0026-895X/91/0394-05$03.00/0

MOLECULAR PHARMACOLOGY, 39:394–398

Correlation Between γ-Aminobutyric Acid$_A$ Receptor Ligand-Induced Changes in *t*-Butylbicyclophosphoro[^{35}S]thionate Binding and $^{36}Cl^-$ Uptake in Rat Cerebrocortical Membranes

WHA BIN IM and DAVID P. BLAKEMAN
CNS Research, The Upjohn Company, Kalamazoo, Michigan 49001
Received August 20, 1990; Accepted December 18, 1990

SUMMARY

We have explored the functional significance of various drug-induced changes in t-[^{35}S]butylbycyclophosporothionate (TBPS) binding to γ-aminobutyric acid$_A$ (GABA$_A$) receptors by comparing them with the actions of the drugs on GABA-induced $^{36}Cl^-$ uptake in rat cerebrocortical membrane preparations. In the presence of micromolar concentrations of GABA, various benzodiazepine receptor agonists, 3α-21-dihydroxy-5α-pregnan-20-one, and pentobarbital inhibited [^{35}S]TBPS binding, whereas ethyl-β-carboline-3carboxylate (β-CCE), an inverse agonist, stimulated it, in general agreement with earlier reports [*Mol. Pharmacol.* **23**:326–336 (1983); *Mol. Pharmacol.* **30**:218–225 (1986)]. The drug-induced changes in [^{35}S]TBPS binding, after normalization with respect to the corresponding action of diazepam, were closely related to the relative ability of the drugs to affect $^{36}Cl^-$ uptake, with a correlation coefficient of 0.98 and a slope of 0.85. Upon abolishment of GABA action by the use of bicuculline, however, all the tested drugs stimulated [^{35}S]TBPS binding to various degrees, and their relative changes displayed a lower correlation coefficient of 0.69, with a slope of 2. In particular, the effects of the anesthetic steroid and pentobarbital on [^{35}S]TBPS binding were markedly altered by GABA, which at 2 μM increased not only their maximal effects, but also their half-maximal concentrations severalfold. On the other hand, GABA did not significantly affect these parameters for diazepam under our experimental conditions. Also, the GABA-independent changes in [^{35}S]TBPS binding produced by various benzodiazepine receptor agonists matched reasonably well the actions of the drugs on $^{36}Cl^-$ uptake, with a correlation coefficient of 0.85 and a slope of 1.0. These data suggest more pronounced functional coupling of the GABA sites with those for the steroid and the barbiturate, as compared with the benzodiazepine site. It appears that the degree of [^{35}S]TBPS binding in the presence of GABA closely reflects the functional state of GABA$_A$ receptors and may be useful for characterization of allosteric interactions between various sites on the receptors.

GABA$_A$ receptors interact with various hypnotic and anxiolytic agents (1–4). In particular, the benzodiazepine binding site is the most well known modulatory site on the receptor and accommodates not only classical benzodiazepines but also chemical agents of diverse structures (5, 6). Many studies have been carried out to obtain therapeutically important information on the affinities and efficacies of various drugs interacting with the GABA$_A$ receptor, using equilibrium binding studies. The types of efficacy tests include measurements of changes in the ligand affinity in the presence of GABA (7–9) or, more recently, modulation of TBPS binding to GABA$_A$ receptors (10–13). TBPS, a cage convulsant, binds with high affinity to the picrotoxin binding site that is presumably located near the mouth of the chloride ionophore of the GABA$_A$ receptor complex (10). Its binding is known to be allosterically affected by GABA, benzodiazepines, β-carbolines, pyrazolopyridine, cyclopyrrolone, imidazopyridines, barbiturates, metabolites of progesterone, and avermectin B (10–17). In particular, GABA has been reported to play a pivotal role by reversing the stimulatory effects of benzodiazepine agonists and the inhibitory actions of inverse agonists (12, 18). Both GABA-dependent and -independent actions of benzodiazepine receptor ligands on [^{35}S]TBPS binding, however, have been suggested to be related to the general *in vivo* behavioral pharmacological actions of the drugs (11, 12). To understand the functional significance of the changes in [^{35}S]TBPS binding, in this study we have characterized the effects of various drugs on [^{35}S]TBPS binding in purified rat cerebrocortical synaptosomal membranes in the presence of excess bicuculline, or of defined levels of GABA, and directly compared their ability to stimulate GABA-mediated $^{36}CL^-$ uptake in rat cerebrocortical synaptoneurosomes.

