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Specific Binding of the Novel Na^+ Channel Blocker PD85,639 to the α Subunit of Rat Brain Na^+ Channels

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SUMMARY

The local anesthetic-like Na^+ channel-blocking drug [^{3}H]PD85639 [α-([4-^{3}H]phenyl)-*N*-[3-(2,6-dimethyl-1-piperizinyl)-α-propyl][4-^{3}H]benzeneacetamide] binds specifically to receptor sites on Na^+ channels in intact synaptosomes and synaptosomal membranes, purified and reconstituted Na^+ channels, and type IIA Na^+ channel α subunits expressed in the transfected Chinese hamster ovary cell line CNaIIA-1. No specific binding was observed in nontransfected CHO-K1 cells, confirming the specificity of binding to Na^+ channels. Two classes of binding sites that differed in affinity and dissociation rate were observed in all three preparations. In synaptosomes, the high affinity sites had K_d values of 3–20 nM and a B_{max} of approximately 0.2 pmol/mg, whereas the low affinity sites had K_d values of 0.4–20 μM and a B_{max} of approximately 5 pmol/mg. Binding of PD85,639 was inhibited by the local anesthetics tetracaine, bupivacaine, and mepivacaine at concentrations in the same range as those that inhibit Na^+ channels. Tetracaine did not affect the dissociation rate of PD85,639, consistent with competitive binding of these two drugs at the same receptor site. In contrast, binding of PD85,639 was unaffected by the anticonvulsants phenytoin and carbamazepine, which also inhibit Na^+ channels. Veratridine and batrachotoxin, which bind at neurotoxin receptor site 2 on Na^+ channels, inhibited specific PD85,639 binding completely. PD85,639 accelerated dissociation of specifically bound batrachotoxin, consistent with an indirect allosteric interaction between these two compounds. Thus, like local anesthetics, PD85,639 inhibits binding of batrachotoxin by an allosteric mechanism. The results indicate that PD85,639 binds specifically to a local anesthetic receptor site on the Na^+ channel α subunit that is allosterically linked to neurotoxin receptor site 2. PD85,639 may be a useful molecular probe of this important drug receptor site on the Na^+ channel.

The voltage-dependent Na^+ channel is an integral plasma membrane protein responsible for the increase in Na^+ permeability during the rapidly rising phase of the action potential of excitable cells (1). Na^+ channels isolated from rat brain consist of three nonidentical glycoprotein subunits, a 260-kDa α subunit that is covalently attached to a 33-kDa β2 subunit and noncovalently associated with a 36-kDa β1 subunit (2). Four different rat brain α subunit cDNAs, which have >85% amino acid sequence identity, have been isolated (3, 4). mRNA encoding the α subunit of the rat brain Na^+ channel alone can direct the synthesis of functional Na^+ channels in *Xenopus* oocytes (3, 4) and mammalian somatic cells (5, 6).

Local anesthetics are a class of clinically important drugs that reversibly block the propagation of action potentials in excitable membranes. Their action has been ascribed to general membrane perturbation or to direct interaction with a receptor site on the Na^+ channel (reviewed in Ref. 7). Electrophysiological studies of the voltage- and frequency-dependent block of Na^+ channels by local anesthetics have provided persuasive but indirect evidence that the locus of local anesthetic action is the Na^+ channel protein itself (7). The locus of local anesthetic action must be on the α subunit, because Na^+ currents in transfected cells that express only the α subunit of the rat brain Na^+ channel are subject to voltage- and frequency-dependent block by local anesthetic-like drugs, as in neurons (8).

The observed voltage- and frequency-dependent block of Na^+ currents by tertiary amine local anesthetics has been analyzed in terms of two different models of local anesthetic action. According to the modulated receptor model, local anesthetic drugs have different affinities for distinct Na^+ channel conformational states and cause conformational transitions resulting in voltage- and frequency-dependent Na^+ channel block (7, 9). According to the guarded receptor hypothesis, local anesthetic drugs do not distinguish different Na^+ channel conformations but channel conformation controls the accessibility to the drug of the local anesthetic receptor residing on the cytoplasmic side of the channel pore (7, 10). Electrophysiological evidence for single (9, 11, 12) and multiple (13, 14) local anesthetic receptors

ABBREVIATIONS: BTx, batrachotoxin; CHO, Chinese hamster ovary; STx, saxitoxin; TTx, tetrodotoxin; PbTx, brevetoxin; BSA, bovine serum albumin; BTx-B, batrachotoxinin A 20α-benzoate; LqTx, *L. quinquestriatus quinquestriatus* toxin V; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

on Na^+ channels has been reported. These issues concerning the molecular nature of the local anesthetic receptor on the Na^+ channel remain to be resolved using alternate experimental approaches.

Biochemical evidence also supports the hypothesis that the locus of local anesthetic action is the Na^+ channel. Local anesthetics inhibit veratridine- and BTx-stimulated $^{22}Na^+$ influx into rat brain synaptosomes (15–18). These neurotoxins persistently activate Na^+ channels by binding to neurotoxin receptor site 2 (19). Local anesthetics also allosterically inhibit [^{3}H]BTx binding to Na^+ channels in synaptosomes (20, 21). A more direct biochemical approach to study the Na^+ channel local anesthetic receptor is to characterize binding of radiolabeled local anesthetics to various Na^+ channel preparations. This approach may allow identification of the local anesthetic receptor site by photoaffinity labeling and peptide mapping. However, a lack of suitable high affinity radiolabeled ligands has made such biochemical studies difficult. Two laboratories have characterized the binding of [^{3}H]tetracaine to rat brain synaptosome preparations (22–24), but conclusive evidence that [^{3}H]tetracaine specifically binds to Na^+ channels rather than other membrane components was not presented. Compound PD85,639, a potent inhibitor of veratridine-activated Na^+ channels (25), blocks Na^+ currents mediated by type IIA Na^+ channel α subunits expressed in a somatic cell line in a frequency- and voltage-dependent manner, like local anesthetics (26). In this study, we have characterized the binding of [^{3}H]PD85,639 [α-([^{3}H]phenyl)-*N*-[3-(2,6-dimethyl-1-piperizinyl)-α-propyl][^{3}H]benzeneacetamide] to rat brain synaptosomes, synaptosomal membranes, purified rat brain Na^+ channels reconstituted into phospholipid vesicles, and membranes prepared from CHO cells transfected with a cDNA encoding the type IIA α subunit of the Na^+ channel. Our results indicate that [^{3}H]PD85,639 specifically binds to a Na^+ channel local anesthetic receptor site and is a suitable high affinity ligand to directly study this receptor.

