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Inhibition of Binding of [^{3}H]Batrachotoxinin A 20-α-Benzoate to Sodium Channels by the Anticonvulsant Drugs Diphenylhydantoin and Carbamazepine

MAX WILLOW AND WILLIAM A. CATTERALL

Department of Pharmacology, SJ-30, University of Washington, School of Medicine, Seattle, Washington 98195

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SUMMARY

The effects of a number of clinically effective anticonvulsants on the binding of ligands to receptors associated with voltage-sensitive sodium channels in rat brain synaptosomes has been examined. Diphenylhydantoin (IC_{50} = 40 μM) and carbamazepine (IC_{50} = 131 μM) inhibited the binding of [^{3}H]batrachotoxinin A 20-α-benzoate (BTX-B), which binds to a receptor site responsible for the activation of sodium channel ion flux. Although the effects of diphenylhydantoin on BTX-B binding were not voltage-dependent, the IC_{50} for carbamazepine inhibition of BTX-B binding was 3-fold lower when synaptosomes were incubated under depolarizing conditions. Comparison of the IC_{50} values with mean brain levels of these drugs achieved during prevention of seizures indicates that diphenylhydantoin and carbamazepine occupy 35% and 25%, respectively, of these receptor sites associated with sodium channels at mean therapeutic brain concentrations. Phenobarbital (IC_{50} = 2.60 mM) and diazepam (IC_{50} = 152 μM) also inhibited BTX binding, but the concentrations required were substantially in excess of effective brain levels which protect against experimentally induced seizures in laboratory animals. Trimethadione, ethosuximide, and sodium valproate did not alter BTX-B binding at concentrations up to 1 mM. Scatchard analysis of BTX-B binding to synaptosomes shows that both diphenylhydantoin and carbamazepine reduce receptor affinity without altering maximal binding capacity. The reduction in receptor affinity in the presence of these agents appears to be due to an increased rate of ligand dissociation from the receptor-ligand complex, suggesting an indirect allosteric mechanism for anticonvulsant inhibition of BTX-B binding. The rate of association of BTX-B with the receptor complex is unaltered in the presence of diphenylhydantoin or carbamazepine. Diphenylhydantoin and carbamazepine did not alter basal ^{125}I-labeled scorpion toxin binding to synaptosomes in the absence of batrachotoxin. However, in the presence of batrachotoxin (1.25 μM), diphenylhydantoin and carbamazepine inhibited the batrachotoxin-dependent increase in scorpion toxin binding (IC_{50} values = 100 μM and 260 μM, respectively) in a concentration-dependent manner, providing further evidence that the effects of these agents are mediated at the alkaloid toxin binding site. None of the anticonvulsants examined in the present study affected [^{3}H]saxitoxin binding. The results of this study suggest that diphenylhydantoin and carbamazepine, both of which possess similar therapeutic profiles in the management of grand mal and partial seizures, may exert their effects by interacting with receptor sites involved in the activation of voltage-sensitive sodium channels. A classification of anticonvulsant drugs with respect to sites and mechanisms of action is proposed.

INTRODUCTION

The molecular mechanisms underlying the pharmacological actions of many of the clinically effective anticonvulsants are at present poorly understood. However, there is evidence that some classes of anticonvulsants may effectively raise seizure threshold by interacting with voltage-sensitive sodium channels responsible for the propagation of action potentials in myelinated and nonmyelinated nerve fibers. DPH[1] has been shown to block sodium conductance in a variety of preparations, including squid axons (1) and myelinated frog nerve (2). In addition, the influx of radiolabeled sodium into stimulated lobster nerve fibers is inhibited in the presence of

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[1] The abbreviations used are: DPH, diphenylhydantoin; CBZ, carbamazepine; BTX, batrachotoxin; BTX-B, batrachotoxinin A 20-α-benzoate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; BSA, bovine serum albumin; GABA, γ-aminobutyric acid.

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DPH (3). CBZ, an iminostilbene derivative possessing a therapeutic profile similar to that of DPH in the management of grand mal and partial seizure disorders (4), exerts depressant effects on action potentials recorded in sciatic nerve fibers (5). These effects appear to be due to a reduction in sodium conductance, as has been observed in voltage clamped giant axons (6).

Pinched-off nerve endings (synaptosomes) provide a useful preparation with which to study the effects of DPH, CBZ, and other anticonvulsant agents on the properties of sodium channels in mammalian brain. Synaptosomes retain many of the functional properties of intact nerve terminals, including ion transport (7), membrane potential (8), neurotransmitter release (9), and sodium channels (10). Previous studies using neurotoxin binding assays (10–12) and ion flux measurements (13) have revealed that sodium channels in synaptosomes have properties similar to those in intact nerve and cultured neuroblastoma cells (for a review, see ref. 14). Such studies reveal three distinct neurotoxin receptor sites associated with sodium channels. Receptor site 1 binds the water-soluble inhibitors tetrodotoxin and saxitoxin (11). Grayanotoxin and the alkaloid toxins veratridine, batrachotoxin, and aconitine, which cause persistent activation of sodium channels, bind to neurotoxin receptor site 2 (12). The low molecular weight polypeptides, scorpion toxin and sea anemone toxin, bind to neurotoxin receptor site 3 and both slow sodium channel inactivation and enhance persistent activation by neurotoxins which bind to receptor site 2 (10, 11). DPH blocks veratridine-activated sodium channels in squid giant axon (15) and competitively inhibits sodium ion flux activated by batrachotoxin in neuroblastoma cells (16). The present study examines the effects of a number of clinically effective anticonvulsants on the binding of radiolabeled neurotoxins to sodium channels in synaptosomes in order to define further the mechanism of action of these agents.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were obtained from the following sources: tetrodotoxin from Calbiochem (San Diego, Calif.); veratridine from Aldrich Chemical Company (Milwaukee, Wisc.); scorpion (*Leiurus quinquestriatus*) venom and bovine serum albumin from Sigma Chemical Company (St. Louis, Mo.). Batrachotoxin was a gift from Dr. John Daly (Laboratory of Bioorganic Chemistry, National Institutes of Health), and [^{3}H]batrachotoxinin A 20-α-benzoate was kindly supplied by Dr. George Brown (University of Alabama, Birmingham, Ala.). The preparation and purification of BTX and [^{3}H]BTX-B has been described elsewhere (17). Saxitoxin was supplied by the National Institutes of Health and tritiated as previously reported (11). Scorpion toxin was purified and labeled with ^{125}I using a lactoperoxidase-catalyzed iodination as previously described (18, 19). DPH, CBZ, diazepam, phenobarbital, ethosuximide, trimethadione, and valproic acid were gifts from Drs. A. Camerman and R. H. Levy (University of Washington, Seattle, Wash.). Solutions of DPH and CBZ were prepared by dissolving the compounds in ethanol followed by dilution into aqueous medium with gentle warming. These solutions were stable for at least 1 hr and were used immediately. Control experiments showed that the final concentration of ethanol (0.1%) had no effect. All other standard laboratory reagents were of analytical grade.

