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PERTURBATION OF BENZODIAZEPINE RECEPTOR BINDING BY PYRAZOLOPYRIDINES INVOLVES PICROTOXININ/BARBITURATE RECEPTOR SITES[1]

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Abstract

The two pyrazolopyridines, etazolate (SQ20009) and cartazolate (SQ65396), enhance the binding of [^{3}H]diazepam to benzodiazepine receptor sites in rat brain. This enhancement is due to a change in affinity without a change in maximal binding. Pentobarbital also enhances [^{3}H]diazepam binding by lowering the K_D (Leeb-Lundberg, F., A. Snowman, and R. W. Olsen (1980) Proc. Natl. Acad. Sci. U. S. A. *77*: 7468–7472). Pentobarbital gives a maximal enhancement of benzodiazepine binding slightly greater than that induced by etazolate, but maximal concentrations of etazolate cannot increase further the maximal enhancement by pentobarbital. By contrast, the enhancement of benzodiazepine binding by γ-aminobutyric acid (GABA) is fully additive with the effect of either etazolate or pentobarbital. The enhancement by γ-aminobutyric acid is blocked specifically by the two γ-aminobutyric acid mimetic compounds, imidazole acetic acid and 4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol (THIP), compounds which do not inhibit the enhancement of diazepam binding by pentobarbital or etazolate. The effects of etazolate and pentobarbital, but not those of GABA, can be blocked competitively by 1 to 10 μM picrotoxinin. Etazolate and cartazolate, like pentobarbital (Ticku, M. K., M. Ban, and R. W. Olsen (1978) Mol. Pharmacol. *14*: 391–402), competitively inhibit α-[^{3}H]dihydropicrotoxinin binding with potencies correlating well with their stimulatory properties on benzodiazepine binding. These results suggest that pyrazolopyridines, like pentobarbital, stimulate [^{3}H]diazepam binding through a picrotoxinin-sensitive site which is distinct from the γ-aminobutyric acid receptor site. These *in vitro* interactions between the etazolate and benzodiazepine receptor sites can be modulated also by the GABA antagonist, bicuculline, consistent with the existence of a complex containing three receptors (GABA, benzodiazepines, and etazolate/barbiturates/picrotoxinin). Bicuculline reverses completely the effects of etazolate and GABA on benzodiazepine binding but reverses only partially the enhancement by pentobarbital. This difference between the effects of pentobarbital and etazolate, as well as differences in their maximal enhancing effects mentioned above, suggests that pentobarbital may have an action similar to etazolate on some bicuculline-sensitive benzodiazepine binding sites, but, in addition, pentobarbital can enhance some other benzodiazepine sites in a manner which is neither mimicked by etazolate nor blocked by bicuculline.

Significant evidence now exists for pharmacologically relevant benzodiazepine receptors in the mammalian central nervous system (CNS). Correlation has been made between the potency of a series of benzodiazepine compounds, such as muscle relaxants, anxiolytics, and anticonvulsants, and their affinity for the "receptor" binding sites (Squires and Braestrup, 1977; Möhler and Okada, 1977). These binding sites can be modulated by γ-aminobutyric acid (GABA) receptor agonists (Tallman et al., 1980), an effect which further increases the physiological significance of these receptors, since the coupling of benzodiazepine and GABA binding sites *in vitro* is consistent with neuropharmacological evidence that benzodiazepine action *in vivo* appears to involve a potentiation of GABAergic inhibitory synaptic transmission (Haefely et al., 1979; Costa and Guidotti, 1979; Tallman et al., 1980). Recently, several other agents have been reported to modulate benzodiazepine binding. For example, chloride ions modulate benzodiazepine binding (Costa et al., 1979), suggesting that the GABA receptor-coupled chloride ion channel might be involved in the binding site of benzodiazepines. Furthermore, the anxiolytic pyrazolopyridines, etazolate, cartazolate (Beer et al., 1978; Williams and Risley, 1979), and tracazolate (ICI 136,753; Salama and Meiners, 1980), as well as anesthetic barbi-

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turates (Leeb-Lundberg et al., 1980), modulate benzodiazepine binding. These effects are dependent on chloride and other anions able to penetrate GABA-activated ion channels (Supavilai and Karobath, 1979; Leeb-Lundberg et al., 1980). All of these agents are able to enhance the affinity of the benzodiazepine receptor for its ligands. We recently have characterized barbiturate enhancement in some detail and suggest that these drugs act at a picrotoxinin-sensitive site (Leeb-Lundberg et al., 1980). This study further examines the effects of pyrazolopyridines on benzodiazepine binding and their relationship to the other modulators. Our results indicate that one of the primary sites of action in the CNS for these drugs is the picrotoxinin binding site. This would further support the existence of a CNS depressant drug receptor site related to the GABA-regulated chloride ion channel.

Materials and Methods

N-[*methyl*-^{3}H]Diazepam (83.5 Ci/mmol) was purchased from New England Nuclear and α-[^{3}H]dihydropicrotoxinin (DHP, 15 Ci/mmol) was custom synthesized by Amersham. Benzodiazepines were kindly donated by Dr. H. Möhler and Dr. W. Schlosser of Hoffmann-La Roche. Etazolate (SQ20009) and cartazolate (SQ65396) were gifts of E. R. Squibb & Sons, Inc. TETS (tetramethylenedisulfotetramine) was the gift of Dr. J. Casida, Berkeley, CA. Picrotoxinin was obtained from picrotoxin (Sigma) by column chromatography. Dihydropicrotoxinin was obtained from picrotoxinin by reduction under H_2 gas with Pt/C (5%) as a catalyst, as previously described (Ticku et al., 1978). THIP (4,5,6,7-tetrahydroisoxazolo[5,4-*c*]pyridin-3-ol) was a gift of Dr. P. Krogsgaard-Larsen, Copenhagen. All other materials were obtained from commercial sources.

