0026-895X/86/030218-08$02.00/0

MOLECULAR PHARMACOLOGY, 30:218–225

Modulation of the Chloride Ionophore by Benzodiazepine Receptor Ligands: Influence of γ-Aminobutyric Acid and Ligand Efficacy

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Received September 30, 1985; Accepted June 12, 1986

SUMMARY

t-Butylbicyclophosphorothionate (TBPS) produces dose-dependent enhancement of [^{3}H]propyl β-carboline-3-carboxylate ([^{3}H]PCC, 40 pM) binding to the benzodiazepine$_1$ (BZ_1) receptor subtype in hippocampus. Furthermore, TBPS enhancement of [^{3}H]PCC binding was antagonized by micromolar concentrations of γ-aminobutyric acid (GABA) in a way reversible by bicuculline. BZ receptor ligands that are "GABA positive" (i.e., enhance GABA neurotransmission) allosterically inhibited [^{35}S]TBPS binding, whereas "GABA-negative" ligands (i.e., inhibit GABA neurotransmission) produced the opposite effect. The efficacy of the ligands as modulators of [^{35}S]TBPS binding was consistent with their reported *in vivo* pharmacology. The effects of positive and negative ligands on [^{35}S]TBPS binding were modulated by micromolar concentrations of GABA. Examination of the kinetics of [^{35}S]TBPS binding suggested the presence of slowly and rapidly dissociating components. The GABA-positive clonazepam stabilized the rapidly dissociating component of [^{35}S]TBPS binding, whereas methyl β-carboline-3-carboxylate had a similar effect on the slowly dissociating component. It is speculated that the slowly dissociating component of [^{35}S]TBPS binding is associated with a closed chloride channel, whereas the opposite is proposed for the rapidly dissociating component. The differential effects of GABA-positive versus GABA-negative ligands on [^{35}S]TBPS binding and the modulatory effect of GABA provide further evidence to suggest that [^{35}S]TBPS labels a site near the chloride ionophore linked to the GABA-BZ receptor complex.

Considerable evidence has accumulated to suggest that the clinically useful BZs interact with the central nervous system in a highly specific manner, namely, by enhancing the effects of the major inhibitory neurotransmitter, GABA (1, 2). At the membrane level, BZ modulation of GABAergic function may result in part from changes in the kinetics of chloride channel opening (3). The ability of BZs to influence GABA neurotransmission is thought to occur via an allosteric link between the BZ and GABA receptors (4, 5).

Recently, Squires *et al.* (6) presented evidence to suggest that the cage convulsant, TBPS, specifically labels a site allosterically coupled to a chloride ionophore associated with the $GABA_A$-BZ receptor complex. Subsequently, several *in vitro* studies appeared demonstrating the ability of various BZ receptor ligands to modulate [^{35}S]TBPS binding in rat brain (7–9). Furthermore, these studies suggested that the pharmacological potency and efficacy of various BZ receptor ligands could be predicted by their ability to modulate [^{35}S]TBPS binding. Lawrence *et al.* (9) demonstrated that the rank order potency of certain GABA-positive BZs (i.e., those BZs that produce antianxiety, anticonvulsant, and sedative-hypnotic effects) as modulators of [^{35}S]TBPS binding correlated with their *in vivo* antimetrazol potencies. Moreover, they demonstrated that agonist BZs require the presence of micromolar quantities of GABA to modulate [^{35}S]TBPS in a manner consistent with their presumed role as "GABA potentiators." In the present study, we extend the findings of Lawrence *et al.* (9) by examining: 1) the ability of certain non-BZ compounds to modulate [^{35}S]TBPS via the BZ receptor in a pharmacologically relevant fashion (i.e., consistent with their *in vivo* effects), 2) the effect of GABA on GABA-negative ligand (i.e., those BZ receptor ligands that produce anxiety or convulsions) modulation of [^{35}S]TBPS binding and the specificity of the GABA effect, 3)

This research was supported by United States Public Health Service Grants MH-27257 (H. I. Y.) and MH-30626 (H. I. Y.). K. W. G. is a recipient of a Faculty Development Award in Basic Pharmacology from the Pharmaceutical Manufacturers Association Foundation. H. I. Y. is a recipient of a Research Scientist Development Award, Type II (MH-00095) from the National Institute of Mental Health.

A preliminary report of this work was presented at the Fall 1984 Meeting of the American Society for Pharmacology and Experimental Therapeutics, Indianapolis, Indiana.

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ABBREVIATIONS: BZ, benzodiazepine; GABA, γ-aminobutyric acid; TBPS, *t*-butylcyclophosphorothionate; EDTA, ethylenediaminetetraacetate; PCC, propyl β-carboline-3-carboxylate; FLU, flunitrazepam; MCC, methyl β-carboline-3-carboxylate; ECC, ethyl β-carboline-3-carboxylate.

the mechanism(s) underlying the effects of GABA-positive versus GABA-negative ligands on [^{35}S]TBPS binding, and 4) the presence of functional coupling between the BZ_1 receptor subtype and a [^{35}S]TBPS-labeled chloride ionophore.

