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[^{3}H]Phenytoin Identifies a Novel Anticonvulsant-Binding Domain on Voltage-Dependent Sodium Channels

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SUMMARY

The voltage-dependent sodium channel has been proposed as a specific target for the actions of the anticonvulsant drug phenytoin. Working at 0–4°, we previously reported the existence of specific [^{3}H]phenytoin binding sites in rat brain membranes. In the present study, the binding of [^{3}H]phenytoin was assessed at 22°, a temperature favorable to the binding of sodium channel ligands. At 22°, the site had a K_d of 1.5 μM, which is in the relevant therapeutic concentration range for anticonvulsant activity (1–10 μM), and a calculated B_{max} of 4.5 pmol/mg of protein, which is similar to previous estimates of sodium channel concentration in brain membranes. In competition experiments, specific [^{3}H]phenytoin binding was found to be inhibited by drugs that interact with the sodium channel, including antiarrhythmics, local anesthetics, anticonvulsants, and site-specific neurotoxins (the steroidal alkaloid activators, β-scorpion venoms, and brevetoxin-3). Diazepam, used clinically in the management of tonic-clonic status epilepticus, and flunarizine, a calcium channel blocker with anticonvulsant activity, potentiated [^{3}H]phenytoin binding at micromolar concentrations. Other drugs and ligands, including neurotransmitters, neuromodulators, and ligands for other ion channels, had no effect. Depolarization with KCl showed [^{3}H]phenytoin binding to be voltage sensitive. Experiments with batrachotoxin (a specific site 2 toxin) and anticonvulsants demonstrated that the interactions between these compounds and the [^{3}H]phenytoin binding site are allosteric in nature. These results provide direct evidence that phenytoin interacts with the voltage-dependent sodium channel and indicate that such interactions take place at therapeutic concentrations. They support previous proposals, based on toxin-binding and electrophysiological studies, that the therapeutic effects of phenytoin result from a selective inhibition of voltage-dependent sodium flux.

Phenytoin is the drug of choice for all types of epilepsy except absence seizures (1) and is one of the most widely prescribed agents in modern medicine (2, 3). Electrophysiological studies have suggested that phenytoin limits sustained repetitive neuronal firing via inhibition of voltage-dependent sodium flux (4–13). These effects are observed within the drug's therapeutic range of 1–10 μM (14–17). Biochemical studies, however, have generally failed to show a significant action of the drug within the therapeutic range. Catterall and colleagues have reported that phenytoin displaces the hydrophobic alkaloid neurotoxin BTX from binding site 2 (18) on the voltage-dependent sodium channel (9, 19, 20), but these interactions take place only at supratherapeutic concentrations (IC_{50} = 30–40 μM).

In 1981, Burnham *et al.* (21) reported the existence of a specific and saturable [^{3}H]phenytoin binding site in rat brain homogenates. This finding was subsequently confirmed by other investigators (22–25). Binding to the [^{3}H]phenytoin site occurs with a K_d of approximately 5 μM (21), making it one of the few biochemical actions of phenytoin reported within the therapeutic concentration range of the drug. The present study was designed to determine whether the [^{3}H]phenytoin site might be a domain on the voltage-dependent sodium channel. Experiments were conducted at 22°, a temperature commonly used in electrophysiological studies and favorable to the binding of ligands to the voltage-dependent sodium channel (26, 27). Previous studies of [^{3}H]phenytoin binding had been conducted at 0–4° (21–25).

Materials and Methods

Preparation of synaptoneurosomes. Synaptoneurosomes were prepared from the neocortex of male Long-Evans rats (200–250 g) using the method of Hollingsworth *et al.* (28). Briefly, brains were placed on ice, and the whole neocortex was quickly removed and placed in 10 ml of homogenization buffer (10 mM Tris, 250 mM sucrose, pH 7.4 at 4°), supplemented with protease inhibitors (10–25 μg/ml soybean trypsin inhibitor, 10 mM benzamidine, and 5 mM EDTA). The tissue was gently

Portions of this work have been presented in abstract form (40, 41). J.F. was funded by a fellowship from the Bloorview Epilepsy Program.

ABBREVIATIONS: BTX, batrachotoxinin A 2,4-dimethyl pyrrole-3-carboxylate; BSA, bovine serum albumin; [^{3}H]BTX-B, [^{3}H]batrachotoxinin A 20-α-benzoate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Lqq, *Leiurus quinquestriatus quinquestriatus*; α-MEM, α-minimum essential medium; FBS, fetal bovine serum.

