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COMPETITION FOR DIAZEPAM RECEPTOR BINDING BY DIPHENYLHYDANTOIN AND ITS ENHANCEMENT BY γ-AMINOBUTYRIC ACID

Godfrey Tunnicliff & Julie A. Smith

Evansville Center for Medical Education
Indiana University School of Medicine
8600 University Boulevard, Evansville, IN 47712

and

That T. Ngo

Faculty Research Facility
University of California
Irvine, CA 92717

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SUMMARY

The anticonvulsant diphenylhydantoin competitively inhibited the binding of [^{3}H]diazepam to synaptic membranes of rat cerebral cortex. The K_i was calculated as 0.9 μM. In the presence of γ-aminobutyric acid the K_i was reduced to 0.3 μM. A similar change in K_i was also observed in the presence of muscimol, a potent agonist of γ-aminobutyric acid. γ-Aminobutyric acid also reduced the dissociation constant (K_d) of diazepam binding from 16.7 nM to 3.9 nM. Thus it appears that diphenylhydantoin and diazepam are binding at the same site and the presence of γ-aminobutyric acid increases the affinity of the receptor for each of the drugs. These observations indicate that the anticonvulsant effects of diphenylhydantoin and diazepam may be related to an interaction with a common binding site and to the modification of this binding site by γ-aminobutyric acid.

INTRODUCTION

For several decades diphenylhydantoin (DPH) has been the most popular drug used in the control of grand mal epilepsy. In addition, this drug has been prescribed for myokymia [1] and cardiac arrhythmias [2]. Despite its long history the mechanism of action of DPH is unknown. There are indications, however, that it can interfere with ion flux across neural membranes. For instance, calcium influx is inhibited [3], as is that of sodium into stimulated nerve cells [4]. Recently

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Perry *et al* [5] reported that diphenylhydantoin blocks passive resting sodium channels in the giant squid axon.

Diazepam is a drug that is widely used in the treatment of anxiety. However, like DPH it also possesses important anticonvulsant properties. The exact nature of its action is still unclear. There is considerable evidence, however, that this drug interacts with γ-aminobutyric acid, the major inhibitory neurotransmitter in the brain. Electrophysiological studies have demonstrated that diazepam potentiates the inhibition produced by γ-aminobutyric acid [6,7]. In addition, the binding of [^{3}H]diazepam to receptors is stimulated in the presence of γ-aminobutyric acid [8,9].

We now present evidence that diphenylhydantoin can inhibit the binding of diazepam to synaptic membranes and that γ-aminobutyric acid markedly enhances this inhibition.

MATERIALS AND METHODS

5,5-Diphenylhydantoin was purchased from Sigma Chemical Company, St. Louis, MO. Muscimol was supplied by Research Organics Inc., Cleveland, OH. Diazepam was kindly donated by Hoffmann La Roche, Nutley, NJ. [^{3}H]Diazepam (36 curies/mmol) was obtained from Amersham, Arlington Heights, IL.

Preparation of membranes. The cerebral cortex from male Sprague Dawley rats was homogenised in 9 vol of 0.32 M sucrose containing 1 mM HEPES buffer (pH 7.4) and centrifuged at 4°C for 10 min at 1000 g. The supernatant was diluted 4-fold with 50 mM TRIS citrate buffer (pH 7.4) and centrifuged at 30,000 g for 20 min at 4°C. The precipitate was suspended in 20 vol (based on the original weight of tissue) of the TRIS buffer and recentrifuged as previously. This procedure was repeated 3 times to ensure thorough washing of the membranes. The pelleted membranes were stored overnight at -20°C.

