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A Novel Drug Binding Site on Voltage-Gated Sodium Channels in Rat Brain

Dieter R. Riddall, Michael J. Leach, and John Garthwaite

Wolfson Institute for Biomedical Research, University College London, London United Kingdom (D.R.R., J.G.); and School of Science and School of Pharmacy, University of Greenwich at Medway, Chatham Maritime, United Kingdom (M.J.L.)

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ABSTRACT

The effectiveness of several antiepileptic, analgesic, and neuroprotective drugs is attributable to state-dependent inhibition of voltage-gated sodium channels. To help characterize their site and mode of action on sodium channels, a member of the lamotrigine family, *R*-(−)-2,4-diamino-6-(fluromethyl)-5-(2,3,5-trichlorophenyl)-pyrimidine (BW202W92), was radiolabeled and used as a binding ligand in rat forebrain synaptosomes. Although the level of specific [^{3}H]BW202W92 binding in a standard incubation medium was relatively poor, low concentrations of tetrodotoxin (EC_{50} = 2–3 nM) greatly enhanced the binding, apparently by increasing the affinity of the binding sites. Tetrodotoxin-dependent binding was stereoselective (the less active enantiomer, *S*-(−)-2,4-diamino-6-(fluromethyl)-5-(2,3,5-trichlorophenyl)-pyrimidine (BW203W92), was up to 30-fold less potent, depending on conditions) and was extremely sensitive to inhibition by raised K^+ concentration (IC_{50} = 5.9 mM), an effect that was ascribed to changes in membrane potential. In addition, the binding was inhibited by sodium channel neurotoxins acting on sites 3 and 4, but it was resistant to batrachotoxin (site 2) and brevetoxin (site 5). Several drugs acting on sodium channels displaced tetrodotoxin-dependent [^{3}H]BW202W92 binding, and most of those tested showed different affinities under depolarized (100 mM K^+) and polarized (1 mM K^+) conditions. In a subset of compounds for which data were available, binding affinity in depolarized synaptosomes correlated well with apparent affinity for the inactivated state of sodium channels. The [^{3}H]BW202W92 binding site is novel and is likely to represent a pharmacologically important site of action of drugs on voltage-gated sodium channels in the brain.

Voltage-gated sodium channels are the signature ion channels of excitable cells. The channels are large and complex proteins that open transiently upon membrane depolarization, giving the upstroke of the action potential (Catterall, 2000). A wide range of compounds that modulate the activity of voltage-gated sodium channels show therapeutic utility as local anesthetics, antiarrhythmics, anticonvulsants, and analgesics, and additional compounds are being developed for treatment of neurodegenerative and bipolar disorders (Clare et al., 2000). The molecular mechanism(s) underlying these disparate therapeutic activities remains unclear.

To date, nine isoforms of the pore-forming α-subunits are recognized, and these subunits exist together with smaller auxiliary β-subunits to make up the functional channels (Catterall, 2000). During their normal operation, the channels switch between a variety of states, broadly categorized as open, closed, and inactivated. Thus, the different therapeutic utilities of sodium channel inhibitors may reflect preferential drug binding to the one or more channel isoforms and/or to particular states of the channel that become more prevalent under pathophysiological conditions. In functional terms, the drugs generally stabilize channel inactivation with little or no activity on other states. In this way, they enable normal sodium channel function to continue at negative membrane potentials while dampening activity as the membrane becomes more depolarized, when more channels adopt the inactivated state (Ragsdale et al., 1991; Rogawski and Loscher, 2004).

The traditional pharmacology of voltage-gated sodium channels is built on the six distinct binding sites for a range of naturally occurring toxins, most of whose molecular tar-

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ABBREVIATIONS: [^{3}H]BTX-B, [^{3}H]batrachotoxinin-A 20-α-benzoate; BW202W92, *R*-(−)-2,4-diamino-6-(fluromethyl)-5-(2,3,5-trichlorophenyl)-pyrimidine; BW203W92, (*S*)-(−)-2,4-diamino-6-(fluromethyl)-5-(2,3,5-trichlorophenyl)-pyrimidine; BW4030W92, (*R*)-(−)-2,4-diamino-6-(fluoromethyl)-5-(2,3-dichlorophenyl)-pyrimidine; BW4082W92, (*S*)-(+)-2,4-diamino-6-(fluoromethyl)-5-(2,3-dichlorophenyl)-pyrimidine; BW227C89, 2,4-diamino-6-(methyl)-5-(2,6-dichlorophenyl)-pyrimidine; MK-801, (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5,10-imine maleate; YC-1, 3-(5′-hydroxymethyl-2′-furyl)-1-benzylindazole; PD85,639, α-phenyl-*N*-(3-(2,6-dimethyl-1-piperizinyl)α-propyl)benzeneacetamide; WIN 17317-3; 1-benzyl-7-chloro-4-*n*-propylimino-1,4-dihydroquinoline hydrochloride.

gets have been mapped to differing domains of the α-subunits (Cestele and Catterall, 2000). Site 1 is for tetrodotoxin, saxitoxin, and μ-conotoxin, which inhibit sodium flux through the channel pore. Batrachotoxin and veratridine act on site 2 to stabilize the channel in the open state. Site 3 is the binding site for α-scorpion and sea anemone toxins, which slow inactivation (and potentiate the action of the toxins at site 2). The β-scorpion toxins act on site 4 to shift activation to more negative membrane potentials. Site 5 is for brevetoxins, which also shift activation to more negative membrane potentials (and enhance toxin binding at sites 2 and 4). Finally, the δ-conotoxins bind to site 6 and slow inactivation in a manner similar to that of the α-scorpion toxins.

A common feature of several anticonvulsant and local anesthetic drugs is that they can allosterically affect site 2, and accordingly, the binding of [^{3}H]batrachotoxinin-A 20-α-benzoate ([^{3}H]BTX-B) has often been used as a tool for the discovery of new drugs (Clare et al., 2000). Moreover, mutational analysis has indicated that the drug binding site is located in the inner cavity of the channel pore, close to the batrachotoxin binding site (Linford et al., 1998). As a complementary approach, characterization of the binding sites for drugs themselves should provide a powerful way to help define how and where they act on the channels. Hitherto, however, only limited studies of this type have been carried out with ligands such as [^{3}H]tetracaine (Grima et al., 1986; Reith et al., 1987), [^{3}H]PD85,639 (Thomsen et al., 1993), [^{3}H]phenytoin (Francis and Burnham, 1992), and [^{3}H]lifarizine (MacKinnon et al., 1995).

A chemically distinct family of compounds emerged from the Wellcome Research Laboratories with the discovery of the sodium channel inhibitor lamotrigine (Lamictal), a drug that is now widely used to treat epilepsy and bipolar disorder (Clare et al., 2000). A related compound, sipatrigine (BW619C89), also inhibits sodium channels and shows powerful neuroprotective properties, whereas others, such as BW4030W92 and BW227C89, are analgesic (Clare et al., 2000; Liu et al., 2003). One of the most potent members of the lamotrigine family is the compound BW202W92, which is a very effective neuroprotectant in models of stroke and which electrophysiological studies have confirmed to be a potent and selective inhibitor of voltage-gated sodium channels (Caputi et al., 2001). We report here on the binding of [^{3}H]BW202W92 and find that it engages a novel site on sodium channels in rat brain synaptosomes.

