MOLECULAR PHARMACOLOGY, 14, 256-265

Properties of γ-Aminobutyric Acid Receptor Binding with (+)-[^{3}H]Bicuculline Methiodide in Rat Cerebellum

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(Received May 25, 1977)

(Accepted October 24, 1977)

SUMMARY

MÖHLER, HANNS & OKADA, TOSHIKAZU (1978) Properties of γ-aminobutyric acid receptor binding with (+)-[^{3}H]bicuculline methiodide in rat cerebellum. *Mol. Pharmacol.*, 14, 256-265.

Specific binding of (+)-[^{3}H]bicuculline methiodide ([^{3}H]BCM) to synaptic membranes of rat cerebellum, which most likely represents an interaction with the γ-aminobutyric acid (GABA) receptor, has been characterized further. Keeping the membranes stored frozen prior to the binding assay, as routinely done, slightly reduced (10–20%) the capacity of [^{3}H]BCM specific binding compared with freshly prepared membranes, and had a negligible effect on the affinity of BCM or GABA for the [^{3}H]BCM binding site, as shown by their K_i values is competing for [^{3}H]BCM specific binding: for BCM, K_i fresh = 270 ± 25 nM, and K_i frozen = 218 ± 21 nM; for GABA, K_i fresh = 490 ± 50 nM, and K_i frozen = 420 ± 55 nM. Specific [^{3}H]BCM binding was saturable, with an apparent dissociation constant (K_d) of 380 ± 20 nM. The maximal amount of specifically bound [^{3}H]BCM was 4.5 ± 0.2 pmoles/mg of protein. The amount of [^{3}H]BCM specifically bound was proportional to protein concentration and showed a broad pH optimum (pH 7-9). Similar amounts of [^{3}H]BCM were specifically bound at 25° and 37°. Equilibrium between the specific binding sites and [^{3}H]BCM was reached within 10 min. Incubation of the membranes at temperatures above 37° or with Triton X-100 resulted in a marked decrease of [^{3}H]BCM specific binding. Specific binding of [^{3}H]BCM was enhanced in the presence of SCN^-, I^-, or ClO_4^- in the incubation medium, amounting to as much as 50% of total binding. The results are compatible with a GABA receptor model with two binding sites, represented by specific [^{3}H]GABA binding and specific [^{3}H]BCM binding, respectively. The binding sites may reflect two conformational states of the GABA receptor, an agonist and an antagonist conformation.

INTRODUCTION

Binding studies with synaptic membranes may make possible the biochemical identification of neurotransmitter receptors by using labeled receptor agonists or antagonists as ligands (1). In previous attempts to identify the GABA[2] receptor, [^{3}H]GABA was used as ligand. GABA, however, also binds to glial and neuronal uptake sites and to enzymes metabolizing GABA. Thus Na^+-dependent [^{3}H]GABA binding (2, 3), in contrast to Na^+-independent [^{3}H]GABA binding, may not be

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[2] The abbreviations used are: GABA, γ-aminobutyric acid; BCM, (+)-bicuculline methiodide.

0026-895X/78/0142-0256$02.00/0

related to the GABA receptor (4).

(+)-Bicuculline or the chemically more stable (5) (+)-bicuculline methiodide appears to be more suitable as a ligand for GABA receptor binding studies. These compounds antagonize reversibly and rather selectively the synaptic action of GABA (6–11) without affecting the uptake or release of GABA or enzymes metabolizing GABA (4, 12–14). In fact, we have found that the characteristics of specific [^{3}H]BCM binding to rat cerebellar synaptic membrane fractions are compatible with the properties of the synaptic GABA receptor expected from neurophysiological evidence (15): specific [^{3}H]BCM binding was stereospecific and localized mainly in the synaptic membrane fraction. Among a variety of compounds tested, only GABA, GABA agonists, and GABA antagonists were competitors for [^{3}H]BCM specific binding sites. Their affinities were comparable to their respective neurophysiological potencies in mimicking or antagonizing the synaptic action of GABA (15).

In the present report we describe further characteristics of specific [^{3}H]BCM binding to the synaptic membrane fraction from rat cerebellum: The saturability of the specific (+)BM-binding sites, their temperature stability and susceptibility to detergents, and the influence of pH and ions on specific [^{3}H]BCM binding. A model of GABA receptor function is proposed.

