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Voltage Activation of Purified Eel Sodium Channels Reconstituted into Artificial Liposomes†

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ABSTRACT: We report here a characterization of the voltage-activated behavior of sodium channels purified from the electroplax of *Electrophorus electricus*. Single-channel activity in response to depolarizing pulses was recorded from patches excised from liposomes containing the reconstituted channel. Strong hyperpolarizations were required to elicit channel activity. Channels exhibited two typical gating patterns. They either would open in brief bursts upon depolarization and then inactivate (fast) or would stay opened for prolonged periods that frequently lasted several consecutive depolarizations and showed intense flickering (slow). The single-channel conductance estimated from the slope of the *I-V* curves ranged between 15 and 30 pS under several experimental conditions. Channels gating in either mode, fast or slow, were indistinguishable in terms of their sizes. No clear difference in their mean open times was observed. In addition to the two gating patterns, we also found a very clear tendency of the channels to stay quiet for long periods.

In the past several years, voltage-sensitive sodium channels have been isolated biochemically from a variety of electrically excitable tissues, including eel electroplax (Agnew et al., 1978; Miller et al., 1983; Norman et al., 1983; James et al., 1989), rat and rabbit skeletal muscle (Barchi et al., 1980; Barchi, 1983; Kraner et al., 1985), chicken heart (Lombet & Lazdunski, 1984), and rat brain (Hartshorne & Catterall, 1981, 1984; Barhanin et al., 1983). One question which has remained largely unanswered was whether these purified proteins could be reassembled into artificial membranes and induced to function normally in response to changes in the transmembrane potential. Single-channel recording by patch-clamp techniques offers extremely sensitive measurements of channel gating and conductance. The present report describes progress in applying patch-clamp recording techniques to the characterization of pure sodium channel molecules which have been isolated from eel electroplax and reconstituted into artificial liposomes. This approach is demonstrated to be suitable for detailed characterization of the conductance and gating properties of these molecules under well-defined conditions of membrane protein and lipid composition.

Sodium channels from all tissues so far examined contain a large heavily glycosylated peptide, or α-subunit, of

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270 000–300 000 daltons, 20–30% of which is carbohydrate [e.g., see Barchi (1988) and Trimmer and Agnew (1989)]. This is the sole constituent of the electroplax protein (Miller et al., 1983; James et al., 1989), while channels from skeletal muscle and brain also contain one or two smaller glycopeptides, or β-subunits (Barchi, 1983; Kraner et al., 1985; Hartshorne & Catterall, 1984). With two exceptions (Rosenberg et al., 1984b; Tanaka et al., 1986), previous biophysical studies of purified preparations have involved characterization of channels incorporated into planar lipid bilayers (Hanke et al., 1984; Hartshorne et al., 1985; Furman et al., 1986; Recio-Pinto et al., 1987; Corbett & Krueger, 1988; Shenkel et al., 1989). The large size of the bilayer, however, severely limits the measurements that can be made. Capacitance currents obscure small currents through transiently activated channels, the charging time of the membrane is long relative to the time required to activate the channel, and the noise limits the frequency of channel gating that can be monitored. Because of these limitations, channels have been primarily studied under the influence of powerful activating neurotoxins such as batrachotoxin (BTX)[1] and veratridine (VTN). These toxins produce chronically, as opposed to transiently activated channels (Quandt & Narahashi, 1982; Krueger et al., 1983). However, most of the biophysical properties of the toxin-treated channels are altered. BTX, in particular, removes normal fast and slow inactivation, alters the voltage dependence of activation, and reduces both the conductance and permeation selectivity of the channel (Quandt & Narahashi, 1982; Krueger et al., 1983; Huang et al., 1984; Green et al., 1987; Correa et al., 1989b; Garber & Miller, 1987). Furthermore, as confirmed in the present studies, BTX can force inactive—and possibly damaged—channels into a high level of uniform, albeit anomalous, activity.

In a previous study, Rosenberg et al. (1984b) demonstrated that electroplax sodium channels, purified and reconstituted into liposomes, could be activated by voltage alone. Single-channel events were measured in patches excised from liposomes expanded by freezing and thawing. Macroscopic currents reconstructed from single-channel currents exhibited clear voltage-dependent activation and subsequent inactivation. The currents were selective for Na over K, with magnitude and mean channel lifetimes consistent with those expected for conventional sodium channels. Technically, these experiments were difficult to perform, and no similar reports, until now, have appeared from this or other laboratories.

In the studies described here, we have identified and overcome many of the technical obstacles that interfere with making these measurements. A new lectin affinity isolation procedure makes it possible to rapidly isolate the protein (James et al., 1989). Improved reconstitution procedures have been established that leave the protein at controlled high, uniform surface abundance in the liposomal membrane. Methods for inducing fusion of small vesicles into large liposomes suitable for patch-clamp recording have been employed (Correa & Agnew, 1988). Activation with BTX has been used to confirm the abundance and orientation of the channels in the excised patches, and channel activity, measured under the influence of voltage alone, has been characterized.

With these improved techniques, we find it is routinely possible to excise patches containing optimal numbers of channels (1–5) for study at a single-channel level. In the absence of neurotoxins, channels may be activated by voltage alone. Overall, the probability of channels being activated by voltage is much less than in other preparations. This reduced level of activity is apparently caused by the tendency of channels to enter a quiescent state. When active, however, the channels have a high probability of opening with each test depolarization. Active channels appear to exhibit one of two types of gating, defined as fast and slow, based on the time course of inactivation of the reconstructed macroscopic currents. The "fast" mode exhibits voltage-dependent activation and subsequent inactivation, similar to that reported by Rosenberg et al. (1984b). In the "slow" gating mode, the channels exhibit a strongly voltage-dependent activation followed by a very slow inactivation time course. These characteristics and their relationship to the normal physiological characteristics of the native channel are discussed. A preliminary account of these findings has been reported (Correa et al., 1989a).

