[³⁵S]-t-BUTYLBICYCLOPHOSPHOROTHIONATE BINDING SITES ARE CONSTITUENTS OF THE γ -AMINOBUTYRIC ACID BENZODIAZEPINE RECEPTOR COMPLEX

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Abstract

t-Butylbicyclopophosphorothionate (TBPS), a derivative of potent GABA antagonistic cage convulsants, has recently been introduced (Squires, R. F., J. E. Casida, M. Richardson, and E. Saederup (1983) *Mol. Pharmacol.* 13: 326-336) as ligand for a GABA-A receptor-linked drug receptor. Using conventionally prepared washed membrane fractions from rat cerebral cortex, we have confirmed that in the presence of 200 mM NaBr [³⁵S]TBPS binds to a high affinity population of binding sites (K_d 26 ± 5 nM) and that muscimol inhibits [³⁵S]TBPS binding (IC_{50} $0.32 \mu M$) allosterically. In 200 mM NaCl the apparent affinity of [³⁵S]TBPS binding sites is lower (K_d 60 ± 5 nM), and muscimol has biphasic effects with stimulation at low concentrations of muscimol (EC_{50} $0.023 \mu M$) followed by inhibition at high concentrations (IC_{50} $0.72 \mu M$). Both base line [³⁵S]TBPS binding (in 200 mM NaCl) and muscimol inhibition of [³⁵S]TBPS binding (in 200 mM NaBr) are bidirectionally modulated by the occupancy of benzodiazepine receptors with its ligands. Benzodiazepine receptor agonists, regardless of their structure, enhance and inverse benzodiazepine receptor agonists inhibit base line [³⁵S]TBPS binding and muscimol inhibition of [³⁵S]TBPS binding. Fourteen ligands for benzodiazepine receptors display a similar *in vitro* profile as benzodiazepine receptor agonists or inverse benzodiazepine receptor agonists on [³⁵S]TBPS binding as their anti- or proconvulsive effects *in vivo* suggest (Jensen, L. H., E. N. Petersen, and C. Braestrup (1983) *Life Sci.* 33: 393-399). That [³⁵S]TBPS binding sites are constituents of a GABA benzodiazepine receptor complex is also suggested by a number of membrane pretreatments. After photoaffinity labeling of benzodiazepine receptors with flunitrazepam, [³⁵S]TBPS binding sites became insensitive to modulation by flunitrazepam but not by 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylic acid methyl ester. Pretreatment of membranes with Ag⁺ ions, which leads to the sole appearance of high affinity [³H]muscimol binding sites, to high affinity muscimol stimulation of [³H]flunitrazepam binding, and to a loss of allosteric modulation of these binding sites by etazolate (SQ 20009) or isopropylbicyclopophosphate (IPTBO), results in the disappearance of [³⁵S]TBPS binding. Pretreatment of membranes with Triton X-100, which perturbs the GABA and benzodiazepine receptor binding in a mode similar to Ag⁺ ions, also leads to the disappearance of [³⁵S]TBPS binding. Thus [³⁵S]TBPS binding sites can be inactivated separately from GABA and benzodiazepine receptors, and this inactivation goes parallel with perturbation of GABA and benzodiazepine receptor binding.

The CNS depressants etazolate and pentobarbital differ in their effects on [³⁵S]TBPS binding when compared to picrotoxinin and IPTBO. The latter compounds inhibit [³⁵S]TBPS binding unperturbed by the occupancy of benzodiazepine receptors. Etazolate and pentobarbital (in 200 mM NaCl) have biphasic effects on [³⁵S]TBPS binding with stimulation at low concentrations (EC_{50} $0.4 \mu M$ and $60.4 \mu M$) and inhibition (IC_{50} $12.9 \mu M$ and $550 \mu M$) at higher concentrations with Hill numbers of 1.5 and 2.7. The potencies of etazolate or pentobarbital as inhibitors of [³⁵S]TBPS binding (in 200 mM NaBr) are bidirectionally modulated by the occupancy of benzodiazepine receptors with agonists or with inverse agonists. These observations are not compatible with the concept that these CNS depressants and CNS convulsants act on an identical drug receptor.

