Pharmacology Biochemistry & Behavior, Vol. 21, pp. 151–158, 1984. © Ankho International Inc. Printed in the U.S.A. 0091-3057/84 $3.00 + .00

Differential Interactions of GABA Agonists, Depressant and Convulsant Drugs with [^{35}S]-t-Butylbicyclophosphorothionate Binding Sites in Cortex and Cerebellum

MAHARAJ K. TICKU*† AND REBBAPRAGADA RAMANJANEYULU*

*Departments of *Pharmacology and †Psychiatry, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284*

Received 21 March 1984

TICKU, M. K. AND R. RAMANJANEYULU. *Differential interactions of GABA agonists, depressant and convulsant drugs with [^{35}S]-t-butylbicyclophosphorothionate binding sites in cortex and cerebellum.* PHARMACOL BIOCHEM BEHAV **21**(1) 151–158, 1984.—Effects of three GABA agonists, four GABA antagonists and convulsants (picrotoxinin, α-dihydropicrotoxinin [DHP], pentamethylenetetrazole [PTZ] and isopropylbicyclophosphate ester) and three depressant drugs (pentobarbital, (+)etomidate and etazolate) were investigated on [^{35}S]t-butylbicyclophosphorothionate (TBPT) in cortex and cerebellum. All the convulsants tested were equipotent in inhibiting [^{35}S]TBPT binding in cortex and cerebellum. Convulsants like picrotoxinin inhibited [^{35}S]-TBPT binding competitively in both cortex and cerebellum. In contrast, γ-aminobutyric acid (GABA) agonists (muscimol, GABA and 4,5,6,7-tetrahydroisoxazol[5,4-*C*]pyridine-3-ol [THIP]), and depressants like etazolate, (+)etomidate and pentobarbital were more potent inhibitors of [^{35}S]TBPT binding in cerebellum than in cortex. GABA inhibition of [^{35}S]TBPT binding appears to be mediated through a low-affinity site. GABA and pentobarbital inhibited [^{35}S]TBPT binding in cortex and cerebellum noncompetitively. Depressants like pentobarbital appear to interact with the TBPT sites allosterically. These results suggest that depressant and convulsant drugs that modulate GABAergic transmission interact differently with the TBPT binding sites in cortex and cerebellum.

GABA receptor complex Convulsants Depressants Allosteric interactions

THE benzodiazepine-GABA receptor-ionophore complex is an oligomeric allosteric protein complex consisting of GABA receptor sites, benzodiazepine sites and picrotoxinin sites, which are coupled to the chloride ionophores. Several lines of electrophysiological and biochemical studies have demonstrated that several classes of structurally unrelated convulsant, depressant, anticonvulsant and anxiolytic drugs may act by modulating GABAergic transmission. *In vitro* radioligand binding studies have demonstrated that drugs which affect GABAergic transmission bind to one of the sites of the oligomeric GABA receptor complex and, in turn, they modulate the binding of ligands to other sites [1, 5, 7, 8, 10–12, 17–21, 23–36].

Based on earlier observations that cage convulsant bicyclophosphate esters which are convulsants and GABA antagonists [3], and inhibit [^{3}H]DHP binding [32], a new radioligand, TBPT, has become available for the picrotoxinin site of the benzodiazepine-GABA receptor-ionophore complex [25]. Since differences in the properties of the GABA/benzodiazepine complex in cerebellum have been reported [1, 2, 7, 12, 19, 22, 29, 35], we have compared the ability of GABA agonists, convulsant and depressant drugs which modulate GABAergic transmission to interact with the [^{35}S]TBPT binding sites in cerebellum and cerebral cortex.

METHOD

Materials

TBPT (25.6–95 Ci/mmol) was purchased from New England Nuclear (Boston, MA). (+)Etomidate was a gift from Dr. J. Leysen (Janssen Pharmaceuticals, Belgium); etazolate from Squibb and Sons, Inc. (Princeton, NJ); bicyclophosphate esters from Dr. J. Casida (Berkley, CA); Methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate (DMCM) and Ethyl-β-carboline-3-carboxylate (β-CCE) from Dr. C. Braestrup (Soeborg, Denmark); RO15-1788 from Dr. Scott (Hoffman-LaRoche, Nutley, NJ); THIP from Sandoz (East Hanover, NJ); and 3α-hydroxy-16-imino-5β--17-aza-androstan-11-one (R-5135) from Dr. Peter Hunt (Romanville, France). Picrotoxinin, GABA, pentamethylenetetrazole, phenobarbital and pentobarbital were purchased from Sigma Chemical Company (St. Louis, MO). Depending upon the solubility, ligands were dissolved in either water or di-

methylsulfoxide (DMSO). DMSO, at concentrations used for the binding studies (1.0%), did not affect the basal [^{35}S]TBPT binding.

Tissue Preparation

All experiments were done using male Sprague-Dawley rats (125–200 g). Tissue for TBPT binding studies were prepared by a modification of the procedure of Squires *et al.* [25], as described [20]. Stated briefly, rats were decapitated, brains removed and cerebral cortex and cerebellum dissected and pooled when necessary. These regions were homogenized in 25 vol of cold 1 mM EDTA solution (pH 7.0) using a Teflon-glass homogenizer (14–16 strokes) and centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 140,000 g for 30 min to obtain the mitochondrial plus microsomal (P_2 + P_3) fraction. The (P_2 + P_3) fraction was resuspended in 25 vol of 1 mM EDTA (pH 7.0) in water, homogenized and dialyzed overnight (12–15 hr) against 50 vol of double-distilled water at 0–4°C. The EDTA treatment and dialysis against water are necessary to remove endogenous GABA [25]. Following dialysis, the membrane suspension was pelleted at 140,000 g, resuspended by homogenization in 0.32 M sucrose and frozen. On the day of the experiment, the tissue was thawed at room temperature, centrifuged, washed once with buffer (5 mM Tris HCl, 0.2 M KBr, pH 7.5) and resuspended at a protein concentration of 0.3–0.6 mg/ml for the binding studies.