Experimental Procedures

Materials. PD85,639 was prepared by catalytic reductive exchange of the paradibrominated analog. The specific activity was approximately 45 Ci/mmol. [^{3}H]STx (20–40 Ci/mmol) was prepared as described previously (27) or was purchased from Amersham Corp. [^{3}H]BTx-B (40–50 Ci/mmol) was purchased from New England Nuclear (Boston, MA). TTx was purchased from Calbiochem (La Jolla, CA). BTx was a gift from John Daly (Laboratory of Biooganic Chemistry, National Institutes of Health, Bethesda, MD). *Leiurus quinquestriatus quinquestriatus* venom was purchased from Latoxan (Rosans, France) and LqTx was purified as described previously (28, 29). *Centruroides suffusus suffusus* toxin IV was a gift from Dr. Herve Rochat (Laboratory of Biochemistry, University of Marseille, Marseille, France). PbTx-2 and PbTx-3 (from the dinoflagellate *Ptychodiscus brevis*) were gifts from Dr. Daniel Baden (Department of Biochemistry and Molecular Biology, University of Miami, Miami, FL). Etidocaine, prilocaine, tocainide, tetracaine, mepivacaine, and bupivacaine were gifts from Astra Pharmaceutical Products, Inc. (Worcester, MA). Verapamil and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

[^{3}H]PD85,639 binding to rat brain synaptosomes and synaptosomal membranes. Rat brain synaptosomes were prepared as described (30) and stored at −80° until needed. Synaptosomal membranes were prepared by homogenization of synaptosomes in 5 mM Tris buffer, pH 8.8, stirring for 45 min on ice, centrifugation at 15,000 × *g* for 30 min at 4°, and resuspension of the pellet in ice-cold standard assay buffer containing 130 mM choline chloride, 50 mM HEPES-Tris, 5.5 mM glucose, 0.8 mM $MgSO_4$, 5.4 mM KCl, and 1 mg/ml BSA, adjusted to pH 9.0 by addition of 10 N NaOH or Tris base. Binding assays were initiated by the addition of 250–500 μg of synaptosomes or synaptosomal membrane protein (10–20 μl) to 160–170 μl of standard assay buffer containing 2.35–2.5 nM [^{3}H]PD85,639 (2 nM final concentration) and 20 μl of the indicated concentrations of drugs. The assays were performed at pH 9.0 to increase specific binding (see Results). Reactions were incubated for 30 min at 25° with agitation. The incubation was terminated by dilution of the reaction mixture with 4 ml of ice-cold wash buffer containing 163 mM choline chloride, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 5 mM HEPES-Tris, pH 7.4, and 1 mg/ml BSA and filtering over GF/C glass fiber filters under vacuum. The filters were washed four more times with ice-cold wash buffer. Filters were counted by scintillation counting in 10 ml of Ecolume scintillation fluid. Nonspecific binding was determined in the presence of 100 μM unlabeled PD85,639 and accounted for 10–25% of total binding. Approximately 75% of nonspecific binding was to filters alone. Filter washes were carried out as close to 0° as possible and were complete within 20 sec. Nevertheless, it is likely that up to 25% of the low affinity component of specific PD85,639 binding may have been lost during filtration, considering its half-time for dissociation of 0.7–1.0 min at 4° (see Results).

[^{3}H]STx binding to rat brain synaptosomes. [^{3}H]STx saturation isotherms for binding to Na^+ channels in rat brain synaptosomes were measured as described previously (31). Briefly, 200–250 μg of protein (10 μl) were added to 190 μl of standard assay buffer, pH 7.4, containing the indicated concentrations of [^{3}H]STx. The reaction was incubated for 30 min at 25° and bound [^{3}H]STx was measured as described for [^{3}H]PD85,639 binding to synaptosomes. Nonspecific binding was determined in the presence of 1 μM TTx and was 5–10% of the total binding. The B_{max} for STx in this preparation was approximately 2.7 pmol/mg of protein.

[^{3}H]BTx-B binding to rat brain synaptosomes. [^{3}H]BTx-B binding to rat brain synaptosomes was quantitated as described previously (19). The binding assay was initiated by the addition of 200–250 μg of synaptosome protein (10 μl) to 170 μl of standard assay buffer, pH 7.4, containing 11.8 nM [^{3}H]BTx-B (10 nM final concentration), 1.1 μM TTx, 0.35 μM LqTx, 1 mg/ml BSA, and 20 μl of the indicated concentrations of drugs. Incubations were performed for 60 min at 37°. Filtration and filter washes were performed as described for [^{3}H]PD85,639 binding to synaptosomes. Nonspecific binding was determined in the presence of 300 μM veratridine and was 10–20% of total binding.

[^{3}H]PD85,639 binding to purified Na^+ channels reconstituted into phospholipid vesicles. Rat brain Na^+ channels purified through the step of chromatography on wheat germ agglutinin-Sepharose (32), at concentrations of 200–300 pmol/ml, were reconstituted into phosphatidylcholine/phosphatidylethanolamine vesicles as described previously (33). Binding assays were initiated by addition of 50–100 μl of vesicles to 50–100 μl of standard assay buffer containing 4–8 nM [^{3}H]PD85,639 (2–4 nM final concentration), 1 mg/ml BSA, and the indicated concentrations of drugs. The reaction mixture was incubated for 45 min at 25°. Measurement of bound ligand was carried out as described for synaptosome binding except that GF/F filters were used instead of GF/C filters. Nonspecific binding was determined in the presence of 100 μM PD85,639 and was 15–30% of total binding.

Cell culture and cell membrane preparation. CNaIIA-1 cells were derived from a CHO cell line (CHO-K1; American Type Culture Collection) transfected with the vector ZEM2580 containing a cDNA encoding the rat brain type IIA Na^+ channel α subunit. The vector places the Na^+ channel cDNA under the control of the mouse metallothionein promoter and the vector also contains a neomycin resistance gene, which confers resistance to the antibiotic G418. A more detailed description of this cell line and cell culture methods are given elsewhere (6, 8).

For membrane preparations, cells were scraped from Petri dishes, transferred to a glass homogenizer, and homogenized by 30 strokes with a Teflon pestle (4°) in 50 mM Tris buffer, pH 7.4. Disrupted cells were then centrifuged at 1000 × *g* for 10 min (4°), the supernatant was saved, and the pellet was resubjected to homogenization and centrifugation as described above. Combined supernatants were then centrifuged at 30,000 × *g* for 45 min (4°) and the resultant pellet was resuspended in standard assay buffer, pH 7.4, without BSA. Membranes were used the same day they were prepared.

PD85,639 binding to cell membranes. [^{3}H]PD85,639 binding to CHO-K1 and CNaIIA-1 cell membranes was performed as described for synaptosomes and synaptosomal membranes except that 300–600 μg of membrane protein were added to each tube. The Na^+ channel density in CNaIIA-1 cells is approximately 100 fmol/mg of cell protein, as measured by STx binding at 20 nM. Nonspecific binding was determined in the presence of 100 μM PD85,639 and was 10–30% of total binding.

Data analysis. [^{3}H]STx binding isotherms were analyzed and binding parameters were determined by the nonlinear least squares curve-fitting program GraphPAD (Institute for Scientific Information). [^{3}H]PD85,639 saturation data were fit using LIGAND, a nonlinear least-squares curve-fitting computer program (34). Data were fit to both one- and two-site models, and the fit having the lower squared sum of residuals was chosen as the best fit. Curves for drug competition for [^{3}H]PD85,639 and [^{3}H]BTx binding were also evaluated using the nonlinear least squares curve-fitting program GraphPAD. Data were fit to one- and two-component competition functions, and the fit having the lowest squared sum of residuals was determined to be the best fit. Equilibrium binding parameters for [^{3}H]PD85,639 saturation isotherms were determined using the nonlinear least-squares curve-fitting program LIGAND (34). [^{3}H]PD85,639 association and dissociation time course data were fit to one- and two-exponential functions using GraphPAD, and the fit giving the lowest squared sum of residuals was selected as the best fit of the data. In each case we have used a binding model with two noninteracting sites to derive an empirical description of the results. However, as considered in the Discussion, it is likely that a more complex binding mechanism with interconvertible sites is involved.

