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Isolation of Two Saxitoxin-Sensitive Sodium Channel Subtypes from Rat Brain with Distinct Biochemical and Functional Properties

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Summary. Two different ^{3}H-saxitoxin-binding proteins, with distinct biochemical and functional properties, were isolated from rat brain using a combination of anion exchange and lectin affinity chromatography as well as high resolution size exclusion and anion exchange HPLC. The alpha subunits of the binding proteins had different apparent molecular weights on SDS-PAGE (Type A: 235,000; Type B: 260,000). When reconstituted into planar lipid bilayers, the two saxitoxin-binding proteins formed sodium channels with different apparent single-channel conductances in the presence of batrachotoxin (Type A: 22 pS; Type B: 12 pS) and veratridine (Type A: 9 pS; Type B: 5 pS). The subtypes were further distinguished by scorpion (*Leiurus quinquestriatus*) venom which had different effects on single-channel conductance and gating of veratridine-activated Type A and Type B channels. Scorpion venom caused a 19% increase in single-channel conductance of Type A channels and a 35-mV hyperpolarizing shift in activation. Scorpion venom doubled the single-channel conductance of Type B channels and shifted activation by at least 85 mV.

Key Words sodium channels · scorpion venom · veratridine · planar bilayer · channel subtypes · reconstitution

Introduction

Voltage-gated sodium channels are responsible for the regenerative influx of sodium ions that underlies action potential generation in a wide variety of electrically excitable cells. Using binding of tritiated saxitoxin (STX) or tetrodotoxin (TTX) as an assay for the channel protein, voltage-gated sodium channels have been purified from electric eel electroplax (Agnew et al., 1978), rat brain (Hartshorne & Catterall, 1984), rat and rabbit skeletal muscle (Barchi, 1983; Kraner, Tanaka & Barchi, 1985) and from cardiac tissue (Lombet & Lazdunski, 1984). The subunit composition of the sodium channels from different sources varies, but all possess an alpha subunit with a molecular weight of approximately 260,000 (Agnew, 1984; Catterall, 1986). The presence or number of additional beta subunits varies with tissue source (Barchi, 1983; Miller, Agnew & Levinson, 1983; Hartshorne & Catterall, 1984; Kraner et al., 1985). Purified sodium channels have been shown to retain many of their original functional properties as determined by ion flux measurements of reconstituted channels in vesicles (Tamkun & Catterall, 1981; Rosenberg, Tomiko & Agnew, 1984*a*; Furman et al., 1986), single-channel recording from channels reconstituted in planar lipid bilayers (Hartshorne et al., 1985; Keller et al., 1986; Recio-Pinto et al., 1987) and single-channel recordings obtained by patch clamping giant liposomes containing reconstituted channels (Rosenberg et al., 1984*b*; Correa & Agnew, 1988).

Although voltage-gated sodium channels from various sources have similar functional properties, some differences have been found, particularly with regard to STX and TTX sensitivity. For example, TTX-insensitive and TTX-sensitive Na channels appear at different times during nerve and muscle development (Sherman et al., 1983; Strichartz, Bar-Sagi & Prives, 1983; Frelin et al., 1984; Haimovich, Tanaka & Barchi, 1986). Denervation of skeletal muscle induces the synthesis and subsequent appearance of new TTX-insensitive Na channels in sarcolemma (Thesleff, Vyskogil & Ward, 1974; Barchi & Weigle, 1979; Rogart & Regan, 1985). Voltage-clamp studies on normal and denervated skeletal muscle have described kinetic differences in the macroscopic currents produced by TTX-sensitive and insensitive Na channels (Pappone, 1980). Single-channel studies by Weiss and Horn (1986) revealed different single-channel conductances and voltage dependence of activation for these subtypes.

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Additional evidence for the existence of multiple Na channel subtypes in the same tissue comes from studies in which different but highly homologous sodium channel genes were cloned from rat brain (Noda et al., 1986; Kayano et al., 1988). Transcripts from all three genes have been shown to be TTX-sensitive when expressed in *Xenopus* oocytes (Stuhmer et al., 1987; Suzuki et al., 1988). Purified Na channel preparations from rat brain appear to consist of some mixture of at least two and probably three of these TTX-sensitive sodium channel subtypes (Gordon et al., 1987) by immunological criteria. Recently, Barres, Chun and Corey (1989) demonstrated that a novel TTX-sensitive Na channel type with slower gating kinetics than neuronal Na channels is present in rat brain glial cells. It is not known if this channel correlates with one of the three types described in molecular biological studies (Noda et al., 1986; Kayano et al., 1988).

In this report we describe the biochemical and functional characterization of two distinct STX-sensitive sodium channel subtypes isolated from rat brain. These subtypes, designated Type A and Type B, differ in their elution on anion exchange chromatography and have alpha subunits with different apparent molecular weights on SDS-PAGE. When reconstituted in planar lipid bilayers, the two Na channel subtypes differ in their apparent single-channel conductance in the presence of veratridine (VER) or batrachotoxin (BTX) and differ in the response of the veratridine-modified channel to scorpion venom.

Materials and Methods

Protease Inhibitors

Protease inhibitors were included in all solutions used in the purification procedure. Pepstatin A (1 μM in Me_2SO), phenylmethylsulfonyl fluoride (0.1 mM in acetone) and 1,10-phenanthroline (1 mM in acetone) were prepared fresh at the beginning of each experiment.

Measurement of ^{3}H-STX Binding

Method 1 (Rapid Gel Filtration)

Solubilized sodium channels were assayed for ^{3}H-STX binding by rapid gel filtration. Two-ml columns of Sephadex G-50 were equilibrated with 100 mM KCl, 20 mM histidine, 0.1% Triton X-100, spun at 1000 × *g* for 15 sec, and refrigerated. Column fractions were assayed in a solution containing 3 nM ^{3}H-STX, 100 mM KCl, 20 mM histidine, 11 mM EDTA, 0.1% Triton X-100, 0.025% egg phosphatidylcholine (PC), and protease inhibitors, pH 7.0 at 4°C. Nonspecific binding was determined in the presence of 3 μM STX. Following a 25-min incubation with ^{3}H-STX, the samples were loaded onto the prespun Sephadex G-50 columns, and centrifuged at 1000 × *g* for 20 sec. The eluant (150 μl) was counted by liquid scintillation.

Method 2 (Glass Fiber Filter Filtration)

This method was used for either native membrane vesicles or purified sodium channels which had been reconstituted into phospholipid vesicles. Incubation conditions were similar to those in Method 1, except for the composition of the dilution buffer (100 mM KCl, 20 mM histidine, pH 7.0) and the concentration of ^{3}H-STX (10 nM). Unlabeled STX was used to determine nonspecific binding. Following a 30-min incubation, the samples were filtered through three stacked glass fiber filters. The filters were then washed three times with 4.5 ml of the KCl buffer and counted by liquid scintillation.

Protein Assay

Protein was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Preparation of Brain Membranes

Crude membrane vesicles were prepared from rat brain as described by Krueger et al. (1979). Briefly, rat brains were homogenized in a large volume of isotonic sucrose using a cavitating tissue disrupter (Tekmar Tissumizer, Cincinnati, OH) and the homogenate was subjected to differential centrifugation. The 1000 × *g* and the 10,000 × *g* pellets were discarded and the 100,000 × *g* pellet (*P*3) was resuspended in 0.4 M sucrose at approximately 10 mg protein per ml. This material had a specific activity of 5–10 pmol ^{3}H-STX binding sites per mg protein.