Preparation of synaptosomes. Synaptosomes were prepared from whole rat brain according to a modification of the method of Gray and Whittaker (20). The brains of male Sprague-Dawley rats were removed and homogenized in ice-cold 0.32 M sucrose using 10 complete excursions in a motor-driven Teflon-glass homogenizer. All subsequent procedures were performed at 4°. The homogenate (10%, w/v) was centrifuged at 1,000 × *g* for 10 min and the resultant supernatant (S_1) was retained. The pellet (P_1) consisting mainly of sedimented nuclei, blood cells, and broken cellular fragments, was resuspended (10%, w/v) in 0.32 M sucrose and recentrifuged as above. The supernatant (S_2) was combined with S_1 and centrifuged at 17,000 × *g* for 60 min. The supernatant was discarded and the pellet (P_3) was resuspended in 0.32 M sucrose containing 5 mM K_2HPO_4 (pH 7.4) to a volume of 10 ml. The resuspended P_3 fraction was then layered onto a discontinuous gradient consisting of 10-ml layers of 1.2, 1.0, 0.8, 0.6, and 0.4 M sucrose containing 5 mM K_2HPO_4 (pH 7.4). The gradient was centrifuged at 100,000 × *g* (maximal radius, SW 25.2 rotor) for 105 min. The synaptosome fraction sedimenting at the 1.0–1.2 M sucrose interface was collected and diluted to 0.32 M by the dropwise addition of 5 mM K_2HPO_4 (pH 7.4) during constant stirring. The diluted synaptosomal fraction was centrifuged at 40,000 × *g* for 45 min and the resultant pellet was resuspended in standard incubation medium (consisting of 5.4 mM KCl, 0.8 mM $MgSO_4$, 5.5 mM glucose, 50 mM Hepes-Tris (pH 7.4), and 130 mM choline chloride) to a final protein concentration of 35 mg/ml using a loose-fitting glass-glass homogenizer. The synaptosomes were divided into 0.5-ml aliquots and slowly frozen over 30 min on dry ice prior to storage in liquid nitrogen. Previous studies have shown that there is no appreciable loss of toxin binding activity or $^{22}Na^+$ flux activity using this procedure (13).

Measurement of radiolabeled neurotoxin binding. Specific binding of ^{125}I-labeled scorpion toxin, [^{3}H]saxitoxin, and [^{3}H]BTX-B was measured as described previously (10–12). Prior to use, an aliquot of frozen synaptosomes was thawed at 36° for 5 min and then stored on ice. The synaptosomes were diluted 4-fold in standard incubation medium to a final protein concentration of 1.10 mg/ml in the assay. ^{125}I-Labeled scorpion toxin binding was measured by incubating synaptosomes for 10 min at 36° in the standard incubation medium containing 25 mM KCl and 105 mM choline chloride plus ^{125}I-labeled scorpion toxin (0.1 nM), tetrodotoxin (1 μM), BSA (1 mg/ml) in the absence or presence of batrachotoxin (1.25 μM) and varying concentrations of added drug. Nonspecific binding of ^{125}I-labeled scorpion toxin was determined in the presence of 200 nM unlabeled scorpion toxin. Binding reactions were initiated by the addition of 25 μl of the synaptosomal suspension to 175 μl of the reaction mixture. Samples were rapidly mixed and, following incubation (10 min), the reaction was stopped by the addition of 3 ml of ice-cold wash medium consisting of choline chloride (163 mM), 5 mM Hepes-Tris (pH 7.4), $CaCl_2$ (1.8 mM), $MgSO_4$ (0.8 mM), and BSA (1 mg/ml). The synaptosomes were immediately collected on glass-fiber filters (Whatman GF/C) under vacuum and washed

three times with 3 ml of wash medium. ^{125}I bound to filters was determined in a gamma scintillation spectrometer.

[^{3}H]Saxitoxin binding was measured in a similar manner to that described above. The reaction mixture contained standard incubation medium plus [^{3}H]saxitoxin (1.0 nM), BSA (1 mg/ml), and varying concentrations of added drugs. Binding was measured for 10 min at 36°. Nonspecific binding of [^{3}H]saxitoxin was determined in the presence of tetrodotoxin (1 μM). Filters containing trapped synaptosomes were suspended in liquid scintillation fluid, and ^{3}H bound was determined in a liquid scintillation spectrometer.

[^{3}H]BTX-B binding was studied by incubating synaptosomes for 30 min at 36° in the standard incubation medium plus [^{3}H]BTX-B (10 nM), tetrodotoxin (1 μM), scorpion toxin (0.3 or 2 μM), BSA (1 mg/ml), and varying concentrations of added drugs. Nonspecific binding of [^{3}H]BTX-B was determined in the presence of 0.3 mM veratridine. [^{3}H]BTX-B bound was determined as above. In some experiments, KCl (135 mM) was substituted for choline chloride in the standard incubation medium in order to depolarize synaptosomes.

