Purified and Unpurified Sodium Channels from Eel Electroplax in Planar Lipid Bilayers

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ABSTRACT Highly purified sodium channel protein from the electric eel, *Electrophorus electricus*, was reconstituted into liposomes and incorporated into planar bilayers made from neutral phospholipids dissolved in decane. The purest sodium channel preparations consisted of only the large, 260-kD tetrodotoxin (TTX)-binding polypeptide. For all preparations, batrachotoxin (BTX) induced long-lived single-channel currents (25 pS at 500 mM NaCl) that showed voltage-dependent activation and were blocked by TTX. This block was also voltage dependent, with negative potentials increasing block. The permeability ratios were 4.7 for Na^+:K^+ and 1.6 for Na^+:Li^+. The midpoint for steady state activation occurred around −70 mV and did not shift significantly when the NaCl concentration was increased from 50 to 1,000 mM. Veratridine-induced single-channel currents were about half the size of those activated by BTX. Unpurified, nonsolubilized sodium channels from *E. electricus* membrane fragments were also incorporated into planar bilayers. There were no detectable differences in the characteristics of unpurified and purified sodium channels, although membrane stability was considerably higher when purified material was used. Thus, in the eel, the large, 260-kD polypeptide alone is sufficient to demonstrate single-channel activity like that observed for mammalian sodium channel preparations in which smaller subunits have been found.

INTRODUCTION

In many excitable tissues, the rising phase of action potentials is mediated by ionic currents through voltage-dependent sodium channels. All published polypeptide compositions of the tetrodotoxin (TTX)/saxitoxin-binding component of the sodium channel have in common a large, 260-kD glycoprotein (Catterall, 1986), first characterized in eel electroplax (Agnew et al., 1978). Additional lower-molecular-weight polypeptides have been reported for preparations of rat and rabbit muscle (Kraner et al., 1985; Tanaka et al., 1986) and rat brain (Hartshorne and Catterall, 1984), but not for eel (Norman et al., 1983; Elmer

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et al., 1985; Agnew et al., 1986; Duch, 1986). Elmer et al. (1985) did not detect any low-molecular-weight polypeptides in rat brain. This variability in polypeptide composition could reflect tissue or species differences, isolation of different subtypes of sodium channels (Noda et al., 1986*a*), or the loss and/or breakdown of polypeptides during purification.

Different approaches are available to address the issue of the functional significance of the various polypeptide subunits. Functional sodium channels have been expressed in amphibian oocytes injected with messenger RNA coding for only the large polypeptide of a mammalian brain channel (Noda et al., 1986*b*; Goldin et al., 1986). Nevertheless, small peptides endogenous to the oocyte could have combined with the product of the exogenous message to form a functional channel (Noda et al., 1986*b*; Goldin et al., 1986). In a complementary approach, the single-channel properties of purified, well-characterized protein preparations can be examined in lipid bilayer membranes of known composition (Rosenberg et al., 1984*b*; Hartshorne et al., 1985; Furman et al., 1986; Keller et al., 1986; Levinson et al., 1986).

In the present study, sodium channels from *Electrophorus electricus* were purified using new methods to yield reconstituted preparations of unprecedented recovery (25–35%) and specific activity (1,900 pmol TTX bound/mg protein), in which a single 260-kD peptide was the sole or dominant component. We describe in detail the functional characteristics of this polypeptide in lipid bilayers, as well as those derived from unpurified electroplax membrane fragments. We conclude that the 260-kD glycopeptide preparation from eel electroplax is sufficient to express sodium channel functionality in lipid bilayers, and further that these channels differ little from those of mammalian nerve or muscle. Preliminary accounts have appeared elsewhere (Duch et al., 1986; Levinson et al., 1986; Recio-Pinto et al., 1986).

METHODS

Preparation of Sodium Channel Material

The TTX-binding component from *E. electricus* was purified using the detergent CHAPS (3-[3-cholamidopropyl]-dimethylammonio-1-propane sulfonate; Calbiochem-Behring Corp., La Jolla, CA) and asolectin/phosphatidylcholine (1:1) mixtures as previously described (Duch, 1986; Duch and Levinson, 1987).

Reconstitution into lipid vesicles for fusion with planar bilayers was carried out by placing purified protein fractions in Spectrapor (Fisher Scientific Co., Pittsburgh, PA) 32-mm standard cellulose dialysis tubing (molecular weight cutoff, 12,000–14,000) and dialyzing against 2 liters of 150 mM sucrose, 67.5 mM $Tris_2$-sulfate, 0.5 mM $MgCl_2$, and 25 mM HEPES/Tris, pH 7.4 at 4°C. The dialysis buffer was changed every 8 h for 3 d. The use of this buffer increased the probability of stable channel activity in the bilayer compared with other reconstitution buffers (Duch, 1986; Duch and Levinson, 1987).

The purest preparations contained only a single polypeptide with an apparent molecular weight of 260 kD (Fig. 1). In some preparations, smaller polypeptides (200 and 45 kD) were sometimes present, but always made up <10% of the total purified protein. These smaller polypeptides have also been found in muscle preparations, where they are believed to represent proteolytic fragments of the large, 260-kD glycoprotein (Tanaka et al., 1986).

No peptides with apparent molecular weights from 30 to 40 kD were found in any of the purified eel preparations.

The post-reconstitution specific activities of the four preparations (preparations 1–4) fused with planar bilayers were 1,550, 2,080, 2,000, and 1,800, respectively, yielding an average of 1,860 pmol TTX bound/mg protein. This value represents ~50% of the theoretical value for a 100% pure and functional preparation (~3,846 pmol TTX bound/mg protein based on a molecular weight of 260 kD). Preparation 1 was eluted only once, and preparations 2–4 were eluted twice through a Sepharose 6B column. The average loss of specific activity during reconstitution was only 13%. This represents an improvement over previously reported reconstitution systems using SM-2 Biobeads and freeze-thaw steps, where ≥50% of the TTX-binding capacity is lost during reconstitution (Weigele and Barchi, 1982; Talvenheimo et al., 1982; Rosenberg et al., 1984*a*; Elmer et al., 1985). Reconstituted vesicles were either kept on ice and used for a period between 7 and 14 d after the initial channel extraction or frozen and stored in liquid nitrogen. When kept on ice, channel activity in planar bilayers was generally lost toward the end of

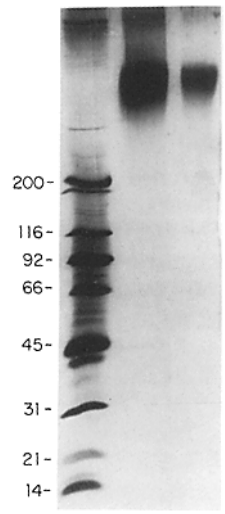

FIGURE 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of purified protein fractions that had been reconstituted by dialysis (chemical purity). Lane 1 contains standards, while lanes 2 and 3 contain the purified, reconstituted protein preparation. Lane 2 had twice as much protein added as lane 3 in order to make the presence of any contaminating peptides more distinguishable. A densitometry scan of lane 3 revealed only the single large polypeptide seen on the gel. This preparation had a specific activity of 1,800 pmol TTX bound/mg protein after reconstitution.

