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INTERACTION OF DIPHENYLHYDANTOIN AND BENZODIAZEPINES IN THE CNS

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SUMMARY

Using extracellular unit recording and microiontophoretic techniques, the anticonvulsant diphenylhydantoin (DPH) was found to increase the physiological efficacy of the benzodiazepines. This increased biological effect could be correlated with an enhanced specific binding of benzodiazepines measured in vivo following pretreatment of rats with DPH. The increased binding of benzodiazepines is due to an increase in the total number of benzodiazepine binding sites without an alteration in the affinity of these sites for [^{3}H]diazepam. The data show that the effects of DPH on benzodiazepine binding are qualitatively different and independent from the effects of γ-aminobutyric acid. Based on the dose–response relationship between benzodiazepine binding effects and the anticonvulsant activity of DPH and reports of other convulsant, anticonvulsant compounds which alter benzodiazepine binding, it is suggested that the benzodiazepine binding site may be relevant to convulsant–anticonvulsant activity.

INTRODUCTION

Diphenylhydantoin (DPH; Dilantin; 5,5-diphenyl-2,4-imidazolidinedione) and diazepam (Valium; 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2H-1,4-benzodiazepine-2-one) are used clinically both acutely and chronically to control some forms of seizure[3,24]. Although the mechanism for the anticonvulsant activity of these compounds is unknown, it has been suggested that they may exert their anti-epileptic effects through interactions with the same or similar sites in the CNS[7,22].

Several studies have described a functional interaction between the benzodiaze-

pines (BZ) and γ-aminobutyric acid (GABA), which results in a facilitation of GABA-mediated inhibition[8,9,11,15,16,21]. This facilitated inhibition can be correlated with a GABAergic activation of [^{3}H]diazepam (or [^{3}H]flunitrazepam) binding due to an increased affinity[13,39,43] of a specific high-affinity binding site for BZ in brain[25,35]. The increased affinity of this binding site was demonstrated following both the in vitro addition of GABA to a binding assay[39,43] and in vivo pretreatment of animals with GABA agonists and drugs which increased brain GABA levels[13,40]. In addition, Guidotti et al.[14] have demonstrated an activation of GABA binding mediated by BZ through an endogenous modulator, further supporting an interaction between GABA and the BZs.

Since it has been postulated that the anticonvulsant DPH also facilitates neuronal inhibition in the CNS[2,5,10,32,44], one possible site of action for DPH is the GABA/BZ system. In this series of experiments, we have investigated possible interactions between DPH and the GABA/BZ systems in brain using electrophysiological and binding techniques. Our findings indicate that DPH does enhance the ability of BZ to inhibit neuronal cell firing and suggest that this effect of DPH is due to increased binding of BZ. The data show that the effects of DPH on BZ binding are qualitatively different and independent from GABA. However, functionally, such alterations in BZ binding could still result in facilitated neuronal inhibition at BZ-coupled GABAergic neurons.

EXPERIMENTAL PROCEDURES

Single cell recording and microiontophoresis

Previous single unit recording and microiontophoretic experiments have shown that dorsal raphe (5-HT) cells in the midbrain of chloral hydrate-anesthetized rats (Sprague–Dawley/Taconic Farms; 220–260 g) are responsive to the iontophoretic application of both 5-HT and GABA and have been used to demonstrate a direct interaction between GABA and the BZs[11]. In the present studies similar extracellular recording techniques using 2 mm glass micropipettes filled with 2% Pontamine sky blue dye in 2 M sodium chloride solution were used to determine the effects of DPH (100 mg/kg administered i.p. 1 h prior to recording) on the firing rate of raphe cells and a combination of DPH pretreament with an i.v. dose of diazepam. In addition, direct application of 5-HT (0.05 M, pH 4.0 serotonin creatinine sulfate, Sigma), GABA (0.01 M in 0.2 M NaCl, pH 4.0; Sigma) and DPH (0.12 M in 0.15 M NaCl, pH 11; Parke-Davis and Company) or flurazepam (0.1 M, pH 4.0; Hoffman-LaRoche, Inc.) onto 5-HT cells was accomplished using 5-barrel microelectrodes introduced into the region of the dorsal raphe nucleus. Drugs were ejected by turning the direct current source from 4 to 20 nA of retaining current to 1 to 10 nA of ejecting current. In a separate experiment, possible iontophoretic effects of a pH 11 NaOH solution were investigated on dorsal raphe firing. In this system ejection currents greater than 10 nA resulted in nonspecific current and pH effects. At the conclusion of each recording experiment, a 16 μA cathodal current was passed through the recording electrode for 10 min to deposit a discrete spot of Pontamine sky blue dye[6]. The location of the electrode tip