Materials and Methods

Materials. BW202W92, BW203W92, BW4030W92, and its enantiomer BW4082W92 were supplied at greater than 98.6% chiral purity by Synnovation Ltd. (Epsom, UK), who also supplied BW227C89. Lamotrigine and sipatrigine were provided by the Chemistry Division, Wolfson Institute for Biomedical Research (London, UK). [^{3}H]BTX-B was purchased from PerkinElmer Life and Analytical Sciences (Beaconsfield, UK) at a specific activity of 49 Ci/mmol. [^{3}H]BW202W92 was synthesized by GE Healthcare (Little Chalfont, UK) from 2,4-diamino-5-(2,3,5-trichlorophenyl)-pyrimidine-6-carboxaldehyde (supplied by Greenwich Chemicals, Chatham, UK) by reduction and fluorination. The resulting racemic mixture was separated by high-performance liquid chromatography, and the resulting [^{3}H]BW202W92 was supplied as an ethanolic solution (at greater than 95% purity) with a specific activity of 18 Ci/mmol. Batrachotoxin was a gift from Dr. John Daly (National Institutes of Health, Bethesda, MD). Tetrodotoxin citrate, riluzole, and MK-801 were from Tocris Cookson Inc. (Bristol, UK) and YC-1 was from Axxora (UK) Ltd. (Nottingham, UK). All other special chemicals and toxins were obtained from Sigma Chemical (Poole, Dorset, UK). The brevetoxin used was *Ptychodiscus brevis* toxin-2.

Preparation of Rat Forebrain Synaptosomes. Experiments were performed using forebrain (whole brain less cerebellum and medulla) from Male Wistar rats weighing 175 to 250 g. All efforts were made to reduce the number of animals used, and all experiments were carried out in accordance with the UK Animals (Scientific Procedures) Act, 1986 and the European Community Council Directive of 24 November 1986 (86/609/EEC). After killing of animals by stunning and decapitation, crude forebrain synaptosomes (heavy and light mitochondrial fraction containing synaptosomes) were prepared as described previously (Garthwaite et al., 2002). In brief, forebrain was transferred to a glass Potter vessel at a final concentration of 10% (w/v) in 0.25 M sucrose and homogenized, using a Teflon pestle, by eight up-and-down strokes of a Braun Potter S motor-driven homogenizer set to 900 rpm. The homogenate was centrifuged at 1036*g* at 4°C for 10 min, and the supernatant was collected. The remaining pellet was resuspended, as described above, in fresh ice-cold 0.25 M sucrose, and the centrifugation step was repeated. The supernatant fractions were pooled and centrifuged at 45,000*g* at 4°C for 15 min, and the resulting pellet resuspended in assay buffer (see below). Lysed synaptosomal membranes were prepared by resuspending the above-mentioned synaptosomal pellet in 20 volumes of ice-cold water using an Ultra-Turrax homogenizer (IKA-Werke GmbH & Co. KG, Staufen, Germany). After 30-min storage on ice, the homogenate was centrifuged at 15,000*g* at 4°C for 20 min, and the resulting supernatant and loose buffy-coat layer were removed by careful aspiration, combined, and centrifuged at 50,000*g* at 4°C for 15 min. The resulting pellet was resuspended in assay buffer (see below).

Binding Studies. Binding of [^{3}H]BW202W92 (usually 4–10 nM for displacement studies and up to 300 nM for saturation analysis) was carried out using 14-ml polypropylene test tubes and was initiated by the addition of 12.5 mg (approximately 500 μg of protein) original wet weight of tissue to tubes that contained [^{3}H]BW202W92 (incubation concentration measured independently by radioactivity counting), tetrodotoxin (1 μM unless otherwise indicated), and compounds under test, in a final volume of 1 ml. Assays were carried out in buffer that consisted of 50 mM HEPES (adjusted to pH 7.4 with Tris base), 5.5 mM D-glucose, 0.8 mM $MgSO_4$ and either 1 mM KCl and 134 mM choline chloride or 100 mM KCl and 35 mM choline chloride. Changes in KCl or NaCl concentration were balanced by corresponding changes in the concentration of choline chloride such that the molarity of the buffer remained constant. Samples were mixed and then incubated for 40 min at 25°C (unless indicated otherwise). Incubations were terminated by the addition of 5 ml of ice-cold wash buffer consisting of 163 mM choline chloride, 1.8 mM $CaCl_2$ and 0.8 mM $MgSO_4$ in 5 mM HEPES buffer, pH 7.4, followed immediately by vacuum filtration through GF/C glass fiber filters (Whatman, Maidstone, UK) using a Brandel cell harvester (Brandel Inc., Gaithersburg, MD). A further 2 × 5 ml of ice-cold wash buffer was added to each tube, and the vacuum filtration step was repeated. The GF/C glass fiber filters containing bound [^{3}H]BW202W92 were transferred to minivials, and 4 ml of Picofluor^{40} liquid scintillant was added using a Brandel deposit/dispense system. Radioactivity was measured using a liquid scintillation counter (Beckman Coulter, Inc., Fullerton, CA), and cpm was converted directly to dpm via reference to appropriate quench parameters.

Binding studies with [^{3}H]BTX-B were carried out using normal assay buffer as described above with the addition of 100 μg/ml α-scorpion toxin (unless stated otherwise) in an incubation volume of 0.25 ml for 90 min at 2°C.

[^{14}C]Guanidinium Ion Flux. Veratrine-evoked uptake of [^{14}C]guanidinium ions was carried out as described previously (Garthwaite et al., 2002).

Data Analysis. Data are presented as mean ± S.E.M. unless indicated otherwise. Inhibition studies were carried out using either single or duplicate samples encompassing an appropriate range of compound concentrations (usually 12). For comparative purposes, most experiments were carried out in parallel such that identical drug, toxin, and synaptosome preparations were used. IC_{50} values were computed from $\log_{10}$ concentration-effect curves using a four-parameter logistic equation: $y = A + B/(1 + \exp(-C(x - IC_{50})))$, where A is nonspecific binding, B is specific binding, and C is binding slope. Saturation binding parameters were computed by plotting ^{3}H-ligand concentration (measured by counting radioactivity) versus bound ligand according to the Hill equation: $y = (B_{max}[L]^{nH})/(K_D{}^{nH} + [L]^{nH})$, where y is specifically bound ligand, [L] is ^{3}H-ligand concentration, and n_H is the Hill coefficient. If displacement binding slopes were not significantly different from 1, then parameters were recomputed with Hill slope set to 1. Observed on-rates (K_{obs}) were computed from plots of specific binding (in the presence of 1 μM tetrodotoxin) versus time (t) at varying ligand concentrations using the equation $y = A(1 - \exp(-K_{obs}t))$, where y is specific binding and A is maximal binding. On- and off-rates were derived by plotting K_{obs} versus ligand concentration (L) according to the equation $K_{obs} = k_{+1}L + k_{-1}$, where k_{+1} is the on-rate constant (in nanomolar per minute) and k_{-1} is the off-rate constant (per minute); the K_D value was calculated from the ratio k_{-1}/k_{+1}. All statistical analyses used Student's (two-way) t test.