There is evidence from biochemical and electrophysiological experiments that benzodiazepines exert their

pharmacological effects in the brain by enhancing the actions of the inhibitory neurotransmitter GABA (Costa et al., 1975; Haefely et al., 1975). Later, benzodiazepines have been shown to interact with their brain-specific

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drug receptors (Möhler and Okada, 1977; Squires and Braestrup, 1977), which appear to be constituents of certain types of GABA receptors (Karobath and Sperk, 1979; Tallmann et al., 1978), the GABA-benzodiazepine-chloride ionophore receptor complex (Supavilai and Karobath, 1981; Olsen and Leeb-Lundberg, 1981). The investigation of GABA and benzodiazepine binding sites and their interactions with the various components of the GABA benzodiazepine receptor complex has been possible by the availability of suitable ligands for these binding sites. However, the investigation of the drug receptor of GABA-regulated chloride ion channels has been very difficult since the only available ligand, [^3H] dihydropicrotoxinin, has very high unspecific binding to brain membrane fractions (Ticku et al., 1978). But recently Squires et al. (1983) have investigated the binding of [^{35}S]-t-butylbicyclophosphorothionate ([^{35}S]TBPS), a derivative of a series of potent GABA antagonistic cage convulsants (Bowery et al., 1976). They demonstrated that [^{35}S]TBPS binds with high affinity to brain-specific binding sites linked to GABA receptors with biochemical and pharmacological properties similar, but not identical, to [^3H]dihydropicrotoxinin binding sites (Squires et al., 1983). We now report that these [^{35}S]TBPS binding sites are subject to allosteric modulations by drugs which act at the GABA-benzodiazepine-chloride ionophore receptor complex.

Materials and Methods

Membrane preparation. Membranes from the cerebral cortex of male Wistar rats (150 to 200 gm) were prepared by homogenization and recentrifugation at least five times with ice-cold 50 mM Tris-HCl buffer, pH 7.1, as described in detail elsewhere (Placheta and Karobath, 1980). These membrane suspensions were kept frozen overnight and were used for binding experiments after one further centrifugation in 50 mM Tris-citrate buffer, pH 7.5.

In some experiments, aliquots of the membranes were pretreated with 0.05% Triton X-100 as described (Placheta and Karobath, 1980). In other experiments, aliquots of the membrane fractions resuspended in 50 mM Tris-citrate buffer were pre-incubated for 15 min at 23°C with 100 μM AgNO_3 , washed twice in the same buffer at 0°C, and then used for binding experiments (Supavilai et al., 1982). Pretreatment of membranes by photoaffinity labeling with 20 nM unlabeled flunitrazepam was performed by incubating membrane suspensions in ice-cold 50 mM Tris-citrate buffer, pH 7.5, for 20 min in the absence (controls) or presence of long wavelength UV light (Karobath and Supavilai, 1982). Membrane suspensions were then washed twice in 50 mM Tris-citrate buffer, pH 7.5, to remove nonincorporated flunitrazepam (Hirsch, 1982).

Standard [^{35}S]TBPS binding procedure. Membrane suspensions containing the equivalent of about 4 mg of fresh tissue were incubated essentially as described by Squires et al. (1983) at 23°C for 90 min in 50 mM Tris-citrate buffer, pH 7.5, with the appropriate concentrations of drugs to be tested and 2 nM [^{35}S]TBPS (68.7 and 20.3 Ci/mmol; New England Nuclear Corp.). Final incubation volume was 1 ml. Triplicate incubations were

performed with SEM <5%. Separation of membrane-bound from free ligand was performed by rapid filtration through Whatman GF/B glass fiber filter discs (1 cm diameter) followed by two washings with 4.5 ml of ice-cold 50 mM Tris-HCl buffer, pH 7.1. Radioactivity on the filter discs was determined by liquid scintillation counting. Nonspecific [^{35}S]TBPS binding was defined as that in the presence of 30 μM isopropyl bicyclophosphate (IPTBO), and it constituted approximately 10% of total [^{35}S]TBPS binding.

Kinetic analysis of equilibrium binding experiments was performed with affinity spectra (Tobler and Engel, 1983). In these experiments, the binding of 2 nM [^{35}S]TBPS was determined in the absence or in the presence of increasing concentrations of unlabeled TBPS (1, 2, 5, 10, 20, 50, 100, 200, 500, and 1000 nM).