Measurement of [^{3}H]*diazepam binding.* The frozen membranes were suspended in 50 mM TRIS-citrate buffer (2.5 ml per 1 g of tissue). Twenty five microlitres of membranes (about 0.3 mg protein) was transferred to 0.975 ml of TRIS citrate-buffer containing 1.4 nM [^{3}H]diazepam (70,000 dpm). The mixture was incubated for 30 min at 23°C prior to centrifugation for 10 min in an Eppendorf 3200 centrifuge. The pellet was washed twice with 1.5 ml of the TRIS citrate buffer. The membranes were dissolved in 0.3 ml of tissue

solubiliser (NCS, Amersham) and, after the addition of 10 ml of toluene containing 0.1% PPO and 0.03% POPOP, were counted in a Nuclear Chicago Unilux IIA. Binding of diazepam was taken as that radioactivity able to be displaced by 10 μM non-radioactive diazepam.

Protein assay. The method of Lowry [10] was followed. Bovine serum albumin was used as a standard.

RESULTS

Effects of DPH and analogues on diazepam binding.

Synaptic membranes were incubated in the absence or presence of 10 μM DPH and several structurally related compounds. The results are presented in Table 1. Diphenylhydantoin gave rise to an 83% inhibition of binding. Acetyl-2-thiohydantoin and phenyl-5-ureido-oxadiazole inhibited binding by 31% and 29% respectively. The remainder of the compounds produced no inhibition.

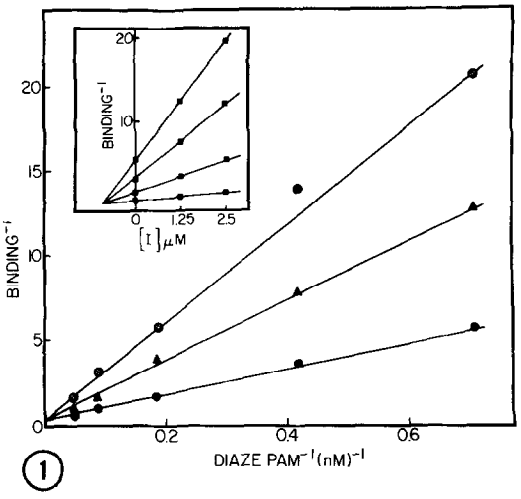
Competitive inhibition of diazepam binding by DPH.

Membranes were incubated with [^{3}H]diazepam over a ligand concentration range of 1.4×10^{-9} to 21.4×10^{-9}M. In some experiments 1.25 μM or 2.5 μM DPH was present. The results were plotted by the method of Lineweaver and Burk [11] and

TABLE 1. Effects of DPH and related compounds on the binding of diazepam to rat synaptic membranes.

Compound (10 μM)	% Inhibition
Diphenylhydantoin	83.1 ± 6.9
Acetyl-2-thiohydantoin	31.0 ± 2.7
Phenyl-5-ureido-oxadiazole	29.2 ± 3.2
Barbituric acid	1.3 ± 0.2
Pentobarbital	0.8 ± 0.2
Uric acid	5.1 ± 2.6
Allantoin	-3.3 ± 0.5

Membranes (about 0.3 mg protein) were incubated in 1.4 nM [^{3}H]diazepam at 23°C for 30 min in the absence or presence of each compound. The values represent the mean (±S.E.M.) of 3 experiments.



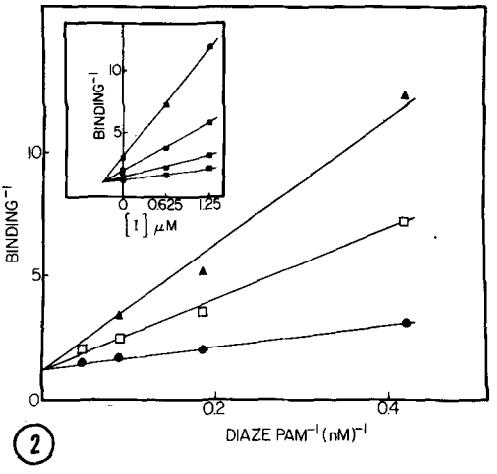


Fig. 1. Double reciprocal plot of the effects of DPH on diazepam binding to synaptic membranes over a ligand concentration range. Membranes were incubated at 23°C for 30 min in the absence (closed circles) or presence of DPH (triangles = 1.25 μM; stars = 2.5 μM). The inset is the data shown as a Dixon plot. Each point is the mean of 3 experiments. Binding = pmol per mg protein.

