WIN 17317-3, a New High-Affinity Probe for Voltage-Gated Sodium Channels†

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ABSTRACT: The iminodihydroquinoline WIN 17317-3 was previously shown to inhibit selectively the voltage-gated potassium channels, $K_v1.3$ and $K_v1.4$ [Hill, R. J., et al. (1995) *Mol. Pharmacol. 48*, 98−104; Nguyen, A., et al. (1996) *Mol. Pharmacol. 50*, 1672−1679]. Since these channels are found in brain, radiolabeled WIN 17317-3 was synthesized to probe neuronal K_v1 channels. In rat brain synaptic membranes, [^{3}H]WIN 17317-3 binds reversibly and saturably to a single class of high-affinity sites (K_d 2.2 ± 0.3 nM; B_{max} 5.4 ± 0.2 pmol/mg of protein). However, the interaction of [^{3}H]WIN 17317-3 with brain membranes is not sensitive to any of several well-characterized potassium channel ligands. Rather, binding is modulated by numerous structurally unrelated sodium channel effectors (e.g., channel toxins, local anesthetics, antiarrhythmics, and cardiotonics). The potency and rank order of effectiveness of these agents in affecting [^{3}H]WIN 17317-3 binding is consistent with their known abilities to modify sodium channel activity. Autoradiograms of rat brain sections indicate that the distribution of [^{3}H]WIN 17317-3 binding sites is in excellent agreement with that of sodium channels. Furthermore, WIN 17317-3 inhibits sodium currents in CHO cells stably transfected with the rat brain IIA sodium channel with high affinity (K_i 9 nM), as well as agonist-stimulated ^{22}Na uptake in this cell line. WIN 17317-3 interacts similarly with skeletal muscle sodium channels but is a weaker inhibitor of the cardiac sodium channel. Together, these results demonstrate that WIN 17317-3 is a new, high-affinity, subtype-selective ligand for sodium channels and is a potent blocker of brain IIA sodium channels.

Recently, the properties of a novel, small molecule potassium channel inhibitor, termed WIN 17317-3,[1] were described in some detail. This substituted iminodihydroquinoline is a high-affinity blocker of two voltage-gated potassium channels: $K_v1.3$ (expressed heterologously or in human T-lymphocytes; K_i of 80−200 nM; *1*−*3*) and $K_v1.4$ (K_i of 300 nM; *3*). Strikingly, WIN 17317-3 is very selective among potassium channels since it blocked with much lower potency all other types of potassium channels tested, including voltage-gated (e.g., K_v1, K_v3, and K_v4 family members; $K_i > 10\ \mu$M) and calcium-activated (e.g., low and high conductance representatives; $K_i > 10\ \mu$M) channels (*3*). WIN 17317-3 inhibits binding of [^{125}I]charybdotoxin to $K_v1.3$ in human T-lymphocyte membranes and in functional studies decreases IL-2 production from activated T-lymphocytes in a dose-dependent manner ($IC_{50} \sim 1\ \mu$M), supporting the idea that $K_v1.3$ inhibitors may act as novel immunosuppressants.

In the present study, we report on the synthesis of [^{3}H]-WIN 17317-3 and its interaction with rat brain synaptic plasma membrane vesicles. Using radioligand binding techniques, we show that [^{3}H]WIN 17317-3 binds selectively and with high affinity to voltage-gated sodium channels in brain; no interaction with $K_v1.3$ or $K_v1.4$ channels is detectable. Consistent with this finding, WIN 17317-3 blocks brain IIA sodium channels in both electrophysiological and ^{22}Na flux experiments. Further binding and functional studies demonstrate that WIN 17317-3 has a similar high affinity for the skeletal muscle sodium channel but that this compound interacts more weakly with the cardiac sodium channel. WIN 17317-3, therefore, represents a new high-affinity ligand for voltage-gated sodium channels that can differentiate members of this ion channel superfamily in native tissues. A preliminary report of these findings has appeared (*4*).

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[1] Abbreviations: ATX II, sea anemone toxin II; BSA, bovine serum albumin; BTX, batrachotoxin; [^{3}H]BTX-B, [^{3}H]batrachotoxin A20 α-benzoate; CHO cells, Chinese hamster ovary cells; ChTX, charybdotoxin; DDT, dichlorodiphenyltrichloroethane; HEK, human embryonic kidney cells; MgTX, margatoxin; STX, saxitoxin; TEA, tetraethylammonium; TTX, tetrodotoxin; WIN 17317-3, 1-benzyl-7-chloro-4-(*n*-pentylimino)-1,4-dihydroquinoline hydrochloride; PEI, poly(ethylenimine).

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EXPERIMENTAL PROCEDURES

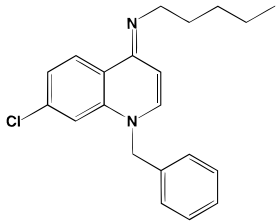
Materials. Crude venom from the scorpion *Leiurus quinquestriatus hebraeus*, tetrodotoxin (TTX), veratridine, aconitine, carbamazepine, carbamazepine 10,11-epoxide, tetraethylammonium chloride (TEA), flunarizine, cinnarizine, amiodarone, ethosuximide, and sodium valproate were purchased from Sigma (Vienna, Austria) and [^{3}H]batrachotoxin A20 α-benzoate (40−50 Ci/mmol; [^{3}H]BTX-B) and ^{22}NaCl from NEN Life Science Products (Boston, MA); BTX and sea anemone toxin II (ATX II) were generous gifts from Prof. Ernst Habermann (Giessen, Germany). Lidocaine, procaine, and quinidine were obtained from Astra (Sodertalje, Sweden) and DPI 201-106 and isradipine from Sandoz (Basel, Switzerland); BDF 8784 was from Beiersdorf (Hamburg, Germany). Phenytoin, (+)-*cis*-diltiazem, and gabapentin were provided by Goedecke (Freiburg, Germany), and phenytoin was from Gerot Pharma (Vienna, Austria). Prajmaline and ajmaline were obtained through Giullini Pharma (Hannover, Germany). Propafenone and devapamil were gifts from Knoll (Ludwigshafen, Germany), tolbutamide was from Hoechst (Frankfurt, Germany), and disopyramide was from Roussel-Uclaf (Romainville, France). Diazepam and lifarizine were obtained from Hofmann-La Roche (Basel, Switzerland, and Palo Alto, CA), and tetrandrine and correolide were provided by Merck (Rahway, NJ). Cocaine was a gift from Dr. Battista (Department of Forensic Medicine, Innsbruck, Austria). Brevetoxin A was purchased from Calbiochem (San Diego, CA). Minoxidil was obtained from Upjohn (Kalamazoo, MI), pimozide, flupentixol, mexiletine, and fluspirilene were from Janssen (Beerse, Belgium), felbamate was from AESCA (Traiskirchen, Austria), lamotrigine was from Glaxo (Stevenage, England), and PD85,639 was from Parke-Davis (Morris Plains, NJ). Pentobarbital was purchased from Merck (Darmstadt, Germany), and DDT, deltamethrin, and permethrin were from Riedel-de-Haën (Hannover, Germany).

Synthesis and Radiolabeling of WIN 17317-3. All materials that were commercially available were used without further purification. The 4-pentenylamine was prepared according to described methods (*5*).