Chemicals and reagents. [^{35}S]TBPS (68.7 and 20.3 Ci/mmol) was purchased from New England Nuclear (Boston). Drugs were kindly donated by the following persons and institutions: diazepam, clonazepam, flunitrazepam, oxazepam, and Ro 15-1788 by H. Möhler, Hofmann-La Roche, Basle, Switzerland; propyl- β -carboline-3-carboxylate (β -CCPr), methyl- β -carboline-3-carboxylate (β -CCM), ethyl- β -carboline-3-carboxylate (β -CCE), β -carboline-3-carboxyl acid ethyl ester methylamide (FG 7142), and 6,7-dimethoxy-4-ethyl- β -carboline-3-carboxylic acid methyl ester (DMCM), by C. Braestrup, St. Hans Hospital, Roskilde, Denmark; Cl 218-872 by American Cyanamide (Pearl River, NY); IPTBO by J. Collins, City of London Polytechnic (London); TBPS by R. Squires (Orangeburg, NY); etazolol (SQ 20009) and SQ 20006 by S. Lucania, E.R. Squibb and Sons, Inc. (Princeton, NY); R 5135 from P. Hunt, Roussel Uclaf (Romainville, France). All other chemicals and reagents were of highest purity commercially available.

Results

Influence of muscimol and salts. In our membrane preparations [^{35}S]TBPS binds to a single population of binding sites which exhibit a higher affinity when the binding experiments are performed in the presence of 200 mM NaBr (K_d 26 ± 5 nM; B_{max} 178 ± 11 fmol/mg of tissue wet weight) than in the presence of 200 mM NaCl (K_d 60 ± 5 nM; B_{max} 193 ± 30 fmol/mg of tissue wet weight).

As has been described (Squires et al., 1983), the stereospecific interaction of GABA-like compounds with GABA recognition sites leads to an allosteric inhibition of [^{35}S]TBPS binding. Similar to these results we find inhibition of [^{35}S]TBPS binding by muscimol (IC_{50} 323 ± 15 nM; mean $\pm$ SEM; $n = 14$) in incubations performed in the presence of 200 mM NaBr (Fig. 1). However, when NaBr is reduced to 100 mM (not shown) or when it is replaced by 200 mM NaCl, biphasic actions of muscimol with high affinity stimulation (EC_{50} 23 ± 4 nM; mean $\pm$ SEM; $n = 10$) and low affinity inhibition (IC_{50} 724 ± 69 nM; $n = 10$) are observed (Fig. 2). The EC_{50} and the IC_{50} values for muscimol correspond to the apparent K_d values of high and low affinity [^3H]muscimol binding in the same assay conditions (Supavilai et al., 1982). In the presence of R 5135, a potent, bicuculline-like GABA antagonist (Hunt and Clement-Jewery, 1981), the bi-

Figure 1. Concentration response curves of muscimol alone and in the presence of drugs acting on benzodiazepine receptors on $[^{35}\text{S}]$ TBPS binding to membranes of rat cerebral cortex. The binding of 2 nM $[^{35}\text{S}]$ TBPS in the presence of 200 mM NaBr was investigated as described under "Materials and Methods." The results are mean values of triplicate incubations with SEM <5%. This experiment was repeated twice with similar results. *Flu*, flunitrazepam.

Figure 2. Biphasic concentration response curves of muscimol alone and in the presence of three different concentrations of R 5135 on $[^{35}\text{S}]$ TBPS binding. The binding of 2 nM $[^{35}\text{S}]$ TBPS was investigated in the presence of 200 mM NaCl as described under "Materials and Methods." The experiment was repeated twice with similar results. *R 5135*, 3 α -hydroxy-16-imino-5 β -17-aza-androstan-11-one.

phasic effects of muscimol on $[^{35}\text{S}]$ TBPS binding persist with the concentration response curves of muscimol shifted to the right (Fig. 2).

