γ -Aminobutyric Acid- and Benzodiazepine-induced Modulation of [^{35} S]-t-Butylbicyclophosphorothionate Binding to Cerebellar Granule Cells

VITTORIO GALLO,¹ BRADLEY C. WISE, FLORA VACCARINO, AND ALESSANDRO GUIDOTTI²

Laboratory of Preclinical Pharmacology, National Institute of Mental Health, Saint Elizabeths Hospital, Washington, D.C. 20032

Abstract

t-Butylbicyclophosphorothionate (TBPS) is a bicyclopophosphate derivative with potent picrotoxin-like convulsant activity that binds with high affinity and specificity to a Cl^- channel-modulatory site of the γ -aminobutyric acid (GABA)/benzodiazepine receptor complex. Using intact cerebellar granule cells maintained in primary culture, we have studied the modifications induced by GABA and diazepam on the ion channel-modulatory binding site labeled by [^{35} S]TBPS. At 25°C, and in a modified Locke solution, the [^{35} S]TBPS specific binding, determined by displacing the radioligand with an excess (10^{-4} M) of picrotoxin, was approximately 70% of the total radioactivity bound to the cells. [^{35} S]TBPS specific binding was saturable with a K_d of approximately 100 nM, a B_{max} of approximately 440 fmol/mg of protein, and a Hill coefficient of 1.18. Neither cerebellar astrocytes maintained in culture for 2 weeks nor a neuroblastoma cell line (NB-2A) exhibited any specific [^{35} S]TBPS binding. Muscimol (0.3 to 5 μM) enhanced and bicuculline (0.1 to 5 μM) inhibited [^{35} S]TBPS specific binding to intact cerebellar granule cells. The effect of muscimol and bicuculline on [^{35} S]TBPS binding was noncompetitive. Muscimol (0.1 to 5 μM) reversed bicuculline inhibition in a dose-dependent fashion but failed to reverse picrotoxin-induced inhibition. [^{35} S]TBPS binding was also modulated by benzodiazepine receptor ligands. The binding was increased by diazepam and decreased by 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylic acid methylester. Muscimol (0.05 μM) failed to reverse bicuculline inhibition in the absence of diazepam, but it became effective in the presence of 0.1 to 1 μM diazepam. This effect of diazepam was blocked by the benzodiazepine antagonist, ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazole 1,5a-1,4-benzodiazepine-3-carboxylate (Ro 15-1788). These results confirm that [^{35} S]TBPS binds to the modulatory site of the GABA/benzodiazepine/ionophore complex and suggest that GABA/benzodiazepine receptor stimulation exposes to the outer surface of the cell membrane those TBPS recognition sites that under resting conditions are located in the inner membrane portion of the Cl^- ionophore.

A specific ionophore activated by the inhibitory neurotransmitter

γ -aminobutyric acid (GABA) changes neuronal membrane excitability by regulating Cl^- ion conductance. This ionophore operates within the regulatory domain of a receptorial system currently termed the GABA/benzodiazepine receptor complex (Costa and Guidotti, 1979; Olsen, 1982; Guidotti et al., 1983; Barnard et al., 1984; Olsen et al., 1984). This complex modulates the conformational changes of the ionophore protein, thereby orchestrating the opening and closing of the Cl^- channel. Several specific ligands of the benzodiazepine recognition site can regulate the efficiency whereby GABA changes the conformation of the ionophore. Thus, these ligands can increase or decrease GABA-mediated responses by altering the kinetics of the channel activity rather than the conductance of activated channels (Study and Barker, 1981; Segal and Barker, 1984; Bormann and Clapham, 1985). The Cl^- ionophore in addition to the GABA/benzodiazepine receptorial domain has another important domain, probably located in the inner portion of the ionophore and distal from the outer surface of the neuronal membrane, that recognizes ^3H -dihydropicrotoxin (Ticku et al., 1978; Ticku and Olsen, 1979). However, the possibility of using this probe to study the latter receptorial domain has been hampered by the high nonspecific binding of this ligand.