Solubilization of the Sodium Channel

All manipulations were carried out at 4°C. The crude vesicle preparation (*P*3) from rat brain was added to a solution containing 100 mM KCl, 10 mM HEPES, 1% Triton X-100, 0.1% PC, and protease inhibitors, with a final volume of approximately 150 ml and protein concentration of 4 mg/ml. Following stirring for 30 min, the mixture was centrifuged in a Beckman Ti-70.1 rotor at 40,000 rpm for approximately 30 min ($\omega^2 t = 2.91 \times 10^{10}$). The supernatant, containing the solubilized STX-binding sites, was retained. The pH of the solubilized material was adjusted to pH 6.5, and $CaCl_2$ was added to a final concentration of 10 mM.

DEAE Ion Exchange Column

DEAE-Fast Flow Sepharose (100–150 ml packed bed volume, 15 × 800 mm) was equilibrated with two column volumes of 160 mM KCl, 20 mM histidine, 0.1% Triton X-100, 0.025% PC, 10 mM $CaCl_2$, and protease inhibitors, pH 6.5. The solubilized material was loaded onto the column and washed with 150–200 ml of equilibration solution. The column was batch eluted at 2.5

ml/min with a jump in KCl concentration to 300 mM. The fractions (5 ml) containing protein greater than 0.5 mg/ml were pooled.

Wheat Germ Agglutinin Column

The pH of the pooled DEAE eluate was raised to pH 7.0 by the addition of histidine, calcium was chelated by the addition of 1 M EDTA, pH 7.0, such that the final EDTA concentration was 11 mM (causing the pH to drop to 6.5) and 1 M KH_2PO_4, pH 7.0, was added to a final concentration of 100 mM (pH of solution returned to 7.0). Wheat germ agglutinin Sepharose was prepared using a combination of the methods of March, Parikh and Cuatrecasas (1974) and those described by the manufacturer (Cell Affinity Chromatography, 1980). The pooled fractions from the DEAE column were loaded onto the WGA column (30–55 ml packed volume, 15 × 300 mm) which had been equilibrated with 0.5 M KCl, 20 mM HEPES, 0.1% Triton X-100, 0.025% PC, and protease inhibitors, pH 7.0. The resin was then washed with 50 ml of equilibration buffer, followed by a wash with 0.5 M KCl, 20 mM HEPES, 10 mM $CaCl_2$, 0.1% Triton X-100, 0.025% PC, and protease inhibitors, pH 7.0. The column was eluted (2.5 ml/min) with 0.4 M NaCl, 20 mM HEPES, 10 mM $CaCl_2$, 0.1% Triton X-100, 0.025% PC, 60 mM N-acetylglucosamine and protease inhibitors, pH 7.0. Fractions containing protein greater than 0.5 mg/ml were pooled and concentrated by centrifugation for 50 min at 4000 rpm in Centricon 30 concentrators. This procedure concentrated the fractions 10- to 12-fold such that the final volume was 1.1–1.5 ml.

HPLC Size Exclusion Column

All solutions used with the high performance liquid chromatography (HPLC) columns were passed through 0.45 μm filters prior to use. One ml of the concentrated WGA eluate was loaded onto the HPLC size exclusion columns (two Beckman Spherogel TSK-G 4000 columns, 21.5 × 300 mm, in series following a TSK guard column), equilibrated with 100 mM KCl, 20 mM histidine, 0.1% Triton X-100, 0.025% PC, 10 mM $CaCl_2$, and protease inhibitors, pH 6.5. The column was eluted with the equilibration buffer at 2 ml per min; the first 100 ml contained no ^{3}H-STX binding activity and was discarded. Fractions with STX binding (Method 1) greater than 5 pmol/ml were pooled.

HPLC DEAE Column

The HPLC DEAE column (LKB DEAE 5-PW, 7.5 × 80 mm) was equilibrated with 100 mM KCl, 20 mM histidine, 0.1% Triton X-100, 0.025% PC, 10 mM $CaCl_2$ and protease inhibitors, pH 6.5. The pooled fractions from the HPLC size exclusion column were loaded directly onto the HPLC DEAE column. The column was eluted with a 55-ml gradient from 0.1 M KCl to 0.25 M KCl at 0.3 ml per min. Fractions were assayed for STX binding (Method 1) and pooled for reconstitution.

Reconstitution of Purified Sodium Channels

The pH of the pooled samples was adjusted to pH 7.0 by histidine addition after Ca^{2+} was chelated by addition of EDTA. The pooled fractions were then mixed with a concentrated lipid suspension to give final concentrations of 3 mg/ml PC, 0.75% Triton X-100, 1 mM $CaCl_2$, 100 mM KCl, 20 mM histidine and protease inhibitors, pH 7.0. This mixture was then incubated with gentle agitation with polystyrene beads (Amberlite XAD-2, Sigma) for 3 hr and the reconstituted vesicles were separated from the beads by passage through a sintered glass filter. ^{3}H-STX binding of the reconstituted vesicles was determined by Method 2. Reconstituted vesicles were stored in small aliquots at −70°C. Rapid freezing in acetone/dry ice prior to storage at −70°C resulted in a greatly reduced probability of incorporation of purified sodium channels in planar lipid bilayers.

SDS-PAGE

SDS gel electrophoresis was run according to methods previously described (Rudolph & Krueger, 1979). Using an acrylamide to N,N′-methylene-*bis*-acrylamide ratio of 37.5 to 1, the lower gel was formed with a linear gradient of 7–20% acrylamide solution and a 3% solution was used for the stacking gel. Samples were heated to 100°C in the presence of sample buffer for 1 min and, in some cases, iodoacetamide was added in fourfold excess of mercaptoethanol. Gels were silver stained according to the method of Wray et al. (1981).

Planar Bilayer Formation and Channel Incorporation

Planar bilayers were formed by drawing a membrane-forming solution containing 39 mg/ml 1-palmitoyl-2-oleoyl-phosphatidylethanolamine and 26 mg/ml bovine brain phosphatidylserine in decane across a 250 μm hole in a polycarbonate partition (Mueller et al., 1963). Identical solutions (containing 250 mM NaCl, 75 μM $CaCl_2$, 50 μM EGTA, 2 mM $MgCl_2$, 10 mM HEPES-Tris, and 100 μM veratridine or 120 nM batrachotoxin) were present on both sides of the bilayer.

Incorporation of reconstituted sodium channels was achieved using a modification of the fusion technique (Miller, 1978). Reconstituted vesicles were mixed 1 : 1 with 1.2 M sucrose, 20 mM histidine (pH 7.0) and subjected to two rapid freeze-thaw cycles. For all experiments with purified material, a small amount of the mixture was taken up into a glass microliter volumetric pipette and was gently blown onto a preformed bilayer. Upon incorporation of channels, the conductance of the membrane increased in steps, each level indicating the incorporation of at least one channel. The number of channels in the bilayer was determined by the number of discrete current levels seen when the probability of channel opening was high. In this report, only bilayers containing 1–2 channels were analyzed.