Materials and Methods

Preparation of rat cerebrocortical synaptosomal membranes. The rat membranes were prepared from brain cortices of male Sprague-

ABBREVIATIONS: GABA, γ-aminobutyric acid; TBPS, *t*-butylbicyclophosphorothionate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 5-α-THDOC, 3α,21-dihydroxy-5α-pregnan-20-one; CHAPS, 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate; β-CCE, ethyl-β-carboline-3-carboxylate.

Dawley rats (150–180 g). The tissues were homogenized in 10 volumes of ice-cold homogenizing buffer containing 300 mM sucrose, 10 mM HEPES, pH 7.4, 0.1 mM EDTA, 1 μM pepstatin A, and 3 μg/ml leupeptin, using a Dounce homogenizer with a tight-fitting pestle (15 strokes). The homogenates were centrifuged at 3,000 × *g* for 15 min, and the supernatant was collected and centrifuged at 40,000 rpm for 35 min in a 60 Ti rotor. The membranous pellets were resuspended in 20 volumes of 5 mM Tris·HCl, pH 8.0, 1 μM pepstatin A, 3 μg/ml leupeptin, were incubated for 10 min on ice, and were rehomogenized. The membranes were recovered by ultracentrifugation as described above, resuspended in the sucrose homogenizing buffer, and further resolved by sedimentation through a sucrose density gradient consisting of 0.4 M, 0.8 M, and 1.1 M sucrose solutions buffered with 5 mM HEPES/Tris, pH 7.4 (19). After centrifugation at 100,000 × *g* for 60 min in a SW 27 rotor, the membranes located between the 0.4 and 1.1 M sucrose solutions were collected, washed, and stored in sucrose homogenizing buffer supplemented with glycerol to a final concentration of 20% (v/v), at −80°.

Preparation of rat cerebrocortical synaptoneurosomes. The synaptoneurosomes were prepared following procedures reported elsewhere (20). Briefly, cerebral cortices from five male Sprague-Dawley rats were suspended in 30 ml of ice-cold solution containing 118 mM NaCl, 5 mM KCl, 1.8 mM $MgSO_4$, 2.5 mM $CaCl_2$, 20 mM HEPES/Tris, pH 7.0 (buffer A), and 10 mM D-glucose; they were homogenized with a loose-fitting pestle and then by five strokes with a tight-fitting pestle. The suspension was diluted to 160 ml with buffer A and filtered through three layers of nylon cloth and a 10-μm Millipore filter (LC type). The filtrates were centrifuged at 3000 × *g* for 15 min. The membrane pellets were washed once and resuspended in buffer A to a final protein concentration of 8 mg/ml.

Measurements of [^{35}S]TBPS binding. The ligand binding was measured in medium containing 2 nM [^{35}S]TBPS, unless specified otherwise, 50 μg of membrane proteins, 1 M NaCl, and 10 mM Tris·HCl, pH 7.4, in a total volume of 500 μl. Drugs were added as concentrated methanolic solutions. The level of methanol did not exceed 0.2% and was maintained constant in all tubes. The mixtures were incubated for 120 min at 24° and filtered over a Whatman GF/B filter under vacuum. The filters were washed three times with 4 ml of 1 M NaCl, 10 mM Tris·HCl buffer, pH 7.4, and counted for [^{35}S]TBPS radioactivity. Nonspecific [^{35}S]TBPS binding was estimated in the presence of 2 μM unlabeled TBPS and was subtracted to compute specific binding.

Measurements of $^{36}Cl^-$ uptake. The ion uptake in rat cerebrocortical synaptoneurosomes was measured by a rapid filtration technique, using Whatman GF/B filters, as described elsewhere (21). A typical incubation medium contained 0.2 μCi of $Na^{36}Cl$/ml, 118 mM NaCl, 5 mM KCl, 1.8 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 20 mM HEPES/Tris, pH 7.0, with or without test drugs. Drugs were added as concentrated methanol solutions; the level of methanol did not exceed 0.4% and was maintained constant in all tubes. The membrane suspensions were preincubated for 5 min at 30°. The reaction was initiated by mixing of equal volumes (125 μl) of the membrane suspension (1 mg of protein) and the reaction mixture containing $^{36}Cl^-$, at 30°. After 5 sec, the reaction was terminated by addition of ice-cold NaCl incubation buffer containing 1 mM 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (22). The mixture was filtered over a Whatman GF/B filter under vacuum, and the filter was washed four times with 5 ml of ice-cold NaCl incubation buffer. The radioactivity on the filter was counted in the presence of Instagel (15 ml).