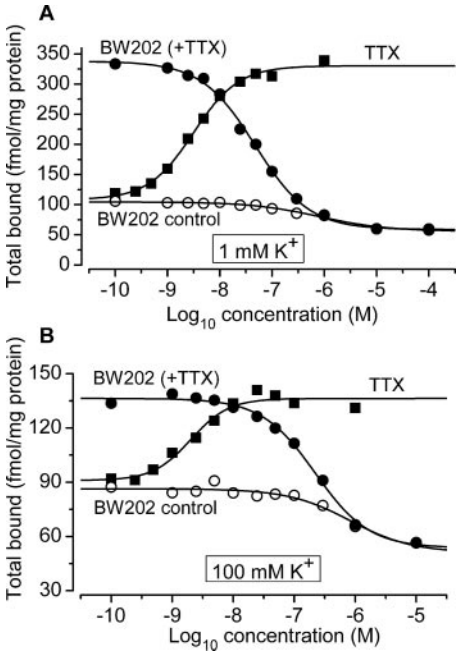

Fig. 1. Effect of tetrodotoxin (TTX) and BW202W92 (BW202) on the binding of [^{3}H]BW202W92 to rat forebrain synaptosomes under normal (1 mM K^+) and depolarizing (100 mM K^+) conditions. Experiments were carried by incubating synaptosomes for 40 min at 25°C using the same drug or toxin solutions and a common precursor pool of forebrain synaptosomes. Representative concentration-effect relationships for tetrodotoxin (squares) and for BW202W92 in the presence (●) or absence (○) of 1 μM TTX are shown under normal (A) and depolarizing (B) conditions. Data are the means of duplicate measurements, and in this example, the concentrations of [^{3}H]BW202W92 were 9.3 and 8.8 nM in A and B, respectively.

Results

Initially, [^{3}H]BW202W92 was disappointing as a ligand in rat brain synaptosomes because the level of specific binding (displaceable by BW202W92 itself) was only approximately 40% of the total (Fig. 1, A and B). Two manipulations changed this picture: addition of tetrodotoxin and varying the K^+ concentration.

Effect of Tetrodotoxin. On addition of 1 μM tetrodotoxin at a low K^+ concentration (1 mM), the specific binding of [^{3}H]BW202W92 was enhanced 7-fold (Fig. 1A). This effect of tetrodotoxin persisted at high K^+ concentration (100 mM), although the total specific binding was then reduced by approximately 60% (Fig. 1B). The potency of tetrodotoxin for enhancing the binding was very similar at 1 and 100 mM K^+, with the EC_{50} values both being 2 to 3 nM (Table 1). Thus, the total specific [^{3}H]BW202W92 binding is the sum of tetrodotoxin-dependent sites (85% at 1 mM K^+; 65% at 100 mM K^+) and tetrodotoxin-independent sites (15 and 35%, respectively). The effect of tetrodotoxin was maximal at 1 μM (at 1 or 100 mM K^+), and so this concentration was subsequently used routinely.

Effect of K^+. Binding of [^{3}H]BW202W92 in the presence of tetrodotoxin was extremely sensitive to the external K^+ concentration (Fig. 2A). Starting at 1 mM K^+, the binding was inhibited by K^+ with an IC_{50} of 5.9 ± 0.6 mM (n = 6) and a slope of 1.83 ± 0.04. Inhibition was maximal at 100 mM K^+ at which concentration total binding was reduced by approximately 60%. Binding in the absence of tetrodotoxin was also partially sensitive to K^+, with the IC_{50} value being approximately 10 mM (Fig. 2A).

Under the assay conditions used (choline as the main cation, no Na^+), the membrane potential of synaptosomes should be approximately Nernstian with respect to the external K^+ concentration. Assuming an internal K^+ concentration of 100 mM (Blaustein and Goldring, 1975), the membrane potential should fall from −116 mV at 1 mM K^+ to 0 mV at 100 mM K^+. The IC_{50} value in the presence of tetrodotoxin (5.9 mM) should correspond to a membrane potential of approximately −70 mV. Although the dependence on K^+ suggests that the level of binding depends on membrane potential, another possible interpretation is that it is inhibited by K^+ independently of the resultant membrane potential changes. Depolarizing the synaptosomes by the usual alternative methods (increasing the permeability to Na^+) could not be carried out with the standard Na^+-free buffer so, instead, the incubation temperature was varied.

TABLE 1

Effect of sodium channel toxins on the binding of [^{3}H]BW202W92 to rat forebrain synaptosomes

Synaptosomes were incubated for 40 min at 25°C. Data for batrachotoxin, veratrine, α-scorpion venom, and β-scorpion venom were obtained in the presence of 1 μM tetrodotoxin. Values are presented as mean ± S.E.M.

Toxin (K^+ conc)	EC_{50} or IC_{50}	Binding Slope	n
Tetrodotoxin (1 mM K^+)	EC_{50} = 2.9 ± 0.2 nM	1.05 ± 0.05	4
Tetrodotoxin (100 mM K^+)	EC_{50} = 2.2 ± 0.2 nM	1.39 ± 0.09	5
Batrachotoxin (1 mM K^+)	<10% inhibition at 10 μM		2
Veratrine (1 mM K^+)	IC_{50} = 31 ± 5 μg/ml	1.19 ± 0.08	3
α-Scorpion venom (1 mM K^+)	IC_{50} = 3.6 ± 1.8 μg/ml	2.29 ± 0.41	3
β-Scorpion venom (1 mM K^+)	IC_{50} = 1.2 ± 0.5 μg/ml	1.95 ± 0.16	3

When incubated in a medium in which choline is the major cation, as here, the synaptosomal membrane potential rapidly dissipates at 37°C but becomes progressively sustained as the temperature is reduced (Gilles et al., 2001). The effect of temperature on the time courses of specific binding of [^{3}H]BW202W92 in the presence of 1 μM tetrodotoxin is shown in Fig. 2B. At 4°C, binding approached a maximum after 120-min incubation, whereas a similar plateau level was reached by 30 min at 25°C. Binding at 25°C then remained stable for a further 20 min before gradually declining. At 37°C, a peak of binding occurred after 10 min but then it rapidly became reduced. The peak at 37°C was less than 50% of the maximal specific binding observed at the lower temperatures. These changes in [^{3}H]BW202W92 binding closely follow the changes in synaptosomal membrane potential with time at similar temperatures (Gilles et al., 2001). Finally, lysis of the synaptosomes seemed to mimic the effect of 100 mM K^+ in that specific tetrodotoxin-dependent binding of [^{3}H]BW202W92 was reduced and was of relatively low affinity (Fig. 3A), with the IC_{50} for displacement by BW202W92 being 214 ± 1 nM compared with 34 ± 1 nM when the same synaptosomes were kept intact (cf. below). In addition, there was little or no inhibition of binding by K^+ in the lysed synaptosomes (Fig. 3B). Hence, it is reasonable to attribute the inhibitory effect of raised K^+ under normal conditions (intact synaptosomes at 25°C in the presence of tetrodotoxin) to membrane depolarization.

To measure maximal specific binding, the concentration of [K^+] was routinely kept at 1 mM and, as appropriate, measurements were made in the presence and absence of 1 μM tetrodotoxin, with the samples being incubated for 40 min at 25°C.

Effect of Na^+. One explanation for the stimulatory effect of tetrodotoxin on [^{3}H]BW202W92 binding might be that choline (the main cation in the standard synaptosome incubation medium) normally inhibits the binding, and tetrodotoxin alleviates this inhibitory effect by preventing access of choline to the binding site. This was examined by progressively substituting Na^+ for choline to give a range of Na^+ concentrations (5–129 mM). In the absence of tetrodotoxin, there was no effect on total (or nonspecific) [^{3}H]BW202W92 binding (Fig. 3C), ruling out this explanation. In the presence of tetrodotoxin, Na^+ partially inhibited [^{3}H]BW202W92 binding (IC_{50} = 40 ± 1 mM; Fig. 3C), possibly by causing a small depolarization or by directly interfering with the binding of tetrodotoxin or [^{3}H]BW202W92.