Materials and Methods

Channel Purification and Reconstitution. Channels were purified and reconstituted as described elsewhere (James et al., 1989; Correa & Agnew, 1988). Briefly, the sodium channels from the main electric organ of the eel *Electrophorus electricus* were solubilized in 1% (w/v) Lubrol-PX and purified by ion-exchange chromatography with DEAE-Sephadex and by affinity chromatography with a resin containing sialic acid specific agglutinin from *Limax flavus* (LFA), covalently coupled to Sepharose 6B. Sodium channels can be eluted at high concentrations (100–500 μg of protein/mL) from the LFA resin, in the presence of low concentrations of free lipid and detergent (0.183 mg/mL phosphatidylcholine and 1 mg/mL Lubrol PX, respectively). The binding activities of the LFA fractions were measured by Sephadex G-50 assays (Agnew et al., 1978) and were always 1000–2000 pmol of [^{3}H]TTX binding sites/mg of protein. It represents ~30% of the theoretical maximum, 4800 pmol/mg, calculated from the binding of one [^{3}H]TTX binding site per 208 000 Da of the pure polypeptide chain. After dialysis against a high-Na^+, high-Ca^{2+} solution (400 mM NaCl, 1 mM $CaCl_2$, 0.02% NaN_3, and 10 mM Tris-HCl, pH 7.2; 250× the sample volume 3 times, each lasting an hour), peak LFA fractions were supplemented stepwise with additional lipid (phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine, PE/PS/PC, 5:4:1; Avanti Polar Lipids, Inc., Birmingham, AL) in the form of sonicated liposomes (40 mg/mL lipid) prepared in the same solution. Each addition was followed by mixing and incubation on ice for 10 min. All sonications were performed in a bath sonicator with the samples in an Ar or N_2 atmosphere at room temperature. Channels were then reconstituted by removal of the detergent by adsorption to Bio-Beads SM2 (0.3 g/mL) for 3 h. Purification, dialysis, and reconstitution steps were carried out at 4–5 °C.

Fusion of the Reconstituted Liposomes for Patch-Clamp Recordings. Samples were prepared for efficient liposome fusion (Correa & Agnew, 1988) by additional lipid supplementation (sonicated PE/PS/PC liposomes) to a final concentration of 10 mg/mL, brief sonication (less than 1 s), and slow freezing by storage at −80 °C. For patch-clamp recordings, samples were thawed at room temperature, without agitation. An aliquot (2–5 μL) of the unperturbed sample was applied to the surface of the glass bottom of the recording chamber and diluted 20-fold by slow addition of 10 mM Tris-HCl, pH 7.2; after 5–10 min, or when vesicles had efficiently swollen, they were diluted further with the same solution containing 250 mM NaCl which was the regular bath solution. Pipets were filled with the Tris-HCl solution and chloride salts of Na^+ (5 or 25 mM) and either K^+, Rb^+, or

[1] Abbreviations: MOT, mean open time; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; BTX, batrachotoxin; VTN, veratridine; Tris, tris(hydroxymethyl)aminomethane.

Cs^+ (245 or 225 mM, respectively). The abundance of reconstituted Na^+ channels in the patches was estimated with the aide of batrachotoxin (BTX), a generous gift from Dr. John Daly (National Institutes of Health).

Electrical Recording. High-resistance seals of more than 10 GΩ were formed by release of slight positive pressure applied to the patch pipet before immersion. Sometimes very slight suction was required. Patch pipets were pulled from Corning 7052 glass (Garner Glass Co., Claremont, CA). Tip resistances were of the order of 10 MΩ. Conventional patch-clamp arrangements following Hamill et al. (1981) were used. The set-ups have been previously described (Bezanilla, 1987; Rosenberg et al., 1984b, Correa & Agnew, 1988). Data were acquired at either 20, 50, or 100 μs/point (1200 points per record) depending on the pulse length (20, 50, or 100 ms), and filtered through a 4- or 8-pole Bessel filter at 5-kHz bandwidth. Channel activity was sought for at least 20 min (sometimes 40–60 min elapsed) before discarding the patch as inactive; during this period, positive and negative holding potentials, 70–120 mV, were imposed, and pulses to different potentials were applied every 1–1.5 s. The data presented here were obtained from patches that lasted from 12 min to 4 h. Normally, channel activity was seen within 2–10 min after seal formation. In some occasions, however, 30–40 min passed before any channel activity was observed. The bulk of the data analyzed comes from patches that lasted from 40 min to 4 h, yielding over 1000 traces. Experiments shorter than 40 min (or less than 1000 records) contributed mainly to single-channel conductance calculations. For analysis, control records made from the average of blank records were subtracted from the individual traces. Standard analysis programs were used to determine open and closed levels following the 50% threshold criterion. The total number of channels in a patch was defined as the maximum number of simultaneous openings observed in an experimental run. The computation of the average number of openings per trace excludes blank records. Mean open times were calculated as the time open weighted by the open level and divided by the number of closings. Fits of the open time distributions of the linear and/or logarithmic data were done excluding traces with multiple openings. Only sets of data containing 500 or more transitions were considered for fits of open time distributions.

Voltage and current signs follow the physiological conventions: outward current is positive, and voltage is measured as the potential inside of the cell minus the potential outside. The inside of the cell was assigned as the side containing the intracellular portion of the protein as judged by its gating behavior. In the experiments reported here, the intracellular side of the channel was exposed to the bath.

Results

Initially, patches excised from the swollen liposomes usually did not exhibit channel activity in response to simple conventional depolarization regimens. This was not surprising: we anticipated that sodium channels which have undergone solubilization and reconstitution, and which have, in effect, experienced prolonged depolarization, might enter slow inactivated states, or might be partially denatured. To test whether potentially active channels were routinely present in the excised patches, therefore, we resorted to pharmacological activation.

Surface Abundance of Functional Channels. The lipid-soluble alkaloid-like toxin BTX is a powerful activator of sodium channels. Among its effects, BTX removes both fast and slow inactivation and shifts the activation midpoint to hyperpolarizing potentials. At potentials more positive than −60 mV (cellular orientation), channels are open >90% of the time. Thus, addition of BTX provided a convenient means of assessing the abundance and orientation of channels in the excised membranes.

In the presence of BTX, some patches exhibited as many as 10 channels. Most frequently, one to four channels were encountered. As have been previously reported by our laboratory (Correa & Agnew, 1988), the behavior of BTX-treated purified eel channels was uniform and consistent with that of channels from either native membranes or purified preparations, studied in planar lipid bilayers (Krueger et al., 1983; Hartshorne et al., 1985; Furman et al., 1986; Green et al., 1987; Recio-Pinto et al., 1987; Behrens et al., 1989; Shenkel et al., 1989). The conductances determined from the slopes of I–V relationships varied between 10 and 16 pS in symmetrical 250 mM NaCl, which are about half those reported previously by Rosenberg et al. (1984b). Although BTX-modified channels were encountered in both orientations, they were most frequently found to be oriented with their intracellular side facing the bath solution (inside-out). These results provided strong evidence that sodium channels were present in essentially every excised patch and that they had been successfully reconstituted at high surface densities.

