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A novel drug binding site on voltage-gated sodium channels in rat brain

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Running title: Novel drug binding site on voltage-gated sodium channels

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Abbreviations

BW202W92, R-(-)-2,4-diamino-6-(flurometyl)-5-(2,3,5-trichlorophenyl)-pyrimidine; BW203W92, S-(-)-2,4-diamino-6-(flurometyl)-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; [^{3}H]BTX-B, [^{3}H]batrachotoxinin-A 20-α-benzoate; 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.

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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, BW202W92, was radiolabeled and used as a binding ligand in rat forebrain synaptosomes. Whilst 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, 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 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 was 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.

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Introduction

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 which modulate the activity of voltage-gated sodium channels show therapeutic utility as local anesthetics, anti-arrhythmics, anticonvulsants and analgesics, and further compounds are being developed for treatment of neurodegenerative and bipolar disorders (Clare *et al.*, 2000). The molecular mechanism(s) underlying these disparate therapeutic activities remain 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 whilst 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 targets 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

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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 (Liu *et al.*, 2003; Clare *et al.*, 2000). One of the most potent members of the lamotrigine family is the compound BW202W92 (R-(-)-2,4-diamino-6-(fluromethyl)-5-(2,3,5-trichlorophenyl)-pyrimidine) 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.

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Materials and Methods

Materials. BW202W92, BW203W92 (S-(-)-2,4-diamino-6-(flurometyl)-5-(2,3,5-trichlorophenyl)-pyrimidine), BW4030W92 (R-(-)-2,4-diamino-6-(fluoromethyl)-5-(2,3-dichlorophenyl)-pyrimidine) and its enantiomer BW4082W92 (S-(+)-2,4-diamino-6-(fluoromethyl)-5-(2,3-dichlorophenyl)-pyrimidine) were supplied at greater than 98.6% chiral purity by Synnovation Ltd (Epsom, UK), who also supplied BW227C89 (2,4-diamino-6-(methyl)-5-(2,6-dichlorophenyl)-pyrimidine. 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 Sciences (Beaconsfield, UK) at a specific activity of 49 Ci/mmol. [^{3}H]BW202W92 was synthesised by Amersham Life Science (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 into its enantiomers by HPLC using chiral 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 (NIH, Bethesda, Maryland, USA). Tetrodotoxin citrate, riluzole and (5*S*,10*R*)-(+)-5-methyl-10,11-dihydro-5*H*-dibenzo[a,d]cyclohepten-5,10-imine maleate (MK-801) were from Tocris Cookson Ltd (Bristol, UK) and 3-(5'-hydroxymethyl-2'-furyl)-1-benzylindazole (YC-1) was from Axxora (UK) Ltd (Nottingham, UK). All other special chemicals and toxins were obtained from Sigma-Aldrich (Poole, 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 175-250 g. All efforts were made to reduce the number of animals used and all experiments were

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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). Following killing of animals by stunning and decapitation, crude forebrain synaptosomes (heavy and light mitochondrial fraction containing synaptosomes) were prepared as described (Garthwaite *et al.*, 2002). Briefly, 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 8 up-and-down strokes of a Braun Potter S motor-driven homogenizer set to 900 r.p.m. The homogenate was centrifuged at 1036 g at 4 °C for 10 min and the supernatant collected. The remaining pellet was resuspended, as above, in fresh ice-cold 0.25 M sucrose and the centrifugation step 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 synaptosomal pellet in 20 volumes of ice-cold water using an Ultra-Turrax homogenizer. 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 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 (approx 500 µg protein) original wet weight of tissue to tubes which 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 either in buffer which 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

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corresponding changes in the concentration of choline chloride such that the molarity of the buffer remained constant. Samples were mixed, incubated for 40 min at 25 °C (unless indicated otherwise) and incubations 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 Whatman GF/C glass fibre filters using a Brandel™ cell harvester. A further 2 x 5 ml of ice-cold wash buffer was added to each tube and the vacuum filtration step repeated. The GF/C glass fibre filters containing bound [^{3}H]BW202W92 were transferred to minivials and 4 ml Picofluor^{40} liquid scintillant added using a Brandel™ deposit/dispense system. Radioactivity was measured using a Beckman Liquid Scintillation Counter and cpm 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 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.