*Synthesis of Precursors. (A) 7-Chloro-4-(4-pentenylamino)quinoline (**1**).* To a solution of 5 g (25 mmol) of 4,7-dichloroquinoline in 100 mL of ethanol was added 2.5 g (30 mmol) of 5-amino-1-pentene. The reaction was stirred at reflux for 24 h. The residue was treated with 100 mL of 1 N NaOH and extracted with three portions of ethyl acetate. The combined organic layers were washed with brine, dried over K_2CO_3, and concentrated. The residue was purified by flash chromatography on silica using 30% ethyl acetate/hexane to give 3.5 g of the title compound (58%): ^{1}H NMR ($CDCl_3$) δ 1.80 (m, 4 H), 2.20 (m, 2H), 3.30 (m, 2 H), 4.90−5.10 (m, 2H), 5.25 (s, br, 1H), 5.70−5.90 (m, 1H), 6.39 (d, 5.3 Hz, 1H), 7.32 (d, J = 8.9 Hz, 1H), 7.68 (d, J = 8.8 Hz, 1H), 7.92 (s, 1H), 9.49 (d, J = 5.4 Hz, 1H); mass spectrum (APCI) *m*/*e* 246, 248 (M + NH_4).

*(B) 1-Benzyl-7-chloro-4-(4-pentenylimino)-1,4-dihydroquinoline Hydrobromide (**2**).* To a solution of 2 g (8.1 mmol) of **1** and 1.59 g (9.7 mmol) of NaI in 25 mL of acetone was added 1.5 g (9 mmol) of benzyl bromide. The solution was stirred at reflux for 18 h and then allowed to cool to room temperature. The mixture was filtered, and the solid residue was washed with acetone. The residue was dissolved in 25 mL of hot ethanol, and the solution was made basic with 2 M NaOH. The cooled mixture was concentrated under vacuum, and the aqueous solution was partitioned between ethyl acetate and water. The aqueous layer was washed with two portions of ethyl acetate, and the combined organic extracts were washed with brine, dried over K_2CO_3, and concentrated. The oily residue was dissolved in a hot solution of HBr in ethanol. The product crystallized upon cooling at 4 °C for 18 h and then was filtered and dried to afford 1.2 g of white crystals (35%): ^{1}H NMR (DMF-d_7) δ 1.95 (m, 2H), 2.27 (m, 2H), 3.76 (m, 2H), 5.01 (d, J = 8 Hz, 1H), 5.10 (d, J = 10 Hz, 1H), 5.97 (m, 1H), 6.53 (s, 2H), 7.22(d, 7.8 Hz, 1H), 7.35−7.46 (m, 5H), 7.83 (d, J = 7.8 Hz, 1H), 8.24 (s, 1H), 9.01−9.07 (m, 2H); mass spectrum (APCI) *m*/*e* 354, 356 (M + NH_4).

Table 1: Inhibition of [^{3}H]WIN 17317-3 Binding to Rabbit Skeletal Muscle Microsomal Membranes



compound	IC_{50} (nM)	slope
WIN 17317-3	3.14	0.83
aconitine	90000	0.75
flunarizine	22	0.67
phenytoin (stimulation)	32000	1.36
lifarizine	42	0.69

*Hydrogenation Procedures. (A) 1-Benzyl-7-chloro-4-(pentylimino)-1,4-dihydroquinoline Hydrobromide (WIN 17317-3; **3**; Table 1*). A solution of 5 mg of **2** in methylene chloride was shaken with 8 mg of tris(triphenylphosphine)rhodium-(I) chloride (Wilkinson's catalyst) in polystyrene-bound beads under 40 psi of H_2 for 8 h. The sample was monitored by HPLC (1:1 0.1 N NaOAc:CH_3CN, Zorbax RX-C_{18}, 4.6 mm × 25 cm) until the reduction was complete: **2**, R_f = 7.6 min; **3** R_f = 10.2 min (flow = 1.1 mL/min). The catalyst was removed by filtration and the solution evaporated to give a residue that was purified by HPLC to give 4.5 mg of **3** as the acetate salt. The sample was converted to the HBr salt in two steps as described for **2**: ^{1}H NMR (DMF-d_7) δ 0.90 (t, 7.5 Hz, 3H), 1.40 (m, 4H), 1.74 (m, 2H), 3.62 (m, 2H), 6.10 (s, 2H), 7.21 (d, 7.8 Hz, 1H), 7.31−7.70 (m, 5H), 7.78 (d, J = 7.8 Hz, 1H), 8.21 (s, 1H), 9.07 (m, 1H), 9.20 (m, 1H); mass spectrum (APCI) *m*/*e* 356, 358 (M + NH_4). This product was identical by HPLC and ^{1}H NMR spectra to WIN 17317-3 prepared by starting from pentylamine.

(B) [^{3}H]WIN 17317-3. A solution of 5 mg of **2**, DMF (0.8 mL), and resin-bound Wilkinson's catalyst (10 mg) was degassed and stirred at room temperature under 422 mmHg of carrier-free tritium gas. The reaction was stopped after 4.5 h, and any unreacted tritium gas was returned to the storage tank. The reaction mixture was passed through a syringeless filtering device, and the catalyst residue was washed with methanol (3 × 20 mL). The filtrate was concentrated under reduced pressure and purified by HPLC chromatography (Zorbax RX-C_{18}, acetonitrile:water:TFA, 30:70:0.1). The product peak was concentrated and further

purified by passing through a Waters C_{18} Sep-Pak cartridge system. A total of 23 mCi of 98% radiochemically pure [^{3}H]-WIN 17317-3 was obtained. The specific activity of [^{3}H]-WIN 17317-3 was 28.5 Ci/mmol.

[^{3}H]WIN 17317-3 Binding to Plasma Membranes Preparations. Rat brain synaptosomal plasma membrane vesicles were prepared as previously described (*6*). Purified sarcolemmal membranes were obtained from porcine cardiac muscle (*7*) or rabbit skeletal muscle (*8*) using established procedures. Binding assays were initiated by the addition of membrane protein (25−35 μg of protein) to an assay buffer consisting of 20 mM Tris-HCl (pH 7.4), 0.1% bacitracin, 1−1.5 nM [^{3}H]WIN 17317-3, and various concentrations of test drugs (1 mL final assay volume). Nonspecific binding was defined in the presence of 1 μM WIN 17317-3, and incubations were carried out at 22−25 °C, typically for 120 min. All serial drug dilutions were performed using DMSO. A final DMSO concentration of 1% was never exceeded in the binding reaction, and this concentration was without any effect on [^{3}H]WIN 17317-3 binding. Bound ligand was separated by single point filtration of the sample through Whatman GF/C filters presoaked for 30 min in 0.3% poly(ethylenimine) (PEI). The filters were washed three times with 3 mL of ice-cold filtration buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Triton-X100), and filter-retained radioactivity was determined by liquid scintillation measurements.

[^{3}H]BTX-B Binding to Rat Brain Synaptosomal Plasma Membranes. The interaction of [^{3}H]BTX-B with rat brain membranes was measured as described (*9*, *10*). Nonspecific binding was determined in the presence of 100 μM aconitine. Incubations were carried out for 60 min at 37 °C before the reaction was terminated by filtration of the sample through GF/C filters presoaked in 0.5% PEI for at least 60 min. Samples were washed three times with 3 mL of ice-cold buffer consisting of 10% (w/v) poly(ethylene glycol) 6000, 10 mM Tris-HCl, pH 7.4, and 10 mM $MgCl_2$ before radioactivity was determined by liquid scintillation techniques.

Analysis of Binding Data. Radioligand binding studies were analyzed as described previously to determine equilibrium and kinetic ligand binding parameters (*6*). Data from competition experiments were fit by computer to the general dose−response equation (*11*). Protein concentration was determined using bovine serum albumin (BSA) as a protein standard (*12*).