MATERIALS AND METHODS

Preparation of membrane fractions. Cerebellar crude synaptic membrane fractions were prepared according to Zukin *et al.* (17). Briefly, male SPF rats (150–200 g, Füllinsdorf) were decapitated and the cerebella were rapidly removed and homogenized in 15 volumes of ice-cold 0.32 M sucrose solution with about 10 strokes in a Potter-Elvehjem glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 1000 × *g* for 10 min, and the supernatant was centrifuged at 20,000 × *g* for 20 min in order to obtain the crude mitochondrial pellet, which was resuspended in 20 volumes of distilled water and dispersed with a Brinkmann Polytron PT-10 (setting 6) for 30 sec. (Instead of the Polytron, a Potter-Elvehjem glass homogenizer fitted with a glass pestle could also be used for the hypoosmotic shock without any detectable difference in the binding characteristics of the final membrane fraction.) The resulting suspension was centrifuged at 8000 × *g* for 20 min. The supernatant was collected, and the pellet was carefully rinsed with the supernatant fluid to collect the soft, buffy upper layer of the pellet. The combined supernatant fraction was centrifuged at 48,000 × *g* for 20 min. In some cases this crude synaptic membrane fraction was immediately suspended in 50 mM Tris-HCl buffer, pH 7.4, for the binding assay ("fresh membranes"). Routinely, however, the membrane fraction was stored at −30° for at least 18 hr ("frozen membranes"). For the binding assay frozen pellets were resuspended in distilled water, maintained at room temperature for 20 min, centrifuged at 48,000 × *g* for 20 min, and resuspended in 50 mM Tris-HCl buffer, pH 7.4.

Binding assay. In the routine [^{3}H]BCM binding assay aliquots of previously frozen crude synaptic membrane fractions (2 mg of membrane protein) were placed in a shaking incubator at 25° for 15 min in 2 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaSCN and 5 nM [^{3}H]BCM in the absence and presence of 50 μM GABA. The assay was terminated by centrifugation at 48,000 × *g* for 10 min. The supernatant was decanted, and the pellet was rinsed with 5 ml and then 10 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.4. After solubilization of the pellet in 2 ml of Protosol, scintillant was added and radioactivity was counted (counting efficiency, 38%).

The binding observed in the presence of high concentrations of GABA (routinely 50 μM) was termed nonspecific. This nonspecific binding was subtracted from the binding observed in the absence of GABA (total binding) to obtain the binding termed specific. Determinations were routinely done in triplicate, with standard errors of the mean of less than 2%.

Protein determination. Protein was de-

termined according to Lowry *et al.* (18).

Stability of [³H]BCM. [³H]BCM (specific radioactivity, 8.0 Ci/mmole), prepared as described by Möhler and Okada (15), was more than 99% radiochemically pure as judged by thin-layer chromatography on silica gel (Eastman, sheet No. 6061, activated at 100°) in three solvent systems (methanol-acetone-HCl, 90:10:4, R_F 0.52; 1-butanol-glacial acetic acid-H_2O, 4:1:1, R_F 0.36; 1-butanol-ethanol-H_2O, 5:2:2, R_F 0.11). The R_F values corresponded to those of nonradioactive BCM visualized by Dragendorff reagent. [³H]BCM remained radiochemically pure for at least 8 months under storage at −60° in 50% (v/v) methanol. Possible contamination with ^3H_2O was less than 1% as judged from the radioactivity that could be evaporated from [³H]BCM samples.

There was no degradation of free or bound [³H]BCM during the binding assay. After termination of the assay aliquots of the supernatant were tested by paper chromatography (Whatman No. 1) in three different solvent systems (1-butanol-glacial acetic acid-H_2O, 4:1:1; 1-butanol-ethanol-H_2O, 5:2:2; methanol-acetone-HCl, 90:10:4). The radiaoctivity in the supernatant migrated as a single peak identical with that of nonradioactive BCM and the original [³H]BCM. The radioactivity bound in the pellet after rinsing was tested by suspending the pellet in methanol (2 ml/2 mg of protein), which extracted 95% of the radioactivity bound to the membranes. After centrifugation aliquots of the methanol extract were tested by paper chromatography as described above. No radioactivity except that of [³H]BCM could be detected.