To determine the effect of thiocyanate on GABA inhibition of [^{35}S]TBPT binding, the EDTA-treated and dialyzed (P_2 + P_3) fraction was incubated at 37°C for 20 min and centrifuged. The tissue was then resuspended in the buffer and the incubation with [^{35}S]TBPT was carried out in the absence or presence of 50 mM ammonium thiocyanate (SCN^-).

Some experiments were conducted using the following membrane preparations (without EDTA treatment): the mitochondrial plus microsomal (P_2 + P_3) fraction was prepared and subjected to two osmotic shock treatments and three buffer washes (0.2 M KBr, 5 mM Tris HCl, pH 7.5) and frozen. On the next day, the tissue was thawed, centrifuged and washed twice prior to the binding studies (frozen preparation, see Table 3); the (P_2 + P_3) fraction was subjected to two osmotic shock treatments, two freeze-thaw cycles and five buffer washes, as described elsewhere for GABA binding studies ([5]; GABA preparation, Table 2).

Binding Studies

Routinely aliquots (0.2–0.5 mg protein/ml) of cerebral cortex or cerebellum (P_2 + P_3) fraction in 5 mM Tris HCl (pH 7.5) containing 0.2 M KBr were incubated with or without other drugs and 2 nM [^{35}S]TBPT for 100 min at 24°C. Following incubation, 250 μl aliquots were filtered on Whatman GF/B filters and washed rapidly twice with 3.5 ml of buffer. The filters were dried and radioactivity determined in 3 ml of 2,5-diphenyl-oxazole/BBS (Bio-Solv; Beckman) scintillation cocktail. Nonspecific binding obtained in the presence of 10^{-5} M picrotoxinin represented 12±4% of the total radioactivity. Protein was estimated by the method of Lowry *et al.* [14]. Since the β-emission energies of [^{35}S] and [^{14}C] are in the same range (E_{mean}=0.049 Mev), the counting efficiency was determined by the external standard method using [^{14}C]-toluene and was 80±2%.

For Scatchard plots, the concentration of [^{35}S]TBPT was varied (1, 2, 5, 10, 20, 40, 80 and 120 nM). The K_D and B_{max} values were obtained by the linear regression of the Scatchard data. IC_{50} values were obtained using 6–8 concentrations of the ligands. All experiments were done in triplicate. The statistical significance of the results was calculated using the Student's *t*-test.

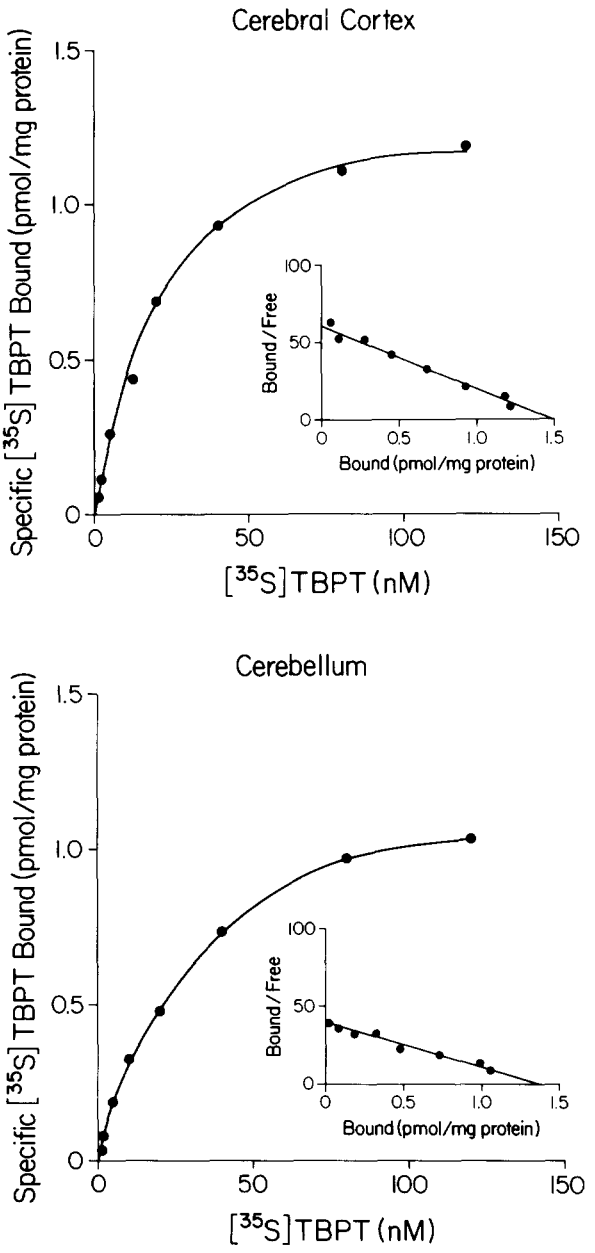

FIG. 1. Saturation binding isotherms and Scatchard plots of [^{35}S]TBPT binding to cerebral cortex and cerebellum. Aliquots of EDTA treated and dialyzed (P_2 + P_3) membrane homogenates were incubated with various concentrations of [^{35}S]TBPT (1–120 nM) in absence and presence of 10^{-5} M picrotoxinin for 100 min at 24°C, as described in the text. The results are typical of a single experiment, which was replicated several times. The K_D and B_{max} values were obtained by linear regression and are summarized in Table 5.

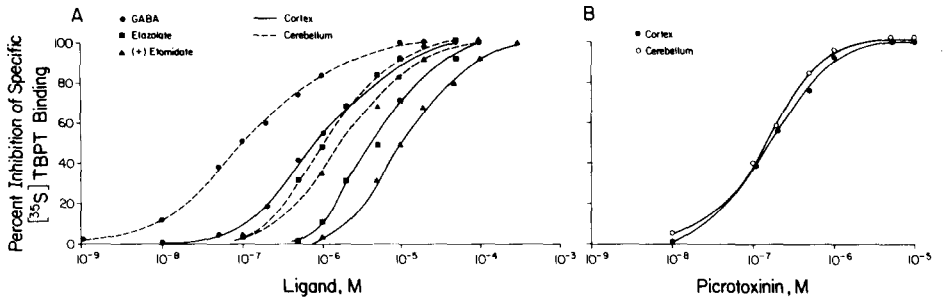

FIG. 2. Inhibition of specific [^{35}S]TBPT by GABA etazolate and (+)etomidate (2A) and picrotoxinin (2B) in cerebral cortex and cerebellum. Results are the mean values of at least three experiments.