Influence of the occupancy of benzodiazepine receptors. Recently it has been proposed that drugs acting on benzodiazepine receptors can have agonistic (benzodiazepine-like) effects or inverse benzodiazepine agonistic effects (like some β -carbolines and DMCM) (Polc et al., 1982). In addition, a third class of ligands has been proposed, the benzodiazepine receptor antagonists, which have no marked pharmacological effects of their own but which antagonize the actions of benzodiazepine receptor agonists and of inverse benzodiazepine receptor agonists (Polc et al., 1982). When we investigated the actions of benzodiazepine receptor agonists on $[^{35}\text{S}]$ TBPS binding in the presence of 200 mM NaBr, we observed only weak effects of benzodiazepine agonists on $[^{35}\text{S}]$ TBPS binding (Fig. 1, see ordinate line), which is in agreement with previous observations (Squires et al., 1983). However, DMCM (Fig. 1), a proposed inverse benzodiazepine receptor agonist (Jensen et al., 1983), and to a lesser extent the partial inverse agonists β -CCM, β -CCE, FG 7142, and 2-phenylpyrazolo [4,3-*c*]quinolin-3-(5H)-one (CGS 8216) slightly decrease $[^{35}\text{S}]$ TBPS binding (not shown). These actions are more pronounced when these drugs are reinvestigated in incubation media which contain 200 mM NaCl. It can be seen from Table I that benzodiazepine receptor agonists stimulate and inverse benzodiazepine receptor agonists inhibit $[^{35}\text{S}]$ TBPS binding. Ro 15-1788, a benzodiazepine receptor antagonist, only slightly stimulates $[^{35}\text{S}]$ TBPS binding (Table I), but it is able to antagonize the stimulatory action of flunitrazepam and the inhibitory effects of DMCM (Fig. 3). The latter compound, like β -CCM, in high concentrations inhibits $[^{35}\text{S}]$ TBPS binding to a lesser extent than at lower concentrations (Table I).

TABLE I

Effect of ligands for benzodiazepine receptors on base line $[^{35}\text{S}]$ TBPS binding

Binding experiments with 2 nM $[^{35}\text{S}]$ TBPS were performed as described under "Materials and Methods" in the presence of 200 mM NaCl.

Addition	μM	$[^{35}\text{S}]$ TBPS Binding: Percentage of Control (Mean $\pm$ SEM; $n \geq 3$)
None (control)		100 $\pm$ 2
Flunitrazepam	0.5	172 $\pm$ 5
Diazepam	1	160 $\pm$ 7
Clonazepam	0.2	156 $\pm$ 7
Oxazepam	1	152 $\pm$ 3
Zopiclone	1	173 $\pm$ 2
Cl 218-872	10	144 $\pm$ 4
CGS 9896	0.1	117 $\pm$ 5
Ro 15-1788	0.2	120 $\pm$ 10
β -CCPr	0.2	106 $\pm$ 7
CGS 8216	0.1	95 $\pm$ 5
β -CCE	0.2	89 $\pm$ 1
FG 7142	2.5	91 $\pm$ 1
β -CCM	0.1	83 $\pm$ 5
β -CCM	1	98 $\pm$ 3
DMCM	0.1	71 $\pm$ 5
DMCM	1	71 $\pm$ 6
DMCM	10	93 $\pm$ 4

In addition to these actions on base line [35 S]TBPS binding, the occupancy of benzodiazepine receptors with their ligands leads also to a perturbation of the allosteric actions of muscimol on [35 S]TBPS binding (Fig. 1). The investigation of 14 drugs acting on benzodiazepine receptors reveals that benzodiazepine receptor agonists facilitate, and inverse benzodiazepine receptor agonists inhibit the action of muscimol on [35 S]TBPS binding (Table II). Ro 15-1788 and β -CCPr lead only to a weak facilitation of the allosteric effects of muscimol. All drugs do not alter the Hill number of the inhibitory effect of muscimol (1.6 ± 0.1 ; $n = 4$) on [35 S]TBPS binding significantly.

Influence of CNS depressants. The CNS depressant etazolate (SQ 20009), which inhibits [3 H]dihydropicrotoxinin and [35 S]TBPS binding (Leeb-Lundberg et al., 1980; Squires et al., 1983), has been shown to perturb GABA and benzodiazepine receptor binding (Beer et al., 1978; Williams and Risley, 1979) at a site which is sensitive to antagonism by picrotoxin and cage convulsants (Supavilai and Karobath, 1979; Leeb-Lundberg et al., 1981). In confirmation of the results presented by Squires et al. (1983), we observe inhibition of [35 S]TBPS binding by etazolate ($IC_{50} 2.8 \pm 0.2 \mu M$; $n = 5$) and by pentobarbital ($IC_{50} 260 \pm 27 \mu M$; $n = 5$) in incubations performed in the presence of 200 mM NaBr (Fig. 4). SQ 20006, a centrally inactive analogue of etazolate which does not alter [3 H]muscimol (Placheta and Karobath, 1980) or [3 H]flunitrazepam binding (Supavilai and Ka-