was then confirmed histologically and compared with the previously established location of 5-HT-containing neurons in the dorsal raphe nucleus[1].

Systemic administration of drugs

Drugs, administered by the i.p. route, include diphenylhydantoin sodium (Dilantin, 0.5, 5, 10 and 50 mg/ml, Parke-Davis and Company) and SKF525A (25 mg/ml, Smith, Kline and French Company).

When drugs were administered i.v. they were given via the tail vein. These compounds include diazepam (Valium, 5 mg/ml, Hoffmann-LaRoche Inc.), diazepam vehicle (Hoffman-LaRoche, Inc.), picrotoxin (3 mg/ml, Sigma Chemical Co.), and D-lysergic acid diethylamide bitartrate (LSD, 50 μg/ml).

Receptor binding studies

Binding of [^{3}H]diazepam to cortical membrane fractions prepared from rat brain was assessed as previously described[13]. Cortical membrane fractions were prepared by centrifuging homogenates at 18,000 $\times$ *g* for 10 min. Membrane pellets were washed, centrifuged and resuspended 5 times with 50 vol. (w/v) of ice-cold Tris·HCl (0.05 M, pH 7 at 25 °C). This extensive washing procedure resulted in the removal of $>$ 99.93% of exogenously added GABA and indicates that less than 1 nM GABA would remain in the washed membranes. The washed tissue was finally resuspended in 50 vol. of buffer for assay (1 ml) as previously described[13]. Non-specific binding was defined as binding occurring in the presence of 10^{-5} M chlordiazepoxide. Unless otherwise indicated, the concentration of [^{3}H]diazepam used in this study was 0.5 nM.

In vivo labeling experiments

In vivo labeling of specific BZ binding in brain was carried out as described and validated previously[40]. DPH (100 mg/kg) or vehicle (dilute NaOH, pH 11) was administered i.p. 60 min prior to the injection of [^{3}H]diazepam. Each animal then received an i.v. injection of 200 μCi of [^{3}H]diazepam (representing 60 μg in a vol of 1 ml; New England Nuclear, Inc.) 5 min prior to sacrifice. Animals were decapitated, then brains removed and immediately homogenized in 150 vol. Tris·HCl, pH 7.4 containing 10^{-5} M chlordiazepoxide; 2 ml aliquots were immediately filtered to assess total[^{3}H]diazepam bound and an additional 2 ml aliquot was filtered at 20 min to assess non-specific binding.

RESULTS

Electrophysiological studies

One hour after an intraperitoneal injection of DPH (100 mg/kg) animals were anesthetized and prepared for unit recording in the dorsal raphe nucleus. Spontaneous firing rates of 5-HT cells (0.5–2/sec) were not found to be altered from control rats by this treatment (control (11) = 1.3 $\pm$ 0.1/sec; DPH (8) = 1.3 $\pm$ 0.2/sec). In addition, diazepam alone (in cumulative doses of up to 4 mg/kg, i.v.) did not alter baseline firing rates of these cells (Fig. 1A). However, following DPH pretreatment, 1.0 mg/kg of i.v.