White Sprague-Dawley rats (200 to 300 gm) were decapitated and their brains were removed rapidly and bathed in ice cold 0.32 M sucrose. Cerebral cortex then was homogenized in 20 vol of 0.32 M sucrose in a glass homogenizer fitted with a Teflon pestle (12 passes, 400 rpm). The homogenate was centrifuged at 2000 rpm (1000 $\times$ *g*) for 10 min at 0 to 4°C (Beckman JA 17 rotor). The pellet (P_1) was discarded, and the supernatant fraction was centrifuged at 45,000 rpm (140,000 $\times$ *g*) for 45 min (Spinco rotor 60 Ti). The pellet (P_2 + P_3) then was treated in two different ways, depending on the binding assay to be done. Membranes for DHP binding were homogenized in ice cold buffer (0.2 M NaCl, 20 mM sodium phosphate, pH 7.0 $\pm$ 0.1) using the glass homogenizer and centrifuged at 45,000 rpm for 15 min. The P_2 + P_3 pellet was resuspended to a final concentration of 2 to 3 mg/ml of protein in the same buffer. Membranes for diazepam binding were homogenized (Ultra-Turrax tissue disrupter; 5 sec at 40%) in 25 vol of ice cold double distilled water (osmotic shock) and centrifuged at 45,000 rpm for 45 min. The pellet was washed again in buffer. This additional procedure is intended to lower membrane GABA content below the micromolar concentrations which affect benzodiazepine binding (Tallman et al., 1980), while minimizing damage to the fragile binding sites for picrotoxinin and barbiturates (Ticku et al., 1978). In some cases, where indicated, the membranes were treated further by a freeze-thaw, multiple wash procedure to remove thoroughly endogenous GABA (Napias et al., 1980; Karobath et al., 1979). The pellets were resuspended to a final protein concentration of 0.5 to 2.0 mg/ml in 0.2 M NaCl, 20 mM sodium phosphate, pH 7.0.

DHP binding. Aliquots of the membrane suspension were incubated in quadruplicate for 15 min at 0°C with 50 nM [^{3}H]DHP with and without drugs in a total volume of 1 ml. At the end of the incubation, the membranes were pelleted rapidly (20,000 rpm in Beckman rotor JA 20.1; 50,000 $\times$ *g*), and the vials were rinsed twice with 3 ml of ice cold buffer without disturbing the pellet. The membranes were solubilized overnight at room temperature in 0.2 ml of Soluene 350:toluene (1:1). Then 3 ml of toluene containing 0.5% 2,5-diphenyloxazole (PPO) were added and radioactivity was measured in a Beckman model LS-3155T scintillation counter (efficiency 30%). Background was estimated with 0.1 mM nonradioactive DHP and contributed about 75% of the radioactivity.

[^{3}H]Diazepam binding. Aliquots of the membrane suspension were incubated in triplicate for 60 min at 0°C with 0.5 nM [^{3}H]diazepam with and without drugs in a total volume of 1 ml. At the end of the incubation, the membranes were trapped rapidly on Whatman GF/B filters. An additional 2 ml of saline (0.2 M NaCl) were added twice to the vial and poured onto the filter. The filters were dried and put into plastic vials. Five milliliters of Aquasol:toluene (2:1) (New England Nuclear) were added, and radioactivity was determined as above (efficiency 45%). Background was estimated with 10 μM nonradioactive diazepam and normally contributed about 10% of the total radioactivity.

Results

The anxiolytic pyrazolopyridine compound, etazolate, markedly enhanced the specific binding of [^{3}H]diazepam. At a single constant concentration of [^{3}H]diazepam, etazolate increased binding up to a maximal 82% over base line, that is, 182% of control (average 75 $\pm$ 10%, n = 7) at 10 μM etazolate and a half-maximal effect at 1 μM (Table I*A*); concentrations over 10 μM gave less enhancement.

Pentobarbital and related barbiturates also enhance benzodiazepine binding *in vitro* (Leeb-Lundberg et al., 1980). Pentobarbital enhancement of [^{3}H]diazepam binding in the absence and presence of etazolate (10 μM) is shown in Figure 1. The maximal effect of pentobarbital was roughly 120% (115 $\pm$ 10%, n = 10) increase over base line, with no downturn in the enhancement even up to 3 mM pentobarbital. The maximal effect of pentobarbital was consistently somewhat greater than that of etazolate, but the stimulation by maximal concentrations of both drugs added together did not exceed that of pentobarbital alone. This suggests that the two agents may act via a common mechanism, at least in part.

The inhibitory neurotransmitter, GABA, also enhances the binding of benzodiazepines *in vitro* (Tallman et al., 1980). If, however, a maximal concentration of GABA (10 μM, enhancement 75%; Table I*B*) was added together with a maximal concentration of etazolate (10 μM, enhancement 68%), the effect was additive (128% enhancement). Likewise, the effect of pentobarbital (100

TABLE I

Interaction of various drugs with pyrazolopyridine and barbiturate enhancement of benzodiazepine binding

Rat brain membranes were prepared and assayed for [^{3}H]diazepam binding as described under "Materials and Methods" using 0.5 nM [^{3}H]diazepam. Each value is typical of at least two determinations in triplicate which varied by <10%. The names and sources of all drugs are given in the text.