TABLE II
Effect of ligands for benzodiazepine receptors on muscimol-induced inhibition of [35 S]TBPS binding

The binding of 2 nM [35 S]TBPS in the presence of 200 mM NaBr was determined as described under "Materials and Methods." IC_{50} values for muscimol have been calculated without consideration of base line changes, which tends to reduce the effects of the drugs. For example, IC_{50} values for muscimol, when corrected for base line changes, are $0.12 \pm 0.01 \mu M$ in the presence of $1 \mu M$ diazepam and $0.82 \pm 0.05 \mu M$ in the presence of $0.5 \mu M$ DMCM.

Addition	μM	IC_{50} Muscimol (Mean $\pm$ SEM; $n \geq 3$)
None		0.30 ± 0.02
Flunitrazepam	0.5	0.16 ± 0.01
Diazepam	1	0.15 ± 0.02
Clonazepam	0.2	0.18 ± 0.02
Oxazepam	1	0.16 ± 0.01
Zopiclone	1	0.13 ± 0.03
CI 218-872	10	0.16 ± 0.02
CGS 9896	0.1	0.26 ± 0.01
Ro 15-1788	0.2	0.25 ± 0.03
β -CCPr	0.2	0.27 ± 0.02
CGS 8216	0.1	0.38 ± 0.03
β -CCE	0.2	0.40 ± 0.02
FG 7142	2.5	0.40 ± 0.04
β -CCM	0.2	0.49 ± 0.01
DMCM	0.5	0.58 ± 0.03

Figure 3. Inhibition by Ro 15-1788 or DMCM of [35 S]TBPS binding stimulated by flunitrazepam and stimulation by Ro 15-1788 or flunitrazepam of [35 S]TBPS binding inhibited by DMCM. Binding experiments in 50 mM Tris-citrate buffer, pH 7.5, containing 200 mM NaCl and 2 nM [35 S]TBPS were performed as described under "Materials and Methods." The experiment is typical of three. *Flu*, flunitrazepam.

Figure 4. Effects of etazolate and pentobarbital on [35 S]TBPS binding. Experiments were performed as described under "Materials and Methods." The experiment is typical of three. *Eta*, etazolate; *Pento*, pentobarbital.

robath, 1980), but which has equipotent actions on cAMP phosphodiesterase (A. Free, Squibb Institute for Medical Research, personal communication), fails to influence [35 S]TBPS binding in concentrations up to $100 \mu M$ (not shown). However, in the presence of 200 mM NaCl, etazolate and pentobarbital have unexpectedly biphasic actions on [35 S]TBPS binding with stimulation at lower concentrations ($EC_{50} 0.4 \pm 0.1 \mu M$; $n = 3$ and $60.4 \pm 6 \mu M$; $n = 3$, respectively) and inhibition at higher concen-

trations (IC_{50} $12.9 \pm 3 \mu\text{M}$; $n = 3$ and $550 \pm 50 \mu\text{M}$; $n = 3$, respectively) (Fig. 4). The Hill number for etazolate inhibition is 1.5 ± 0.1 ($n = 5$), and that for pentobarbital inhibition is 2.7 ± 0.2 ($n = 5$). The inhibitory effects of pentobarbital occur at high concentrations, which may lead to an unspecific perturbation of membranes. Therefore, we have investigated $[^{35}\text{S}]\text{TBPS}$ binding after exposure of membrane fractions with even higher concentrations of pentobarbital (incubation for 30 min at 23°C with 2 mM pentobarbital followed by two washings in order to remove potential dissociated membrane constituents and pentobarbital) and find unchanged binding after this procedure (Table III). In contrast to the CNS

depressants etazolate and pentobarbital, the convulsants picrotoxinin and IPTBO have only inhibitory effects on $[^{35}\text{S}]\text{TBPS}$ binding regardless of whether 200 mM NaBr or NaCl is present (Fig. 5). These observations indicate differences in the interaction of CNS depressants and CNS convulsants with $[^{35}\text{S}]\text{TBPS}$ binding sites.