14 d. This correlated with an increasing inability to obtain measurable toxin-mediated ion fluxes into reconstituted vesicles, even though TTX binding was stable throughout this period (Duch, D. S., unpublished observation). Preparations kept in liquid nitrogen displayed normal, though reduced, activity even after 18 mo of storage.

Unpurified, nonsolubilized sodium channels in membrane fragments from eel electroplax were prepared as described (Miller et al., 1983), except that after the 48,000-*g* centrifugation step, the membranes were resuspended in the sucrose buffer described above. This unpurified preparation was stored in liquid nitrogen before use. It had 70–100 pmol TTX binding per milliliter of preparation as compared with 40–300 pmol TTX binding per milliliter of the highly purified preparation.

Bilayer Procedures

For the studies of single-channel characteristics, planar bilayer techniques were used (Urban et al., 1980; Green et al., 1987*a*). The organic solvents and salts were at least

reagent grade (Fisher Scientific Co., Pittsburgh, PA, and VWR Scientific Div., Univar, San Francisco, CA). The alkali metal chlorides were roasted for at least 24 h at 500–600°C. TTX was purchased from Sigma Chemical Co., St. Louis, MO. All solutions were prepared using doubly distilled water and buffered with 10 mM HEPES at pH 7.4 (from United States Biochemical Corp., Cleveland, OH). Experiments were performed at room temperature (22–25°C).

Symmetrical Teflon bilayer chambers with 5-ml compartments were as designed by Andersen (1983). Contaminated or leaky chambers were cleaned as follows (Andersen, 1983): sonication in petroleum ether, three water rinses, sonication in ethanolic NaOH, three water rinses, and sonications in ethanol (70%), ethanol (30%), water, and acetone (stage I cleaning). Before and after each experiment, the chambers were cleaned using successive rinses of water, ethanol, and chloroform/methanol (2:1 vol/vol); this sequence was repeated three times (stage II cleaning). Acetone, chloroform, and methanol were distilled before use.

Channel currents were recorded under voltage-clamp conditions using a standard current-to-voltage amplifier (Urban et al., 1980) with a 10^{10}-Ω feedback resistor and a 150-Hz corner frequency. Stripchart records were typically filtered at 50 Hz (eight-pole Bessel filter) and analyzed by hand. Before each experiment, DC and AC calibration was verified with an equivalent circuit consisting of a parallel combination of a 10^{11}-Ω resistor (1%) and a 310-pF polystyrene capacitor (2.5%), and a power spectrum was obtained. Coiled wire Ag/AgCl electrodes (asymmetries <1 mV) made direct contact with the electrolyte, the *trans* chamber being at virtual ground.

Planar bilayers were formed from neutral phospholipid solutions containing (4:1) phosphatidylethanolamine and phosphatidylcholine (Avanti Polar Lipids, Inc., Birmingham, AL) in decane (5% wt/vol, 99.9% pure; Wiley Organics, Columbus, OH). Bilayer membranes were usually large (200–400 pF). Activating alkaloid toxins, 1 μM batrachotoxin (BTX) (John W. Daly, National Institutes of Health, Bethesda, MD) or 100 μM veratridine (Sigma Chemical Co.), were present only in the *trans* chamber and were allowed to equilibrate with the bilayer for 20–40 min. A background conductance of 5–20 pS and a noise level comparable to that of the equivalent circuit were verified before any channel material was used.

The channel preparation was added to the *cis* chamber either by following established procedures (Krueger et al., 1983) or by placing the tip of a Pasteur or Eppendorf pipette containing 0.1–0.5 μl of channel material (alone or mixed 1:1 with the phospholipid solution) directly above the partition hole. The latter method was generally used for material stored in liquid nitrogen. To aid incorporation of channel activity into the planar bilayer, square pulses (±100 mV, 1 Hz) were used. Incorporation of channel activity was achieved without prior freeze-thawing of the reconstituted vesicles (Rosenberg et al., 1984*b*; Hartshorne et al., 1985), without creating osmotic gradients (Hartshorne et al., 1985; Furman et al., 1986) or adding divalent cations (Hanke et al., 1984; Hartshorne et al., 1985), and without cholesterol (Hanke et al., 1984) in the planar lipid bilayer. Additional channel incorporations usually occurred when large potentials were applied during the course of an experiment, making it difficult to analyze TTX-block data or to follow the gating characteristics of the same channels with time. The electrophysiological sign convention was used; the extracellular side of the channels was determined by their gating characteristics and by TTX block. Purified sodium channels could orientate in either direction in the planar bilayer, regardless of the procedure used for the addition of the channel material.

Single-channel conductances were obtained by using the linear regression analysis of the current-voltage curves. In multiple-channel membranes, the number of channels was estimated by dividing the total conductance (corrected for the background) by the

predominant single-channel conductance. When measuring TTX block, each holding potential was applied for 10–20 min in order to control for time-dependent changes in TTX-binding parameters, which appear to occur after applying a voltage change across the channels (unpublished observation). When gating characteristics were measured, membrane potentials were held for 10–20 s and incremented or decremented monotonically in 20- and 10-mV steps. Time-averaged currents were recorded simultaneously by splitting the output from the amplifier and filtering one channel at 1 Hz (Andersen and Muller, 1982). These traces were sampled every second, and the mean value of the continuous sample points at a constant membrane potential gave the time-averaged single-channel current. These data were further transformed as described in the Results. They were displayed together with the calculated curves, which were adjusted by hand. Analysis