Miscellaneous assays. The amount of protein in membranes was determined by the method of Lowry *et al.* (23), using bovine serum albumin for calibration. Analysis of binding data was carried out using a computer program of linear and nonlinear regressions for one of multiple classes of binding sites. The reported values for binding capacities in the study were obtained using a model for a single class of sites, which best fit our data in this study.

Results

Estimation of the concentration of endogenous external GABA. Because endogenous external GABA in brain membranes is known to inhibit [^{35}S]TBPS binding to $GABA_A$ receptors (10), it is useful to know its level in the purified synaptosomal membrane preparations. To this end, excess bicuculline was employed to obtain maximal [^{35}S]TBPS binding in the membranes (Fig. 1A). Then the percentage changes in [^{35}S]TBPS binding were measured in the presence of various concentrations of exogenous GABA (Fig. 1B). The GABA dose-response data were fitted to the equation

$$I/I_{max} = [GABA]^n/([IC_{50}]^n + [GABA]^n) \quad (1)$$

where I is the degree of inhibition of [^{35}S]TBPS binding at a given concentration of GABA, I_{max} is the maximal inhibition, IC_{50} is the GABA concentration for half-maximal inhibition, and n is the degree of cooperativity. The data were fitted to the equation via least squares minimization, assuming an endogenous external GABA concentration varying from 0 to 3 μM at intervals of 0.2 μM. Estimation of the endogenous external GABA concentration as 1 μM gave the least sum of squares value in the data-fitting process. On the basis of this value, the IC_{50} for GABA was 2.3 ± 0.1 μM, and n was 2.0 ± 0.1. According to this method of estimation, endogenous external GABA, from preparation to preparation, was reasonably constant, 1.0 ± 0.3 μM (three measurements).

Characterization of the actions of various drugs on [^{35}S]TBPS binding. Fig. 2 shows that diazepam, Ro 15-1788, and β-CCE, in the presence of excess bicuculline, stimulated [^{35}S]TBPS binding maximally up to 13.7 ± 0.7, 5 ± 1.5, and 5 ± 1.3%, respectively, as compared with the value observed with bicuculline alone. In the presence of GABA (1 μM endogenous plus 1 μM exogenous), the [^{35}S]TBPS binding decreased to 60% of that observed with bicuculline. Diazepam enhanced the inhibitory effect of GABA, as did Ro 15-1788, albeit to a lesser degree; the maximal level of inhibition was 22 ± 1.5% for diazepam and 6 ± 2.1% for Ro 15-1788, as compared with that observed with GABA alone. Various agonists for the benzodiazepine site, like diazepam, showed GABA-independent stim-

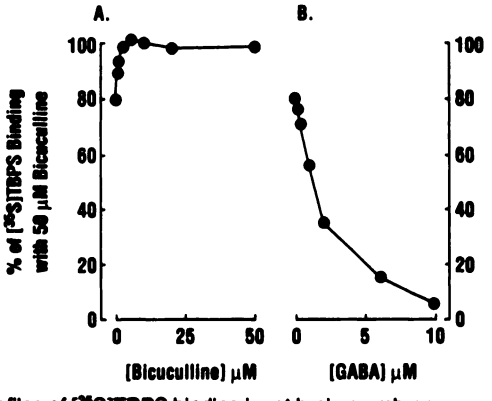

Fig. 1. Profiles of [^{35}S]TBPS binding in rat brain membranes as a function of bicuculline and exogenous GABA concentrations. [^{35}S]TBPS binding was measured in rat purified synaptosomal membranes in the presence of the indicated concentrations of bicuculline (A) or in the presence of exogenous GABA in the synaptosomal membranes (B). The reaction was carried out at 23° for 120 min. The concentration of [^{35}S]TBPS was 2 nM. The rest of the experimental conditions were as described in Materials and Methods. The data represent means of triplicate measurements, with experimental errors less than 10%.