Channel Activity Induced by Membrane Depolarization. We next proceeded to search for the activity of channels in the absence of activating neurotoxins. The orientation of the channels in the patches was inferred from the voltage dependence of the gating. We always recorded from patches with channels oriented inside-out (the internal side of the channel facing the bath solution). This was due mainly to instability of the seals when held at very negative potentials (in the pipet) which were required to elicit purely voltage-activated channel activity in this preparation. Inside-out patches were generally stable for 30 min to several hours.

We found that the overall probability of encountering a channel activated by voltage alone was much less than in the presence of BTX. However, when an active channel was observed, it was generally active in several successive depolarizations. The activity would then often disappear for seconds to many minutes, before resuming (see below). Approximately, 40% of the patches exhibited channel activity (17 out of 39). The results we describe here correspond to 10 patches derived from at least 3 different purified channel preparations.

The general, characteristic behavior of the voltage-activated purified eel Na channel is illustrated in Figure 1. In part A is shown an array of 40 consecutive traces (out of 102 recorded under the same conditions) obtained by pulsing to +60 mV for 45 ms from a holding potential of −120 mV. The patch contained two channels as estimated from the maximum number of simultaneous openings observed throughout the experiment (3728 total records). Two major conclusions can be made from data such as those depicted in this figure. First, channels exhibited two types of gating behavior (Figure 1A). In the first mode of gating, the channels opened for long periods sometimes lasting the length of the pulse (see traces 3–10, left column; also Figure 3B). These long openings were interrupted by several brief closures revealing a burstlike activity. Events of this sort very frequently occurred in clusters of several consecutive traces. The second type of gating pattern was characterized by briefer openings which preferentially occurred at the beginning of the depolarizing pulse (see traces 16–20, left column; also Figure 3A). Short reopenings during the pulse were commonly seen. Trends of traces containing both short and long openings, similar to that shown in the right

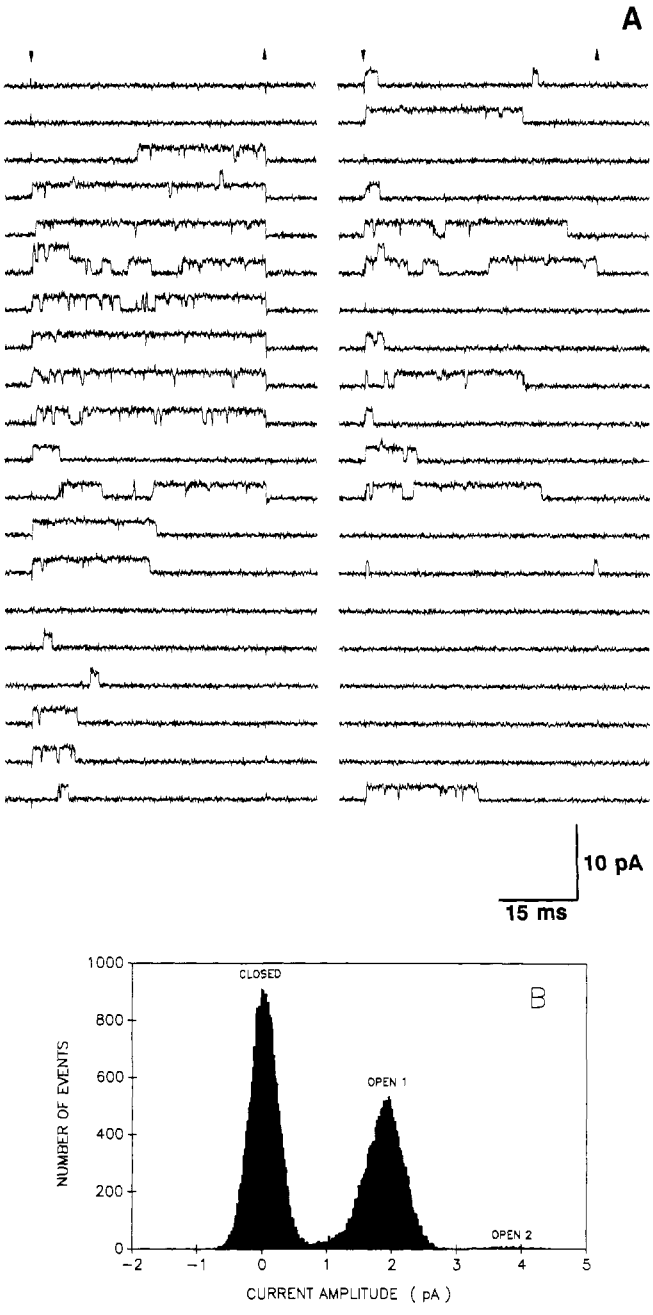

FIGURE 1: Single-channel activity activated by voltage alone. (A) The array shown corresponds to consecutive records at +60 mV. The holding potential was −120 mV. The arrows at the top of each column indicate the onset and the offset of the depolarizing pulse applied every 1.5 s and lasting 45 ms. Illustrated are the two types of gating patterns as well as the silent, nonconductive periods described in the text. Recordings were made in 250 mM NaCl/0.5 mM $CaCl_2$ in the bath and in 225 mM KCl/25 mM NaCl in the pipet. Data were acquired at 50 μs per point. Filter: 5 kHz. File: NANMY22E. (B) The current amplitude histogram shown here corresponds to the entire set of data at +60 mV (286 events). To reduce the base-line noise, only traces with events were considered. The levels at which the closed, first open, and second open distributions peaked are marked in the figure.

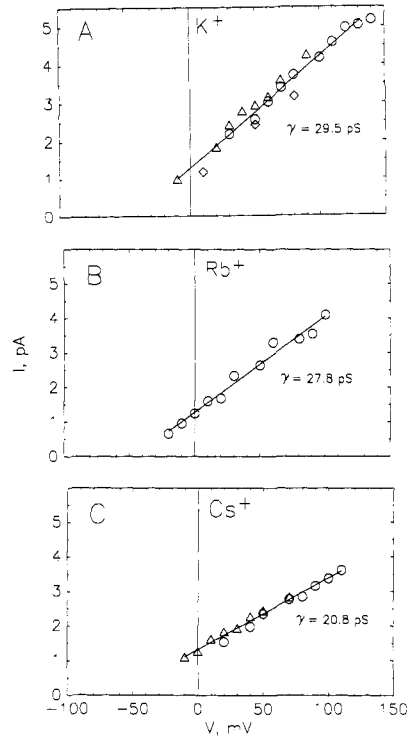

FIGURE 2: Single-channel conductance. The current to voltage relation of the conduction through the eel Na^+ channel is illustrated for three different experimental conditions. The internal side of the channel was exposed to 250 NaCl; the external solution varied in composition. Different symbols in each panel represent data from different patches. Parts A, B, and C show the I–V relations in external 245 mM K^+, Rb^+, or Cs^+ and 5 mM NaCl, respectively. The slope conductances indicated in each panel were calculated from the linear fit by least squares to the data. The values on the abscissa are the applied potentials, not corrected for junction potentials.

column of the figure, were also frequent. The events from either type of gating were indistinguishable from each other on the basis of their sizes as illustrated in Figure 1B by the amplitude histogram of the data from the same experiment. The amplitude distribution of the OPEN 1 state is skewed toward the closed state indicating unresolved flickering.