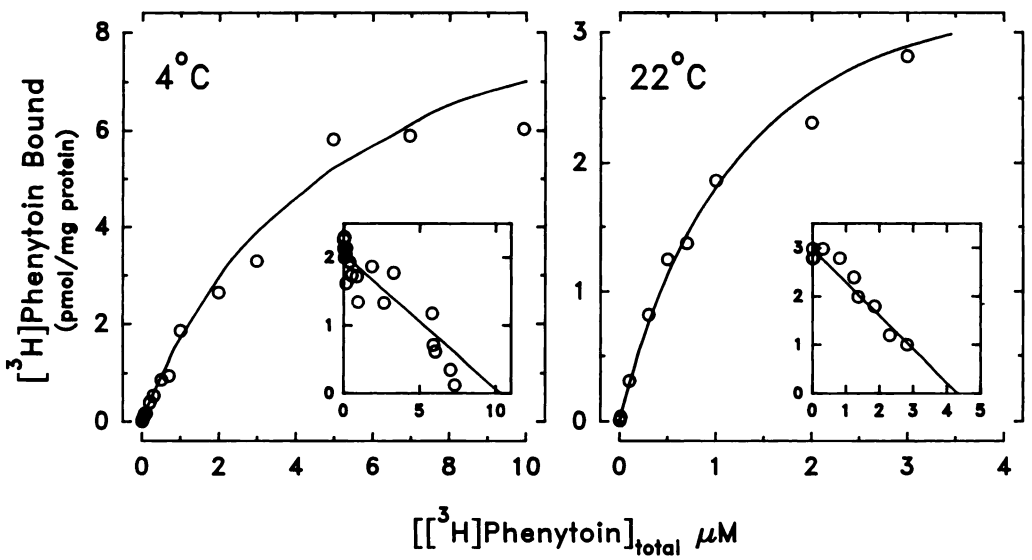

Fig. 1. Saturable and specific [^{3}H]phenytoin binding in rat brain neocortical synaptoneurosomes at 4° and 22°. The figure represents saturation isotherms, with Scatchard transformations of both the data points and the fitted curve from the saturation data (*insets*). The *ordinate* for the Scatchard plots has units of bound/total (fmol/mg of protein/nM); the *abscissa* has units of bound (pmol/mg of protein). *Left*, saturable binding at 4°. These data are the means of four separate experiments, each conducted in triplicate. *Right*, saturable binding at 22°. These data are the means of five separate experiments, each conducted in triplicate. The fitted curves represent the best fit of these data to a model describing a single population of binding sites (see Ref. 29 for details).

homogenized with 10 up-down strokes in a glass-glass homogenizer, and the suspension was then filtered through four layers of nylon (160-μm mesh). The homogenizer was rinsed with an additional 25 ml of buffer and this rinse buffer was also filtered through the nylon mesh. The combined filtrate was then passed through a 10-μm polytetrafluoroethylene filter. The resulting filtrate was centrifuged at 1000 × *g* for 15 min. The supernatant was discarded and the pellet was resuspended in 35 ml of HEPES-Tris buffer (50 mM HEPES, 130 mM choline chloride, 5.5 mM D-glucose, 0.8 mM magnesium sulfate, 5.4 mM potassium chloride, adjusted to pH 7.4 at 4° with Tris base), supplemented with protease inhibitors as in the homogenization buffer. The suspension was then centrifuged at 1000 × *g* for 15 min. The resulting pellet was resuspended in 16 ml of assay buffer (HEPES-Tris buffer minus protease inhibitors, pH 7.4 at 22°), giving a final protein concentration of 1–2 mg/ml, and was kept on ice. Immediately before each assay, the synaptoneurosomes were warmed to 22° in a 35° shaking water bath and mechanically dispersed (Polytron, setting 6 for 10 sec).

Cell culture. SK-N-MC human neuroblastoma cells were grown in 150-mm × 25-mm plastic culture dishes in α-MEM, supplemented with FBS (10% final concentration), 100 IU/ml penicillin, and 100 μg/ml streptomycin, and maintained at 37° in a humidified 5% CO_2 environment. Initially, cells were grown for 2–3 weeks, being reseeded when necessary. Thereafter, cells were harvested for experiments by standard procedures every third or fourth day, at which time the cultures were confluent. Before each experiment, cells were maintained in suspension in α-MEM, without FBS or antibiotics, in a 37° shaking water bath in a 5% CO_2/95% O_2 environment. For each assay, the cell suspension was centrifuged at 1000 × *g* for 5 min. The supernatant was discarded and the pellet was gently resuspended in 16 ml of assay buffer by trituration using a sterile plastic Pasteur pipette. This suspension was then passed through a 37-μm nylon mesh to remove any cell debris. An aliquot of this final suspension was reserved for determining cell viability using trypan blue dye exclusion. Viability was typically 70–80%. Approximately 1.2 × 10^6 viable cells were added to each assay tube.

[^{3}H]Phenytoin binding assay. Binding assays were performed using a modification of the method of Burnham *et al.* (21). Toxins and drugs were prepared as concentrated ethanolic stocks, unless otherwise indicated. Unlabeled phenytoin was prepared as a 1 mM alkaline aqueous stock solution. Dilutions were made using HEPES-Tris buffer (as described above, pH 7.4 at 22°), supplemented with 3 mg/ml BSA (1 mg/ml final concentration in assay tube). In saturation experiments, assay tubes contained either 200 μl of HEPES-Tris-BSA buffer (total binding) or unlabeled phenytoin dilution (1 nM to 50 μM). In competition experiments, assay tubes contained either 200 μl of HEPES-Tris-BSA buffer (total binding) or the appropriate unlabeled toxin or drug dilution. Nonspecific [^{3}H]phenytoin binding in competition studies was measured in the presence of 50 μM unlabeled phenytoin. For both saturation and competition studies, all assay tubes contained 200 μl of [^{3}H]phenytoin (3 nM final concentration; 40–50 Ci/mmol). Assays were initiated by the addition of 200 μl of tissue (synaptoneurosomal membranes or SK-N-MC neuroblastoma cell suspension) and were incubated for 40 min at 22°. Assays were terminated by rapid filtration of the samples through Whatman GF/C glass microfiber filters. Filters were washed with a single volume (8 ml, dispensed in 4 sec) of ice-cold wash buffer (50 mM Tris, 5 mM EDTA, pH 7.4 at 0–4°), air dried, and equilibrated overnight (at least 12 hr at room temperature) in 5 ml of scintillation cocktail. Samples were vigorously shaken and then counted in a Beckman LS5000TA scintillation counter at 50–60% efficiency.

Depolarization experiments. For assays addressing the effects of depolarization on specific [^{3}H]phenytoin binding, choline chloride in the assay buffer (130 mM) was replaced with KCl, resulting in a final KCl concentration of 135.4 mM.

Data analysis. Saturation and competition data were analyzed using a weighted, nonlinear, least squares regression program (LIGAND) (29) on an IBM-PC microcomputer. Where appropriate, goodness of fit was assessed using an extra sum of squares *F* test (29).