TABLE 1

IC_{50} VALUES OF CONVULSANT AND DEPRESSANT DRUGS AND GABA ON [^{35}S]TBPT BINDING

Ligand	Cerebral Cortex		Cerebellum	
	IC_{50} (μM)	Slope Factor	IC_{50} (μM)	Slope Factor
I. Convulsants:				
Isopropylbicyclophosphate ester (3)	0.25 ± 0.06	1.17 ± 0.03	0.19 ± 0.04	0.98 ± 0.06
Picrotoxinin (4)	0.35 ± 0.07	0.97 ± 0.07	0.40 ± 0.06	0.99 ± 0.05
α-Dihydropicrotoxinin (3)	1.21 ± 0.04	1.11 ± 0.03	1.63 ± 0.06	1.09 ± 0.05
Pentamethylene-tetrazole (3)	610 ± 14	0.98 ± 0.10	625 ± 12	1.00 ± 0.07
II. GABA Agonists				
Muscimol (3)	0.19 ± 0.01	1.03 ± 0.09	0.03 ± 0.01†	0.79 ± 0.01
GABA (3)	0.77 ± 0.05	1.17 ± 0.13	0.12 ± 0.03†	0.78 ± 0.07
THIP (3)	7.21 ± 0.81	0.79 ± 0.05	0.84 ± 0.06†	0.70 ± 0.09
III. Depressants:				
Etazolate (3)	5.0 ± 1.1	1.11 ± 0.21	1.1 ± 0.04*	1.07 ± 0.04
(+)Etomidate (4)	12.5 ± 2.3	1.03 ± 0.16	2.91 ± 1.60†	0.75 ± 0.08
Pentobarbital (3)	169 ± 30	1.65 ± 0.57	55 ± 6*	1.56 ± 0.18

IC_{50} values were obtained by graphic analysis, using six to eight concentrations of the displacing ligand (see Fig. 2). Slope factors were obtained by plotting log (B_0/B_D^{-1}) against log [displacer], where B_0 and B_D represent the binding in the absence and presence of the displacer. Binding assay conditions were as described in Table 1 and in the Method section.

*$p<0.005$, as compared to cerebral cortex.

†$p<0.001$, as compared to cerebral cortex.

RESULTS

Specific [^{35}S]TBPT Binding in Cortex and Cerebellum

In agreement with the results of Squires *et al.* [25], we have recently observed that the specific binding of [^{35}S]TBPT to whole brain membranes was maximal in buffer containing 0.2 M KBr. Furthermore, we observed that both the mitochondrial (P_2) and microsomal (P_3) fraction had significant [^{35}S]TBPT binding [20]. All experiments in the present study were done using (P_2 + P_3) fraction of cerebral cortex and cerebellum in buffer containing 0.2 M KBr. Figure 1 shows typical saturation isotherms and Scatchard plots of [^{35}S]TBPT binding to cerebral cortex (1A) and cerebellar membranes (1B). [^{35}S]TBPT binds to a single site in both cerebral cortex and cerebellum with an apparent K_D value of 25–30 nM. Initial experiments with various ligands that inhibit [^{35}S]TBPT binding revealed that GABA and pentobarbital but not picrotoxinin were more potent in inhibiting [^{35}S]TBPT binding in cerebellum than in cortex.

Interaction of Convulsants, GABA Agonists and Depressant Drugs with [^{35}S]TBPT Binding Sites in Cerebral Cortex and Cerebellum

Figure 2 shows the concentration-dependent inhibition of specific [^{35}S]TBPT binding from cortex and cerebellum by GABA, etazolate, (+)etomidate and by picrotoxinin. Figure 2A shows that GABA, etazolate and (+)etomidate were more potent in displacing [^{35}S]TBPT binding in cerebellum than in cortex. In contrast, picrotoxinin gave similar inhibition curves in cortex and cerebellum (2B).

Table 1 compares the IC_{50} values and slope factors of four convulsant, three depressant drugs and three GABA agonists on [^{35}S]TBPT binding in cortex and cerebellum. Four GABA antagonists and convulsants (isopropylbicyclophosphate ester, picrotoxinin, DHP and PTZ) were equipotent in inhibiting [^{35}S]TBPT binding in both cerebral cortex and cerebellum. In contrast, three depressant drugs (etazolate, (+)etomidate and pentobarbital) which are

TABLE 2

INHIBITION OF SPECIFIC [^{35}S]TBPT BINDING USING DIFFERENT TISSUE PREPARATIONS

	Percent Inhibition of Specific [^{35}S]TBPT BINDING					
	EDTA Preparation*		Frozen Preparation†		GABA Preparation‡	
Ligand	Cortex	Cerebellum	Cortex	Cerebellum	Cortex	Cerebellum
5×10^{-7} M GABA	37 ± 5 (5)	71 ± 5 (6)	34 ± 4 (4)	64 ± 4 (4)	46 ± 7 (3)	76 ± 6 (3)
10^{-5} M (+)Etomidate	46 ± 11 (4)	76 ± 5 (5)	38 ± 4 (4)	74 ± 3 (4)	44 ± 3 (3)	71 ± 5 (3)
5×10^{-7} M Picrotoxinin	56 ± 4 (4)	49 ± 5 (4)	52 ± 8 (5)	60 ± 9 (4)	46 ± 6 (5)	49 ± 5 (4)

The binding was measured as described in the Method section, using 2 nM [^{35}S]TBPT.

The values represent the mean ± S.D. of the number of experiments indicated in the parentheses.

*Refers to the regular EDTA-treated and dialyzed tissue preparation, as described in the Method section.

