Site of anticonvulsant action on sodium channels: Autoradiographic and electrophysiological studies in rat brain

(batrachotoxin/phenytoin/carbamazepine/veratridine/hippocampal slice)

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ABSTRACT The anticonvulsants phenytoin and carbamazepine interact allosterically with the batrachotoxin binding site of sodium channels. In the present study, we demonstrate an autoradiographic technique to localize the batrachotoxin binding site on sodium channels in rat brain using [³H]batrachotoxinin-A 20- α -benzoate (BTX-B). Binding of [³H]BTX-B to brain sections is dependent on potentiating allosteric interactions with scorpion venom and is displaced by BTX-B ($K_d \approx 200$ nM), aconitine, veratridine, and phenytoin with the same rank order of potencies as described in brain synaptosomes. The maximum number of [³H]BTX-B binding sites in forebrain sections (≈ 1 pmol/mg of protein) also agrees with biochemical determinations. Autoradiographic localizations indicate that [³H]BTX-B binding sites are not restricted to cell bodies and axons but are present in synaptic zones throughout the brain. For example, a particularly dense concentration of these sites in the substantia nigra is associated with afferent terminals of the striatonigral projection. By contrast, myelinated structures possess much lower densities of binding sites. In addition, we present electrophysiological evidence that synaptic transmission, as opposed to axonal conduction, is preferentially sensitive to the action of aconitine and veratridine. Finally, the synaptic block produced by these sodium channel activators is inhibited by phenytoin and carbamazepine at therapeutic anticonvulsant concentrations. Thus, these anticonvulsants may limit seizure spread not only by affecting all-or-none conduction by axonal sodium channels but also by modulating graded aspects of synaptic transmission.

Recent studies indicate that the anticonvulsants phenytoin and carbamazepine interact directly with sodium channels. Using radioligand binding techniques, Catterall and associates have found an allosteric interaction between these drugs and the batrachotoxin (BTX) binding site of sodium channels from rat brain (1, 2). Phenytoin and carbamazepine block the influx of sodium into rat brain synaptosomes elicited by BTX, which activates sodium channels (3). Electrophysiological studies of neuroblastoma cells demonstrate a frequency- and voltage-dependent blockade of sodium currents by both of these anticonvulsants (4, 5). These findings suggest that blockade of sodium channel activity by these agents underlies their anticonvulsant actions.

A growing body of evidence has also focused attention on heterogeneity among sodium channels. Sodium channels from brain and skeletal muscle possess markedly different sensitivity to crotoxin and geophyllotoxin (6, 7), and antisera against the sodium channel purified from rat brain crossreact poorly with sodium channels from peripheral nerve or skeletal muscle (8). Distinguishable populations of sodium currents have also been found in invertebrate and peripheral nerve preparations (9, 10). The presence of distinct

sodium currents in several types of mammalian brain neurons (11–15) and the identification of three distinct sodium channel messenger RNAs from rat brain (16) underscore the heterogeneity among sodium channels. Accordingly, in assessing the mechanism of action of anticonvulsants, it is desirable to investigate their interaction with brain sodium channels. Since anticonvulsants allosterically affect the BTX binding site, we have conducted autoradiographic studies using [³H]batrachotoxinin-A 20- α -benzoate (BTX-B) to characterize the distribution of these sites in brain and find evidence of dense synaptic localizations. Furthermore, electrophysiological experiments using the hippocampal slice preparation indicate prominent actions of sodium channel activators on synaptic transmission and inhibition of these actions by phenytoin and carbamazepine at therapeutic anticonvulsant concentrations.