Materials. [^{3}H]Phenytoin (40–50 Ci/mmol) was obtained from New England Nuclear (Boston, MA). BTX was a generous gift from Dr. John W. Daly (Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health). Soybean trypsin inhibitor, HEPES, saxitoxin, and brevetoxin were obtained from Calbiochem (La Jolla, CA). Diazepam was kindly donated by Hoffman-La Roche (Basel, Switzerland). Millex and 10-μm polytetrafluoroethylene (LCWP 047 00) filters were from Millipore (Mississauga, Ontario, Canada). Cytoscint scintillation cocktail was obtained from ICN (Irvine, CA). Male Long-Evans rats (200–250 g) were purchased from Charles River (Montreal, Canada). SK-N-MC human neuroblastoma cells were from the American Type Culture Collection (Rockville, MD). α-MEM was from Tissue Culture Media Preparation Services, University of Toronto. FBS and antibiotics were from GIBCO (Burlington, Ontario, Canada). (±)-BAYK8644 was provided by Miles Research Laboratories (New Haven, CT). Carbamazepine was donated by Geigy Pharmaceuticals (Mississaugua, Ontario, Canada). Ethosuximide was provided by Parke-Davis (Brockville, Ontario, Canada). Mephenytoin was from Sandoz (Montreal, Canada). Ethotoin was from Abbott Laboratories (Chicago, IL). All other compounds and chemicals were purchased from either Sigma (St. Louis,

TABLE 1

Screening of various compunds for activity versus 3 nM [^{3}H] phenytoin at 22°

A. Compounds that inhibit specific [^{3}H]phenytoin binding

Anticonvulsants	Antiarrhythmics	Ca^{2+} channel ligands
Phenytoin[a]	Quinidine[d]	Verapamil[d]
Mephenytoin[b]	Clofilium[d]	Diltiazem[d]
Ethotoin[b]	Na^+ channel toxins	Miscellaneous
Carbamazepine[b]	BTX[e]	Chlorpromazine[d]
Phenobarbital[b]	Aconitine[d]	
Local anesthetics	Veratridine[b]	
Bupivacaine[c]	Cs ScVe[f,j]	
Dibucaine[c]	Ts ScVe[f]	
Lidocaine[c]	Aa ScVe[f]	
RAC109 I[c]	PbTx-3[e]	
RAC109 II[c]	Benzamil[d]	
Tetracaine[c]		

B. Compounds that potentiate specific [^{3}H]phenytoin binding

Anticonvulsants	Ca^{2+} channel ligands
Diazepam[a]	Flunarizine[d]

C. Compounds that do not affect specific [^{3}H]phenytoin binding

Na^+ channel toxins	Anticonvulsants	Miscellaneous
STX[e]	Valproate[h]	Adrenaline[d]
TTX[e]	Ethosuximide[d]	Noradrenaline[d]
Lqq ScVe[g]	Antiarrhythmics	Serotonin[d]
Ca^{2+} channel ligands	Amiodarone[d]	Dopamine[d]
Nifedipine[d]	Lorcainide[d]	Carbachol[d]
Nitrendipine[d]	Bretylium[d]	Nicotine[d]
(±)-BAYK8644[d]	Disopyramide[d]	Histamine[d]
K^+ channel ligands	Propranolol[d]	Adenosine[d]
TEA[h]	Procainamide[d]	GABA[d]
4-AP[h]	Local anesthetics	Glutamate[d]
Apamin[i]	Procaine[c]	Aspartate[d]
		Glycine[d]
		Kainic acid[d]
		Strychnine[d]
		NMDA[d]
		Morphine[d]
		Ouabain[d]
		DDT[d]

[a–j] Ligand concentrations used were as follows: *a*, 50 μM; *b*, 300 μM; *c*, 2 mM; *d*, 100 μM; *e*, 1 μM; *f*, 1 mg/ml; *g*, 25 μg/ml; *h*, 1 mM; *i*, 5 μM.

[j] Cs, *Centruroides sculpturatus*; Ts, *Tityus serrulatus*; Aa, *Androctonus australis*; ScVe, scorpion venom; PbTx-3, *Ptychodiscus brevis* toxin 3; STX, saxitoxin; TTX, tetrodotoxin; TEA, tetraethylammonium chloride; DDT, 1,1-bis(*p*-chlorophenyl)-2,2,2-trichloroethane; GABA, γ-amino-*n*-butyric acid; NMDA, *N*-methyl-D-aspartate; 4-AP, 4-aminopyridine.

MO) or Research Biochemicals (Natick, MA) and were of the highest available grade.

Results

[^{3}H]Phenytoin binds to a saturable site at 22°. Initial experiments were designed to characterize [^{3}H]phenytoin binding at 22°, a temperature favorable to the binding of sodium channel ligands (26). Fig. 1 presents a saturation isotherm, with Scatchard (30) transformation, for specific [^{3}H]phenytoin binding at 4° and 22° to synaptoneurosomes prepared from rat whole neocortex. At 22°, nonlinear regression analysis (29) indicates the existence of a single population of binding sites with an equilibrium dissociation constant (K_d) of 1.5 μM and a maximal density (B_{max}) of 4.5 pmol/mg of protein. At 4°, [^{3}H] phenytoin binds with a K_d of 5.5 μM and a B_{max} of 10 pmol/mg of protein. These latter data approximate the results of previous experiments conducted at 0–4° (21). Thus, at 22° [^{3}H]phenytoin continues to bind to a specific and saturable site with a K_d that falls within the therapeutic concentration range of the drug.

The [^{3}H]phenytoin site is a domain on the voltage-dependent sodium channel. The effects of a wide variety of ligands on [^{3}H]phenytoin binding at 22° were subsequently investigated in competition experiments involving single high doses of each compound. As indicated by Table 1, most of the drugs thought to interact with the sodium channel (31) displayed competition for the [^{3}H]phenytoin site. These included the local anesthetics, the sodium channel-active antiarrhythmics (class 1) (32, 33), and channel-specific toxins and venoms (18, 31, 34). Antiarrhythmics with actions unrelated to the sodium channel and channel toxins related to site 1 (tetrodotoxin and saxitoxin) and site 3 (Lqq scorpion toxin) did not compete. Two compounds, diazepam and flunarizine, potentiated specific [^{3}H]phenytoin binding. A variety of other drugs and ligands, including ligands for the other voltage-dependent ion channels, had no effect. These data strongly suggest that the [^{3}H]phenytoin binding domain is a site on the voltage-dependent sodium channel.