	Additions	[^{3}H]Diazepam Binding (% of Control)
A	1. None	100
	2. Etazolate (0.1 μM)	113
	3. Etazolate (0.3 μM)	122
	4. Etazolate (1 μM)	146
	5. Etazolate (3 μM)	175
	6. Etazolate (10 μM)	182
	7. Etazolate (30 μM)	150
B.	1. None	100
	2. Etazolate (10 μM)	168
	3. GABA (10 μM)	175
	4. IAA (100 μM)	128
	5. Etazolate (10 μM) + GABA (10 μM)	228
	6. Etazolate (10 μM) + IAA (100 μM)	190
	7. Etazolate (10 μM) + GABA (10 μM) + IAA (100 μM)	201
C.	1. None	100
	2. Pentobarbital (100 μM)	151
	3. GABA (50 μM)	169
	4. Pentobarbital (100 μM) + GABA (50 μM)	228
D.	1. None	100
	2. Etazolate (5 μM)	177
	3. Pentobarbital (100 μM)	146
	4. Bicuculline (10 μM)	86
	5. THIP (100 μM)	89
	6. Etazolate (5 μM) + bicuculline (10 μM)	86
	7. Pentobarbital (100 μM) + bicuculline (10 μM)	108
	8. Etazolate (5 μM) + THIP (100 μM)	184
	9. Pentobarbital (100 μM) + THIP (100 μM)	144
	10. Bicuculline (10 μM) + THIP (100 μM)	75
	11. Etazolate (5 μM) + bicuculline (10 μM) + THIP (100 μM)	91
	12. Pentobarbital (100 μM) + bicuculline (10 μM) + THIP (100 μM)	93

μM, enhancement 51%; Table IC) was additive with that of GABA (50 μM, enhancement 69% alone, 128% with pentobarbital).

The Scatchard plots in Figure 2 further demonstrate this point and also show that the enhancement of diazepam binding by etazolate (as well as GABA and pentobarbital) involves a decrease in K_D without a change in the number of binding sites, B_{max}. Maximal etazolate (10 μM) decreased the K_D for [^{3}H]diazepam binding to fresh washed rat cortex membranes from 2.33 to 1.2 nM (Fig. 2, legend). GABA alone (10 μM) reduced the control K_D to 0.94 nM, and pentobarbital alone (500 μM) reduced it to 0.86 nM. Etazolate and pentobarbital together decreased the K_D to 0.91 nM (slightly lower than etazolate alone, but not lower than pentobarbital alone); etazolate and GABA together lowered the K_D to 0.66 nM (lower than either drug alone and about 3.5-fold over control).

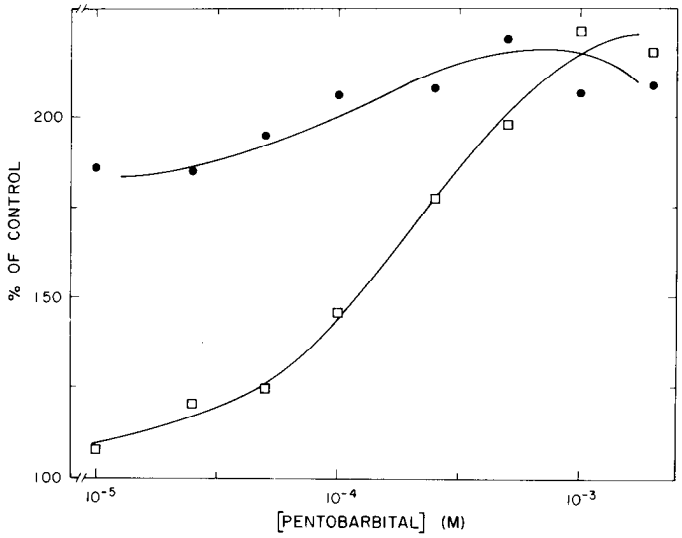

Figure 1. Enhancement of [^{3}H]diazepam binding by pentobarbital in the presence and absence of etazolate. [^{3}H]Diazepam binding was measured as described under "Materials and Methods" with 0.5 nM [^{3}H]diazepam, and varying concentrations of pentobarbital, with (●) and without (□) constant etazolate (10 μM). Each *point* is the average of triplicate determinations with variations of ≤5% and is typical of at least three experiments which varied by ±10%.