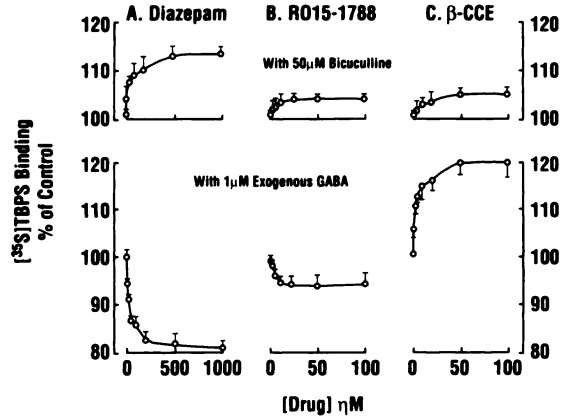

Fig. 2. Effects of diazepam, Ro 15-1788, and β-CCE on [^{35}S]TBPS binding in purified synaptosomal membranes in the presence of 50 μM bicuculline or 1 μM exogenous GABA. The drug effects were expressed as a percentage of [^{35}S]TBPS binding observed in the presence of bicuculline (*upper*) or in the presence of exogenous GABA (*lower*). The level of [^{35}S]TBPS binding was reduced by about 40% with 1 μM exogenous GABA. The conditions for [^{35}S]TBPS binding were the same as described in the legend to Fig. 1 and as fully described in Materials and Methods. The data represent means ± standard deviations from three experiments, consisting of duplicate measurements each.

TABLE 1

Comparison of the relative changes in [^{35}S]TBPS binding and the relative ability to stimulate $^{36}Cl^-$ uptake for various $GABA_A$ receptor ligands

[^{35}S]TBPS binding at 2 nM in rat cerebrocortical synaptosomal membranes was measured in the presence of 50 μM bicuculline or 1 μM exogenous GABA, with the drugs at 1 μM, except pentobarbital (100 μM). $^{36}Cl^-$ uptake in rat cerebrocortical synaptoneurosomes was measured in the presence of 5 μM exogenous GABA with 1 μM benzodiazepine receptor ligands, 100 μM pentobarbital, or 1 μM 5-α-THDOC. Their effects were normalized with respect to the corresponding action of diazepam. The data represent means ± standard deviations from at least three experiments, consisting of triplicate measurements for $^{36}Cl^-$ uptake and duplicate measurements for [^{35}S]TBPS binding.

	[^{35}S]TBPS binding		GABA-mediated $^{36}Cl^-$ Uptake
	Without GABA	With GABA	
	% of diazepam		
Diazepam	100 ± 15	100 ± 12	100 ± 15
Alprazolam	133 ± 13	117 ± 3	113 ± 10
Flunitrazepam	75 ± 3	87 ± 12	114 ± 16
Triazolam	125 ± 4	131 ± 4	168 ± 31
Zolpidem	112 ± 6	95 ± 10	121 ± 16
Zopiclone	63 ± 5	106 ± 8	109 ± 21
Ro 17-1812	35 ± 7	71 ± 5	76 ± 21
Ro 15-1788	29 ± 7	28 ± 9	5 ± 5
ZK 93426	11 ± 6	4 ± 5	2 ± 29
U-78875	5 ± 7	6 ± 12	7 ± 12
β-CCE	36 ± 11	−91 ± 11	−22 ± 12
Pentobarbital	92 ± 21	290 ± 15	227 ± 32
5-α-THDOC	131 ± 30	475 ± 45	459 ± 64

ulatory and GABA-dependent inhibitory effects on [^{35}S]TBPS binding. Table 1 shows the effects of the drugs, expressed as a percentage of the corresponding action by diazepam, at a concentration of 1 μM. On the other hand, β-CCE stimulated [^{35}S]TBPS binding up to 20 ± 2.5% in the presence of GABA. These data are qualitatively in agreement with the earlier report by Gee *et al.* (12), where the GABA-free condition was achieved with CHAPS solubilization of brain membranes.

Analysis of the dose-response profiles for diazepam, using Eq. 1 (inhibition as well as stimulation), showed that GABA did not significantly decrease the half-maximal concentration for diazepam; its value was 37 ± 7 and 32 ± 4 nM in the absence and presence of GABA, respectively. The cooperativity factor was 0.96 regardless of the presence of GABA. Similar analysis with β-CCE showed its half-maximal concentration to be 2.9 ± 0.7 nM in the presence of GABA, with a cooperativity factor of 1. The other data were not analyzed because of marginal effects and large variations.

Fig. 3 shows the results from similar studies with pentobarbital, 5-α-THDOC, and ethanol. In the presence of bicuculline, pentobarbital and 5-α-THDOC also increased [^{35}S]TBPS binding maximally up to 14 ± 1.4 and 20 ± 1.3%, respectively, with half-maximal concentrations of 19 ± 4.3 μM and 97 ± 18 nM, respectively. With GABA, they inhibited [^{35}S]TBPS binding completely, with an IC_{50} value of 89 ± 29 μM for pentobarbital and 340 ± 39 nM for 5-α-THDOC. The cooperativity factors ranged from 0.96 to 1.03, regardless of the presence of GABA. Ethanol inhibited [^{35}S]TBPS binding in the presence of bicuculline and GABA and showed no signs of saturation in its effect at concentrations of up to 10% (v/v).