Materials and Methods

Tissue preparation. The cerebral cortex and hippocampus from male Sprague-Dawley rats (175–300 g) were removed over ice immediately following decapitation. Each brain area was suspended in 0.32 M sucrose (J. T. Baker Chemical Co., Phillipsburg, NJ) by gentle homogenization (10%, w/v) with a Teflon-coated pestle followed by centrifugation at 1,000 × *g* for 10 min. The supernatant was carefully decanted and centrifuged at 9,000 × *g* for 20 min. The resultant P_2 pellet was resuspended in a 50 mM Na/K phosphate (J. T. Baker Chemical Co.) buffer (pH 7.4) + 200 mM NaCl (J. T. Baker Chemical Co.) as a 10% (w/v) homogenate. A solubilized receptor complex was prepared by resuspending the P_2 pellet in 40 mM 3-(3-cholamidopropyl)-dimethylammonio 1-propanesulfonate (CHAPS, Sigma Chemical Co., St. Louis, MO), in a 50 mM Na/K phosphate buffer (pH 7.4) containing 2 mM EDTA (Sigma Chemical Co.), 0.2 mM benzethonium chloride (Sigma Chemical Co.), 0.2 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co.), 0.4 mg/ml of NaN_3 (J. T. Baker Chemical Co.), and 40 μg/ml of bacitracin (Sigma Chemical Co.). The 10% (original wet weight/v) suspension was allowed to incubate at 0–4° for 60 min with occasional gentle agitation. The soluble fraction was obtained by centrifugation of the suspension at 100,000 × *g* for 60 min. The resultant supernatant was applied to a Sephadex G-50 (Sigma Chemical Co.) column to remove endogenous GABA. The "GABA-free" supernatants were used immediately in binding assays.

[^{35}S]TBPS, [^{3}H]PCC, [^{3}H]FLU, and [^{3}H]Ro15 1788 binding assays. Binding assays using P_2 preparations follow a previously described method (9). Briefly, 2 nM [^{35}S]TBPS (40–90 Ci/mmol, New England Nuclear Corp., Boston, MA, apparent K_d ~ 20 nM) was incubated with 100-μl aliquots of tissue homogenate, 10-μl aliquots of various concentrations of BZ receptor ligands (dissolved in dimethyl sulfoxide, J.T. Baker Chemical Co.) in a final volume of 1 ml. Fifty mM Na/K phosphate buffer (pH 7.4) + 200 mM NaCl was used in all assays. Nonspecific binding was defined as binding in the presence of 2 μM TBPS. Assays were terminated after a 90-min incubation at 25° by rapid filtration through Whatman GF/B glass fiber filters. In assays using the soluble receptor preparation, 300-μl aliquots of GABA-free soluble fraction were incubated with 2 nM [^{35}S]TBPS and various BZ receptor ligands. The final assay volume of 1 ml was filtered through polyethyleneimine (Sigma Chemical Co.) treated Whatman GF/B glass fiber filters. Three-hr pretreatment of filters with 0.3% polyethyleneimine as described by Bruns *et al.* (10) allowed the soluble receptor complex to be trapped upon filtration.

Hippocampal or cortical P_2 homogenates (100 μl) were incubated with either 40 pM or 0.5 nM [^{3}H]PCC (90 Ci/mmol, New England Nuclear Corp., apparent K_{d1} ~ 0.5 nM, K_{d2} ~ 10 nM) in the presence or absence of various concentrations of TBPS dissolved in dimethyl sulfoxide. Binding in the presence of 1 μM clonazepam was considered nonspecific. The effect of GABA on TBPS modulation of [^{3}H]PCC binding was evaluated by the addition of 100 μM GABA (Sigma Chemical Co.). Specificity of the GABA effect was determined by the ability of either 10 μM baclofen or (+)-bicuculline (Sigma Chemical Co.) to block the GABA effect. Bicuculline and baclofen at the concentrations used had no effect on [^{3}H]PCC binding. Incubations were maintained for 90 min at 0° followed by a rapid filtration through Whatman GF/B glass fiber filters. In assays using [^{3}H]FLU (75 Ci/mmol, New England Nuclear Corp., apparent K_d ~ 1 nM) or [^{3}H]Ro15 1788 (87 Ci/mmol, New England Nuclear Corp., apparent K_d ~ 1 nM) to label BZ receptors, a 0.5 nM concentration of each radioligand was incubated with 100 μl of cortical P_2 homogenate and various concentrations of TBPS at 25° for 60 min. Nonspecific binding was defined as binding in the presence of 1 μM clonazepam. Assays were terminated as described for [^{35}S]TBPS binding assays using cortical P_2 homogenates. Filter-bound radioactivity in all binding assays was quantitated by liquid scintillation spectrophotometry.

[^{35}S]TBPS binding kinetics. Dissociation kinetics under control conditions were determined by preincubating cortical P_2 homogenates with 2 nM [^{35}S]TBPS for 90 min (steady state conditions) in the presence of 10 μM GABA. This concentration of GABA was added to avoid individual variations in GABA content while at the same time maintaining a reasonable amount of [^{35}S]TBPS binding (i.e., GABA inhibits [^{35}S]TBPS binding). The effect of a GABA-positive or GABA-negative ligands on dissociation was determined by including 1 μM MCC, respectively, during the preincubation period. Binding in the presence of 2 μM cold TBPS was defined as nonspecific. Following the 90-min preincubation (time 0), 2 μM TBPS was added to initiate dissociation. [^{35}S]TBPS binding as a percentage of specifically bound [^{35}S]TBPS at time 0 was determined at various time intervals (16 time points) and plotted according to the equation $\ln(B_e/B_t) = -k_{-1}t$, where B_e is the amount of specifically bound [^{35}S]TBPS at time 0, B_t is the specific binding at time t, and k_{-1} is the dissociation rate constant (11). The data were evaluated by nonlinear regression analysis using a computerized iterative procedure to resolve the apparent dissociation rate constants. The presence of dissociation from two sites was determined by the extent of the reduction in the residual variance of fit compared to dissociation from a single-site model using a partial F test with acceptance criteria set at $p < 0.01$ (12).