Receptor Autoradiography. Male Spraque-Dawley rats (250−300 g) were sacrificed by cervical dislocation; their brains were rapidly removed and placed for 90 s in isobutane, chilled to −40 °C. Thereafter, the brains were stored for 30 min at −30 °C in a sealed vial. Sections (20 μm) were cut on a cryostat microtome (Leitz, Germany) and thaw-mounted onto gelatin-coated slides. Sections were labeled in 118 mM NaCl, 4.8 mM KCl, 1.3 mM $MgSO_4$, 1.2 mM $CaCl_2 \cdot 2H_2O$, 1.2 mM KH_2PO_4, 15 mM $NaHCO_3$, 10 mM Tris-HCl, pH 7.4, 50 mM glucose, and 0.1% BSA for 1 h at 22 °C at a K_d concentration of [^{3}H]WIN 17317-3 (2−3 nM). Nonspecific binding was determined in a series of adjacent sections by inclusion of 5 μM WIN 17317-3. Thereafter, the sections were rinsed for 1 h in ice-cold 20 mM Tris-HCl, pH 7.4, and 150 mM NaCl and then rapidly dried in a stream of cold air. Autoradiograms were obtained by exposing the sections to a sheet of film (Hyperfilm-^{3}H, Amersham) for 10−15 days and developed using Kodak D-19 developer.

^{22}Na Flux Experiments. CHO cells stably expressing the rat brain IIA sodium channel α-subunit (*13*) were obtained from W. A. Catterall, University of Washington, Seattle (*14*). HEK cells stably expressing the human heart sodium channel, hH1a, were provided by H. Hartmann, Baylor College of Medicine, Houston, TX (*15*). ^{22}Na uptake studies were carried out essentially as previously described (*16*) with some minor modifications: cells were seeded in 96-well plates at a density of ∼100 000 cells/well 24 h before the experiment was performed. Cells were washed twice with 200 μL of uptake medium (buffer A) consisting of 10 mM HEPES/Tris-HCl, pH 7.5, 140 mM choline chloride, 5.6 mM KCl, 1.8 mM $MgCl_2$, 0.8 mM $CaCl_2$, 10 mM glucose, 1 mM ouabain, and 1 mg/mL BSA. Buffer A containing various drugs with 2 mM NaCl (^{22}Na at 0.1 μCi/well, 50 uL assay volume) was added, and the cell layer was washed 8 min later. Cells were washed three times with 200 μL of 10 mM HEPES/Tris-HCl, pH 7.5, 163 mM choline chloride, 1.8 mM $MgCl_2$, and 0.8 mM $CaCl_2$, and the cell-trapped radioactivity was determined by liquid scintillation measurements to assess the initial rate of ^{22}Na uptake.

Electrophysiological Studies. Voltage-clamp studies were conducted by employing the whole cell voltage clamp technique using a Dagan 3900A amplifier (Dagan Instruments, Minneapolis, MN) with CHO cells stably expressing rat brain IIA sodium channels. The pipet solution consisted of (in mM) sodium glutamate, 100; NaCl, 20; NaF, 20; $MgCl_2$, 1; $CaCl_2$, 0.9; HEPES, 10; and BAPTA, 11; the solution was adjusted to pH 7.2. The extracellular solution consisted of (in mM) CsCl, 150; NaCl, 5; $MgCl_2$, 0.5; $CaCl_2$, 1.8; and either HEPES or CAPS, 10; for experiments at pH 7.5 or 9.8, respectively. WIN 17317-3 was diluted from a stock solution of 2 mM drug in DMSO. Experiments were conducted at 21−24 °C. Data were acquired using the program Pulse, and most analysis was performed with the companion program Pulsefit (Instrutech Instruments, Great Neck, NY). Linear leak and capacity currents were subtracted by P/5 steps from −100 mV. Data were sampled at 50 or 100 kHz and filtered (f_c, −3 db) at 10 kHz.

RESULTS

Binding of [^{3}H]WIN 17317-3 to Membranes Prepared from Native Tissues. Exposure of rat brain synaptosomal membranes to increasing concentrations of [^{3}H]WIN 17317-3 resulted in concentration-dependent association of ligand with this preparation (Figure 1A). In the presence of 1 μM WIN 17317-3, radioactivity associated with membranes was a linear function of ligand concentration, and at a K_d concentration of both radioligand and receptor, it was less than 5% of total binding. Specific binding, defined as the difference between total and nonspecific binding, is saturable, and a Scatchard analysis of these data indicates the presence of a single class of binding sites with a K_d of 2.2 ± 0.3 nM and a B_{max} of 5.4 ± 0.2 pmol/mg of protein ($n = 4$). In agreement with equilibrium saturation experiments, unlabeled WIN 17317-3 displaced binding of its tritiated congener with a K_i value of 1.3 nM and a pseudo-Hill slope of 1.0 (Figure 1B). Identical experiments were performed with porcine cardiac sarcolemmal membranes, but no specific [^{3}H]WIN

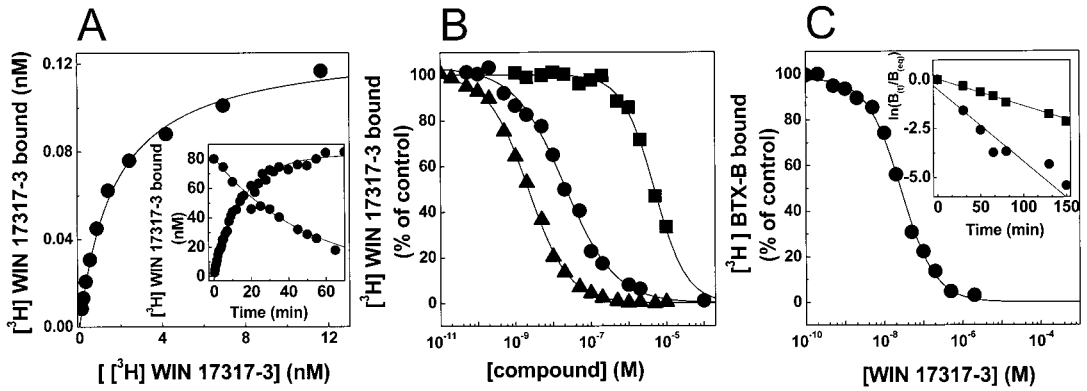


FIGURE 1: Binding of [^{3}H]WIN 17317-3 and [^{3}H]BTX to rat brain synaptosomal membrane vesicles. (A) Saturation binding analysis. Rat brain membrane vesicles were incubated with increasing concentrations (0.015−12 nM) of [^{3}H]WIN 17317-3. Specific binding was assessed from the difference between total and nonspecific binding. For this experiment, a K_d of 1.7 nM and a B_{max} of 4.5 pmol/mg of protein were measured. Inset: Kinetic studies. Association kinetics: 0.26 nM of rat brain synaptic membrane receptor was incubated with 1.2 nM [^{3}H]WIN 17317-3 for the indicated periods of time. An association rate constant (k_{+1}) of 2.33×10^5 M^{-1} s^{-1} was measured. Dissociation kinetics: After rat brain membranes were incubated with 1.2 nM [^{3}H]WIN 17317-3 for 2 h at 22 °C, dissociation was initiated by dilution for the indicated periods of time. In this experiment, a dissociation rate constant (k_{-1}) of 4×10^{-4} s^{-1} was determined. (B) Modulation of [^{3}H]WIN 17317-3 binding to rat brain synaptosomal plasma membrane vesicles. All binding assays were performed as described in Experimental Procedures. Displacement curves were fit by computer to the general dose−response curve. The corresponding IC_{50} values and pseudo-Hill slopes are given in parentheses: WIN 17317-3 (▲, $IC_{50} = 2.2$ nM, $n_H = 0.86$); amiodarone (●, $IC_{50} = 20$ nM, $n_H = 0.75$); BTX (■, $IC_{50} = 4.8$ μM, $n_H = 1.04$). (C) Effect of WIN 17317-3 on [^{3}H]BTX binding. Binding assays were performed as described in Experimental Procedures. The mean IC_{50} value of two to three independent experiments is given in Results; $IC_{50} = 25.6$ nM, $n_H = 0.96$. Inset: Dissociation kinetics. After rat brain membranes were incubated with [^{3}H]BTX-B for 2 h at 22 °C, dissociation was initiated by addition of 100 μM aconitine alone (■) or 100 μM aconitine together with 3 μM WIN 17317-3 (●) for the indicated periods of time. [^{3}H]BTX-B dissociation occurred as a monoexponential function in the control with a $t_{1/2}$ of 55 min ($k_{-1} = 1.8 \times 10^{-4}$ s^{-1}) and in the presence of WIN 17317-3 with a $t_{1/2}$ of 12 min ($k_{-1} = 9.2 \times 10^{-4}$ s^{-1}), respectively.