MATERIALS AND METHODS

[³H]BTX-B Autoradiography. Rat brain sections (10 μ m) were prepared as described (17). Sections were labeled with [³H]BTX-B (New England Nuclear; 50 Ci/mmol; 1 Ci = 37 GBq) by incubating them at 37°C in buffer A (50 mM Tris-HCl, pH 7.7/100 mM KCl) containing 0.5 mg of scorpion venom (*Leiurus quinquestratus*, Sigma) per ml and 50 nM [³H]BTX-B. Nonspecific binding was assessed by adding 10 μ M BTX-B (gift from J. W. Daly). To conserve on [³H]BTX-B and scorpion venom, sections were incubated horizontally and covered with 100 μ l of labeling solution per section. Coverslips were used to slow evaporation. Sections were then washed in buffer A at 4°C. In initial studies, forebrain sections (two per slide) were wiped from the slides with glass fiber filters (GF/C, Whatman) and assayed by scintillation spectroscopy. The pharmacologic specificity of [³H]BTX-B binding was assessed by adding veratridine (Sigma), aconitine (Sigma), or phenytoin (Elkins-Sinn, Cherry Hill, NJ) to the incubation solution. Autoradiograms were prepared by exposing LKB Ultrofilm to labeled brain sections for 4–5 weeks at 4°C. Quantitative analysis of autoradiograms was performed with a computerized microdensitometer (Loats Associates, Westminster, MD). Excitotoxin lesions of the striatum, substantia nigra, and cerebellum were performed as described (17) and animals were sacrificed after 5 days for autoradiographic studies.

Electrophysiological Recordings from Hippocampal Slices. Slices were obtained from male Sprague-Dawley rats using techniques that have been described in detail (18, 19). One 400- μ m-thick slice was held submerged in the recording chamber at 30°C. Temperature was regulated by a heating-cooling module (Cambion, Cambridge, MA) and was monitored within 1 mm of a slice by a hypodermic thermistor

FIG. 1. Association (Lower) and wash (Upper) curves for [³H]BTX-B binding to rat brain sections. Forebrain sections were incubated in the presence of 50 nM [³H]BTX at 37°C and tissue binding was determined by scintillation spectroscopy. Nonspecific binding was determined in the presence of 10 μ M BTX-B or 500 μ M veratridine.

probe. Other slices were maintained in an incubation chamber at room temperature. The standard physiological saline was saturated with 95% O₂/5% CO₂ and consisted of (in mM) NaCl, 130; KCl, 5; CaCl₂, 2.5; MgSO₄, 1.5; NaH₂PO₄, 1.25; NaHCO₃, 24; and glucose, 10. The recording chamber provides constant perfusion and allows for drug application by switching between salines by means of a valve.

Field potential recordings were made from the CA1 pyramidal cell layer with fiber-filled glass microelectrodes that contained 2 M NaCl and had impedances of 5–15 M Ω at 135

Table 1. Pharmacology of [³H]BTX-B binding to brain sections

Drug	Concentration, μ M	% inhibition of [³ H]BTX-B binding
BTX-B	1	85
	0.5	60
	0.1	20
Veratridine	500	100
	100	80
	10	40
Aconitine	100	100
	10	80
	240	80
Phenytoin	80	50

Brain sections were incubated in buffer containing 50 nM [³H]BTX-B, 0.5 mg of scorpion venom per ml, and competing ligand. Binding was determined either by microdensitometry of autoradiograms (BTX-B data) or by scintillation spectroscopy of brain section wipes. Nonspecific binding was defined as the radioactivity remaining in the presence of 10 μ M BTX-B and was typically 20% of total binding. Values reported are the mean, rounded to the nearest multiple of five, of two or more experiments that varied by <15% from the mean.

Hz. Field potentials were elicited routinely at 10- to 90-sec intervals by 50- μ sec pulses from a bipolar stimulating electrode located in the stratum radiatum near the junction of CA1 and CA3. Stimulation voltage was routinely adjusted to a level just below that producing maximal population spike amplitude. Data were collected by a Nicolet 2090 digital oscilloscope and recorded on a Gould 60000 X-Y recorder. Carbamazepine analogues were obtained from CIBA-Geigy.

Measurement of [³H]BTX-B Binding. [³H]BTX-B binding to rat brain was measured by using a modification of published techniques (2, 20). Fresh Sprague-Dawley rat forebrains were homogenized in 10 vol of ice-cold 0.32 M sucrose by 10 strokes with a motordriven Teflon-on-glass pestle (Wheaton, Millville, NJ). The homogenate was centrifuged 10 min at 1500 $\times$ g and the supernatant was pelleted at 9000 $\times$ g for 20 min. This pellet (crude synaptosomes) was resuspended in 5