†Refers to the (P_2 + P_3) fraction that was subjected to two osmotic shock treatments, followed by three buffer washes with 5 mM Tris HCl, 200 mM KBr (pH 7.5), freezing and two buffer washes on the day of the assay.

‡Refers to GABA preparation, as described elsewhere [5], which involves subjecting the (P_2 + P_3) fraction to two osmotic shock treatments, two freeze-thaw cycles and a total of five washes (5 mM Tris HCl, 200 mM KBr, pH 7.5) washes prior to the binding studies.

TABLE 3

EFFECT OF KBr CONCENTRATION ON THE POTENCY OF GABA TO INHIBIT [^{35}S]TBPT BINDING

	IC_{50} GABA (μM)	
KBr Concentration	Cerebral Cortex	Cerebellum
50 mM	0.18 ± 0.02	0.05 ± 0.03
100 mM	0.21 ± 0.04	0.07 ± 0.03
200 nM	0.77 ± 0.05	0.12 ± 0.03

Cerebral cortex and cerebellar membrane homogenates were incubated with 2 nM [^{35}S]TBPT and various concentrations of KBr for 100 min, as described in the text. The values represent the mean ± S.D. of at least three experiments.

TABLE 4

EFFECT OF 50 mM AMMONIUM THIOCYANATE ON THE POTENCY OF GABA TO DISPLACE [^{35}S]TBPT BINDING

	GABA IC_{50} (μM)	
Brain Region	$-SCN^-$	$+SCN$
Cerebral Cortex	0.77 ± 0.05	1.09 ± 0.07
Cerebellum	0.12 ± 0.03	0.24 ± 0.04

IC_{50} values for GABA were determined in the presence and absence of 50 mM ammonium thiocyanate (SCN^-). Values are the mean ± S.D. of three separate experiments, each performed in triplicate.

TABLE 5

EFFECT OF VARIOUS MODULATORS ON THE BINDING CONSTANTS OF [^{35}S]TBPT BINDING

	Cerebral Cortex		Cerebellum	
	K_D	B_{max}	K_D	B_{max}
Treatment	(nM)	(pmol/mg protein)	(nM)	(pmol/mg protein)
None	26.8 ± 7.6 (8)	1.36 ± 0.19	32.7 ± 2.3 (4)	1.26 ± 0.18
5×10^{-7} M GABA	23.5 ± 3.5 (3)	0.92 ± 0.09†	26.9 ± 5.4 (3)	0.44 ± 0.11†
*Pentobarbital	28.7 ± 6.0 (3)	0.65 ± 0.11†	33.4 ± 4.7 (3)	0.52 ± 0.09†
5×10^{-7} M Picrotoxinin	61.5 ± 6.4 (3)†	1.49 ± 0.33	62.8 ± 4.6 (3)‡	1.43 ± 0.26

Scatchard plots of [^{35}S]TBPT to cerebral cortex and cerebellar membranes were determined in the absence and presence of various ligands. The concentrations of [^{35}S]TBPT used were 1, 2, 5, 10, 20, 40, 80 and 120 nM and nonspecific binding was determined in the presence of 10^{-5} M picrotoxinin. In some cases, the 120 nM point was omitted.

*The concentrations of pentobarbital used were 2×10^{-4} M for cerebral cortex and 10^{-4} M for cerebellum.

K_D and B_{max} values were obtained by the linear regression of the Scatchard data. The values are the mean ± S.D. of the number of experiments indicated in the parentheses, each done in triplicate.

†$p<0.001$, as compared to control.

TABLE 6

EFFECTS OF ANTAGONISTS OF GABA AND BENZODIAZEPINE TO PREVENT INHIBITION OF [^{35}S]TBPT BINDING

Inhibitor	Percent of Control [^{35}S]BPT Binding				
	Alone	10^{-8} M R-5135	10^{-5} M Bicuculline Methiodide	10^{-7} M DMCM	10^{-8} M RO15-1788
I. Cerebral Cortex:					
5×10^{-7} M GABA (5)	56 ± 4	82 ± 5*	110 ± 12*	50 ± 5	52 ± 4
10^{-4} M Pentobarbital (6)	60 ± 6	71 ± 4*	88 ± 6*	57 ± 3	59 ± 4
10^{-5} M (+)Etomidate (3)	60 ± 5	76 ± 4*	93 ± 8*	58 ± 4	53 ± 4
5×10^{-7} M Picrotoxinin (3)	44 ± 7	40 ± 6	42 ± 5	43 ± 7	46 ± 5
7×10^{-4} M PTZ (3)	53 ± 6	44 ± 8	47 ± 4	46 ± 5	44 ± 3
II. Cerebellum:					
5×10^{-7} M GABA (5)	30 ± 4	82 ± 6*	91 ± 4*	28 ± 6	31 ± 7
10^{-4} M Pentobarbital (4)	40 ± 7	72 ± 8*	86 ± 14*	36 ± 4	39 ± 3
10^{-5} M (+)Etomidate (3)	28 ± 6	51 ± 5‡	73 ± 9*	29 ± 3	27 ± 4
5×10^{-7} M Picrotoxinin (3)	48 ± 4	40 ± 3	46 ± 6	41 ± 3	40 ± 5
7×10^{-4} M PTZ (3)	53 ± 6	48 ± 4	51 ± 6	43 ± 5	42 ± 5

Cerebral cortex or cerebellum membranes were incubated with 2 nM [^{35}S]TBPT, inhibitors (GABA, pentobarbital, (+)etomidate, picrotoxinin and PTZ) and antagonists (R-5135, bicuculline methiodide, DMCM and RO15-1788) at 25°C for 100 min, as described in the Method section. The results are the means ± S.D. the number of experiments in the parenthesis.

*$p>0.005$; †$p>0.02$; ‡$p>0.01$ when compared to alone group.

known to facilitate GABAergic transmission, were more potent (three-to-five-fold) inhibitors of [^{35}S]TBPT binding in cerebellum than in cortex. The Hill coefficient of pentobarbital inhibition of [^{35}S]TBPT was ~1.5, whereas for other compounds, this values was ≤1.0. Likewise, GABA agonists (muscimol, THIP and GABA) were more potent inhibitors of [^{35}S]TBPT binding in cerebellum than in cortex. GABA was six-fold more potent in displacing [^{35}S]TBPT binding in cerebellum (IC_{50}=0.12 μM) than in cortex (IC_{50}=0.77 μM).