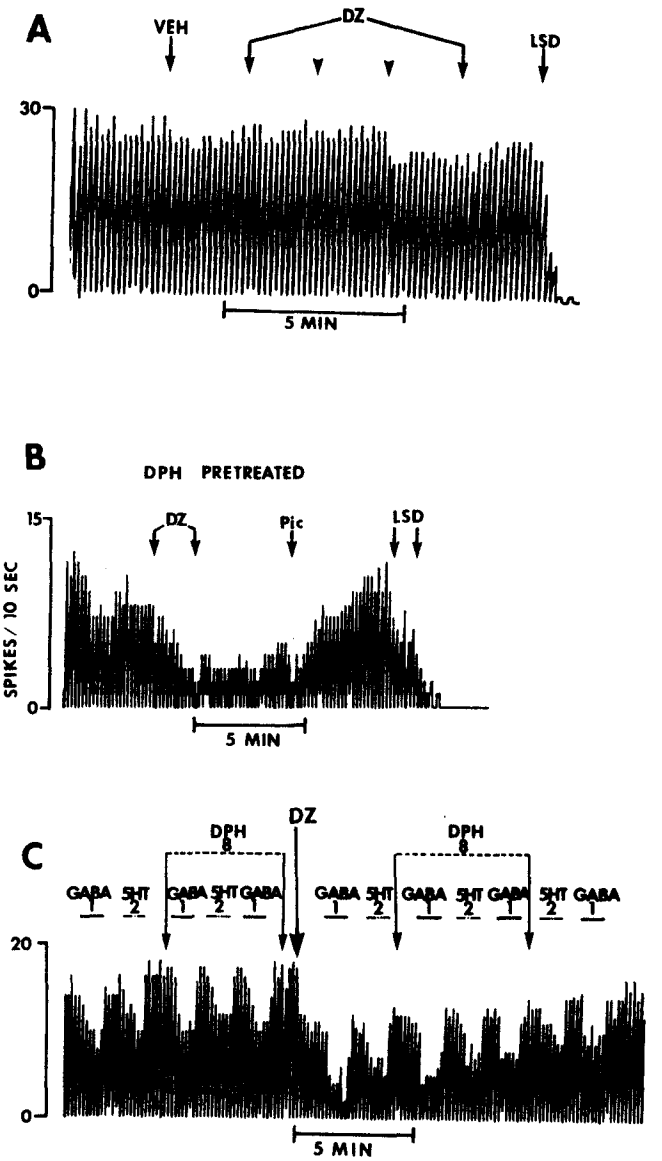

Fig. 1. A: firing rate histogram of dorsal raphe cell. No significant change in baseline firing rate was observed following administration of diazepam vehicle (VEH, 0.2 ml i.v.) or diazepam (DZ, 0.5, 0.5, 1 and 2 mg/kg i.v. at arrows) while LSD (10 μg/kg i.v.) produces its normal depressant effect. B: rat was pretreated with DPH (100 mg/kg, i.p.) 1 h prior to recording. Following i.v. injections of diazepam (DZ, 0.5 + 0.5 mg/kg at arrows) firing rate of dorsal raphe cell was significantly inhibited while a subconvulsant dose of picrotoxin (Pic, 0.6 mg/kg, i.v.) reversed this inhibition. Cell was inhibited by LSD (10 μg/kg + 10 μg/kg, i.v. at arrows). C: submaximal inhibition of dorsal raphe firing was produced by 5-HT (2 nA) ejected for 60 sec (broken line) and GABA (1 nA) ejected for 60 sec (solid line). The simultaneous iontophoresis of DPH (8 nA) for 300 sec (dotted line) had no effect on either the spontaneous activity of the cell or the responses to 5-HT and GABA. Following 1.0 mg/kg i.v. injection of DZ (large arrow) subsequent responses to GABA were enhanced, independent of further DPH iontophoresis.

TABLE I

Effects of in vivo administration of various doses of diphenylhydantoin on cortical [^{3}H]diazepam binding

Treatment	*[^{3}H]Diazepam specifically bound§§§ (cpm/incubation)*	*% Change from control*
Vehicle (13)	1187 ± 21	—
1 mg/kg§ (5)	1243 ± 27	+ 5%
10 mg/kg§ (5)	1361 ± 42*	+ 15%
20 mg/kg§ (5)	1320 ± 34*	+ 11%
100 mg/kg§§ (8)	1442 ± 46**	+ 21%

§ Drugs were injected intraperitoneally and animals were killed 30 min later. Cortical tissue was rapidly dissected and homogenized. After removal of brain, cortical membrane fractions were prepared as described in Experimental Procedures.

§§ When a dose of 100 mg/kg of DPH was administered, animals were killed 60 min after the injection.