More recently, these difficulties have been overcome by the discovery (Squires et al., 1983) of [^{35} S]-t-butylbicyclophosphorothionate ([^{35} S]TBPS) (a cage convulsant of the bicyclopophosphate family) which binds with high affinity and great specificity to a membrane recognition site similar if not identical to that labeled by dihydropicrotoxin (Ticku and Maksay, 1983; Squires et al., 1984; Supavilai and Karobath, 1984). Using TBPS, several groups have now studied the characteristics of the Cl^- ionophore channel and its interaction with GABA and benzodiazepine recognition sites, both in crude synaptic membranes and in highly purified and solubilized preparations of the GABA/benzodiazepine receptor complex (Squires et al., 1983; Ticku and Maksay, 1983; Barnard et al., 1984; Olsen et al., 1984; Supavilai and Karobath, 1984). However, the use of crude brain membranes or the purified GABA/benzodiazepine/ Cl^- ionophore complex represents a limitation for a biochemical study of the allosteric regulation of an ion channel. For this type of study nerve cells maintained in culture offer the advantage of being an intact system in which the cell membrane is in direct contact with the extracellular environment and therefore available for binding studies to surface receptors (Hosli et al., 1980; Ticku et al., 1980; White et al., 1981). In this study we used primary cultures of rat cerebellar interneurons which have been shown to be greatly enriched in granule cells (Gallo et al., 1982; Levi et al., 1984) and which differentiate morphologically (Kingsbury et al., 1985) and biochemically (Balazs et al., 1982; Gallo et al., 1982, 1985; Levi et al., 1984). Both autoradiographic (Mohler et al., 1981) and biochemical studies (Meier and Schousboe, 1982; Allen and Dutton, 1984) have previously indicated that cerebellar granule cells possess a high density of GABA and benzodiazepine receptors. Therefore, we approached the study of [^{35} S]TBPS binding in these

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¹ Present address: Laboratorio di Fisiopatologia, Istituto Superiore Sanita, Viale Regina Elena 299, 00161 Roma, Italy.

² To whom correspondence should be addressed.

cells in order to be able to characterize its modulation by GABA and benzodiazepine agonists and antagonists in an intact, differentiated, and homogeneous population of neurons.

Materials and Methods

Cell cultures

Cerebellar neuronal cultures were obtained from 8-day-old rats as previously described (Wilkin et al., 1976; Levi et al., 1984). Cells were resuspended and seeded in the following culture medium: Eagle's basal medium (BME) (Flow Laboratories), 10% fetal calf serum (FCS) (Grand Island Biological Co. (GIBCO)), 25 mM KCl (final concentration), 2 mM glutamine, and 100 μ g/ml of gentamycin (GIBCO). Cells were plated (2.5×10^6 cells/dish) onto 35-mm Falcon dishes coated with 5 μ g/ml of poly-L-lysine ($M_r = 70,000$ to $150,000$) and were incubated at 37°C in an atmosphere of 5% CO₂/95% air. Arabinosylcytosine (ara-C; 10 μ M) was added to the cultures after 16 hr *in vitro* to inhibit the replication of non-neuronal cells. Granule cells were used after 8 days *in vitro*.

Cerebellar astrocytic cultures were prepared as described previously (Woodhams et al., 1981) and plated on plastic 35-mm Falcon dishes. The culture medium was BME containing 10% FCS, 2 mM glutamine, and 100 μ g/ml of gentamycin. The KCl concentration in this medium was 4.7 mM and no ara-C was added to these cultures. Astroglial cultures were used after 14 days *in vitro*.

Neuroblastoma cells (NB-2A) were cultured in Dulbecco's modified Eagle's medium (Flow Laboratories) containing 10% FCS and 50 units/ml of penicillin and 50 μ g/ml of streptomycin (GIBCO). Cells were subcultured 10 times and finally plated on 35-mm dishes 2 days before the binding experiment.

[³⁵S]TBPS binding studies

Intact cells. TBPS binding studies were carried out on monolayer cell cultures plated in 35-mm dishes. Intact cells were washed twice with 2 ml of Locke's solution (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO₃, 2.3 mM CaCl₂, 1.0 mM MgCl₂, 5.6 mM glucose, 5 mM HEPES, pH 7.4) and then incubated at 25°C with 1 ml of Locke's solution for the appropriate time in the presence of [³⁵S]TBPS. To stop the assay the cells were rapidly washed (three washes in 8 sec) and resuspended in 1 ml of 0.1 N NaOH. After 2 hr at 0°C, 0.5 ml was counted for radioactivity determination and 0.1 ml was used for protein estimation (Lowry et al., 1951).