FIG. 2. Autoradiographic localization of [³H]BTX-B binding sites in coronal rat brain sections. Regions shown as white are enriched in binding, which is high throughout grey matter areas, including neocortex (N), globus (G) and ventral pallidum (V), thalamus (T), hypothalamus (Hy), hippocampus (H), central grey (C), substantia nigra (S), and molecular layer of the cerebellum (M). Regions with dense neuronal layers such as the stratum pyramidale of the hippocampus or granule cell layer of the cerebellum (GC) are not enriched in binding sites. Myelinated structures such as the corpus callosum (CC), anterior commissure (A), and medial lemniscus (ML) display much lower levels of [³H]BTX-B binding.

vol of buffer B (130 mM choline chloride/50 mM Tris-HCl, pH 7.7/5.5 mM glucose/0.8 mM MgCl₂/5.4 mM KCl/1 mg of bovine serum albumin per ml/1 μ M tetrodotoxin) with 3 strokes of a Dounce homogenizer. Binding assays included 150 μ l of crude synaptosomes, 10 μ g of scorpion venom per ml, and 10 nM [³H]BTX-B in a total volume of 250 μ l of buffer B. Assays were incubated 45 min at 37°C, stopped by rapid filtration over polyethyleneimine (0.1%)-soaked glass fiber filters (Schleicher & Schuell), and washed three times with 4 ml of 163 mM choline chloride/5 mM Tris-HCl, pH 7.7/1 mg of bovine serum albumin per ml. Radioactivity remaining on the filters was assayed by scintillation spectroscopy. Non-specific binding, determined in the presence of 20 μ M BTX-B, was typically 25% of total [³H]BTX-B binding. For competition binding assays, stock solutions of drugs were prepared in dimethyl sulfoxide and control assays included identical volumes of dimethyl sulfoxide (about 1%).

RESULTS

Autoradiographic Localization of [³H]BTX-B Binding Sites in Rat Brain. Since the BTX binding site appears to be closely linked to the anticonvulsant actions of phenytoin and carbamazepine, we sought to study these sites autoradiographically by adapting the methods used to measure [³H]BTX-B binding in homogenates (2, 20). Incubation of brain sections with [³H]BTX-B in the presence of scorpion venom produces saturable binding (Fig. 1) that is displaceable with veratridine, aconitine, or phenytoin (Table 1). Competition binding analysis with unlabeled BTX-B indicates an affinity constant (K_d) of $\approx$ 200 nM and a B_{max} of $\approx$ 1 pmol/mg of protein, which agrees with values reported for binding to brain synaptosomes (2, 20).

The autoradiographic localization of these [³H]BTX-B binding sites (Fig. 2) is heterogeneous with high levels in the globus and ventral pallidum, substantia nigra, molecular layer of cerebellum, thalamus, neocortex, and brain stem central grey. Layers containing densely packed neuronal cell bodies, such as the stratum pyramidale of the hippocampus and granule cell layer of the cerebellum, display fewer sites. Lowest levels of binding are found in white matter tracts, such as the medial lemniscus, corpus callosum, and anterior commissure. [³H]BTX-B binding is displaced uniformly throughout the brain by phenytoin, indicating that various regions do not differ in their sensitivity to anticonvulsants.

To determine the neuronal elements containing [³H]BTX-B binding sites, we performed autoradiography following selective excitotoxin lesions. Unilateral lesions of the caudate with quinolinic acid produce complete loss of [³H]BTX-B binding to the ipsilateral substantia nigra ($n = 3$, Fig. 3),

FIG. 3. Excitotoxin lesion of the striatum markedly reduces [³H]BTX-B binding in the ipsilateral substantia nigra. Horizontal brain sections were prepared from rats following a unilateral striatal lesion with quinolinic acid. (A) [³H]BTX-B autoradiography demonstrating reduced binding in the substantia nigra (Δ) ipsilateral to the lesion (*). (B) Toluidine blue stain of the same section demonstrating tissue symmetry.

FIG. 4. Veratridine reversibly blocks synaptic transmission in the hippocampus. Stimulation of the stratum radiatum elicits extracellular field potentials recorded from the CA1 pyramidal cell layer. The stimulus artifact (Δ) is followed by a fiber spike (*) and population spike (p). Application of 2 μ M veratridine (VTR) for 30 min abolishes the population spike in a reversible manner. The fiber spike is relatively resistant to veratridine. Recovery is observed 90 min later.

indicating that these sites are contained on nerve endings of striatonigral afferents. Conversely, lesions of nigral neurons do not significantly alter [³H]BTX-B binding in the substantia nigra or caudate ($n = 2$). In the cerebellum, kainic acid lesions ($n = 2$) reduce [³H]BTX-B binding only in the immediate vicinity of the injection site, where granule and Purkinje cells are lesioned, and not in the surrounding area, where Purkinje cells are selectively affected (data not shown). Accordingly, the high level of [³H]BTX-B binding sites in the molecular layer of the cerebellum is likely associated predominantly with the densely packed parallel fiber network.