The second major observation concerns the periods during which no channel activity was observed. These silent periods lasted from a couple of records (see right column, Figures 1A and 3A), up to several seconds (we have recorded as many as 32 consecutive blank records), with the result that the overall probability of opening was very small at each potential within the voltage range studied. Large negative holding potentials, sometimes together with hyperpolarizing prepulses, were required to observe channel activity and, many times, reactivated channels that had entered the long-lasting silent, nonconductive states. Also on very few occasions channel activity was seen with more positive holding potentials (−80 mV) but opening events were very infrequent. Therefore, only holding potentials from −90 to −140 mV were used.

Channel activity was studied at pulse potentials from −10 to +140 mV. Under the experimental conditions used, events at voltages more negative than −10 mV were seldom clear enough to resolve from base-line noise. The current through the channel was a linear function of voltage within this voltage range as illustrated in Figure 2. Here are shown the I–V relationships obtained in experiments in which the ionic composition of the external (pipet) solution was varied (different symbols represent data from different patches). The conductance estimated from the slope of the linear part of the I–V curves varied slightly with the composition of the external media. Values of 29.5, 27.8, and 20.8 pS were obtained in K^+, Rb^+, and Cs^+ (Figure 2A,B,C, respectively). The conductance became smaller as the main external cation was changed to a more impermeant cation as measured for other Na^+ channels [e.g., see Hille (1984)].

The macroscopic currents reconstructed from the average of traces containing either or both modes of channel gating varied depending on the contribution of each type of opening pattern. Figures 3–5 illustrate this more clearly. The top part