The kinetics of association under control conditions were determined by incubating 2 nM [^{35}S]TBPS with cortical P_2 homogenates in the presence of 10 μM GABA at 25° for 0.25–90 min. Association in the presence of 1 μM clonazepam or MCC was carried out in a similar fashion. Samples were filtered at various time points and filter-bound radioactivity was determined as previously described. Association of 2 nM [^{35}S]TBPS to specific sites in cortical P_2 fractions was plotted according to the pseudo-first order equation (11):

$$\ln \frac{B_e - B_t}{B_e} = -(k_{+1}[L] + k_{-1})t$$

in which B_e and B_t are the concentrations of ligand-receptor complex at steady state and at time t during incubation, respectively. $[L]$ is the free ligand concentration and was approximately 2 nM since less than 2% of the total [^{35}S]TBPS added was bound. The k_{+1} (association rate constant) value was determined from the slope (k_{ob}) of the plot of

$$\ln \frac{B_e - B_t}{B_e} \text{ versus time } t \text{ where } k_{+1} = \frac{k_{ob} - k_{-1}}{[L]}.$$

The presence of association to two sites was identified by a method similar to that described for dissociation from two sites (12).

Results

BZ-TBPS receptor interactions. The effect of TBPS on BZ receptor recognition properties was evaluated by observing [^{3}H]FLU and [^{3}H]Ro15 1788 binding in the presence of TBPS (Fig. 1). TBPS produced dose-dependent inhibition of [^{3}H]FLU binding in rat cortex with maximal inhibition of 66 ± 3% of control [^{3}H]FLU binding at 20 μM TBPS. Interestingly, TBPS in concentrations of up to 50 μM resulted in only minimal inhibition of [^{3}H]Ro15 1788 binding (94 ± 1% of control).

When [^{3}H]PCC was used under conditions that allow selective labeling of BZ_1 sites in the hippocampus (13), TBPS produced dose-dependent enhancement of 40 pM [^{3}H]PCC binding (Fig. 2). A similar enhancement by TBPS was observed when 0.5 nM [^{3}H]PCC (labels both BZ_1 and BZ_2 receptor subtypes) was used to label BZ receptors in the hippocampus (data not shown). In the presence of 100 μM GABA, the TBPS/[^{3}H]PCC dose-response curve was shifted to the right (Fig. 2).

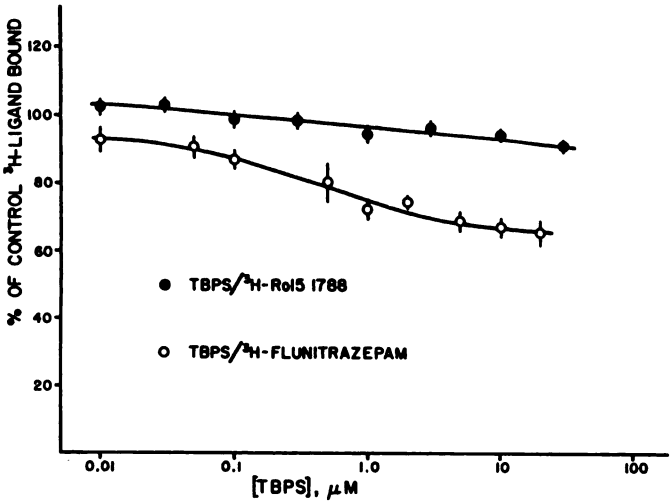

Fig. 1. TBPS modulation of the BZ receptor labeled by [^{3}H]FLU or [^{3}H] Ro15 1788 in rat cerebral cortex. Each *point* represents the mean ± standard error of four to five independent determinations. Data were plotted as log concentration versus percentage of control ^{3}H-ligand bound. Rat cerebral cortex P_2 homogenates were used in all assays. A 0.5 nM concentration of [^{3}H]FLU or [^{3}H]Ro15 1788 was used to label BZ receptors. Nonspecific binding was defined as binding in the presence of 1 μM clonazepam.

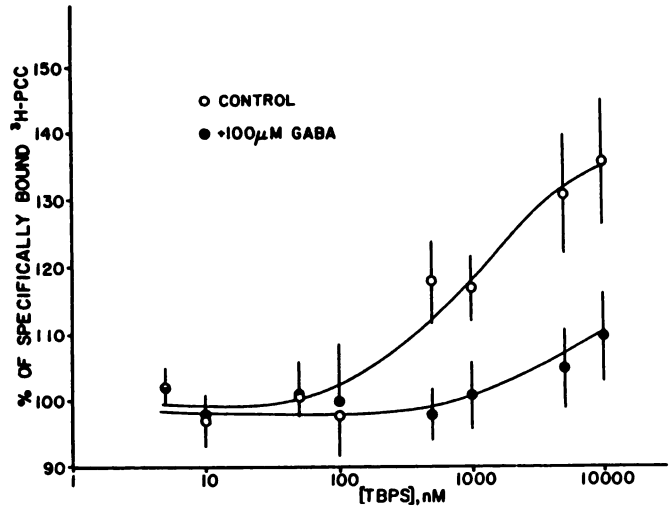

Fig. 2. TBPS modulation of [^{3}H]PCC binding in the presence or absence of 100 μM GABA. Forty pM [^{3}H]PCC was used to selectively label the BZ_1 receptor subtype in rat hippocampal P_2 homogenates. Each *point* represents the mean ± standard error of four independent determinations. Data were plotted as log concentration versus percentage of specifically bound [^{3}H]PCC. Incubations were carried out at 0° for 90 min. Nonspecific binding was defined as binding in the presence of 1 μM clonazepam.

At the highest TBPS concentration (10 μM) used, [^{3}H]PCC binding was 136 ± 10% of control. In contrast, TBPS-enhanced [^{3}H]PCC binding was only 110 ± 7% of control when GABA was present. GABA (100 μM) had no effect on control [^{3}H]PCC binding under the conditions used. The effect of GABA on TBPS enhancement of [^{3}H]PCC binding was abolished in the presence of 10 μM (+)-bicuculline. TBPS also enhanced [^{3}H] PCC (40 pM) binding in cerebral cortex and the effect was GABA sensitive in a manner similar to that observed in the hippocampus (data not shown).