[14C]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 4 parameter logistic equation: $y = A + B/(1+\exp(-C.(x-IC_{50})))$ where A = non-specific binding, B = specific binding and C = binding slope. Saturation binding parameters were computed by plotting specific [^{3}H]ligand concentration (measured by counting radioactivity) versus bound ligand according to the Hill equation: $y = (B_{max}[L]^n)/(K_D{}^n + [L]^n)$, where y = specifically bound ligand, [L] = [^{3}H]ligand concentration and n = Hill

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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: $y = A(1\text{-}exp(-K_{obs}.t))$, where y = specific binding and A = 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} = on-rate constant (nM^{-1} min^{-1}) and k_{-1} = off-rate constant (min^{-1}); the K_D was calculated from the ratio k_{-1}/k_{+1}. All statistical analyses used Students (2-way) t-test.

Results

To begin with, [^{3}H]BW202W92 was disappointing as a ligand in rat brain synaptosomes because the level of specific binding (displaceable by BW202W92 itself) was only about 40 % of the total (Fig. 1A,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 about 60 % (Fig. 1B). The potency of tetrodotoxin for enhancing the binding was very similar at 1 and 100 mM K^+, the EC_{50} values both being 2-3 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

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inhibited by K^+ with an IC_{50} of 5.9 ± 0.6 mM (n = 6) and a corresponding slope of 1.83 ± 0.04. Inhibition was maximal at 100 mM K^+ at which concentration total binding was reduced by about 60 %. Binding in the absence of tetrodotoxin was also partially sensitive to K^+, the IC_{50} being about 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 about -70 mV. While 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.

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 appeared to mimic the

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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), the IC_{50} for displacement by BW202W92 being 214 ± 1 nM compared with 34 ± 1 nM when the same synaptosomes were kept intact (c.f. 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.

In order 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 non-specific) [^{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 centre, 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 accumulation of [^{14}C]guanidinium ions and inhibition of [^{3}H]BTX-B binding, the S-enantiomer, BW203W92, was about 8-fold weaker as an inhibitor of sodium channels than the R-enantiomer, BW202W92 (Table 2). In the absence of

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tetrodotoxin, and with either 1 mM or 100 mM K^+, both BW202W92 and BW203W92 inhibited [^{3}H]BW202W92 binding in an apparently competitive manner since the slopes of the inhibition curves were not significantly different from 1 (Fig. 4A,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} for BW202W92 in 1 mM K^+ was greatly decreased to about 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 about 0.6 µM to about 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 (about 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 if the binding sites are similar, a direct comparison of the properties of the binding of the two ligands was made. Whilst 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.

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5A,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. 5A,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 non-competitive 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), the extrapolated IC_{50} value in the presence of tetrodotoxin being about 100 µM, whereas it was some 1000-fold more potent at displacing the binding of [^{3}H]BTX-B (Fig. 5C,D). Veratrine (another site 2 toxin) displaced the binding of both ligands but was about 10-fold more efficacious against [^{3}H]BTX-B binding (Fig. 5C,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) 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 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. Non-specific

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binding (the linear phase of the saturation isotherm) was unaffected by addition of tetrodotoxin or K^+ and was approximately 9.5 fmol/mg 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 Methods) gave Hill slopes which were not significantly different from 1, so parameters were re-computed 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 protein. Under depolarizing conditions (100 mM K^+) B_{max} was non-significantly changed (20 % reduction) whereas the K_D was lowered by a factor of 5, to about 100 nM.

Effect of antiepileptics, local anaesthetics and other drugs. To help understand the pharmacological relevance of the $[^3H]$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 inhibited the binding concentration-dependently, including lamotrigine itself (K_I = 1.8 µM) and sipatrigine (K_I = 0.4 µM). 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 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 (amongst other effects), both inhibited binding whereas the

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neuroprotectant clomethiozole, a GABA receptor agonist with no reported activity on sodium channels, was inactive at 100 µM. An assortment of other drugs previously reported 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).