[^{3}H]Phenytoin binding is depolarization sensitive. Electrophysiological experiments have shown that the actions of phenytoin on sodium channels are voltage dependent (4–13). We, therefore, studied the effects of KCl-induced depolarization in our [^{3}H]phenytoin binding assay. The conditions used for depolarization (see Materials and Methods) have previously been shown to affect activator toxin binding to the sodium channel (19, 26). In synaptoneurosomal membranes there was no significant effect of depolarization on specific [^{3}H]phenytoin binding (Fig. 2A). In contrast, in SK-N-MC human neuroblastoma cells (Fig. 2B) voltage sensitivity was observed. Control [^{3}H]phenytoin binding in cells was monophasic and displayed a lower affinity than that seen in synaptoneurosomes (the calculated K_d in cells was 20 μM and the B_{max} was approximately 5 pmol/10^6 cells). Depolarization resulted in a conversion of the monophasic control [^{3}H]phenytoin binding curve to a biphasic curve. The higher affinity site had a K_d of 700 nM and a B_{max} of approximately 19 fmol/10^6 cells, whereas the lower affinity site had a K_d of 30 μM and a B_{max} of approximately 22 pmol/10^6 cells. Thus, [^{3}H]phenytoin binding appears to be depolarization sensitive.

Site 2 ligands interact with the [^{3}H]phenytoin binding site. The effects of site 2 ligands, and particularly BTX, on specific [^{3}H]phenytoin binding were subsequently investigated in more detail (Fig. 3). Allosteric interactions between phenytoin and the prototypical site 2 ligand [^{3}H]BTX-B (35) have been reported in past studies (19). Calculated equilibrium inhibition constants, K_i (36), for three site 2 ligands were 2.5 μM for BTX, 5.5 μM for aconitine, and 104 μM for veratridine. This rank order of inhibition is similar to that previously reported for the inhibition of the binding of [^{3}H]BTX-B (26), i.e., BTX > aconitine > veratridine. BTX competition with [^{3}H]phenytoin was subsequently found to be temperature dependent. BTX was an effective competitor at 22° but was ineffective at 4° (Fig. 4). As suggested by previous toxin studies (19, 33), the phenytoin binding site appears to be distinct from the BTX binding site. Scatchard analysis of [^{3}H]phenytoin binding in synaptoneurosomes in the presence of 1 μM unlabeled BTX showed a decrease in both K_d and B_{max}, compared with control (Fig. 5), indicating that the interaction between the BTX site and the [^{3}H]phenytoin site was allosteric in nature. Also, although the order of potency is similar for the two sites, the K_i values of the alkaloid toxins are up to 50-fold higher in com-

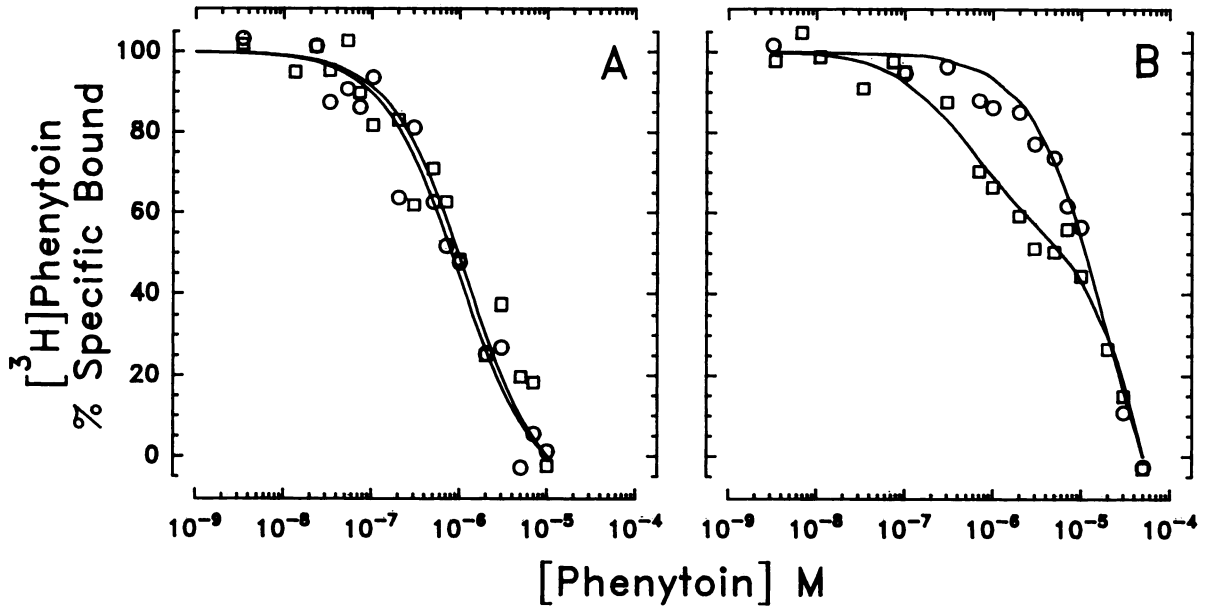

Fig. 2. Effects of KCl-induced depolarization on specific [^{3}H]phenytoin binding at 22°. A, Binding in neocortical synaptoneurosomes. ○, Control; □, depolarized. These data are the means of five experiments. Fitted curves represent the best fit of these data to a model describing a single population of binding sites. B, Binding in SK-N-MC neuroblastoma cells. ○, Control. These data are the means of four experiments. The fitted curve represents the best fit of these data to a model describing a single population of binding sites. □, Depolarized. These data are the means of six experiments. The fitted curve represents the best fit of these data to a model describing two significantly different ($p < 0.05$) populations of binding sites. In all cases, experiments were conducted in triplicate.