17317-3 binding was observed in this preparation. In skeletal muscle microsomes, [^{3}H]WIN 17317-3 bound with a K_d of 9.4 ± 1.3 nM and a B_{max} of 9.1 ± 1.3 pmol/mg of protein ($n = 3$; not shown). Unlabeled WIN 17317-3 displaced binding of ligand to skeletal muscle membranes with a K_i value of 3.1 ± 1.1 nM (Table 1).

The kinetics of [^{3}H]WIN 17317-3 binding to rat brain synaptic plasma membranes were measured to determine whether ligand association occurs through a simple bimolecular reaction. The data presented in Figure 1A (inset) indicate that incubation of [^{3}H]WIN 17317-3 with rat brain membranes results in time-dependent ligand association that approached equilibrium after ca. 120 min at room temperature. Subjecting these data to transformation according to the second-order rate equation yields a linear dependence, and the slope of this line gives k_{+1}. The mean association rate constant, k_{+1}, is 3.36×10^5 M^{-1} s^{-1} ($n = 3$). [^{3}H]WIN 17317-3 dissociation, as initiated by dilution, displays a simple monoexponential relationship with a $t_{1/2}$ of ca. 29.5 min (k_{-1} 3.9×10^{-4} s^{-1}; Figure 1A, inset). The K_d calculated from these kinetic constants (k_{-1}/k_1) is 1.16 nM, a value in good agreement with that determined under equilibrium binding conditions (2.2 nM).

Pharmacological Characterization of [^{3}H]WIN 17317−3 Binding. To determine whether [^{3}H]WIN 17317-3 binding in brain or skeletal muscle membranes occurs in potassium channels, we tested several well-characterized potassium channel ligands (e.g., ChTX, MgTX, TEA, correolide, tolbutamide, and minoxidil) in the binding reaction. None of these agents had any effect on [^{3}H]WIN 17317-3 binding, even when tested at saturating concentrations (data not shown). In striking contrast, sodium channel site 2 ligands did affect [^{3}H]WIN 17317-3 binding to rat brain membranes (BTX, $IC_{50} = 4.8$ μM, Figure 1B; aconitine, $IC_{50} = 8.2$ μM, Table 2; veratridine, $IC_{50} = 8.4$ μM, Table 2). Site 1 (TTX), 3, and 5 ligands (e.g., the α-scorpion toxins, sea anemone toxin 2, and brevetoxin A, all at 1 μM) had no effect on binding (Table 2), while site 6 ligands (e.g., permethrin, deltametrin, and DDT) inhibit binding weakly with apparent IC_{50} values >50 μM (Table 2). To determine whether the binding site labeled by WIN 17317-3 is reciprocally coupled to the sodium channel site 2, [^{3}H]BTX-B was used as the sodium channel ligand, and the effects of WIN 17317-3 on this binding reaction were investigated. WIN 17317-3 completely inhibited [^{3}H]BTX-B binding with an IC_{50} value of 25.6 nM (Figure 1C). Experiments using lifarizine and phenytoin served as controls in the [^{3}H]BTX-B binding protocol. Each control ligand displaced [^{3}H]BTX-B completely with IC_{50} values of 86 nM and 97 μM, respectively, in good agreement with their previously reported IC_{50} values for inhibiting binding of this radioligand (48.9 nM and 20.4 μM, respectively; *17*). The kinetics of [^{3}H]BTX-B dissociation were measured in the absence or presence of WIN 17317-3 as a way of determining whether the interaction between the two ligands is competitive or allosteric (Figure 1C, inset). Under control conditions, [^{3}H]BTX-B dissociation, initiated by the addition of aconitine, occurred as a monoexponential function with a $t_{1/2}$ of 53 min ($k_{-1} = 2.2 \times 10^{-4}$ s^{-1}), while in the presence of both aconitine and WIN 17317-3, ligand dissociation was significantly increased ($t_{1/2} = 13$ min, $k_{-1} = 8.7 \times 10^{-4}$ s^{-1}). This pattern is consistent with the idea that WIN 17317-3 decreases [^{3}H]BTX-B affinity for the sodium channel through a negative allosteric mechanism affecting ligand dissociation.

The effects of various antiarrhythmic compounds, cardiotonic agents, local anesthetics, anticonvulsants, voltage-gated calcium channel modulators, and the neuroprotectant, lifarizine, on [^{3}H]WIN 17317-3 binding to rat brain synaptosomal plasma membranes were also investigated (see Table 2). Lifarizine ($IC_{50} = 4.8$ nM) was one of the most potent

Table 2: Inhibition of [^{3}H]WIN 17317−3 Binding to Rat Brain Synaptosomal Membrane Vesicles

compound	IC_{50} (nM)	slope	compound	IC_{50} (nM)	slope
sodium channel effectors			calcium channel modifiers		
TTX	>10000	nd[a]	(±)-devapamil	112	0.83
α-scorpion toxin	>1000	nd	(+)-tetrandrine	365	0.72
sea anemone toxin II	>100	nd	(+)-*cis*-diltiazem	17000	0.84
brevetoxin	>100	nd	flunarizine	4.8	0.96
BTX	4800	1.04	cinnarizine	4.1	0.67
aconitine	8200	0.82	pimozide	4.4	0.62
veratridine	8400	1.02	fluspirilene	19.7	0.50
permethrin	50000	1.41	flupentixol	943	0.61
DDT	>50000	1.43	local anesthetics		
antiarrhythmic agents			PD 85.639	220	0.84
amiodarone	20	0.75	cocaine	7700	0.78
propafenone	120	0.79	lidocaine	61000	0.81
prajmaline	770	1.01	procaine	280000	0.85
quinidine	1000	0.85	antiepileptic and neuroprotective agents		
ajmaline	2700	0.83	valproate	>100000	nd
mexiletine	4800	0.78	ethosuccimide	>100000	nd
disopyramide	40000	1.3	pentobarbital	>100000	nd
cardiotonic and calcium channel modifiers			diphenylhydantoin	>100000	nd
(±)-DPI 201−106	4.8	0.82	carbamazepine	>100000	nd
(+)-DPI 201−106	6.4	0.82	diazepam	>100000	nd
(−)-DPI 201−106	5.4	0.80	phenytoin (stimulation)	3000	0.87
(±)-BDF 8784	4.6	0.68	lamotrigene	90000	nd
isradipine	>1000	1.97	gabapentin	>100000	nd
(−)-devapamil	71	0.77	felbamate	>100000	nd
(+)-devapamil	175	0.88	lifarizine	4.8	0.81

[a] Not determined.