Nonspecific binding was taken as [³⁵S]TBPS bound to the cells in the presence of 10^{-4} M unlabeled TBPS, *n*-butylbicyclophosphate, or picrotoxin. The binding of the ³⁵S-ligand to the plastic dish was evaluated by control samples without cells, and the nonspecific binding to the cells was calculated after subtracting the counts bound to the plastic dishes. The binding of TBPS to the dish was insensitive to picrotoxin or to unlabeled bicyclophosphate and represented 10 to 15% of the amount of labeled [³⁵S]TBPS bound nonspecifically to the cells. Unless otherwise stated, a 30-min incubation time was chosen to study the specific binding of [³⁵S]TBPS.

Crude P₂ membranes. When the binding of [³⁵S]TBPS was carried out in disrupted membrane preparations, the cells were harvested in H₂O, homogenized with a Polytron (setting 6), centrifuged at 48,000 $\times$ g, and frozen overnight. After washing the membranes three times with either 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, or Locke's solution, aliquots of the membranes (100 to 250 μ g of protein) were incubated with [³⁵S]TBPS for 90 min at 25°C either in 200 mM KBr, 5 mM Tris-HCl, pH 7.5, 1 mM EDTA, or Locke's solution. The reaction was terminated by filtration through Whatman GF/C filters.

High pressure liquid chromatography (HPLC) analysis of TBPS

[³⁵S]TBPS purity after binding to the cell cultures was determined by HPLC. Aliquots of NaOH extract of cells or supernatant medium from cells which were incubated with different concentrations of TBPS (5 to 200 nM) were brought to pH 3 with concentrated HCl and injected into an HPLC system equipped with a 4 mm $\times$ 25 cm Bio-Rad C-18 reverse phase column maintained at 25°C.

The column was equilibrated with 0.1% trifluoroacetic acid in water and then eluted with a linear gradient of acetonitrile in H₂O from 0 to 60% in 60 min, at a flow rate of 1 ml/min. [³⁵S]TBPS was retained by the column and eluted as a single peak around 40 min.

GABA determination

Cells were homogenized in 0.4 M HClO₄/50 nmol of citrulline/ml as standard and were centrifuged at 33,000 $\times$ g for 15 min. The supernatant was injected into an HPLC system equipped with 3.1 mm $\times$ 25 cm Bio-Rad

Aminex A9 cation exchange resin column maintained at 25°C. Amino acids were eluted from the column by a step gradient buffer and quantified fluorimetrically as described by Schmid et al. (1980).

Materials

Poly-L-lysine, ara-C, and picrotoxin were from Sigma Chemical Co. (St. Louis, MO). Bicuculline methiodide was from Pierce Chemical Co. (Rockford, IL), and diazepam was from Hoffman-La Roche (Basel, Switzerland). [³⁵S]TBPS (55.3 Ci/mmol) and unlabeled TBPS were from New England Nuclear (Boston, MA). Muscimol was a generous gift of Dr. P. Krosgaard-Larsen (Royal Danish School of Pharmacy, Copenhagen, Denmark). *n*-Butylbicyclophosphate was a generous gift of Dr. J. Collins (Department of Chemistry, City of London Polytechnic, London, England).

Results

Characteristics of [³⁵S]TBPS binding to cerebellar granule cells. [³⁵S]TBPS specific binding to an intact monolayer of 8-day-old cerebellar cultured cells (measured in the presence of 10^{-4} M unlabeled TBPS or picrotoxin at 25°C; see "Materials and Methods") rapidly increases with time of incubation and reaches equilibrium after approximately 15 min (Fig. 1). The binding was saturable for concentrations of TBPS higher than 200 nM (Fig. 1), and the ratio of total versus nonspecific binding was approximately 3:1 for concentrations of ligand between 1.25 and 50 nM (see Fig. 1).