RESULTS

Comparison of [³H]BCM binding to fresh and frozen membranes. Storing cerebellar membrane fractions at −30° had no marked effect on the characteristics of [³H]BCM binding (Fig. 1). The K_i values for the inhibition of specific [³H]BCM binding by unlabeled BCM were very similar for fresh (K_i = 270 ± 25 nM) or previously frozen (24 hr) membranes (15) (K_i = 218 ± 21 nM). Likewise, the K_i values for the inhibition of specific [³H]BCM binding by GABA were very similar using fresh (K_i = 490 ± 50 nM) or previously frozen (24 hr) membranes (K_i = 420 ± 55 nM). The capacity for both total and specific [³H]-BCM binding was reduced by 10% and 20% in membranes previously frozen for 30 min and 7 days, respectively, compared with fresh membranes (Table 1).

TABLE 1

Comparison of specific [³H]BCM binding to fresh and previously frozen membranes

Cerebellar synaptic membranes were prepared as described in MATERIALS AND METHODS, and a portion was assayed immediately in the routine binding assay (2 mg of protein, 50 mM Tris-HCl buffer, pH 7.4, 50 mM NaSCN, 5 nM [³H]BCM in the absence and presence of 50 μM GABA), whereas the other portions were kept frozen at −30° for various time intervals prior to the routine binding assay. The values are means ± standard errors of three experiments, each with triplicate determinations.

Time frozen	[³H]BCM specifically bound	
	dpm/assay	*% total binding*
0	3065 ± 163	34.4 ± 0.1
30 min	2608 ± 119	29.8 ± 0.8
1 day	2407 ± 71	29.2 ± 0.3
7 days	2392 ± 34	29.8 ± 0.2

Saturation of specific [³H]BCM binding. Specific [³H]BCM binding was saturable with increasing concentrations of [³H]BCM, in contrast to the nonspecifically bound [³H]BCM, which increased linearly (Fig. 2A). Scatchard analysis of the data for specific [³H]BCM binding resulted in a straight line (Fig. 2B), indicating that a single population of binding sites apparently was involved, with a dissociation constant (K_d) for BCM of 380 ± 20 nM. The maximal number of specific binding sites was 4.5 ± 0.2 pmoles/mg of protein. There was no evidence of another high-affinity BCM binding site from saturation experiments with [³H]BCM concentrations between 5 and 50 nM.

In order to evaluate the possibility of a cooperative interaction among BCM binding sites, the data for saturation of specific [³H]BCM binding from Fig. 2A were analyzed by Hill plots. A straight line was obtained with a Hill coefficient (n) of 0.99

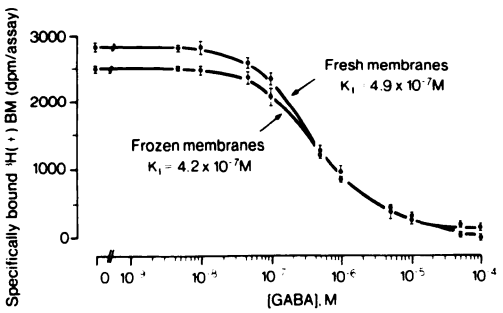

FIG. 1. *Competition by GABA for [³H]BCM specific binding sites of fresh and previously frozen cerebellar membranes*

The binding assay was performed in triplicate by incubating fresh or previously frozen (−30°, 24 hr) cerebellar synaptic membranes (2 mg of protein) for 15 min at 25° in 2 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaSCN, 5 nM [³H]bicuculline methiodide [³H(+)BM], and increasing concentrations of GABA. The 50% inhibitory concentration, IC_{50}, was calculated by log logit analysis and converted to K_i according to the equation $K_i = IC_{50}/(1 + C/K_d)$, where C is the concentration of radioactive ligand and K_d is its apparent dissociation constant.

± 0.01. In agreement with this value, a Hill coefficient of 0.95 ± 0.02 was found (Fig. 3) when the data (15) for the displacement of [³H]BCM by increasing concentrations of unlabeled BCM were used for calculation. Displacement of [³H]BCM by increasing concentrations of GABA, however, resulted in a Hill coefficient of 0.73 ± 0.03 (Fig. 3), which was significantly ($p < 0.01$) lower than that for BCM.

Specificity of [³H]BCM specific binding sites. It was shown previously that among a variety of compounds tested only GABA, GABA agonists, and GABA antagonists are bound to specific BCM binding sites with high affinity (15). This is supported further by the findings that the GABA-like compound *trans*-3-aminocyclopentane-1-carboxylic acid (19) inhibited specific [³H]BCM binding with a K_i of 1.23 ± 0.03 μM and that tubocurarine, a nonspecific GABA antagonist (20), inhibited binding with a K_i of 8.9 ± 0.6 μM. Furthermore, it was established that BCM did not inhibit the uptake of [³H]GABA (5 nM) in crude synaptosomal preparations from rat cerebellum, indicating that BCM, like bicuculline itself (17), was not bound to presynaptic GABA uptake sites.[3] In addition, [³H]BCM is not taken up into cerebellar synaptosomal preparations,[3] indicating that there are no specific uptake sites for BCM.