Data Acquisition and Analysis

The current across the bilayer was measured and command voltages applied to the bathing solutions via a pair of Ag/AgCl electrodes connected to a homemade voltage-clamp circuit (French et al., 1986). The side to which the reconstituted vesicles were added was designated the *cis* side; the opposite side, *trans*, was held at virtual ground. Data were recorded on videotape. Stored records were filtered at 50–60 Hz on playback onto a strip chart recorder.

Table 1. Purification of STX-binding proteins*

Step	Specific activity (pmol STX bound/mg)
Solubilized *P*3	4.2 ± 0.23 (n = 11)
DEAE Sepharose	43 ± 3.5 (n = 11)
WGA Sepharose	328 ± 28 (n = 10)
HPLC size exclusion	1115 ± 88 (n = 10)
HPLC DEAE	2570 ± 235 (n = 4)

* ^{3}H-STX binding was determined at four different dilutions of each pooled fraction and averaged. Each value is the mean (±SEM) specific activity of n experiments. The value for HPLC DEAE column was obtained by averaging specific binding of all five pooled fractions (as shown in Fig. 1).

Results

Purification

Two distinct saxitoxin-binding proteins from rat brain were purified using a combination of liquid chromatography (anion exchange and lectin affinity chromatography modified from Hartshorne & Catterall, 1984) combined with HPLC (size exclusion and anion exchange with gradient elution) steps for high resolution separation. Table 1 shows the specific activity (pmol of ^{3}H-saxitoxin bound/mg protein) of the pooled active fractions following each purification step. The specific activity shown for the final HPLC DEAE purification step is an average of all pooled fractions and does not reflect individual specific activities for the two saxitoxin-binding proteins. Theoretical specific activity of the pure sodium channel is approximately 3200 pmol ^{3}H-saxitoxin bound/mg protein ignoring the contribution of carbohydrates to the molecular weight. An average value obtained in our studies was 2570 (Table 1); however, using a 15% correction for underestimate of equilibrium saxitoxin binding (Hartshorne & Catterall, 1984), an average final value obtained in this study was 2955 pmol/mg, similar to that obtained in other laboratories.

In the final purification step, HPLC DEAE, separation of two different ^{3}H-STX-binding proteins was achieved using a high resolution gradient elution. Column fractions were assayed for ^{3}H-STX binding as described in Materials and Methods (Method 1). The elution profile was broad (Fig. 1), suggesting the presence of more than one ^{3}H-STX-binding component. Fractions were pooled as indicated (PF1, PF2, PF3, PF4, and PF5), run on SDS-PAGE and/or reconstituted into vesicles for functional assays in planar bilayers. The gel profile for the five pooled fractions from a typical purification is shown at the top of Fig. 1 (*−IODO*). The alpha subunits from those fractions which elute early from

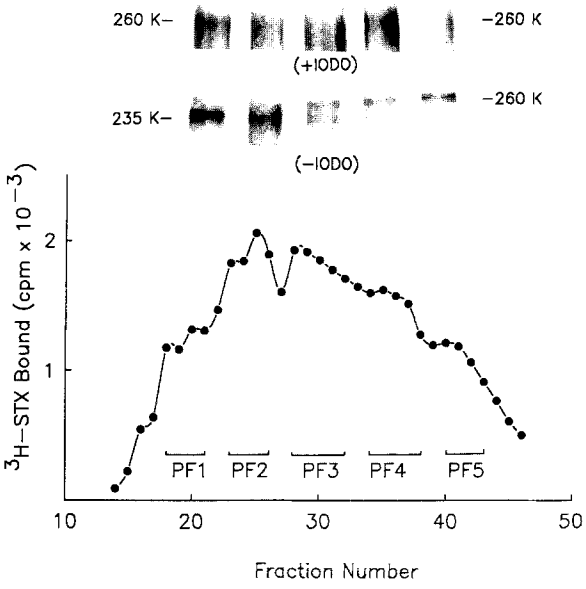

Fig. 1. Elution profile from HPLC TSK DEAE-5PW. ^{3}H-STX binding (mean specific binding, three replicates, two purifications) is shown for eluted fractions from the final (HPLC DEAE) purification step with corresponding positions of pooled fractions. Fractions 10–18 and 47–50 contained 1.5 ml; all other fractions contained 0.6 ml. Alpha subunits associated with each pooled fraction are shown above (SDS-PAGE, 7–20% gradient gel) in the presence (*+IODO*) and absence (*−IODO*) of excess iodoacetamide

the DEAE column have a lower apparent molecular weight (235,000) than those fractions which elute late (260,000). PF1 and PF2 contained only the lower molecular weight alpha subunit, PF3 and PF4 contained a mixture of the two alpha subunits, and PF5 contained only the alpha subunit of higher apparent molecular weight. Treatment with excess iodoacetamide following boiling causes both alpha subunits to run at 260,000 (Fig. 1, *+IODO*). This finding suggests that the lower apparent molecular weight of the alpha subunit which eluted first from this column was caused by the reformation of disulfide bridges, a process that is blocked by alkylation with iodoacetamide.

Functional Characterization

Veratridine–Modified Na Channel Subtypes

Each of the five pooled fractions from the final HPLC anion exchange step was reconstituted into planar bilayers for functional analysis. Figure 2 shows representative single-channel current records from the pooled fractions at +45 and −45 mV in the presence of 100 μM veratridine. The pooled fractions which contained the alpha subunit with the lower