Other methods. Synaptosomal protein concentration was measured according to the method of Peterson (21). Except where mentioned, the data presented are the results of a single experiment that are representative of four or more similar experiments. Each data point is the mean of three to six replicate determinations. In all experiments, standard errors of the mean were less than 10% of the mean binding observed. Linear segments on Scatchard plots and kinetic experiments were computed by linear regression. The IC_{50} determinations were calculated by log-probit analysis according to the method of Finney (22).

RESULTS

Anticonvulsant inhibition of [^{3}H]BTX-B binding to synaptosomes. In a previous study of the effects of several antiarrhythmic drugs on neurotoxin binding and action at sodium channel receptor sites in neuroblastoma cells, it was found that DPH was a competitive inhibitor of batrachotoxin action at neurotoxin receptor site 2 but had no effect on saxitoxin or scorpion toxin binding to neurotoxin receptor sites 1 and 3 (16). Recently, methods have been developed to measure specific binding of [^{3}H]BTX-B to neurotoxin receptor site 2 on sodium channels in synaptosomal membranes (12). In this binding assay, scorpion toxin is used to decrease the K_D for BTX-B from approximately 1 μM to 70 nM through the allosteric interaction between neurotoxin receptor sites 2 and 3 (14). Sodium-free medium and tetrodotoxin are used to block ion transport through sodium channels activated by BTX-B during the binding measurement. Under these conditions, specific binding of [^{3}H]BTX-B to sodium channels comprises 70%–80% of total [^{3}H]BTX-B binding and can be accurately estimated (12). Since this binding requires scorpion toxin (12), a highly specific sodium channel ligand (10, 13, 19), it is unlikely that binding to calcium channels or other synaptosomal components makes a significant contribution to the data.

The effect of a number of anticonvulsants on the binding of [^{3}H]BTX-B (10 nM) to synaptosomes in the presence of 0.3 μM scorpion toxin is illustrated in Fig. 1A and B. DPH and CBZ, both of which are effective in the management of grand mal and partial seizure disorders, inhibited binding in a concentration-dependent manner with IC_{50} values of 40.8 ± 2.5 μM (n = 4) and 158 ± 14 μM (n = 4), respectively (Fig. 1A). Trimethadione, ethosuximide, and sodium valproate did not affect [^{3}H]BTX-B binding at concentrations up to 500 μM (Fig. 1A). Diazepam (IC_{50} = 152 ± 14 μM, n = 5) and phenobarbital

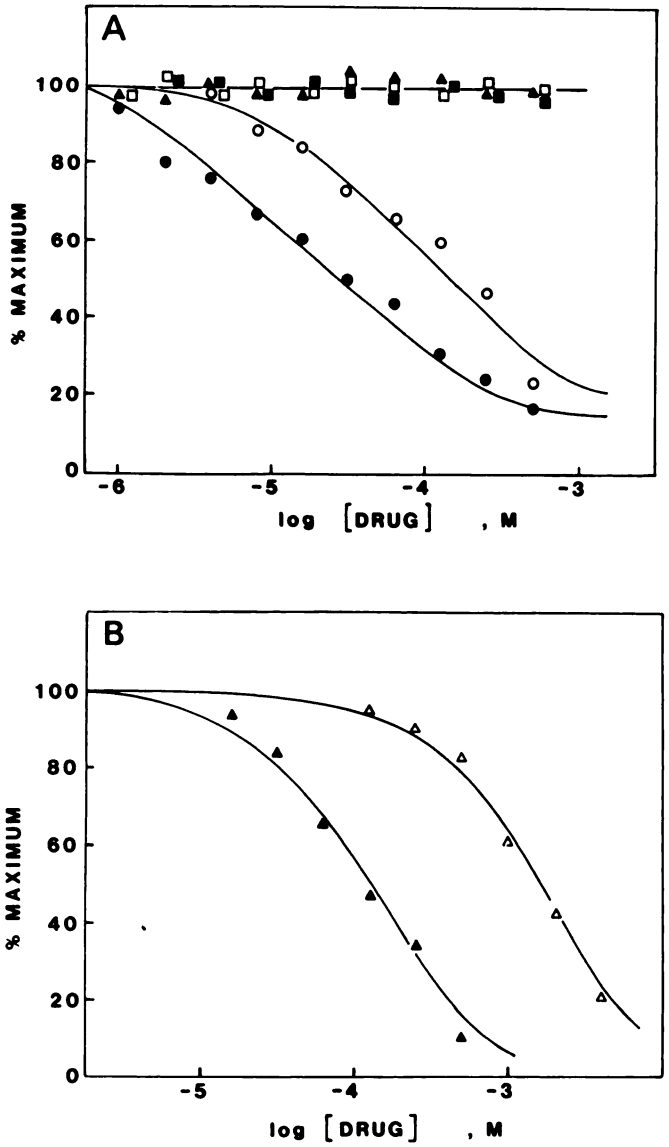


FIG. 1. *Effect of anticonvulsants on the binding of [^{3}H]BTX-B to intact synaptosomes*

A. Effect of DPH, CBZ, trimethadione, ethosuximide, and sodium valproate. Synaptosomes were incubated in the presence of 10 nM [^{3}H]BTX-B (in the presence of 0.3 μM scorpion toxin) and varying concentrations of DPH (●), CBZ (○), trimethadione (▲), ethosuximide (□), or sodium valproate (■) for 30 min. Binding was then measured as described under Experimental Procedures.

B. Effect of diazepam and phenobarbital. Synaptosomes were incubated in the presence of 10 nM [^{3}H]BTX-B (in the presence of 0.3 μM scorpion toxin) and varying concentrations of diazepam (▲) or phenobarbital (△) for 30 min. Binding was then measured as described under Experimental Procedures.

(IC_{50} = 2.60 ± 0.25 mM, n = 5) also inhibited [^{3}H]BTX-B binding (Fig. 1B).

Table 1 compares IC_{50} values for block of [^{3}H]BTX-B binding by the different anticonvulsants with the estimates of brain levels (micromoles per kilogram wet weight) of drug achieved during effective prevention of maximal electroshock seizures in rats. Of the seven drugs tested, only DPH and CBZ block [^{3}H]BTX-B binding to a statistically significant fraction of sodium channel receptor sites at their mean therapeutic levels. From the data of Fig. 1, these drugs block 35% and 25%, respectively, of [^{3}H]BTX-B binding at their mean therapeutic brain levels.