Stereoselectivity of [^{3}H]BW202W92 Binding. BW202W92 possesses a chiral center, offering the advantage of determining enantioselectivity of the binding site, which, if demonstrated, provides a good indication of specificity. Based on two criteria, namely, inhibition of veratrine-stimulated

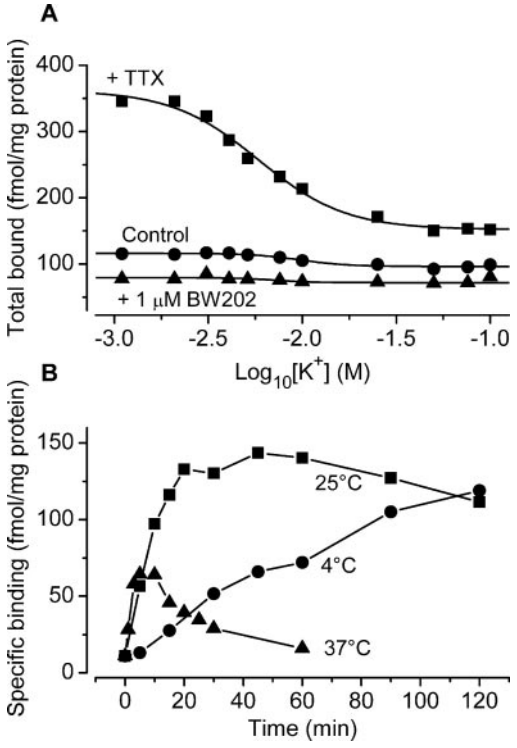

Fig. 2. Effects of K^+ concentration and temperature on the binding of [^{3}H]BW202W92 to rat forebrain synaptosomes. A, synaptosomes were incubated for 40 min at 25°C in varying concentrations of K^+ with the media being kept isotonic. Incubations were carried out either in the presence and absence of 1 μM tetrodotoxin (TTX) or in the presence of 1 μM BW202W92. Data are the means of duplicate measurements with an incubation concentration of [^{3}H]BW202W92 of 8.7 nM. B, time course of total specific binding of [^{3}H]BW202W92 to rat forebrain synaptosomes at the indicated temperatures in the presence of both 1 mM K^+ and 1 μM tetrodotoxin. The radioligand concentration was 4.4 nM, and data are the mean specific binding (total binding less binding in presence of 1 μM BW202W92) of duplicate incubations at each time point.

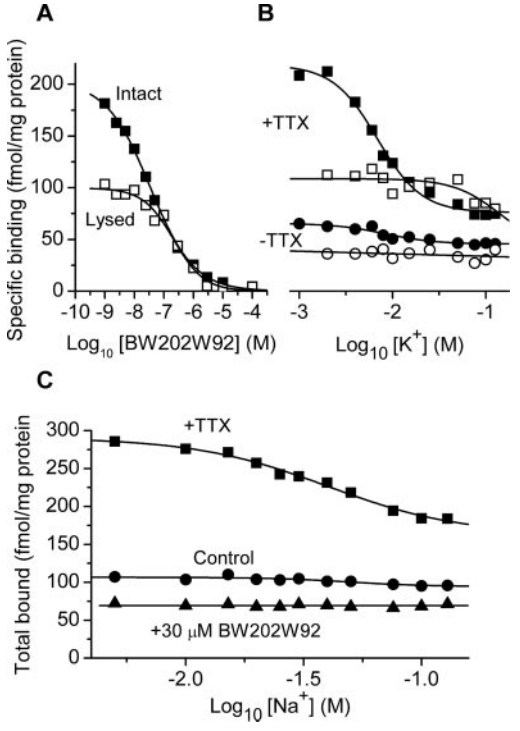

Fig. 3. Effect of lysis and Na^+ on [^{3}H]BW202W92 binding to rat forebrain synaptosomes. A, inhibition curves for BW202W92 in the same batch of synaptosomes kept intact (■) or lysed (□). B, in the same experiment as A, intact (filled symbols) or lysed (open symbols) synaptosomes were incubated with varying K^+ concentrations in the absence (circles) or presence (squares) of 1 μM tetrodotoxin (TTX). Points in A and B are the means of duplicate determinations, the ligand concentration being 7.4 nM. C, effect of substituting Na^+ for choline on [^{3}H]BW202W92 binding in the absence (control) or presence of 1 μM TTX (squares) or 30 μM BW202W92 (triangles). Points are the means of two experiments, each carried out in duplicate on different batches of synaptosomes (ligand concentration is 7.6 nM); errors (S.D.) are within the dimensions of the symbols.

accumulation of [^{14}C]guanidinium ions and inhibition of [^{3}H]BTX-B binding, the *S*-enantiomer, BW203W92, was approximately 8-fold weaker as an inhibitor of sodium channels than the *R*-enantiomer, BW202W92 (Table 2). In the absence of tetrodotoxin, and with either 1 mM or 100 mM K^+, both BW202W92 and BW203W92 inhibited [^{3}H]BW202W92 binding in an apparently competitive manner because the slopes of the inhibition curves were not significantly different from 1 (Fig. 4, A and B; Table 2). Displacement of this component by both enantiomers was of relatively low affinity (near 1 μM) and was unaffected by K^+, but there was a 7-fold degree of selectivity for the *R*-enantiomer (BW202W92).

In the presence of 1 μM tetrodotoxin, the IC_{50} value for BW202W92 in 1 mM K^+ was greatly decreased to approximately 50 nM (Fig. 4A; Table 2). The slope was reduced to below 1 under these conditions, presumably reflecting the presence of both lower affinity tetrodotoxin-independent and higher affinity tetrodotoxin-dependent sites. Under depolarizing conditions (100 mM K^+), the effect of 1 μM tetrodotoxin was less marked, but the affinity of BW202W92 was again increased, this time from approximately 0.6 μM to approximately 0.2 μM (Fig. 4B; Table 2). This interaction was characterized by a binding slope not significantly different from 1, probably reflecting the relatively small separation (approximately 3-fold) between the affinities for tetrodotoxin-independent and tetrodotoxin-dependent binding sites under these conditions. In contrast to the effects seen with BW202W92, displacement by BW203W92 in the presence of 1 μM tetrodotoxin was relatively little affected by K^+. Under normal conditions (tetrodotoxin; 1 mM K^+), the affinity for BW202W92 was some 32-fold higher than that for BW203W92, and the effect of depolarization was to reduce this ratio to 12, primarily through a decreased affinity for BW202W92.

Effect of Sodium Channel Toxins. Many drugs acting on voltage-gated sodium channels inhibit the binding of [^{3}H]BTX-B, and the same was true of BW202W92 (Table 2). To determine whether the binding sites are similar, a direct comparison of the properties of the binding of the two ligands was made. Although tetrodotoxin markedly enhanced the binding of [^{3}H]BW202W92 (as before), it had little or no effect on the binding of [^{3}H]BTX-B (Fig. 5, A and B). In contrast, α-scorpion venom (a site 3 neurotoxin) greatly enhanced the binding of [^{3}H]BTX-B but, in the absence of tetrodotoxin, had a small inhibitory effect on the binding of [^{3}H]BW202W92 (Fig. 5, A and B). In the presence of tetrodotoxin, however, the binding of [^{3}H]BW202W92 was markedly inhibited by α-scorpion venom and also by the site 4 toxin, β-scorpion venom (Fig. 5E; Table 1). The slopes of both inhibition curves were significantly greater than unity, indicating a noncompetitive interaction, and both toxins gave incomplete inhibition, amounting to 85 ± 4% (n = 3) and 67 ± 1% (n = 3) of specific binding, respectively.