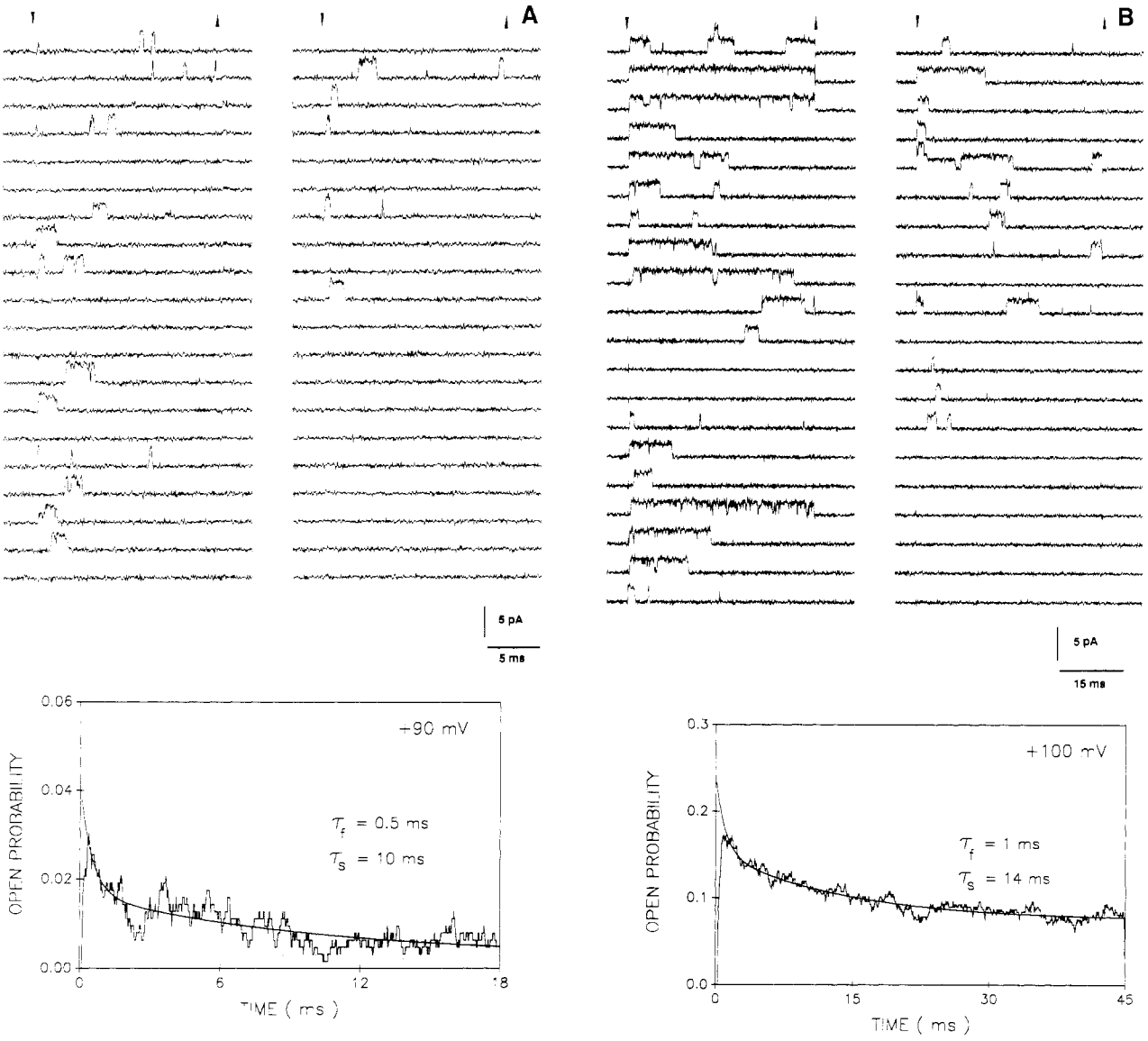

FIGURE 3: Reconstructed macroscopic currents and first latencies related to both types of gating patterns. (A) Top: Array of consecutive records showing channel activity at +90 mV during an 18-ms depolarization, indicated by the arrows, from a holding of −120 mV with an applied 50-ms prepulse to −150 mV. Current direction follows the usual convention: upward inflections are outward. Channels in the patch: 2 (2844 records). Sampling: 20 μs/point. Cycle period: 2 s. Filter: 5 kHz. Solutions (mM): 250 NaCl internal, 245 CsCl–5 NaCl external. Bottom: Reproduction from the single-channel data of the macroscopic current expressed as the probability of opening as a function of time during the pulse. Data from 314 records (involving 490 transitions) including blanks were averaged. The solid line is the best fit of the falling phase to two exponentials. τ_f and τ_s are the time constants obtained through the fit and correspond to the fast and slow exponential components of the decay in the current. The maximum probability (0.03025) occurred at 0.5 ms. File: NANJL4B. (B) Single-channel data at +100 mV during a 45-ms pulse (holding and prepulse conditions same as above). Number of channels: 2 (3596 records). Sampling: 50 μs/point. Cycle period: 1.5 s. Filter: 5 kHz. Solutions (mM): 250 NaCl–0.5 $CaCl_2$ internal, 225 KCl–25 NaCl external. Bottom: Similar to part A (bottom); corresponding average current during the 45-ms pulse to +100 mV in terms of the open probability determined from a total of 172 records (1345 transitions), blanks included. The peak probability of 0.17151 was established in 0.85 ms. File: NANMY22F.

of Figure 3 shows two sets of representative consecutive records used to create the average currents shown underneath each array. The resulting macroscopic currents are represented as the probability of being open as a function of time within the pulse. In part A, we show results from a patch with channel activity predominantly like that of a conventional Na^+ channel. The patch was pulsed to +90 mV for 18 ms, from a holding potential of −120 mV, with a 50-ms prepulse to −150 mV. The average current exhibits a fast rising phase and peaks at 0.5 ms. The decay of the current could be fit by two exponentials with time constants of 0.51 ms (τ_f) and 10.01 ms (τ_s). Inactivation, as shown in the ensemble record, was not complete. The average number of openings per trace was 2.29. The mean open time (MOT) was 0.44 ms. In part B, we illustrate data from another patch held at −120 mV and pulsed to +100 mV for 45 ms after a 50-ms prepulse to −150 mV. As expected from the average gating behavior seen in these records, the reconstructed macroscopic current peaked rapidly (0.85 ms) to its maximum probability and decayed very slowly during the course of the depolarizing pulse. The time course of the falling phase could be fit by two exponentials with time constants of 1.02 ms (τ_f) and 13.88 ms (τ_s). The average openings per trace were 6.29, approximately triple that of the example in part A. The MOT was 2.21 ms, about 5 times that in part A. Both the bursting activity and the prolonged open time would contribute to the slow decay of this current. This macroscopic current, in short, resembles that produced by channels in native cellular membranes which have been treated to remove fast inactivation (see also Discussion).

The possibility that the slow decay of the average curves was being determined by late openings was explored. The accumulated first latency curves corresponding to the data in

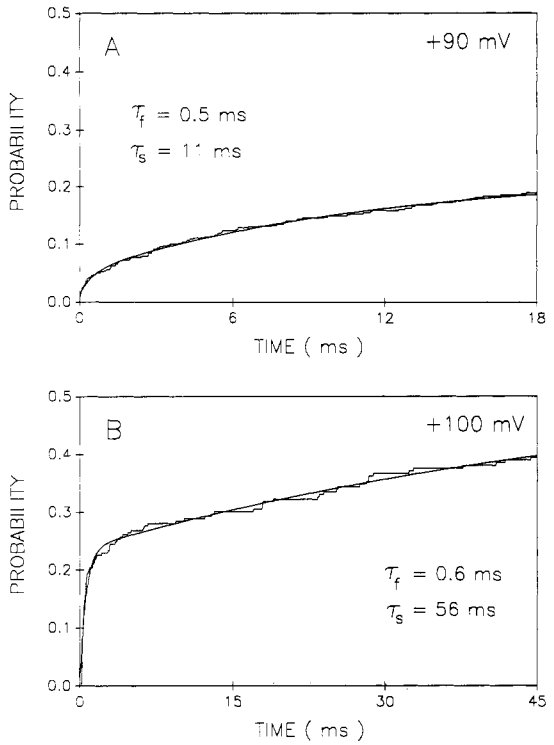

FIGURE 4: Accumulated times to the first opening as a function of the time during the pulse. (A) Data at +90 mV corresponding to part 3A. (B) Data from part 3B. The solid lines are fits of the whole curves to two exponentials. The indicated τ values are the fitted fast and slow time constants. Same conditions as previous figure.

Figure 3 are shown in Figure 4. These curves were fit to two exponentials and the resulting time constants compared to the ones obtained from the fit of the decay of the corresponding, reproduced macroscopic currents shown in Figure 3, bottom. Time constants of 0.50 ms (τ_f) and 11.19 ms (τ_s) were found to describe the fast and slow components of the first latency at +90 mV, part A. The coincidence in the values of the time constants suggests that the time course of inactivation at +90 mV (Figure 3A, bottom) could be accounted for by late openings. However, since the slow component of the decay of the macroscopic current could be well fit with a wide range of time constants due to the scatter of the data (values from 10 to 100 ms were seen), an unequivocal correlation between inactivation of the current and first latency will not be attempted. The fast and slow components of the first latency curve in part B were well fit with τ's of 0.59 and 55.95 ms, respectively. Though of the same order of magnitude, these values do not agree with those found to fit well the time course of inactivation of the current (Figure 3B, bottom). These results suggest that the slow time course of inactivation is due primarily to channels closing and reopening multiple times during the pulse, failing to enter an absorbing inactivated state. The analysis of several sets of data (see also Figures 5 and 6) consistently did not show a simple correlation between the time to the first opening and the falling phase of the macroscopic currents.

Some large variations in gating behavior were frequently observed within an experiment. In Figure 5, we illustrate this point by showing data obtained at different intervals with the same patch. Parts A and B of the figure show the currents obtained at 0 and +20 mV after the average of 474 and 472 records, respectively, including blanks. Both sets of data were recorded after a prepulse to −150 mV from the holding potential of −120 mV. Parts C and D show the corresponding accumulated first-latency graphs. The solid lines drawn in