Effects of incubation time, protein concentration, pH, and temperature on specific [³H]BCM binding. The incubation time selected for the binding assay was 15 min, since at that time equilibrium between the ligand and the binding sites had been reached (Fig. 4). Specific [³H]BCM binding increased linearly with protein concentration up to 2.5 mg of membrane protein per assay (Fig. 5). The pH optimum of specific [³H]BCM binding was broad, ranging from pH 7 to at least pH 9 (Fig. 6). The amount of [³H]BCM specifically bound increased with incubation temperature, reaching a plateau at 25° that was maintained to at least 37° (Fig. 7).

Temperature stability and detergent susceptibility of [³H]BCM specific binding sites. The temperature stability of [³H]BCM specific binding sites was examined by maintaining suspensions of previously frozen membranes in 50 mM Tris-HCl buffer, pH 7.4, at various temperatures for 10 min prior to the 15-min routine binding assay at 25°. At temperatures exceeding 37° there was a steep decrease in the amount of specifically bound [³H]BCM (Fig. 8).

Prior treatment of freshly prepared membrane fractions with increasing concentrations of Triton X-100 (30 min, 37°) resulted in a dramatic decrease in the amount of specifically bound [³H]BCM (Table 2). This could have been due at least partly to the solubilization of membrane components, since the amount of membrane protein that could be recovered by centrifugation decreased with increasing Triton X-100 concentration (Table 2). Similar results were obtained after treating previously frozen membranes with Triton X-100.

Influence of ions on specific [³H]BCM binding. Specific [³H]BCM binding was especially pronounced in the presence of

[3] T. Okada and H. Möhler, manuscript in preparation.

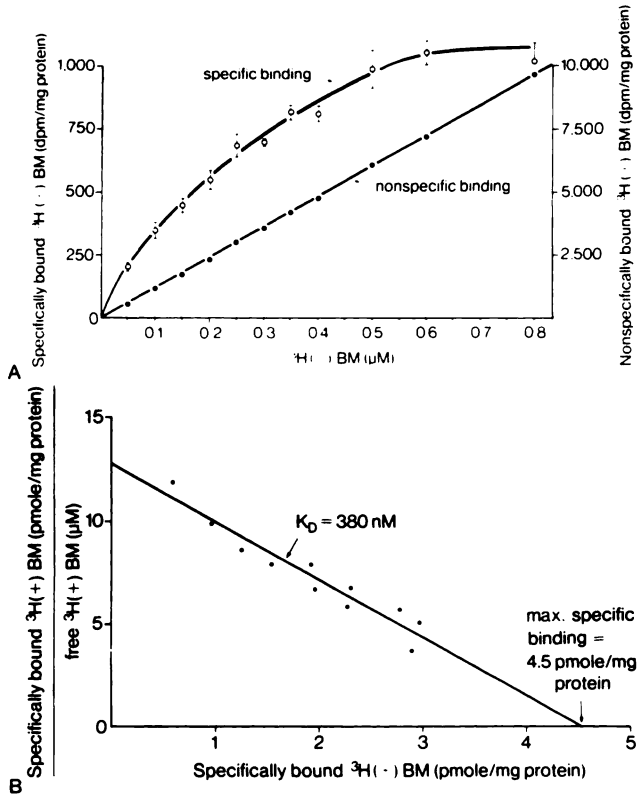

FIG. 2. *Saturation of specific [³H]BCM binding with increasing concentrations of [³H]BCM (A) and Scatchard plot of specific [³H]BCM binding (B)*

Cerebellar synaptic membranes (2 mg of protein) were incubated in triplicate in 2 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaSCN and increasing concentrations of [³H]bicuculline methiodide [³H(+)BM] in the absence and presence of 50 μM GABA, as described in MATERIALS AND METHODS. The values observed in the presence of 50 μM GABA are termed nonspecific. This nonspecific binding was subtracted from the binding observed in the absence of GABA (total binding) in order to obtain specific binding. For the values of nonspecifically bound [³H]BCM the standard errors of the mean were less than 1%, corresponding to about the diameter of the points shown in the graph. For the Scatchard plot the data for specific [³H]BCM binding from Fig. 2A were used. The K_d value for BCM was found to be 380 ± 20 nM (five experiments). The specific radioactivity of [³H]BCM was lowered 50-fold in this experiment by adding unlabeled BCM. Note the different scales of the abscissae in Fig. 2A.