BZ receptor ligand modulation of [^{35}S]TBPS binding. The effects of various non-BZ ligands on [^{35}S]TBPS binding in rat cortex were compared to the effects of clonazepam and Ro15 1788 (Fig. 3, A and B). It is evident from the data in Fig. 3 that known GABA-positive ligands produced at least 10% inhibition of [^{35}S]TBPS binding in the range of concentrations examined. The maximum degree of inhibition of [^{35}S]TBPS binding produced by the three compounds was different. The greatest inhibitory effect on [^{35}S]TBPS binding was produced by the strongly GABA-positive ligand clonazepam at 10 μM, where [^{35}S]TBPS binding was 76.7 ± 1% of control. Analysis of variance of the inhibition data for Ro15 1788 between 0.1 and 1 μM indicated that the degree of inhibition produced was not statistically significant ($p > 0.05$) and thus represented a maximum degree of inhibition (mean = 91% of control). Similar evaluation of the inhibitory effects of CL 218872 on [^{35}S]TBPS binding indicated that the degree of inhibition was similar (analysis of variance, $p > 0.05$) from 1 to 10 μM. The maximum inhibitory effect observed was reduction to 85% (mean value from 1 to 10 μM CL 218872) of control. However, at 10 μM CL 218872, the degree of inhibition of [^{35}S]TBPS binding was not significantly different from that observed at 10 μM clonazepam (Student's *t* test, $p > 0.05$). Thus, the rank order of efficacy for these compounds as inhibitors of [^{35}S]TBPS binding is clonazepam = CL 218872 > Ro15 1788.

The quinoline derivatives, PK 8165 and PK 9084, showed a unique profile. Both compounds produced slight inhibition of [^{35}S]TBPS binding at low concentrations (10–300 nM) and enhancement of [^{35}S]TBPS binding at concentrations in excess of 300 nM (Fig. 3A, data not shown for PK 8165). At the highest concentration (100 μM) of PK 8165 and PK 9084 examined, enhancement of [^{35}S]TBPS binding in excess of 125% of control was observed. Evaluation of the effects of concentrations in excess of 100 μM was impossible because of insolubility in the buffer media. The overall rank order of efficacy is clonazepam = CL 218872 > Ro15 1788 > PK 8165 = PK 9084 for the inhibition of [^{35}S]TBPS binding in rat cerebral cortex.

The methyl ester, MCC, a GABA-negative ligand for BZ receptors, produced dose-dependent enhancement of [^{35}S] TBPS binding in rat cortex (Fig. 3B). In the range of MCC concentrations examined, a maximum enhancement of 118 ± 4% of control was observed at 0.1 μM. The ethyl ester (ECC), a slightly GABA-negative ligand, produced a small but significant enhancement of [^{35}S]TBPS binding at 1 μM relative to the effect observed at 0.1 μM. In contrast, the propyl ester (PCC) produced a slight enhancement of [^{35}S]TBPS binding from 0.03 to 0.1 μM. The enhancement produced by ECC and PCC over the range of concentrations tested was <8% above control [^{35}S]TBPS binding (data not shown). For the β-carboline analogues evaluated, the rank order of efficacy in producing enhancement of [^{35}S]TBPS binding is MCC > ECC ≅ PCC.

Effect of clonazepam and MCC on the kinetics of [^{35}S] TBPS binding in cerebral cortex. The manner by which BZ receptor ligands with "positive"(GABA positive) or "negative" (GABA negative) efficacy allosterically modulate [^{35}S] TBPS binding was evaluated by observing the effects of clonazepam or MCC on the kinetics of [^{35}S]TBPS binding. Dissociation data under the various conditions were plotted as ln (B_e/B_t) versus time. The plots were multiphasic, suggesting dissociation from multiple sites or components (Fig. 4). Similarly, dissociation of [^{35}S]TBPS in the presence of 1 μM clonazepam or MCC also occurred from two sites or states of the ionophore. The dissociation rate constants (k_{-1}) for the slow