Since the affinity of BW202W92 (in the presence of tetrodotoxin) was reduced by depolarizing the synaptosomes with 100 mM K^+, we examined if 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 10-20 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 of 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 assuming that tetrodotoxin is simply a pore blocker. It is important to note that agents inhibiting tetrodotoxin-dependent binding (e.g. toxins, K^+, competitive inhibitors) also had qualitatively similar effects on tetrodotoxin-independent binding, suggesting that tetrodotoxin

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does not artificially expose the binding sites but rather 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 which are converted to inactivated states (Madeja, 2000; Strichartz *et al.*, 1987). 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 appears 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 prominently were α- and β-scorpion venoms, which act on sites 3

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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). Conceivably, 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). Clearly, 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).

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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 ($R = 0.87$, $P < 0.0001$). As 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).

While the [^{3}H]BW202W92 binding site is novel, the major question is whether or not 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 6 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

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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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Footnotes

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Figure Legends

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 (filled circles) or absence (open circles) 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 nM and 8.8 nM in A and B, respectively.

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 (filled squares) or lysed (open squares). 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 tetrodotoxin (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 = 7.6 nM); errors (S.D.) are within the dimensions of the symbols.

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.

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,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,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 tetrodotoxin (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

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presence (filled symbols) of 1 µM tetrodotoxin (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-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.

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.

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Tables

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.

Toxin (K^+ concentration)	EC_{50} or IC_{50} ± S.E.M.	Binding slope ± S.E.M.	*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

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TABLE 2

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

Assay	BW202W92 IC_{50} (µM)	Binding slope	*n*	BW203W92 IC_{50} (µM)	Binding slope	*n*	Ratio[b]
[^{3}H]BW202W92 1 mM K^+ + TTX[c]	0.048 ± 0.006	0.81 ± 0.03	6	1.5 ± 0.3	0.91±0.02	4	32
[^{3}H]BW202W92 1 mM K^+	0.73 ± 0.09	0.94 ± 0.17	5	4.4 ± 1.1	1.31±0.30	4	6.0
[^{3}H]BW202W92 100 mM K^+ + TTX[c]	0.20 ± 0.02	0.95 ± 0.03	4	2.4 ± 0.6	0.91±0.01	3	12
[^{3}H]BW202W92 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]Values are means ± S.E.M. [b]Ratio of IC_{50} values for BW203W92 relative to BW202W92.

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

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TABLE 3

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

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

[a]Data are means ± S.E.M. [b]$P < 0.05$ versus value at 1 mM K^+. The concentration of tetrodotoxin was 1 µM in all cases.

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TABLE 4

Comparative drug potencies against [^{3}H]BW202W92 and [^{3}H]BTX-B binding

Compound class	Compound	BW202W92 binding K_i (μM) ± S.E.M.	BW202W92 binding slopes ± S.E.M.	*n*	[^{3}H]BTX-B binding IC_{50} (μM) ± S.E.M. (*n*)	Mean literature [^{3}H]BTX-B binding IC_{50} or K_I (μM)[a]
Lamotrigine	BW202W92	0.037 ± 0.001	0.81 ± 0.03	6	4.4 ± 0.3 (3)	4.4[b]
family	BW203W92	1.2 ± 0.2	0.91 ± 0.02	4	33 ± 9 (2)	33[b]
	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[b,c,d]
	Lamotrigine	1.8 ± 0.2	0.86 ± 0.05	5	63 (1)	121[b,e,f]
Other	Phenytoin	9 ± 1	1.06 ± 0.08	4		53[f,g,h,i,j]
anticonvulsants	Carbamazepine	36 ± 3	1.01 ± 0.17	3		374[f,h,i,j,k]
	Felbamate	>100		1		
	Valproate	>100		2		>1000[i]
	Ethosuccimide	>100		1		>1000[i]
	Gabapentin	>100		1		
	Vigabatrin	>100		1		
	Zonisamide	>100		1		