Electrophysiological Recordings. Veratridine and aconitine, sodium channel activators that act at the BTX site of the channel, slowly and reversibly decrease the population spike amplitude recorded in the CA1 pyramidal cell layer of the hippocampus (Fig. 4). Both drugs spare the fiber spike even when the population spike is completely abolished (Figs. 4 and 6), suggesting that conduction in afferent fibers is selectively resistant to these drugs. Blockade of synaptic responses by veratridine or aconitine is accelerated by increased stimulation frequency (Table 2), consistent with their use-dependent action on sodium channel function described in other preparations (21, 22). To examine the interaction of phenytoin and carbamazepine with the BTX site on brain sodium channels, we pretreated slices with these anticonvulsant drugs for 20 min prior to application of aconitine or veratridine. Consistent with previous reports, phenytoin and carbamazepine have little effect on the population spike (23, 24). However, phenytoin and carbamazepine markedly slow the actions of the sodium channel

Table 2. Suppression of population spike by veratridine and aconitine: Dependence on concentration and stimulation interval

Drug	Concentration, μ M	Stimulation interval, sec	Latency, min
Veratridine	1	90	50 $\pm$ 5
	1	5	35 $\pm$ 5
	2	90	30 $\pm$ 2
	2	10	20 $\pm$ 2
	0.1	10	40 $\pm$ 5
Aconitine	0.5	10	20 $\pm$ 2
	1	10	10 $\pm$ 2

CA1 neurons were stimulated orthodromically at intervals varying from 5 to 90 sec in the presence of bath-applied veratridine or aconitine. The time interval following drug application until the population spike is completely abolished is reported as the latency. Data are mean values of at least three experiments $\pm$ SEM.

FIG. 5. Time-dependent reduction of population spike amplitude by veratridine. CA1 hippocampal neurons were stimulated orthodromically every 90 sec and the population spike amplitude was recorded as a function of time in the presence of 2 μ M veratridine (VTR). Thirty micromolar carbamazepine (CBZ) retards the inhibitory action of veratridine on the population spike. Data on each curve are from single experiments.

activators (Figs. 5 and 6). Therapeutic concentrations of other anticonvulsants, diazepam (2 μ M) and phenobarbital (100 μ M), do not affect [3 H]BTX-B binding (1) and do not

FIG. 6. Carbamazepine inhibits the action of veratridine. CA1 hippocampal neurons were stimulated orthodromically every 90 sec. Bath-applied veratridine (VTR, 2 μ M) results in a progressive loss of the population spike, which is completely abolished by 30 min. Carbamazepine (CBZ), but not the structurally related compound CGP 5924, inhibits this action of veratridine. Only the fiber spike (*) remains at 30 min in slices treated with veratridine or veratridine and CGP 5924.

FIG. 7. Dose-dependent inhibition of veratridine by phenytoin (PT), carbamazepine (CBZ), and CGP 5924. CA1 hippocampal neurons were stimulated orthodromically every 90 sec and the effects of 2 μ M veratridine on the population spike were determined at 30 min in the presence of test drugs. Ten micromolar phenytoin or carbamazepine significantly inhibits veratridine, whereas threshold effects of CGP 5924 occur at 50 μ M. Data are mean values of at least three experiments that varied by <10% of the mean.

alter veratridine's action. The effect of carbamazepine is apparent at 10 μ M (2.4 μ g/ml) (Fig. 7) and is maximal at 30 μ M. Similarly, phenytoin's effect is readily detectable at 10 μ M (2.5 μ g/ml).

To further ensure that blockade of veratridine by carbamazepine is related to its interaction with sodium channels, we tested several analogues of carbamazepine (25) for their ability to block veratridine's action. The rank order potencies of these analogues parallel their affinity for the [3 H]BTX-B binding site of the sodium channel (Table 3). For example, CGP 5924, which is inactive in displacing [3 H]BTX-B binding, is weak in the electrophysiological assay, whereas CGP 9055 is more potent than carbamazepine in binding and electrophysiological assays.