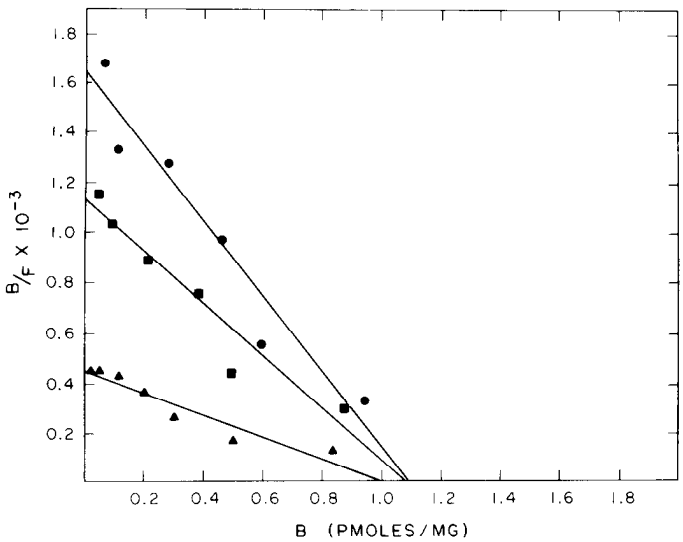

Figure 2. Scatchard plot of [^{3}H]diazepam binding to rat cortex in the absence and presence of drugs. [^{3}H]Diazepam concentrations were varied from 0.05 to 50 nM, determining nondisplaceable background with 10 μM nonradioactive diazepam. Brain membranes were prepared as described under "Materials and Methods." ▲, Control; ■, with 500 μM pentobarbital and 10 μM etazolate; ●, with 10 μM GABA and 10 μM etazolate. The *solid lines* represent computer-fitted linear regressions. The result is typical of five experiments. Kinetic constants (K_D (nanomolar concentrations) and B_{max} (picomoles per mg)) of presented data and additional controls are: control (2.33, 0.99); 10 μM etazolate (1.2, 1.05); 500 μM pentobarbital (0.86, 0.99); 10 μM GABA (0.94, 1.06); 500 μM pentobarbital and 10 μM etazolate (0.91, 1.08); 10 μM GABA and 10 μM etazolate (0.66, 1.09). *B/F*, bound/free.

Imidazole acetic acid (IAA) is a GABA mimetic able to stimulate benzodiazepine binding to a low but significant extent, but additionally, this drug will reverse the enhancement by GABA in a manner suggestive of partial agonists (Karobath et al., 1979; Braestrup et al., 1979). This drug was used to determine whether the effect of etazolate was independent of that of GABA. Saturating levels of IAA (100 μM) reversed the effect of GABA, either in the absence or presence of etazolate, but did not affect the action of etazolate. The level of enhancement of benzodiazepine binding by a combined addition of IAA, etazolate, and GABA together was equal to the effect of etazolate and IAA in the absence of GABA (Table I*B*). Another GABA analogue which does not enhance [^{3}H]benzodiazepine binding but can reverse the effect of GABA is the compound THIP. This agent also failed to inhibit the etazolate or pentobarbital enhancement of benzodiazepine binding (Table I*D*). Thus, the action of etazolate appears different from that of GABA.

The GABA antagonist, bicuculline, inhibits GABA-stimulated [^{3}H]diazepam binding (Tallman et al., 1980; Karobath et al., 1979; Braestrup et al., 1979). This drug also inhibited stimulation of benzodiazepine binding by both pentobarbital and etazolate (Table I*D*). However, the GABA analogues, IAA and THIP, did not reverse the effects of pentobarbital or etazolate (Table I*D*), indicating that the latter compounds do not act at the GABA receptor site. Assuming that bicuculline and IAA both act at the GABA receptor site, the bicuculline reversal of etazolate enhancement of diazepam binding must be indirect. High concentrations of IAA and THIP apparently did not reverse the action of bicuculline; however, a dose-response curve for the bicuculline effect (Fig. 3) revealed that bicuculline was very potent and present in concentrations greatly exceeding its IC_{50} value in the experiments described in Table I. Bicuculline at 10 to 100 nM inhibited the enhancement of benzodiazepine binding by GABA, etazolate, and pentobarbital (Fig. 3). However, the inhibition by bicuculline of pentobarbital enhancement, but not that of GABA or etazolate, did not reach 100% but leveled out at about 70% reversal (four experiments) under these conditions. The plateau effect is not yet understood completely but again is consistent with an indirect interaction between the binding sites for bicuculline and pentobarbital.

The stimulation of [^{3}H]diazepam binding by etazolate was inhibited by picrotoxinin in a dose-dependent manner, with 50% inhibition at 6 μM (5 ± 3 μM, n = 3) (Fig. 4). Tetramethylenedisulfotetramine (TETS) and RO5-3663, two convulsants known to act at the picrotoxinin binding site (Olsen et al., 1980; Ticku and Olsen, 1979; Leeb-Lundberg et al., 1981), also inhibited etazolate-enhanced benzodiazepine binding (Table II). Due to an additional inhibition of base line diazepam binding, it was difficult to establish accurate IC_{50} values for the inhibition by these compounds. The experiments described in Table II were carried out with frozen-thawed, thoroughly washed membranes to minimize the endogenous GABA content. The harsher treatment resulted in a smaller but still significant (38 ± 5%, n = 4) enhancement by 10 μM etazolate than that seen with fresh rat cortex.

Etazolate displaced specific α-[^{3}H]dihydropicrotoxinin (DHP) binding (Fig. 5). This inhibitory property was shared with the somewhat more potent pyrazolopyridine, cartazolate. Etazolate and cartazolate inhibited [^{3}H]DHP binding with 50% displacement at 8 ± 3 μM and 0.5 ± 0.2 μM (n = 3), respectively. Figure 6 shows a double reciprocal plot of [^{3}H]DHP binding with and without etazo-