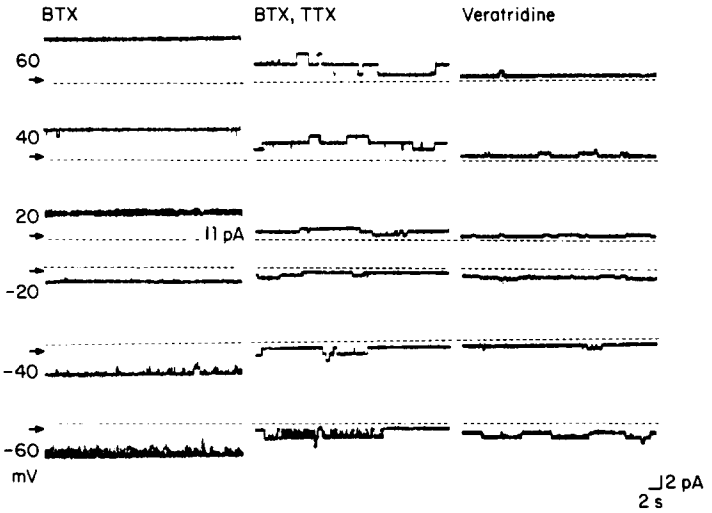

FIGURE 2. Activator-modified single-channel current transitions of purified sodium channels in symmetrical 500 mM NaCl at various membrane potentials. (*Left*) Membrane with three BTX-modified sodium channels. The channels remained mostly open at positive and lower negative potentials, and began to gate at higher negative potentials. (Note the change in current scale for the +20-mV tracing.) (*Middle*) Same membrane and channels as in the left column after the addition of TTX (180 nM). (*Right*) Another membrane with at least two veratridine-modified sodium channels. Potentials refer to the *cis* chamber; the orientation of the sodium channels was not determined. The arrows indicate baseline (0 mV) current.

of variance and the Bonferroni procedure for the modified *t* test (Dunn and Clark, 1974; Wallenstein et al., 1980) were used to compare experimental groups.

RESULTS

Single-Channel Conductances

The number of channels incorporated into lipid bilayers varied from 1 to ~200. Membranes with a low number of channels were chosen for measurements of single-channel current transitions. BTX-modified, purified sodium channels remained mostly open, except at higher negative potentials (Fig. 2). In symmetrical NaCl solutions, the current-voltage relationship was symmetrical, and the single-

channel conductance was independent of membrane potential (Fig. 3). Channels observed for >5 h showed no obvious deterioration and no change in their current-voltage relationship.

TTX blocked sodium channels in an all-or-none manner and only when added to the extracellular side of the channel. Submaximal TTX concentrations did not change the magnitude of the single-channel current transitions (Figs. 2 and 3 and Table I). TTX block was voltage dependent, with negative potentials increasing block (Fig. 2). Veratridine-modified sodium channels showed a smaller single-channel conductance (Fig. 3 and Table I) and many more closures than channels activated by BTX (Fig. 2). The frequency and duration of these closures appeared to be voltage dependent (unpublished observation).

Purified and unpurified BTX-modified sodium channels had the same single-channel conductance and showed the same small reduction in conductance when the salt concentration was lowered (Fig. 4 and Table II). The averages have been

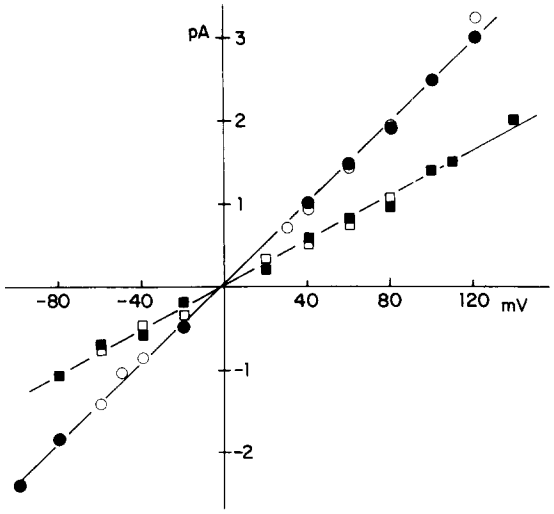

FIGURE 3. Activator-modified single-channel current transitions of purified sodium channels as a function of membrane potential in symmetrical 500 mM NaCl. The slope fitted by linear regression gave the single-channel conductance. For BTX-modified sodium channels in the absence (filled circles) and in the presence (open circles) of TTX (42 nM) the slope was 25 pS ($r = 0.998$). For veratridine-modified sodium channels (two membranes, open and filled squares), the slope was 13 pS ($r = 0.996$).

calculated by weighting the single-channel conductance data either by the number of channels or by the number of membranes. The first method gives equal weight to each channel. This method could give misleading results if, for example, channel behavior were affected by variable amounts of native lipids or other contaminating substances that might be incorporated into bilayers together with sodium channels. The second method indicates how much the single-channel conductance varied between membranes. Both methods gave quite similar results. There were no detectable differences between purifications (Table I) and, in addition, the single-channel conductances were quite similar for purified and unpurified channels (Fig. 5 and Table II). About 30% of the formed membranes contained channel activity regardless of whether purified (43 out of 132 membranes) or unpurified (21 out of 69 membranes) material was used. However, membrane stability was considerably higher when purified material was used (Table II): membranes containing purified channels lasted approximately seven times longer.

Single-channel conductances of 12–14 pS were observed in all preparations, but less frequently than, and often together with, 25-pS channels. These small channels were blocked by TTX (not shown) and showed gating behavior (Fig. 9), as expected for a sodium channel. Channels usually opened to their maximal conductance, and occasionally to submaximal conductance levels (for example, 5 pS). However, these openings were infrequent and were not characterized in this study. At 20 mM NaCl, small and large conductances were observed together

TABLE I

Single Sodium Channel Conductances for Various Purified and Unpurified Materials

NaCl	P/U	Number of membranes	Number of channels per membrane	*g*	TTX
mM				*pS*	*nM*
100	P2	1	3	19	—
	P2	1	3	21	42*
	P4	3	1.7	20	—
	P4	1	2	16	60
	U	2	6	17	—
	U	1	1	16	45
500	P1	1	3	24	—
	P1	2	3	25	180–720*
	P2	2	5	25	—
	P2	1	5	24	42*
	P4	2	1.5	24	—
	U	5	3.6	25	—
	U	1	3	23	45
Veratridine					
500	P2	3	6	12	—
	P3	4	11.2	13	—

The symmetrical salt concentrations are given in the first column, followed by the sodium channel preparation used (P1–P4 are purified material from preparations 1–4; U indicates unpurified material). Columns 4 and 5 give the average number of channels within those membranes and the average single-channel conductance (*g*) determined for each membrane by using linear regression analysis of the current-voltage curves. When used, the concentration of TTX is indicated in the last column.

* Same membrane and channels as in the previous line.

in all three experiments. They could represent either subconductance states or separate channels.