the monovalent anions SCN^-, I^-, and ClO_4^- in the incubation medium (Table 3). In comparison with the 50 mM SCN^- routinely used in the binding assay, more specific binding sites were accessible in the presence of ClO_4^-, while I^- was less effective than SCN^-. The effect of ClO_4^- was remarkable: at 200 mM ClO_4^- specific [³H]BCM binding accounted for 50% of total binding. Other anions tested were much less effective than SCN^- or were without effect.

Monovalent cations had little effect on [³H]BCM binding, since total and specific [³H]BCM binding were unchanged using either NaSCN, NH_4SCN, or KSCN (50 mM) in the incubation medium (Table 3). However, the addition of divalent cations to 50 mM NaSCN decreased total [³H]BCM binding without decreasing specific [³H]BCM binding. Thus, in the presence of 10 mM $MnCl_2$ and 50 mM NaSCN, the amount of specifically bound [³H]BCM reached 39% of total [³H]BCM binding, compared with 30% in the presence of 50 mM NaSCN alone, the condition routinely used in the binding assay. It remains to be established whether the effects of the

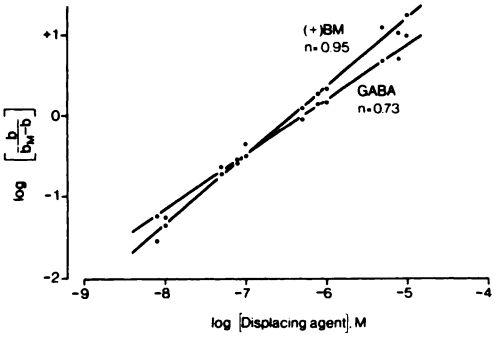

FIG. 3. *Hill plots of competition for [³H]BCM specific binding sites by BCM and GABA*

For the calculation of the Hill plots, the data on competition for [³H]BCM specific binding sites by [³H]BCM and unlabeled bicuculline methiodide [(+)BM] and GABA were taken from ref. 15, Fig. 1 and Fig. 2, respectively. Cerebellar synaptic membranes (2 mg of protein) were incubated at 25° for 15 min in 2 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaSCN, 5 nM [³H]BCM, and increasing concentrations of GABA or BCM as displacing agents. b is picomoles of [³H]BCM specifically bound; b_M is the maximal number of specific binding sites for [³H]BCM (picomoles per milligram of membrane protein). The points are the means from triplicate determinations, with standard errors of the mean ranging from 0.1% to 2.0%.

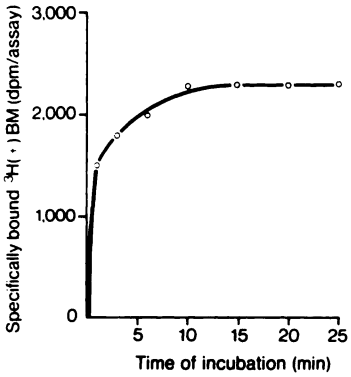

FIG. 4. *Time dependence of specific [³H]BCM binding*

Cerebellar synaptic membranes (2 mg of protein) were incubated for various times at 25° in 2 ml of Tris-HCl buffer, pH 7.4, containing 50 mM NaSCN and 5 nM [³H]bicuculline methiodide [³H(+)BM] in the presence and absence of 50 μM GABA as described in MATERIALS AND METHODS. The points are the means of two experiments, each performed in triplicate, with standard errors of less than 2%.

different ions on [³H]BCM specific binding reflect alterations in the affinity or the accessibility of BCM specific binding sites.

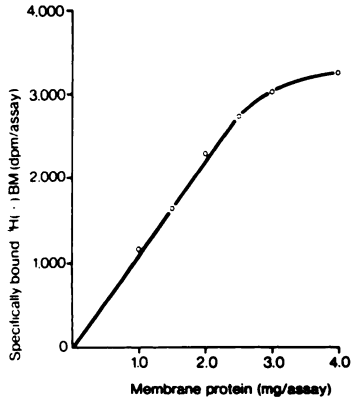

FIG. 5. *Protein dependence of specific [³H]BCM binding*

Various amounts of cerebellar synaptic membranes were incubated for 15 min at 25° in 2 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 50 mM NaSCN and 5 nM [³H]bicuculline methiodide [³H(+)BM] in the presence and absence of 50 μM GABA. The points are the means (SEM < 4%) of three experiments, each performed in triplicate.