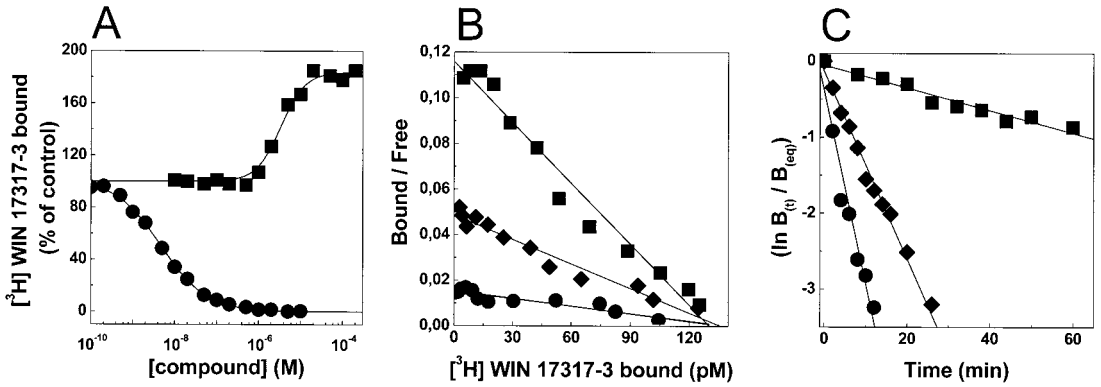


FIGURE 2: Allosteric modulation of [^{3}H]WIN 17373-3 binding in rat brain synaptosomal plasma membranes. (A) Displacement curves. [^{3}H]WIN 17317-3 binding to rat brain synaptosomal plasma membrane vesicles was measured in the absence or presence of different compounds as described in Experimental Procedures: phenytoin (■, EC_{50} = 3.0 μM, n_H = 1.81, maximal stimulation = 180% of control binding); (±)-DPI 201-106 (●, IC_{50} = 4.8 nM, n_H = 0.82). (B) Equilibrium saturation experiments. Rat brain membranes were incubated with increasing concentrations (0.06−15.7 nM) of [^{3}H]WIN 17317-3. Specific binding was assessed from the difference between total and nonspecific binding. For this experiment, a K_d of 2.8 nM was measured under control conditions, and specific binding data (◆) are presented in the form of a Scatchard representation. Data derived from coincubation with 20 nM DPI 201-106 (●, K_d = 9.6 nM) or 20 μM phenytoin (■, K_d = 1.31 nM) are also shown. (C) Dissociation kinetics. [^{3}H]WIN 17317-3 dissociation was initiated by addition of either 3 μM WIN 17317-3 (◆, $k_{-1} = 2.67 \times 10^{-3}$ s^{-1}) or 3 μM WIN 17317-3 together with 1 μM DPI 201-106 (●, $k_{-1} = 5.3 \times 10^{-3}$ s^{-1}) or with 100 μM phenytoin (■, $k_{-1} = 5 \times 10^{-4}$ s^{-1}). A semilogarithmic representation of each first-order dissociation reaction is presented.

inhibitors of binding, as was the antiarrhythmic agent, amiodarone (IC_{50} = 20 nM; Figure 1B). The cardiotonic sodium channel agonist, DPI 201-106 (*18*−*21*), represents an additional class of high-affinity inhibitor of [^{3}H]WIN 17317-3 binding (IC_{50} = 4.8 nM; Figure 2A). This compound caused a 3.4-fold decrease in radioligand affinity (Figure 2B), most likely due to increase in the rate of the [^{3}H]WIN 17317-3 dissociation noted in the presence of DPI 201-106 (Figure 2C). These data indicate that DPI 201-106 is a negative allosteric modulator of the [^{3}H]WIN 17317-3 binding reaction. The local anesthetics PD 85.639, cocaine, lidocaine, and procaine inhibited [^{3}H]WIN 17317-3 binding completely with IC_{50} values of 220 nM, 7.7 μM, 61 μM, and 280 μM, respectively (Table 2). These inhibition constants are in excellent agreement with those previously reported for inhibition of [^{3}H]BTX-B binding in this tissue (*17*, *22*−*25*).

A number of antiepileptic drugs (for reviews see refs *26* and *27*) were also evaluated for their effects on [^{3}H]WIN 17317-3 binding in rat brain membranes (Table 2). However, only lamotrigene and phenytoin, for which direct binding to voltage-gated sodium channels has previously been demonstrated (*28*), had an effect on the binding reaction. Phenytoin stimulated [^{3}H]WIN 17317-3 binding up to 196 ± 8% of control values (EC_{50} = 3 μM, n = 3; Figure 2A, Table 2), and this effect was due to a 2-fold increase in radioligand affinity (Figure 2B). Phenytoin caused a decrease of 5.3-fold in the kinetics of [^{3}H]WIN 17317-3 dissociation Figure 2C), consistent with its modulation of ligand binding through a positive allosteric interaction.

To investigate whether the molecular pharmacology of the binding site for WIN 17317-3 in brain is unique to neuronal membranes, [^{3}H]WIN 17317-3 binding to rabbit skeletal muscle microsomal membranes was also monitored (Table 1). The sodium channel agonist, aconitine, displaced ligand binding to skeletal muscle membranes with an IC_{50} of 90 μM, while lifarizine was also ca. 10-fold weaker as an inhibitor of [^{3}H]WIN 17317-3 binding in this tissue (IC_{50} = 42 nM) as compared to brain. Similarly, the stimulatory effect of phenytoin on [^{3}H]WIN 17317-3 binding was 10-fold weaker than that observed in brain membranes (Table 1). The results of these and saturation binding experiments suggest that WIN 17317-3 interacts with neuronal and skeletal muscle sodium channel isoforms by similar mechanisms but that several modulators of voltage-gated sodium channels show selectivity for neuronal vs skeletal muscle channels.

To complete the pharmacological characterization of [^{3}H]-WIN 17317-3 binding in rat brain synaptic membranes, the effects of various voltage-gated calcium channel modulators were also examined in the binding reaction (*29*, *30*). Except for dihydropyridines, many of these compounds completely inhibited ligand binding, displaying a wide range of potencies in blocking the binding reaction. The respective inhibitory properties of these compounds (IC_{50} and slope values) are listed in Table 2.

Distribution of [^{3}H]WIN 17317-3 Binding Sites in Rat Brain. A second line of evidence for a selective interaction of [^{3}H]WIN 17317-3 with voltage-gated sodium channels in rat brain is provided by the distribution of this compound's binding sites. The location of sites that are labeled by [^{3}H]-WIN 17317-3 correlates exactly with that of either [^{3}H]TTX or [^{3}H]STX binding sites in rat brain sections (*31*−*33*). [^{3}H]-WIN 17317-3 binding sites are present in all brain areas (Figure 3), although they are predominant in regions with dense axodendritic synaptic connections. The cerebral cortex, the hippocampal formation, and the substantia nigra, *pars reticulata* expressed a high site density of [^{3}H]WIN 17317-3 binding, and strikingly, the most intense autoradiographic staining was observed in the cerebellar molecular layer.

Inhibition of Voltage-Gated Sodium Channels by WIN 17317-3. A third, independent line of evidence for an interaction between WIN 17317-3 and voltage-gated sodium channels was obtained from functional studies using either ^{22}Na flux or electrophysiological protocols to monitor channel activity. Stable expression of rat brain IIA and human heart hH1a sodium channels has previously been described (*14*, *15*, *34*). Basal influx of ^{22}Na in both cell lines is very low. ^{22}Na influx (TTX blockable uptake of ^{22}Na as measured by both initial rate and steady-state accumulation) can be stimulated 2−3-fold by either veratridine or deltamethrin. ATX II is only weakly active. However, each agonist will stimulate ^{22}Na uptake to a larger degree when the experiment is carried out in the presence of the other two agents. Thus, as shown in Figure 4A, ATX II and deltamethrin, each assayed with a fixed concentration of the other two agonists present, stimulate uptake of ^{22}Na in a concentration-dependent manner, albeit with different efficacies. Veratridine functions similarly when monitored with ATX II (300 nM) and deltamethrin (100 μM) present; EC_{50} = 30 μM (data not shown). To achieve near-maximal rates of ^{22}Na accumulation, all three agonists were combined, and this condition was used to assess the ability of WIN 17317-3 to inhibit sodium channel activity. As a control, TTX completely suppressed ^{22}Na uptake mediated by addition of ATX II, veratridine, and deltamethrin, displaying the expected selectivity with IC_{50}'s of 5.6 nM and 4.5 μM for rat brain IIA and human heart sodium channels, respectively (Figure 4B). WIN 17317-3 also blocked agonist-activated ^{22}Na influx with respective IC_{50} values of 750 nM and 9.7 μM for neuronal and cardiac sodium channels (n = 8, Figure 4B), while no effect was observed on basal ^{22}Na influx in these cell lines. Under such conditions, other known voltage-gated sodium channel blockers (e.g., phenytoin) were also effective in blocking agonist-stimulated ^{22}Na influx (not shown).