Miscellaneous. Protein concentration was determined by the method of Lowry *et al.* (35), with BSA as a standard.

Results

Influence of pH on [^{3}H]PD85,639 binding to rat brain synaptosomes and reconstituted Na^+ channels. The partition coefficient of PD85,639 for octanol/phosphate buffer at pH 7.4 is 1.16, indicating that it is a lipophilic drug with substantial solubility in nonaqueous phases. Its pK_a in 67% dimethylformamide/water is 9.3, indicating that nearly all the drug is protonated at physiological pH. The uncharged species presumably can diffuse readily through the plasma membrane of synaptosomes and gain access to the Na^+ channel local anesthetic receptor that is thought to reside on the cytoplasmic side of the channel pore; access of the charged species to the receptor would be expected to be slower (7, 9). Increasing the pH of the assay buffer from 6.5 to 9.0 increased total binding to synaptosomes and decreased nonspecific binding (Fig. 1A). Specific binding to synaptosomes was typically 75–90% of total binding when measured at pH 9.0. Both nonspecific and total binding were linear between 100 and 625 μg of synaptosomal protein/tube (data not shown).

In contrast, total binding of [^{3}H]PD85,639 to Na^+ channels reconstituted into phospholipid vesicles was not greatly affected by assay pH (Fig. 1B). Nonspecific binding decreased slightly as the pH was increased to pH 9.0. Specific binding was

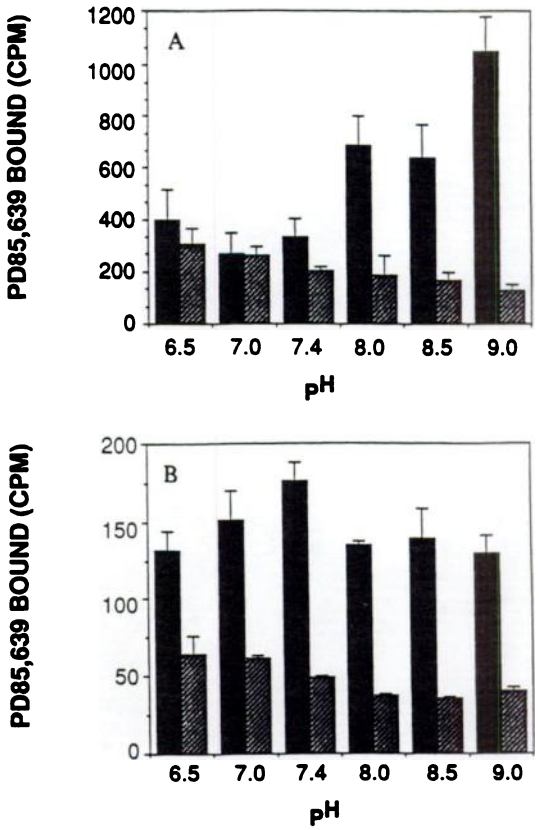

Fig. 1. Influence of pH on [^{3}H]PD85,639 binding to synaptosomes and reconstituted Na^+ channels. Rat brain synaptosomes (A) and reconstituted vesicles (B) were incubated in assay buffer at the indicated pH and binding assays were performed as described in Experimental Procedures. ■, Total binding; ▨, nonspecific binding. Data are from a single representative experiment of three that gave similar results, and each *bar* is the mean ± standard error of triplicate determinations.

normally 60–80% of total binding when measured at pH 9.0. Comparison of [^{3}H]PD85,639 binding to 100 μl of control phospholipid vesicles and vesicles reconstituted with Na^+ channels indicated that [^{3}H]PD85,639 did not appreciably bind to vesicles alone. The relatively small effect of pH on [^{3}H]PD85,639 binding to reconstituted Na^+ channels, compared with synaptosomes, may be due to more rapid permeability of the charged form through the vesicle membrane or to the presence of Na^+ channels reconstituted in an inside-out orientation (approximately 40% of total; data not shown). The results with reconstituted vesicles suggest that the dependence on pH is not an intrinsic property of the binding of PD85,639 to its receptor site but instead is due to the increased access of the uncharged form of the compound to its receptor site in synaptosomes at pH 9.0.

PD85,639 competition for [^{3}H]PD85,639 binding to synaptosomes, synaptosomal membranes, and reconstituted Na^+ channels. Binding of 2 nM [^{3}H]PD85,639 to Na^+ channels is rapid, and the initial phase of drug dissociation is also rapid (see below). The binding assay described in Experimental Procedures allows measurement of specific binding of PD85,639 without substantial loss of specifically bound drug during the washing procedures. PD85,639 competition curves for binding of 2 nM [^{3}H]PD85,639 to rat brain synaptosomes and synaptosomal membranes were shallow and extended over >2 log units (Fig. 2A). Computer analysis of competition curves

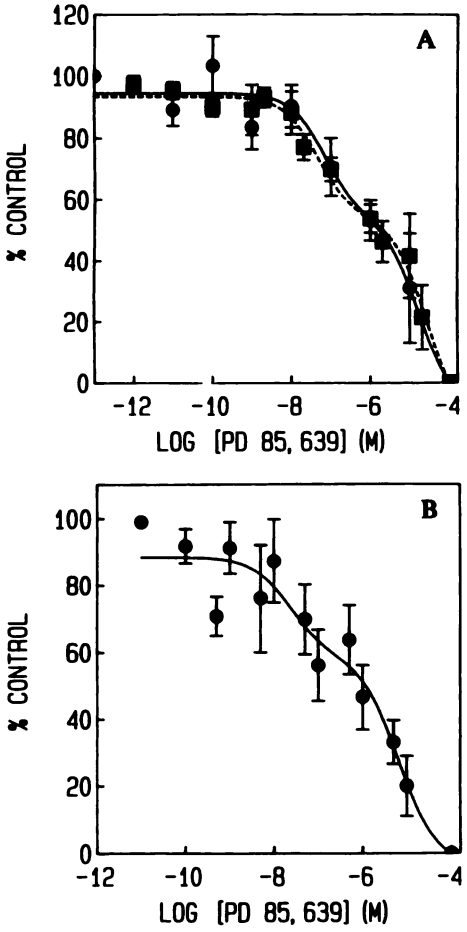

Fig. 2. PD85,639 competition for [^{3}H]PD85,639 binding to rat brain synaptosomes, synaptosomal membranes, and reconstituted Na^+ channels. A, PD85,639 competition for binding of 2 nM [^{3}H]PD85,639 to synaptosomes (●) and synaptosomal membranes (■) was performed at pH 9.0 as described in Experimental Procedures. PD85,639 dilutions were made in standard assay buffer. Data are expressed as percentage of control, and each *data point* is the mean ± standard error of data from five independent experiments, in which triplicate determinations were made at each drug concentration. *Curves* represent statistically best fits of competition data to a two-site model of binding, as described in Experimental Procedures. B, PD85,639 competition for binding of 2 nM [^{3}H]PD85,639 to Na^+ channels reconstituted into phospholipid vesicles was performed at pH 9.0 as described in Experimental Procedures. PD85,639 dilutions were made in standard assay buffer. Data are expressed as percentage of control, and *points* represent the mean ± standard error of data from five independent experiments, in which triplicate determinations were made at each drug concentration. The *drawn curve* is the best fit of data to a two-site model of binding, as described in Experimental Procedures.