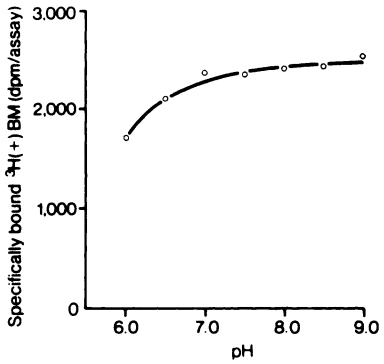

FIG. 6. *Effect of pH on specific [³H]BCM binding*

Cerebellar synaptic membranes (2 mg of protein) were incubated at 25° for 15 min in 2 ml of 50 mM Tris-HCl buffer of different pH values containing 50 mM NaSCN and 5 nM [³H]bicuculline methiodide [³H(+)BM] in the presence and absence of 50 μM GABA as described in MATERIALS AND METHODS. Each point represents the mean of two experiments, each performed in triplicate, with standard errors of less than 2%.

DISCUSSION

It was important to establish that the characteristics of the specific BCM binding sites would not be affected by keeping—as routinely done—the membranes stored frozen prior to the binding assay. The finding that the affinity of the specific BCM binding sites for GABA and BCM

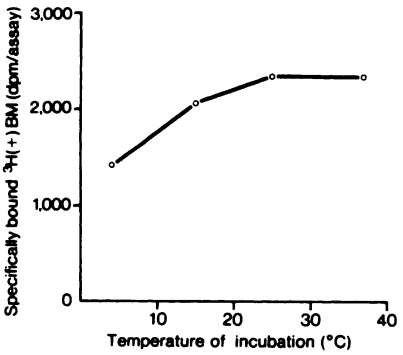

FIG. 7. *Effect of incubation temperature on specific [³H]BCM binding*

Cerebellar synaptic membranes (2 mg of protein) were incubated at various temperatures for 15 min in 2 ml of 50 mM Tris-HCl buffer, pH 7.4, 50 mM NaSCN, and 5 nM [³H]bicuculline methiodide [³H(+)BM] in the presence and absence of 50 μM GABA as described in MATERIALS AND METHODS. Each point represents the mean (SEM < 4%) of three experiments, each performed in triplicate.

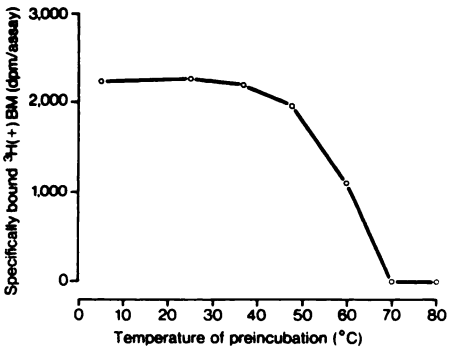

FIG. 8. *Temperature stability of [³H]bicuculline methiodide [³H(+)BM] specific binding sites*

The cerebellar synaptic membrane suspension in 50 mM Tris-HCl buffer, pH 7.4, prepared as described in MATERIALS AND METHODS ("frozen membranes"), was incubated at various temperatures for 10 min prior to the 15-min routine binding assay at 25°. Each point represents the mean of two experiments, each performed in triplicate, whose standard errors were less than 2%.

was not markedly affected by this treatment (Fig. 1) seems to justify the use of membranes stored frozen. Furthermore, only a small loss in the number of specific BCM binding sites resulted from this treatment (20% after keeping the membranes frozen for 7 days) (Table 1 and Fig. 1).

Evidence has been presented previously that specific [³H]BCM binding to cerebellar synaptic membranes most likely represents an interaction with the synaptic GABA receptor (15, 16). The finding that specific [³H]BCM binding is saturable (Fig. 2) supports this concept. The dissociation constant of BCM of 380 ± 20 nM (Fig. 2) obtained in saturation experiments is, as expected, similar to the K_i value of BCM (218 ± 21 nM) determined in experiments in which [³H]BCM specific binding was inhibited by increasing concentrations of unlabeled BCM (15). The discrepancy between K_d and K_i values may be due partly to an inaccuracy in the former value, since at higher [³H]BCM concentrations specific binding decreases as percentage of total binding (Fig.) 2A).