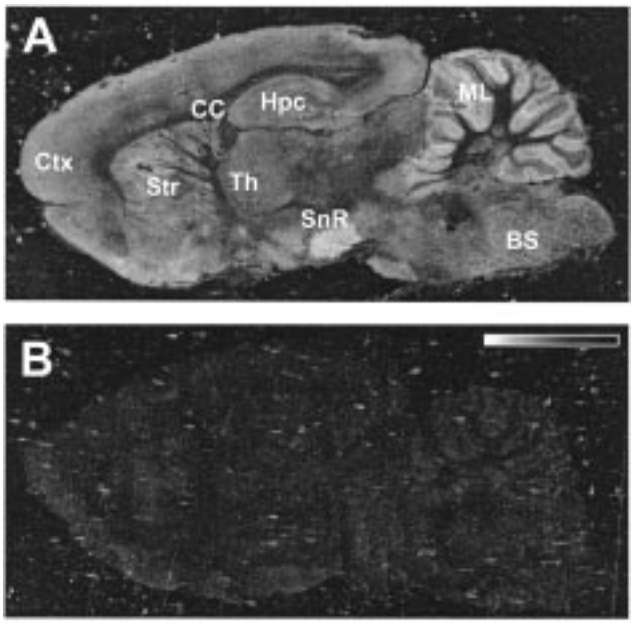


FIGURE 3: Distribution of [^{3}H]WIN 17317-3 binding in rat brain. Autoradiographic localization of [^{3}H]WIN 17317-3 binding sites in sagittal cryostat sections of rat brain. Sections were incubated with [^{3}H]WIN 17317-3 (2−3 nM) in the absence (A) or presence (B) of nonradiolabeled WIN 17317-3. The dark areas denote high grain densities, indicating high binding site densities. Regions of the brain are labeled as follows: Ctx, cortex; Str, striatum; Th, thalamus; CC, corpus callosum; Hpc, hippocampus; SnR, substantia nigra, *pars reticulata*; ML, cerebellar molecular layer; BS, brain stem.

The potency of WIN 17317-3 is substantially lower as a blocker of ^{22}Na flux than would be predicted by binding studies. This difference may be due to a number of factors, such as the nonequilibrium nature of the uptake assay or sodium channel activators decreasing potency of WIN 17317-3 through allosteric interactions. To determine whether potency is decreased by including channel stimulators in this protocol, experiments were repeated after the concentration of each stimulator was reduced by 3-fold. In rat brain IIA expressing cells, the IC_{50} of WIN 17317-3 was not altered. Attempts to further reduce the stimulator concentrations employed were unsuccessful due to collapse of the ^{22}Na influx signal. However, the potency of WIN 17317-3 was increased ca. 10-fold by preincubating these cells with inhibitor for 30 min before addition of the agonist cocktail and ^{22}Na. Similar results were obtained with the human heart hH1a cell line. These data demonstrate that WIN 17317-3 can inhibit voltage-gated sodium channel activity in agonist-mediated flux assays but that its potency

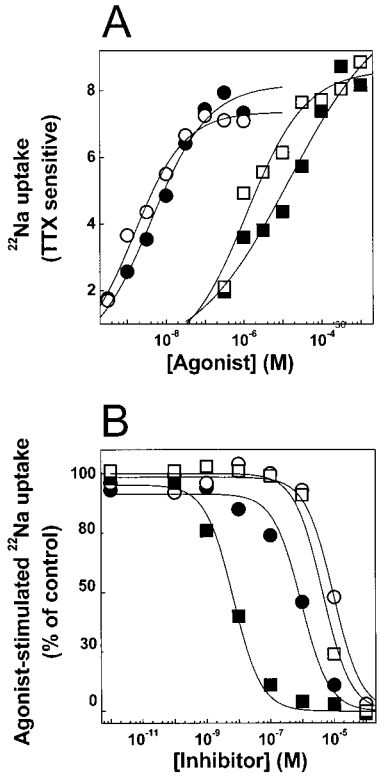


FIGURE 4: Effect of WIN 17317-3 on ^{22}Na uptake through rat brain IIA and human cardiac hH1a sodium channels. (A) Pharmacology of ^{22}Na flux. Uptake of ^{22}Na was monitored as described under Experimental Procedures. The concentration dependence for stimulation of ^{22}Na uptake (initial rates, determined at an 8 min time point; TTX-sensitive accumulation) was determined for ATX II (in the presence of 100 μM veratridine and 100 μM deltamethrin; ●, EC_{50} = 4 nM) or deltamethrin (in the presence of 100 μM veratridine and 300 nM ATX II; ■, EC_{50} = 20 μM) in CHO cells expressing rat brain IIA sodium channels. In parallel experiments, the respective values for stimulating ^{22}Na uptake in HEK cells expressing human heart hH1a sodium channels were determined for ATX II (○, EC_{50} = 1.5 nM) or deltamethrin (□, EC_{50} = 2 μM) using the same conditions described above. (B) Inhibition of ^{22}Na fluxes by WIN 17317-3. Channel stimulator (300 nM ATX II, 100 μM deltamethrin, and 100 μM veratridine; used in combination) mediated ^{22}Na uptake (TTX-sensitive window) was monitored in either the absence or presence of WIN 17317-3. The IC_{50} values determined for inhibition by WIN 17317-3 from an average of eight separate experiments were 750 nM for cells expressing neuronal IIA sodium channels (●) and 9.7 μM for cells expressing human heart hH1a sodium channels (○). The respective values for inhibition of ^{22}Na flux by TTX from an average of eight separate experiments are 5.6 nM for cells expressing brain IIA sodium channels (■) and 4.5 μM for cells expressing human heart hH1a sodium channels (□).

in such experiments is clearly protocol dependent.

CHO cells stably transfected with the rat brain IIA sodium channel display TTX-sensitive sodium currents. Electrophysiological experiments were designed to detect high-affinity sodium channel block consistent with the [^{3}H]WIN 17317-3 binding studies. Potent block of rat brain IIA Na channels by WIN 17317-3 is observed at an extracellular pH of 9.8 (Figure 5). A high pH was tested because the p*K* of this compound is ca. 10.5 (K. M. Rupprecht, unpublished observations). Local anesthetics are often inactive as extracellular cations but active when they are applied intracellularly or when the neutral form of the molecule can partition into the cell membrane (*35*). A significant fraction of WIN 17317-3 will be neutral only at high extracellular pH, and this condition will facilitate membrane penetration of inhibitor in acutely performed experiments. Figure 5A shows the onset of block by 100 nM drug when the holding potential (V_h) is −70 mV; ca. 90% of the current is blocked. Drug block was partially reversed by holding at more negative potentials. Figure 5B shows the steady-state availability for the same experiment that is recorded in Figure 5A. Drug was allowed to equilibrate for 5 s at each voltage before each test pulse. The solid lines in panel B of Figure 5 indicate the best fit by a Boltzmann equation; the midpoint potential of this distribution is shifted −10 mV to more negative voltages by 100 nM drug. The effects of drug on channel availability are similar to those of lidocaine and other local anesthetics and can be accounted for by a form of the modulated receptor theory which postulates that drug binds more potently to inactivated channels than to channels in the rested state (*35*, *36*). The apparent binding constant for the inactivated state (K_I) is determined by