Such differences are also observed when the effects of these drugs on $[^{35}\text{S}]\text{TBPS}$ binding are investigated under conditions where benzodiazepine receptors are occupied with their ligands. It can be seen from Figure 6 that

TABLE III
Effects of membrane pretreatments on $[^{35}\text{S}]\text{TBPS}$ binding
Membrane pretreatment and binding experiments with 2 nM $[^{35}\text{S}]\text{TBPS}$ were performed in 50 mM Tris-citrate buffer containing 200 mM NaBr as described under "Materials and Methods."

Pretreatment	Addition	Specific $[^{35}\text{S}]\text{TBPS}$ Binding (Mean $\pm$ SEM, $n \geq 3$) fmol/mg of tissue wet weight
None	None	10.50 ± 0.65
None	Flunitrazepam, 0.5 μM	12.35 ± 0.17
None	DMCM, 0.5 μM	7.56 ± 0.29
Photoaffinity labeling with flunitrazepam, 20 nM	None	10.40 ± 0.36
Photoaffinity labeling with flunitrazepam, 20 nM	Flunitrazepam, 0.5 μM	11.04 ± 0.28
Photoaffinity labeling with flunitrazepam, 20 nM	DMCM, 0.5 μM	8.23 ± 0.28
Pentobarbital, 2 mM	None	9.82 ± 0.45
Triton X-100, 0.05%	None	0
AgNO_3 , 100 μM	None	0.34 ± 0.24

Figure 5. Effect of picrotoxinin and IPTBO on the binding of 2 nM $[^{35}\text{S}]\text{TBPS}$. Experiments were performed as described under "Materials and Methods." The experiment is typical of three. Picro, picrotoxinin.

Figure 6. Modulation of etazolate (a) or pentobarbital- (b) induced inhibition of $[^{35}\text{S}]\text{TBPS}$ binding by ligands for benzodiazepine receptors. The binding of 2 nM $[^{35}\text{S}]\text{TBPS}$ in 50 mM Tris-citrate buffer, pH 7.5, containing 200 mM NaBr was determined as described under "Materials and Methods." The experiment is typical of three. Flu, flunitrazepam.

flunitrazepam increases and DMCM decreases the inhibitory effects of etazolate and pentobarbital on [35 S]TBPS binding, with Ro 15-1788 being almost ineffective. In contrast, the inhibitory effects of picrotoxinin or IPTBO (not shown) are not modulated by these drugs (Fig. 7).

Influence of photoaffinity labeling of benzodiazepine receptors with flunitrazepam. The irreversible binding of flunitrazepam by photoaffinity labeling into one of the four postulated benzodiazepine binding sites of the GABA benzodiazepine receptor complex (Möhler et al., 1980) leads to changes in the properties of the three remaining sites (Hirsch, 1982). The affinity of benzodiazepines is decreased about 100-fold, whereas that of benzodiazepine receptor antagonists or of inverse benzodiazepine receptor agonists is not markedly altered (Karobath and Supavilai, 1982). Thus, if [35 S]TBPS labels a drug receptor of the GABA benzodiazepine receptor complex, then photoaffinity labeling with flunitrazepam should inhibit the allosteric effects of flunitrazepam on [35 S]TBPS binding. The results of such experiments with photoaffinity-labeled membranes demonstrate that flunitrazepam, in concentrations which are effective in untreated membranes (Fig. 1), has lost its ability to alter muscimol inhibition of [35 S]TBPS binding (Fig. 8) and to alter base line [35 S]TBPS binding (Table III), whereas the effects of DMCM are not altered (Fig. 8).

Influence of pretreatment of membranes with AgNO₃ or with Triton X-100. Pretreatment of membranes with AgNO₃, followed by subsequent washing to remove non-bound Ag⁺ ions, has been shown to perturb the GABA benzodiazepine receptor complex. Only high affinity [3 H]muscimol binding can be found in such membranes, and

Figure 7. Lack of a modulatory effect of flunitrazepam, Ro 15-1788, and DMCM on picrotoxinin inhibition of [35 S]TBPS binding. The binding of 2 nM [35 S]TBPS in 50 mM Tris-citrate buffer, pH 7.5, containing 200 mM NaBr was determined as described under "Materials and Methods." The experiment is typical of three. *Flu*, flunitrazepam.