indicated that the data were statistically better fit to a model in which there were two populations of [^{3}H]PD85,639 binding sites. A high affinity component with an EC_{50} of 56 ± 23 nM (40%) and a lower affinity component with an EC_{50} of 20 ± 6 μM (60%) were observed in experiments using synaptosomes. Similar EC_{50} values of 17 ± 13 nM (39%) and 10 ± 6 μM (61%) were observed using lysed synaptosomal membranes instead of intact synaptosomes. PD85,639 competition for [^{3}H]PD85,639 binding to reconstituted Na^+ channels was also best fit by a two-site model of binding, and computer-derived EC_{50} values of 23 ± 17 nM (32%) and 6.6 ± 3.3 μM (67%) were determined (Fig. 2B). Thus, PD85,639 competition data are qualitatively similar for all three Na^+ channel preparations evaluated and indicate the presence of two populations of binding sites that differ in affinity.

PD85,639 competition experiments were also performed at more physiological pH values (pH 7.4) in synaptosomes. Specific binding was 50–60% of total binding at this pH. Competition curves were biphasic, with EC_{50} values of 4.9 ± 14 nM (28%) and 3.2 ± 1.1 μM (72%) for high and low affinity components, respectively (Table 1). Both EC_{50} values are lower than those observed in assays performed at pH 9.0, but the proportions of high and low affinity components are comparable.

Saturation isotherms for [^{3}H]PD85,639 and [^{3}H]STx binding to rat brain synaptosomes. Three independent saturation experiments indicated that there were two populations of binding sites labeled by [^{3}H]PD85,639 in rat brain synaptosomes. A representative saturation isotherm and Scatchard transformation are shown in Fig. 3. Data were fit to both one- and two-site models using LIGAND, and the two-site fit was statistically better ($p < 0.05$). Average K_d values of 3 ± 1.2 nM and 428 ± 89 nM and B_{max} values of 0.2 ± 0.03 pmol/mg and 5 ± 1 pmol/mg were determined for high and low affinity sites, respectively (three experiments). The value of B_{max} observed for the sum of the two classes of sites (5.2 pmol/mg) is similar to the concentration of Na^+ channels in synaptosomes, as measured by STx binding (31). These results are consistent with the conclusion that both the high and low affinity sites reflect binding to Na^+ channels.

Kinetics of [^{3}H]PD85,639 binding to rat brain synaptosomes and reconstituted Na^+ channels. Initial time course measurements of [^{3}H]PD85,639 binding to rat brain synaptosomes at 25° indicated that binding was too rapid ($t_{1/2}$ < 0.5 min) to be measured manually. Therefore, association time courses were quantitated at 4° instead. Association time course data were best fit by a single-exponential rate function, and a computer-derived half-time of 1.2 ± 0.46 min was obtained, based on time points between 0.5 and 15 min (Fig. 4A). However, binding decreased by 30–50% after 15 min of incubation and then remained stable for 3 hr of incubation. The rates of [^{3}H]PD85,639 binding to synaptosomal membranes at pH 9.0 or to synaptosomes at pH 7.4 were similar to those observed using synaptosomes at pH 9.0 (data not shown). However, binding to synaptosomal membranes did not decrease with longer incubations and remained stable between 2 and 180 min. The half-time of [^{3}H]PD85,639 binding to reconstituted Na^+ channels was slower, with a half-time of 8.7 ± 1.1 min (Table 2), and levels of binding remained stable for up to 180 min of incubation.

The time course of [^{3}H]PD85,639 dissociation from rat brain

TABLE 1

Summary of PD85,639 competition experiments

All curves fit a two-site model better than a one-site model of binding.

Preparation	n[a]	Site 1		Site 2	
		EC_{50}	Proportion	EC_{50}	Proportion
		nM	%	μM	%
Synaptosomes, pH 9.0	5	56 ± 23	40	20 ± 6	60
Synaptosomes, pH 7.4	3	5 ± 14	28	3 ± 1	72
Synaptosomal membranes, pH 9.0	5	17 ± 13	39	10 ± 6	61
Reconstituted Na^+ channels, pH 9.0	5	19 ± 13	32	6 ± 3	67

[a] *n*, number of experiments.

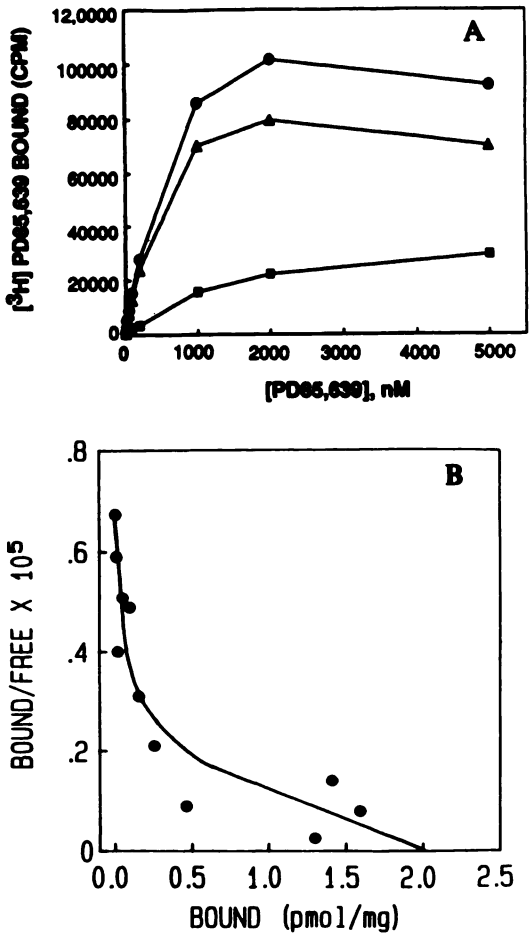

Fig. 3. Saturation binding of [^{3}H]PD85,639 to rat brain synaptosomes. A, Representative saturation isotherm for [^{3}H]PD85,639 binding to rat brain synaptosomes at pH 9.0. Bound [^{3}H]PD85,639 was determined as described in Experimental Procedures. Total (●), nonspecific (■), and specific (▲) binding is indicated. [^{3}H]PD85,639 stock was isotopically diluted with unlabeled PD85,639 and binding was measured between 0.1 and 20 μM. B, Scatchard transformation of the saturation data of A. Equilibrium parameters determined using the nonlinear least squares curve-fitting computer program LIGAND are presented in the text.