The results of the [³⁵S]TBPS binding experiments analyzed by Scatchard and Hill plot (Fig. 1) revealed a single binding component (slope, 1.18; $r = 0.99$) with a K_d between 100 and 110 nM and a B_{max} value of approximately 440 fmol/mg of protein. Specific [³⁵S]TBPS binding was equally and maximally blocked by excess unlabeled TBPS, *n*-butylbicyclophosphate, and picrotoxin. Table I shows the K_i values of these three different ligands for the TBPS-binding site. TBPS was the most potent, whereas *n*-butylbicyclophosphate was two orders of magnitude less potent than TBPS. Picrotoxin shows an intermediate potency between the two bicyclophosphates. Using picrotoxin, maximal inhibition of binding was achieved with concentrations greater than 5×10^{-5} M. Therefore, for routine experiments 10^{-4} M picrotoxin was used as displacer to determine [³⁵S]TBPS specific binding. In order to establish the specific [³⁵S]TBPS binding under standard assay conditions, labeled ligand and picrotoxin were added at the same time. However prelabeling the cells with 10 nM [³⁵S]TBPS for 15, 30, or 45 min before the addition of 10^{-4} M picrotoxin resulted in the same number of apparent [³⁵S]TBPS-binding sites as that measured in cells incubated without preincubation under standard assay conditions.

[³⁵S]TBPS specific binding was found to be temperature dependent since, when cells were incubated for 30 min with 10 nM [³⁵S]TBPS at 0°C, the specific binding was reduced by 60% compared to binding at 25°C. Furthermore, the total omission of Na⁺ ions from the incubation medium by replacement with choline produced a 50% decrease in the amount of [³⁵S]TBPS specifically bound to the

TABLE I
Apparent inhibitory constant (K_i) of various convulsants for [³⁵S]TBPS binding to intact cerebellar granule cells

K_i values were calculated according to the method of Cheng and Prusoff (1973). $K_i = IC_{50}/(1 + L/K_d)$; where L is the concentration of the radioactive ligand and K_d is the value of the dissociation constant as calculated by Hill plot analysis in Figure 1. Drugs were tested using six concentrations in triplicate with 10 nM [³⁵S]TBPS as ligand. The mean values of the specific [³⁵S]TBPS binding at various drug concentrations were used to calculate the IC_{50} values by log PROBIT analysis. For each drug concentration the SEM was less than 5%.

Compound	K_i (μ M)
TBPS	0.094
Picrotoxin	0.230
<i>n</i> -Butylbicyclophosphate	6.410

Figure 1. Characteristics of $[^{35}\text{S}]$ TBPS binding to intact cerebellar granule cells. A, Plot showing nonspecific binding (binding measured in the presence of 10^{-4} M picrotoxin) and specific binding (total binding minus nonspecific binding) as a function of $[^{35}\text{S}]$ TBPS concentration. Incubation time was 30 min. B, Scatchard plot of the specific binding data. C, Hill plot of the same data. D, Plot showing the time course of total, specific and nonspecific binding of $[^{35}\text{S}]$ TBPS (10 nM). Each point represents the mean value obtained from a total of six to eight culture dishes processed in two separate experiments. The dishes were from two different culture batches. For each point the SE was less than 5% of the mean value.

nerve cells (TBPS binding using 10 nM $[^{35}\text{S}]$ TBPS was 35 ± 2.8 fmol/mg of protein in control and 17 ± 2.1 fmol/mg of protein in the absence of Na^+). HPLC analysis of the $[^{35}\text{S}]$ TBPS present in the medium or bound to the cells at the end of the incubation indicates that $[^{35}\text{S}]$ TBPS has not been metabolized as more than 95% of the radioactivity emerges from the HPLC reverse phase column as a single peak with 40% acetonitrile. The binding of the radioactive labeled bicyclic phosphoramide was also studied in other cell cultures. Cerebellar astroglial-enriched cultures, containing 85 to 90% glial fibrillary acidic protein-positive cells (Woodhams et al., 1981), did not bind $[^{35}\text{S}]$ TBPS specifically (31 ± 2 and 30 ± 1.5 fmol/mg of protein in the absence and presence of 10^{-4} M picrotoxin, respectively; $n = 4$, 25 nM $[^{35}\text{S}]$ TBPS), and specific binding of this ligand was also absent in the NB-2A neuroblastoma cell line (29 ± 1 and 28.5 ± 1 fmol/mg of protein in the presence and absence of 10^{-4} M picrotoxin, respectively; $n = 3$, 25 nM $[^{35}\text{S}]$ TBPS).