$$K_I = D/[(1 + D/K_R)\exp(-\Delta V_h/k) - 1]$$

where K_I and K_R are the dissociation constants for the inactivated and rested states, respectively, *D* is the concentration of drug, ΔV_h is the shift in midpoint potential, and *k* is the mean slope factor. K_R is calculated from the amount of block at very negative holding potentials for which all channels are in the rested state. For the experiment shown in Figure 5B, K_R = 310 nM and K_I = 9.3 nM. The block shown in Figure 5A is consistent with this value of K_I. The value for binding to the inactivated channel state compares favorably to the binding constant measured in membranes which are depolarized and are, therefore, expected to contain sodium channels which are fully inactivated. There is no significant effect of pH on equilibrium [^{3}H]WIN 17317-3 binding, as monitored upon long-term incubations, in the range 6.5−10 (n = 4, not shown). Interestingly, when agonist-mediated ^{22}Na influx experiments of the type described in Figure 4B are repeated at pH 9.0 with either rat brain IIA or human heart hH1a cells, the potency of WIN 17317-3 is increased 10-fold (not shown). Therefore, in two independent protocols that acutely measure sodium channel activity, raising the pH of the incubation medium increases the apparent inhibitory potency of WIN 17317-3.

DISCUSSION

WIN 17317-3 is a previously described, selective, high-affinity blocker of the voltage-gated potassium channels, K_v1.3 and K_v1.4. In the present study, interaction between this drug and rat brain membranes has been characterized. [^{3}H]WIN 17317-3 binds saturably and reversibly and with very good signal-to-noise ratio to a single class of binding sites that display the pharmacological signature of voltage-gated sodium channels. Similar data were obtained with skeletal muscle but not with cardiac sarcolemmal membranes. Strikingly, [^{3}H]WIN 17317-3 does not appear to interact with potassium channels in either rat brain, heart, or skeletal muscle membrane preparations.

Several lines of evidence indicate a selective interaction of WIN 17317-3 with voltage-gated sodium channels in rat brain and rabbit skeletal muscle membranes. The density of sodium channels in these preparations, as measured by independent methods (*37*, *38*), correlates with the density

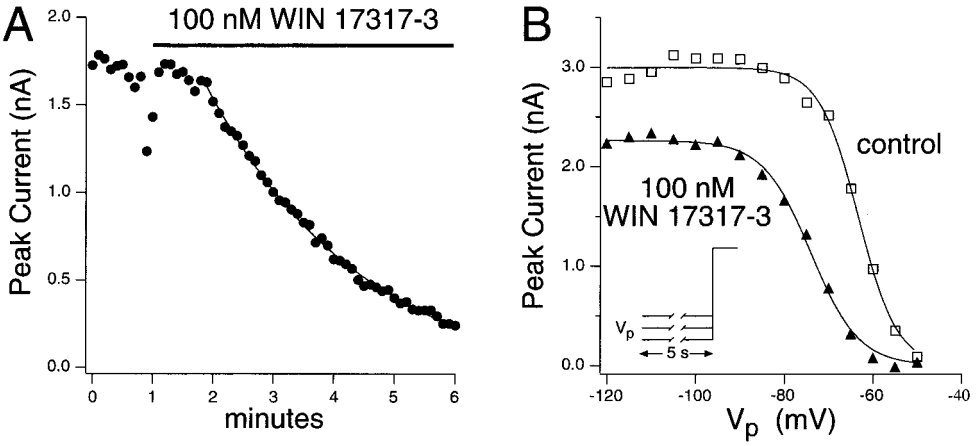


FIGURE 5: Effect of WIN 17317-3 on rat brain IIA sodium channels. (A) Time course of channel block. CHO/IIA cells were voltage clamped as described in Experimental Procedures. WIN 17317-3 (100 nM) was applied to the bath, and sodium currents were recorded with time by stepping from a holding potential of −70 to 0 mV. The pH of the bath solution was 9.8. (B) Steady-state voltage dependence of the block by WIN 17317-3. Drug binding was allowed to equilibrate for 5 s at each voltage before a test pulse was applied. Peak current is plotted vs prepulse potential (V_p): □, control; ▲, 100 nM WIN 17317-3. See inset for pulse protocol. The pH of the bath solution was 9.8.

of [^{3}H]WIN 17317-3 binding sites. For example, in brain the B_{max} of [^{3}H]WIN 17317-3 binding sites is virtually identical to that reported for [^{3}H]STX, suggesting that [^{3}H]-WIN 17317-3 binds with a 1:1 stoichiometry to voltage-gated sodium channels. WIN 17317-3 blocks binding of the well-characterized sodium channel ligand, [^{3}H]BTX-B, through an allosteric interaction. In addition, many well-known sodium channel effectors modulate [^{3}H]WIN 17317-3 binding; some function through allosteric mechanisms. The distribution of [^{3}H]WIN 17317-3 sites in brain mirrors that of voltage-gated sodium channels as ascertained using either [^{3}H]TTX or [^{3}H]STX binding protocols. In functional experiments, WIN 17317-3 blocks rat brain IIA sodium channels expressed in CHO cells in either flux or electrophysiological paradigms. Remarkably, no high-affinity [^{3}H]-WIN 17317-3 binding was observed to voltage-gated sodium channels in cardiac sarcolemmal membranes. This suggests that WIN 17317-3 posseses selectivity for neuronal and skeletal muscle sodium channels over the cardiac channel isoform. Consistent with this postulate, WIN 17317-3 is ca. 10-fold less potent as an inhibitor of agonist-mediated ^{22}Na uptake into HEK cells expressing the human cardiac channel. Together, these data indicate that WIN 17317-3 is a novel high-affinity probe for voltage-gated sodium channels and that it can differentiate members of this ion channel superfamily.

In brain, WIN 17317-3 potently inhibits binding of [^{3}H]-BTX-B, a ligand which is routinely used to identify unknown sodium channel effectors. Interaction between WIN 17317-3 and site 2 of the sodium channel appears to be allosteric because WIN 17317-3 accelerates [^{3}H]BTX-B dissociation 4-fold over control values. Conversely, all sodium channel site 2 ligands tested (e.g., batrachotoxin, veratridine, aconitine) inhibit [^{3}H]WIN 17317-3 binding to rat brain membranes with IC_{50}'s in the low micromolar range, values close to reported affinities of these compounds for the sodium channel (*10*). Similar data were obtained for [^{3}H]WIN 17317-3 binding to skeletal muscle membranes. However, [^{3}H]WIN 17317-3 binding in brain is not affected by scorpion venom (which contains site 3 selective toxins), the site 5 toxin, brevetoxin A, or the site 1 ligand, TTX. This latter finding is consistent with a known lack of effect of TTX on binding of many different ligands to rat brain sodium channels (e.g., [^{3}H]BTX-B, *10*; [^{125}I]ATX II, *39*; [^{3}H]-lifarizine, *17*; [^{3}H]phenytoin, *28*; [^{3}H]PD85,639, *25*).

There is good correlation between effects of many structurally distinct sodium channel modulators (e.g., anticonvulsants, antiarrhythmics, local anesthetics, and cardiotonics) in terms of their ability to displace [^{3}H]BTX-B and their efficacy as modulators of [^{3}H]WIN 17317-3 binding. For example, binding of both [^{3}H]WIN 17317-3 and [^{3}H]-BTX-B is displaced by local anesthetics with an identical rank order of potency (i.e., PD 85,639 > quinidine > cocaine > lidocaine) and similar IC_{50} values (*24*, *25*, *40*). Furthermore, many neuroprotective agents (e.g., lifarizine, flunarizine, and cinnarizine) demonstrate potent interactions in both binding assays, yielding low nanomolar IC_{50} values (*17*).