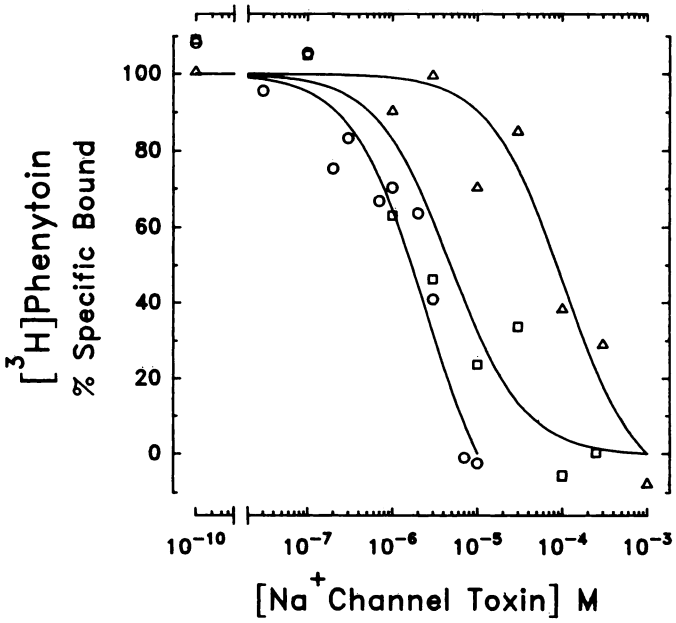

Fig. 3. Inhibition of specific [^{3}H]phenytoin binding to rat brain neocortical synaptoneurosomes by alkaloid sodium channel neurotoxins. Competition curves represent BTX (○), aconitine (□), and veratridine (△) and are each the means of six experiments, each conducted in triplicate. *Point on the abscissa labeled* 10^{-10}, total radioligand binding (i.e., no competitor). The fitted curves represent the best fit of these data to a model describing a single population of binding sites.

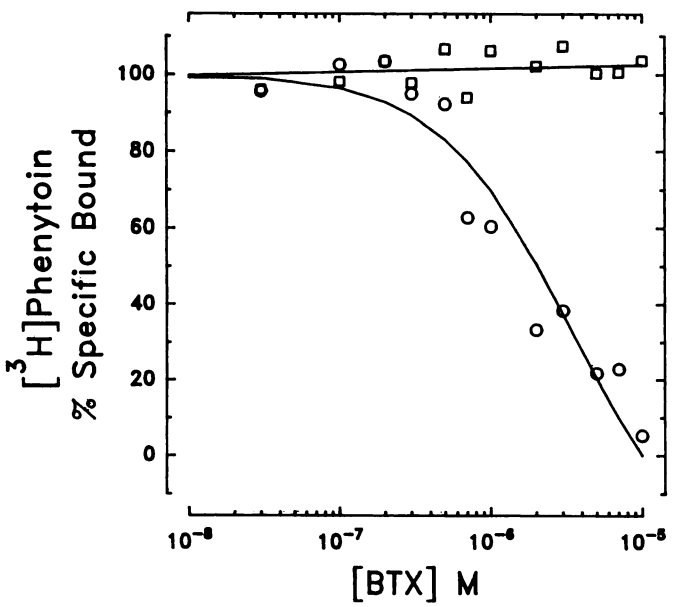

Fig. 4. Effects of incubation temperature on BTX competition for [^{3}H] phenytoin binding sites. Curves represent BTX competition at 4° (□) and at 22° (○). These data are the means of four experiments, each conducted in triplicate. The fitted curve for BTX competition at 22° represents the best fit of these data to a model describing a single population of binding sites.

petition with [^{3}H]phenytoin than in competition with [^{3}H] BTX-B (Fig. 3; see Ref. 26).

Clinically relevant anticonvulsants inhibit specific [^{3}H]phenytoin binding. Past experiments, conducted at 0–4°, indicated that the clinically effective hydantoin anticonvulsants inhibited the binding of [^{3}H]phenytoin with a rank order appropriate to their therapeutic actions (21). Competition by hydantoins was also seen under the present assay conditions (Table 1), and a similar rank order was observed, i.e., phenytoin > mephenytoin > ethotoin (Fig. 6). Scatchard analysis, however, indicated that this competition, like the competition observed with alkaloid channel ligands, was allosteric in nature and also that it occurred only at high concentrations (Fig. 5). Approximate K_i values were 300 μM for mephenytoin and 350

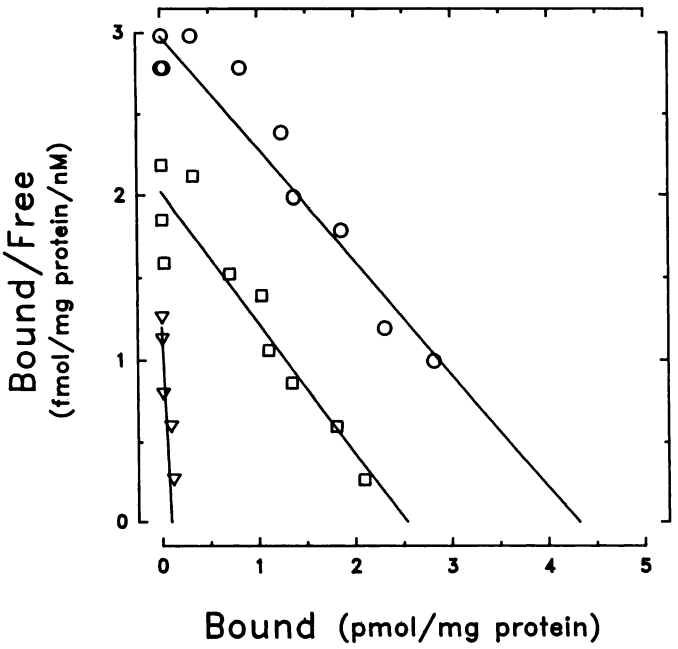

Fig. 5. Allosteric modulation of specific [³H]phenytoin binding to rat brain neocortical synaptoneurosomes by anticonvulsants and neurotoxins. These data are the means of three to six experiments, each conducted in triplicate, and represent Scatchard transformations of the mean data and fitted curves from the corresponding saturation experiments. The fitted curves represent the best fit of these data to a model describing a single population of binding sites. ○, Control [³H]phenytoin binding. ▽, Specific [³H]phenytoin binding in the presence of 1 μM BTX. □, Specific [³H]phenytoin binding in the presence of 300 μM mephenytoin.