Modulation of TBPS binding by GABA and benzodiazepines. Granule cell-enriched primary cultures and their crude membrane preparations show both GABA and benzodiazepine high affinity,

saturable binding sites (Meier and Schousboe, 1982; Allen and Dutton, 1984). Moreover, as expected by the presence of a few GABAergic interneurons that take up $[^3\text{H}]\text{GABA}$ (Currie and Dutton, 1980; Aloisi et al., 1985), these cultures also contain GABA (8.2 ± 0.3 nmol/mg of protein; $n = 5$). Therefore, in a subsequent series of experiments we studied the relationship among GABA, benzodiazepines, and TBPS-binding sites in the cultured cells. It has been described in brain membranes (Squires et al., 1983; Supavilai and Karobath, 1984) that micromolar concentrations of muscimol (a GABA receptor agonist) lead to an allosteric inhibition of $[^{35}\text{S}]$ TBPS binding. Similar to these results, we find that 5 μM muscimol produced a significant inhibition of $[^{35}\text{S}]$ TBPS binding to cerebellar neuronal culture membranes assayed in the presence of 200 mM KBr (TBPS binding using 2.5 nM $[^{35}\text{S}]$ TBPS was 68 ± 4 fmol/mg of protein in control and 36 ± 1.5 fmol/mg of protein in 5 μM muscimol-treated membranes; $n = 4$) or in membranes assayed in the presence of Locke's solution (TBPS binding using 5 nM $[^{35}\text{S}]$ TBPS was 110 ± 10 fmol/mg of protein in control and 25 ± 5 fmol/mg of protein in 5 μM muscimol-treated membranes; $n = 3$). The inhibitory

Figure 2. Muscimol-induced enhancement of [^{35} S]TBPS binding in intact cultures treated with and without bicuculline. Control cultures or cultures treated with 5 μ M bicuculline methiodide just prior to receiving increasing concentrations of muscimol were incubated together with 12.5 nM [^{35} S]TBPS. Each point is the mean $\pm$ SEM obtained from a total of six to eight dishes processed in two separate experiments. The dishes were from two different culture batches. *, $p < 0.05$ when compared with cultures without muscimol.

Figure 3. Bicuculline-induced inhibition of [^{35} S]TBPS binding to intact cerebellar granule cells. Each histogram represents the mean value $\pm$ SEM obtained from a total of six to nine dishes processed in three separate experiments. The dishes were from three separate culture batches. Water-soluble bicuculline methiodide was used in the experiments. The concentration of [^{35} S]TBPS was 12.5 nM.

effect of muscimol was blocked by the GABA antagonist bicuculline methiodide added to the incubation medium in a concentration of 5 μ M. Bicuculline (5 μ M) itself increases the binding of [^{35} S]TBPS in the presence of 200 mM KBr from 68 to 150 fmol/mg of protein. In contrast to these experiments with crude membrane preparations, [^{35} S]TBPS binding to intact cultured cells was increased by muscimol. The maximal effect of muscimol was observed at concentrations in the range of 1 to 5 μ M (Fig. 2). Higher muscimol concentra-

TABLE II

Bicuculline-induced decrease in the density of specific [^{35} S]TBPS-binding sites to intact cerebellar granule cells

$B_{\max}$ (fmol/mg of protein) values were estimated from Scatchard analyses; K_d (nM) and Hill coefficient values were estimated from Hill plots. The results are the average of two experiments, each run in triplicate. In each experiment, the SE of the mean was less than 5%. [^{35} S]TBPS was used in concentrations ranging from 5 to 200 nM. Bicuculline is bicuculline methiodide.

Treatment	$B_{\max}$	K_d	Hill Slope
Control	420	95	1.06
Bicuculline (0.5 μ M)	270	98	1.08

TABLE III

Modulation by benzodiazepine receptor ligands of [^{35} S]TBPS binding to intact cerebellar granule cells

Eight-day-old cultures were incubated with 15 nM [^{35} S]TBPS and the various additions. Each point is the mean $\pm$ SEM obtained from a total of six dishes processed in two separate experiments.