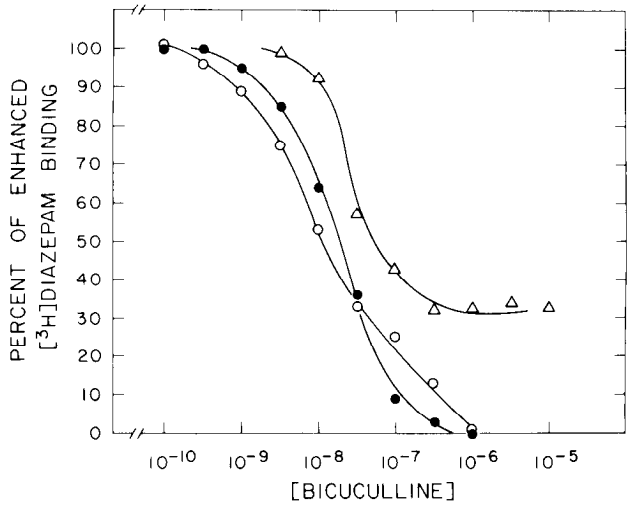

Figure 3. Concentration dependence of bicuculline reversal of benzodiazepine binding enhancement by various agents. Rat cortex membranes were prepared and assayed as described under "Materials and Methods" with constant [^{3}H]diazepam at 0.5 nM. The enhancement of benzodiazepine binding by 10 μM etazolate (○), 10 μM GABA (●), and 100 μM pentobarbital (△) was determined in the absence and presence of varying concentrations of bicuculline (diluted into the assays from a 5 mM stock solution at pH 3 just prior to addition of the membrane suspension). The enhancement over base line benzodiazepine binding by the three drugs was normalized to 100% and was actually equal to 68% (etazolate), 71% (GABA), and 55% (pentobarbital). Each *point* is the mean of triplicates which varied by <10%, and each result is typical of at least four experiments. The IC_{50} values for bicuculline reversal of drug enhancement were 11, 19, and 30 nM, respectively.

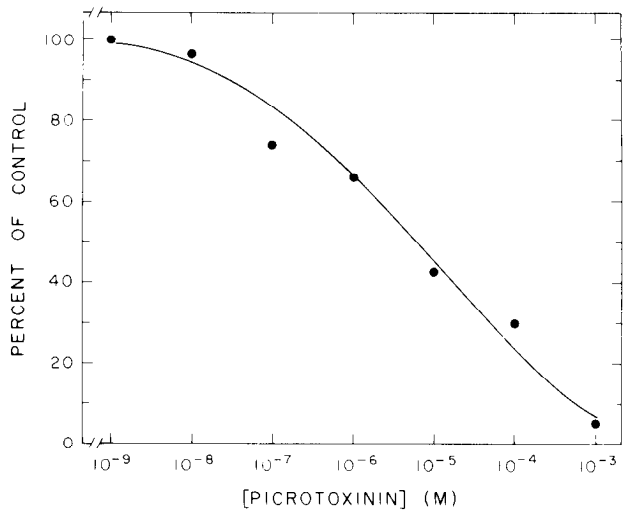

Figure 4. Inhibition by picrotoxinin of etazolate—enhancement of [^{3}H]diazepam binding. Membranes were prepared and assayed as described under "Materials and Methods" with a constant concentration (10 μM) of etazolate and various concentrations of picrotoxinin. The experiment is typical of three trials, and the IC_{50} value was 6 μM.

TABLE II

Interaction of picrotoxin-like convulsants with pyrazolopyridine enhancement of benzodiazepine binding

Rat brain membranes (fraction $P_2 + P_3$) were prepared by freezing and thawing and five buffer washes (20 mM sodium phosphate, 0.2 M NaCl, pH 7.0 ± 0.1). [^{3}H]Diazepam binding was done as described under "Materials and Methods" using 0.5 nM [^{3}H]diazepam. Each number is typical of at least two determinations in triplicate which varied by <10%. The names and sources of all drugs are given in the text.

Additions	[^{3}H]Diazepam Binding (% of Control)
1. None	100
2. Etazolate (10 μM)	133
3. TETS (0.05 μM)	99
4. TETS (0.5 μM)	98
5. TETS (5 μM)	97
6. TETS (50 μM)	76
7. RO5-3663 (0.1 μM)	99
8. RO5-3663 (1 μM)	99
9. RO5-3663 (10 μM)	91
10. RO5-3663 (100 μM)	57
11. Etazolate (10 μM) + TETS (0.05 μM)	127
12. Etazolate (10 μM) + TETS (0.5 μM)	124
13. Etazolate (10 μM) + TETS (5 μM)	121
14. Etazolate (10 μM) + TETS (50 μM)	98
15. Etazolate (10 μM) + RO5-3663 (0.1 μM)	127
16. Etazolate (10 μM) + RO5-3663 (1 μM)	119
17. Etazolate (10 μM) + RO5-3663 (10 μM)	112
18. Etazolate (10 μM) + RO5-3663 (100 μM)	82

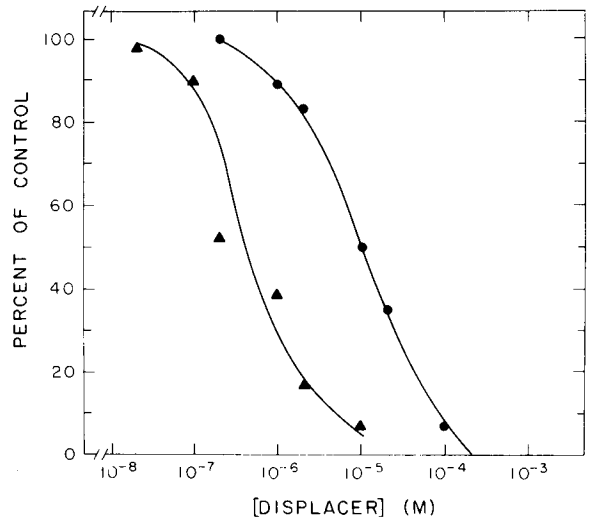

Figure 5. Displacement of specific DHP binding by etazolate (SQ20009) and cartazolate (SQ65396). Membranes were prepared and assayed as described under "Materials and Methods" without and with various concentrations of displacing agents. ●, Etazolate; ▲, cartazolate. The experiments are typical of three trials, and the IC_{50} values were 10 and 0.4 μM, respectively.

late, suggesting a competitive type of inhibition. These results, taken together, suggest that the pyrazolopyridines perturb benzodiazepine binding via picrotoxinin/barbiturate receptor sites, which may be coupled to, but are distinct from, GABA receptor sites.