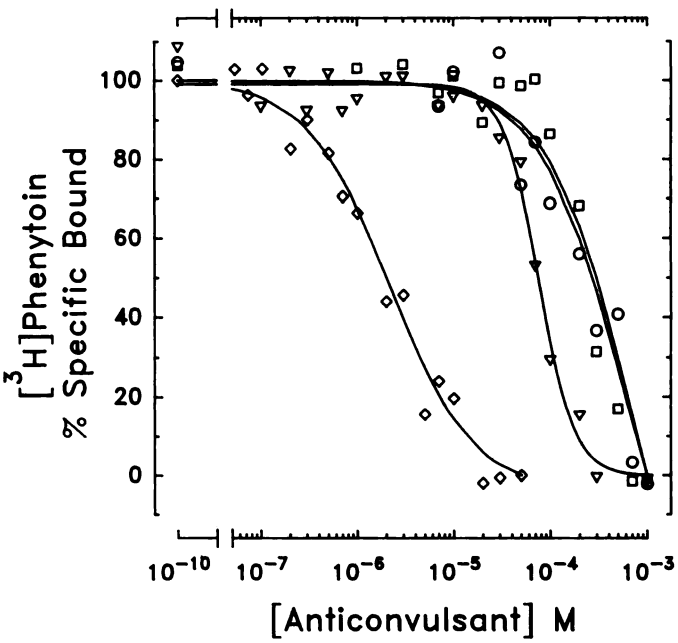

Fig. 6. Inhibition of specific [³H]phenytoin binding to rat brain neocortical synaptoneurosomes by anticonvulsant drugs. Competition curves represent phenytoin (◇), carbamazepine (▽), mephenytoin (○), and ethotoin (□) and are each the means of six to eight separate experiments, conducted in triplicate. *Point on the abscissa labeled* 10^{-10}, total binding (i.e., no competitor). The fitted curves represent the best fit of these data to a model describing a single population of binding sites. The phenytoin curve is included for comparative purposes. The calculated K_i for phenytoin is 2 μM, which is similar to the K_d value of 1.5 μM obtained in Fig. 1.

μM for ethotoin. Similar inhibition was observed with carbamazepine, an anticonvulsant iminostilbene (Fig. 6). This action of carbamazepine was not previously seen at 0–4° (21). The approximate K_i for carbamazepine was 80 μM. These data suggest that mephenytoin, ethotoin, and carbamazepine bind to the voltage-dependent sodium channel but that they bind at sites distinct from the [³H]phenytoin site.

Discussion

[³H]Phenytoin binds to rat brain voltage-dependent sodium channels. It has been proposed that phenytoin limits sustained repetitive neuronal firing by inhibiting voltage-dependent sodium flux (4–13). The interactions of phenytoin with known toxin binding sites on the sodium channel, however, occur well above the therapeutic concentration range of 1–10 μM (1, 10, 14–17). The present study was designed to determine whether the [³H]phenytoin binding site, with a reported K_d of 5 μM (21), might represent a novel binding domain on the voltage-dependent sodium channel.

Working at 22°, we found that a number of ligands that specifically bind to the sodium channel inhibited [³H]phenytoin binding, whereas a variety of other ligands and toxins had no effect. These data strongly suggest that the [³H]phenytoin binding site lies on the voltage-dependent sodium channel. This localization is consistent with the high maximal density found for the [³H]phenytoin site (4.5 pmol/mg of protein), a density that falls within the range reported for sodium channel ligands (26), and also with previous electrophysiological and toxin studies that have suggested an interaction of phenytoin with the voltage-dependent sodium channel (4–13, 19, 20, 33). The effects of the calcium channel ligands verapamil, diltiazem, and flunarizine likely reflect direct high dose actions of these compounds at sodium channels rather than L-type calcium channels. The potentiating actions of diazepam have been described previously (22, 23, 25, 37) and may also reflect an allosteric action at the sodium channel.

Specific [³H]phenytoin binding was found to be sensitive to tonic KCl-induced depolarization, with the appearance of higher affinity binding in intact cells. The electrophysiological actions of phenytoin in intact cell preparations are enhanced by depolarization (7–9, 12). However, phenytoin inhibition of [³H]BTX-B binding to sodium channels in synaptosomes was not significantly affected by depolarization (19). This latter finding could be due to the fact that homogenates are partially depolarized initially and that under these conditions phenytoin binding would not be affected by further depolarization. The neuroblastoma cells used in the present studies retain a resting membrane potential closer to that found in intact neurons. Thus, the effects of depolarization on [³H]phenytoin binding that are not seen in homogenates (Fig. 2A) are revealed in the depolarized cell preparation (Fig. 2B).

Scatchard analyses showed that the interactions of phenytoin and the site 2 ligands were allosteric in nature, indicating that the [³H]phenytoin site is a separate domain, distinct from the [³H]BTX-B site. This was confirmed by the finding that the K_i values of site 2 toxins were much higher against [³H]phenytoin binding than against [³H]BTX-B binding. Studies of the effects of phenytoin on [³H]BTX-B binding have similarly reported allosteric interactions (19, 20, 33). Investigations related to the nature of the site 4 β-scorpion venoms and

brevetoxin interactions with the [^{3}H]phenytoin site are currently underway.

What is the relation of binding to the pharmacological activity of phenytoin? Electrophysiological and toxin-binding studies have suggested that phenytoin limits sustained repetitive neuronal firing by stabilizing the voltage-dependent sodium channel in its inactivated state (6, 14). This action should have little effect on normal rates of neuronal firing but should limit firing in the high frequency range that occurs during seizures. Our present findings now indicate that the [^{3}H]phenytoin site, which has a K_d in the therapeutic concentration range of the drug, is a site on the voltage-dependent sodium channel. We, therefore, postulate that phenytoin exerts its therapeutic actions by binding to the [^{3}H]phenytoin site and that this binding serves to increase the amount of time the channel spends in its inactivated state. This, therefore, mediates the effects of phenytoin on repetitive firing, which also occur at therapeutic drug concentrations (14).