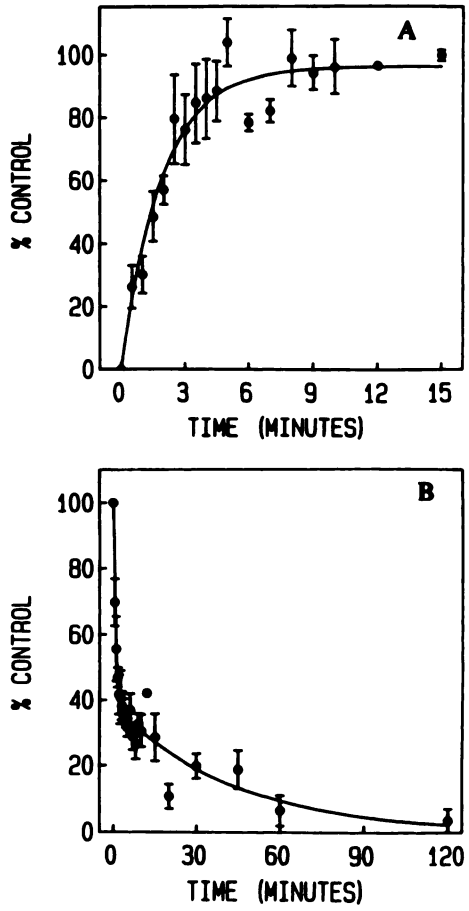

Fig. 4. Kinetics of binding of [^{3}H]PD85,639 to rat brain synaptosomes. A, Binding of 2 nM [^{3}H]PD85,639 to rat brain synaptosomes was measured at 4° and pH 9.0 between 0.5 and 180 min. At indicated times, aliquots were removed and bound [^{3}H]PD85,639 was determined as described in Experimental Procedures. Time course data evaluated between 0.5 and 15 min are shown. The percentage of control based on specific binding at 15 min was determined for each time point, and *data points* represent the mean ± standard error of pooled data from five independent experiments. Data were then fit to monoexponential and biexponential rate functions as described in Experimental Procedures, and the *drawn curve* is the best fit of data to the monoexponential rate function. B, Dissociation of 2 nM [^{3}H]PD85,639 from rat brain synaptosomes was initiated by the addition of 100 μM PD85,639 (1% of total volume) after a 30-min incubation at 4° and pH 9.0. [^{3}H]PD85,639 bound between 0.5 and 120 min was quantitated as described in Experimental Procedures. Percentage of control for each time point was calculated based on binding at the zero time point, and each *data point* represents the mean ± standard error of data from five independent experiments. Data were fit to both monoexponential and biexponential rate functions, as described in Experimental Procedures, and the *drawn curve* is the best fit of data using the biexponential rate function.

synaptosomes at 4° was best fit by a biexponential rate function (Fig. 4B). The fast and slow components of [^{3}H]PD85,639 dissociation occurred with half-times of 0.7 ± 0.4 min and 45 ± 9 min, respectively. Dissociation of [^{3}H]PD85,639 from reconstituted Na^+ channels was also biexponential, with half-times of 1 min and 97 min for the fast and slow phases of dissociation, respectively (Table 2). The amplitudes of the fast and slow components of dissociation were 60–70% and 30–39%, respectively. The proportion of the low affinity component of displacement observed in competition studies (Table 1) was identical to the proportion of fast [^{3}H]PD85,639 dissociation and, conversely, the proportion of the high affinity component of competition correlated with the proportion of slow dissociation. The 50–100-fold difference in dissociation rates of the rapidly and slowly dissociating components of specifically bound PD85,639 can account for most of the difference in affinity between the high and low affinity sites.

[^{3}H]PD85,639 binding to cells transfected with type IIA Na^+ channel α subunit. [^{3}H]PD85,639 did not appreciably bind to membranes prepared from CHO cells (Fig. 5A), which contain only low levels of endogenous Na^+ channels (5, 6). However, [^{3}H]PD85,639 bound specifically to membranes prepared from CHO cells transfected with cDNA encoding the type IIA α subunit (CNaIIA-1). PD85,639 competition for binding of 2 nM [^{3}H]PD85,639 occurred over 4 log units, and computer analysis of the data indicated that the curves were best fit by a two-site model of binding. EC_{50} values of 2 ± 0.8 nM and 736 ± 373 nM were determined for high and low affinity components, respectively (Fig. 5B). These values are lower than those observed for other Na^+ channel preparations at pH 9.0 but are close to values obtained when assays were performed at pH 7.4 in synaptosomes (Table 1). The proportions of high and low affinity components of binding competition were 46

TABLE 2
Summary of the kinetics of [^{3}H]PD85,639 binding

Association time course Preparation	n[a]	k_f	$t_{½}$				
		min^{-1}	min				
Synaptosomes	5	0.71 ± 0.45	1.2 ± 0.46				
Reconstituted Na^+ channels	2	0.08 ± 0.01	8.7 ± 1.1				
Dissociation time course Preparation	**n**	**Fast**			**Slow**		
		k_f	$t_{½}$	A_f	k_s	$t_{½}$	A_s
		min^{-1}	min	%	min^{-1}	min	%
Synaptosomes	6	1.6 ± 1.4	0.7 ± 0.4	61	0.022 ± 0.01	45 ± 9	39
Synaptosomes + 100 μM tetracaine	1	1.3	0.53	53	0.030	23	47
Reconstituted Na^+ channels	1	0.73	1.0	70	0.007	97	30

[a] *n*, number of experiments.

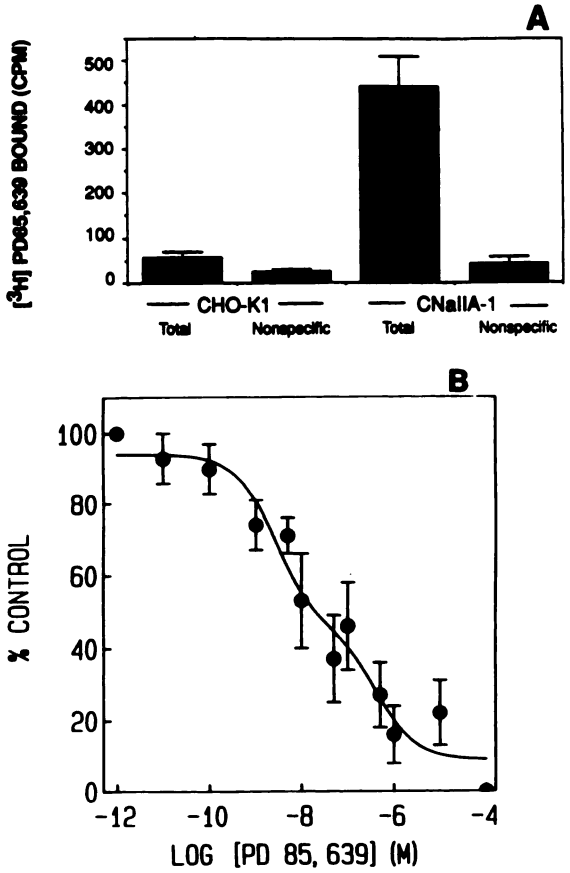

Fig. 5. [^{3}H]PD85,639 binding to CHO-K1 AND CNaIIA-1 cell membranes. A, Binding of 2 nM [^{3}H]PD85,639 to CHO-K1 and CNaIIA-1 cell membranes. Bound [^{3}H]PD85,639 was measured at pH 9.0 as described in Experimental Procedures. Each *bar* represents the mean ± standard error of triplicate determinations for a single representative experiment. B, PD85,639 competition for [^{3}H]PD85,639 binding to CNaIIA-1 cell membranes. Bound [^{3}H]PD85,639 in the absence and presence of the indicated concentrations of PD85,639 was measured at pH 9.0 as described in Experimental Procedures. The percentage of control was calculated based on specific binding in the absence of drug, and each *data point* represents the mean ± standard error of data from independent experiments, in which triplicate determinations were made at each drug concentration. Data were fit to one- and two-site models of binding, and the *drawn curve* is the best fit of data to the two-site model.

and 53%, respectively. These results indicate that equilibrium binding of [^{3}H]PD85,639 to CNaIIA-1 cell membranes containing only type IIA α subunits is quantitatively similar to binding to other Na^+ channel preparations studied that contain additional α subunit subtypes as well as β1 and β2 subunits.