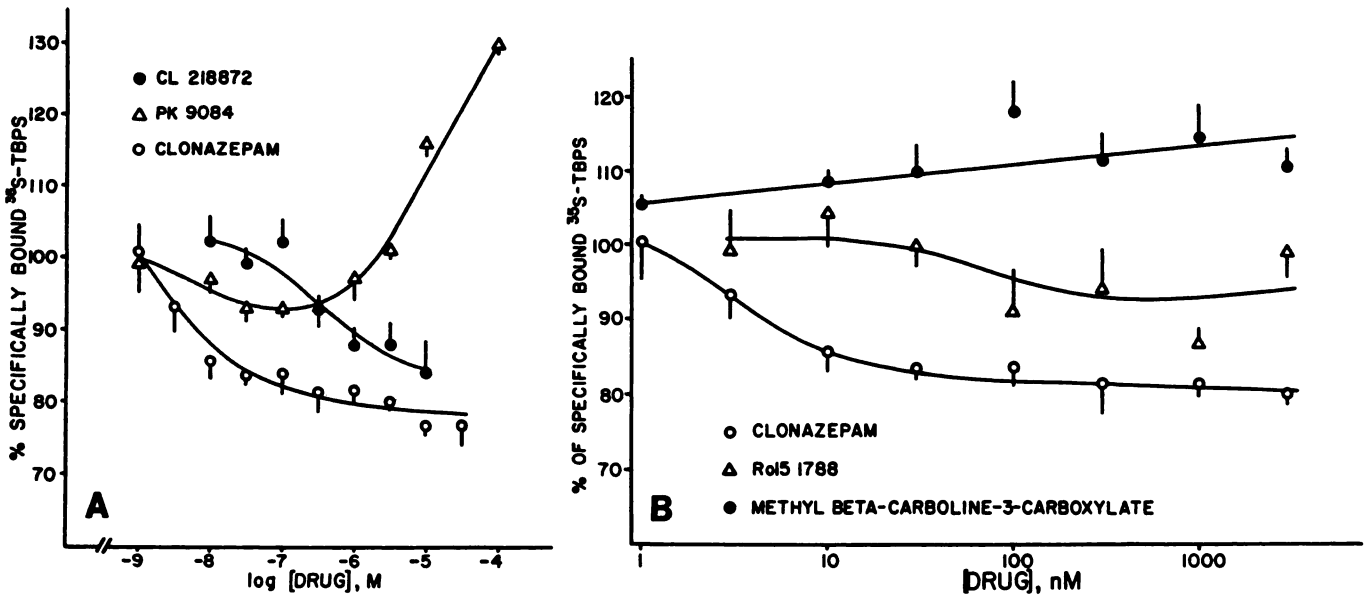

Fig. 3. Modulation of [^{35}S]TBPS binding by the non-BZs, CL 218872 and PK 9084, compared to the effects of clonazepam in rat cerebral cortex (A). Comparison of the modulatory effects of Ro15 1788 and MCC are depicted in B. All *points* represent the mean ± standard error of four to eight independent determinations. Data were plotted as log drug concentration versus percentage of specifically bound [^{35}S]TBPS. Two nM [^{35}S]TBPS was used in all assays at 25°. Nonspecific binding was defined as binding in the presence of 2 μM TBPS.

(S) and rapid (R) components and the proportion of S and R components in the presence or absence of clonazepam or MCC are shown in Table 1. Half-lives for each of the components in the presence of clonazepam or MCC did not vary significantly from control ($p > 0.05$, Student's t test). Consequently, the calculated k_{-1} values were also similar (Table 1). In contrast, the relative proportions of [^{35}S]TBPS-labeled S and R components in the presence of clonazepam or MCC were significantly different from control. Under control conditions, approximately 29% of the sites labeled by 2 nM [^{35}S]TBPS corresponded to the S component. In the presence of 1 μM clonazepam, only 5% of the sites labeled by [^{35}S]TBPS belong to the S component. The GABA-negative ligand, MCC, elicited the opposite effect. MCC increased the proportion of the S component to 60% of the total number of sites specifically labeled by 2 nM [^{35}S]TBPS.

The association rate constants (k_{+1}) for the binding of 2 nM [^{35}S]TBPS in the presence or absence of clonazepam and MCC were estimated from the time course of binding (Fig. 5). The total amount of [^{35}S]TBPS bound in these studies was less than 2% of the total radioligand added. The total number of specific sites bound by 2 nM [^{35}S]TBPS in the presence of MCC was greater than control, whereas the opposite occurred in the presence of clonazepam (Fig. 5). Estimates of the k_{+1} value were derived from the kinetics of association in the presence of MCC or clonazepam. Association of [^{35}S]TBPS was curvilinear when plotted as $\ln(B_e - B_t/B_e)$ versus time and could be resolved into rapidly and slowly associating complexes in the presence of MCC or clonazepam, respectively (Table 2). In contrast, using steady state conditions and a broad range of [^{35}S]TBPS concentrations, no evidence for multiple [^{35}S]TBPS sites was observed.

Influence of GABA on MCC enhancement of [^{35}S]TBPS binding in rat cerebral cortex. The effect of MCC on [^{35}S]TBPS binding in a soluble preparation rendered "GABA-free" by Sephadex G50 column chromatography is shown in Fig. 6. As in the membrane preparation, MCC produced dose-dependent enhancement of [^{35}S]TBPS (2 nM) binding between 0.1 and 10,000 nM. Maximum enhancement of 142 ± 11% of control was observed at 3 μM MCC when GABA was absent. GABA and MCC produce opposite effects on [^{35}S]TBPS binding, yet the combined effect of 10 μM GABA and MCC on this binding was a net potentiation of MCC enhancement of [^{35}S]TBPS binding (Table 3). The effect of GABA was antagonized by 10 μM (+)-bicuculline but not by 10 μM baclofen (Table 3). The specificity of the GABA effect was similar when clonazepam was used to modulate [^{35}S]TBPS binding (Table 3).

Discussion

The present study provides additional evidence in support of the hypothesis that [^{35}S]TBPS labels a site associated with the chloride ionophore linked to both BZ and $GABA_A$ receptors. Interactions between the BZ receptor and the [^{35}S]TBPS binding site occur in the presence of concentrations of GABA-positive or GABA-negative BZ receptor ligands that are consistent with their *in vivo* potency. The observation that GABA-positive ligands inhibit [^{35}S]TBPS binding in a manner similar to that of the GABA-mimetics (6), whereas the opposite is observed with GABA-negative ligands, lends further support to the pharmacological relevance of the BZ receptor-[^{35}S]TBPS site interaction. Similarly, perturbation of the binding domain of the BZ receptor by TBPS is most effectively recognized by BZ receptor ligands that possess intrinsic activity (Fig. 1). In this regard, the pharmacological relevance of the findings by other investigators showing enhancement of [^{35}S]TBPS binding by GABA-positive ligands and inhibition by GABA-negative compounds is difficult to rationalize (7, 8).

