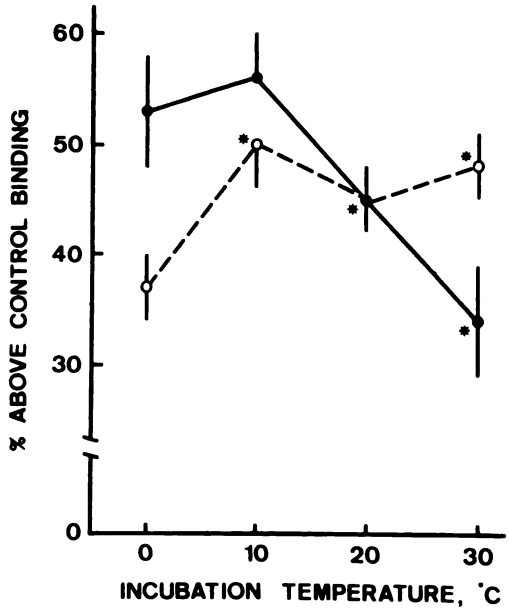

FIG. 5. *Temperature dependence of the enhancement of 4 nM [^{3}H]DZ binding by 10 μM GABA and 5 mM $NiCl_2$*

○, Binding in the presence of 10 μM GABA; ●, binding in the presence of 5 mM $NiCl_2$. Points represent the means ± standard error of the mean of 7–11 experiments. Asterisks indicate significant difference from value at 0° ($p < 0.025$, Student's *t*-test).

that, even in the presence of 10 μM GABA or 5 mM $NiCl_2$, there are no abrupt transitions in the plots, in contrast to the van't Hoff plots for [^{3}H]DZ in Fig. 2. The van't Hoff plots for the displacement of [^{3}H]DZ by β-CCE (data not shown) were also unbroken.

Effect of temperature on GABA and Ni^{2+} modulation. The enhancement of 4 nM [^{3}H]DZ binding by 10 μM GABA increases with increasing temperature (Fig. 5), and appears constant above 10°. Increased GABA enhancement of benzodiazepine binding with increasing temperature has been reported by others (18, 24). It should be noted that this effect is on only a single, low concentration of [^{3}H]DZ and should be contrasted with the effect of GABA on the [^{3}H]DZ equilibrium binding constants illustrated in Fig. 2. The Ni^{2+} stimulation of 4 nM [^{3}H]DZ binding decreased with increasing temperature, dropping from 53% at 0° to 34% at 30°. The inhibitory effect of Ni^{2+} on [^{3}H]PrCC binding is also reduced as temperature is increased (see Fig. 3). Although similar findings have been reported for chloride ion (150 mM NaCl) (24), nickel apparently does not interact with the chloride ion channel, since its effects could not be blocked by picrotoxinin or 4,4-diisothiocyanostilbene-2,2′-disulfonic acid (25).

DISCUSSION

The purposes of these experiments were to characterize the thermodynamic parameters of binding for various compounds to the benzodiazepine receptor and to determine whether this type of biophysical analysis would be useful in identifying compounds that antagonize the benzodiazepines.

We found that binding for all compounds, in the absence of added modulators, was entropy-driven, with ΔS°

values ranging from 5 to 73 cal/mole Kelvin in crude whole rat brain membranes (Table 2) and from 6 to 18 cal/mole Kelvin in thrice-washed membranes (Table 4). For those compounds (desmethylmedazepam and triazolam) whose binding was endothermic and whose affinities increased with increasing temperatures, favorable energy changes ($\Delta G°$) were maintained because of large favorable entropy changes. In the absence of added regulatory factors, however, the benzodiazepine antagonists β-PrCC and β-CCE could not be distinguished from other compounds on the basis of the signs or magnitudes of their enthalpy or entropy changes.