According to Scatchard analysis (Table 2), the stimulatory action of diazepam and 5-α-THDOC in the presence of bicuculline was due to enhanced affinity for TBPS, without notable effects on its B_{max}; the K_d for TBPS was decreased from 26 ± 2.2 to 20.5 ± 0.9 and 19.5 ± 0.6 nM by diazepam and 5-α-THDOC, respectively. On the other hand, GABA at micromolar concentrations decreased both the affinity and the B_{max} for TBPS; the K_d increased from 26 ± 2.2 to 30 ± 1.2 and 65 ± 4.8 nM as the addition changed from bicuculline to 1 or 3 μM exogenous GABA, respectively, and the corresponding changes in the B_{max} were from 8.2 ± 0.4 to 6.6 ± 0.2 and 5.4 ± 0.3 pmol/mg of protein. Diazepam at 1 μM enhanced the inhibitory effect of GABA by further increasing the K_d for TBPS from 30 ± 1.2 (with 1 μM exogenous GABA) to 38 ± 2.1 nM, whereas β-CCE reduced the inhibitory effect of GABA by decreasing the K_d for TBPS to 26 ± 1.1 nM, a value similar to that observed with bicuculline, 26 ± 2.2 nM. Both drugs, however, showed no measurable effect on the B_{max} for TBPS. Pentobarbital and 5-α-THDOC differed from benzodiazepines in that they potentiated the inhibitory action of GABA by decreasing both the affinity and the B_{max} for TBPS; the K_d for TBPS increased

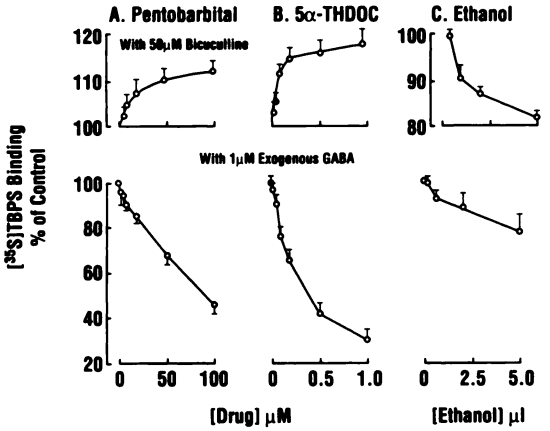

Fig. 3. Effects of pentobarbital, 5-α-THDOC, and ethanol on [^{35}S]TBPS binding in purified synaptosomal membranes in the presence of 50 μM bicuculline or 1 μM exogenous GABA. The drug effects were expressed as a percentage of [^{35}S]TBPS binding in the presence of bicuculline alone (*upper*) or GABA alone (*lower*). The conditions for [^{35}S]TBPS binding were the same as described in the legend to Fig. 1 and in Materials and Methods. The data represent means ± standard deviations from three experiments, consisting of duplicate measurements each.

TABLE 2

Alterations in the equilibrium binding parameters for [35S]TBPS by various $GABA_A$ receptor ligands, in the presence of bicuculline or exogenous GABA

[35S]TBPS binding was measured in purified rat cerebrocortical synaptosomal membranes in the presence of 50 μM bicuculline or 1 or 3 μM exogenous GABA, with or without the indicated concentrations of drugs. The concentration of TBPS ranged from 2 to 300 nM, and nonspecific binding was subtracted. Scatchard analysis was fit best to a single class of sites. The rest of the experimental conditions were as described in Materials and Methods. The data represent estimates ± standard errors obtained from two experiments, consisting of duplicate measurements each.

	K_d	B_{max}
	nM	pmol/mg of protein
Bicuculline (50 μM)	26.0 ± 2.2	8.2 ± 0.4
Bicuculline (50 μM) + diazepam (1 μM)	20.5 ± 0.9	8.1 ± 0.7
Bicuculline (50 μM) + 5-α-THDOC (1 μM)	19.5 ± 0.6	8.3 ± 0.3
Exogenous GABA (1 μM)	30.0 ± 1.2	6.6 ± 0.2
Exogenous GABA (1 μM) + diazepam (1 μM)	38.0 ± 2.1	6.3 ± 0.2
Exogenous GABA (1 μM) + β-CCE (0.2 μM)	26.0 ± 1.1	6.7 ± 0.2
Exogenous GABA (1 μM) + pentobarbital (80 μM)	55.0 ± 3.6	4.7 ± 0.3
Exogenous GABA (1 μM) + 5-α-THDOC (0.5 μM)	48.0 ± 2.8	4.6 ± 0.2
Exogenous GABA (3 μM)	65.0 ± 4.8	5.4 ± 0.3

from 30 ± 1.2 (1 μM exogenous GABA) to 55 ± 3.6 and 48 ± 2.8 nM in the presence of pentobarbital (80 μM) and 5-α-THDOC (500 nM), respectively, and the B_{max} decreased from 6.6 ± 0.2 to 4.7 ± 0.3 and 4.6 ± 0.2 pmol/mg of protein, respectively.