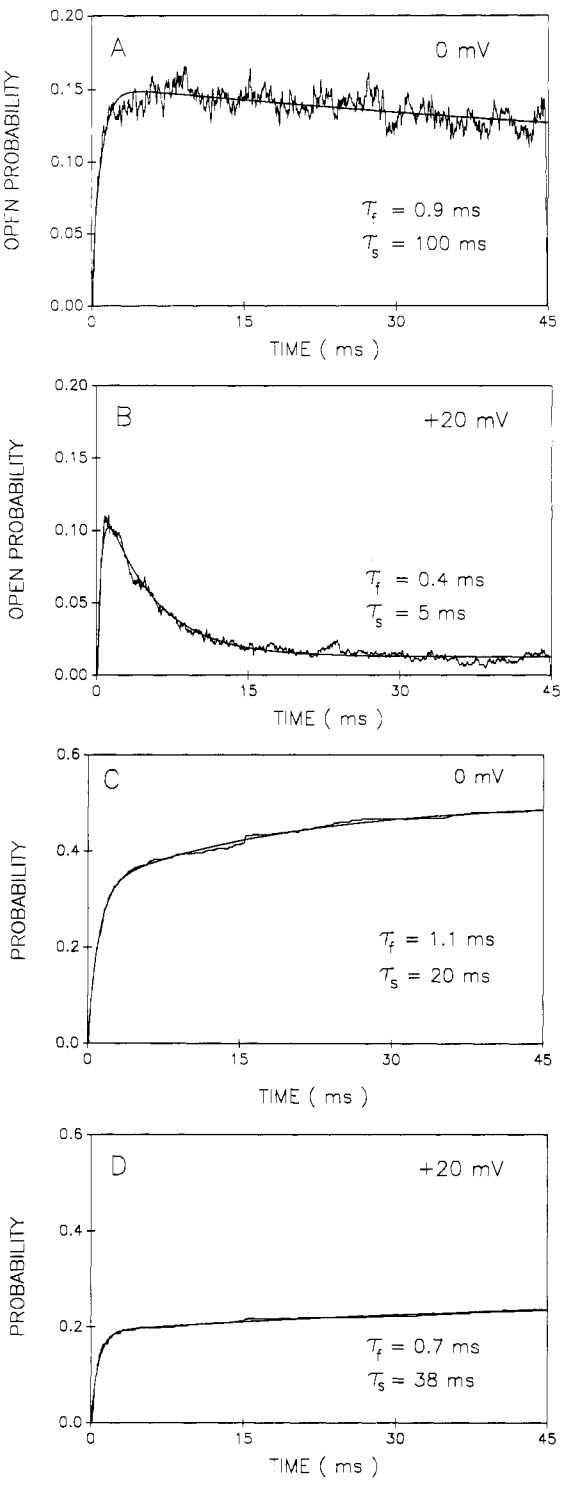

FIGURE 5: Consequences of the variability in the gating pattern: ensemble currents and first latencies. Parts A and C contain data from a patch held at −120 mV, prepulsed to −150 mV, and pulsed to 0 mV during 45 ms every 0.5 s. The number of records and of events analyzed were 474 and 9435, respectively. In parts B and D are illustrated single-channel data recorded from the same patch under the same protocol but pulsed to +20 mV (473 records, 1197 events). Parts A and B illustrate the ensemble open probabilities along the length of the pulse and superimposed (solid lines) the reproduction of the whole curves from the fit of two exponentials. The values of τ in each panel represent the calculated time constants for the fast (τ_f) and slow (τ_s) components which in this example correspond to the rising and falling phases of the curves. Parts C and D, on the other hand, document the observed times to first openings for each set of data. The whole curves were again fitted to two exponentials. Solutions (mM): 250 NaCl internal, 245 CsCl–5 NaCl external. Number of channels: 2 (3792 records) Sampling: 50 μs/point. File: NANJU11E (A and C) and NANJU11D (B and D).

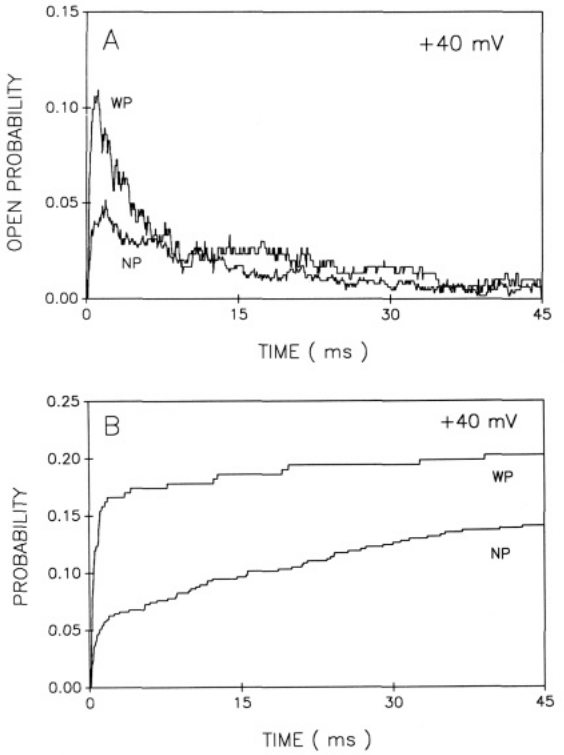

FIGURE 6: Effect of hyperpolarization on the single-channel activity. The data shown in this figure were recorded from the same patch. Panel A illustrates the ensemble currents at +40 mV with (WP) or without (NP) an applied hyperpolarizing prepulse to −150 mV during 50 ms, from a holding of −120 mV. Pulses lasted 45 ms. The number of records and of transitions involved in the averages shown were (respectively) 321 and 686 in the trace without a prepulse and 151 and 550 with the prepulse. The peak probabilities were 0.05 at 1.95 ms (NP) and 0.11 at 1.20 ms (WP). The fit of the inactivation time courses to two exponentials (not shown) gave the following time constants: for NP, τ_f and τ_s equaled 0.636 and 14.28 ms, respectively; for WP, τ_f was 2.299 ms and τ_s was 27.90 ms. The values of the MOT for the two sets were 1.23 ms without the prepulse and 1.17 ms with the prepulse. The average openings per trace were 4.11 (NP) and 5.04 (WP). (B) Plotted are the first latencies measured at +40 mV before and after the application of the prepulse to −150 mV. Again NP refers to *no prepulse* and WP to *with prepulse*. The best fit of the data to two exponentials according to the expression $A_0 + A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$, where $A_2 = A_0 + A_1$, gave the following coefficients and time constants for NP: $A_0 = 0.173$, $A_1 = -0.054$, $\tau_1 = 0.599$, $A_2 = -0.119$, $\tau_2 = 32.98$; the respective values for WP were $A_0 = 0.220$, $A_1 = -0.167$, $\tau_1 = 0.534$, $A_2 = -0.052$, $\tau_2 = 37.01$. File: NANJU11C.