Fig. 2. Double reciprocal plot of the effects of DPH on diazepam binding to synaptic membranes over a ligand concentration range in the presence of 10 μM γ-aminobutyric acid. Membranes were incubated at 23°C for 30 min in the absence (closed circles) or presence of DPH (squares = 0.625 μM; triangles = 1.25 μM). The inset is the data shown as a Dixon plot. Each point is the mean of 3 experiments. Binding = pmol per mg protein.

shown in Fig. 1. It is evident that diphenylhydantoin competitively inhibits the binding of diazepam. The data were replotted by the method of Dixon [12] (inset in Fig. 1) and revealed a $K_i = 0.9$ μM. The dissociation constant (K_d) for the diazepam binding was calculated as 16.7 nM.

Enhancement of DPH inhibition by γ-aminobutyric acid and muscimol.

The previous experiment was repeated in the presence of γ-aminobutyric acid (10 μM). This time the concentrations of DPH were reduced to 1.25 μM and 0.625 μM. Once again DPH produced a competitive inhibition of [^{3}H]diazepam binding (Fig. 2).

However, the K_i was reduced by a factor of three to 0.31 μM. Furthermore, the K_d for diazepam binding was reduced by a similar degree (from 16.7 nM to 3.9 nM).

If muscimol, a γ-aminobutyric acid agonist, was used in place of γ-aminobutyric acid in the above experiment, a K_i of 0.35 μM and a K_d of 6.6 nM were obtained.

DISCUSSION

It is clear from our experiments that there is an interaction between DPH, diazepam and γ-aminobutyric acid – two anticonvulsants and an amino acid intimately linked to convulsions [13-15]. Diphenylhydantoin and diazepam appear to be competing for the same receptor site although the affinity for diazepam is about 60 times that for DPH (16 nM compared with 900 nM). That diazepam and diphenylhydantoin interact with the same binding site is not unexpected in light of a close resemblance in their molecular conformations [16]. Each of these drugs also possess important antiepileptic actions thus the fact they seem to share a common receptor is highly significant.

γ-Aminobutyric acid is the major inhibitory neurotransmitter in the mammalian brain [17]. Evidence is available that a reduction in brain levels of this amino acid is related to the onset of experimental seizures [13,14]. Further, blockage of the γ-aminobutyric acid receptor induces convulsions [15]. On the other hand, increases in brain γ-aminobutyric acid concentrations tend to protect against seizures [18]. It is known that γ-aminobutyric acid can enhance diazepam receptor binding [8,9], an observation confirmed in the present study. The fact that γ-aminobutyric acid and its agonist muscimol also stimulated the inhibition of diazepam binding by DPH is strong support for the idea that DPH and diazepam bind to the same site.

Moreover the increase in affinity induced by γ-aminobutyric acid is about the same (3 to 4-fold) for both drugs. It is inviting to speculate that the observed γ-aminobutyric acid-elicited increase in DPH and diazepam binding is associated with the anticonvulsant actions of these compounds. The mechanism of the affinity changes brought about by γ-aminobutyric acid is unknown. However, it has been postulated that the diazepam and γ-aminobutyric acid receptors are adjacent to each other and that when the neurotransmitter occupies its binding site the shape of the diazepam receptor is altered so that a better fit occurs [8].

Of late a report has appeared stating that pretreatment of rats with diphenylhydantoin enhanced diazepam receptor binding [19]. These results do not necessarily disagree with ours since the two studies differ conciderably. It is feasible that occupancy of the diazepam binding site by DPH *in vivo* leads to affinity changes at the receptor.

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