Batrachotoxin was an extremely weak displacer of the binding of [^{3}H]BW202W92 (with or without tetrodotoxin), with the extrapolated IC_{50} value in the presence of tetrodotoxin being approximately 100 μM, whereas it was some 1000-fold more potent at displacing the binding of [^{3}H]BTX-B (Fig. 5, C and D). Veratrine (another site 2 toxin) displaced the binding of both ligands but was approximately 10-fold more efficacious against [^{3}H]BTX-B binding (Fig. 5, C and D; Table 1). Finally, the binding of [^{3}H]BTX-B was significantly enhanced by the site 5 toxin brevetoxin (5 μM) both in the presence and absence of α-scorpion venom (100 μg/ml),

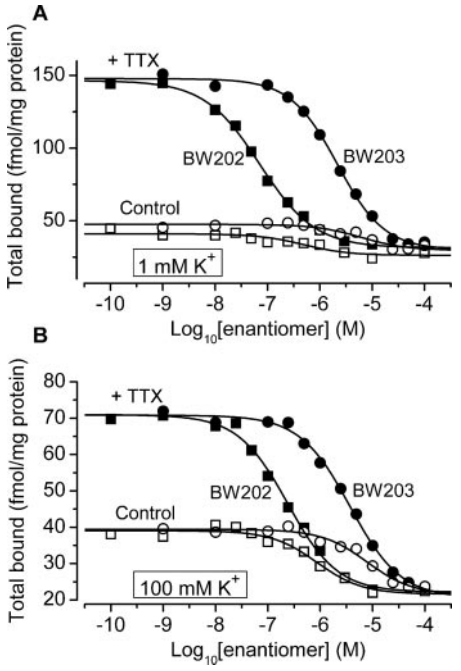

Fig. 4. Stereoselectivity of the binding of [^{3}H]BW202W92 to rat forebrain synaptosomes. The data are from a single representative experiment and are means of duplicate measurements for each concentration of compound tested. A, displacement of [^{3}H]BW202W92 (4.4 nM) under normal conditions (1 mM K^+) by BW202W92 (BW202; squares) and BW203W92 (BW203; circles) in the absence (control; open symbols) and presence (filled symbols) of 1 μM tetrodotoxin (TTX). B, as in A but under depolarizing conditions (100 mM K^+) with the radiolabeled ligand concentration being 3.7 nM.

TABLE 2

Stereo selectivity of BW202W92 over BW203W92 for inhibition of [^{3}H]BW202W92 binding and in other sodium channel assays in rat forebrain synaptosomes

Values are means ± S.E.M. Ratio indicates IC_{50} values for BW203W92 relative to BW202W92.

Assay	BW202W92 IC_{50}	Binding Slope	n	BW203W92 IC_{50}	Binding Slope	n	Ratio
	μM			μM			
[^{3}H]BW202W92							
1 mM K^+ + TTX[a]	0.048 ± 0.006	0.81 ± 0.03	6	1.5 ± 0.3	0.91 ± 0.02	4	32
1 mM K^+	0.73 ± 0.09	0.94 ± 0.17	5	4.4 ± 1.1	1.31 ± 0.30	4	6.0
100 mM K^+ + TTX[a]	0.20 ± 0.02	0.95 ± 0.03	4	2.4 ± 0.6	0.91 ± 0.01	3	12
100 mM K^+	0.64 ± 0.13	0.89 ± 0.08	4	5.0 ± 1.5	0.99 ± 0.01	3	7.8
[^{14}C]Guanidinium ion flux	2.0 ± 0.2	0.88 ± 0.03	9	15 ± 4	1.13 ± 0.05	4	7.5
[^{3}H]BTX-B	4.4 ± 0.3	0.81 ± 0.03	3	33 ± 9	0.80 ± 0.06	2	7.5

[a] The tetrodotoxin (TTX) concentration was 1 μM.

whereas, in the same experiment, brevetoxin had no significant effect on the binding of [^{3}H]BW202W92 (Fig. 5F).

Measurement of K_D and B_{max}. The rates of binding of [^{3}H]BW202W92 to tetrodotoxin-dependent sites were computed from individual time courses of specific binding at varying ligand concentrations (Fig. 6). Association and dissociation rate constants were derived from these data, and their ratio was used to determine the dissociation constant (K_D) of binding. The dissociation and association rate constants were 0.0849 ± 0.004 min^{-1} and 0.0029 ± 0.0004 nM^{-1} min^{-1}, respectively (±S.D.; n = 2), giving a K_D of 29 ± 6 nM (±S.D.; n = 2). A representative saturation analysis of the binding of [^{3}H]BW202W92 under both normal and depolarizing conditions, with and without 1 μM tetrodotoxin, is shown in Fig. 7. Nonspecific binding (the linear phase of the saturation isotherm) was unaffected by addition of tetrodotoxin or K^+ and was approximately 9.5 fmol/mg of protein/nM. Although tetrodotoxin-independent saturable binding was present, it was not possible to obtain reliable estimates of the binding parameters for these sites. Fitting tetrodotoxin-dependent specific binding to the Hill equation (see *Materials and Methods*) gave Hill slopes that were not significantly different from 1, so parameters were recomputed with Hill slopes set to 1. The results obtained in this manner were very similar to those obtained using Scatchard analysis (Fig. 7; Table 3). The K_D values obtained for tetrodotoxin-dependent binding (1 mM K^+) using saturation or Scatchard analysis (21 nM) were similar to those obtained using rate methodology (29 nM; see above), and total binding (B_{max}) amounted to 827 fmol/mg of protein. Under depolarizing conditions (100 mM K^+), B_{max} was nonsignificantly changed (20% reduction), whereas the K_D value was lowered by a factor of 5, to approximately 100 nM.

Effect of Antiepileptics, Local Anesthetics, and Other Drugs. To help understand the pharmacological relevance of the [^{3}H]BW202W92 binding site, displacement of the binding by representatives of various drug classes was tested in the presence of tetrodotoxin and at 1 mM K^+ (Table 4). Compounds of the lamotrigine family, including lamotrigine itself (K_I = 1.8 μM) and sipatrigine (K_I = 0.4 μM), inhibited the binding concentration dependently. The antiepileptic/analgesic compound BW4030W92 exhibited a K_I (0.25 μM) 30-fold lower than its *S*-enantiomer (BW4082W92), confirming the stereoselectivity of the binding site. The antiepileptic drugs phenytoin (K_I = 9 μM) and carbamazepine (K_I = 36 μM), which act on voltage-gated