Ion Selectivity

The ion selectivity of BTX-modified sodium channels was established by measuring reversal potentials under asymmetrical ion conditions. The relative Na^+:Li^+ selectivity was determined as shown in Fig. 6. From the reversal potential of 10.8 mV, the Goldman-Hodgkin-Katz equation gave a permeability ratio of

1.6 for Na^+:Li^+. In Fig. 6, the slope conductance in the negative voltage range (0 to −100 mV; the chamber containing LiCl was negative with respect to the chamber containing 500 mM NaCl) increased continuously as the membrane potential became more negative. In the voltage range from −50 to −100 mV, the slope conductance, 22 pS (correlation coefficient $r = 0.964$), was close to the one measured in symmetrical 500 mM NaCl solutions (25 pS; see Table II) and therefore reflects mainly Na^+ current from the chamber containing only Na^+ to the chamber containing Li^+. The slope conductance in the positive voltage range (20–100 mV) was constant and significantly lower (16 pS; $r = 0.989$) than the slope conductance measured in symmetrical 500 mM NaCl solutions. The ratio

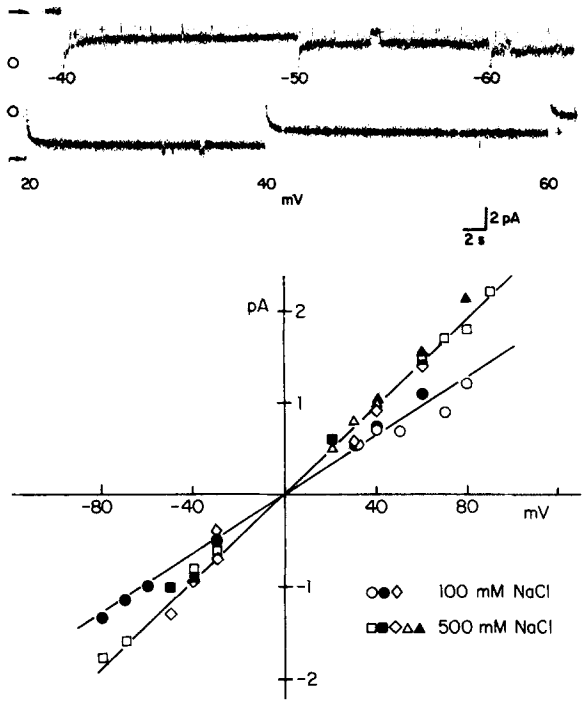

FIGURE 4. BTX-modified single-channel current transitions of unpurified sodium channels as a function of membrane potential in symmetrical 100 and 500 mM NaCl. At 100 mM NaCl, the slope was 16 pS ($r = 0.994$), and at 500 mM NaCl, the slope was 24 pS ($r = 0.998$). The top part of the figure shows the single-channel transitions at different membrane potentials for a membrane with two channels (corresponding to the filled squares). Each symbol represents a different experiment.

of these slope conductances is 1.6, the same as the permeability ratio calculated from the reversal potential.

The relative Na^+:K^+ selectivity was measured in a similar way by using 450 mM KCl instead of 450 mM LiCl (Levinson et al., 1986). Under these conditions, the single-channel currents reversed when the chamber containing 500 mM NaCl became 31.4 ± 2.4 mV more negative than the other chamber (mean ± SD for four membranes). The calculated permeability ratio for Na^+:K^+ was 4.7, which is similar to the value of 7 obtained by Rosenberg et al. (1984*b*) for unmodified channels (Table V). The slope conductance in the positive voltage range (the chamber containing KCl was positive with respect to the chamber containing 500 mM NaCl; Fig. 7 in Levinson et al., 1986) decreased continuously as the

TABLE II

Average Single Sodium Channel Conductances and Lifetimes for Membranes Containing Purified and Unpurified Material

NaCl		n_1	g	n_2	g	SD	Time	SD
mM			*pS*		*pS*		*min*	
100	P	10	19	5	19	2	150	80
	U	13	16	3	16*	1	20‡	8
500	P	16	25	5	25	1	160	90
	U	21	24	6	25*	2	23‡	20
Veratridine								
500	P	73	12	9	12§	1		

The single-channel conductances were either weighted by the number of channels present in a membrane or each membrane contributed equally toward the average. The lifetime of a membrane was measured from the time a channel(s) incorporated to the time the membrane broke (spontaneously or during manipulations) or when the membrane became too noisy to resolve single-channel events. n_1 is the number of channels; n_2 is the number of membranes; SD is the standard deviation; P is purified material; U is unpurified material.

* Not significantly different from the immediately preceding group ($P > 0.05$).

‡ $P < 0.01$ between the group and the immediately preceding group.

§ $P < 0.01$ between groups with veratridine and BTX at 500 mM NaCl.

membrane potential became more positive, approaching 6 pS ($r = 0.939$) between +40 and +120 mV. This value is similar to the 5.7 pS reported by Garber and Miller (1987) recorded in symmetrical KCl. The average slope conductance in the negative voltage range (−30 to −100 mV) was 15 ± 2 pS (mean ± SD for three membranes). This value is similar to that previously reported for unmodified and BTX-modified sodium channels in physiological salt solutions (reviewed in Andersen et al., 1986), but considerably lower than that measured in symmetrical 500 mM NaCl solutions (25 pS; Table V). This may reflect block of sodium currents by K^+ (Green et al., 1987*a*; Garber and Miller, 1987).

Steady State Activation

The fractional open time, f_o, of BTX-modified sodium channels showed voltage dependence (Figs. 1 and 7–10). Time-averaged current traces were simultane-

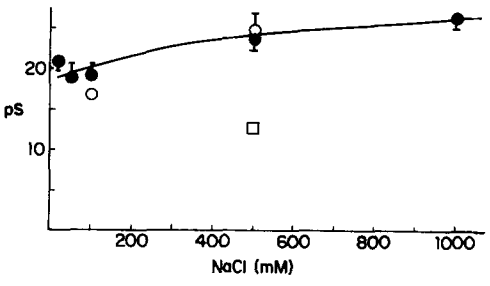

FIGURE 5. Activator-modified single-channel conductances as a function of symmetrical NaCl concentrations. The circles show the BTX-modified purified (filled) and unpurified (open) sodium channels. The open square shows the veratridine-modified purified sodium channels. The data points represent the averages and standard deviations of various membranes. The line was drawn by hand.