Inhibition by local anesthetics of [^{3}H]PD85,639 binding to rat brain synaptosomes and reconstituted Na^+ channels. Local anesthetic drugs inhibited [^{3}H]PD85,639 binding to both synaptosomes and reconstituted Na^+ channels when tested at a concentration of 100 μM (Fig. 6). Preincubation of synaptosomes or reconstituted vesicles with drug before initiation of binding assays by addition of [^{3}H]PD85,639 did not result in greater levels of inhibition, indicating that local anesthetic binding equilibrium is achieved during the binding assay (Fig. 6). Inhibition of binding by local anesthetics was concentration dependent (Table 3), with K_i values ranging from 31 to 314 μM for tetracaine, bupivacaine, and mepivacaine. Competition curves for these three local anesthetics were fit by a single-site model, indicating that they have comparable affinity for both the high and low affinity sites occupied by PD85,639. This suggests that the unique structural features of PD85,639 are required to distinguish the high and low affinity forms of its receptor site.

Addition of 100 μM tetracaine simultaneously with 100 μM unlabeled PD85,639 did not accelerate [^{3}H]PD85,639 dissociation, suggesting that both drugs compete for binding to the same site (Table 2). In contrast to these results with local anesthetics, the antiepileptic drugs phenytoin and carbamazepine (100 μM) had no effect on [^{3}H]PD85,639 binding to rat brain synaptosomes, indicating that the receptor site for anticonvulsant agents differs from the receptor site(s) for PD85,639 and local anesthetics on Na^+ channels (data not shown).

Influence of neurotoxins on [^{3}H]PD85,639 binding to rat brain synaptosomes and reconstituted Na^+ channels. Neurotoxins that interact with five distinct neurotoxin receptor sites on Na^+ channels were evaluated for their influence on [^{3}H]PD85,639 binding to both rat brain synaptosomes and reconstituted Na^+ channels (Fig. 7A). Only BTx and veratridine, which bind to neurotoxin receptor site 2, significantly decreased binding to both Na^+ channel preparations; other neurotoxins that bind to sites 1, 3, 4, and 5 had no consistent effect. Inhibition of binding by BTx and veratridine was concentration dependent, with EC_{50} values of 7 ± 1 μM and 11 ± 5 μM, respectively, at pH 9.0 (Fig. 7B). LqTx, which allosteri-

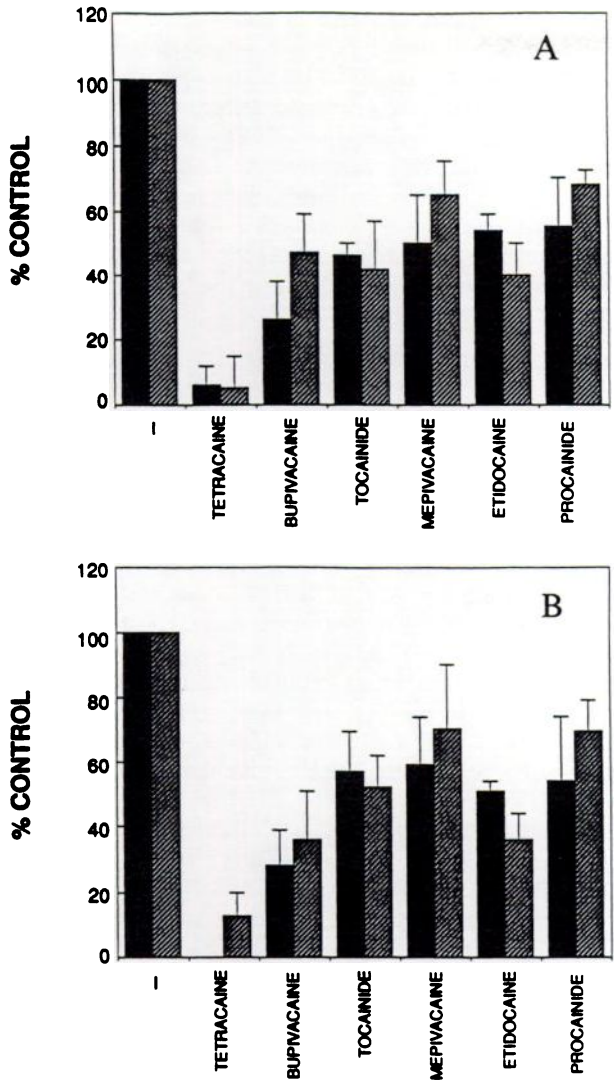

Fig. 6. Inhibition by local anesthetics of [^{3}H]PD85,639 binding to rat brain synaptosomes and reconstituted Na^+ channels. The influence of local anesthetics on binding of [^{3}H]PD85,639 to synaptosomes (A) and reconstituted vesicles (B) at pH 9.0 is shown. ■, Data from experiments in which synaptosomes and vesicles were not preincubated with drug before initiation of the assay; ▨, experiments in which drug was preincubated for 30 min at 25° before initiation of the assay by addition of [^{3}H]PD85,639. Bound [^{3}H]PD85,639 was determined as described in Experimental Procedures. Data are expressed as percentage of control of specific binding without added drug, and *data points* represent the mean ± standard error of data from two independent experiments, in which triplicate determinations were made. All drugs were tested at 100 μM and 1% ethanol was present in the control and drug incubations.

TABLE 3

Local anesthetic competition for [^{3}H]PD85,639 binding to synaptosomes

Data were fit to one-site and two-site competition curves and the statistically better fit is shown.

Drug	n*	EC_{50}
		μM
Tetracaine	4	31 ± 6
Bupivacaine	3	86 ± 10
Mepivacaine	3	314 ± 162

* *n*, number of experiments.

cally enhances BTx binding to synaptosomes (19), had no effect alone on [^{3}H]PD85,639 binding but did increase the potency of BTx to inhibit [^{3}H]PD85,639 binding. Lower EC_{50} values of 355 ± 89 nM and 1 ± 0.2 μM were obtained for BTx and