To determine if the potency differences observed with GABA agonists and depressants were due to the tissue preparation utilized in our study (i.e., EDTA treatment), we compared the ability of GABA, one depressant and one convulsant, to inhibit [^{35}S]TBPT binding using two other tissue preparations. Table 2 shows that GABA and (+)etomidate, but not picrotoxinin, were more potent inhibitors of [^{35}S]TBPT binding in cerebellum than in cortex, using other tissue preparations.

Table 3 shows that the potency of GABA to inhibit [^{35}S]TBPT in both cortex and cerebellum was dependent upon KBr concentration. At all three concentrations of KBr examined, GABA was always more potent in inhibiting [^{35}S]TBPT binding in cerebellum than in cortex. However, the reversal by KBr of GABA's inhibition of [^{35}S]TBPT binding was more potent in cortex than in cerebellum (Table 3), suggesting a differntial sensitivity to KBr in these regions.

Effect of Ammonium Thiocyanate on GABA Inhibition of [^{35}S]TBPT Binding

To determine whether the inhibition of [^{35}S]TBPT binding by GABA was mediated by the high or the low affinity GABA receptor site, we examined the effect of ammonium thiocyanate (SCN^-), which eliminates the high affinity GABA receptor sites [4]. Table 4 compares the IC_{50} values of GABA to inhibit [^{35}S]TBPT binding in cerebral cortex and cerebellum in the absence and presence of SCN^-. The data in Table 4 indicates that inhibition of [^{35}S]TBPT binding by GABA appears to be mediated through a low-affinity site.

Effect of GABA Agonists, Depressant and Convulsant Drugs on the Binding Constants of [^{35}S]TBPT Binding

Table 5 summarizes the effect of various ligands on the binding constants of [^{35}S]TBPT binding in cerebral cortex and cerebellum. GABA, while having no significant effect on the K_D value, decreased the B_{max} of [^{35}S]TBPT in cerebral cortex from a control value of 1.36±0.19 pmol/mg protein to 0.92±0.09 pmol/mg protein ($p<0.001$), and in cerebellum from a control value of 1.26±0.18 pmol/mg protein to 0.44±0.11 pmol/mg protein ($p<0.001$). Thus, GABA appears to inhibit [^{35}S]TBPT binding in both cerebral cortex and cerebellum noncompetitively. Pentobarbital, like GABA, decreased the B_{max} of [^{35}S]TBPT binding in both cortex and cerebellum, suggesting a noncompetitive type of interaction (Table 5). Picrotoxinin (5×10^{-7} M) decreased the affinity of [^{35}S]TBPT binding in cerebral cortex from a control value of 26.8±7.6 nM to 61.5±6.4 nM ($p<0.001$) and in cerebellum from a control value of 32.7±2.3 nM to 62.8±4.6 nM ($p<0.001$) without significantly altering the B_{max}. Thus, picrotoxinin inhibits [^{35}S]TBPT binding in both cortex and cerebellum, apparently competitively.

Effect of Various Antagonists of the Benzodiazepine-GABA Receptor-Ionophore Complex on the Inhibition of [^{35}S]TBPT Binding in Cerebral Cortex and Cerebellum

To further characterize the interaction of various ligands with the [^{35}S]TBPT binding site, we investigated the effect of bicuculline methiodide and R-5135 (ligands which interact with the GABA recognition site) and RO15-1788 and DMCM (benzodiazepine antagonists) on these interactions. Table 6 shows that GABA antagonists bicuculline methiodide (10^{-5} M) and R-5135 (10^{-8} M) reversed the inhibition of [^{35}S]TBPT binding by depressants like pentobarbital and (+)etomidate and GABA but not that produced by convulsants like picrotoxinin and PTZ in cerebral cortex and cerebellum. Bicuculline methiodide was slightly more potent in reversing GABA effect in cortex than the cerebellum. In contrast,

benzodiazepine antagonists RO15-1788 and DMCM did not reverse the inhibitory effect of GABA, depressant or convulsant drugs on TBPT binding in cerebral cortex or cerebellum (Table 6). To further characterize the ability of bicuculline methiodide to prevent inhibition of TBPT binding by GABA and pentobarbital in cortex and cerebellum, we studied the concentration-dependent effect of these antagonists. Figure 3 shows concentration-dependent effect of bicuculline methiodide to prevent inhibition of [^{35}S]TBPT binding by GABA and pentobarbital in cortex and cerebellum. Bicuculline methiodide, in a concentration-dependent manner, prevented inhibition of [^{35}S]TBPT binding by GABA and pentobarbital in both cortex and cerebellum (Fig. 3).

DISCUSSION

Various components of the oligomeric GABA receptor complex represent potential sites of action for several classes of structurally unrelated convulsants, depressants, anticonvulsants and anxiolytic drugs. It is becoming apparent that besides having recognition sites for GABA, this receptor complex has other modulatory sites which bind benzodiazepine agonists and antagonists, β-carbolines, picrotoxinin and related cage convulsant bicyclophosphate esters, sedative-hypnotic barbiturates, nonbenzodiazepine anxiolytics like pyrazolyridines and nonbarbiturate hypnotics.

[^{35}S]TBPT has been shown to bind to a single site to rat brain membranes, with properties similar, but not identical, to that of [^{3}H]DHP [20,25]. In addition, the [^{35}S]TBPT binding sites appear to be associated with GABA receptor and chloride-ionophores ([25]; this study). A variety of convulsant, depressant and anxiolytic drugs that have been previously shown to inhibit [^{3}H]DHP binding [30–32] also inhibit [^{35}S]TBPT binding to rat brain membranes [20, 21, 25]. All of these results support the notion that [^{35}S]TBPT apparently binds to the picrotoxinin site of the benzodiazepine-GABA receptor-ionophore complex.