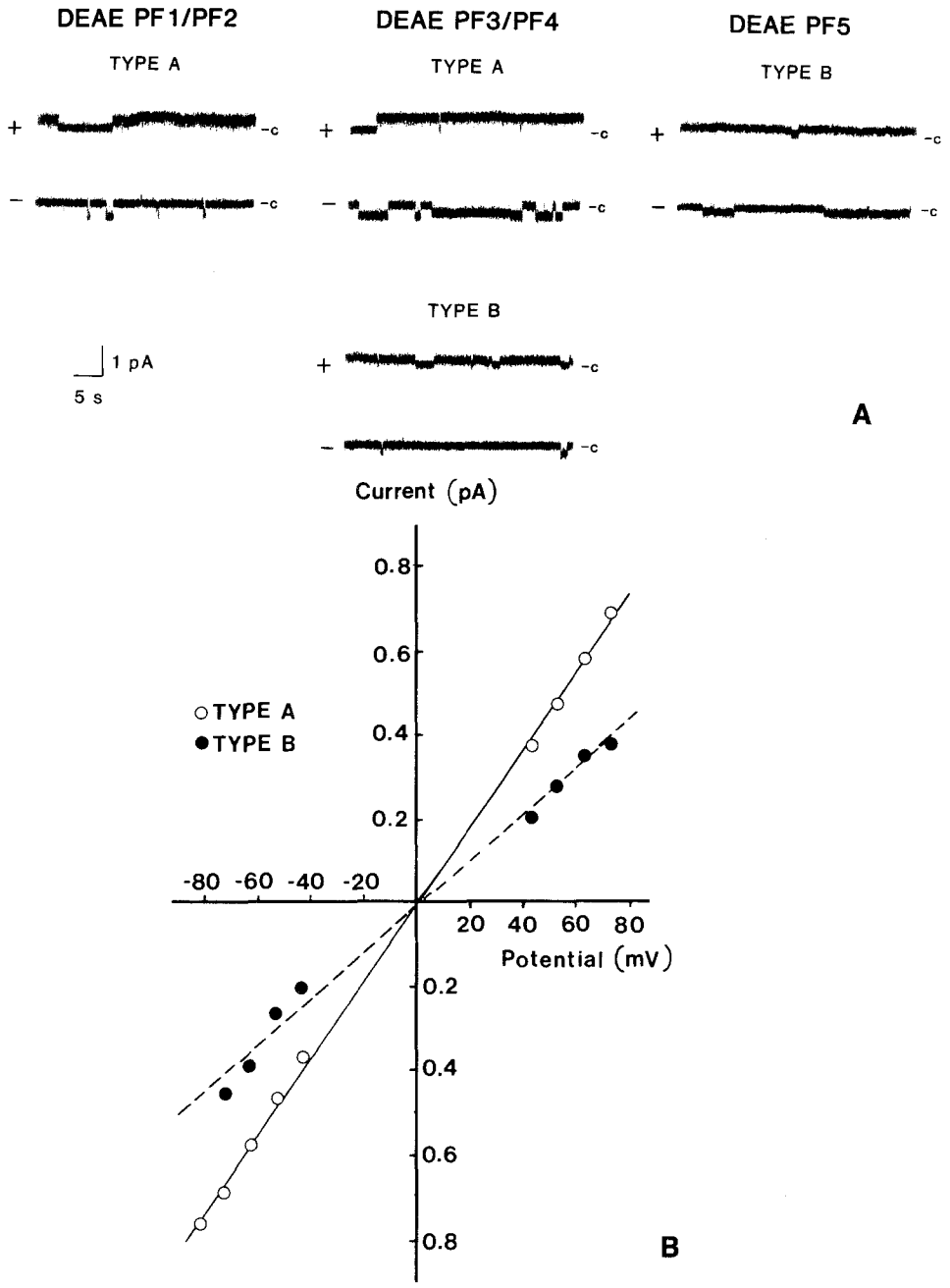

Fig. 2. (*A*) Incorporation of reconstituted DEAE pooled fractions into planar lipid bilayers in the presence of 100 μM veratridine. Experimental conditions are described in Materials and Methods. Single-channel current records are shown at +45 and −45 mV for pooled fractions (PF) 1 and 2, PF3 and 4, and PF5. Those fractions which elute first and have an alpha subunit with a lower apparent molecular weight in the absence of iodoacetamide (PF 1 and 2) displayed a single-channel conductance of approximately 9 pS (Type A channels). PF 3 and PF4, which contained a mixture of 235 and 260 kD alpha subunits in the absence of iodoacetamide, displayed both 9- and 5-pS conductances (Type A and Type B channels). Pooled fraction 5, which contained only the 260-kD alpha subunits in the absence of iodoacetamide, produced channels with a conductance of 5 pS (Type B). Records were filtered at 50 Hz. (*B*) Current-voltage relations of Type A and Type B channels in the presence of 100 μM veratridine. Type A channel (solid line, open circles) and Type B channel (dashed line, filled circles) current-voltage relations were linear with conductances of 9.5 and 4.8 pS, respectively

apparent molecular weight (PF1/PF2) formed functional sodium channels (designated Type A channels) of similar conductance (8.5–10 pS). Type A channels sometimes entered subconductance states (primarily at positive potentials) with current levels $\frac{2}{3}$ and/or $\frac{1}{3}$ the normal single-channel current level (Corbett & Krueger, 1989). Pooled fraction 5, which contained only the larger alpha subunit, formed only low conductance (4–6 pS) sodium channels (Type B). Pooled fractions 3 and 4, which contained a mix-

Table 2. Occurrence of Type A *vs.* Type B veratridine-activated Na channels at different levels of purification[a]

	P3	Partially purified	HPLC DEAE column				
			PF1	PF2	PF3	PF4	PF5
Type A (8.5–10 pS)	80%	64%	100%	100%	60%	41%	—
Type B (4–6 pS)	20%	36%	—	—	40%	59%	100%
n	75	40	11	8	10	21	22

[a] The number of times a Type A channel (8.5–10 pS) was incorporated in a planar lipid bilayer *vs.* a Type B channel (4–6 pS) in the presence of 100 μM veratridine was tallied. *P3* is prior to solubilization; *partially purified* includes channels reconstituted from pooled fractions in either the WGA column or HPLC size exclusion column. The number of channels incorporated is shown as *n*. The majority of bilayer membranes tallied in this study contained single channels; membranes containing more than two channels were not tallied.

ture of the two alpha subunits, produced both Type A and Type B sodium channels. The current-voltage relationship is shown in Fig. 2*B* for Type A (PF2: 9.5 pS) and Type B (PF5: 4.8 pS) sodium channels. The number of times that Type A and Type B Na channels were incorporated into planar bilayers is reported in Table 2 for each of the DEAE pooled fractions as well as crude and partially purified preparations. Both large (Type A) and small (Type B) conductance channels were observed in both crude membrane fractions from brain and partially purified preparations (WGA and HPLC size exclusion pooled fractions). The frequency of Type B channel incorporation increased following solubilization.

The voltage-dependent gating of Type A and Type B channels was similar. In both cases, channel openings at depolarized potentials tended to occur in long bursts with flickering in the open state. In the hyperpolarized range, channel openings were rare, with long closings between openings. Due to the small single-channel conductance under these experimental conditions (symmetrical 250 mM NaCl), we were not able to study channel properties between ±40 mV, where the driving force on Na^+ is small. On the assumption that the maximal probability of the channel being open (P_o) approaches 1, the plot of P_o at different membrane potentials (V_m) can be fit to a Boltzmann relation to obtain estimates of $V_{0.5}$ and gating charge. The Type A channels had a $V_{0.5}$ of -44 ± 2.7 mV and an apparent gating charge of 2.8 ± 0.4 (SEM; $n = 6$); the Type B channels had a $V_{0.5}$ of -32 ± 2.6 mV and an apparent gating charge of 2.5 ± 0.7 (SEM; $n = 4$). Due to the limited number of experiments performed, we cannot be sure that the differences in $V_{0.5}$ are significant. Thus, the primary functional distinction between the two channel types in the presence of veratridine alone is that Type A channels have approximately twice the conductance of Type B channels.

Batrachotoxin-Modified Na Channels

Figure 3*A* shows single-channel current records for Type A channels (DEAE PF2) and Type B channels (DEAE PF5) in the presence of 120 nM batrachotoxin. The Type A channels had about twice the single-channel conductance of the Type B channels. In both cases, the current-voltage plots were linear, with a conductance of 22 pS for the Type A channel and 12 pS for the Type B channel (Fig. 3*B*). Both channel types were blocked by the addition of 5 nM STX (Type A: $K_i = 0.4$ nM at -60 mV, $K_i = 34$ nM at $+60$ mV; Type B: $K_i = 0.67$ nM at -45 mV, $K_i = 1$ nM at $+45$ mV).

The number of times a Type A (20–30 pS) *versus* Type B (10–17 pS) batrachotoxin-modified channel was incorporated into the bilayer from crude brain membrane vesicles, partially purified and purified preparations is shown in Table 3. As with VER-activated sodium channels (Table 2), the low conductance BTX-activated channels are present in crude brain membranes and the percentage of low conductance BTX-modified channels increased following solubilization.