DISCUSSION

The major finding of the present study is that the brain sodium channel BTX binding site is enriched in synaptic regions and

Table 3. Comparison of drug effects on [3 H]BTX-B binding and veratridine suppression of CA1 population spike

Drug	% inhibition of [3 H]BTX-B binding by drugs at 125 μ M	Inhibition of veratridine suppression of population spike by drugs at 30 μ M
Phenytoin	75	+++
CGP 8853	60	+++
CGP 9055	58	+++
GP 37375	42	++
CGP 7137	40	++
Carbamazepine	31	++
GP 49023	18	+
CGP 10,000	18	+
CGP 19077	12	+
GP 47779	9	+
CGP 5924	0	-

Phenytoin, carbamazepine, and a series of carbamazepine analogues were screened for inhibition of [3 H]BTX-B binding to crude brain synaptosomes. Data are reported as the % inhibition of binding at a single drug concentration (125 μ M) and are mean values of three experiments that varied by <5% from the mean. In the hippocampal slice preparation, bath application of 2 μ M veratridine abolishes the population spike in 30 min when the stimulation interval is 90 sec. Drugs were bath-applied 20 min prior to addition of 2 μ M veratridine and the population spike amplitude was determined 30 min later. The percentage of the control population spike amplitude at 30 min is reported as +++, >80%; ++, 40–80%; +, 10–40%; -, <10%. Analogues were assayed a minimum of three times with similar results. Phenobarbital (100 μ M) or diazepam (2 μ M) is inactive in the paradigm. Structural formulae of carbamazepine analogues appear in ref. 25.

that phenytoin and carbamazepine exert prominent actions on this site at therapeutic (26) anticonvulsant concentrations. Autoradiographic studies of the [^3H]BTX-B binding sites are validated by their close correspondence to previous biochemical measurements. The rank order of potencies of BTX-B, aconitine, veratridine, and phenytoin at this site and the number of binding sites measured autoradiographically and biochemically are comparable (1, 2, 20). Scorpion venom, which markedly increases the affinity of BTX-B binding sites (2), is required to detect these sites autoradiographically. Finally, the patterns of BTX-B and tetrodotoxin binding sites determined autoradiographically match closely (27). Very high levels of binding are associated with terminals of the striatonigral pathway. This localization of [^3H]BTX-B binding sites fits with the presence of substantial levels of these sites in purified brain synaptosomal preparations (2) and may account for the ability of phenytoin and carbamazepine to affect transmitter release (23, 28).

Electrophysiological experiments conducted in the *in vitro* hippocampal slice preparation demonstrate inhibitory actions of veratridine and aconitine. Furthermore, phenytoin and carbamazepine block the inhibitory action of veratridine at therapeutic anticonvulsant concentrations. The close correlation between the potencies of a series of carbamazepine analogues at the [^3H]BTX-B binding site and their activities in blocking veratridine's action in the hippocampal slice indicates that their protective effect on synaptic transmission is mediated by interactions with the sodium channel. The effectiveness of relatively low concentrations of these anticonvulsants in veratridine-treated hippocampal slices may reflect an enhancement of their potencies with membrane depolarization. Frequency- and depolarization-dependent increases in anticonvulsant potency have been demonstrated and may confer selectivity of these agents for epileptic tissue (3–5, 28). Our findings complement the seminal observations of others who have demonstrated the ability of phenytoin and carbamazepine to block sodium currents in neuroblastoma cells (3–5) and to selectively inhibit high-frequency repetitive firing of sodium action potentials (29, 30). The localization of sodium channels in synaptic zones suggests that anticonvulsants may limit excitability not only by affecting all-or-none high-frequency impulse conduction by axonal sodium channels but also by modulating graded aspects of synaptic transmission.

At present, it is not clear whether the effectiveness of carbamazepine in other clinical syndromes, such as trigeminal neuralgia and bipolar affective illness (31), also stems from its action on sodium channels. We have found that the potencies of several carbamazepine analogues differ markedly at the BTX site. Clinical studies aimed at determining the clinical activity of carbamazepine analogues with varying potency at this site may help clarify its mechanism of action in these syndromes.

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