In this study, we compared the interaction of GABA agonists, convulsants which inhibit GABAergic transmission, and depressant drugs which facilitate GABAergic transmission, with the [^{35}S]TBPT binding in cerebral cortex and cerebellum. Several studies have shown that picrotoxinin and DHP [16, 18, 23, 24], cage convulsant isopropylbicyclophosphate esters [3] and PTZ [23,37] are GABA antagonists, and inhibit [^{3}H]DHP binding [30–32]. These antagonists inhibited [^{35}S]TBPT binding in cerebral cortex and cerebellum with similar IC_{50} values and slope factors close to unity. Isopropylbicyclophosphate was the most potent, followed by picrotoxinin, DHP and PTZ. Scatchard analysis indicated that picrotoxinin inhibits [^{35}S]TBPT binding competitively in both cerebral cortex and cerebellum. Likewise, we have recently reported that PTZ and tetrazole analogues inhibit [^{35}S]TBPT binding in whole rat brain membranes apparently competitively and with potencies which correlate with their convulsant activity [21]. These results suggest that GABA antagonist and convulsant drugs (picrotoxinin, cage convulsants and PTZ) bind to the picrotoxinin site and that they may block GABAergic transmission by acting at this site.

In contrast to convulsants, the three GABA agonists examined were more potent in inhibiting [^{35}S]TBPT binding in cerebellum than in cortex (Table 1). In the presence of thiocyanate, which eliminates the high affinity GABA receptor sites [4], GABA was still able to inhibit [^{35}S]TBPT binding. This finding suggests that the GABA apparently interacts with the [^{35}S]TBPT through a low-affinity site. The low affinity GABA receptor sites are also coupled to the benzodiazepine binding sites [4,5]. It may be pointed out that the displacing potency of GABA agonists decreases in the presence of thiocyanate, while those of GABA antagonists are higher in the presence of thiocyanate [4,15].

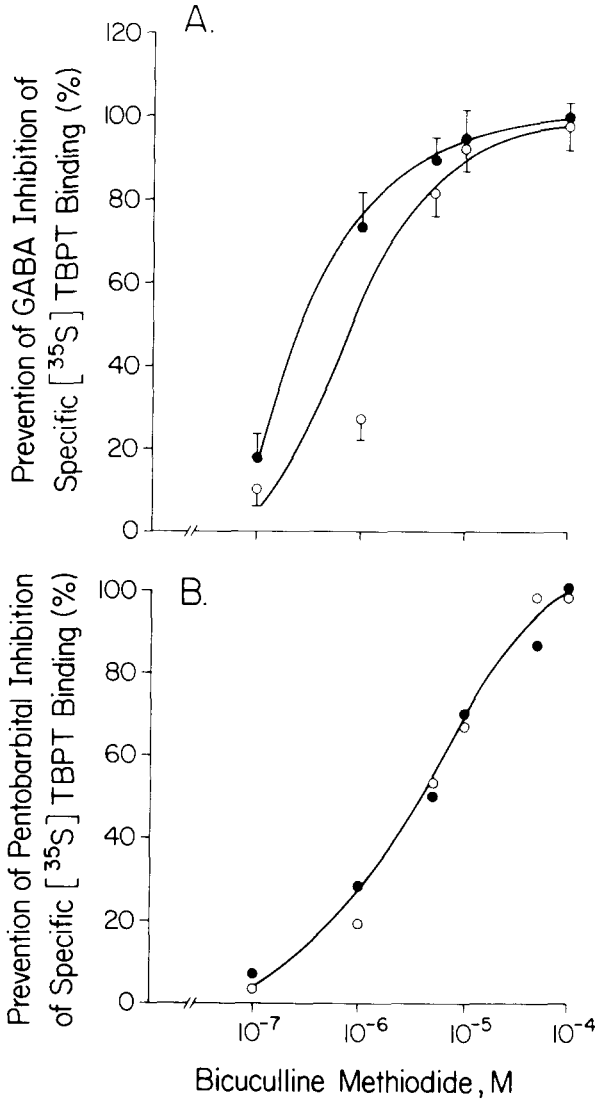

FIG. 3. Effect of bicuculline methiodide on the inhibition of specific [^{35}S]TBPT binding by GABA (5×10^{-7} M; top) and pentobarbital (2×10^{-4} M, bottom) in cerebral cortex (●—●) and cerebellum (○—○). Aliquots of membrane homogenates were incubated with 2 nM [^{35}S]TBPT in the absence and presence of GABA or pentobarbital for 100 min at 24°C, and assayed as described in the text. The results are the mean±SD of four experiments for Fig. 3A and mean values of four experiments for Fig. 3B.

In contrast to convulsants, but like GABA agonists, the three depressant drugs (pentobarbital, (+)etomidate and etazolate) exhibited potency differences in inhibiting [^{35}S]TBPT binding in cerebral cortex and cerebellum. Etazolate, (+)etomidate and pentobarbital were three- to six-fold more potent inhibitors of [^{35}S]TBPT binding in cerebellum than in cerebral cortex (Table 1). The Hill coefficient for

pentobarbital inhibition of [^{35}S]TBPT binding (~1.5) may be due to its racemic nature and its dual convulsant/depressant activity [6]. It is very likely that the (+)isomer, which has some excitatory activity, competes directly with the convulsant (TBPT) site, whereas the depressant (−)isomer displaces allosterically from the depressant site. Other depressant ligands used in our study are not racemic mixtures, are devoid of this dual behavior and give Hill coefficient values of ≤1.0. Scatchard analysis on the effect of GABA and depressant drugs with the [^{35}S]TBPT binding sites revealed further differences in their interactions as compared to convulsants. GABA inhibited [^{35}S]TBPT binding noncompetitively in both cerebral cortex and cerebellum. This finding is consistent with the notion that GABA and picrotoxinin bind to two distinct but coupled sites. Depressant barbiturates like pentobarbital also inhibited [^{35}S]TBPT binding noncompetitively in cortex and cerebellum. These results indicate that pentobarbital does not bind directly to the TBPT site, but to an allosteric site. A previous report from our laboratory indicated a uncompetitive inhibition of [^{35}S]TBPT binding by pentobarbital and (+)etomidate in whole brain membranes [20]. This discrepancy may be due to the fact that we performed detailed Scatchard analysis using [^{35}S]TBPT concentrations in the ranges of 1–120 nM in the present study, versus 1–40 nM in the previous study. This point is especially valid since the K_D of TBPT is ~25 nM.