§§§ [^{3}H]Diazepam binding was assessed after 0.25 nM [^{3}H]diazepam was added to the incubation mixture.

* $P < 0.02$ vs vehicle control.

** $P < 0.001$ vs vehicle control.

diazepam was able to significantly inhibit (n = 8) spontaneous firing of the dorsal raphe cells; 0.6 mg/kg of i.v. picrotoxin was able to reverse this inhibition (Fig. 1B). Picrotoxin has been previously shown not to affect spontaneous firing at this dose[12,42]. Direct microiontophoretic application of 5-HT and GABA produced inhibition of firing as previously reported[11,12,42]. However, in contrast to studies with the BZs[11] which alter responsiveness to GABA but not 5-HT, the simultaneous iontophoretic application of DPH did not alter the inhibition in cell firing rates produced by either of these putative transmitters (n = 9 cells in 6 rats) (data not shown). In fact, when DPH was iontophoresed onto raphe cells before and after an intravenous dose of diazepam (1.0 mg/kg), only the post-benzodiazepine GABA responses were potentiated, independent of the iontophoretic application of DPH (n = 5 cells in 3 rats, Fig. 1C). These data indicate that, unlike BZ, DPH does not affect the GABA inhibitory response directly.

Binding studies

When animals were pretreated with DPH (100 mg/kg, i.p.) for 1 h, BZ binding in washed cortical membranes prepared from these rats was increased (Table I). A large dose of DPH (100 mg/kg, i.p.) was used in most of these experiments in order to demonstrate the enhanced binding effects most clearly. However, dose–response relationships have been obtained to show that these effects on BZ binding are dose-dependent and significant following anticonvulsant doses (10, 20, 100 mg/kg) of DPH, while not significantly altered by a subanticonvulsant dose (1 mg/kg) of DPH[36,41] (Table I). The times were chosen to correlate with peak anticonvulsant effects as reported for maximal electroshock seizure tests[29,36].

The increase in specific BZ binding observed after pretreatment of animals with

DPH can be attributed to an increase in the total number of BZ binding sites without a significant change in their affinity for [^{3}H]diazepam (Fig. 2). In these experiments, cortices from 5 vehicle-injected control and 5 DPH-treated rats were assayed individually for binding following the addition of 0.5 nM of [^{3}H]diazepam. Cortical membranes from all animals in each treatment group were then pooled to assess binding at various concentrations of [^{3}H]diazepam. In this study, the K_D observed in control rats was 5.4 $\pm$ 0.1 nM with total diazepam binding of 1498 $\pm$ 39 fmol/incubation. After pretreatment for 1 h with DPH (100 mg/kg, i.p.) the observed K_D was 5.8 $\pm$ 0.3 nM and the total binding of diazepam was 2045 $\pm$ 98 fmol/incubation. This represents an