GABA (10 μM) and $NiCl_2$ (5 mM) had differential effects on the binding of [^{3}H]DZ and the β-carbolines to thrice-washed membranes. GABA enhanced the affinity of the receptor for [^{3}H]DZ between 0° and 30° (Table 3), although the stimulation at 30° was less than that at lower temperatures. On the other hand, GABA had no significant effect on either the binding of [^{3}H]PrCC or the displacement of [^{3}H]DZ binding by β-PrCC or β-CCE (Table 4). Like GABA, Ni^{2+} enhanced the affinity of the receptor for [^{3}H]DZ, but the stimulation at 30° was less than that at lower temperatures. In contrast, 5 mM $NiCl_2$ inhibited the displacement of [^{3}H]DZ binding by β-PrCC and β-CCE and raised nonspecific binding for [^{3}H]PrCC by over 400%. The highest concentration of $NiCl_2$ that could be used without significantly affecting [^{3}H]PrCC nonspecific binding was 0.1 mM. At this concentration, Ni^{2+} increased the K_D and B_{max} for [^{3}H]PrCC binding at 0° by 5-fold and 3-fold, respectively. These differences between the sensitivities of [^{3}H]DZ and the β-carbolines to GABA and Ni^{2+} modulation may reflect binding to different subclasses of the benzodiazepine, as has been suggested (21, 25).

GABA and Ni^{2+} also significantly affected the thermodynamic parameters of binding. Between 0° and 20° both modulators induced a higher affinity state for the receptor; enthalpy and entropy increased for [^{3}H]DZ binding (Table 3), and binding was entropy-driven. At around 20°, however, there were abrupt transitions in the [^{3}H]DZ van't Hoff plots when GABA or Ni^{2+} was present (Fig. 2). There was a loss of entropy of 18–32 cal/mole Kelvin which was compensated for by a change in enthalpy of −5 to −9 kcal/mole, and the driving force for binding changed to enthalpic. Whether these transitions to a more stabilized state represent membrane lipid phase shifts (26), conformational changes resulting from a direct effect on the receptor, or a reversible coupling/uncoupling of benzodiazepine and GABA receptors (27), or perhaps generation of a signal through phosphorylation (28) or methylation, is unknown.

It is important to note that there were no transitions in the van't Hoff plots for the displacement of [^{3}H]DZ by β-PrCC (Fig. 4) or β-CCE (data not shown), even when GABA and Ni^{2+} were present. This antagonism of the [^{3}H]DZ transitions suggests that the binding of these β-carbolines "locks" the receptor into a form that is neither activated by the modulators nor able to undergo transitions. Under all conditions, binding remained entropy-driven. However, preliminary observations indicate that not all classes of benzodiazepine antagonists have the same sensitivities to GABA and Ni^{2+} modulation, although they do antagonize the transitions in [^{3}H]DZ binding.[3]

The effects of GABA and Ni^{2+} on the thermodynamic parameters of [^{3}H]PrCC binding were similar to those for β-PrCC and β-CCE. Although 0.1 mM $NiCl_2$ destabilized binding (entropy increased by 18 cal/mole Kelvin), this effect was compensated for by the 6 kcal/mole enthalpy change (Table 4). GABA only slightly decreased enthalpy (−0.7 kcal/mole) and entropy (−2 cal/mole Kelvin). The van't Hoff plots for [^{3}H]PrCC binding (Fig. 3) were linear at temperatures up to 30° in the absence or presence of the modulators. Whether [^{3}H]PrCC binds to the benzodiazepine receptor and/or to a subclass of that receptor, its binding apparently blocked the GABA/Ni^{2+}-induced higher affinity state of the receptor and also blocked any temperature-facilitated transition of the receptor (up to 30°).

The principal findings of these studies are that (a) in a washed homogenate the presence of 10 μM GABA or 5 mM $NiCl_2$ induces a higher affinity state in the benzodiazepine receptor; (b) binding of [^{3}H]DZ to this "activated" state causes a temperature-facilitated transition in the receptor, which in turn shifts binding from an entropy-driven to an enthalpy-driven process; (c) this transition in [^{3}H]DZ binding can be antagonized by β-PrCC and β-CCE; and (d) there is no similar transition apparent when [^{3}H]PrCC binding is measured under the same conditions. Should these findings generalize to other benzodiazepines and their antagonists, the thermodynamic approach may be useful in discriminating between such compounds *in vitro*. In that regard, other reports have indicated transitions in the van't Hoff plots for [^{3}H]flunitrazepam (29) and [^{3}H]clonazepam (30) binding, and the apparent lack of transitions in the binding of the benzodiazepine antagnoists [^{3}H]Ro 15-1788 (30) and [^{3}H]CGS 8216 (31). These results raise intriguing questions about the relationships of benzodiazepines, their antagonists, and modulators to the receptor, and what the transition at 20° represents. Our data support the suggestion of Marangos *et al.* (32) that modulation of the GABA (or ion)-enhanced state of the benzodiazepine receptor may be more relevant to the actions of receptor ligands than modulation of the unstimulated receptor.

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