Effect of Scorpion Venom on Veratridine-Modified Na Channel Subtypes

Scorpion (*Leiurus quinquestriatus*) venom (LqV) had different effects on veratridine-modified Type A and Type B Na channels, providing further evidence of functional distinction between the two channel types. Figure 4*A* shows single-channel current records of two veratridine-modified Type A Na channels reconstituted in a planar bilayer. As reported previously (Garber & Miller, 1987; Corbett & Krueger, 1989), Na channels open rarely in the presence of veratridine, with bursts of openings lasting

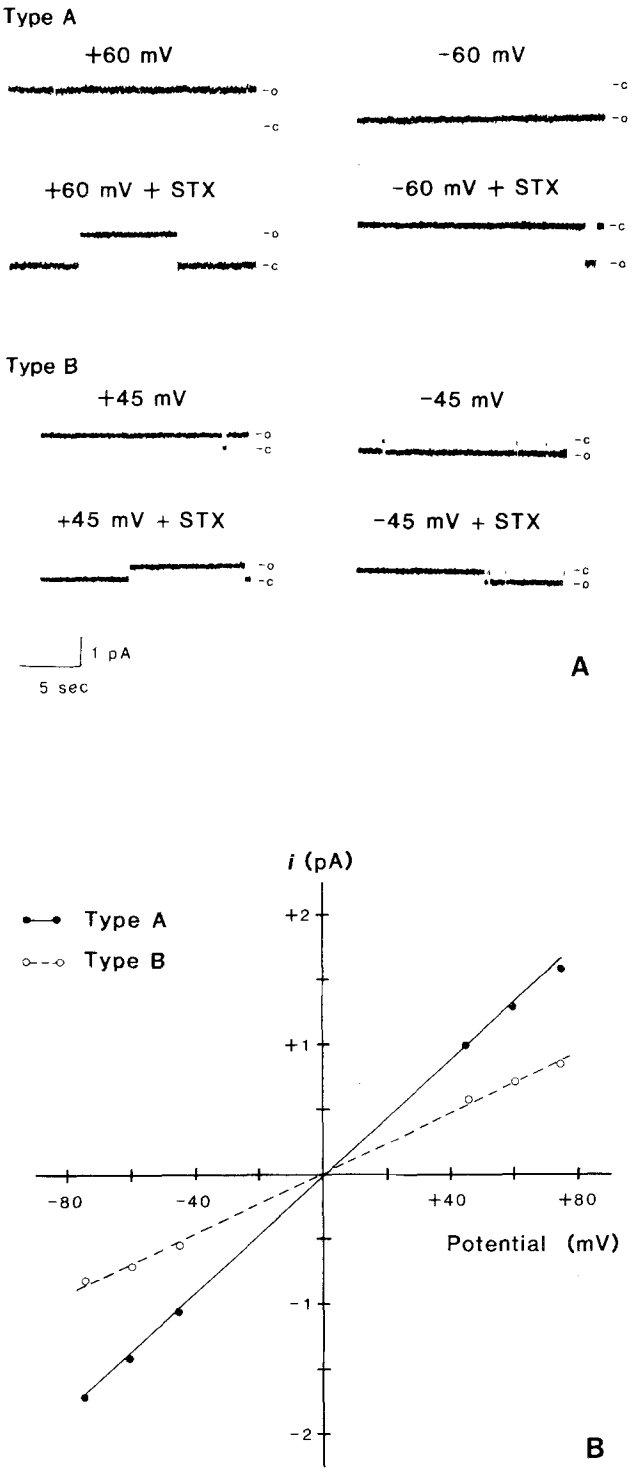

Fig. 3. (*A*) Single-channel current records of BTX-activated Type A (HPLC DEAE PF2) and Type B (HPLC DEAE PF5) channels. 600 nM BTX was present on the *cis* side of the bilayer. Experimental conditions were as described in Materials and Methods. 5 nM STX was added to the extracellular side where indicated. (*B*) Current-voltage relationships for BTX-activated Type A and Type B sodium channels. The single-channel conductances for Type A (solid line, filled circles) and Type B (dashed line, open circles) were 22 and 12 pS, respectively

Table 3. Occurrence of Type A *vs.* Type B batrachotoxin-activated Na channels at different levels of purification[a]

	*P*3	Partially purified	HPLC DEAE	
			PF2	PF5
Type A (20–30 pS)	87%	72%	100%	—
Type B (10–17 pS)	13%	28%	—	100%
n	349	39	5	2

[a] The number of times a Type A channel (20–30 pS) was incorporated into a planar lipid bilayer *versus* a Type B channel (10–17 pS) in the presence of 120 to 600 nM batrachotoxin was tallied. Terms and conditions for inclusion of data are the same as in Table 2.

as long as several seconds. Subconductance levels (Fig. 4*A*, arrowheads) were seen in both the absence and presence of LqV. Openings to a subconductance state account for less than 5% of all of the channel openings in the Type A channel at hyperpolarized potentials.

There was a small increase in apparent single-channel conductance following the addition of scorpion venom (19 ± 1% increase for five paired experiments in five membranes; $P < 0.001$), shown in Fig. 4*B*. The effects of scorpion venom on Type A channels reported here are similar to results obtained with purified alpha scorpion toxin and sea anemone toxin on partially purified Type A sodium channels (Corbett & Krueger, 1989).

The probability of the veratridine-modified Type A channel being open (P_o) as well as the duration of each opening decreased with hyperpolarization (Fig. 4*A* and *C*). P_o was determined such that the total open time of the channel, whether it opened to a full or subconducting state, was divided by the total recording time at that potential. Extracellular LqV increased P_o due to an increase in the open dwell time and shorter intervals between openings. Data from the single-channel current records are shown quantitatively in Fig. 4*C*. In control records (trace *1*), the probability of the channel being open decreases with hyperpolarization. Immediately following addition of scorpion venom (Fig. 4*C*, trace *2*), there is no change in the probability of channel opening at −45 mV. However, P_o increased as the membrane was hyperpolarized. This paradoxical result may reflect the voltage-dependent binding of the toxin to the channel at hyperpolarized potentials. At −85 mV, the probability of the channel opening decreased, reflecting the voltage-dependent gating of the venom-modified channel. Once the channel had been hyperpolarized and the toxin bound, the highest probability of the channel opening was seen at −45 and −55 mV, and decreased with hyperpo-