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Local	Tetracaine	0.070 ± 0.005	0.98 ± 0.02	2		1.5[j,l,m,n,o]
anesthetics	Lidocaine	1.9 ± 0.3	1.01 ± 0.03	3		127[g,h,j,l,m,n]
	Procaine	7.5 ± 0.5	0.91 ± 0.08	4		89[l,m,n,o]
	Procainamide	37 ± 2	0.88 ± 0.01	2		
Other	MK-801	2.3	1.08	1		4.8[g]
neuroprotectants	Riluzole	0.84	0.94	1		40[g]
	Clomethiazole	>100		2		
Miscellaneous	Pimozide	0.03	0.93	1	0.07 ± 0.04 (2)	0.07[b]
	Amiodarone	0.09 ± 0.02	0.90 ± 0.1	2	0.46 + 0.13 (3)	0.27[b,m]
	Phenobarbitone	78	1.00	1		2600[i]
	± propranolol	0.39	1.22	1	6 (1)	11[b,m]
	YC-1	11	0.70	1		27[i]

[a]Where both IC_{50} and K_I values are provided, K_I values are used. [b]Present study; [c]Garthwaite *et al.*, 2002; [d]Grauert *et al.*, 2002; [e]Cheung *et al.*, 1992; [f]Salvati *et al.*, 1999; [g]MacKinnon *et al.*, 1995; [h]Zimanyi *et al.*, 1989; [i]Willow and Catterall, 1982; [j]Lingamaneni and Hemmings, Jr., 2003; [k]Bonifacio *et al.*, 2001; [l]Creveling *et al.*, 1983; [m]Grima *et al.*, 1986; [n]Postma and Catterall, 1984; [o]Reith *et al.*, 1987.

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TABLE 5

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

Compound	IC_{50} 1 mM K^+ (µM)	Slope	IC_{50} 100 mM K^+ (µM)	Slope	IC_{50}, 100 mM K^+ / IC_{50}, 1 mM K^+	Mean apparent K_I for inactivated sodium channels (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[a,b]
Lamotrigine	2.6	0.80	33	1.33	12	17[b,c,d]
Phenytoin	14	1.21	15	1.18	1.0	13[d,e]
Carbamazepine	45	1.33	77	1.19	1.7	29[d,f,g]
Tetracaine	0.07	0.98	0.34	0.72	5.0	6[h]
Lidocaine	2.8	1.00	52	1.16	19	18[e,i]
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	

[a]Xie and Garthwaite, 1996; [b]Liu *et al.*, 2003; [c]Xie *et al.*, 1995; [d]Kuo and Lu, 1997; [e]Ragsdale *et al.*, 1996; [f]Bonifacio *et al.*, 2001; [g]Wang *et al.*, 2002; [h]Li *et al.*, 1999; [i]Kuo *et al.*, 2000.

Slope signifies binding slope.

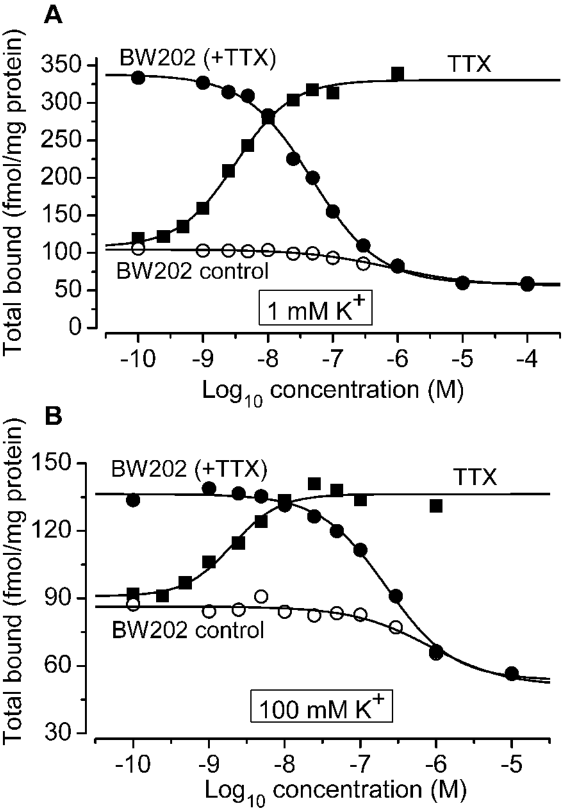
A
BW202 (+TTX)
TTX
BW202 control
1 mM K+
Total bound (fmol/mg protein)
Log10 concentration (M)
350
300
250
200
150
100
50
0
-10
-9
-8
-7
-6
-5
-4
B
BW202 (+TTX)
TTX
BW202 control
100 mM K+
Total bound (fmol/mg protein)
Log10 concentration (M)
150
120
90
60
30
-10
-9
-8
-7
-6
-5