Based upon our earlier study, GABA-positive BZs appear to modulate [^{35}S]TBPS binding in a manner consistent with their presumed role as "GABA potentiators" only when micromolar quantities of GABA are present (9). This observation may

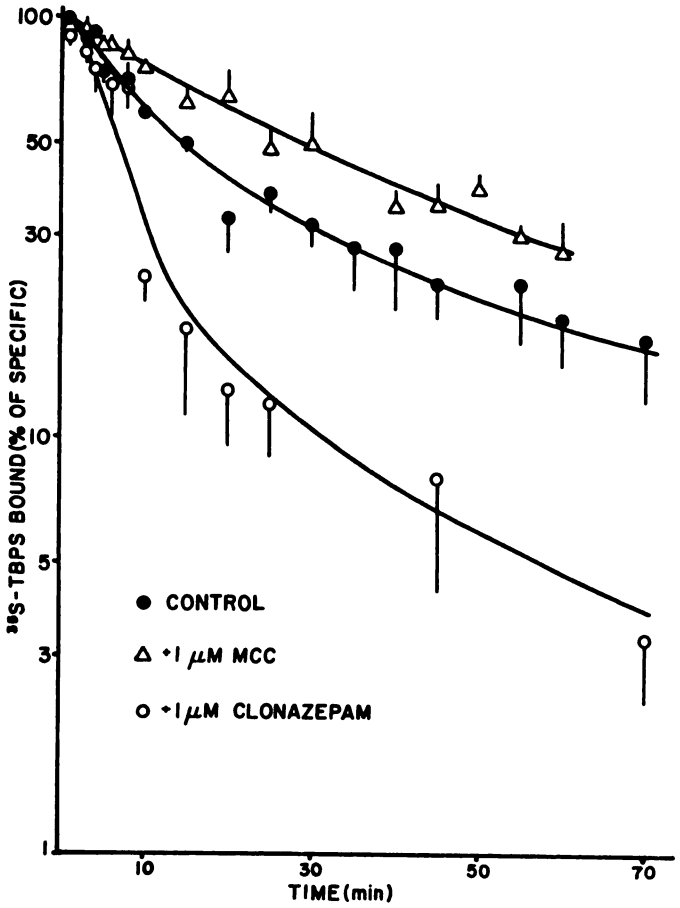

Fig. 4. Time course for the dissociation of 2 nM [^{35}S]TBPS from rat cerebral cortical P_2 preparations in the presence or absence of 1 μM clonazepam or MCC. Each *point* represents the mean ± standard error of four independent determinations. Dissociation was initiated by the addition of 2 μM TBPS following a 90-min incubation at 25°. All assays were performed in the presence of 10 μM GABA.

resolve the question of why other investigators found that GABA-positive BZs such as clonazepam enhance [^{35}S]TBPS binding. Clonazepam produces dose-dependent enhancement of [^{35}S]TBPS binding only in a GABA-free environment, conditions similar to those found in studies using extensively washed and dialyzed membrane preparations (7, 8). MCC enhancement of [^{35}S]TBPS binding in a GABA-free preparation (Fig. 6) in a manner similar to that observed with membrane homogenates suggests that GABA-negative ligands may produce their effect independently of GABA. It is noteworthy that the net result of GABA-induced reductions in the apparent affinities of both MCC and [^{35}S]TBPS for their respective recognition sites is the enhancement of MCC's potency as a facilitator of [^{35}S]TBPS binding (6, 14). Interestingly, MCC produces its effect on [^{35}S]TBPS binding in a way that is consistent with its pharmacological properties (i.e., "a GABA antagonist") with or without GABA. The micromolar concentrations of GABA required to produce the effects observed and the sensitivity of the GABA effect to (+)-bicuculline but not baclofen imply action at a low affinity $GABA_A$ receptor subtype. Overall, these studies further support the concept of an allosteric link between the BZ receptor and a chloride ionophore. Whether the link between these two components of the $GABA_A$-BZ receptor complex is direct or occurs through a $GABA_A$ receptor cannot be discriminated by the present studies.

If GABA-positive ligands are presumed to enhance GABA neurotransmission (i.e., enhance the effects of GABA), then they should modulate the [^{35}S]TBPS-labeled ionophore in the same fashion as GABA (3, 5). Conversely, if GABA-negative ligands act as "GABA antagonists" (i.e., inhibit GABA neurotransmission), then enhancement would be expected. Consistent with these expectations, all BZ receptor ligands examined in the present study behave in the predicted manner. Using the strongly GABA-positive ligand clonazepam as a reference, CL 218872 is less potent an inhibitor of [^{35}S]TBPS but equally efficacious. It is known that CL 218872 is less potent than clonazepam as an anxiolytic and as an antagonist of metrazol-induced seizures in rats (15–17). However, based upon the ability of CL 218872 to inhibit [^{35}S]TBPS binding, it should have a predicted efficacy equal to that of clonazepam. In contrast to previous reports (16), CL 218872 has been observed to produce ataxia comparable to that produced by the strongly GABA-positive BZ diazepam and consistent with its effect on [^{35}S]TBPS binding (17). Several studies now indicate that Ro15 1788 is nearly GABA neutral, with slight GABA-positive agonist activity at the BZ receptor (18–21). The maximum inhibitory effect of Ro15 1788 (91% of control) on [^{35}S]TBPS binding relative to clonazepam is also consistent with slight GABA-positive activity (Fig. 3A).

The case of the two quinoline derivatives, PK 8165 and PK 9084, is novel in that both compounds have bidirectional effects on [^{35}S]TBPS binding. The two phenylquinolines have been reported to possess antianxiety activity devoid of sedative or anticonvulsant effects (22). In contrast to an earlier report (22),

TABLE 1

Dissociation kinetic parameters for the dissociation of 2 nM [^{35}S]TBPS in the presence or absence of 1 μM clonazepam or MCC

[^{35}S]TBPS (2 nM) was brought to steady state conditions in the presence or absence of 1 μM clonazepam or MCC at 25°. GABA (10 μM) was present in all assays. Dissociation was initiated by the addition of 2 μM TBPS. Half-life values were derived from the best fit of the data from four independent determinations using computerized nonlinear regression analysis. Relative proportions of S and R components were similarly derived. The $t_{1/2}$ and percentage of specific sites values represent the mean ± standard error. The dissociation rate constants (k_{-1}) were calculated from the equation $k_{-1} = (0.693/t_{1/2})$. S and R represent the slow and rapid components, respectively.