Effect of membrane depolarization on the inhibition of [^{3}H]BTX-B binding by DPH and CBZ. To examine whether the effects of DPH and CBZ on [^{3}H]BTX-B binding were dependent on membrane potential, parallel experiments were performed examining the effects of these agents in medium containing 5 mM or 135 mM KCl. Previous studies have indicated that the membrane potential of synaptosomes under these conditions is approximately −55 mV and 0 mV, respectively (14). Since the affinity of scorpion toxin binding is voltage-dependent (19), it was necessary to increase the concentration of scorpion toxin to 2 μM in order to ensure that scorpion toxin occupied essentially all sodium channel receptor sites at both membrane potentials (12). Under these conditions, the effects of membrane potential on inhibition of [^{3}H]BTX-B binding should reflect changes in drug affinity. CBZ appeared to be 3-fold more potent in inhibiting [^{3}H]BTX-B binding under depolarizing conditions (IC_{50} = 131 ± 12.7 μM, n = 4) as compared with nondepolarizing conditions (IC_{50} = 394 ± 3 μM, n = 2; see Fig. 2). In contrast, the inhibition of [^{3}H]BTX-B binding by DPH was identical under depolarizing and nondepolarizing conditions (Fig. 2). These results suggest that the mechanisms of interaction of DPH and CBZ with sodium channel receptor site(s) are different.

Comparison of the inhibition curves at 5 mM K^+ in Fig. 2 with those of Fig. 1A reveals a marked difference in steepness. At a scorpion toxin concentration of 0.3 μM as in Fig. 1A, DPH and CBZ give shallow inhibition curves characterized by apparent Hill coefficients of 0.55 and 0.76 respectively. These data suggest a heterogeneous binding affinity for the drugs. In contrast, at 2 μM scorpion toxin as in Fig. 2, the inhibition curves have a hyperbolic shape characterized by apparent Hill coefficients of 0.90 and 0.90, indicating only a single affinity for the drugs. It seems likely that the heterogeneity of binding affinity observed at 0.3 μM scorpion toxin is due to heterogeneity of membrane potential in the synaptosome preparation. If scorpion toxin has an effect of either increasing or decreasing drug binding, sodium channels in partially depolarized synaptosomes will be only partially saturated with scorpion toxin at 0.3 μM, and a heterogeneous drug binding affinity will be observed. At 2 μM scorpion toxin, all channels will be occupied by scorpion toxin and no heterogeneity will be observed. The shift in IC_{50} for CBZ from 131 μM at 0.3 μM scorpion toxin (Fig. 1A) to 394 μM at 2 μM scorpion toxin (Fig. 2) suggests that scorpion toxin reduces the affinity for CBZ. Further resolution of these effects will require direct measurement of DPH and CBZ binding to sodium channels.

TABLE 1

Effect of anticonvulsants on BTX-B binding

Anticonvulsant	IC_{50} (BTX-B binding)	Rat central nervous system levels (ref.)	% Inhibition at mean therapeutic level
	μM	*μmoles/kg*	
DPH	40	10–20 (23)	35
CBZ	131	10–50 (23)	25
Phenobarbital	2600	10–80 (23)	1.7
Diazepam	152	0.5–1.0 (24)	0.3
Sodium valproate	NA[a]	300–600 (25)	0
Trimethadione	NA	≥1000 (26)[b]	0
Ethosuximide	NA	600–700 (27)	0

[a] NA denotes no effect on [^{3}H]BTX-B binding at concentrations up to 1 mM.

[b] Rat central nervous system levels unavailable. Denotes approximate central nervous system levels for therapeutic doses in mice (300 mg/kg) based on data obtained with higher doses (1000 mg/kg).

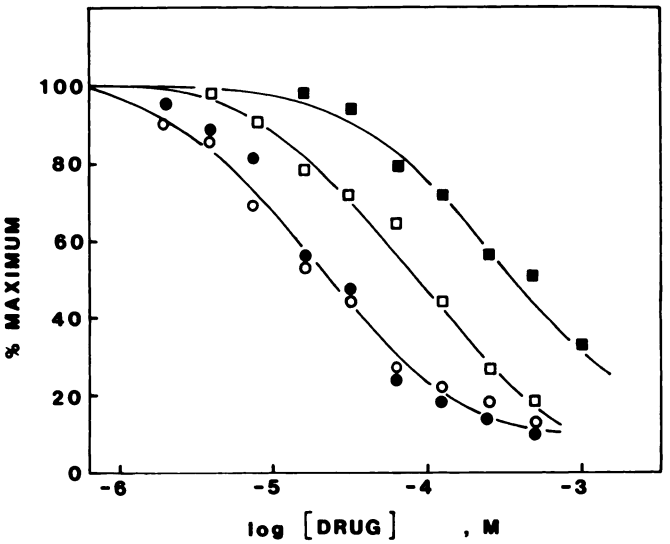


FIG. 2. *Effect of DPH and CBZ on the binding of [^{3}H]BTX-B to synaptosomes under depolarizing and nondepolarizing conditions*

Synaptosomes were incubated in the presence of 10 nM [^{3}H]BTX-B (scorpion toxin concentration = 2 μM) and varying concentrations of DPH (●) and CBZ (■) (medium containing 5 mM KCl) or DPH (○) and CBZ (□) (medium containing 135 mM KCl). Binding was then measured as described under Experimental Procedures.

Effect of DPH and CBZ on kinetic and equilibrium properties of BTX-B binding. Scatchard analysis of BTX-B binding over an extended concentration range (Fig. 3A and B) shows that this ligand binds to a single class of high-affinity binding sites, in agreement with earlier studies (12). DPH (50 μM) and CBZ (200 μM) increase the K_D for [^{3}H]BTX-B from 125 nM to 256 nM or 357 nM, respectively (Fig. 3). The maximal binding capacity for BTX-B (2.2 pmoles/mg) is not significantly affected by DPH or CBZ. These results indicate a competitive mechanism for inhibition of BTX-B binding by DPH and CBZ.