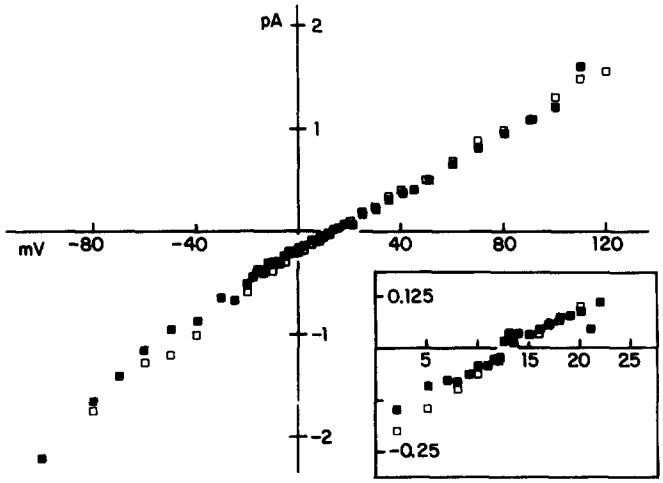

FIGURE 6. Na^+:Li^+ selectivity of BTX-modified single sodium channels. Single-channel currents reversed when the bilayer chamber containing 500 mM NaCl became 10.8 mV more negative than the chamber filled with 450 mM LiCl and 50 mM NaCl. The extracellular aspect of the sodium channel faced the 500 mM NaCl solution; the potentials refer to the electrophysiological sign convention (T = 23°C). Two different membranes are shown, one with one (filled squares) and the other with three (open squares) channels.

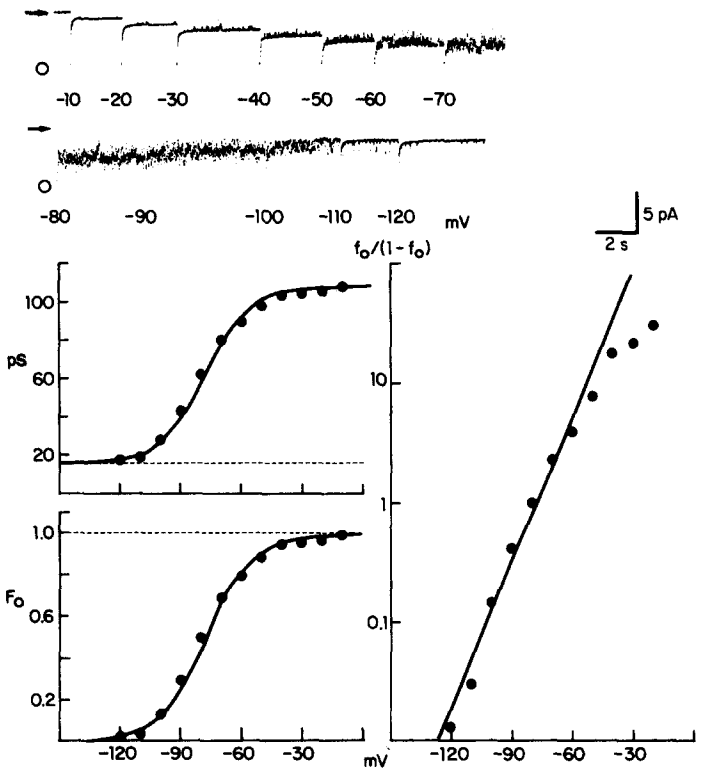

FIGURE 7.

ously recorded and converted into averaged conductances (Fig. 7). The fractional open time, f_o, was obtained by subtracting the background conductance (bilayer and leak) from the averaged conductance and dividing by the number of channels and the single-channel conductance. The plot of the fractional open time as a function of membrane potential (Fig. 7) gave the midpoint value for the steady state activation curve, which was also determined by fitting the data to the following equation (for a two-level Boltzmann distribution):

$$f_o = 1/\{1 + \exp(-z_a F[V - V_a])/RT\}, \quad (1)$$

where z_a is the valence of the apparent gating charge, F is the Faraday constant, V is the membrane potential, V_a is the midpoint potential of the activation curve, R is the gas constant, and T is the absolute temperature. A plot of the logarithm of the ratio of fractional open and closed times as a function of membrane potential (Fig. 7) showed both the steepness of the activation curve and how well the data fitted Eq. 1.

When measuring the steady state activation curves for the BTX-modified sodium channels, three problems became apparent. First, a variably large rightward shift in the activation curve occurred whenever a channel, closed by a descending (hyperpolarizing) potential sequence, was reopened by an ascending (depolarizing) potential sequence (Fig. 8). This hysteresis indicates a dependence of channel gating behavior on both the applied potential and its previous "history." For this reason, several minutes were allowed to elapse between activation curve measurements, and only curves obtained during the descending phase of the potential sequence were used in the averages. Second, shifts (average of 10 mV for 16 membranes) of the steady state activation curves were observed when the same sequence of membrane potentials was repeated. These shifts showed no obvious trends, could go back and forth, and occurred also (sometimes more than once) during the recordings of steady state activation curves. Third, both short (fast flicker) and longer channel closures (>0.2 s) were observed in the gating regions, the occurrence of long closures being quite variable. Some of the long closures were observed also outside the gating range (Fig. 8) and seemed unrelated to gating; others occurred predominantly within the gating range (Fig. 9). As there appeared to exist no obvious criteria for dissecting out the long closures, they were not corrected for; thus, they also contributed to the

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FIGURE 7. (*opposite*) Gating of BTX-modified purified sodium channels in symmetrical 500 mM NaCl. The membrane contained four sodium channels, which appeared to gate at different membrane potentials. The current traces are shown on the top, and the analysis of the steady state activation is shown in the three graphs below. The upper left-hand graph plots the time-averaged total conductance as a function of membrane potential; the background (membrane) conductance is indicated by the dashed line. The lower left-hand graph shows the fractional open time as a function of membrane potential, and indicates that the midpoint of the activation curve occurred at −78 mV. The curves were fitted to Eq. 1. The right-hand graph is a logarithmic plot of the ratio of fractional open over the fractional closed time ($1 - f_o$). From its slope, an apparent gating charge of 2.4 electronic charges was determined; the deviations at low and high ratios reflect mainly experimental uncertainty. The arrows indicate baseline (0 mV) current; the letter "O" indicates the direction of channel openings.

time-averaged conductances and hence to the steady state activation curves.