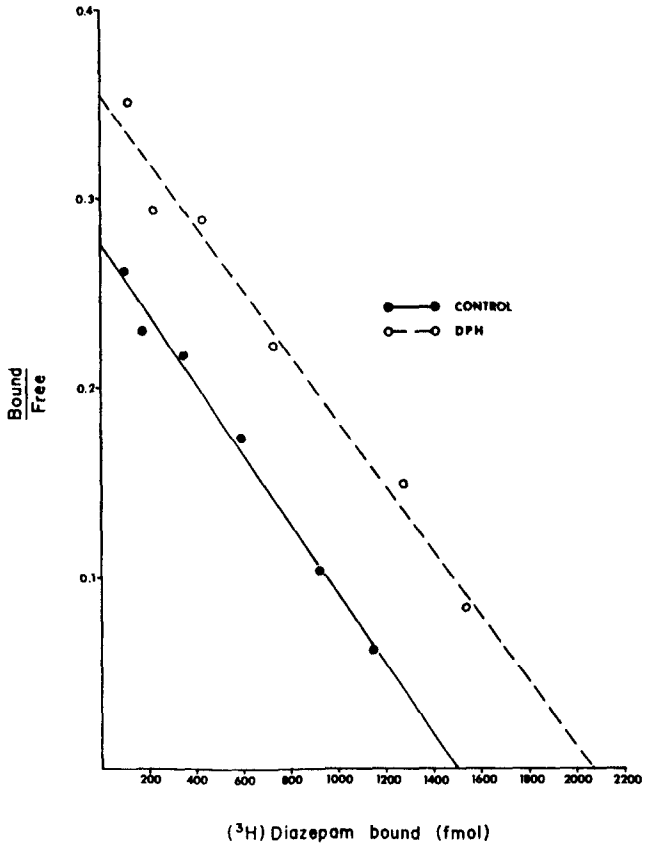

Fig. 2. Scatchard analysis of [^{3}H]diazepam binding in rats pretreated for 60 min with a single i.p. injection of dilute NaOH (pH 11) or DPH (100 mg/kg in solution at pH 11; n = 5). Assays were carried out on cortical membranes as described in experimental procedures except that the concentration of [^{3}H]diazapam was varied as indicated,

$$\text{(Bound/Free} = \frac{\text{[}^3\text{H]diazepam specifically bound}}{\text{Total [}^3\text{H]diazepam in incubation - [}^3\text{H]diazepam bound to filters}}\text{)}.$$

Pooled tissue samples from DPH-treated or control animals were run in quadruplicate, S.E.M. < 3%. Error bars were within the area of the point symbols in the figure. At a point corresponding to the addition of 500 fmol [^{3}H]diazepam, samples from each animal were analyzed individually (n = 5 per treatment group). These values ± S.E.M. were 98 ± 3 fmol/incubation for control animals and 124 ± 1 fmol/incubation for DPH-treated animals ($P < 0.001$).

TABLE II

Effect of diphenylhydantoin on binding in vivo of specifically bound [^{3}H]diazepam

Animals were pretreated for 1 h with diphenylhydantoin (100 mg/kg, i.p.) and then injected i.v. with [^{3}H]diazepam (200 μCi/rat). Animals were maintained at 37 °C and killed 5 min following injection of the label. Brains were homogenized in 150 vol. (w/v) of ice-cold Tris·HCl buffer (pH 7.4) containing 10^{-5} M chlordiazepoxide and aliquots were filtered immediately and at 20 min to assess non-specific binding as described in Experimental Procedures and ref. 40.

	[^{3}H]Diazepam binding (fmol/2 ml)
Control (n = 8)	1438 ± 132
Diphenylhydantoin (n = 6)	2008 ± 164

increase of 36% in the total number of binding sites without significantly changing the affinity for [^{3}H]diazepam. In 4 separate replications, the increase in the total number of binding sites following this DPH pretreatment ranged from 20 to 40%.

Using an in vivo labeling technique previously described[40], specific binding of [^{3}H]diazepam was determined in control animals and animals treated with DPH 1 h prior to the i.v. administration of [^{3}H]diazepam (60 μg/kg containing 200 μCi of [^{3}H]-diazepam). As shown in Table II, DPH (100 mg/kg, i.p.) significantly enhanced the amount of specifically bound [^{3}H]diazepam in brain.

TABLE III

Effects of diphenylhydantoin on [^{3}H]diazepam binding

In vivo drug pretreatment	*In vitro addition*	*[^{3}H]Diazepam binding (fmol/incubation)*	*% Change from control*
Experiment 1§			
Control (6)	—	116 ± 3	—
	+ GABA (10^{-5} M)	137 ± 3*	+17%
	+ DPH (10^{-5} M)	107 ± 5	−8%
	+ DPH (10^{-4} M)	79 ± 5**	−32%
DPH (100 mg/kg i.p.) (6)	—	151 ± 4**	+30%
	+ GABA (10^{-5} M)	178 ± 3**	+53%
Experiment 2§§			
SKF525A (5)		145 ± 8	—
SKF525A + DPH (5)		203 ± 7*	+40%

* $P < 0.02$ vs control.

** $P < 0.005$ vs control.

§ Rats received DPH (100 mg/kg, i.p.) or vehicle. One hour later animals were killed, the cortex removed, homogenized and washed cortical membrane fractions were prepared as described in Experimental Procedures. Compounds added in vitro were included in the incubation mixture and [^{3}H]diazepam binding was assayed as described in Experimental Procedures.