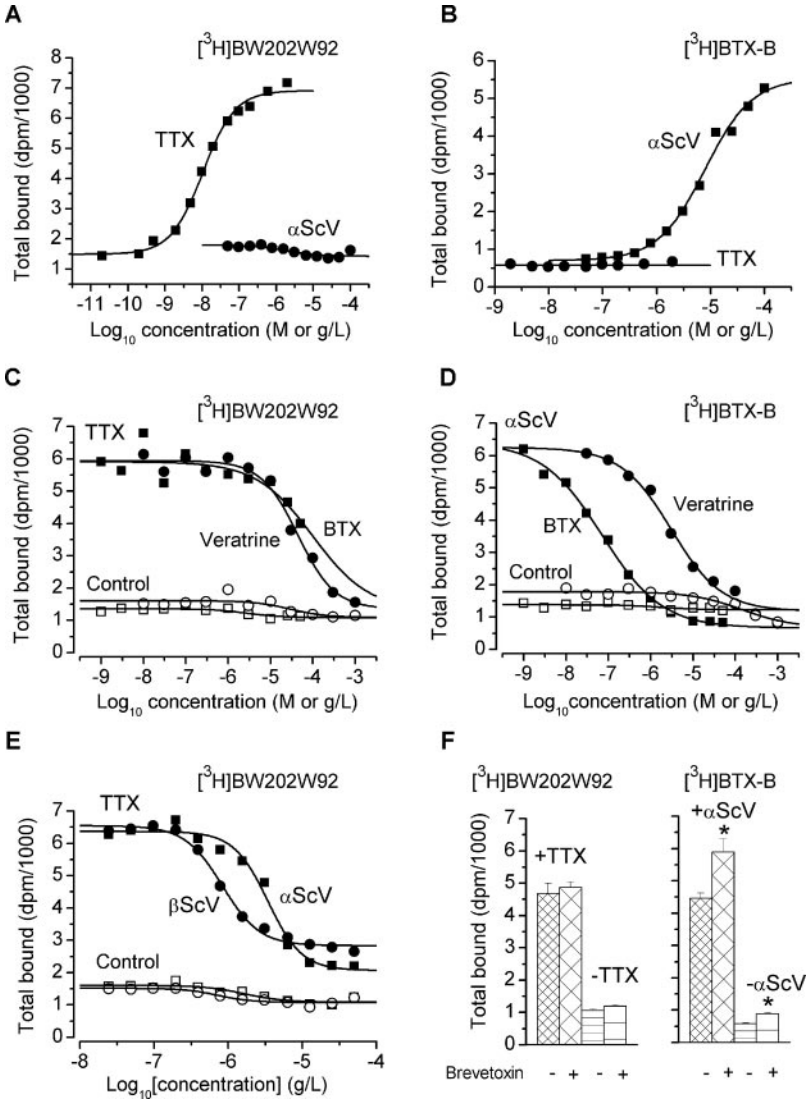

Fig. 5. Effect of sodium channel toxins on the binding of [^{3}H]BW202W92 and [^{3}H]BTX-B to rat forebrain synaptosomes. Where data for both ligands are presented, experiments were carried out in parallel with the same toxin solutions, synaptosome preparations, and assay buffers. A and B, effect of tetrodotoxin (TTX) and α-scorpion venom (αScV) on the binding of [^{3}H]BW202W92 (A) and [^{3}H]BTX-B (B). C and D, effect of batrachotoxin (BTX; squares) and veratrine (circles) on the binding of [^{3}H]BW202W92 in the absence (control; open symbols) and presence (filled symbols) of 1 μM TTX (C) and on the binding of [^{3}H]BTX-B (D). E, effect of α-scorpion venom (αScV; squares) and β-scorpion venom (βScV; circles) on the binding of [^{3}H]BW202W92 in the absence (control; open symbols) and presence (filled symbols) of 1 μM TTX. F, effect of brevetoxin (5 μM) on the binding of the two ligands in the presence or absence of the indicated toxins. Data in A to E are the means of duplicate measurements for each concentration of toxin tested; those in F are the mean ± S.E.M of six replicate incubations.

sodium channels, also displaced the binding, whereas antiepileptics acting through other mechanisms (e.g., valproate and gabapentin) had no significant effect at concentrations up to 100 μM. Local anesthetics also displaced the binding, and of these, tetracaine was the most potent, with a K_I of 63 nM. MK-801 and riluzole, neuroprotectants that inhibit sodium channels (among other effects), both inhibited binding, whereas the neuroprotectant clomethiozole, a GABA receptor agonist with no reported activity on sodium channels, was inactive at 100 μM. An assortment of other drugs reported previously to inhibit sodium channels also inhibited [^{3}H]BW202W92 binding, including the compound YC-1, which is better known as a sensitizer of nitric oxide-activated guanylyl cyclase activity (Garthwaite et al., 2002).

Because the affinity of BW202W92 (in the presence of tetrodotoxin) was reduced by depolarizing the synaptosomes with 100 mM K^+, we examined whether this also applied to other drugs acting at the same site (Table 5). As indicated by the IC_{50} values, the affinities of some compounds such as phenytoin and carbamazepine were little changed. At the other extreme, the affinities of lidocaine, lamotrigine, and procaine were reduced 10- to 20-fold. In between, a 3- to 5-fold reduction was observed for BW202W92, sipatrigine, and tetracaine.

Discussion

The results provide evidence that BW202W92 binds selectively to voltage-gated sodium channels in rat brain synaptosomes, that the binding site is stereoselective and distinct from that of all other ligands examined to date, and that it may correspond to a target for a variety of drugs whose therapeutic utility is ascribed to sodium channel inhibition.

A key ingredient found to augment specific binding was the site 1 toxin tetrodotoxin whose potency matched its low nanomolar potency for inhibiting sodium currents in brain neurons (Madeja, 2000). This result, together with the effects of sodium channel neurotoxins acting at other sites (3 and 4), provides compelling evidence that the binding site is associated with sodium channels. Nevertheless, the effect of tetrodotoxin on the binding is unexpected if tetrodotoxin is simply a pore blocker. It is important to note that agents inhibiting tetrodotoxin-dependent binding (e.g., toxins, K^+, and competitive inhibitors) also had qualitatively similar effects on tetrodotoxin-independent binding, suggesting that tetrodotoxin does not artificially expose the binding sites but rather

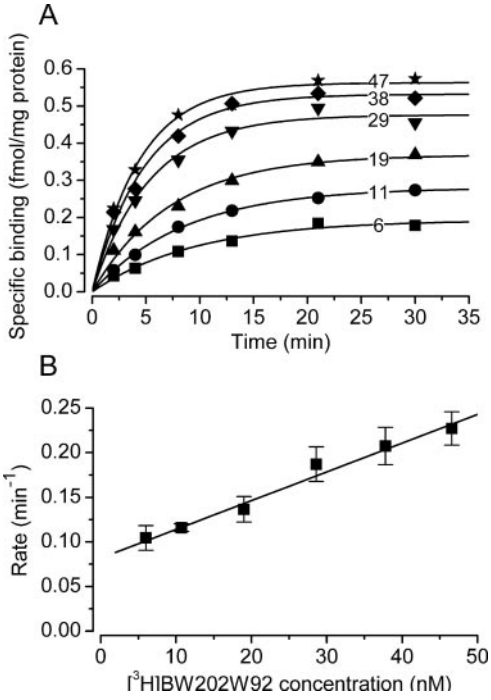

Fig. 6. Kinetics of the binding of [^{3}H]BW202W92 to rat forebrain synaptosomes in the presence of 1 μM tetrodotoxin. A, time courses of specific binding of [^{3}H]BW202W92. The synaptosomes were incubated with the indicated concentrations of [^{3}H]BW202W92 at 25°C for varying times up to 30 min in the presence of 1 mM [K^+] and 1 μM tetrodotoxin, with or without 1 μM unlabeled BW202W92. Specific binding (total binding less binding in the presence of 1 μM BW202W92) is presented as the mean of duplicate measurements for each time point at each ligand concentration. B, rate constants for the fits in A are plotted together with their computer-generated errors against the respective [^{3}H]BW202W92 concentrations to obtain the kinetic parameters (see *Materials and Methods*).