At 50 μM, DPH (Fig. 4) does not influence the rate of association of [^{3}H] BTX-B binding to the receptor complex. CBZ (200 μM) was also without effect on the asso-

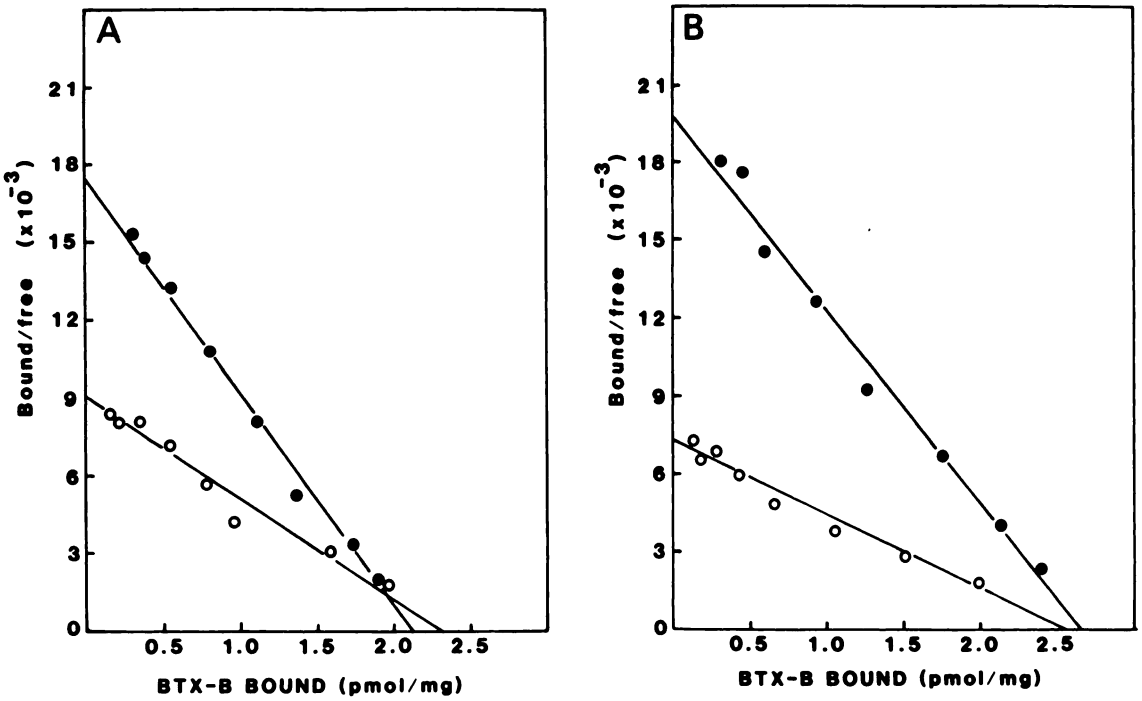


FIG. 3. *Scatchard analysis of [^{3}H]BTX-B binding to synaptosomes*

A. Binding of 10 nM [^{3}H]BTX-B was measured in standard incubation medium (scorpion toxin concentration = 2 μM) in the absence (●) or presence (○) of 50 μM DPH, with increasing concentrations of unlabeled BTX as described under Experimental Procedures.

B. Binding of 10 nM [^{3}H]BTX-B was measured in incubation medium containing 135 mM (scorpion toxin concentration = 2 μM) in the absence (●) or presence (○) of 200 μM CBZ, with increasing concentrations of unlabeled BTX as described under Experimental Procedures.

ciation kinetics of [^{3}H]BTX-B (data not shown). In contrast, both DPH (200 μM) and CBZ (300 μM) accelerate the rate of dissociation of [^{3}H]BTX-B from the steady-state ligand-receptor complex following the addition of 200 μM veratridine (Fig. 5A and B). The dissociation rate constant (k_{-1}) is increased from 0.0086 min^{-1} to 0.014 min^{-1} in the presence of DPH (Fig. 5A) and from 0.0090 min^{-1} to 0.0196 min^{-1} in the presence of CBZ (Fig. 5B). These kinetic data are not consistent with a simple competitive inhibition mechanism in which the drugs bind at the BTX-B receptor site, since this mechanism predicts a reduction in the rate of binding with no effect on the rate of dissociation of the toxin-receptor complex. The data indicate that DPH and CBZ are indirect allosteric competitive inhibitors of BTX-B binding.

The time course of dissociation of the bound BTX-B appears biphasic, as if 10%–15% of the toxin-receptor complex dissociates rapidly (Fig. 5). More detailed kinetic experiments are required to determine the basis of this apparent kinetic heterogeneity. However, the data of Fig. 5 indicate that the acceleration of BTX-B dissociation by DPH and CBZ affects mainly the slower phase of dissociation.

Effect of DPH and CBZ on the binding of scorpion toxin to synaptosomes. Binding of scorpion toxin to neurotoxin receptor site 3 on the sodium channel can be measured directly using ^{125}I-labeled scorpion toxin (10). In synaptosomes, scorpion toxin binding is enhanced by batrachotoxin (12). The enhancement is greater in depolarized synaptosomes. Thus, in medium containing 25 mM K^+ to partially depolarize synaptosomes, the effect of DPH and CBZ on scorpion toxin binding can be measured directly and compared with the effect of these drugs on the batrachotoxin enhancement of scorpion toxin binding. The results of Fig. 6 show no effect of DPH on specific scorpion toxin binding at concentrations as high as 0.5 mM (Fig. 6, ▲). In the presence of 1.25 μM batrachotoxin, scorpion toxin binding is enhanced 2- to 3-fold in medium containing 25 mM KCl (Fig. 6, ●). DPH produced a concentration-dependent inhibition of that fraction of ^{125}I-labeled scorpion toxin binding which is enhanced by the alkaloid toxin with an IC_{50} = 100 μM (Fig. 6). These data indicate a selective effect of DPH on batrachotoxin binding. Similar results were obtained with CBZ (IC_{50} = 260 μM; data not shown).