Conditions	$t_{1/2}$		k_{-1}		Total percentage of specific sites	
	S	R	S	R	S	R
	min		min^{-1}			
Control	55 ± 9	4.2 ± 1	0.01265	0.1662	29 ± 2	75 ± 2
+1 μM Clonazepam	77 ± 15	7.9 ± 3	0.0090	0.0880	5 ± 1[a]	93 ± 1[a]
+1 μM MCC	43 ± 7	3.0 ± 1	0.0162	0.231	60 ± 3[a]	40 ± 3[a]

[a] Significantly different from control at $p < 0.001$ by Student's t test.

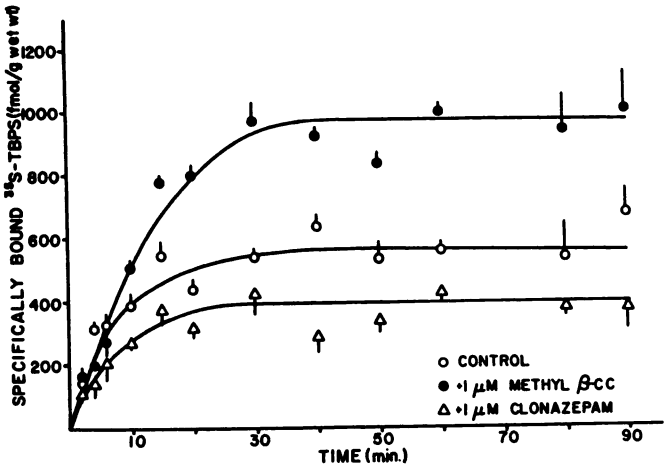

Fig. 5. Time dependence of 2 nM [^{35}S]TBPS binding in rat cerebral cortex. Effects of clonazepam and methyl β-carboline-3-carboxylate (methyl β-CC) on the time course are compared to control binding. Each *point* represents the mean ± standard error of four independent determinations. Nonspecific binding was defined by 2 μM TBPS and was stable over the entire time course. Incubations were performed in the presence of GABA (10 μM) and maintained for up to 90 min at 25°.

TABLE 2

The kinetic parameters for the association of 2 nM [^{35}S]TBPS in the presence of 1 μM clonazepam or MCC

The association rate constants (k_{+1}) were calculated from the equation $k_{+1} = (k_{ob} - k_{-1})/L$, where k_{ob} is the slope of the plot of $\ln[(B_e - B_t)/B_e]$ versus time, derived by computerized nonlinear regression analysis. Association was carried out in the presence of 10 μM GABA. The k_{ob} values are expressed as the mean ± standard error of four independent determinations.

Condition	k_{ob}	k_{+1}
	min^{-1}	min^{-1}
+1 μM MCC	0.066 ± 0.001	0.025
+1 μM Clonazepam	0.090 ± 0.020	0.001

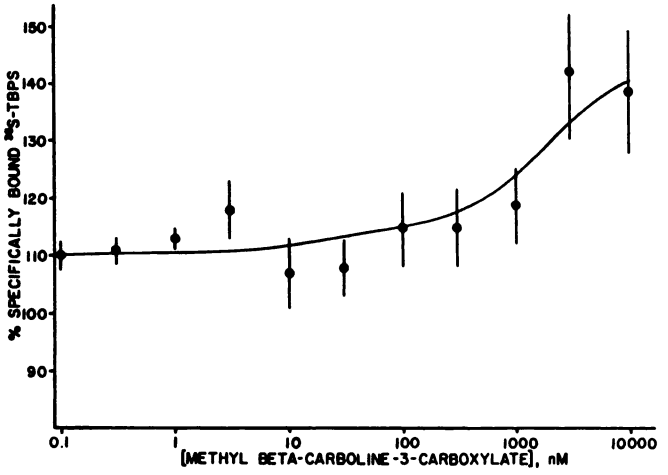

Fig. 6. Modulation of 2 nM [^{35}S]TBPS binding by MCC in the GABA-free soluble fraction extracted from rat cerebral cortex. Each *point* represents the mean ± standard error of four independent determinations. Data were plotted as log MCC concentration versus percentage of specifically bound [^{35}S]TBPS. Soluble fraction was rendered GABA-free by Sephadex G-50 column chromatography. Nonspecific binding was defined as binding in the presence of 2 μM TBPS. All incubations were maintained at 25° for 90 min.

File and co-workers (23, 24) found that both PK 8165 and PK 9084 have little or no anxiolytic activity and may indeed have proconvulsant or convulsant actions. Entirely consistent with these observations, both PK 8165 and PK 9084 were found to produce slight inhibition of [^{35}S]TBPS at low concentrations (i.e., GABA-positive-like activity) and enhancement of binding (i.e., GABA negative-like) at high concentrations (>300 nM).

The manner in which GABA-negative ligands modulate [^{35}S]TBPS binding is also consistent with their pharmacological profile as anxiogenics and convulsants. The most pronounced enhancement of [^{35}S]TBPS binding was produced by the strongly GABA-negative ligand, MCC (Figs. 3B and 6). MCC produced dose-dependent enhancement of [^{35}S]TBPS binding over a broad concentration range. The β-carbolines, ECC and PCC, produced slight enhancement of [^{35}S]TBPS binding over the range of concentrations tested. Behavioral studies suggest that both ECC and PCC are anxiogenic (25–28).

The kinetics of [^{35}S]TBPS binding examined in the presence of a GABA-positive or a GABA-negative ligand revealed information regarding the possible mechanism(s) by which the binding of [^{35}S]TBPS is modulated. Under control conditions, [^{35}S]TBPS appears to bind to two different conformations or states of the ionophore as suggested by the multicomponent association and dissociation curves. Other investigators have also observed curvilinear dissociation plots and have suggested that [^{35}S]TBPS dissociates from multiple sites or conformations of the ionophore (6, 29). Recently, Tehrani *et al.* (30) reported the presence of two binding sites for [^{35}S]TBPS in extensively washed rat cerebral cortex discriminated by Scatchard analysis (30). Our inability to observe multiple sites may stem from differences in the method of tissue preparation and assay conditions. Thus, the data from the present study do not resolve the existence of different conformations of the [^{35}S]TBPS site versus heterogeneity of sites (i.e., structurally distinct sites), nor does it identify the relationship, if any, between the kinetically determined sites and the two sites identified by Scatchard analysis of steady state binding data (30).