Fig. 1

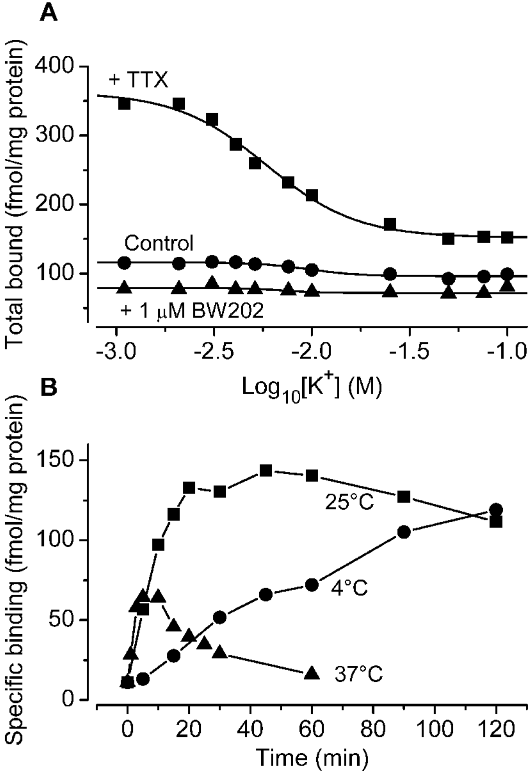
A
Total bound (fmol/mg protein)
400
300
200
100
+ TTX
Control
+ 1 μM BW202
-3.0
-2.5
-2.0
-1.5
-1.0
Log10[K+] (M)
B
Specific binding (fmol/mg protein)
150
100
50
0
25°C
4°C
37°C
0
20
40
60
80
100
120
Time (min)