Effect of anticonvulsants on the specific binding of [^{3}H]saxitoxin to synaptosomes. At concentrations which inhibit [^{3}H]BTX-B binding by approximately 50%, there is no effect of DPH, CBZ, phenobarbital, or diazepam on saxitoxin binding to synaptosomes (Table 2). These data also provide evidence for a selective effect of DPH and CBZ on binding at neurotoxin receptor site 2 on the sodium channel.

DISCUSSION

The present study suggests that DPH and CBZ may exert an inhibitory effect on voltage-sensitive sodium channels in mammalian brain at concentrations which are pharmacologically relevant to their anticonvulsant action (see Table 1). Our measurements of inhibition of

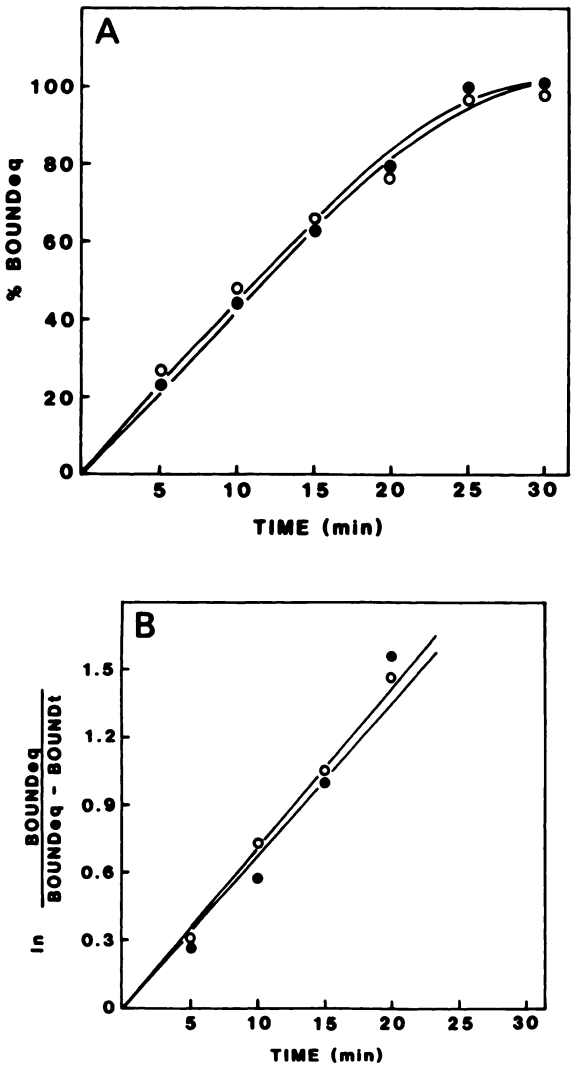


FIG. 4. *Time course of association of the [^{3}H]BTX-B/receptor complex*

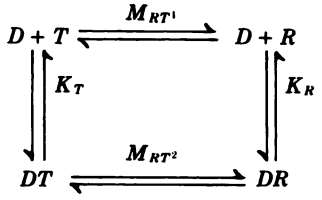
A. Synaptosomes were incubated for the indicated times with 10 nM [^{3}H]BTX-B (in the presence of 0.3 μM scorpion toxin) in the absence (●) and presence (○) of 50 μM DPH. At each time point, binding was measured as described under Experimental Procedures.

B. Data from Fig. 4A presented in logarithmic form.

[^{3}H]BTX-B binding provide quantitive estimates of the K_D for binding of these two drugs at their site of action on sodium channels in mammalian brain, allowing direct comparison of their affinity at these sites with brain concentrations achieved during anticonvulsant therapy. This comparison indicates that DPH and CBZ occupy 35% and 25%, respectively, of their sites of action on sodium channels during anticonvulsant therapy. Earlier studies have shown that DPH inhibits $^{22}Na^+$ flux activated by the alkaloid toxins batrachotoxin and veratridine in cultured mouse neuroblastoma cells (16). Taken together with electrophysiological data demonstrating the inhibitory effects of these agents on sodium currents in a variety of nonmammalian myelinated and unmyelinated nerve preparations (1, 2, 5, 6), these results suggest that 25%–35% of brain sodium channels are inhibited during anticonvulsant therapy with DPH and CBZ. It seems likely that modulation of sodium channel function plays an important role in the anticonvulsant action of these two drugs.

Two different lines of evidence show that DPH and CBZ are indirect allosteric competitive inhibitors of BTX-B binding rather than direct competitive inhibitors which bind at a common receptor site. Studies of DPH inhibition of neurotoxin binding and action in neuroblastoma cells indicated a competitive pattern of inhibition of persistent activation by the full agonist batrachotoxin but a mixed competitive/noncompetitive pattern of inhibition for the partial agonist veratridine (16). These results are expected for an allosteric inhibition mechanism (28). In the present study, we have shown that DPH and CBZ block BTX-B binding by increasing K_D with no change in binding capacity. These results indicate a competitive inhibition of BTX-B binding by DPH and CBZ. Analysis of the effects of DPH and CBZ on the kinetics of BTX-B binding support the conclusion that these drugs act by an indirect allosteric mechanism. DPH and CBZ have no effect on the rate of formation of the BTX-B/receptor complex, in contrast to the reduction in association rate expected for a simple competitive inhibition mechanism. In addition, both drugs accelerated the dissociation of the BTX-B/receptor complex, providing direct evidence for an allosteric competitive inhibition mechanism. The increased rate of dissociation is observed in the presence of a saturating concentration of veratridine, a simple competitive inhibitor of binding, so that the effects of DPH and CBZ on rebinding of dissociated [^{3}H]BTX-B can be ignored.