GABA-mediated $^{36}Cl^-$ uptake. It has been shown that GABA dose-dependently stimulated $^{36}Cl^-$ uptake in rat cerebrocortical synaptoneurosomes, and this GABA action was influenced by $GABA_A$ receptor ligands (4, 21, 22, 24). In this study, for instance, diazepam at a concentration of 1 μM stimulated $^{36}Cl^-$ uptake by 4.8 ± 0.7 nmol/5 sec·mg of protein in the presence of 5 μM exogenous GABA. Table 1 lists the effects of various ligands for the benzodiazepine site (1 μM), pentobarbital (100 μM), and 5-α-THDOC (1 μM) on $^{36}Cl^-$ uptake, normalized with respect to the action by diazepam at a concentration of 1 μM. The concentration of pentobarbital was limited to 100 μM in this study, because higher concentrations (500 μM) reduced $^{36}Cl^-$ uptake due to rapid desensitization (21).

Correlation between [35S]TBPS binding and $^{36}Cl^-$ uptake data. Linear regression analysis was performed on the relative changes in [35S]TBPS binding and $^{36}Cl^-$ uptake induced by the various drugs, as shown in Table 1. The relative changes in [35S]TBPS binding produced by all the tested drugs in the presence of GABA were closely related to the abilities of the drugs to stimulate GABA-mediated $^{36}Cl^-$ uptake, with a correlation coefficient of 0.98 and a slope of 0.85 (Fig. 4), whereas the relative TBPS changes in the presence of bicuculline showed a lower correlation coefficient (0.69) and a slope of 2. If we excluded the data on pentobarbital, 5-α-THDOC, and β-CCE from the analysis, the relative TBPS shifts among the benzodiazepine receptor ligands in the presence of bicuculline were reasonably well matched, with the $^{36}Cl^-$ uptake data showing a correlation coefficient of 0.85 and a slope of 1.0, whereas their counterparts in the presence of GABA displayed a correlation coefficient of 0.95 and a slope of 1.1.

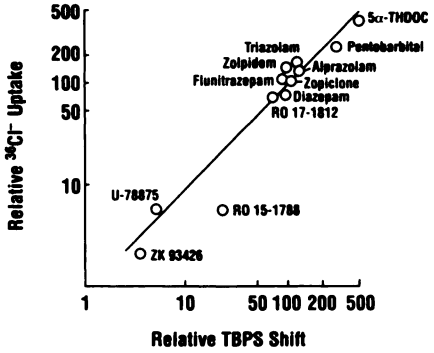

Fig. 4. Plot showing the correlation between the relative changes in TBPS binding in the presence of GABA (2 μM) and the relative ability to stimulate GABA-mediated $^{36}Cl^-$ uptake, for various $GABA_A$ receptor ligands. The effects of the compounds listed in Table 1 on [35S]TBPS binding and $^{36}Cl^-$ uptake, normalized with respect to diazepam (100%), were plotted on a log-log scale, and the line was fitted by eye. β-CCE data were excluded from the plot but included in computing the correlation coefficient.

Discussion

Allosteric actions of $GABA_A$ receptor ligands on [35S]TBPS binding in brain membranes have been of considerable interest to pharmacologists, because of the potential utility of these data to predict the pharmacological efficiency of the ligands (10–18). Earlier, Squires *et al.* (10) reported that $GABA_A$ receptor agonists inhibited [35S]TBPS binding with a rank order of potency comparable to those found in binding, whereas Supavilai and Karobath (11) reported the stimulatory actions of benzodiazepine receptor agonists on [35S]TBPS binding and suggested their potential for estimating the *in vivo* efficacy of the drugs. Recent studies have pointed out that the effects of benzodiazepines are highly dependent on GABA levels. Gee *et al.* (12) and Martini *et al.* (18) reported that benzodiazepine agonists inhibited and inverse agonists stimulated [35S]TBPS binding in rat cerebrocortical membranes in the presence of physiological levels of GABA but the agonists reversed their effects in GABA-free CHAPS- or Triton X-100-solubilized receptor preparations. They also suggested the consistency of the TBPS data with the *in vivo* pharmacology of the drugs.