The kinetic studies using clonazepam suggest that GABA-positive ligands increase the proportion of chloride ionophores with low affinity for [^{35}S]TBPS, whereas the opposite occurs when a GABA-negative ligand (i.e., MCC) is present. The specificity of these interactions for the BZ receptor is supported by the observation that the effects of these ligands on [^{35}S]TBPS binding occur in a concentration range which is consistent with their apparent K_i values for the BZ receptor under similar conditions. For example, the K_i for clonazepam/[^{3}H]FLU interactions is ~10 nM, a value that is consistent with the reported IC_{50} (concentration at which half-maximal inhibition of [^{35}S]TBPS occurs where half-maximal is ~77% of control) of 15 nM for clonazepam/[^{35}S]TBPS interactions (9). The net effect of GABA-positive and GABA-negative ligands on [^{35}S]TBPS binding may, in part, be related to alterations in the apparent affinity of the ionophore for [^{35}S]TBPS. Perhaps occupation of the BZ receptor by GABA-positive or GABA-negative ligands induces a conformational change in the [^{35}S]TBPS binding site.

Ample evidence exists to suggest the existence of BZ receptor subtypes (31). The functional significance of such receptor subtypes would be enhanced if each subtype were found to be functionally linked to an ionophore. Since PCC shows intrinsic activity by its ability to modulate [^{35}S]TBPS binding, it was

TABLE 3

The effect of (+)-bicuculline and baclofen on GABA-induced enhancement of clonazepam or MCC modulation of [^{35}S]TBPS binding in the soluble fraction of rat cerebral cortex

All values are expressed as the mean ± standard error (n = 4–5) of the percentage of specific [^{35}S]TBPS binding in the absence of a modulator.

Modulator	Control	+ GABA[a]	+ GABA + bicuculline[b]	+ GABA + baclofen[c]
Clonazepam (0.1 μM)	112 ± 7[d]	59 ± 5[e,f]	100 ± 3	62 ± 4
MCC (1 μM)	119 ± 7	253 ± 47[g]	133 ± 10	216 ± 20
MCC (3 μM)	242 ± 11	204 ± 14[h]	ND[i]	ND

[a] GABA concentration was 10 μM.
[b] (+)-Bicuculline concentration was 10 μM.
[c] Baclofen concentration was 10 μM.
[d] Slight enhancement of [^{35}S]TBPS binding occurs in the absence of GABA.
[e] Expressed as a percentage of [^{35}S]TBPS binding in the presence of 10 μM GABA.
[f] Significantly different from control at $p < 0.005$ by Student's t test.
[g] Significantly different from control at $p < 0.05$ by Student's t test.
[h] Significantly different from control at $p < 0.02$ by Student's t test.
[i] ND, not determined.

reasonable to assume that TBPS would modulate [^{3}H]PCC binding if coupling was present. The dual observations of dose-dependent enhancement of 40 pM [^{3}H]PCC binding by TBPS in the hippocampus and the sensitivity of this enhancement to GABA modulation suggests that these BZ_1 receptors are coupled to a chloride ionophore and the chloride ionophore is coupled to a $GABA_A$ site (Fig. 2). However, previous studies by Gee *et al.* (13) suggested that the BZ_1 receptor subtype in the rat hippocampus is not functionally coupled to a GABA site. One possible model that is consistent with the available evidence is a chloride ionophore that is independently modulated via interactions at a $GABA_A$ receptor and a BZ_1 receptor subtype but with no interaction between the GABA and BZ_1 sites. Whether such a receptor-ionophore complex exists and is of functional significance remains to be determined. In contrast, the BZ_2 receptor subtype appears to be coupled to both a chloride ionophore and a GABA receptor (13).

In conclusion, [^{35}S]TBPS binding provides a pharmacologically relevant *in vitro* "response" to occupancy of the BZ receptor by ligands with intrinsic activity. The site on the chloride ionophore labeled by [^{35}S]TBPS appears to exist as two distinct sites or in at least two conformational states. Dissociation kinetics data suggest that GABA-positive ligands stabilize the R conformation, whereas GABA-negative ligands shift the equilibrium in favor of the S conformation. It is tempting to speculate that this arrangement is consistent with the possibility that the S component of the site is associated with a closed chloride channel, whereas the R component is related to an open channel. Consequently, the pharmacological potency and efficacy of a BZ receptor ligand as a modulator of chloride channel function can be predicted by its ability to shift the equilibrium between the two apparent states of the site labeled by [^{35}S]TBPS. Both BZ_1 and BZ_2 receptor subtypes discriminated by PCC in rat hippocampus appear to be coupled to a [^{35}S]TBPS-labeled site. This coupling allows the independent study of the possible differences between the chloride ionophore-BZ receptor interactions associated with the BZ_1 versus BZ_2 receptor subtypes. Both GABA-positive and GABA-negative ligand-induced changes in [^{35}S]TBPS binding are modulated by GABA via a low affinity $GABA_A$ receptor. Finally, [^{35}S]TBPS appears to be a valuable probe for the study of the events involved in the modulation of a site linked to the chloride ionophore associated with the $GABA_A$-BZ receptor complex.

Acknowledgments

We wish to thank Dr. J. E. Casida for his generous gift of TBPS, Hoffmann-LaRoche for the various BZ receptor ligands used in this study, and Lederle Laboratories for CL 218872.

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