The midpoints and apparent gating charge valences of the steady state activation curves from purified and unpurified sodium channels under different ionic conditions are shown in Tables III and IV. Whenever membrane stability permitted, several activation curves were recorded. Not infrequently, membranes would become noisy during some stage of an activation gating measurement, resulting in incomplete steady state activation curves. These were not included in the average, although they gave an indication of whether the activation

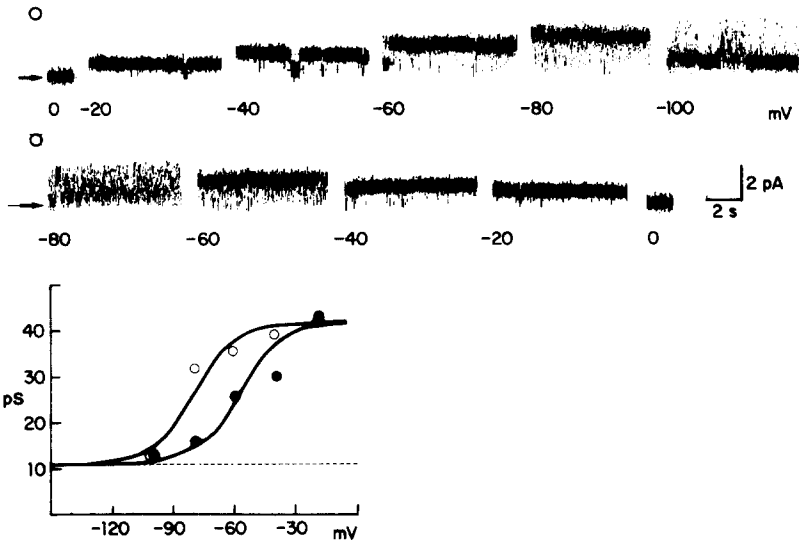

FIGURE 8. Gating of a single BTX-modified purified sodium channel in symmetrical 1,000 mM NaCl. The current traces with the sequence of membrane potentials (0 → −100 → 0 mV) are shown on the top. The various membrane potentials were held for 20 s each. The graph below shows the time-averaged total conductance as a function of membrane potential; the background (membrane) conductance is indicated by the dashed line. The conductance curve shows hysteresis. Hysteresis is also clearly visible in the original tracings when comparing channel gating at the same potential during the ascending (open circles; $z_a = 2.5$; $V_a = -80$ mV) and descending phases (filled circles; $z_a = 2.6$; $V_a = -57$ mV). The arrows indicate baseline (0 mV) current; the letter "O" indicates the direction of channel openings.

midpoint had shifted. Other reasons for obtaining only a single activation curve were (apart from the incorporation of noise into the membrane) additional channel incorporations, channel disappearances, and membrane thickening or breakage. Channels with reduced single-channel conductances (as in Fig. 9) were also included in the average.

The scatter in both the apparent gating charges and midpoints of the steady state activation curves was large and similar for both purified and unpurified sodium channels (Table III). In general, this scatter tended to be larger than the spontaneous shifts seen within the same membrane. Furthermore, different

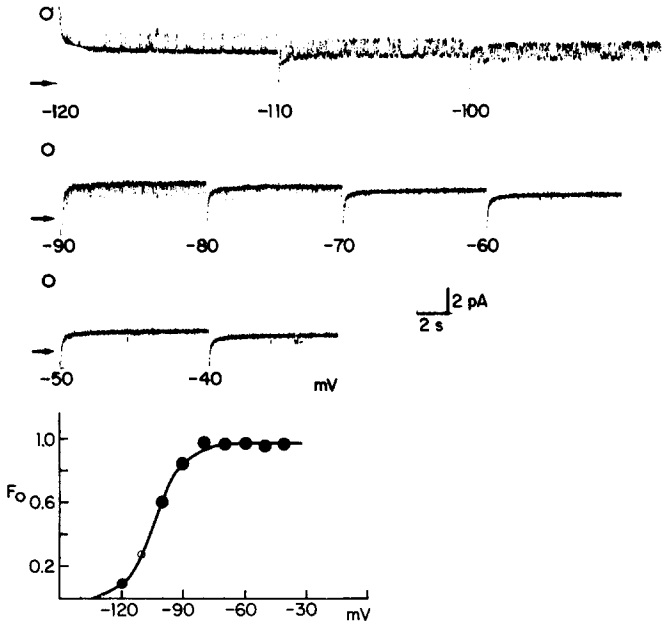

FIGURE 9. Gating of a single BTX-modified purified sodium channel in symmetrical 500 mM NaCl. The current traces are shown on top; the fractional open time as a function of membrane potential is shown below. This channel differs from the majority of other channels by showing a much smaller single-channel conductance (12 pS) and a more hyperpolarized midpoint potential for the steady state activation (−103.5 mV). The two observations may be unrelated. The apparent gating charge, z_a, was 3.7. The arrows indicate baseline (0 mV) current; the letter "O" indicates the direction of channel openings.

TABLE III

Average Sodium Channel Steady State Activation Characteristics for Purified and Unpurified Material

NaCl		n_1	z_a	V_a	n_2	z_a	SD	V_a	SD
mM				*mV*				*mV*	
50	P	5	1.6	−77	2	1.6		−76	
100	P	248	2.0	−71	7	1.8	0.5	−68	9
	U	23	1.9	−78	4	1.9	0.3	−67	17
500	P	23	2.4	−74	7	2.5	0.7	−77	16
	U	137	1.9	−75	11	2.1	0.6	−66	12
1,000	P	28	1.7	−75	4	1.7	0.6	−75	6

The gating characteristics were either weighted by the number of channels present in a membrane or each membrane contributed equally toward the average. Abbreviations are the same as in Table II. There were no statistically significant differences between purified and unpurified material, or between data from various NaCl concentrations ($P > 0.05$).

TABLE IV

Sodium Channel Steady State Activation Characteristics for Purified and Unpurified Material

	≥7 Channels					<7 Channels					All data				
	n	z_a	SD	V_a	SD	n	z_a	SD	V_a	SD	n	z_a	SD	V_a	SD
P+U	14	1.9	0.4	−71	10	21	2.1	0.7	−70	14	35	2.0	0.6	−70	12
P	6	1.9	0.4	−71	5	14	2.1	0.7	−74	13					
U	8	1.9	0.4	−72	13	7	2.2	0.7	−60	10					

Average values for the activation parameters were calculated by pooling all gating data at various NaCl concentrations (same experiments as in Table III) in two separate groups: membranes with seven or more channels, and membranes with fewer than seven channels. z_a is the valence of the apparent gating charge; V_a is the midpoint potential of the steady state activation curves; n is the number of membranes; P is purified material; U is unpurified material.

channels in the same membrane tended to gate at different potentials (Fig. 7; see also Figs. 5 and 6 in Levinson et al., 1986). In some single-channel membranes, gating occurred quite sharply, as in Fig. 9, where the channel was predominantly open at −100 mV and mostly closed at −110 mV. In other membranes (Fig. 7), the transition from mostly open to mostly closed was equally steep (−80 to −100 mV in the upper trace), but longer closures (upper trace,