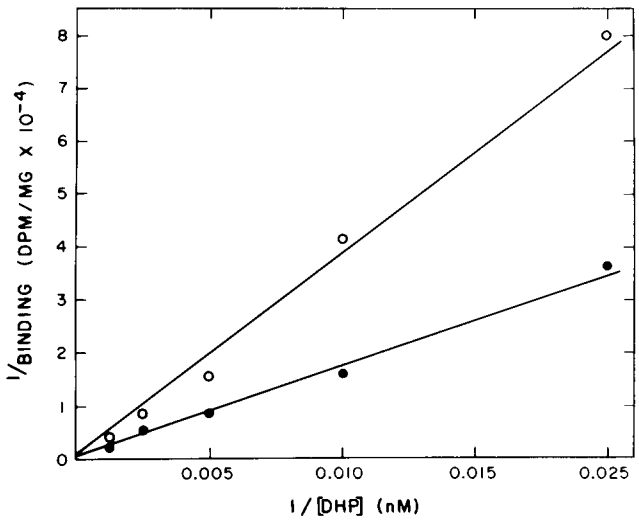

Figure 6. Competitive inhibition of specific DHP binding by etazolate. Rat cortex membranes were prepared and assayed for [^{3}H]DHP binding as described under "Materials and Methods." The concentration of DHP was varied from 10 nM to 10 μM with (○) and without (●) etazolate at 10 μM, and the data are shown as a Lineweaver-Burk plot. The apparent K_D for the single class of DHP binding sites was 1 μM and the apparent K_I for the competitive inhibitor etazolate was 10 μM.

Discussion

Etazolate recently has been found to have potent stimulatory effects on benzodiazepine binding, an action which might be related to its pharmacological properties (Beer et al., 1978; Williams and Risley, 1979; Supavilai and Karobath, 1980). The modulatory role of barbiturates on benzodiazepine binding recently has been characterized in this laboratory (Leeb-Lundberg et al., 1980). Etazolate and pentobarbital appear to have a very similar mechanism of action in this *in vitro* system, at least in part. Maximal concentrations of pentobarbital could elevate further etazolate enhancement of benzodiazepine binding up to the level of the maximal effect of pentobarbital. This elevation by the two drugs together was not fully additive, however, never surpassing the maximal effect of pentobarbital alone. This suggests that the two compounds have a common action, although with different maximal effects.

Under our conditions, the *in vitro* effect of etazolate seems to be different from the enhancement of benzodiazepine binding by GABA. The enhancement by either etazolate or pentobarbital was additive with that of GABA. In addition, the GABA analogue, IAA, was able to reverse the stimulatory effects of GABA, both with and without etazolate present, but IAA did not disturb the etazolate enhancement, either alone or with GABA present. Likewise, the GABA analogue, THIP, reversed the action of GABA but not that of etazolate or pentobarbital.

Bicuculline is able to inhibit GABA- as well as etazolate- and pentobarbital-enhanced benzodiazepine binding. Therefore, it could very well be argued that these two exogenous ligands stimulate benzodiazepine binding simply by increasing the sensitivity of the GABA-benzodiazepine receptor coupling. However, etazolate enhancement is still observed in membranes which have

been thoroughly washed to remove endogenous GABA. Furthermore, any influence from the GABA receptor site would be blocked by IAA or THIP, but these drugs do not disturb etazolate or pentobarbital enhancement. Additionally, bicuculline fails to reverse pentobarbital enhancement completely even at 10 μM, although, at 30 nM, it inhibits a large portion of the pentobarbital effect (Fig. 3). This partial effect indicates that at least some of the pentobarbital sites are not identical with bicuculline sites. That fraction of pentobarbital-enhanced benzodiazepine binding which is blocked by bicuculline probably corresponds to a subpopulation of benzodiazepine receptors which are allosterically coupled to bicuculline (GABA receptor?) sites. These bicuculline-sensitive sites also appear to be perturbed by etazolate, but the bicuculline-insensitive sites which are enhanced by pentobarbital are not perturbed by etazolate. Nevertheless, the effect which etazolate has on benzodiazepine binding resembles more the effect of pentobarbital than that of GABA.

The potency of bicuculline in these experiments is somewhat greater than previously reported for inhibition of GABA binding (Olsen et al., 1978) or GABA enhancement of benzodiazepine binding (Braestrup et al., 1979) and appears to be dependent on brain region and on a high concentration (>0.1 M) of chloride ions (F. Leeb-Lundberg, A. Snowman, and R. W. Olsen, unpublished data). Some anions are known to enhance the affinity of bicuculline for GABA receptor sites (Möhler and Okada, 1978). The apparent inability of 100 μM THIP or IAA to reverse the effects of 10 μM bicuculline is probably due to the high potency of bicuculline under these conditions. Alternatively, bicuculline might act at yet another drug binding site distinct from the GABA receptors (IAA and THIP binding sites).