Phenytoin has been previously described as an allosteric inhibitor of specific [^{3}H]BTX-B binding (19) and of neurotoxin-activated sodium flux (20, 33). The results presented here show that BTX is an allosteric inhibitor of specific [^{3}H]phenytoin binding (Fig. 5). Competition between phenytoin and BTX can be interpreted in terms of an allosteric model for drug binding to selected states of the voltage-dependent sodium channel. BTX (activated state) and phenytoin (inactivated state) bind to and stabilize opposing states of the sodium channel. By considering BTX and phenytoin as "allosteric competitive inhibitors" (19, 20, 33, 38), these ligands can be seen to "compete" for binding to voltage-dependent sodium channels by stabilizing a conformation of the protein that precludes the other ligand from binding. Phenytoin binds to sodium channels and inhibits tracer levels of BTX binding (10 nM [^{3}H]BTX-B, in the presence of the allosteric enhancer Lqq scorpion toxin) (19) at concentrations well above the phenytoin free plasma therapeutic range of 1–10 μM (IC_{50} = 40 μM) (19). Phenytoin binds to sodium channels, in the absence of BTX or Lqq scorpion toxin, with an affinity well within the free plasma therapeutic range (K_d = 1.5 μM; see Fig. 1). BTX inhibits specific [^{3}H]phenytoin binding with a K_i concentration of 2.5 μM (Fig. 3). This is similar to the reported value for the interaction of [^{3}H]BTX-B with sodium channels (K_d = 0.7 μM) (35) in the absence of Lqq scorpion toxin. In Fig. 5, the presence of BTX (in the absence of any Lqq scorpion toxin) at a concentration of 1 μM eliminates a large number of specific [^{3}H]phenytoin binding sites. Taken together, the results presented in this report, and in previous reports, indicate that BTX is more efficient at binding to, and stabilizing, the activated state of the sodium channel than phenytoin is at stabilizing the inactivated state. These effects, however, are temperature dependent, because lower temperatures abolish BTX competition for [^{3}H]phenytoin sites (Fig. 4). The presence of scorpion toxin in some of the BTX assays described above does not detract from this interpretation, because the concentrations of scorpion toxin used do not significantly affect the interactions of phenytoin with sodium channels (Table 1).[1]

Other anticonvulsants also interact with the voltage-dependent sodium channel. In addition to the sodium channel ligands, it was found that the clinically used anticonvulsants mephenytoin, ethotoin, and carbamazepine inhibited [^{3}H]phenytoin binding. Mephenytoin and ethotoin are hydantoins, structurally related to phenytoin (1), whereas carbamazepine is an iminostilbene with a different chemical structure, although similar clinical profile (1). All of these anticonvulsant interactions, like the previously defined interactions of sodium channel toxins, proved to be allosteric in nature. In agreement with past electrophysiological and [^{3}H]BTX-B binding studies (4–13, 19, 20, 31, 33), these data suggest that a variety of clinically used anticonvulsants may interact with the voltage-dependent sodium channel. Just as there are a number of pharmacologically relevant toxin-binding sites on the channel (18), which interact allosterically and affect the stability of the activated state of the channel (39), so there may be a number of pharmacologically relevant anticonvulsant binding sites, which interact allosterically and affect the stability of the inactivated state of the channel. As demonstrated here with phenytoin, radiolabeled derivatives of these other anticonvulsant drugs would permit a direct measurement of their interactions with sodium channels. Experiments designed to test this are currently in progress.

[1] J. Francis and W. B. Burnham, unpublished observations.

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References

1. Rall, T. W., and L. S. Schleifer. Drugs effective in the therapy of the epilepsies, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (A. G. Gilman, L. S. Goodman, T. W. Rall, and F. Murad, eds.), Ed. 7. Macmillan Publishing Company, New York, 446–472 (1985).
2. Tallarida, R. J. *Most Prescribed Drugs (MPD) 1985.* W. B. Saunders Company, Philadelphia, PA (1985).
3. Canadian Compuscript June, 1991. IMS America, Plymouth Meeting, PA (1991).
4. Schwarz, J. R., and W. Vogel. Diphenylhydantoin: excitability reducing action in single myelinated nerve fibres. *Eur. J. Pharmacol.* **44**:241–249 (1977).
5. Macdonald, R. L., and M. J. McLean. Cellular bases of barbiturate and phenytoin anticonvulsant drug action. *Epilepsia* **23** (Suppl. 1):S7–S18 (1982).
6. Courtney, K. R., and E. F. Etter. Modulated anticonvulsant block of sodium channels in nerve and muscle. *Eur. J. Pharmacol.* **88**:1–9 (1983).
7. McLean, M. J., and R. L. Macdonald. Multiple actions of phenytoin on mouse spinal cord neurons in cell culture. *J. Pharmacol. Exp. Ther.* **227**:779–789 (1983).
8. Matsuki, N., F. N. Quandt, R. E. Ten Eick, and J. Z. Yeh. Characterization of the block of sodium channels by phenytoin in mouse neuroblastoma cells. *J. Pharmacol. Exp. Ther.* **228**:523–530 (1984).
9. Willow, M., T. Gonoi, and W. A. Catterall. Voltage clamp analysis of the inhibitory actions of diphenylhydantoin and carbamazepine on voltage-sensitive sodium channels in neuroblastoma cells. *Mol. Pharmacol.* **27**:549–558 (1985).
10. Yaari, Y., M. E. Selzer, and J. H. Pincus. Phenytoin: mechanisms of its anticonvulsant actions. *Ann. Neurol.* **20**:171–184 (1986).
11. Worley, P. F., and J. M. Baraban. Site of anticonvulsant actions on sodium channels: autoradiographic and electrophysiological studies in rat brain. *Proc. Natl. Acad. Sci. USA* **84**:3051–3055 (1987).
12. Quandt, F. N. Modification of slow inactivation of sodium channels by phenytoin in neuroblastoma cells. *Mol. Pharmacol.* **34**:557–565 (1988).
13. Schwarz, J. R., and G. Grigat. Phenytoin and carbamazepine: potential- and frequency-dependent block of Na currents in mammalian myelinated nerve fibres. *Epilepsia* **30**:286–294 (1989).
14. Macdonald, R. L., and M. J. McLean. Anticonvulsant drugs: mechanisms of action. *Adv. Neurol.* **44**:713–735 (1986).
15. Kilpatrick, C. J., S. Wanwimolruk, and L. M. H. Wing. Plasma concentrations of unbound phenytoin in the management of epilepsy. *Br. J. Clin. Pharmacol.* **17**:539–546 (1984).
16. Rimmer, E. M., D. C. Buss, P. A. Routledge, and A. Richens. Should we routinely measure free plasma phenytoin concentrations? *Br. J. Clin. Pharmacol.* **17**:99–102 (1984).
17. Brodie, M. J., S. E. Muir, E. Agnew, G. J. A. Macphee, G. Volo, E. Teasdale, and P. Macpherson. Protein binding and CSF penetration of phenytoin following acute oral dosing in man. *Br. J. Clin. Pharmacol.* **19**:161–168 (1985).