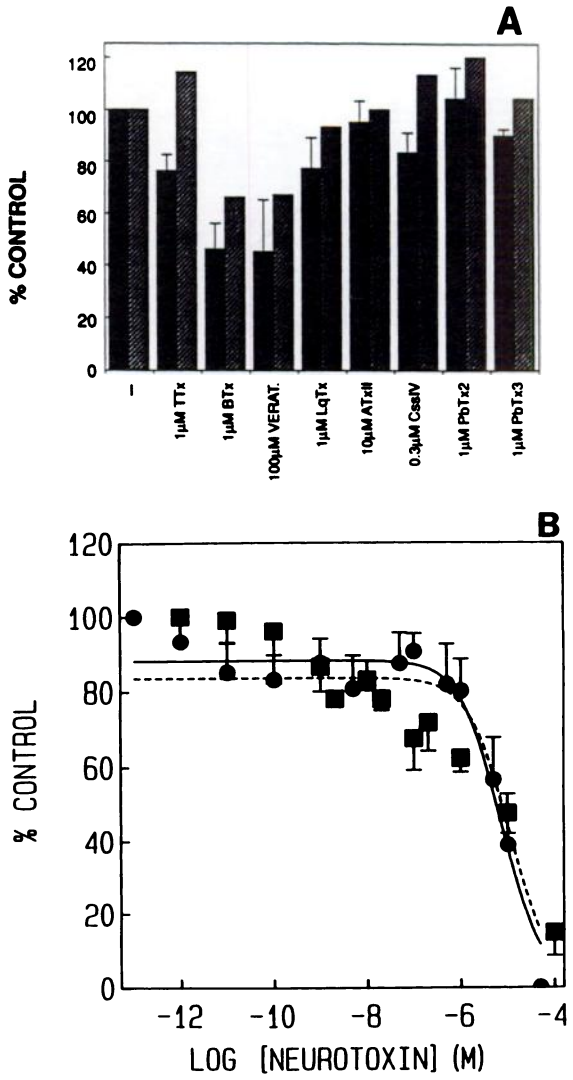

Fig. 7. Influence of Na^+ channel neurotoxins on [^{3}H]PD85,639 binding to rat brain synaptosomes and reconstituted Na^+ channels. A, Specific binding of [^{3}H]PD85,639 to synaptosomes (■) and reconstituted vesicles (▨) in the absence and presence of various neurotoxins. The binding assay was performed at pH 9.0 as described in Experimental Procedures, except that synaptosomes and reconstituted vesicles were preincubated for 30 min with neurotoxin before addition of [^{3}H]PD85,639, followed by an additional 30-min incubation. Data are expressed as percentage of control specific binding (no neurotoxin added), and each *bar* represents the mean ± standard error of two independent experiments in which triplicate determinations were made for synaptosomes and one experiment in which triplicate determinations were made using reconstituted vesicles. *VERAT.*, veratridine; *ATxII*, toxin II from the sea anemone *Anemonia sulcata*; *CssIV*, toxin IV from the scorpion *C. suffussus suffusus*. B, Concentration dependence of BTx (●) and veratridine (■) inhibition of [^{3}H]PD85,639 binding to synaptosomes. Bound [^{3}H]PD85,639 was determined as described in Experimental Procedures, except that incubations were at pH 7.4 to prevent degradation of BTx and assays were incubated for 60 min. The percentage of control specific binding was calculated (based on binding without added drug) at each drug concentration, and *data points* represent the mean ± standard error of data from two independent experiments, in which triplicate determinations were made. *Curves* represent fits of data to a one-site model of binding, as described in Experimental Procedures.

veratridine, respectively, in the presence of 0.3 μM LqTx, as expected from the positive allosteric interaction between neurotoxin receptor sites 2 and 3 (data not shown).

Inhibition by PD85,639 of [^{3}H]BTx binding to rat brain synaptosomes. Like local anesthetics (20, 21), PD85,639 inhibited [^{3}H]BTx binding to rat brain synaptosomes in a concentration-dependent manner, and an EC_{50} of 46 ± 5 nM was determined from computer analysis of the inhibition curve (Fig. 8A). To determine whether inhibition of BTx binding by PD85,635 is direct or allosteric, the effect of 100 μM PD85,639 on the rate of [^{3}H]BTx dissociation was evaluated (Fig. 8B). The half-time for [^{3}H]BTx dissociation in the absence of PD85,639 was 37 min. In the presence of 100 μM PD85,639, the half-time decreased 4-fold, to 8 min. Kinetic data in the absence and presence of PD85,639 were best fit by a monoexponential rate function. Acceleration of [^{3}H]BTx dissociation by PD85,639 indicates that the drug inhibits [^{3}H]BTx binding by an indirect allosteric mechanism, rather than by direct binding to neurotoxin receptor site 2. This is similar to the allosteric inhibition of BTx binding by local anesthetics (21).

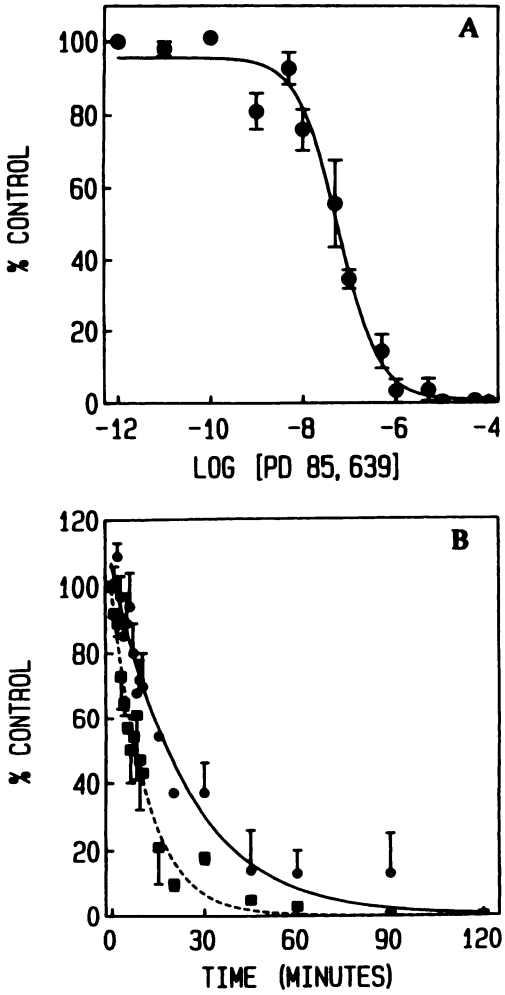

Fig. 8. Influence of PD85,639 on [^{3}H]BTx binding to rat brain synaptosomes. A, Concentration-dependent inhibition of [^{3}H]BTx binding to synaptosomes mediated by PD85,639. Bound [^{3}H]BTx in the presence of the indicated concentrations of PD85,639 was measured as described in Experimental Procedures and percentage of the control specific binding in the absence of PD85,639 was calculated. *Data points* represent the mean ± standard error of data from three different experiments, in which triplicate determinations were made at each drug concentration. The *drawn curve* represents the best fit of data to a one-site model of binding, as described in Experimental Procedures. B, Acceleration of [^{3}H]BTx dissociation by PD85,639. After a normal assay incubation, dissociation of [^{3}H]BTx was initiated by the addition of 300 μM veratridine alone (●) or simultaneously with 100 μM PD85,639 (■). Aliquots were taken between 0.5 and 120 min and bound [^{3}H]BTx was quantitated as described in Experimental Procedures. The percentage of control specific binding based on specific binding at zero time was calculated for each time point, and *points* represent the mean ± standard error of data obtained from three separate experiments. The *drawn curve* represents a computer fit of data to a monoexponential rate function, as described in Experimental Procedures.