Neither etazolate nor pentobarbital appears to act even indirectly via the GABA recognition site. The results presented here strongly suggest that the primary site of action of etazolate is a site related to the picrotoxinin-sensitive chloride ion channel. The effect of etazolate, like that of pentobarbital, could be completely and competitively blocked (Leeb-Lundberg et al., 1980) by picrotoxinin in a dose-dependent manner (Fig. 4) and with a potency correlating well with the affinity for [^{3}H]DHP binding sites, considering that saturating levels of etazolate had to be displaced (Leeb-Lundberg and Olsen, 1980; Olsen and Leeb-Lundberg, 1981; Olsen, 1981). The picrotoxin reversal of etazolate enhancement of benzodiazepine binding, which was shown initially by Supavilai and Karobath (1979), provides the first link between the picrotoxinin and benzodiazepine receptors. Picrotoxin does not inhibit GABA enhancement of benzodiazepine binding (Tallman et al., 1980). Both etazolate (Supavilai and Karobath, 1979) and pentobarbital (Leeb-Lundberg et al., 1980) effects were dependent on anions and specific for those anions that are able to penetrate GABA-activated chloride ion channels. This phenomenon of anion dependence, likewise, is not shared by GABA, at least not to the same extent (F. Leeb-Lundberg, A. Snowman, and R. W. Olsen, unpublished data).

The stimulatory effect of etazolate was inhibited also by the cage convulsant, TETS, and the convulsant benzodiazepine, RO5-3663, two drugs previously reported to act at the picrotoxinin binding site (Olsen et al., 1980; Ticku and Olsen, 1979; Leeb-Lundberg et al., 1981; Leeb-Lundberg and Olsen, 1980; Olsen and Leeb-Lundberg, 1981). Further evidence for an interaction with a picrotoxinin-sensitive site by the pyrazolopyridines comes from the evidence that both etazolate and cartazolate inhibit [^{3}H]DHP binding in a competitive manner and with potencies correlating well with their potencies for stimulating benzodiazepine binding. Pentobarbital has been shown previously (Ticku and Olsen, 1978) to inhibit DHP binding in a competitive manner with a potency (K_I = 50 μM) which also agrees with its effective concentration (EC_{50} = 100 μM) for enhancing benzodiazepine binding (Leeb-Lundberg et al., 1980). The latter action of a series of barbiturates has been demonstrated to correlate well with pharmacological activity for these drugs.

Earlier studies found no direct interaction between picrotoxinin and GABA receptor binding (Olsen et al., 1978), but recent studies show that GABA binding can be perturbed by drugs which bind to picrotoxinin-sensitive sites, namely, pentobarbital (Johnston and Willow, 1981; Olsen, 1981) and etazolate (Placheta and Karobath, 1980). Furthermore, barbiturates (Skolnick et al., 1980) and etazolate (Supavilai and Karobath, 1980) enhance, while the convulsant benzodiazepine (RO5-3663) reverses (O'Brien and Spirt, 1980) the ability of GABA to enhance benzodiazepine binding; GABA analogues enhance the ability of etazolate to enhance benzodiazepine binding (Supavilai and Karobath, 1980). Therefore, at least some GABA and picrotoxinin binding sites appear to be coupled in the membrane via a complex interaction involving some benzodiazepine receptors.

Interestingly, both benzodiazepine (Tallman et al., 1980) and picrotoxinin binding sites (Olsen et al., 1980; Olsen and Leeb-Lundberg, 1980) are inhibited by high concentrations of some naturally occurring purines and related compounds, and these agents also allosterically perturb GABA receptor binding (Ticku and Burch, 1980). Benzodiazepines at micromolar concentrations inhibit picrotoxinin binding (Olsen et al., 1980; Leeb-Lundberg et al., 1981). Although the picrotoxinin binding sites and high affinity benzodiazepine binding sites appear distinct on specificity grounds, these effects may represent further examples of allosteric interaction between the two populations.

Obviously there are more complicated interactions occurring at the GABA/benzodiazepine receptor complex than can be solved by a simple two-unit model. The evidence suggests a coupling between at least three drug receptors: those for GABA, benzodiazepines, and picrotoxinin. At least two types of drugs can interact at the picrotoxinin-sensitive site. One class of drugs (like etazolate and pentobarbital) is able to enhance the affinity for benzodiazepines. A second class of drugs (like picrotoxinin and related convulsants) will not affect the affinity of benzodiazepines but will rather block the effects of the former class of drugs.

References

Beer, B., C. A. Klepner, A. S. Lippa, and R. F. Squires (1978) Enhancement of ^{3}H-diazepam binding by SQ 65396: A novel anti-anxiety agent. Pharmacol. Biochem. Behav. *9:* 849–851.

Braestrup, C., M. Nielsen, P. Krogsgaard-Larsen, and E. Falch (1979) Partial agonists for brain GABA/benzodiazepine receptor complex. Nature *280:* 331–333.

Costa, E., and A. Guidotti (1979) Molecular mechanisms in the receptor action of benzodiazepines. Annu. Rev. Pharmacol. Toxicol. *19:* 531–545.

Costa, T., D. Rodbard, and C. B. Pert (1979) Is the benzodiazepine receptor coupled to a chloride anion channel? Nature *277:* 315–317.

Haefely, W., P. Polc, R. Schaffner, H. H. Keller, L. Pieri, and H. Möhler (1979) Facilitation of GABAergic transmission by drugs. In *GABA-Neurotransmitters*, P. Krogsgaard-Larsen, J. Scheel-Kruger, and H. Kofod, eds., pp. 357–375, Munksgaard, Copenhagen.

Johnston, G. A. R., and M. Willow (1981) Barbiturates and GABA receptors. In *GABA and Benzodiazepine Receptors*, E. Costa, G. DiChiara, and G. Gessa, eds., pp. 191–198, Raven Press, New York.