§§ Animals were injected with SKF-525A (50 mg/kg, i.p.) 50 min prior to receiving DPH (100 mg/kg, i.p.) or vehicle. One hour following DPH injection rats were killed and [^{3}H]diazepam binding was assayed in washed cortical membranes.

Consistent with electrophysiological data, this enhanced binding does not appear to be a direct action of DPH on BZ binding since DPH added directly to the in vitro assay did not enhance BZ binding (Table III, Experiment 1). In fact, when 1×10^{-4} M DPH was added to the assay mixture binding was significantly inhibited. The discrepancy between direct application via microiontophoresis or in vitro addition versus in vivo pretreatment might suggest the formation of some active metabolite of DPH. However, this possibility was excluded by data (Table III, Experiment 2) showing that DPH increased binding in vivo despite pretreatment of animals with SKF525A (50 mg/kg, i.p., given 1 h prior to DPH)[4] at a dose which prevents DPH metabolism by liver hydroxylating enzymes[18,26].

DPH does not appear to enhance binding simply by increasing brain GABA levels since the effect of the in vitro addition of GABA (1×10^{-5} M) is additive with DPH pretreatment (Table III, Experiment 1). The increase in BZ binding following DPH treatment is qualitatively different (increase in number of binding sites; Fig. 2) from the increased binding observed after the addition of GABA (increase in apparent affinity[39,43]). In addition, repeated washing of cortical homogenates from DPH pretreated animals does not abolish the enhanced BZ binding in contrast to our previous observations following pretreatment with the GABA catabolic inhibitor aminooxyacetic acid[13], whose effects are entirely reversible by washing. Further, DPH does not seem to function as a GABAmimetic since, as observed by others[27], under our treatment conditions using the method of Snodgrass[34] we did not observe significant alterations in [^{3}H]muscimol binding by either in vitro additions or in vivo pretreatments of animals with DPH (Table IV).

DISCUSSION

Our results indicate that one site of action of DPH in brain is the BZ binding site. We have used electrophysiology and in vivo labeling to demonstrate that this interaction is relevant in the intact animal and have assessed alterations in binding by

TABLE IV

Effect of diphenylhydantoin on [^{3}H]muscimol binding in cerebral cortex

In vivo pretreatment (n)*	*In vitro addition*	*[^{3}H]Muscimol binding (fmol/incubation)*
Control (7)	—	361 ± 15
Control (7)	DPH (10^{-5} M)	320 ± 12
DPH (100 mg/kg, i.p.) (7)	—	336 ± 26

* Animals received DPH (100 mg/kg, i.p.) or vehicle. One hour later rats were killed, the cortex removed, homogenized and washed cortical membrane fractions were prepared as described by Snodgrass[34]. Where in vitro additions were made, DPH (final concentration 1×10^{-5} M) was added to a control cortical membrane preparation immediately prior to the incubation.

** Binding was assayed using 120 nM [^{3}H]muscimol in the incubation mixture. No significant differences were seen for any DPH treatment.

examining changes in in vitro BZ binding following pretreatment of animals with DPH in vivo.

In electrophysiological experiments, pretreatment of animals with DPH did not significantly alter the activity of dorsal raphe cells, at least within 1–2 h after treatment. This lack of effect of non-toxic doses of DPH on the spontaneous firing rate of dorsal raphe cells has also been observed in cerebellar Purkinje cells[19,30,31]. However, following DPH pretreatment, a dose of diazepam which was without effect in control animals caused a marked inhibition of neuronal activity in treated rats. This effect is similar to that previously observed following pretreatment with GABAmimetics and agents which increase brain GABA levels[11].

Consistent with this electrophysiological evidence, when animals were pretreated with DPH, a significant enhancement of specific [^{3}H]diazepam binding was observed in vivo. In addition, enhanced BZ binding was also demonstrated in an in vitro BZ binding assay[25,35] using washed cortical membranes prepared from animals pretreated with DPH in vivo. Although preliminary evidence suggested an alteration in the affinity of BZ for its binding site[38], data obtained using more extensive washing procedures indicated that the mechanism by which DPH affects the physiological efficacy of BZ and enhances specific binding of BZ in vivo is through an increase in the total number of BZ binding sites without altering the apparent affinity of [^{3}H]diazepam for these binding sites. Alterations in BZ receptor number, but not affinity, has also been reported following experimentally induced seizures[28]. However, these effects are qualitatively different from changes in affinity observed following GABA agonists or agents which increase brain GABA levels[13,39]. In other transmitter systems, an increase in the number of binding sites has been associated with an increased physiological response[33]. Such an enhanced response is consistent with the electrophysiological and in vivo labeling data presented here.