Discussion

PD85,639 binds directly to Na^+ channel α subunits. Our experiments detected specific, high affinity, [^{3}H]PD85,639 binding to synaptosomes, synaptosomal membranes, reconstituted purified Na^+ channels, and membranes prepared from CHO-K1 cells transfected with rat brain type IIA α subunit cDNA. Specific binding of [^{3}H]PD85,639 to Na^+ channels in CNaIIA-1 cells but not to nontransfected CHO-K1 cells demonstrates that the drug binds specifically to α subunits of Na^+ channels and not to other cellular constituents. Similarly, specific binding to purified and reconstituted Na^+ channels demonstrates that the high affinity binding that we measure is directly to Na^+ channels. The Na^+ channel activators veratridine and BTx completely inhibit [^{3}H]PD85,639 binding, consistent with specific [^{3}H]PD85,639 binding to Na^+ channels. This effect is potentiated by LqTx V, which is known to increase the affinity of veratridine and BTx for neurotoxin receptor site 2 on Na^+ channels through an allosteric interaction (19). In addition, the stoichiometry of binding is consistent with specific binding of PD85,639 to Na^+ channels in synaptosomes and in purified and reconstituted Na^+ channels. Thus, our results provide direct biochemical evidence that local anesthetic drugs indeed bind to a specific receptor site on the α subunit of the Na^+ channel. [^{3}H]PD85,639 should be useful in future studies designed to determine the nature, number, and location of the local anesthetic receptor sites and the relationship of local anesthetic affinity to Na^+ channel conformation.

PD85,639 binds to a local anesthetic receptor site. In the preceding paper, Ragsdale *et al.* (26) show that PD85,639 blocks Na^+ channels in a frequency- and voltage-dependent manner, like local anesthetics. The results presented here support the conclusion that PD85,639 binds to a local anesthetic receptor site on Na^+ channels. Local anesthetics compete effectively for specific binding of PD85,639. The relative potencies of local anesthetics in inhibition of [^{3}H]PD85,639 binding are similar to their abilities to block Na^+ channels and to inhibit [^{3}H]BTx binding (20, 21). Their effect on PD85,639 binding most likely results from direct interaction with the same receptor site as that bound by PD85,639, because tetracaine, a high affinity local anesthetic, inhibits binding of PD85,639 but does not affect its rate of dissociation. Like local anesthetics, PD85,639 inhibits binding of BTx due to a negative allosteric interaction between binding of PD85,639 and binding of BTx at neurotoxin receptor site 2. Inhibition of BTx binding by PD85,639 is 10–100 times more potent than that by local anesthetics, consistent with the conclusion that PD85,639 binds to the local anesthetic receptor with relatively high affinity. Thus, we conclude that PD85,639 binds to a local

anesthetic receptor site on Na^+ channels and can be used as a specific radiolabel for that site.

The relationship between local anesthetic structure and action has been extensively studied by electrophysiological methods. Two theories of local anesthetic action have evolved from these investigations. According to both the modulated receptor (7, 9) and guarded receptor (10) hypotheses of local anesthetic action, charged tertiary amine local anesthetics must transverse the membrane and enter the open ion channel to gain access to the local anesthetic receptor located within the cytoplasmic entry to the pore. Unprotonated hydrophobic tertiary amine local anesthetics can access the receptor by an alternate pathway involving free diffusion through the plasma membrane. Consistent with these previous studies, we have found that specific binding of PD85,639 to Na^+ channels in synaptosomes is enhanced by incubation at pH 9.0, where the drug is predominantly uncharged. The limited access of protonated drugs to the local anesthetic receptor site may explain the increased binding of [^{3}H]PD85,639 to Na^+ channels in intact synaptosomes at pH 9.0, compared with pH 7.4.

Although PD85,639 binds to a local anesthetic receptor site on Na^+ channels, the anticonvulsant drugs phenytoin and carbamazepine do not compete for PD85,639 binding. These results provide direct evidence that local anesthetic and anticonvulsant drugs do not bind to the same receptor site on Na^+ channels. Our results do not exclude the possibility that there may be additional receptor sites for local anesthetic and antiarrhythmic drugs that do not bind PD85,639. We have not yet determined whether all of the major chemical classes of local anesthetic and antiarrhythmic drugs that block Na^+ channels compete for [^{3}H]PD85,639 binding, so there may be one or more additional receptor sites for drugs that block Na^+ channels.

There is heterogeneity in the binding affinity of PD85,639. Both equilibrium and kinetic binding data indicate that two populations of Na^+ channel binding sites are present in all Na^+ channel preparations evaluated. Similar binding heterogeneity has been reported for [^{3}H]tetracaine binding to rat brain synaptosomes (24). Because a single Na^+ channel subtype consisting of only an α subunit is expressed in CNaIIA-1 cells, compared with CHO-K1 cells (6), the heterogeneity of binding affinity cannot result from binding to multiple Na^+ channel α subunit subtypes or to α subunits interacting with different β1 or β2 subunits. These results indicate that both the high and low affinity [^{3}H]PD85,639 binding sites reside on the α subunit.

Although both classes of binding sites appear to reside on a single class of α subunits, there are >10 times as many low affinity binding sites as high affinity binding sites in all the preparations that we studied. The ratios of high and low affinity sites are similar in synaptosomes and purified and reconstituted Na^+ channels and are similar at pH 7.4 and pH 9.0. The similar proportions of binding sites at pH 7.4 and 9.0 indicate that heterogeneity is not related to binding of protonated and unprotonated [^{3}H]PD85,639 with different affinities. It is likely that the high and low affinity binding sites reflect binding to two distinct functional states of the Na^+ channel α subunit that are present in similar ratios in these different membrane preparations. Evidently, this receptor site can assume conformations with high and low affinity for PD85,639. Although the two affinities for PD85,639 likely represent interconvertible forms of a single local anesthetic receptor site, we have used a binding model with two classes of noninterconvertible sites to provide an empirical description of our results. Under most conditions, our results do not precisely fit a strictly defined version of such a noninterconvertible two-site model, because we observe greater occupancy of the low affinity sites by 2 nM [^{3}H]PD85,639 than expected. This apparent discrepancy is likely to be due to interconversion of high and low affinity sites by PD85,639 or by other experimental parameters. Binding assay methods yielding more precise results will be necessary before a more complete kinetic analysis of the binding mechanism would be feasible.

Electrophysiological studies show that local anesthetics may have higher affinity for either activated or inactivated conformations of the Na^+ channel (7). It is likely that the inactivated conformation of the Na^+ channel predominates at the depolarized membrane potentials in all preparations studied here. The dominant, low affinity site observed in these binding studies is likely to correspond to the inactivated state of the Na^+ channel. Because the concentration dependence of inhibition of Na^+ currents by PD85,639 (26) is most closely correlated with the low affinity binding of PD85,639, we assume that binding to the low affinity conformation of the PD85,639 receptor site is responsible for inhibition of Na^+ channels. The nature of the high affinity state observed in binding studies is unknown, but it may represent one of the multiple closed states through which the Na^+ channel passes in the process of activation, a persistently inactivated state reached after slow Na^+ channel inactivation during prolonged depolarization, or a state that is differentially modulated by protein phosphorylation or interaction with guanine nucleotide-binding proteins.

[^{3}H]PD85,639 should be a useful molecular probe for further study of the biochemical and pharmacological properties of the local anesthetic receptor of the Na^+ channel. Further biochemical characterization of the Na^+ channel local anesthetic receptor using [^{3}H]PD85,639 and photoreactive derivatives may provide insight into the relationship between Na^+ channel structure and function at the local anesthetic receptor site and may ultimately lead to the design of therapeutically superior Na^+ channel-blocking drugs.

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