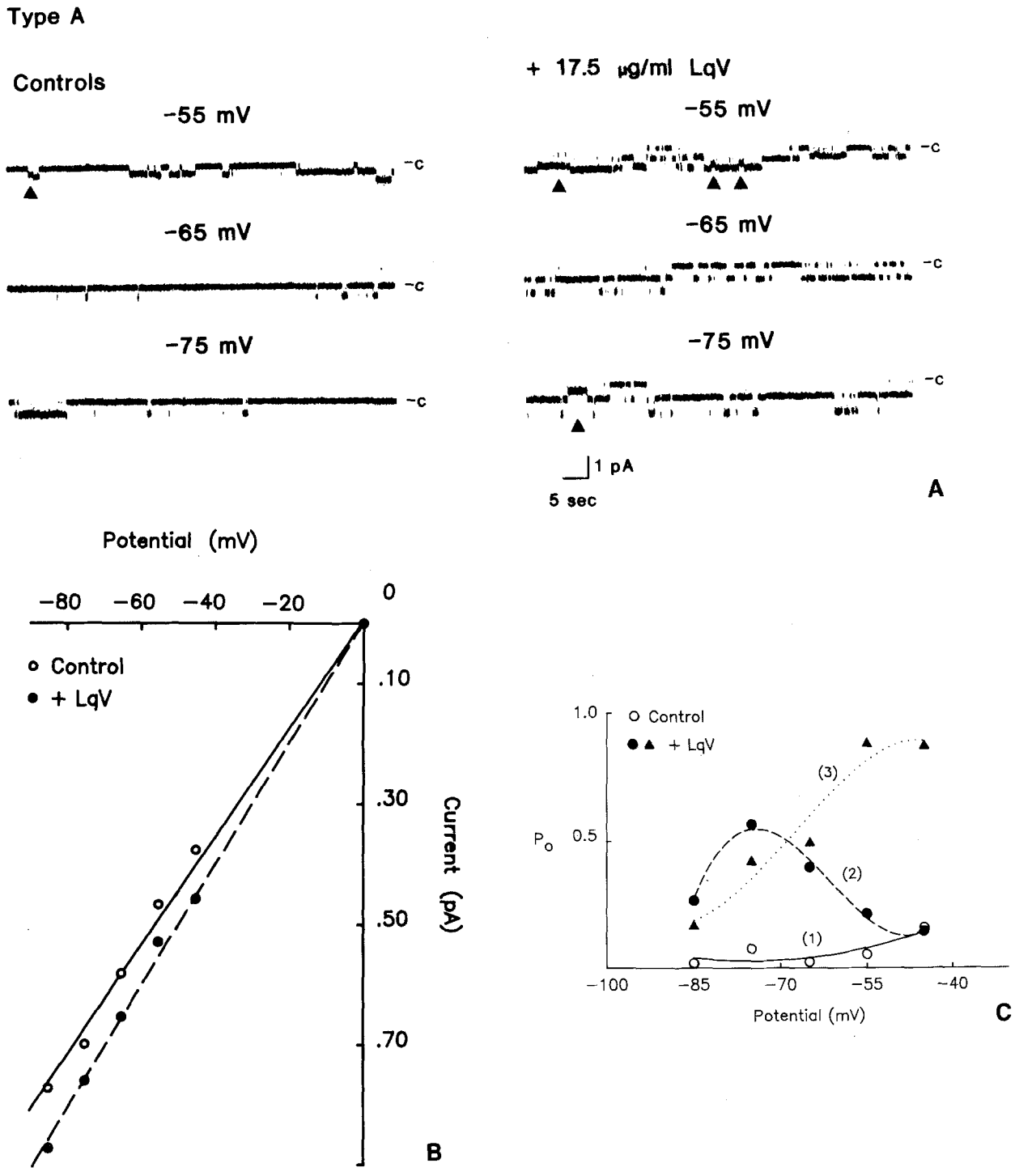

Fig. 4. Effects of LqV on veratridine-activated Type A channels. (*A*) Single-channel current records of Type A channel (DEAE PF2) before and after addition of 17.5 μg/ml scorpion (*Leiurus quinquestriatus*) venom to the extracellular side of the channel. Arrowheads indicate subconductance states present before and after venom addition. Bilayer conditions were as described in Materials and Methods. Records were filtered at 50 Hz. (*B*) Current-voltage relations of Type A channel in the presence (dashed line, filled circles) and absence (solid line, open circles) of scorpion venom. The single-channel conductance in the presence of 100 μM veratridine (control) was 9.1 pS. After LqV addition (7 μg/ml) to the extracellular side, single-channel conductance increased to 10.1 pS. (*C*) Probability of channel opening (P_o) *versus* membrane potential before and after venom addition. P_o was determined as the amount of time that a channel remained open, regardless of the conductance state, divided by the total recording time at any given potential. Trace *1* is the control curve in 100 μM veratridine; trace *2* was determined immediately after venom addition. P_o at each potential was retested following hyperpolarization to −85 mV (trace *3*)

larization, reflecting voltage-dependent gating of the venom-modified channels (Fig. 4*A*: + venom; Fig. 4*C*, trace *3*). Examination of the data at −55 mV in Fig. 4*C* (trace *2*), revealed a stepwise increase in P_o about 80% of the way through the record, possibly reflecting the toxin modification event (*data not shown*). This resulted in an intermediate average P_o value for the entire record at −55 mV.

The effects of scorpion venom on Type B Na channels are shown in Fig. 5. Single-channel current records from a Type B channel in the presence of 100 μM veratridine are shown in Fig. 5*A* (*controls*). As with the Type A channel, the probability of channel opening decreased with hyperpolarization (Fig. 5*A* and *C*: trace *1*). Type B channels, unlike Type A channels, showed no subconductance states from the 5-pS open level. Following addition of scorpion venom, the probability of channel opening increased, as was seen with the Type A channel. In addition, two current levels for channel openings can be seen (Fig. 5*A*, + venom), one at 10 pS and one at 5 pS. The two current level openings seen following LqV addition are shown quantitatively in Fig. 5*B*. These two current levels represent two different conductance states of a single channel for the following reasons. First, it should be noted that there were no second level openings apparent in any of the control records and at the first potential tested (−45 mV) after venom addition, suggesting a single channel in those stages of the experiment. If the 10-pS opening reflects a toxin-modified state, hyperpolarization would be expected to increase the occurrence of 10-pS openings, reflecting the voltage-dependent binding of the toxin to the channel. The following was observed: at −45 mV (before hyperpolarization; Fig. 5*C*: trace *2*) all of the openings were at the 5-pS level and there was no increase in P_o (no toxin modification); at −55 mV, 68% of the openings were at the 10-pS level and there was a dramatic increase in P_o; at −75 mV, 99% of the openings were 10 pS; at −85 mV, 100% of the openings were to the 10-pS level. When −45 mV was retested following hyperpolarization (trace *3*), 52% of the openings were to the 10-pS level. Thus, at potentials where scorpion toxin binding is enhanced (−75 and −85 mV) as demonstrated by increased P_o, a single 10-pS level was observed with full closings to baseline. No partial closings to the 5-pS level were evident from any of the 10-pS openings in over 200 channel closings at −75 and −85 mV. If these records represented two independent 5-pS channels rather than one 10-pS channel, many partial closings to the 5-pS level would be expected. Thus, in the experiment shown in Fig. 5*A*, the bilayer contained only one channel and the 5- and 10-pS levels at −55 and −65 mV represent the unmodified and toxin-modified states, respectively.

Figure 5*C* shows the effect of scorpion venom on the P_o at different potentials. Immediately after the addition of scorpion venom (trace *2*), there is no increase in the P_o at −45 mV, as observed with the Type A channels. With hyperpolarization (trace *2*), the probability of channel opening increased; however, unlike the Type A channel, the probability of channel opening did not decrease when the potential was hyperpolarized from −75 mV. When −45 mV was retested following hyperpolarization (trace *3*), the probability of channel opening was the same as that at −85 mV.