Additions	[^{35} S]TBPS Binding (fmol/mg of protein)	
	Control	Ro 15-1788 (10 μ M)
Control (solvent)	49 $\pm$ 3.0	40 $\pm$ 3.5
Diazepam (1 μ M)	54 $\pm$ 2.2 ^a	39 $\pm$ 1.5
DMCM (1 μ M)	28 $\pm$ 1.0 ^a	40 $\pm$ 3.5

^a $p < 0.01$ when compared with control cells.

Figure 4. Potentiation by diazepam of [^{35} S]TBPS binding to intact cerebellar granule cells. Cells were incubated under standard conditions with solvent (control), 5 μ M bicuculline methiodide, or 5 μ M bicuculline + 0.05 μ M muscimol just before the addition of 10 nM [^{35} S]TBPS. Each value is the mean $\pm$ SEM of triplicate samples. Using 10⁻⁶ M diazepam, results similar to those reported in the figure were obtained in three separate experiments. *, $p < 0.05$ when compared with respective controls.

tions (50 μ M) increased [^{35} S]TBPS binding to a much lesser extent (data not shown). Moreover, in intact cell preparations bicuculline caused a dose-dependent inhibition of TBPS binding (Fig. 3). The effect of bicuculline on the binding was to change the receptor density rather than the affinity of [^{35} S]TBPS for its recognition site (Table II). Muscimol reversed the inhibition produced by bicuculline (Fig. 2), but doses of muscimol which caused an increase and completely reversed the bicuculline inhibition of TBPS specific binding failed to reverse the inhibition caused by 5 μ M picrotoxin.

TBPS binding was modulated by benzodiazepine receptor ligands. Table III shows a 28% stimulation of [^{35} S]TBPS binding induced by 10⁻⁶ M diazepam and a 34% decrease in binding induced

TABLE IV

Blockade by Ro 15-1788 of diazepam plus muscimol-induced increase of [^{35} S]TBPS binding in cerebellar cells incubated with bicuculline

Eight-day-old cultures were incubated with 15 nM [^{35} S]TBPS. Each point is the mean $\pm$ SEM of six dishes processed in the same experiment. Bicuculline methiodide (5 μ M), muscimol (0.05 μ M), and Ro 15-1788 (10 μ M) were added together with [^{35} S]TBPS.

Additions	[^{35} S]TBPS Binding (fmol/mg of protein)		
	Control	Bicuculline + Muscimol	Bicuculline + Muscimol + Ro 15-1788
Control (Solvent)	48 $\pm$ 4.2	19 $\pm$ 0.5	17 $\pm$ 0.5
Diazepam (0.1 μ M)	45 $\pm$ 2.0	30 $\pm$ 3.5 ^a	18 $\pm$ 0.8
Diazepam (1 μ M)	60 $\pm$ 3.8 ^a	41 $\pm$ 4.5 ^a	16 $\pm$ 1.0

^a $p < 0.01$ when compared to the respective group of controls.

Figure 5. Potentiation by diazepam of muscimol-induced increase of [^{35} S]TBPS binding to intact cerebellar granule cells incubated with bicuculline. Cells were incubated with 5 μ M bicuculline methiodide, 10 nM [^{35} S]TBPS, and with increasing concentrations of muscimol. When present, the concentration of diazepam (DIAZ) was 1 μ M. Each point is the mean $\pm$ SEM of triplicate samples. *, $p < 0.01$ when compared with the respective controls.

by 10^{-6} M 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylic acid methylester (DMCM). Ro 15-1788 (ethyl-8-fluoro-5,6-dihydro-5-methyl-6-oxo-4H-imidazole 1,5a-1,4-benzodiazepine-3-carboxylate), a postulated benzodiazepine receptor antagonist (Hunkeler et al., 1981), failed to stimulate or to inhibit TBPS binding per se, but antagonized the stimulatory action of diazepam or the inhibitory effect of DMCM (Table III). In another group of experiments we have found that concentrations of diazepam between 10^{-8} and 10^{-7} M are ineffective in control cultures or in cultures incubated with 5 μ M bicuculline (Fig. 4). However, these doses of diazepam reversed the inhibition of [^{35} S]TBPS binding induced by bicuculline in the presence of a small and, per se, ineffective dose of muscimol (see Fig. 4). Also, this effect of diazepam was blocked by Ro 15-1788 (Table IV). In cell cultures treated with 5 μ M bicuculline the dose-response curve for muscimol stimulation of [^{35} S]TBPS binding was shifted to the left in the presence of 10^{-6} M diazepam; however, the maximum muscimol dose-response curve was not increased (Fig. 5).