The effect of various calcium channel modulators was also investigated on [^{3}H]WIN 17317-3 binding in brain. Respective IC_{50} values of these agents in blocking binding are 10−100-fold less potent when compared with their ability to interact with L-type calcium channels. Recently, evidence has accumulated suggesting that many L-type calcium channel modifiers are also capable of interacting with voltage-gated sodium channels (*41*−*44*). Given structural similarities between these two classes of ion channels, it is expected that some crossover in pharmacology might occur.

Distribution of [^{3}H]WIN 17317-3 binding in rat brain is nearly identical to that reported for either [^{3}H]TTX or [^{3}H]-STX binding in rat, mouse, and human brain (*31*−*33*). Strikingly, all brain regions identified by either [^{3}H]TTX, [^{3}H]STX, or an anti-sodium channel antibody (*45*) are also specifically labeled by [^{3}H]WIN 173717-3, whereas regions that are devoid of voltage-gated sodium channels did not yield any detectable binding signal for this radioligand. The distribution profile of [^{3}H]WIN 17317-3 binding sites is not matched by established regional expression of K_v1.3 or K_v1.4 subunits. Overall expression of K_v1.3 in mammalian brain is very low, while K_v1.4 is not expressed in cerebellum (*46*, *47*). Together, these findings support the lack of interaction of WIN 17317-3 with voltage-gated potassium channels in brain and suggest that, under our experimental conditions,

WIN 17317-3 binds almost exclusively to voltage-gated sodium channels in this tissue.

Conclusive evidence for interaction of WIN 17317-3 with voltage-gated sodium channels comes from ^{22}Na flux and electrophysiological studies employing cell lines stably expressing sodium channel α-subunit isoforms. WIN 17317-3 dose-dependently inhibited agonist-stimulated ^{22}Na influx in cells expressing rat brain IIA and human cardiac hH1a channels. Potency against brain channels (IC_{50} of 750 nM) is ca. 10-fold greater than that against cardiac channels. This inhibition constant is of higher value than the K_d determined from direct [^{3}H]WIN 17317-3 binding to rat brain membranes. However, a cocktail of sodium channel agonists was employed in ^{22}Na flux studies to prevent rapid sodium channel inactivation; one of these agents, veratridine, strongly inhibits binding of WIN 17317-3. Furthermore, inhibition of flux is monitored relatively soon after addition of inhibitor, while binding of WIN 17317-3 requires some time to achieve equilibrium. Indeed, preincubation of cells with inhibitor increases potency in flux protocols. Moreover, WIN 17317-3 most likely exhibits state-dependent channel block; different channel states are present in polarized cells (an agonist-stabilized open channel) and depolarized membranes (inactivated channels), making it difficult to compare IC_{50} and K_d values. It is probable that such flux assays underestimate intrinsic affinity of WIN 17317-3 for sodium channels. Since the predominant isoform of sodium channels in rat brain synaptic membranes is IIA, inhibition of these channels under voltage clamp is another way of comparing potency in ligand binding and functional studies. It was observed that the affinity of drug for sodium channels in certain electrophysiological protocols is very close to the K_d measured for [^{3}H]-WIN 17317-3 binding. Block develops slowly when <1 μM drug is applied to these cells in acute electrophysiological measurements made at physiological pH, and it is difficult to separate block from slow run-down of channel activity that often occurs in the absence of any added drug (not shown). However, potent and rapid block is observed when the neutral form of the drug is formed at high extracellular pH. A similar increase in WIN 17317-3 potency has been observed in flux studies at elevated pH. The main limitation to quantification of sodium current inhibition is that it is unclear whether WIN 17317-3 is active as a neutral molecule or as a cation acting at the intracellular surface. If drug is functioning as a neutral molecule, then effective concentration with 100 nM drug at pH 9.8 is only 16 nM, so that K_R is ca. 50 nM and K_I is ca. 2 nM. Fast onset of block at high pH favors that WIN 17317-3 is acting as a potent neutral molecule to block rat brain IIA sodium channels. WIN 17317-3 has also been reported to inhibit skeletal muscle sodium channels in voltage clamp experiments (*48*).

Although WIN 17317-3 was initially discovered as an inhibitor of the voltage-gated potassium channels, K_v1.3 and K_v1.4, results presented in this study indicate that this compound does not bind to these channels in rat brain membranes at all. This lack of interaction of WIN 17317-3 with neuronal K_v1 channels could be caused by low site density, low ligand affinity, or a combination of such factors. The density of brain K_v1 channels, as determined with the newly identified K_v1 family specific ligand, [^{3}H]dihydrocorreolide (diTC), is within the same range as that of sodium channels (*49*). These channels consist mainly of heteromultimeric structures containing K_v1.1, K_v1.2, and K_v1.4 subunits, with a small contribution of K_v1.3. Although [^{3}H]WIN 17317-3 binds to membranes prepared from cells expressing K_v1.3 channels with a K_d of 2 nM (M. L. Garcia, unpublished observations), the affinity of this ligand for heteromeric channels consisting of WIN 17317-3 sensitive and insensitive subunits has not been determined. However, several lines of evidence (M. L. Garcia, unpublished observations) suggest that the WIN 17317-3 interaction with these heteromeric channels must be of low affinity: (a) diTC binding to K_v1.3 and K_v1.4, but not brain, is inhibited with high affinity (K_i of 10 nM) by WIN 17317-3; (b) binding of peptidyl inhibitors to K_v1.3, but not brain, is inhibited with high affinity (K_i of 2 nM) by WIN 17317-3. Thus, it is possible that the presence of more than one WIN 17317-3 insensitive subunit in a heteromeric K_v1 channel forms the basis for lack of binding of this ligand to K_v1 channels in brain, despite a sufficient receptor density. The same phenomenon may explain why [^{3}H]WIN 17317-3 binding is not detected to K_v1.4 channels in heart or skeletal muscle membrane preparations. A prediction from the current studies is that the density of homomeric K_v1.3 and K_v1.4 channels is very low in brain; previously presented immunoprecipitation results employing K_v1 selective antibodies and solubilized brain synaptic membranes are consistent with this idea (*49*). Data presented in the present study further show that neither correolide, the high-affinity K_v1.3 channel probe MgTX (*50*), nor any other potassium channel ligand tested has any effect on [^{3}H]WIN 17317-3 binding to rat brain membranes. These considerations, taken together with the observations that displacement of [^{3}H]WIN 17317-3 binding by numerous established sodium channel modifiers in brain is always complete, occurs in a strict monophasic fashion, and displays a rank order of potency expected for an interaction with sodium channels (based on inhibitory activities in [^{3}H]BTX-B or [^{3}H]lifarizine binding experiments; *17*, *24*), suggest that [^{3}H]WIN 17317-3 binds with high affinity only to sodium channels in brain.

In summary, the results presented in this study are consistent with [^{3}H]WIN 17317-3 binding selectively to voltage-gated sodium channels in brain and skeletal muscle membrane preparations. WIN 17317-3 displays perhaps the highest affinity for IIA sodium channels of any other previously described synthetic small molecule and appears to define a novel binding site on this channel for potent modulators. [^{3}H]WIN 17317-3 binding could be useful as a diagnostic procedure for identifying agents which interact with sodium channels or as a means of mapping these channels in native tissues. In addition, after complete evaluation of its therapeutic potential, this compound may serve as a template for design of a new generation of sodium channel inhibitors that may ultimately lead to therapeutically superior sodium channel blocking drugs. Further experiments will be required to determine to what extent this ligand can serve as a lead structure in guiding the development of a chemically novel series of compounds for neuroprotective, anticonvulsant, or analgesic (*51*, *52*) applications.

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