each panel are the curves described by fits to two exponentials to the whole curves (rising and falling phases). Immediately apparent to the eye is the difference in the falling phases of the average currents in Figure 5A,B for which a fit to a single exponential gave time constants of 100 ms (0 mV) and 5 ms (+20 mV). The rising phases of the curves follow similar time courses; the time constants were of the same order of magnitude (τ_f's were 0.85 ms for 0 mV and 0.41 ms at +20 mV). The fits to two exponentials of the accumulated latencies to first opening gave fast and slow time constants of 1.05 and 19.7 ms at 0 mV (C) and 0.73 and 37.5 ms at +20 mV (D). The differences here were of the order of a factor of 2 which makes the first latencies at these potentials very similar, in contrast to the large difference observed in the macroscopic currents. Not surprisingly, the average number of openings per trace at 0 mV was very high (27) as opposed to 6 openings per trace found at +20 mV. Again, there was no agreement between the time course of inactivation and the time to first opening at either potential. Also, the differences in the reconstructed curves observed in this figure are not due to an

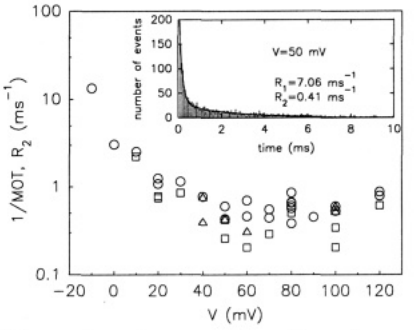

FIGURE 7: Voltage dependence of the reciprocal mean open time (MOT). Main frame: open circles are inverse MOT of events from 15-ms pulses; open triangles come from 45-ms pulses. All the data come from the same patch. Also shown (open squares) are the values of the rate constants of the slowest component obtained from the fit of the open time distributions to two exponentials. Inset: Open time histogram at 50 mV, and fit of the data to two exponentials (solid line). Holding potential: −140 mV. Prepulse: −150 mV (50 ms). Solutions (mM): 250 NaCl–0.5 $CaCl_2$ internal, 225 KCl–25 NaCl external. Files: NANMY22C through L.

increase of depolarization from 0 to +20 mV; slow decays of macroscopic inactivation were observed in this and in other experiments (see, for example, Figure 3B) at more depolarized potentials.

Hyperpolarizing prepulses frequently had clear and dramatic effects on the type of gating observed during a sequence of pulses. An example is illustrated in Figure 6. Part A shows superimposed the average currents obtained at +40 mV from a holding potential of −120 mV, in the absence (trace labeled NP) and presence (trace labeled WP) of a prepulse to −150 mV (50 ms). The traces used to create the averages shown were consecutive records and belong to the same patch as the data shown in the previous figure. The records used to form the NP curve immediately preceded those used in the WP trace. It stands out that channel activity accumulated at the beginning of the pulse when the patch was exposed to the hyperpolarizing prepulse. Also, the probability of opening increased greatly with the prepulse. The average number of openings per trace, the number of transitions involved in the ensemble currents (trace NP had twice the number of records in WP), and the MOT's were the same with or without the prepulse (see figure legend for details). The fit to two exponentials of the falling phases of the reconstructed currents revealed a 4-fold increase in τ_f with a simultaneous 2-fold increase in τ_s after application of the prepulse. Also shown in Figure 6 (part B) are the superimposed accumulated first latencies for each set of data. These curves were best fit with two exponentials with identical time constants, but with exactly opposite coefficients (see figure legend for details). This change is consistent with the observation that channels were opening more often and closer to the onset of the pulse as a consequence of having further hyperpolarized the membrane. This effect, however, was not always seen. Sometimes gating was barely modified by making the holding potential more negative or by imposing a prepulse. Normally, however, further hyperpolarization, through a change in the holding potential or by application of a prepulse, increased the number of events. In one experiment, in 9 out of 12 cases hyperpolarization caused an average increase of 59% (SD <10.8) in the total number of transitions considering all the records at each pulse protocol for each potential (7 potentials).

Open times were voltage dependent as illustrated in Figure 7. In this example, the inverse of the MOT has been plotted over a wide range of pulse potentials (note the log scale in the *y* axis). Open circles are values obtained from events during

15-ms pulses; open triangles, from 45-ms pulses. At potentials from −10 to +50 mV, the inverse MOT decreased (MOT increased) as the membrane was depolarized, as expected from a voltage-dependent deactivation. A minimum was achieved—here at around +50 to +60 mV—and then the curve seems to flatten, or perhaps increase slightly, with further depolarization, as would be expected from a very weakly voltage-dependent inactivation step. The latter could also be due to abnormal inactivation or to a lack of fast inactivation. Compared to other sodium channels, the curve as a whole is displaced by about 50 mV toward more depolarized potentials. Open time distributions like the one shown in the inset were constructed for potentials having more than 500 transitions. A single exponential was not enough to account for the open time distributions. Fits to two exponentials rendered rate constants for the fast (R_1) and the slow (R_2) components of the distributions. Fitted values of R_2 are plotted in the main frame of Figure 7 as open squares. As suggested by the good agreement between the values of R_2 and the inverse mean open times, these latter seem to be primarily determined by the slow component, the major contributor to the open time distributions at all potentials. We also found a high dispersion among the values of the fast rate constants most likely due to limited resolution of durations close to the sampling rates.

Discussion

Our experience has been that with BTX three or four channels were routinely present in an excised patch and that, although orientation was not always random, channels facing both inside-out and outside-out were present. Without BTX, however, the opportunity to study channels oriented in either configuration was not equivalent due to the fact that patches would not tolerate large negative holding potentials (pipet potentials) for periods longer than about 2 min. Therefore, all of the channels from which data are reported were facing inside-out, i.e., their external side facing the inside of the patch pipet. We nevertheless pursued the study of channels facing outside-out and kept the ionic gradient constant with high [Na^+] in the bath and low in the pipet. As a consequence of this experimental arrangement and the polarized stability of the patches, all voltage-activated Na^+ currents studied in the present work have been outward currents measured at potentials between −10 and +150 mV, i.e., mostly all positive potentials (cell convention). Thus, we are aware that a direct comparison at a single-channel level with known properties of other Na^+ channels is not straightforward, but some properties can still be profitably compared, especially with those previously reported by this laboratory (Rosenberg et al., 1984b; Shenkel et al., 1989), with those of the native eel Na^+ channel (Shenkel, 1989) and with those of other Na^+ channels studied under similar experimental arrangements (Adelman & Senft, 1966; Chandler & Meves, 1970a,b; Oxford & Yeh, 1985; Yeh & Oxford, 1985).