The direct in vitro addition of DPH does not lead to enhanced BZ binding. In fact, at high concentrations of DPH [^{3}H]diazepam binding is significantly inhibited. This finding is consistent with electrophysiological data showing a lack of effect following the direct iontophoretic application of DPH and suggests that DPH does not function directly as a BZ agonist or antagonist. While the discrepancy between direct application (via microiontophoresis or in vitro addition) and in vivo pretreatment might suggest the formation of some active metabolite of DPH, pretreatment of animals with SKF525A to inhibit DPH metabolism by the liver[18,26] did not prevent enhanced BZ binding following DPH pretreatment. In fact, SKF525A pretreatment has previously been shown to prolong the anticonvulsant activity but not to alter the ED_{50} for this effect of DPH[37].

In electrophysiological experiments, the ability of DPH to enhance BZ effects was reversed by the GABA antagonist picrotoxin. However, in contrast to data obtained following treatment with BZ in which a specific potentiation of the GABA response was observed[11] no alteration in the neuronal response to GABA (or 5-HT) was observed during DPH iontophoresis. Since these electrophysiological experiments measure the effects of DPH/BZ interactions on a GABAergic response, they indicate that the effects of DPH are at a site different from the GABA site and perhaps distal to

the GABA recognition site. Consistent with these electrophysiological data, repeated washing of cortical homogenates (which has been shown to remove GABA) from DPH-pretreated animals does not abolish the enhanced BZ binding. These data are in contrast to our previous observations following aminooxyacetic acid pretreatment[13] and suggest that DPH does not enhance binding by increasing brain GABA levels. Further, DPH does not seem to function as a GABA mimetic since, as observed by others[27], under our treatment conditions we did not observe any significant alterations in [^{3}H]muscimol binding using either in vitro additions or in vivo pretreatments of animals with DPH. Since the in vitro addition of GABA to cortical membranes from DPH pretreated animals leads to an additive increase in BZ binding, these data indicate separate sites of action for DPH and GABA in BZ binding. Thus, the DPH interaction does not occur at the GABA binding site but is clearly related to the BZ binding site.

In our studies, DPH was found to enhance the ability of BZ to inhibit raphe neuronal firing. A functional result for this interaction would be facilitated GABAergic inhibition, one postulated mechanism for the anticonvulsant properties of DPH[2,10,32,44]. In previous electrophysiological studies, it has been reported that BZ potentiates[21] or prolongs[8] the GABA-mediated postsynaptic inhibition in spinal cord cultures while DPH has been reported to prolong postsynaptic inhibition in the mammalian cortex[31] and GABA-mediated postsynaptic inhibition in invertebrates[2,5]. In addition, qualitatively similar end-effects of BZ and DPH on post-tetanic potentials in various hippocampal regions have been observed[22]. Thus, these electrophysiological data also suggest the possibility of a final common pathway for the action of DPH and BZ.

Since the effects of DPH on BZ binding are dose-related and significant only for doses with anticonvulsant potency[36], these findings may also suggest a correlation between BZ binding effects and the anticonvulsant properties of DPH. An extensive investigation of BZ binding effects and the development of anticonvulsant properties of DPH has recently been completed (Gallager and Mallorga, unpublished observations). In addition, since diazepam, DPH and GABAmimetics[3,17,20,23] have anticonvulsant properties while bicuculline and picrotoxin are convulsants, the effects of these compounds on BZ binding[13,39,40] may suggest a single focal point for their convulsant-anticonvulsant properties. It may, therefore, be possible for one to use BZ binding as an indication of the effectiveness of a drug in a neuronal system, perhaps even as a prediction of convulsant–anticonvulsant activity. One may further speculate that some convulsant disorders could be related to functional or physical alterations in the GABA/BZ binding site complex, or of other transmitters which have input to this complex.

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