Figure 8. Lack of effect of flunitrazepam on muscimol inhibition of [35 S]TBPS binding to membranes of rat cerebral cortex which had been photoaffinity labeled with flunitrazepam. Membrane pretreatment and binding experiments with 2 nM [35 S]TBPS in 50 mM Tris-citrate buffer, pH 7.5, containing 200 mM NaBr were performed as described under "Materials and Methods." The experiment is typical of three. The same investigations with untreated membranes are shown in Figure 1. *Flu*, flunitrazepam.

muscimol stimulates benzodiazepine receptor binding with high affinity (Supavilai et al., 1982). In addition, CNS depressants and CNS convulsants have lost their ability to modulate [3 H]muscimol binding and base line or muscimol-stimulated [3 H]flunitrazepam binding (Supavilai et al., 1982). The investigation of [35 S]TBPS binding to such AgNO₃-pretreated membrane reveals an almost quantitative loss of these binding sites (Table III).

Pretreatment of membranes with Triton X-100 is another perturbation of the GABA benzodiazepine receptor complex which leads to high affinity [3 H]muscimol binding, high affinity stimulation of benzodiazepine binding by GABA (Massotti and Guidotti, 1980), loss of the efficacy of etazolate to modulate [3 H]muscimol or [3 H]flunitrazepam binding (Placheta and Karobath, 1980; Supavilai and Karobath, 1981), and a disappearance of [3 H]dihydropicrotoxinin binding (Ticku and Olsen, 1979). In such Triton X-100-pretreated membranes, no [35 S]TBPS binding is detectable (Table III). This may be the consequence of inactivation or solubilization of [35 S]TBPS binding sites by this treatment.

Discussion

We have investigated the binding of [35 S]TBPS, a new ligand for brain-specific drug receptors linked to GABA receptors, with biochemical and pharmacological properties similar but not identical to [3 H]dihydropicrotoxinin binding sites (Squires et al., 1983). We have used

membranes from the cerebral cortex of rats since in this brain area the majority of GABA binding sites appear to be constituents of the GABA benzodiazepine receptor complex (Palacios et al., 1982). *Our results suggest that $[^{35}\text{S}]$ TBPS binding sites are constituents of the GABA benzodiazepine receptor complex.* This concept is supported by the following observations: (1) Base line and muscimol-inhibited $[^{35}\text{S}]$ TBPS binding is differentially modulated by the occupancy of benzodiazepine receptors with benzodiazepine receptor agonists, antagonists, and inverse benzodiazepine receptor agonists. (2) The irreversible binding of flunitrazepam to benzodiazepine receptors by photoaffinity labeling to one binding site renders adjacent sites into a particular conformation with very low affinity for benzodiazepines (Möhler et al., 1980). In such membranes, flunitrazepam, but not the inverse agonist DMCN, has lost its ability to modulate $[^{35}\text{S}]$ TBPS binding. (3) Pretreatment of membranes with Ag^+ ions or with Triton X-100 abolishes the allosteric effects of etazolate or picrotoxin on $[^3\text{H}]$ muscimol (Placheta and Karobath, 1980) and $[^3\text{H}]$ flunitrazepam binding (Supavilai and Karobath, 1980; Supavilai et al., 1982). In such membranes no $[^{35}\text{S}]$ TBPS binding sites are detectable.

These observations confirm and extend the results of Squires et al. (1983), but there are distinct differences of $[^{35}\text{S}]$ TBPS binding sites in our hands. These include the biphasic actions of a number of agents and differences in the affinity of drugs for $[^{35}\text{S}]$ TBPS binding sites. The reason(s) for these differences is not known but may be related to the different modes of membrane preparations. We investigated membranes which had repeatedly been washed in buffered solution and which have been shown to reveal allosteric interactions between the different constituents of the GABA benzodiazepine receptor complex (Supavilai et al., 1982). On the other hand, Squires et al. (1983) used membranes which had been prepared in EDTA and which had been dialyzed in the presence of EDTA for 2 hr. With the latter membranes, only high affinity (IC_{50} 40 nM) inhibition by muscimol of $[^{35}\text{S}]$ TBPS binding has been observed (Squires et al., 1983), whereas in our investigations muscimol revealed biphasic