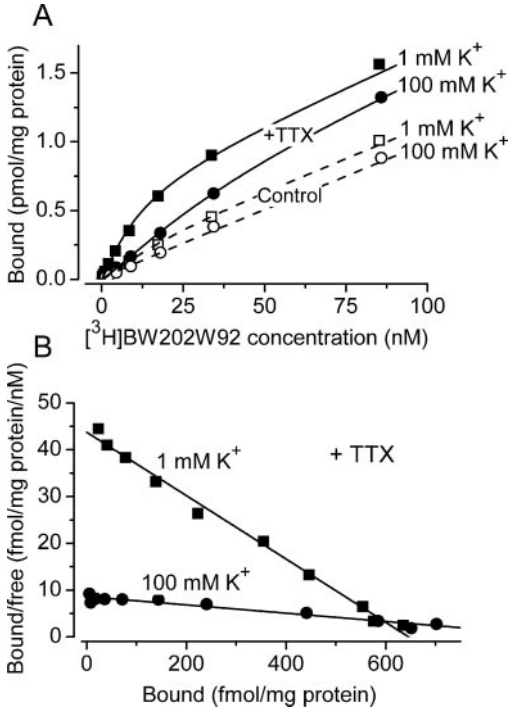

Fig. 7. Saturation analysis of the binding of [^{3}H]BW202W92 to rat forebrain synaptosomes under normal (1 mM K^+; squares) and depolarizing (100 mM K^+; circles) conditions. A, representative saturation curves for the binding in the absence (control; open symbols) and presence (filled symbols) of 1 μM tetrodotoxin (TTX). B, Scatchard plot of tetrodotoxin-dependent specific binding (binding in the presence of 1 μM tetrodotoxin less binding in its absence) under both normal and depolarizing conditions from the data shown in A. Data are the means of duplicate measurements at each concentration of radioligand.

TABLE 3

Parameters of tetrodotoxin-dependent binding of [^{3}H]BW202W92 to rat forebrain synaptosomes under normal and depolarizing conditions

Data are means ± S.E.M.

	Method	K_D	B_{max}	n
		nM	*fmol/mg of protein*	
1 mM K^+	Saturation	21 ± 2	832 ± 129	3
1 mM K^+	Scatchard	21 ± 2	823 ± 108	3
100 mM K^+	Saturation	96 ± 18[a]	648 ± 108	4
100 mM K^+	Scatchard	107 ± 7[a]	660 ± 54	4

[a] $P < 0.05$ versus value at 1 mM K^+. The concentration of tetrodotoxin was 1 μM in all cases.

stabilizes the channel in a natural conformation to which [^{3}H]BW202W92 binds with relatively high affinity. In this respect, tetrodotoxin and saxitoxin, another site 1 toxin, may increase the fraction of channels that is converted to inactivated states (Strichartz et al., 1987; Madeja, 2000). However, this idea is not easily reconciled with the evidence that depolarization of the synaptosomes by elevated [K^+] (in the absence of tetrodotoxin), a procedure that should convert the channels into an inactivated state, did not enhance binding (rather, it had the opposite effect). Hence, in polarized synaptosomes (low [K^+]), the binding is presumably to a closed channel conformation.

The loss of [^{3}H]BW202W92 binding with increasing K^+ concentration was attributable largely to the affinity of the ligand being reduced with membrane depolarization. A qualitatively similar effect of depolarization was observed on the potencies of several other drugs, including some antiepileptics and local anesthetics. At first glance, decreased binding with depolarization seems anomalous because of the abundant evidence that depolarization enhances drug action on sodium channels. This effect is explained by the voltage dependence of the appearance of inactivated state(s) on which the compounds act (Ragsdale et al., 1991; Rogawski and Loscher, 2004). It is commonly assumed that the drugs only bind to the inactivated state, but it is also possible that they bind to the closed state (in keeping with the data here) but in a manner that is functionally inconsequential; only when the channel passes into its inactivated state(s), does stabilization of channel inactivation by the drug become visible electrophysiologically. Such a mechanism would be analogous to β-scorpion toxin being bound to the resting channel and then trapping the voltage sensor once it is mobilized by depolarization, thereby, in this case, enhancing activation (Cestele et al., 1998).

Apart from tetrodotoxin, which acts at site 1, the other toxins found to influence [^{3}H]BW202W92 binding promi-

TABLE 4
Comparative drug potencies against [^{3}H]BW202W92 and [^{3}H]BTX-B binding
Values are presented as S.E.M.

Class and Compound	BW202W92 Binding K_I	BW202W92 Binding Slopes	n	[^{3}H]BTX-B Binding IC_{50} (n)	Mean Literature [^{3}H]BTX-B Binding IC_{50} or K_I[a]	Citation
	μM			μM		
Lamotrigine family						
BW202W92	0.037 ± 0.001	0.81 ± 0.03	6	4.4 ± 0.3 (3)	4.4	Current study
BW203W92	1.2 ± 0.2	0.91 ± 0.02	4	33 ± 9 (2)	33	Current study
BW4030W92	0.25 ± 0.02	0.75 ± 0.10	3			
BW4082W92	8.1	1.00	1			
BW227C89	1.1 ± 0.3	0.91 ± 0.06	2			
Sipatrigine	0.40 ± 0.06	0.94 ± 0.10	6	10 ± 4 (2)	7.0	Garthwaite et al., 2002; Grauert et al., 2002; Current study
Lamotrigine	1.8 ± 0.2	0.86 ± 0.05	5	63 (1)	121	Cheung et al., 1992; Salvati et al., 1999; Current study
Other anticonvulsants						
Phenytoin	9 ± 1	1.06 ± 0.08	4		53	Willow and Catterall, 1982; Zimanyi et al., 1989; MacKinnon et al., 1995; Salvati et al., 1999; Lingamaneni and Hemmings, 2003
Carbamazepine	36 ± 3	1.01 ± 0.17	3		374	Willow and Catterall, 1982; Zimanyi et al., 1989; Salvati et al., 1999; Bonifacio et al., 2001; Lingamaneni and Hemmings, 2003
Felbamate	>100		1			
Valproate	>100		2		>1000	Willow and Catterall, 1982
Ethosuccimide	>100		1		>1000	Willow and Catterall, 1982
Gabapentin	>100		1			
Vigabatrin	>100		1			
Zonisamide	>100		1			
Local anesthetics						
Tetracaine	0.070 ± 0.005	0.98 ± 0.02	2		1.5	Creveling et al., 1983; Postma and Catterall, 1984; Grima et al., 1986; Reith et al., 1987; Lingamaneni and Hemmings, 2003
Lidocaine	1.9 ± 0.3	1.01 ± 0.03	3		127	Creveling et al., 1983; Postma and Catterall, 1984; Grima et al., 1986; Zimanyi et al., 1989; MacKinnon et al., 1995; Lingamaneni and Hemmings, 2003
Procaine	7.5 ± 0.5	0.91 ± 0.08	4		89	Creveling et al., 1983; Postma and Catterall, 1984; Grima et al., 1986; Reith et al., 1987
Procainamide	37 ± 2	0.88 ± 0.01	2			
Other neuroprotectants						
MK-801	2.3	1.08	1		4.8	MacKinnon et al., 1995
Riluzole	0.84	0.94	1		40	MacKinnon et al., 1995
Clomethiazole	>100		2			
Miscellaneous						
Pimozide	0.03	0.93	1	0.07 ± 0.04 (2)	0.07	Current study
Amiodarone	0.09 ± 0.02	0.90 ± 0.1	2	0.46 + 0.13 (3)	0.27	Grima et al., 1986; Current study
Phenobarbitone	78	1.00	1		2600	Willow and Catterall, 1982
(±)-Propranolol	0.39	1.22	1	6 (1)	11	Grima et al., 1986; Current study
YC-1	11	0.70	1		27	Garthwaite et al., 2002

[a] Where both IC_{50} and K_I values were provided, K_I values are used.