Fig. 2

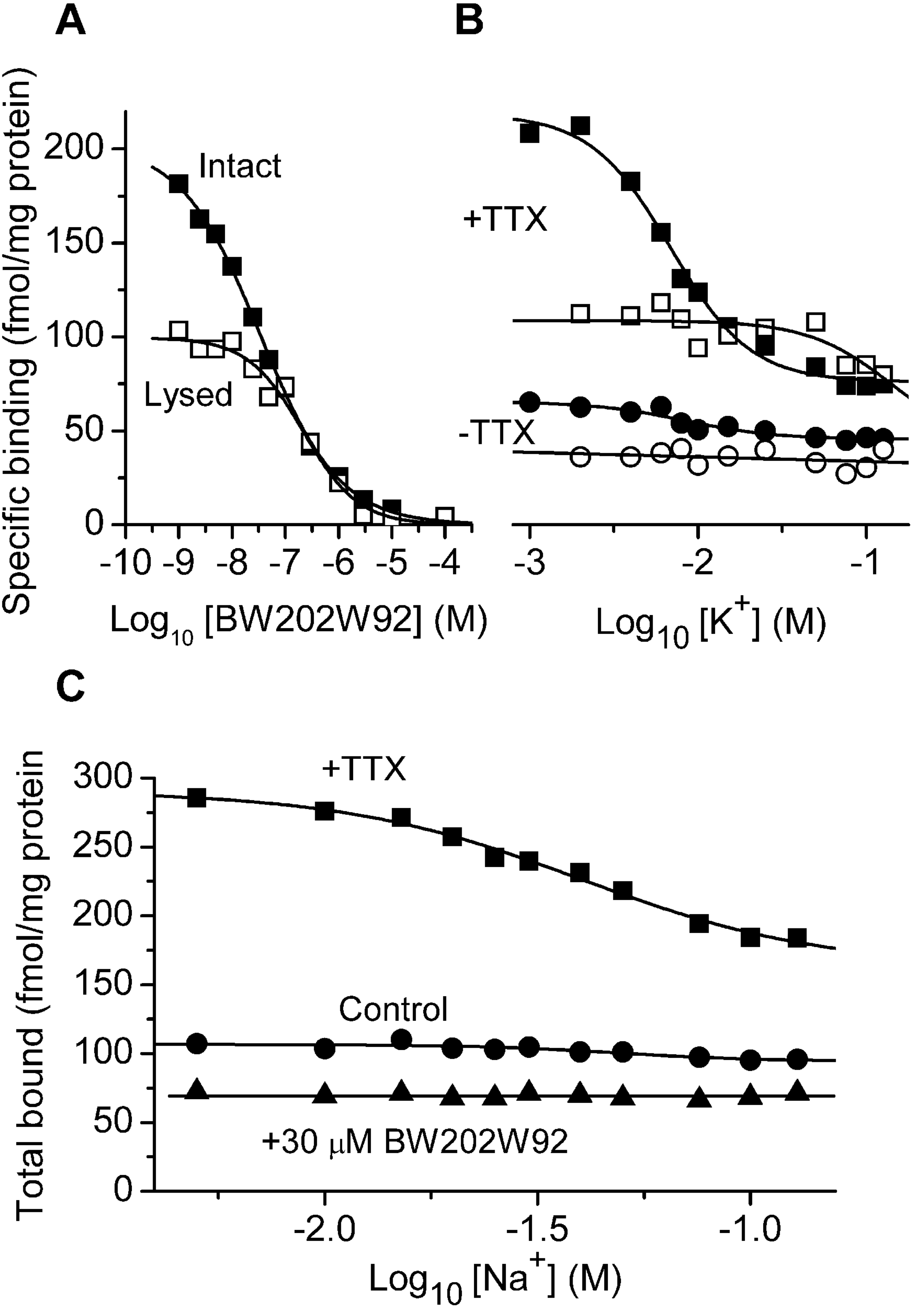
A
B
Specific binding (fmol/mg protein)
Intact
Lysed
+TTX
-TTX
Log10 [BW202W92] (M)
Log10 [K+] (M)
C
Total bound (fmol/mg protein
+TTX
Control
+30 μM BW202W92
Log10 [Na+] (M)

Fig. 3

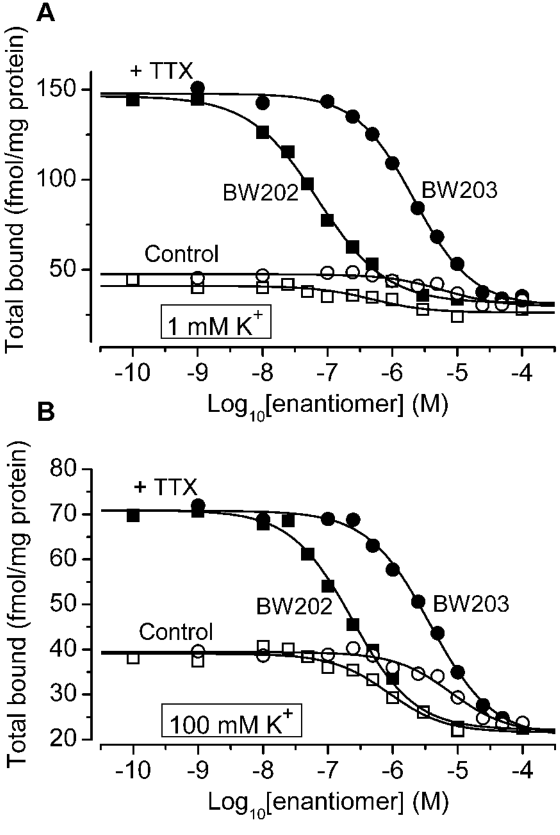
A
Total bound (fmol/mg protein)
150
100
50
+ TTX
BW202
BW203
Control
1 mM K+
-10
-9
-8
-7
-6
-5
-4
Log10[enantiomer] (M)
B
Total bound (fmol/mg protein)
80
70
60
50
40
30
20
+ TTX
BW202
BW203
Control
100 mM K+
-10
-9
-8
-7
-6
-5
-4
Log10[enantiomer] (M)