Discussion

Immunostaining of cerebellar neuronal cultures with a batch of cell markers has shown that 95% of the total cell population consists of tetanus toxin-positive neuronal elements (Kingsbury et al., 1985). About 95% of these neuronal elements are granule cells (Currie and Kelly 1981; Levi et al., 1984), and only a small percentage are GABAergic interneurons as evidenced by glutamic acid decarboxylase-positive immunoreactivity (Aloisi et al., 1985). Cerebellar granule cells either *in vivo* or in culture possess GABA and benzodiazepine recognition sites (Mohler et al., 1981; Meier and Schousboe, 1982; Allen and Dutton, 1984). Thus, the granule cell-enriched cerebellar cultures are an attractive model to study the molecular mechanisms regulating the GABA/benzodiazepine/ Cl^- ionophore receptor complex in a homogeneous and intact population of neurons. We have found in the present studies that cerebellar granule cells maintained in a physiologic milieu at 25°C contain a binding site for TBPS, a Cl^- channel-modulatory ligand. This site is regulated by GABA and benzodiazepine recognition sites. The density of TBPS recognition sites (40 to 80 pmol/gm of wet weight, depending on the amount of GABA present in the incubation medium) measured in 8-day-old cerebellar cultures compares favorably with the high number of TBPS-binding sites measured in membranes obtained from cerebellum of rat brain (Squires et al., 1983) and with the high density of TBPS binding in the granule cell layer as observed by Wamsley et al. (1983) using the autoradiographic binding technique in rat cerebellar slices.

The affinity of the binding for TBPS (100 to 110 nM) in the cerebellar culture preparations is lower than that reported for the binding of TBPS to rat brain crude synaptic membranes treated with 200 mM KBr ($K_d = 25$ nM) (Squires et al., 1983) or 200 mM NaCl ($K_d = 60$ nM) (Supavilai and Karobath, 1984). The lower affinity is not due to metabolism, penetration, or trapping of TBPS by the cells. In fact, more than 95% of the TBPS present in the medium or bound to the cells was found to be authentic TBPS when analyzed by HPLC. Moreover, the binding is temperature and ion dependent, responds to the pharmacological characteristics of specificity including modulation by muscimol, diazepam, and DMCM, and shows constant values under different prelabeling conditions. The binding of [^{35} S]TBPS is also cell specific because it is absent in cultures of astrocytes and in the neuroblastoma NB-2A cell line, but is present to a much lower extent (approximately 1/10 of the level in granule cells) in primary cultures of adrenal chromaffin cells which have recently been reported to have GABA-mediated Cl^- channel opening events (Bormann and Clapham, 1985; Kataoka et al., 1984). It is worth mentioning that the NB-2A cell line also has GABA- and benzodiazepine-binding sites (Baraldi et al., 1979); however, the density of these sites is approximately one-third the density of the sites in granule cells maintained in primary culture when measured under identical experimental conditions (this laboratory: F. Vaccarino, manuscript in preparation). Perhaps, the neuroblastoma NB-2A cell line, due to its oncogenic growth characteristics or to the constant presence of penicillin in the growth medium, has lost the ability to express a GABA/benzodiazepine/ionophore complex with supramolecular characteristics similar to those found in normal neuronal cells.