Discussion

Two Different Na Channels Isolated From Rat Brain

We have isolated two distinct STX-binding proteins from rat brain using a combination of batch elutions with DEAE anion exchange and WGA affinity chromatography followed by high resolution HPLC (size exclusion and DEAE gradient). These STX-binding proteins differed by at least two biochemical and two functional criteria, as discussed below. Both channel types were observed in bilayers to which crude rat brain membrane vesicles had been added (Tables 2 and 3) and thus are not an artifact of solubilization and purification. Type A channels appear to be identical to those previously described in planar bilayers (Hartshorne et al., 1985; French et al., 1986; Green, Weiss & Anderson, 1987); Na channels with the properties of Type B channels have not previously been reported. Type B channels were observed infrequently in crude brain membranes; however, following solubilization and partial purification their appearance in bilayers was enhanced (Tables 2 and 3). Thus, the solubilization procedure may preferentially solubilize Type B channels.

Type A and Type B Channels Have Different Biochemical Properties

Separation of the two STX-binding proteins was achieved by high resolution HPLC anion exchange chromatography (Fig. 1, Tables 2 and 3). The elution behavior on this column is determined primarily by net charge; thus, the Type B channels may have a larger net negative charge resulting in tighter binding to the cationic groups on the column. The Na channel alpha subunit is known to be highly glycosylated with a high density of negatively charged sialic acid residues on the extracellular surface (Miller et al.,

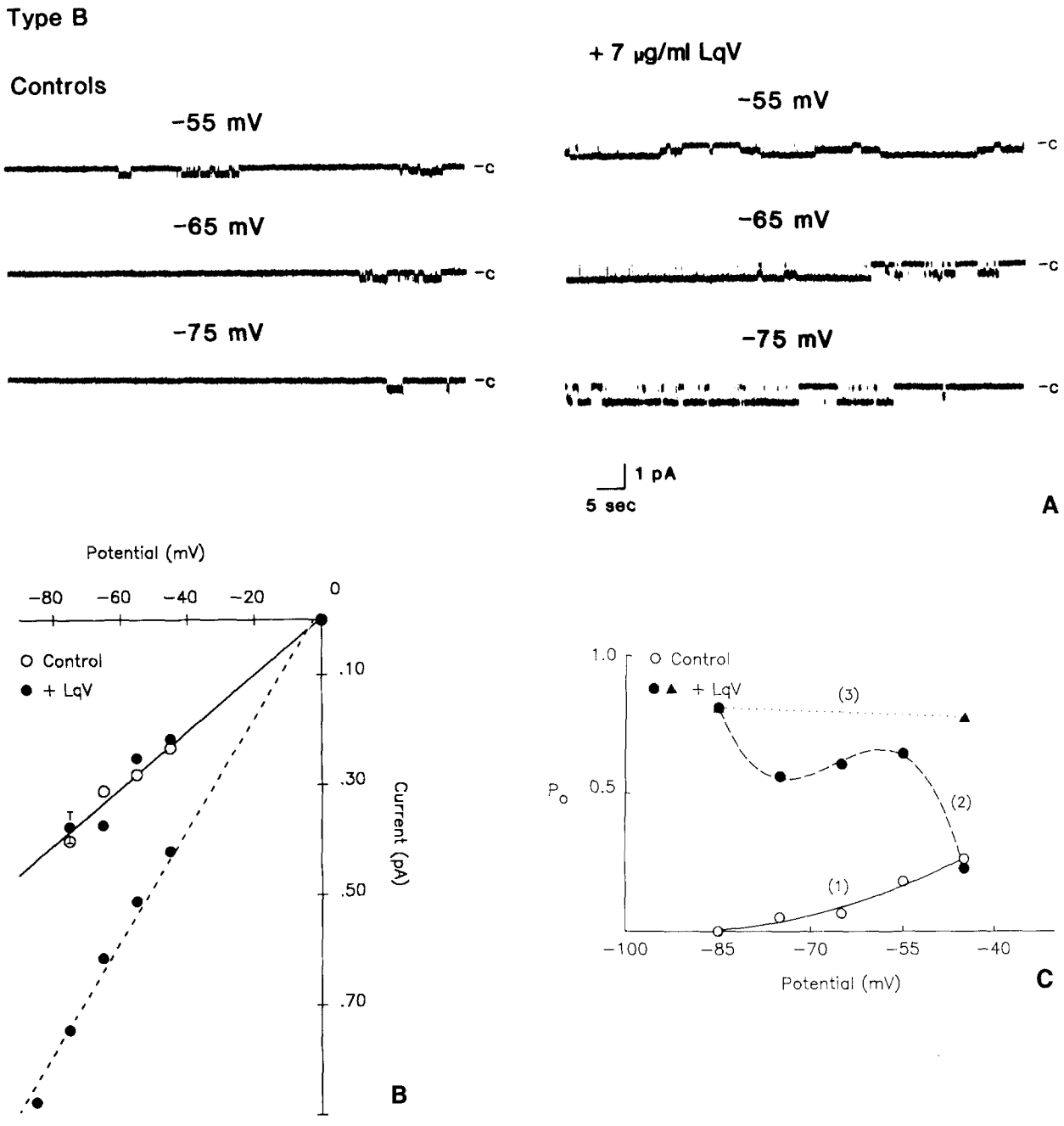

Fig. 5. Effects of LqV on veratridine-activated Type B sodium channels. (*A*) Single-channel current records of Type B (PF5) veratridine-activated Na channel in the presence and absence of 7 μg/ml scorpion (*Leiurus quinquestriatus*) venom. Bilayer conditions were as described in Materials and Methods. In the presence of extracellular LqV, two different unitary current fluctuations were seen corresponding to approximately 5 and 10 pS. Records were filtered at 50 Hz. (*B*) Current-voltage relationship of Type B channel in the absence (○) and presence (●) of scorpion venom. As described in the text, following venom addition, two single-channel current levels were observed, one corresponding to 5.1 pS and one to 10.1 pS. (*C*) Probability of channel opening *versus* membrane potential in the presence and absence of scorpion venom. P_o was determined as described in the legend to Fig. 4 and in the text. Trace *1* shows the control gating in the presence of veratridine alone. The data in trace *2* were measured immediately after venom addition. Following hyperpolarization to −85 mV, P_o values were retested (trace *3*)

1983; Levinson et al., 1986). Differences in the number or three-dimensional configuration of these sialic acid residues could account for this differential chromatographic behavior of Type A and Type B channels. There are differences in net charge among the predicted amino acid sequences of the three cloned rat brain sodium channels (Noda et al., 1986; Kayano et al., 1988).

Type A and Type B channels had molecular weights that appeared to differ by about 25,000 on SDS-PAGE. The absolute molecular weights of peptides in this range are difficult to determine with high precision and thus cannot be directly compared to molecular weights reported by others or to absolute molecular weights calculated from amino acid sequences. We stress that in side-by-side lanes on SDS-PAGE under identical conditions, the two STX-binding proteins are clearly different (Fig. 1). SDS-PAGE separates on the basis of size provided that the proteins are denatured (uncoiled) and have the same charge-to-mass ratios (normally due primarily to bound SDS). Highly glycosylated or fatty-acid acylated proteins, including purified Na channels, do not show typical behavior on SDS-PAGE (Levinson et al., 1986). Thus differences in mobility on SDS-PAGE may reflect differences in glycosylation, retained secondary structure (due to nonreduced or reformed disulfide linkages) as well as actual differences in molecular mass. We observed (Fig. 1) that when treated with excess iodoacetamide before electrophoresis, Type A channels ran at about 260 kD and were then indistinguishable from Type B channels on SDS-PAGE. One of the actions of iodoacetamide involves the alkylation of sulfide groups, which blocks disulfide bridge formation. Other work has shown that proteins which are run in the absence of mercaptoethanol (disulfide bridges intact) have a lower apparent molecular weight than in the presence of mercaptoethanol (Chadwick, Inui & Fleischer, 1988). This result suggests that the two channel subtypes may differ in the number or stability of internal disulfide linkages that determine secondary structure, raising the possibility that the observed differences in electrophoretic behavior of Type A and B channels may be due to differences in protein structure (amino acid sequence) rather than solely to different glycosylation or other post-translational modification.