The broad range of the pH optimum (pH 7–9) of specific [³H]BCM binding (Fig. 6) suggests that the BCM binding site does not contain a component carrying a charged group with a pK in this pH range.

In the presence of ClO_4^- specific [³H]BCM binding amounts to as much as 50% of total binding (Table 3), and thus this anion seems to offer even better conditions for the [³H]BCM binding assay

TABLE 2

Effect of Triton X-100 on [³H]BCM specific binding sites

Suspensions of freshly prepared cerebellar membranes in 50 mM Tris-HCl buffer, pH 7.4, were treated with various concentrations of Triton X-100 for 30 min at 37°, centrifuged at 48,000 × *g* for 20 min, resuspended in Triton-free 50 mM Tris-HCl buffer, pH 7.4, and tested in the routine binding assay (25°, 15 min, in 2 ml of 50 mM Tris-HCl buffer, pH 7.4, 50 mM NaSCN, 5 nM [³H]BCM, in the absence and presence of 50 μM GABA). With increasing Triton X-100 concentrations less membrane protein could be recovered by centrifugation prior to the binding assay, resulting in protein concentrations below 2 mg/assay. The disintegrations per minute of [³H]BCM specifically bound were corrected for 2 mg of protein per assay in these cases.

Triton X-100	Protein content	[³H]BCM specifically bound	
%	*mg/assay*	*dpm/2 mg protein*	*% total binding*
0	2.0	2166	29.0
0.01	1.6	1211	18.4
0.05	1.1	0	
0.10	0.9	0	

TABLE 3

Effects of ions on amount of [³H]BCM specifically bound

Salt[a]	Concentration	Amount of [³H]BCM specifically bound		Salt[a]	Concentration	Amount of [³H]BCM specifically bound	
	mM	*% control*[b]	*% total binding*		*mM*	*% control*[b]	*% total binding*
NaSCN	10	35	11	KCl	2	0	0
	50	100	30		10	9	3
	100	117	33		50	5	2
	200	149	40		100	20	9
NH_4SCN	10	36	10		200	21	10
	50	98	28	$CaCl_2$	2	8	3
	100	106	28		10	20	11
	200	120	32		50	27	16
KSCN	10	37	11		100	40	21
	50	90	26	$MgCl_2$	2	0	0
	100	113	32		10	17	10
	200	130	37		50	30	15
NaOCN	10	4	1		100	26	15
	50	15	5	$MnCl_2$	2	4	7
	100	24	10		10	14	11
	200	34	14		50	19	15
NaN_3	10	20	7		100	29	20
	50	26	10	NaCN	50	19	4
	100	35	14	NH_4Cl	50	13	6
$NaClO_4$	10	51	16	Na_3BO_3	50	13	4
	50	150	37	NaSCN	50		
	100	192	44	$+CaCl_2$	1	95	31
	200	233	50		2	97	33
NH_4I	50	39	14		5	99	35
	100	40	15		10	102	37
	200	60	23	$+MgCl_2$	1	94	30
NaI	50	32	18		2	101	32
	100	41	23		5	92	32
	200	57	28		10	86	32
NaCl	50	7	6	$+MnCl_2$	1	102	32
	100	10	8		2	93	33
	200	13	11		5	93	34
					10	98	39

[a] The following salts were ineffective (less than 8% of control): 50–100 mM $(NH_4)_2SO_4$, sodium citrate, $NaHCO_3$, and Na_2HPO_4; 10–100 mM NaBr, NaF, sodium formate, $(NH_4)_2CO_3$, ammonium acetate, and malic acid; and 50 mM NH_4NO_3.