Our results with various antagonists demonstrated that RO15-1788, DMCM (Table 6) and β-CCE (data not shown) did not prevent inhibition of [^{35}S]TBPT binding by GABA, convulsants or depressants in cerebral cortex or in cerebellum. These results suggest (but do not prove) that while convulsants act directly at the picrotoxin site, GABA and depressants apparently interact *in vitro* with the TBPT site independent of the benzodiazepine binding sites in both cortex and cerebellum.

R-5135 was recently shown to prevent inhibition of [^{35}S]TBPT to brain membranes by depressants but not by convulsants [25]. Our results on R-5135 reversal of inhibition of [^{35}S]TBPT binding by GABA and depressants in cortex and cerebellum are in agreement with this finding. Ability of bicuculline to prevent the inhibition of TBPT binding by pentobarbital in both cortex and cerebellum also suggests that the pentobarbital site is coupled to both GABA and TBPT sites.

In summary, our results indicate that convulsants (picrotoxin, bicyclophosphate esters and PTZ) act directly at the picrotoxinin site, whereas pentobarbital (and other depressant drugs which facilitate GABAergic transmission) interacts with TBPT sites, by binding to an *allosteric site(s)* which is associated with the benzodiazepine-GABA receptor ionophore complex. Pentobarbital, etazolate and (+)etomidate, besides having similar behavioral and pharmacological effects, also have similar inhibitory effects on TBPT binding, and on enhancing effects of GABA and benzodiazepine binding. Furthermore, their enhancing effects can be blocked by picrotoxinin and bicuculline. It is reasonable to speculate that these depressant drugs interact with the picrotoxinin site *(allosterically)* and alter the conformation of GABA and/or benzodiazepine sites. Our results are also consistent with the notion that the picrotoxin site has a pronounced three dimensional structure with an allosteric hydrophobic regulatory site [34]. The nature of this site, as it relates to the binding of convulsant drugs has recently been reviewed [34] and appears to be similar in cerebral cortex and cerebellum. All the convulsants tested in this study meet the steric requirements for this site, whereas depressants do not [34].

Finally, the coupling of various components of the benzodiazepine-GABA receptor-ionophore complex appears to be different in cortex and cerebellum. Several other lines of evidence, including (1) differential distribution of GABA and benzodiazepine binding sites [2,22] and benzodiazepine and TBPT binding sites [7,35] in cerebellum; (2) differential inhibition of pentobarbital stimulation of benzodiazepine binding by bicuculline in cortex and cerebellum [12]; (3) differential stimulation of GABA and benzodiazepine binding by pentobarbital and etomidate in cortex and cerebellum [1, 12, 19, 29]; and (4) predominant presence of Type 1 benzodiazepine receptors in cerebellum [13] support the notion that the properties of the benzodiazepine-GABA receptor complex appear to be different in cerebellum.

ACKNOWLEDGEMENTS

We thank Drs. G. Maksay and W. Morgan for helpful comments, Ms. R. Thyagarajan for excellent technical assistance, and Mrs. M. Wilson for secretarial assistance. This work was supported by National Institute of Health Grant NS-15339.

REFERENCES

1. Ashton, D., R. Geerts, N. C. Waterkey and J. E. Leysen. Etomidate stereospecifically stimulates forebrain but not cerebellar [^{3}H]diazepam binding. *Life Sci* **29:** 2631–2636, 1981.
2. Biggio, C., M. G. Corda, G. DeMontis, E. Stefanini and G. L. Gessa. Kainic acid differentiates GABA receptors from benzodiazepine receptors in the rat cerebellum. *Brain Res* **193:** 589–593, 1980.
3. Bowery, N. G., J. F. Collins, R. G. Hill and S. Pearlson. GABA antagonism as a possible basis for the convulsant action of series of bicyclophosphorous esters. *Br J Pharmacol* **57:** 435p–436p, 1976.
4. Browner, M., J. W. Ferkany and S. J. Enna. Biochemical identification of pharamcologically and functionally distinct GABA receptors in rat brain. *J Neurosci* **1:** 514–518, 1981.
5. Burch, T. P., R. Thyagarajan and M. K. Ticku. Group-selective modification of the benzodiazepine-GABA receptor-ionophore complex reveals that low affinity GABA receptors stimulate benzodiazepine binding. *Mol Pharmacol* **23:** 52–59, 1983.
6. Downes, H., R. S. Perry, R. E. Ostlund and R. Karler. A study of the excitatory effects of barbiturates. *J Pharmacol Exp Ther* **175:** 692–699, 1970.
7. Gee, K. W., J. K. Wamsley and H. I. Yamamura. Light microscopie autoradiographic identification of picrotoxinin/barbiturate binding sites in rat brain with [^{35}S]t-butylbicyclophosphothionate. *Eur J Pharmacol* **89:** 323–324, 1983.
8. Haefely, W., P. Polc, R. Schaffner, H. H. Kller, L. Pieri and H. Möhler. Facilitation of GABAergic transmission by drugs. In: *GABA—Neurotransmitters*, edited by P. Krogsgaard-Larsen, J. Scheel-Kruger and M. Kofod. Copenhagen: Munksgaard, 1979, pp. 357–375.
9. Hunt, P. and S. Clements-Jewery. A steroid derivative, R-5135, antagonizes the GABA/benzodiazepine receptor interactions. *Neuropharmacology* **20:** 357–361, 1981.
10. Leeb-Lundberg, F., A. Snowman and R. W. Olsen. Barbiturate receptor sites are coupled to benzodiazepine receptors. *Proc Natl Acad Sci USA* **77:** 7468–7472, 1980.