We have focused on the alpha subunits in this study. Although some lightly stained protein bands were occasionally observed in the 35–40 kD region on SDS-PAGE, we did not consistently detect beta subunits which would be expected to have only about 10 to 15% of the staining intensity of the alpha subunits. Because our Type A channels have very similar biochemical and functional properties to those of purified and reconstituted rat brain Na channels (Hartshorne et al., 1985; Corbett & Krueger, 1989), we expect Type A channels to be associated with two beta subunits. There has been no evidence suggesting that the presence or absence of beta subunits influences single-channel conductance or voltage-dependent gating, the two functional properties that were studied in this report.

Type A and Type B Channels Have Different Functional Properties

Type A channels had about twice the single-channel conductance of Type B channels in the presence of either veratridine or BTX. The effect of scorpion venom on the single-channel conductances of the two veratridine-activated channel subtypes was, however, dramatically different.

Following the addition of scorpion venom to a single veratridine-activated Type B channel, two current levels were observed, corresponding to single-channel conductances of 5 and 10 pS (Fig. 5*A*). Based on the following, we interpreted the 5- and 10-pS conductances as two different conductance states (toxin-modified and unmodified) of the same channel, rather than two independent channels. At potentials where the binding of scorpion toxin was enhanced (-75 and -85 mV) and channels should have been toxin-modified, over 99% of all channel openings were to the 10-pS level with full closings to baseline evident. One would not expect two independent 5-pS channels to close simultaneously with this frequency (over 200 full 10-pS closings). If this then represents a toxin-induced increase in single-channel conductance, one would expect to observe more of the large conductance openings at membrane potentials where toxin binding would be enhanced (hyperpolarized potentials) and more of the lower conductance openings at more positive potentials, where the toxin readily dissociates. This was, in fact, observed (Fig. 5*A*).

The underlying molecular basis for the differences in single-channel conductance and the different effects of scorpion venom is not known. One possibility is that the pore structures (or charge densities at the pore entrances) of the two channel types are different. Another possibility is that the apparent single-channel conductance of one or both channel types is, in part determined by very rapid gating (flickering) that is too fast to be resolved by our recording system. In this case, the lower apparent single-channel conductance of Type B channels might be due to rapid flickering that is incompletely resolved because of bandwidth limitations. The scorpion venom, then, may suppress that fast gating

component, leading to an increase in the apparent single-channel conductance. Similarly, either an altered permeation pathway or flickering may account for the different apparent single-channel conductances of BTX-activated channel subtypes. Regardless of the mechanism, Type A and Type B channels are always different with respect to apparent single-channel conductance and the effects of scorpion venom. We never observed both Type A and Type B channel properties when there was only a single channel in the bilayer and, thus, the two channel types are not interchangeable nor is one a subconductance state of the other.

In the absence of scorpion venom, the voltage dependence of VER-activated Type A and Type B channels was similar. Type A channels displayed a mean $V_{0.5}$ about 15 mV more negative than Type B channels; however, this is based on a limited number of experiments and may not be significant. The response to scorpion venom, however, was different. Whereas scorpion venom caused a 10- to 30-mV hyperpolarizing shift in the P_o *versus* V_m relation of Type A channels, Type B channel gating was shifted at least 85 mV in the presence of both veratridine and scorpion venom (Figs. 4*C* and 5*C*).

Scorpion venom is known to contain a number of toxins (alpha and beta toxins) as well as phospholipases and proteases which might play some role in modification of sodium channel subtypes. We cannot distinguish between a single venom component having different effects on the two channel subtypes or different components (possibly more than one component) eliciting differential channel behavior. It seems likely that the active component of scorpion venom is the alpha polypeptide toxin (Catterall, 1976). Using partially purified Type A veratridine-activated rat brain Na channels (Corbett & Krueger, 1989), alpha scorpion toxin had effects on both apparent single-channel conductance and P_o *versus* V_m relations similar to those seen with the crude venom (Fig. 4*B* and *C*).

Comparison of Type A and Type B Channels with Previously Described Channel Subtypes

Many excitable tissues, notably cardiac and denervated skeletal muscle have Na channel subtypes with a very low sensitivity to TTX and STX ($K_i > 1$ μM). Neither Type A nor Type B channels are in this toxin-insensitive category because both bind STX with affinities in the nanomolar range. Moreover, we estimate the K_i for block of single-channel currents to be about 1 nM (Fig. 3). Thus, the two STX-sensitive channel subtypes described in this paper are clearly different from those TTX-insensitive subtypes found in cardiac or denervated skeletal muscle.

Rat brain has been shown to contain at least three (R_I, R_{II}, and R_{III}; Noda et al., 1986; Kayano et al., 1988) and possibly four (IIA, Auld et al., 1988) distinct Na channel types. Expression of transcripts from each of these genes in *Xenopus* oocytes revealed that each coded for TTX/STX-sensitive channels. There are slight differences in the predicted protein molecular weights and net charge among these subtypes. However, the calculated molecular weights of these nonglycosylated channel proteins cannot be compared with the apparent molecular weights on SDS-PAGE of the native, heavily glycosylated channel proteins. Catterall and coworkers have reported that purified rat brain Na channels with an alpha subunit molecular weight of 260,000 (using iodoacetamide pretreatment prior to SDS-PAGE) have properties very similar to our Type A channels (Hartshorne et al., 1985). Gordon et al. (1987) demonstrated that this rat brain Na channel preparation contained 18% R_I Na channel and some mixture of R_{II} and R_{III} Na channels by immunological criteria (the antibodies used to distinguish R_{II} Na channels would be expected to cross-react with R_{III} since the antigen had 75% homology between these two types). Although there have been other reports in the literature of low conductance BTX- and VER-activated channels from brain (Duch, Recio-Pinto & Urban, 1987; Green et al., 1987; Duch et al., 1988, 1989), it has not been clear whether these low conductance states are due to multiple substates of a single channel or multiple channel types.

Voltage-clamp analysis of sodium channels expressed in *Xenopus* oocytes from in vitro transcribed mRNA encoding of the alpha subunits of R_I, R_{II} and R_{III} sodium channels from rat brain has revealed no differences in channel activation or inactivation in the absence of neurotoxins (Stuhmer et al., 1987; Suzuki et al., 1988). These results are not inconsistent with our results since the functional differences described here depend on neurotoxin binding and may not reflect differences in the activation or inactivation of the unmodified channels. Thus our Type A and Type B sodium channels may correspond to two of the three rat brain channels for which clones have been isolated and sequenced.

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