Fig. 4

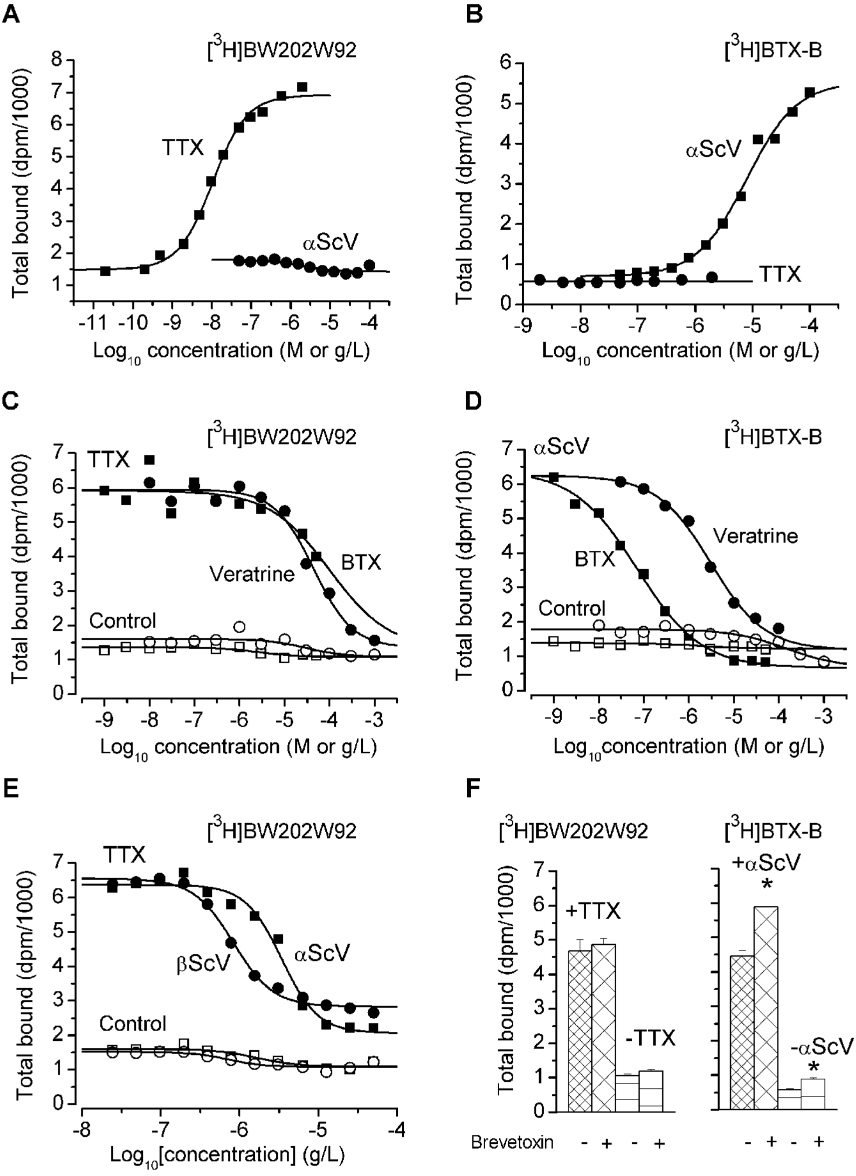
A
[3H]BW202W92
TTX
αScV
Total bound (dpm/1000)
Log10 concentration (M or g/L)
B
[3H]BTX-B
αScV
TTX
C
[3H]BW202W92
TTX
Veratrine
BTX
Control
D
αScV
[3H]BTX-B
BTX
Veratrine
Control
E
[3H]BW202W92
TTX
βScV
αScV
Control
Log10[concentration] (g/L)
F
[3H]BW202W92
[3H]BTX-B
+TTX
-TTX
+αScV
-αScV
*
Brevetoxin
- + - +