11. Leeb-Lundberg, F., A. Snowman and R. W. Olsen. Perturbation of benzodiazepine receptor binding by pyrazolopyridines involves picrotoxinin/barbiturate receptor sites. *J Neurosci* **1:** 471–477, 1981.
12. Leeb-Lundberg, L. M. F. and R. W. Olsen. Heterogeneity of benzodiazepine receptor interactions with γ-aminobutyric acid and barbiturate receptor sites. *Mol Pharmacol* **23:** 315–325, 1983.
13. Lippa, A. S., C. A. Klepner, D. I. Benson, D. J. Critcheet, M. E. Sano and B. Beer. The role of GABA in mediating the anticonvulsant properties of benzodiazepines. *Brain Res Bull* **5:** Suppl 2, 861–865, 1980.
14. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. Protein measurement with the folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
15. Maksay, G. and M. K. Ticku. Diazotization and thiocyanate differentiates agonists from antagonists for the high- and low-affinity receptors of γ-aminobutyric acid. *J Neurochem*, in press, 1984.
16. Nicoll, R. A. and J. M. Wojtowicz. The effects of pentobarbital and related compounds on frog motoneurons. *Brain Res* **191:** 225–237, 1980.
17. Olsen, R. W. GABA-bnezodiazepine barbiturate receptor interactions. *J Neurochem* **37:** 1–13, 1981.
18. Polc, P., E. P. Bonetti, R. Schaffner and W. Haefely. A three-state model of the benzodiazepine receptor explains the interactions between the benzodiazepine antagonist RO15-1788, benzodiazepine tranquilizers, β-carbolines and phenobarbitone. *Naunyn Schmiedebergs Arch Pharmacol* **321:** 260–264, 1982.
19. Quast, U. and O. Brenner. Modulation of [^{3}H]muscimol binding in rat cerebellum and cerebral cortical membranes by picrotoxin, pentobarbital and etomidate. *J Neurochem* **41:** 418–425, 1983.
20. Ramanjaneyulu, R. and M. K. Ticku. Binding characteristics and interactions of depressant drugs with [^{35}S]t-butylbicyclophosphorothionate, a ligand that binds to the picrotoxinin site. *J Neurochem* **42:** 221–229, 1984.
21. Ramanjaneyulu, R. and M. K. Ticku. Interactions of pentamethylenetetrazole and tetrazole analogues with the picrotoxinin site of the benzodiazepine-GABA receptor-ionophore complex. *Eur J Pharmacol* **98:** 337–345, 1984.
22. Simantov, R., M. L. Oster-Granite, R. M. Herndon and S. H. Snyder. Gamma-aminobutyric acid (GABA) receptor binding selectively depleted by viral-induced granule cell loss in hamster cerebellum. *Brain Res* **105:** 365–371, 1976.
23. Simmonds, M. A. Evidence that bicuculline and picrotoxin act at separate sites to antagonize γ-aminobutyric acid in rat cuneate nucleus. *Neuropharmacology* **19:** 39–45, 1980.
24. Simmonds, M. A. Classification of some GABA antagonists with regard to site of action and potency in slices of rat cuneate nucleus. *Eur J Pharmacol* **80:** 347–358, 1982.
25. Squires, R. F., J. E. Casida, M. Richardson and E. Saerderup. [^{35}S]-t-Butylbicyclophosphorothionate binds with high affinity to brain-specific sites coupled to γ-aminobutyric acid A and ion recognition sites. *Mol Pharmacol* **23:** 326–336, 1983.
26. Supavilai, P. and M. Karobath. Stimulation of benzodiazepine receptor binding by SQ20009 is chloride-dependent and picrotoxin-sensitive. *Eur J Pharmacol* **60:** 111–113, 1979.
27. Supavilai, P., A. Mannonen and M. Karobath. Modulation of GABA binding sites by CNS depressants and CNS convulsants. *Neurochem Int* **4:** 259–268, 1982.
28. Tallman, T. F., S. M. Paul, P. Skolnick and D. W. Gallager. Receptors for the age of anxiety. Pharmacology of the benzodiazepines. *Science* **207:** 274–281, 1980.
29. Thyagarajan, R., R. Ramanjaneyulu and M. K. Ticku. Enhancement of diazepam and GABA binding by (+)etomidate and pentobarbital. *J Neurochem* **41:** 578–585, 1983.
30. Ticku, M. K., M. Ban and R. W. Olsen. Binding of [^{3}H]α-dihydropicrotoxinin, a γ-aminobutyric acid synaptic antagonist, to rat brain membranes. *Mol Pharmacol* **14:** 391–402, 1978.
31. Ticku, M. K. and R. W. Olsen. Interaction of barbiturates with dihydropicrotoxinin binding sites related to the GABA receptor-ionophore system. *Life Sci* **22:** 1643–1652, 1978.
32. Ticku, M. K. and R. W. Olsen. Cage convulsants inhibit picrotoxinin binding. *Neuropharmacology* **18:** 315–318, 1979.
33. Ticku, M. K. Interaction of depressant, convulsant and anticonvulsant barbiturates with [^{3}H]diazepam binding sites at the benzodiazepine-GABA receptor-ionophore complex. *Biochem Pharmacol* **30:** 1573–1579, 1981.
34. Ticku, M. K. and G. Maksay. Convulsant/depressant site of action at the allosteric benzodiazepine-GABA receptor-ionophore complex. *Life Sci* **33:** 2636–2375, 1983.
35. Wamsley, J. K., K. G. Gee and H. I. Yamamura. Comparison of the distribution of convulsant/barbiturate and benzodiazepine receptors using light microscopic autoradiography. *Life Sci* **33:** 2321–2329, 1983.
36. Willow, M. and G. A. R. Johnston. Pentobarbitone slows the dissociation of GABA from rat brain synaptosomal binding sites. *Neurosci Lett* **23:** 71–74, 1981.
37. Woodbury, D. Convulsant drugs: Mechanisms of action. In: *Antiepileptic Drugs: Mechanism of Action*, edited by G. H. Glaser, J. K. Penny and D. W. Woodbury. New York: Raven Press, 1980, pp. 249–303.