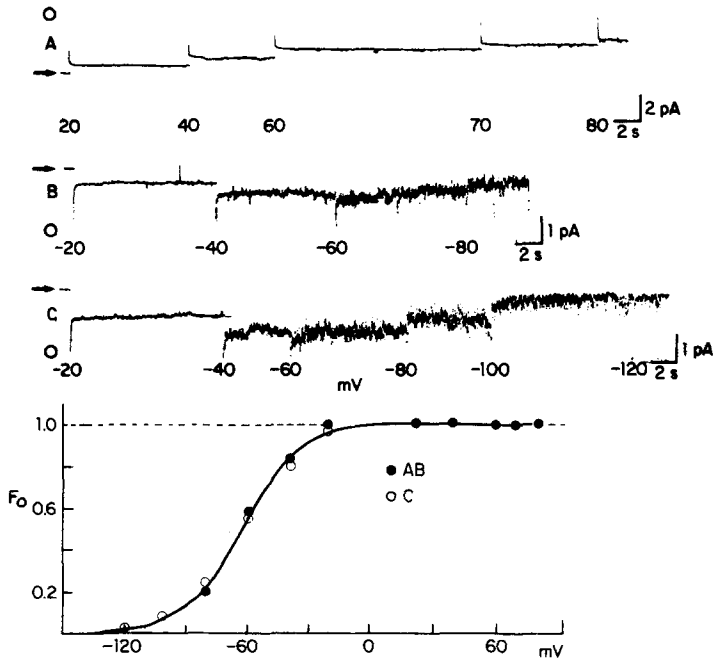

FIGURE 10. Gating of BTX-modified unpurified sodium channels in symmetrical 500 mM NaCl. The current traces are shown on top; the fractional open time as a function of membrane potential is shown below. Channel incorporation occurred in two separate steps: first, an incorporation of ~11 channels (*A*, *B*), followed by a second incorporation of ~9 channels, giving a total of ~20 channels (*C*). $z_a = 1.6$; $V_a = -61$ mV. The arrows indicate baseline (0 mV) current; the letter "O" indicates the direction of channel openings.

−60 mV) or burst activity (upper trace, −100 mV) flattened the activation curve again.

There were no statistically significant differences between the gating characteristics of purified and unpurified sodium channels (Table III). The average midpoint potential value for all the data in Table III was −70 mV (Table IV). For purified channels, the variability (SD) of the data from membranes containing few channels was greater than that for membranes containing many channels. The variability of the data was greater for many-channel membranes containing unpurified material; such membranes therefore do not necessarily yield the population average. This could mean that unpurified channels that incorporated in a single step did not necessarily constitute a random sample of sodium channels. Alternatively, another "factor" could have been incorporated into the membrane together with the unpurified channels, modulating their behavior (surface charge, lipids, etc.). However, we also observed separate incorporations of groups of channels in the same membrane where V_a and the apparent gating charge remained the same (Fig. 10).

DISCUSSION

The problem of the minimal polypeptide composition required for sodium channel function has been difficult to approach experimentally. In the case of mammalian channels, clear evidence has been presented that a multimeric composition is sufficient for channel function in isotopic flux (Tanaka et al., 1983; Tamkun et al., 1984) and bilayer (Hartshorne et al., 1985; Furman et al., 1986) systems. However, the question of whether small polypeptides are necessary remains controversial for several reasons.

First, although oocytes injected with mRNA encoding for the 260-kD rat brain polypeptide expressed functional sodium channels, they could have resulted from the co-assembly of small endogenous oocyte peptides with the rat brain polypeptide. Second, any reconstitution experiment that might show channel inactivation upon dissociation of the small peptides is not definitive if the conditions required for disruption of intermolecular bonds (Messner and Catterall, 1986) are also likely to disrupt putative intramolecular bonds on the large polypeptide. Third, in all previous reconstitution studies, <15% of the channel protein was functional (see the specific activities in Table V). Therefore, the observed sodium transport activity could have resulted from a small subpopulation of channels whose polypeptide composition was different from the majority of nonfunctional channels. This limits correlations between structure and function. Fourth, with final yields of <5% (Table V), the probability increases of selectively examining the properties of a distinct subset of channels, rather than the general properties of all channels.

These shortcomings may be addressed only if the material used is of high chemical and functional purity. In this study, the purity and final yield (Table V) of the preparations examined were significantly better than those of previous studies. This allowed us to be far more confident that the biophysical characteristics of the reconstituted material reflect the function of the large polypeptide alone.

Comparison between Purified and Unpurified Eel Sodium Channels

The single-channel conductances and steady state activation characteristics of purified and unpurified channels showed no statistically significant differences (Tables II and III). These results indicated that sodium channel characteristics were preserved throughout purification. However, bilayers containing unpurified material were usually noisier and less stable than those containing purified material (Table II). This may have been due to the incorporation of other cellular components into the planar lipid bilayers when fusing membrane fragments.

TABLE V

Properties of Various Purified and Unpurified Sodium Channel Preparations in Lipid Bilayers

	Eel (U)*	Eel (P)*	Eel (P)‡	Rat brain (U)§	Rat brain (P)‖	Rat sarcoplasm (U)¶	Rabbit t-tubule (P)**	Dog brain (U)‡‡
Conductance (*pS*)	25	25	20–25	29	25	21	20	25
P_{Na}/P_K		4.7	7	14	7	8.7		5.6
P_{Na}/P_{Li}		1.6				1.1		
$K_{1/2}$ (0.1 M NaCl)		3				16		11
$K_{1/2}$ (0.5 M NaCl)		18				60		35
$V_a \pm$ SD	−66±12	−77±16		−92±10	−91±17	−94	−95; −117	−88±11§§
$z_a \pm$ SD	2.1±6	2.5±0.7		3.9±9	3.8	3.8	3.6; 6.1	3.9±1.2§§
Major peptides (*kD*)		260	270, 95 53, 42		260, 33, 37		260; 38	
Specific activity		1,860	480‖‖		300–900		NA¶¶	
Final yield (%)		25–35	3‖‖		4‖‖		NA	

$K_{1/2}$ is the concentration of TTX (nanomolar) causing channel block half of the time. V_a gives the midpoint potentials (millivolts) of the steady state activation curve. z_a is the effective gating charge of the steady state activation curve. The major peptides present in the preparation were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Specific activity is in picomoles of TTX or saxitoxin bound per milligram of protein. The final yield is the percent of the initially solubilized protein that retained TTX-binding activity after reconstitution.

* This study; Duch and Levinson (1987).

‡ Rosenberg et al. (1948*a*, *b*); Rosenberg (1985).

§ Krueger et al. (1983); French et al. (1986).