An intriguing observation made in these experiments is that GABA has opposite effects on TBPS binding in crude P_2 membranes and in intact cell cultures. When measured in the presence of 200 mM KBr, or in Locke's solution, the binding of TBPS to P_2 membranes obtained from granule cell-enriched cultures is maximal in the absence of GABA. In contrast, the binding of TBPS to intact cell surface receptors is virtually nonexistent in the absence of GABA (see Figs. 2 and 3 where bicuculline almost completely abolishes the binding and muscimol reverses this inhibition in a dose-related fashion) and increases proportionally with the addition of GABA or GABA mimetics in the incubation medium. The results obtained in disrupted membrane preparations confirm and extend previous results (Squires et al., 1983; Ticku and Maksay, 1983; Stephens et

al., 1984; Supavilai and Karobath, 1984) and suggest that in granule cells TBPS-binding sites are constituents of the GABA receptor Cl^- ionophore complex. To explain the absolute dependence on GABA for TBPS binding to intact cerebellar granule cells, we have to consider that GABA receptor-mediated changes of TBPS binding involve modification of the apparent receptor density. In particular, GABA and GABA mimetics unmask and bicuculline masks TBPS (see Table II) binding sites at the cell membrane surface. A possible interpretation of this set of facts is that in the absence of receptor occupancy by GABA, the binding sites for TBPS are buried in the cell membrane structure, perhaps in association with closed Cl^- ionophore pores. TBPS-binding sites become available at the membrane surface when GABA occupies its receptor and subsequently opens the Cl^- ionophore. It follows that in intact cells TBPS will bind to its receptors when the recognition site is in the low affinity state, that being the receptor complex occupied by GABA. This would help to explain the somewhat lower affinity of TBPS binding seen in intact cultured cells compared to disrupted membrane preparations.

Biochemical and electrophysiological experiments suggest that the molecular mechanisms by which benzodiazepines facilitate GABAergic transmission involve an allosteric modification of the GABA receptor system (Study and Barker, 1981; Guidotti et al., 1983; Ferrero et al., 1984; Haefely 1984). Here we show that diazepam (0.01, 0.1, and $1 \mu\text{M}$) fails by itself to enhance or has only marginal enhancing effects on the binding of TBPS to cerebellar granule cells in the presence of a maximal stimulating concentration of muscimol or endogenous GABA. However, a look at the shift to the left by diazepam of the muscimol dose-response curve (Fig. 5) and the observation that this effect of diazepam is blocked by Ro 15-1788 (Table IV) suggest that the GABA receptor-mediated regulation of TBPS binding can be modulated by benzodiazepine receptor occupancy. Furthermore, these results support the concept that, in this intact cell preparation, benzodiazepines facilitate the action of GABA either by increasing the frequency of coupling between GABA recognition sites and the TBPS chloride channel-modulatory sites or by enhancing the affinity of GABA for the GABA_A -binding sites. The existence of an allosteric interaction between GABA and benzodiazepine binding is supported by data obtained in a separate study (F. Vaccarino and A. Guidotti, manuscript in preparation), where it has been observed that [^3H]muscimol and [^3H]benzodiazepines bind to the intact cultures with affinity constants and B_{max} values comparable to those measured in cerebellar granule cell membranes or in rat brain membranes. Furthermore, in intact cerebellar granule cells, the benzodiazepines significantly increase the high affinity binding sites of [^3H]muscimol, and muscimol increases the affinity of the [^3H]benzodiazepines for their recognition sites (F. Vaccarino and A. Guidotti, manuscript in preparation).

To evaluate further the validity of the TBPS binding to cerebellar granule cells as a model to study GABA/benzodiazepine/ Cl^- ionophore protein interaction, we have also studied whether agonists, antagonists, or inverse agonist ligands for benzodiazepine receptors have a bidirectional effect on base line or muscimol-stimulated TBPS binding. Since the binding of TBPS to intact cells is modulated in opposite directions by prototypic anxiolytics or anxiogenic drugs and this modulation is blocked by the benzodiazepine receptor antagonist, Ro 15-1788, it is suggested that TBPS binding to primary cultures of granule cells could be used as a simple *in vitro* test to predict whether a given ligand for the benzodiazepine receptor has agonistic or antagonistic effects *in vivo*.

In conclusion, these experiments indicate that TBPS binding can be studied in membranes or in intact living cultures of rat cerebellar granule cells. However, the difference in binding between the membrane preparation and the intact system points to the importance of the intact system in elucidating the biochemical and physiological mechanisms involved in the allosteric interactions occurring among the different components of the GABA/benzodiazepines/ Cl^- ionophore complex.

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