Karobath, M., R. Placheta, M. Lippitsch, and P. Krogsgaard-Larsen (1979) Is stimulation of benzodiazepine binding mediated by a novel GABA receptor? Nature *278:* 748–749.

Leeb-Lundberg, F., and R. W. Olsen (1980) Picrotoxinin binding as a probe of the GABA postsynaptic membrane receptor-ionophore complex. In *Psychopharmacology and Biochemistry and Neurotransmitter Receptors*, H. I. Yamamura, R. W. Olsen, and E. Usdin, eds., pp. 593–606, Elsevier, New York.

Leeb-Lundberg, F., A. Snowman, and R. W. Olsen (1980) Barbiturate receptor sites are coupled to benzodiazepine receptors. Proc. Natl. Acad. Sci. U. S. A. *77:* 7468–7472.

Leeb-Lundberg, F., C. Napias, and R. W. Olsen (1981) Dihydropicrotoxinin binding sites in mammalian brain: Interactions with convulsant and depressant benzodiazepines. Brain Res., in press.

Möhler, H., and T. Okada (1977) Benzodiazepine receptors: Demonstration in the central nervous system. Science *198:* 849–851.

Möhler, H., and T. Okada (1978) Properties of γ-aminobutyric acid receptor binding with (+)-[^{3}H]bicuculline methiodide in rat cerebellum. Mol. Pharmacol. *14:* 256–265.

Napias, C., M. O. Bergman, P. C. Van Ness, D. V. Greenlee, and R. W. Olsen (1980) GABA binding in mammalian brain: Inhibition by endogenous GABA. Life Sci. *27:* 1001–1011.

O'Brien, R. A., and N. M. Spirt (1980) The inhibition of GABA-stimulated benzodiazepine binding by a convulsant benzodiazepine. Life Sci. *26:* 1441–1445.

Olsen, R. W. (1981) The GABA postsynaptic membrane receptor-ionophore complex: Site of action of convulsant and anticonvulsant drugs. Mol. Cell. Biochem., in press.

Olsen, R. W., and F. Leeb-Lundberg (1980) Endogenous inhibitors of picrotoxinin-convulsant binding sites in rat brain. Eur. J. Pharmacol. *65:* 101–104.

Olsen, R. W., and F. Leeb-Lundberg (1981) Convulsant and anticonvulsant drug binding sites related to GABA-regulated chloride ion channels. In *GABA and Benzodiazepine Receptors*, E. Costa, D. DiChiara, and G. Gessa, eds., pp. 93–102, Raven Press, New York.

Olsen, R. W., M. K. Ticku, P. C. Van Ness, and D. Greenlee (1978) Effects of drugs on γ-aminobutyric acid receptors, uptake, release and synthesis *in vitro*. Brain Res. *139:* 277–294.

Olsen, R. W., F. Leeb-Lundberg, and C. Napias (1980) Picrotoxinin and convulsant binding sites in mammalian brain. Brain Res. Bull. *5* (Suppl. 2)*:* 217–221.

Placheta, P., and M. Karobath (1980) *In vitro* modulation by SQ 20009 and SQ 65396 of GABA receptor binding in rat CNS membranes. Eur. J. Pharmacol. *62:* 225–228.

Salama, A. I., and B. A. Meiners (1980) Enhancement of benzodiazepine binding by the novel anxiolytic ICI 136,753. Soc. Neurosci. Abstr. *6*: 190.

Skolnick, P., S. M. Paul, and J. L. Barker (1980) Pentobarbital potentiates GABA-enhanced ^{3}H-diazepam binding to benzodiazepine receptors. Eur. J. Pharmacol. *65:* 125–127.

Squires, R. F., and C. Braestrup (1977) Benzodiazepine receptors in rat brain. Nature *266:* 732–734.

Supavilai, P., and M. Karobath (1979) Stimulation of benzodiazepine receptor binding by SQ 20009 is chloride-dependent and picrotoxin-sensitive. Eur. J. Pharmacol. *60:* 111–113.

Supavilai, P., and M. Karobath (1980) Interaction of SQ 20009 and GABA-like drugs as modulators of benzodiazepine receptor binding. Eur. J. Pharmacol. *62:* 229–233.

Tallman, T. F., S. M. Paul, P. Skolnick, and D. W. Gallager (1980) Receptors for the age of anxiety: Pharmacology of the benzodiazepines. Science *207:* 274–281.

Ticku, M. K., and T. Burch (1980) Purine inhibition of [^{3}H]γ-aminobutyric acid receptor binding to rat brain membranes. Biochem. Pharmacol. *29:* 1217–1220.

Ticku, M. K., and R. W. Olsen (1978) Interaction of barbiturates with dihydropicrotoxinin binding sites related to the GABA receptor-ionophore system. Life Sci. *22:* 1643–1652.

Ticku, M. K., and R. W. Olsen (1979) Cage convulsants inhibit picrotoxinin binding. Neuropharmacology *18:* 315–318.

Ticku, M. K., M. Ban, and R. W. Olsen (1978) Binding of [^{3}H]α-dihydropicrotoxinin, a γ-aminobutyric acid synaptic antagonist, to rat brain membranes. Mol. Pharmacol. *14:* 391–402.

Williams, M., and E. A. Risley (1979) Enhancement of the binding of ^{3}H-diazepam to rat brain membranes *in vitro* by SQ 20009, a novel anxiolytic, γ-aminobitutyric acid (GABA) and muscimol. Life Sci. *24:* 833–842.