‖ Hartshorne and Catterall (1984); Hartshorne et al. (1985); Keller et al. (1986).

¶ Moczydlowski et al. (1984); Garber and Miller (1987).

** Furman et al. (1986)

‡‡ Weiss et al. (1984); Green et al. (1987*a*, *b*).

§§ Andersen, O. S., personal communication.

‖‖ Calculated from pre-reconstitution data using the estimate given for activity loss during the reconstitution procedures (Talvenheimo et al., 1982; Rosenberg, 1985).

¶¶ Not available.

Comparison with Previous Results for Purified Eel Sodium Channels

Two other studies examined the single-channel characteristics of purified eel sodium channels. Hanke et al. (1984) worked in the absence of any activator toxins in a bilayer setup with a limited frequency response. Only a single-channel conductance of 150 pS was resolved, although 25- and 40-pS channels were reported for sodium channels from rat brain in the same study. In the present study, none of the BTX-modified channels from either purified or unpurified

preparations showed this large single-channel conductance, nor was it found by Rosenberg et al. (1984*b*). They reported a single-channel conductance of 11 pS (90 mM NaCl) for unmodified channels in patch-clamped, freeze-thawed liposomes. Examining BTX-modified channels with the same technique, Rosenberg (1985) measured a more variable conductance of either 10–15 or 20–25 pS (90 mM NaCl). The latter values agree with the conductances predominantly found in this (Table II) and previous (Table V) studies. The smaller channels were present in both the purified (Fig. 9) and the unpurified (not shown) preparations. The consistent presence of both channel conductances is intriguing since only a single cDNA sequence has been reported for the eel sodium channel (Noda et al., 1985). Further studies examining the post-translational modification of the sodium channel protein may help to determine whether the different channel conductances are due to different gene products, are caused by modification of a single polypeptide core, or are induced by other factors such as differential denaturation, sensitivity to alkaloid toxins, or varying lipid environment.

Examination of TTX block of activated channels also showed similarities between this and previous studies. For purified channel material, Rosenberg et al. (1984*a*) initially measured a K_d for TTX binding of 33 nM, and a $K_{1/2}$ for sodium flux inhibition by TTX of 45 nM in 90 mM Na_2PO_4. More recently, using improved methodology, the same laboratory reported a $K_{1/2}$ of 4.3 nM (Tomiko et al., 1986). Examining the purified sodium channels after reconstitution into vesicles, Duch (1986) measured a K_d for TTX of 25 nM in 100 mM Na_2PO_4 for the preparations used in the present studies. Under the same conditions, but using unpurified membrane fragments, Duch (1986) also measured a K_d of 3 nM for TTX binding. These values are comparable to the $K_{1/2}$ of 3 nM at 100 mM NaCl and 18 nM at 500 mM NaCl at 0 mV already reported for the purified channel protein used in our study (Levinson et al., 1986).

Rosenberg et al. (1984*b*) found that BTX-modified sodium channels were mainly closed, even at depolarized potentials. However, in the present bilayer experiments, as well as those reported for mammalian channels (Table V), such channels are open most of the time at equivalent depolarizations. This difference may be related to the different experimental conditions (e.g., transient vs. steady state) and/or recording methods.

Comparison with Sodium Channels from Other Sources

All bilayer studies of BTX-modified sodium channels employing symmetrical ion conditions, including this one, have found symmetrical and linear current-voltage relationships with similar conductance values (Table V), even though the sodium channel protein is presumably highly asymmetric (Levinson et al., 1986). The conductance-concentration relation observed in this article approached saturation at low sodium concentrations (Fig. 4), as expected for a sodium-selective channel and in agreement with previous observations (reviewed in Andersen et al., 1986). The preliminary reports of a small-conductance channel in dog brain (Duch et al., 1987) and partially purified rat brain (Corbett et al., 1986) are in agreement with the properties of the small-conductance channel described here (Fig. 9).

The voltage-dependent block of TTX already reported for the purified channel protein used in this study [correction in Levinson et al., 1986, p. 174: a = 0.74 (0.49) instead of a = 0.71 (0.75)] was similar to that reported for dog brain channels (Green et al., 1987*b*) and to that found for rat muscle channels (Moczydlowski et al., 1984). In addition, the interaction between Na^+ and TTX, as indicated by the increase of the $K_{1/2}$ as the NaCl concentration increased, is similar to that found with other sodium channels (Table V).

The purified eel channel also showed ion-selective properties similar to those of other channels (Table V). Andersen et al. (1986) determined that the permeability ratio did not change with the varying mole fraction at constant ionic strength. However, Garber (1987) found a marked asymmetry in the ion selectivity of the BTX-activated sodium channel. Therefore, some of the apparent discrepancies in Table V may simply reflect different experimental conditions, such as distribution of ionic species.

The gating midpoints of different sodium channels ranged widely, giving rise to large standard deviations. However, the standard deviations were also large in all other studies (Table V; see also French et al., 1986). These observations may correlate with the biochemically observed microheterogeneity, which probably results from protein glycosylation (Levinson et al., 1986). The average of the activation midpoints for individual channels also showed large standard deviations (data not shown). "Spontaneous" shifts in the gating behavior have been observed here and in a number of other bilayer sodium channel systems (French et al., 1986). As expected for a sodium channel, the purified 260-kD polypeptide examined in this article was also activated by veratridine (Fig. 1) at appropriate concentrations. Veratridine-activated channels had roughly half the amplitudes of BTX-activated channels, as first reported for rat muscle channels by Garber and Miller (1987).

There are also differences between sodium channels from eel and other preparations. First, the steady state activation curve of eel channels had lower midpoint potentials (Table V). Second, the eel channel did not show the 20-mV decrease in the steady state activation curve midpoint with an increasing NaCl concentration from 100 to 500 mM, as reported for dog brain channels (Weiss et al., 1984). Finally, the apparent gating charges for both purified and unpurified channels in this study were lower than those reported in previous studies (Table V). However the magnitude of this difference is uncertain, considering the large standard deviations reported (Table V). This disparity could reflect differences in the sodium channel molecules or in the experimental procedures, or both (see Levinson et al., 1986, for further discussion).

In conclusion, the 260-kD polypeptide from the eel electroplax, examined in the absence of other membrane components, showed no significant differences in its properties from those of the unpurified sodium channel from the same tissue examined under identical conditions. These recorded properties were also similar to those of purified and unpurified channels from other tissues, with the exceptions of some aspects of the activation gating. These observations, together with the high functional yields of purified channels and the obvious advantages of stable, well-characterized lipid bilayers, make the present system a useful tool

for future studies of sodium channel behavior and structure-function relationships.

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