actions with high affinity stimulation (EC_{50} 23 nM) and low affinity inhibition (IC_{50} 724 nM). The potency of muscimol to induce its two effects on $[^{35}\text{S}]$ TBPS binding resembles the affinities of the two $[^3\text{H}]$ muscimol binding sites in binding experiments performed at 23°C with the same membrane preparation (Supavilai et al., 1982). The relationship between $[^3\text{H}]$ muscimol binding and actions of muscimol or etazolate on $[^3\text{H}]$ flunitrazepam binding and on $[^{35}\text{S}]$ TBPS binding are listed in Table IV. Thus, conditions which lead to conformational changes of $[^{35}\text{S}]$ TBPS binding sites, either by the interaction of drugs which are considered to act on GABA receptor-regulated chloride ion channels or by membrane pretreatments (Ag^+ , Triton X-100) which inactivate high affinity $[^{35}\text{S}]$ TBPS binding, lead also to alterations of $[^3\text{H}]$ muscimol binding and of muscimol enhancement of benzodiazepine receptor binding. Those observations suggest that high affinity (states of) GABA binding sites can be functionally linked to benzodiazepine receptors under conditions where $[^{35}\text{S}]$ TBPS binding sites are perturbed. In the absence of such perturbing factors, a GABA binding site with low apparent affinity is linked to benzodiazepine receptors (Tallman et al., 1978; Karobath and Sperk, 1979).

The present observations with CNS depressants are difficult to reconcile with the concept of one common drug receptor for those CNS depressants and those CNS convulsants which are thought to exert their pharmacological effects by altering GABA-regulated chloride conductance mechanisms. The biphasic actions of etazolate and of pentobarbital and the Hill number >1 for their inhibitory effects point to a multiplicity of $[^{35}\text{S}]$ TBPS binding sites in a GABA benzodiazepine receptor complex, with cooperativity between these sites. That multiple drug receptors may occur in the GABA benzodiazepine receptor complex has already been suggested by Möhler et al. (1980), who obtained evidence for the existence of four benzodiazepine binding sites per receptor complex. Alternatively, CNS depressants which prolong the opening time of GABA-regulated chloride ion channels (Study and Barker, 1981) and CNS convulsants which decrease permeability at these channels (Barker

TABLE IV
Relationship between $[^3\text{H}]$ muscimol binding, muscimol enhancement of $[^3\text{H}]$ flunitrazepam binding, and $[^{35}\text{S}]$ TBPS binding

Perturbation	$[^3\text{H}]$ Muscimol Binding (or $[^3\text{H}]$ GABA Binding)	Muscimol Enhancement of $[^3\text{H}]$ Flunitrazepam Binding	$[^{35}\text{S}]$ TBPS Binding
None	High and low affinity sites ^a		Detectable ^b
Muscimol		Low affinity ^{c,d}	Stimulation at low concentration followed by inhibition at high concentration
AgNO_3 pretreatment	High affinity only ^e	High affinity ^e	Not detectable
Triton X-100 pretreatment	High affinity binding facilitated ^e	High affinity/ ^f	Not detectable
Etazolate, pentobarbital	High and low affinity binding enhanced ^{e,g}	Medium affinity ^h	Stimulation at low concentrations followed by inhibition
Picrotoxin(in) IPTBO	Inhibition of low affinity binding ^e	Low affinity ^e	Inhibition

^a Enna and Snyder (1977).

^b Squires et al. (1983).

^c Karobath and Sperk (1979).

^d Tallmann et al. (1978).

^e Supavilai et al. (1982).

^f Massotti and Guidotti (1980).

^g Leeb-Lundberg et al. (1981).

^h Supavilai and Karobath (1981).

and MacDonald, 1980) may exert their pharmacological effects at similar but not identical drug receptors. More experimental data are required before these speculations can be confirmed or dismissed.

Finally, the bidirectional effects of ligands for benzodiazepine receptors on baseline or muscimol-inhibited [³⁵S]TBPS binding can be used as a simple *in vitro* test system to indicate whether a given ligand for benzodiazepine receptors has agonistic or inverse agonistic effects *in vivo*. With the 14 compounds investigated, there appears to be a good agreement between the present *in vitro* observations and the pharmacological action of these drugs *in vivo* (Jensen et al., 1983). These results support the concept that drugs acting on benzodiazepine receptors may exhibit a spectrum from full agonists to partial agonists and (partial) inverse agonists (Polc et al., 1982).

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