TABLE 5
Comparative potencies of drugs for [^{3}H]BW202W92 binding at 1 mM and 100 mM K^+
Slope signifies binding slope.

Compound	IC_{50}, 1 mM K^+	Slope	IC_{50}, 100 mM K^+	Slope	$\frac{IC_{50}, 100\ mM\ K^+}{IC_{50}, 1\ mM\ K^+}$	Mean Apparent K_I for Inactivated Sodium Channels	Citation
	μM		μM			μM	
BW202W92	0.05	0.88	0.23	1.30	4.6		
BW203W92	1.5	0.91	2.4	0.9	1.6		
Sipatrigine	0.7	0.98	2.3	0.90	3.0	7	Xie and Garthwaite, 1996; Liu et al., 2003
Lamotrigine	2.6	0.80	33	1.33	12	17	Xie et al., 1995; Kuo and Lu, 1997; Liu et al., 2003
Phenytoin	14	1.21	15	1.18	1.0	13	Ragsdale et al., 1996; Kuo and Lu, 1997
Carbamazepine	45	1.33	77	1.19	1.7	29	Kuo and Lu, 1997; Bonifacio et al., 2001; Wang et al., 2002
Tetracaine	0.07	0.98	0.34	0.72	5.0	6	Li et al., 1999
Lidocaine	2.8	1.00	52	1.16	19	18	Ragsdale et al., 1996; Kuo et al., 2000
Procaine	9.8	0.77	104	1.48	11		
Procainamide	44	0.86	359	1.35	8.2		
YC-1	12	0.70	93	0.94	6.8		

nently were α- and β-scorpion venoms, which act on sites 3 and 4, respectively. Toxins acting on sites 2 and 5 (batrachotoxin and brevetoxin) were inactive at reasonable concentrations. It may not be coincidental that the sites that affect [^{3}H]BW202W92 binding (1, 3, and 4) are located extracellularly, whereas those that do not (sites 2 and 5) are intramembrane domains (Cestele and Catterall, 2000). It is conceivable that the [^{3}H]BW202W92 binding site is extracellular. This notion is inconsistent with the proposed common drug receptor site being located in the inner cavity of the pore, as has been concluded from mutational and other studies (Catterall, 2000; Cronin et al., 2003). However, it is in line with reports that phenytoin, carbamazepine and lamotrigine inhibit sodium channel function when applied extracellularly but not intracellularly (Kuo, 1998) and that mutation of a tryptophan residue located externally in the channel pore abolishes local anesthetic block (Tsang et al., 2005). It is clear that there may be more than one drug receptor on the sodium channel.

The binding site for [^{3}H]BW202W92 differs in several respects from that of other radiolabeled drugs examined so far. Binding sites in brain preparations, deemed to be on sodium channels, have been described for [^{3}H]phenytoin (Francis and Burnham, 1992), [^{3}H]tetracaine (Grima et al., 1986; Reith et al., 1987), [^{3}H]lifarizine (MacKinnon et al., 1995), [^{3}H]PD85,639 (Thomsen et al., 1993), and [^{3}H]WIN 17317-3 (Wanner et al., 1999). The two major differences are with site 1 and 3 toxins, which had no effect on any these previously described binding sites, but, respectively, enhanced and inhibited the binding of [^{3}H]BW202W92. In addition, the standard antiepileptic compounds phenytoin and carbamazepine failed to displace [^{3}H]PD85,639 and [^{3}H]WIN 17317-3 binding, whereas both were effective against [^{3}H]BW202W92 binding. The binding of [^{3}H]phenytoin is complicated by being partly on peripheral-type benzodiazepine receptors (Francis et al., 2000), and doubt has been expressed that the [^{3}H]tetracaine binding site is on sodium channels (Reith et al., 1987).

In common with many other compounds inhibiting sodium channel function, however, is an interaction of BW202W92 with site 2, as shown by its ability to inhibit [^{3}H]BTX-B binding and by compounds inhibiting [^{3}H]BW202W92 binding also inhibiting [^{3}H]BTX-B binding (Table 4). Indeed, there was a good correlation between the potencies of the 16 compounds to inhibit [^{3}H]BW202W92 binding, when assayed at 1 mM K^+, and to inhibit [^{3}H]BTX-B binding ($r = 0.87$; $P < 0.0001$). Because batrachotoxin did not (except at very high concentrations) affect the binding of [^{3}H]BW202W92, the interaction with site 2 is likely to be allosteric, as for many of the other drugs (Linford et al., 1998).

Although the [^{3}H]BW202W92 binding site is novel, the major question is whether the site has any pharmacological relevance as a target for drugs acting on sodium channels. That several chemically distinct sodium channel inhibitors interacted with the binding site at reasonable concentrations (in terms of their biological activity) favors it being a common drug target. In addition, an interesting set of data was for the subset of compounds whose potency at displacing [^{3}H]BW202W92 under polarized (1 mM K^+) and depolarized (100 mM K^+) conditions was directly compared (Table 5). As mentioned above, the relative potencies of the compounds under the two conditions varied greatly and, accordingly, there was no significant correlation between them ($r = 0.53$; $P = 0.11$). For six of the compounds studied, the apparent affinities (K_I values) for the inactivated state of sodium channels have been estimated from electrophysiological analysis (Table 5). The correlation between the potency of these compounds for displacing [^{3}H]BW202W92 binding and K_I is just significant when the binding is measured under polarized conditions ($r = 0.83$; $P = 0.04$) but greatly improves with the binding under depolarized conditions ($r = 0.98$; $P < 0.001$). Although not quantified in terms of K_I, the finding that BW202W92 was 3- to 5-fold more potent than sipatrigine and >20-fold more potent than lamotrigine at inhibiting sodium channel function (Caputi et al., 2001) fits with this correlation. Assuming that the binding under depolarized conditions is to inactivated channels, the correlation between [^{3}H]BW202W92 binding and K_I for the inactivated state(s) becomes coherent.

In conclusion, using [^{3}H]BW202W92 as a ligand, we have identified a novel stereoselective drug binding site on rat brain sodium channels that is intimately influenced by membrane voltage and by toxins acting at sites 1, 3, and 4, and which is a putative target of therapeutically important sodium channel inhibitors. The present data provide no obvious clue as to why different inhibitors acting at the same site display different therapeutic preference, for example for epilepsy, pain, or neurodegeneration. Knowledge of how [^{3}H]BW202W92 binds to different sodium channel isoforms may be helpful in this regard.

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Address correspondence to: Dr. John Garthwaite, Wolfson Institute for Biomedical Research, University College London, Gower St., London WC1E 6BT, UK. E-mail: john.garthwaite@ucl.ac.uk