Our results in this study, with purified synaptosomal membranes in the presence of bicuculline or defined levels of GABA (about 2 μM, including endogenous and exogenous GABA), further confirmed the pivotal role of GABA in the action of benzodiazepine agonists on [35S]TBPS binding, as proposed by the above-mentioned authors, and indicated a similar role for GABA in the action of 5-α-THDOC and pentobarbital on [35S]TBPS binding.

Our analysis indicates that the GABA-dependent changes in [35S]TBPS binding produced by all the tested drugs were closely related to the ability of the drugs to influence GABA-mediated $^{36}Cl^-$ uptake in rat cerebrocortical synaptoneurosomes (Fig. 4). On the other hand, the GABA-independent (stimulatory) actions of 5-α-THDOC and pentobarbital did not match their actions on $^{36}Cl^-$ uptake, although similar stimulatory actions of the various agonists for the benzodiazepine site correlated reasonably well with the anion uptake data (see Table 1). In the case of the steroid and pentobarbital, we found that GABA increased not only their maximal effect on [35S]TBPS binding but also their half-maximal concentrations severalfold, whereas GABA exerted no significant effect on the parameters for

diazepam. One possible explanation is that GABA converts the steroid and pentobarbital binding sites from high to low affinity, but no similar actions with benzodiazepine receptors under our experimental conditions were noted. An alternative possibility of heterogeneous binding sites for the steroid and the barbiturate appears not to be likely, because their dose-response profiles within the range of concentrations used in this study showed no signs of multiphasic actions and displayed a Hill coefficient of 1. In earlier studies, Leeb-Lundberg and Olsen (25) reported that the enhancement of [^{3}H]diazepam binding in rat brain membranes by pentobarbital was partially reversed by bicuculline. The data could be interpreted to mean that the barbiturate affects the diazepam binding site in both GABA-independent and -dependent manners, like [^{35}S]TBPS binding in this study, rather than indicating the presence of multiple barbiturate binding sites. These considerations led us to propose pronounced functional coupling of GABA sites with those for the barbiturate and the steroid, although they share no common site (12), but not with the benzodiazepine site. It appears that the GABA-independent conformational changes induced by benzodiazepine agonists are relevant to the *in vivo* actions of the drugs in the presence of GABA and that GABA-induced reversal of their effects on [^{35}S]TBPS binding could be simply due to coupling of the two independent conformational changes, the GABA and the drug induced.

In short, equilibrium binding properties of TBPS are differentially affected by $GABA_A$ receptor ligands and appear to be useful parameters for understanding the ligand actions on the chloride channel of the receptor complex. Overall, the relative changes in [^{35}S]TBPS binding produced by the ligands in the presence of micromolar levels of GABA seem to be useful for predicting the *in vivo* pharmacological behavioral efficacy of the drugs.