18. Catterall, W. A. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annu. Rev. Pharmacol. Toxicol.* **20**:15–43 (1980).
19. Willow, M., and W. A. Catterall. Inhibition of binding of [^{3}H]batrachotoxinin A 20-α-benzoate to sodium channels by the anticonvulsant drugs diphenylhydantoin and carbamazepine. *Mol. Pharmacol.* **22**:627–635 (1982).
20. Willow, M., E. A. Kuenzel, and W. A. Catterall. Inhibition of voltage-sensitive sodium channels in neuroblastoma cells and synaptosomes by the anticonvulsant drugs diphenylhydantoin and carbamazepine. *Mol. Pharmacol.* **25**:228–234 (1983).
21. Burnham, W. M., L. Spero, M. M. Okazaki, and B. K. Madras. Saturable binding of ^{3}H-phenytoin in rat brain membrane fraction. *Can. J. Physiol. Pharmacol.* **59**:402–407 (1981).
22. Shah, D. S., P. Chambon, and A. Guidotti. Binding of [^{3}H]-5,5-diphenylhydantoin to rat brain membranes. *Neuropharmacology* **20**:1115–1119 (1981).
23. Spero, L. Modulation of specific ^{3}H-phenytoin binding by benzodiazepines. *Neurosci. Lett.* **10**:755–765 (1985).
24. Spero, L. Specific ^{3}H-phenytoin binding in human brain. *Can. J. Physiol. Pharmacol.* **63**:515–516 (1985).
25. Wong, P. T., and W. L. Teo. Diazepam-sensitive specific binding of phenytoin in rat brain. *J. Recept. Res.* **6**:297–309 (1986).
26. Catterall, W. A., C. S. Morrow, J. W. Daly, and G. B. Brown. Binding of batrachotoxinin A 20-α-benzoate to a receptor site associated with sodium channels in synaptic nerve ending particles. *J. Biol. Chem.* **256**:8922–8927 (1981).
27. Brown, G. B. ^{3}H-Batrachotoxinin-A benzoate binding to voltage-sensitive sodium channels: inhibition by the channel blockers tetrodotoxin and saxitoxin. *J. Neurosci.* **6**:2064–2070 (1986).
28. Hollingsworth, E. B., E. T. McNeal, J. L. Burton, R. J. Williams, J. W. Daly, and C. R. Creveling. Biochemical characterization of a filtered synaptoneurosome preparation from guinea pig cerebral cortex: cyclic adenosine 3′:5′-monophosphate-generating systems, receptors, and enzymes. *J. Neurosci.* **5**:2240–2253 (1985).
29. Munson, P. J., and D. Rodbard. LIGAND: a versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
30. Scatchard, G. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**:660–672 (1949).
31. Catterall, W. A. Common modes of drug action on Na^+ channels: local anesthetics, antiarrhythmics and anticonvulsants. *Trends Pharmacol. Sci.* **8**:57–65 (1987).
32. Bigger, J. T., Jr., and B. F. Hoffman. Antiarrhythmic drugs, in *Goodman and Gilman's The Pharmacological Basis of Therapeutics* (A. G. Gilman, L. S. Goodman, T. W. Rall, and F. Murad, eds.), Ed. 7. Macmillan Publishing Company, New York, 748–783 (1985).
33. Catterall, W. A. Inhibition of voltage-sensitive sodium channels in neuroblastoma cells by antiarrhythmic drugs. *Mol. Pharmacol.* **20**:356–362 (1981).
34. Poli, M. A., T. J. Mende, and D. G. Baden. Brevetoxins, unique activators of voltage-sensitive sodium channels, bind to specific sites in rat brain synaptosomes. *Mol. Pharmacol.* **30**:129–135 (1986).
35. Brown, G. B., S. C. Tieszen, J. W. Daly, J. E. Warnick, and E. X. Albuquerque. Batrachotoxinin-A 20-α-benzoate: a new radioactive ligand for voltage sensitive sodium channels. *Cell. Mol. Neurobiol.* **1**:19–40 (1981).
36. Cheng, Y. C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
37. Okazaki, M. M., B. K. Madras, K. E. Livingston, L. Spero, and W. M. Burnham. Enhancement of ^{3}H-phenytoin binding by diazepam and (+)bicuculline. *Life Sci.* **33**:409–414 (1983).
38. Catterall, W. A. Activation of the action potential Na^+ ionophore by neurotoxins: an allosteric model. *J. Biol. Chem.* **252**:8669–8676 (1977).
39. Brown, G. B. Batrachotoxin: a window on the allosteric nature of the voltage-sensitive sodium channel. *Int. Rev. Neurobiol.* **29**:77–116 (1988).
40. Francis, J., L. Spero, and W. M. Burnham. Identification of a hydrophobic domain on voltage-gated sodium channels labelled by [^{3}H]phenytoin. *Soc. Neurosci. Abstr.* **17**:173 (1991).
41. Francis, J., W. M. Burnham, and P. A. Hwang. Identification of a benzodiazepine-sensitive phenytoin binding site on voltage-dependent sodium channels. *Neurology* **42(Suppl. 3)**:363 (1992).

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