The concentration of DPH and CBZ required to accelerate dissociation of the BTX-B/receptor complex is consistent with an allosteric mechanism of drug action. Previous work has shown that batrachotoxin and related lipid-soluble toxins cause persistent activation of sodium channels in neuroblastoma cells and synaptosomes by binding with high affinity to active states of the channel (12, 28). If DPH and CBZ inhibit sodium channel activation by a similar allosteric mechanism, the binding of these drugs to R (active) states of sodium channels should have lower affinity than binding to T (nonconducting) states. Scheme 1 presents such a model, where K_T and K_R are equilibrium dissociation constants for the binding of DPH or CBZ (D) to the T and R states of the sodium channel, respectively. For values of $M_{RT} \gg 1$, K_D for BTX-B is linearly proportional to M_{RT} (12). In a



SCHEME 1

closed set of equilibria as in Scheme 1, $M_{RT^2}/M_{RT^1} = K_R/K_T$. Since DPH and CBZ reduce BTX-B binding at least 6-fold (Figs. 1 and 2), M_{RT^2}/M_{RT^1} and K_R/K_T must be at least 6. Thus, the model predicts that the K_D for binding of DPH and CBZ to sodium channels persistently activated by binding of BTX-B will be 6-fold greater than

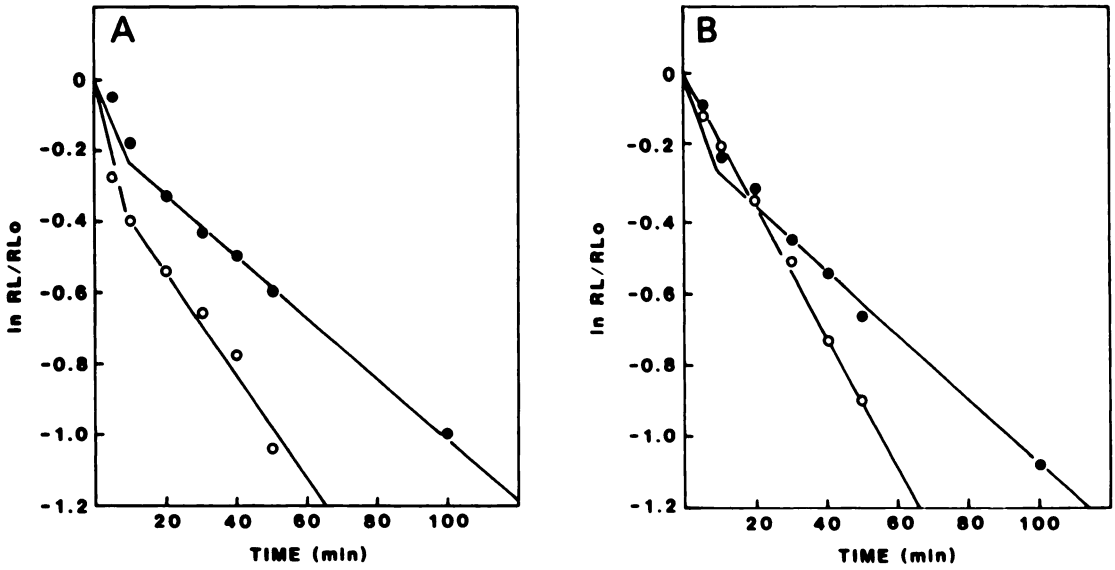


FIG. 5. *Time course of dissociation of the [^{3}H]BTX-B/receptor complex*

A. Synaptosomes were incubated in standard incubation medium for 30 min with 10 nM [^{3}H]BTX-B (scorpion toxin concentration = 0.3 μM), and, at zero time, 200 μM veratridine [in the absence (●) or presence (○) of 50 μM DPH] was added. At the indicated times, samples were removed and bound [^{3}H]BTX-B was determined as described under Experimental Procedures.

B. Experimental conditions were similar to those above, except that, at zero time, 200 μM veratridine [in the absence (●) or presence (○) of 300 μM CBZ] was added.

that to inactive channels. At BTX-B concentrations well below K_D as used here, the IC_{50} values for DPH and CBZ inhibition of [^{3}H]BTX-B binding at equilibrium give estimates of K_D for these drugs binding to inactive (T) states of sodium channels. In contrast, in order for DPH or CBZ to enhance dissociation of bound BTX-B they must bind to active (R) states of sodium channels containing bound [^{3}H]BTX-B. Thus, a 2-fold increase in dissociation rate is expected at 6 · IC_{50} or 240 μM DPH. The data of Fig. 5 show a 1.7-fold increase at 200 μM DPH, in reasonable agreement with this prediction. CBZ enhances BTX-B dissociation 2.1-fold at 300 μM, a somewhat lower concentration than predicted by the model of Scheme 1. However, its binding or action is apparently inhibited by scorpion toxin (compare Figs. 1 and 2), making an exact quantitative agreement of the equilibrium binding data in the presence of 0.3 μM scorpion toxin with the dissociation rate data in the absence of scorpion toxin unexpected. Taken together, the ligand binding data of this report and the ion flux data presented previously (16) provide clear evidence that DPH and CBZ are indirect allosteric inhibitors of toxin binding and action at neurotoxin receptor site 2 on the sodium channel.

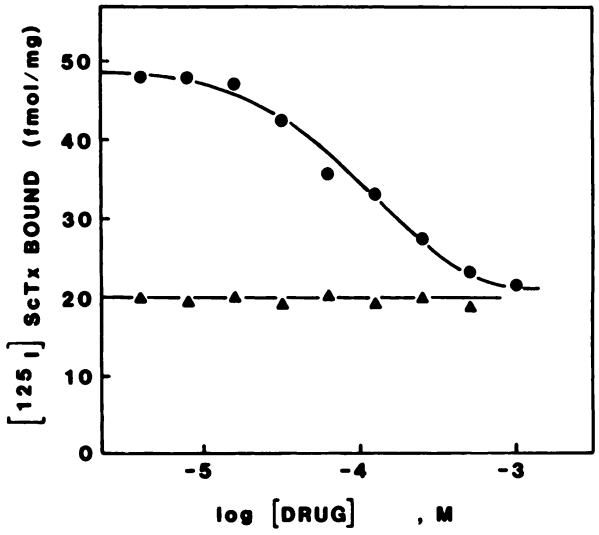


FIG. 6. *Effect of DPH on ^{125}I-scorpion toxin binding*

Synaptosomes were incubated for 10 min in medium containing 0.1 nM ^{125}I-labeled toxin [in the absence (▲) or presence (●) of 1.25 μM unlabeled batrachotoxin] in varying concentrations of DPH. The concentration of KCl in the medium was 25 mM. Binding was then measured as described under Experimental Procedures.