References

1. Tallman, J. F., S. M. Paul, P. Skolnick, and D. W. Gallager. Receptors for the age of anxiety: pharmacology of benzodiazepines. *Science (Washington D. C.)* **207**:274–281 (1980).
2. Olsen, R. W. GABA-benzodiazepine-barbiturate receptor interactions. *J. Neurochem.* **37**:1–13 (1981).
3. Ticku, M. K., and G. Maksay. Minireview: convulsant/depressant site of action at the allosteric benzodiazepine-GABA receptor-ionophore complex. *Life Sci.* **33**:2365–2375 (1983).
4. Majewska, M. D., N. L. Harrison, R. D. Schwartz, J. L. Baker, and S. M. Paul. Steroid hormone metabolites are barbiturate-like modulators of GABA receptors. *Science (Washington D. C.)* **232**:1004–1007 (1986).
5. Costa, E., and A. Guidotti. Molecular mechanisms in the receptor action of benzodiazepines. *Annu. Rev. Pharmacol.* **19**:531–545 (1979).
6. Haefly, W., E. Kyburz, M. Gerecke, and H. Mohler. Recent advances in the molecular pharmacology of benzodiazepine receptors and in the structure-activity relationship of their agonists and antagonists. *Adv. Drug Res.* **14**:166–322 (1985).
7. Braestrup, C., T. Honore, M. Nielsen, E. N. Petersen, and L. H. Jensen. Ligands for benzodiazepine receptors with positive and negative efficacy. *Biochem. Pharmacol.* **33**:859–862 (1984).
8. Karobath, M., P. Supavilai, and P. A. Borea. Distinction of benzodiazepine receptor agonists and inverse agonists by binding studies *in vitro*, in *Benzodiazepine Recognition Site Ligands. Biochemistry and Pharmacology* (G. Biggio and E. Costa, eds.). Raven Press, New York, 37–45 (1983).
9. Kemp, J. A., G. R. Marshall, E. H. F. Wong, and G. N. Woodruff. The affinities, potencies, and efficacies of some benzodiazepine-receptor agonists, antagonists, and inverse-agonists at rat hippocampal $GABA_A$-receptors. *Br. J. Pharmacol.* **91**:601–608 (1987).
10. Squires, R. F., J. E. Casida, M. Richardson, and E. Saederup. [^{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).
11. Supavilai, P., and M. Karobath. Differential modulation of [^{35}S]TBPS binding by the occupancy of benzodiazepine receptors with its ligands. *Eur. J. Pharmacol.* **91**:145–146 (1983).
12. Gee, K. W., L. J. Lawrence, and H. I. Yamamura. Modulation of the chloride ionophore by benzodiazepine receptor ligands: influence of γ-aminobutyric acid and ligand efficacy. *Mol. Pharmacol.* **30**:218–225 (1986).
13. 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**:151–158 (1984).
14. Trifiletti, R. R., A. M. Snowman, and S. H. Snyder. Anxiolytic cyclopyrrolone drugs allosterically modulate the binding of [^{35}S]*t*-butylbicyclophosphorothionate to the benzodiazepine/γ-aminobutyric acid-A receptor/chloride anionphore complex. *Mol. Pharmacol.* **26**:470–476 (1984).
15. Gee, K. W., M. B. Bolger, R. E. Brinton, H. Corini, and B. S. McEwen. Steroid modulation of the chloride ionophore in rat brain: structure-activity requirements, regional dependence and mechanism of action. *J. Pharmacol. Exp. Ther.* **246**:803–812 (1988).
16. Richter, J. A., and H. I. Yamamura. Effects of pentobarbital on *t*-[^{35}S] butylbicyclophosphorothionate and [^{3}H]flunitrazepam binding to membrane bound and solubilized preparations from rat forebrain. *J. Pharmacol. Exp. Ther.* **233**:125–132 (1985).
17. Huidobro-Toro, P., V. Bleck, A. M. Allan, and R. A. Harris. Neurochemical actions of anesthetic drugs on the γ-aminobutyric acid receptor-chloride channel complex. *J. Pharmacol. Exp. Ther.* **242**:963–969 (1987).
18. Martini, C., R. Pacini, G. Giannaccini, and A. Lucacchini. [^{35}S]TBPS binding to purified benzodiazepine-GABA-ionophore receptor complex: the effect of GABA on the modulation exerted by benzodiazepine site ligands. *Neurochem. Int.* **15**:377–379 (1989).
19. Whittaker, V. P. The synaptosome in structural elements of the nervous system, in *Handbook of Neurochemistry* (A. Lajtha, ed.), Vol. 7. Plenum Press, New York, 1–39 (1984).
20. Hollingsworth, E. B., E. T. McNeal, J. L. Burton, R. J. Williams, J. W. Daly, and C. R. Creveling. Biochemical characterization of a filtered synaptoneurosome preparation from guinea pig cerebral cortex. *J. Neurosci.* **5**:2240–2253 (1985).
21. Schwartz, R. D., P. D. Suzdak, and S. M. Paul. *gamma*-Aminobutyric acid-barbiturate-mediated $^{36}Cl^-$ uptake in rat brain synaptoneurosomes. *Mol. Pharmacol.* **30**:419–426 (1986).
22. Im, W. B., D. P. Blakeman, J. P. Davis, and D. E. Ayer. Studies on the mechanism of interactions between anesthetic steroids and γ-aminobutyric acid-A receptors. *Mol. Pharmacol.* **37**:429–434 (1990).
23. 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).
24. Obata, T., and H. I. Yamamura. The effect of benzodiazepines and β-carbolines on GABA-stimulated chloride flux by membrane vesicles from rat cerebral cortex. *Biochim. Biophys. Res. Commun.* **141**:1–6 (1986).
25. Leeb-Lundberg, L. M. F., and R. W. Olsen. Heterogeneity of benzodiazepine receptor interactions with *gamma*-aminobutyric acid and barbiturate receptor sites. *Mol. Pharmacol.* **23**:315–325 (1983).

Send reprint requests to: Wha Bin Im, CNS Research, The Upjohn Company, Kalamazoo, MI 49001.