Fig. 5

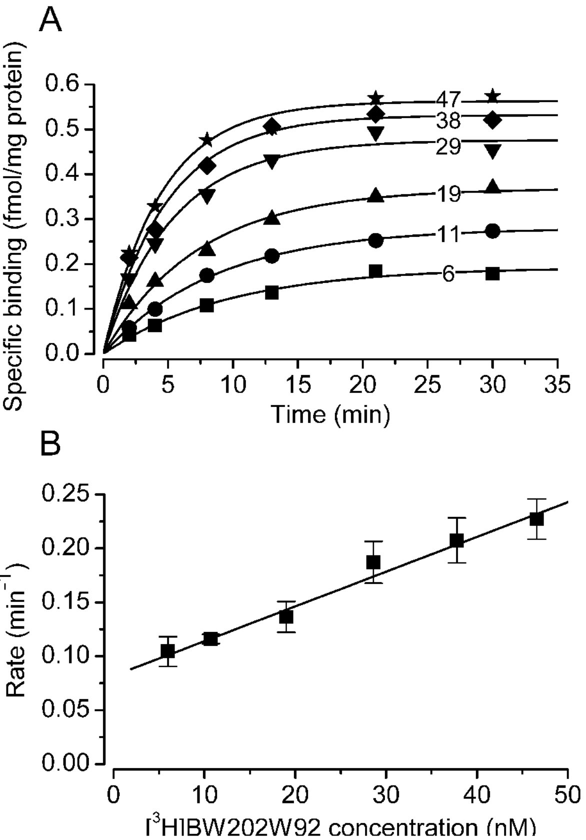
A
Specific binding (fmol/mg protein)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0
5
10
15
20
25
30
35
Time (min)
47
38
29
19
11
6
B
Rate (min^{-1})
0.00
0.05
0.10
0.15
0.20
0.25
0
10
20
30
40
50
[^{3}H]BW202W92 concentration (nM)

Fig. 6

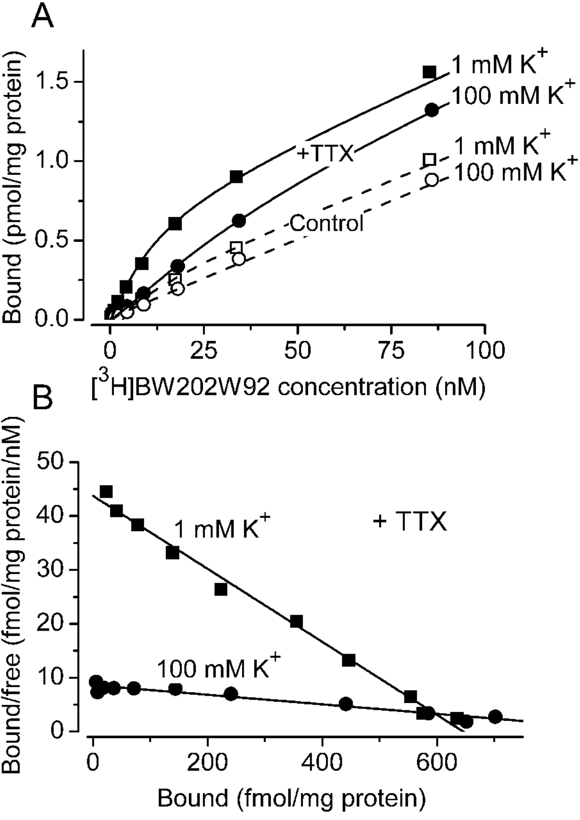
A
1 mM K+
100 mM K+
+TTX
1 mM K+
100 mM K+
Control
Bound (pmol/mg protein)
[3H]BW202W92 concentration (nM)
B
1 mM K+
+ TTX
100 mM K+
Bound/free (fmol/mg protein/nM)
Bound (fmol/mg protein)

Fig. 7