The effects of DPH and CBZ on neurotoxin binding and action at receptor site 2 are specific. Both the binding of saxitoxin to neurotoxin receptor site 1 and the binding of scorpion toxin to neurotoxin receptor site 3 are unaffected by these drugs. However, the enhancement of scorpion toxin binding by batrachotoxin (1.25 μM) is blocked by DPH and CBZ. Assuming a K_D for batrachotoxin of 700 nM (17) in the presence of only tracer

TABLE 2

Effect of anticonvulsants on [^{3}H]saxitoxin (1 nM) binding to synaptosomes

Drug	Concentration	% of Control binding[a] ± SEM	n
	μM		
DPH	50	104 ± 6	4
CBZ	200	103 ± 5	4
Diazepam	200	98 ± 5	4
Phenobarbital	2000	94 ± 8	4

[a] Total and nonspecific saxitoxin binding values in the absence and presence of 1 μM tetrodotoxin were 432 and 30 cpm, respectively.

TABLE 3

Classification of anticonvulsants according to effects on seizures, sodium channels, and GABA receptors

Class	Anticonvulsant	Pharmacological profile[a]				Drug actions at therapeutic concentration	
		Partial seizures	Grand mal	Myoclonic	Petit mal	Sodium channels	GABA receptors[b]
I	Diphenylhydantoin	+	+	−	−	+	−
	Carbamazepine	+	+	−	−	+	NA[c]
II	Phenobarbital	+	+	+	−	−	+
	Benzodiazepines	±	±	+	+	−	+
	Valproate	+	+	+	+	−	+
III	Ethosuximide	−	−	−	+	−	NA
	Trimethadione	−	−	−	+	−	NA

[a] This abbreviated presentation of pharmacological profile is derived from ref. 37.
[b] The data on interaction of these drugs with GABA receptors is taken from refs. 29–36.
[c] NA, Data not available.

concentrations of scorpion toxin (0.1 nM), the calculated K_D for DPH in blocking the batrachotoxin enhancement of scorpion toxin binding is 36 μM, in close agreement with the value of 37 μM derived from direct measurements of [^{3}H]BTX-B binding. Thus, DPH binding at a single site of action blocks both [^{3}H]BTX-B binding and batrachotoxin enhancement of scorpion toxin binding.

Our experiments distinguish DPH and CBZ from the other five anticonvulsant drugs studied, since the other drugs do not have a significant effect on [^{3}H]BTX-B binding at concentrations achieved therapeutically. It is therefore unlikely that these drugs share the action of DPH and CBZ on sodium channels as part of their anticonvulsant action. Other results in the literature support this distinction between classes of anticonvulsants. Barbiturates [including phenobarbital (29)], benzodiazepines [including diazepam (30)], and sodium valproate (31) enhance inhibitory responses to GABA at concentrations that are achieved therapeutically. These effects result, in part, from an enhancement of GABA binding to its receptor (32–34). It is likely that these three classes of anticonvulsants prevent seizures by enhancing inhibitory transmission at GABAergic synapses. However, this in unlikely to be a general mechanism of anticonvulsant action because DPH does not enhance physiological responses to GABA in mammalian sensory neurons (35) and frog motoneurons (36), whereas barbiturates, benzodiazepines, and sodium valproate do. Succinimides and oxazolidinediones have not been reported to have effects on either voltage-sensitive sodium channels or GABA receptors.

In view of the differential profile of clinical effectiveness of the anticonvulsants against specific classes of seizures (37), the differential effects on sodium channels noted in this study, and the differential effects of these drugs on GABA receptors reported in the literature (29–36), we propose that the anticonvulsant drugs can be classified into three groups (Table 3). The Class I anticonvulsants, DPH and CBZ, have a similar spectrum of clinical potency with efficacy primarily against partial seizures and tonic-clonic (grand mal) seizures. We propose that these agents are sodium channel-selective anticonvulsants which modulate sodium channel properties as their principal mechanism of action. The Class II anticonvulsants (phenobarbital, the benzodiazepines, and sodium valproate) have a broad spectrum of clinical potency with significant efficacy against partial seizures, grand mal seizures, myoclonic seizures, and petit mal (absence) seizures (37). On the basis of work in the literature (29–36) we propose that these agents are GABA receptor-selective anticonvulsants which enhance inhibitory synaptic transmission as their principal mechanism of action. The Class III anticonvulsants (ethosuximide, trimethadione, and related succinimides and oxazolidinediones) have a narrow spectrum of clinical effectiveness which is complementary to that of the Class I agents. Their effectiveness is restricted primarily to treatment of petit mal seizures (37). At present, no satisfactory pharmacological mechanism for their action has been proposed.

This proposed classification of anticonvulsant drugs emphasizes the differences in their pharmacological profiles and their mechanisms of action, in contrast to proposals suggesting a single comprehensive mechanism of action applicable to all of these drugs. We believe that this hypothesis is consistent with the existing literature on mechanisms of anticonvulsant action and the clinical experience with use of these drugs. More experimental work is necessary to establish quantitatively the relationships between brain concentrations achieved during therapy and effective concentrations at receptor sites associated with sodium channels or GABA receptors in order to substantiate this classification of anticonvulsant drugs further. In addition, the classification of anticonvulsants according to sites of action will not be complete until a mechanism of action is discovered for the Class III drugs which are specifically effective against petit mal seizures. Further experiments to test the validity of this separation of anticonvulsant drugs into subclasses according to site and mechanism of action are in progress in our laboratory.

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Send reprint requests to: Dr. Max Willow, Department of Pharmacology, SJ-30, University of Washington School of Medicine, Seattle, Wash. 98195.