[b] The amount of [³H]BCM specifically bound in the presence of 50 mM NaSCN (2400 dpm/assay), as used in the routine binding assay, was taken as control (= 100%). The binding assay was performed in triplicate (SEM < 2%) as described in MATERIALS AND METHODS (2 mg of cerebellar synaptic membrane protein in 2 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 5 nM [³H]BCM and various amounts of different salts in the absence and presence of 50 μM GABA). In the absence of added ions specific binding varied between 0% and 4% of total binding.

than the SCN^- used routinely up till now. A related anion effect was originally observed for Na^+-independent specific [³H]-GABA binding (21), where SCN^- and I^- (ClO_4^- was not tested) enhanced the potency of bicuculline in displacing [³H]-GABA while the binding of GABA agonists to [³H]GABA binding sites was not affected. Thus it seems that these ions exert their influence selectively on the GABA antagonist binding site. It is unlikely that the effect of SCN^-, ClO_4^-, and I^- is due exclusively to their chaotropic properties (22), since nitrate, another chaotropic anion, although similarly active in enhancing the potency of bicucul-

line as a competitor for specific Na^+-independent [^{3}H]GABA-binding (21), was ineffective in eliciting specific [^{3}H]BCM binding (Table 3). On the other hand, it is interesting that SCN^- and ClO_4^- inhibit the uptake of I^- into several tissues, e.g., thyroid gland and choroid plexus. In these cases the anion selectivity was attributed to the partial specific volume (23) and/or the hydration energy of these anions (24).

[^{3}H]BCM binding (15), as well as Na^+-independent [^{3}H]GABA binding (4) seems to represent an interaction with the synaptic GABA receptor. It is striking that the [^{3}H]GABA binding site has a higher affinity for GABA agonists than the [^{3}H]BCM binding site: e.g., GABA itself has an IC_{50} of 0.020–0.027 μM for [^{3}H]GABA binding (21, 25) as compared with an IC_{50} of 0.4 μM for [^{3}H]BCM binding (15). On the other hand, the [^{3}H]BCM binding site has a higher affinity for GABA antagonists than the [^{3}H]GABA binding site: e.g., bicuculline has an IC_{50} of 0.07 μM for [^{3}H]BCM binding (15), compared with an IC_{50} of 5–15 μM for [^{3}H]GABA binding (21, 25). This inverse relationship may reflect a GABA receptor existing in two conformations, with the agonist conformation showing a high affinity for GABA agonists and a low affinity for GABA antagonists, while the inverse relationship holds for the antagonist conformation (Fig. 9).

Treating the membrane fraction with Triton X-100 (0.05%) elicits high-affinity binding of [^{3}H]GABA (21, 25) but abolishes [^{3}H]BCM binding (Table 3). This may be explained by a Triton-induced change of the GABA receptor from the antagonist to the agonist conformation. On the other hand, SCN^-, ClO_4^-, and I^-, which enhance [^{3}H]BCM binding (Table 3), may stabilize the antagonist conformation.

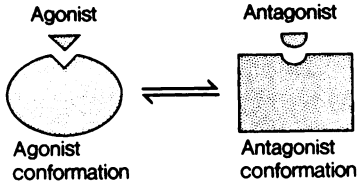

FIG. 9. *A model of GABA receptor function*

If GABA receptor function is based on a conformational change between the agonist and antagonist conformations, an equal number of agonist and antagonist binding sites in different brain regions would be expected. In cerebellar synaptic membrane fractions the maximal number of BCM binding sites (4.5 pmoles/mg of protein) correlates closely with the number of GABA binding sites (3.9 pmoles/mg of protein), which are characterized by a K_d of 27 nM (25). The maximal number of another population of GABA binding sites in cerebullum, characterized by a K_d of 5.9 nM, is somewhat lower (1.35 pmoles/mg of protein) (25). Since the functional significance of the presence of two GABA binding sites is unknown, a definite quantitative correlation between the maximal number of agonist and antagonist binding sites cannot be made at present. For other brain regions—although the maximal number of [^{3}H]GABA and [^{3}H]BCM binding sites is not yet known—the rank order of specific binding of [^{3}H]GABA (21) and [^{3}H]BCM is somewhat similar, except for cerebral cortex (15). Thus, at least in some rat brain areas, GABA receptors may contain varying numbers of GABA agonist or GABA antagonist binding sites, or [^{3}H]-GABA and [^{3}H]BCM may label different types of GABA receptors.

There does not seem to be a cooperative interaction between BCM binding sites, as shown by the Hill coefficient (n = 0.99 or 0.95). However, the Hill coefficient for the competition of GABA and [^{3}H]BCM for specific BCM binding sites (n = 0.73) may indicate a negative cooperative interaction between GABA binding sites and BCM binding sites, which would favor their localization on the same GABA receptor. In view of the possible heterogeneity of GABA receptors (26), however, further evidence is needed to support the proposed model of GABA receptor function.

ACKNOWLEDGMENT

We thank Dr. W. Haefely for critical reading of the manuscript.

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