Density of Channels in the Liposomes. Previous biochemical studies with the reconstituted electroplax protein demonstrated that it was possible to form small liposomes that had a high average density of channels which actively bound [^{3}H]TTX, and mediated neurotoxin-activated fluxes (Rosenberg et al., 1984a; Tomiko et al., 1986). However, a substantial fraction of the protein in the sample was denatured. Denaturation was due primarily to long gel permeation chromatography steps originally used to isolate the channels. The denaturation of channels during isolation has now been largely circumvented with the rapid affinity purification procedures used here (James et al., 1989). In the past, several factors had combined to reduce the average surface abundance of active channels in the reconstituted liposomes. Dilute material eluting from the gel filtration columns had to be concentrated before reconstitution. Such material could not be concentrated (e.g., by pressure dialysis) without accumulating lipid-containing micelles used for stabilization during purification (Agnew & Raftery, 1979). Phosphatidylcholine (PC), optimal for purification buffers, was not ideal for reconstitution (Rosenberg et al., 1984a). This invariably meant that for optimal reconstitution the accumulated PC had to be supplemented with PE and PS, reducing the overall protein to lipid ratio, and consequently the channel surface density. The higher protein concentrations, combined with a higher specific activity, now available require that samples must be actually diluted before reconstitution in order to obtain the appropriate or desired channel densities.

As discussed elsewhere (Correa & Agnew, 1988), an additional technical problem we encountered was that channels were apparently not uniformly distributed in large liposomal structures obtained by simply freezing and thawing (Tank et al., 1982). The encounter frequency of channels in excised patches from frozen–thawed liposomes was much lower than expected based on flux and toxin binding activity (Tomiko et al., 1986). The technical improvements introduced by Correa and Agnew (1988) markedly improved the encounter frequency, making it routinely possible to examine, with patch-clamp techniques, the activity of purified electroplax channels reconstituted into artificial membranes. Because the channels being studied have been subjected to solubilization, biochemical isolation, reconstitution, sonication, and freezing and thawing, it may be expected that some of the behavior, especially at the level of single molecules, may be anomalous.

In the absence of any neurotoxins, channels were, overall, much less likely to be active, and—when activated—displayed somewhat heterogeneous gating behavior. In the presence of BTX, essentially all of the channels appeared to be active and to display uniform gating and conductance properties [for more detail, see Correa and Agnew (1988)]. Although a study of BTX-activated channels is beyond the scope of this paper, it can be said that BTX-modified purified eel channels were comparable to BTX-treated channels studied in planar lipid bilayers reconstituted either from native cell membranes or from reconstituted liposomes (Krueger et al., 1983; Tanaka et al., 1986; Hartshorne et al., 1985; Recio-Pinto et al., 1987; Behrens et al., 1989; Shenkel et al., 1989). They do not compare, however, to the preliminary results reported by Rosenberg et al. (1984b), who observed, in BTX, events with a higher conductance and anomalous gating behavior. Also, in an extensive study of the properties of purified and unpurified BTX-modified eel Na^+ channels in bilayers, Recio-Pinto et al. (1987) have described a high scatter in the values for the midpoint of the activation curve. The variations they reported could well be due to the obvious heterogeneity in the gating of the unmodified channels. Their findings emphasize the usefulness of being able to characterize reconstituted channels under the influence of voltage alone, in the physiological time frame.

Channel Conductance and Selectivity. All the *I–V* curves reported here are from outward currents, primarily carried by sodium. The decrease in conductance observed in the presence of Cs^+ and Rb^+, also seen with internal Ca^{2+} (data not shown), is likely to be due to partial block by these less permeant ions. Reversal potentials, though clearly negative, could not be determined accurately because the measurements did not extend to inward currents. However, by extrapolation, under the ionic conditions tested, the selectivity for Na^+ over

K^+ is at least 5 to 1, as has been observed for purified Na^+ channels with and without treatment with BTX [Rosenberg et al., 1984b; see also Recio-Pinto et al. (1987)]. The estimated selectivities over Cs^+ and Rb^+ were higher. The conductance of 30 pS is within the range of conductances (20–30 pS) measured in the native electrocyte under comparable salt conditions (Shenkel, 1989). Also, a conductance of 30 pS is about double the conductance we observe for the BTX-treated channels. This is consistent with findings in other preparations [e.g., see Correa et al. (1989b)] and agrees well with the ratio between the conductances observed for the trypsin-treated and for the BTX-modified eel purified sodium channels as reported by Shenkel et al. (1989). It is higher, however, than that reported by Rosenberg et al. (1984b). This is most likely due to the difference in salt concentrations (90 mM Na^+ in their experiments), to the presence of milimolar concentrations of divalents, and to a limited resolution (1- vs 5-kHz bandwidth used here).

Gating Behavior. We have observed two types of voltage-activated gating patterns termed *slow* and *fast* on the basis of the time course of activation and inactivation of the reconstructed macroscopic currents. In fast gating, currents showed rapid rising phases and clear evidence of channel inactivation. Together with the report by Rosenberg et al. (1984b), this is the only demonstration of inactivation for any purified sodium channel preparation. In slow gating, the currents showed variable rising phases and very slow inactivation. Also, the voltage dependence of the gating processes, of both fast and slow currents, is displaced toward more positive potentials than observed for other sodium channels. The voltage dependence of the mean open times speaks for a normal voltage-dependent deactivation and a weakly voltage-dependent inactivation. Clear separation of fast and slow currents was very infrequent in a run; most often, a combination of both gating patterns was found.

How Normal Are These Channels? Compared to inward sodium currents studied under physiological conditions in most native cells, in particular, electrocytes (Nakamura et al., 1965; Shenkel, 1989), the inactivation process observed here is more incomplete, and its time course is slower at all potentials. At the single-channel level, the lengthened time courses do not seem to result from delayed onset of activation (Aldrich et al., 1983), but from several repeated openings before the channel inactivates. The inactivation of Na^+ currents in cells of muscle origin is known to be slower, a consequence also of the bursting nature of those channels [e.g., see Kirsch and Brown (1989)]. It is also well-known that inactivation of sodium currents at positive potentials tends to be incomplete [e.g., see Bezanilla and Armstrong (1977)]. However, our results would be more appropriately compared to sodium currents studied under similar conditions as those used in the experiments reported here, i.e., high [Na^+] inside and a reversed gradient. Under such conditions, outward sodium currents exhibit clear slow and incomplete inactivation [e.g., see Adelman and Senft (1966), Chandler and Meves (1970a,b, Oxford and Yeh (1985), and Yeh and Oxford (1985)]. One possible explanation is the coexistence of two functionally distinct sodium conductance pathways as suggested by Chandler and Meves (1970a); Oxford and Yeh (1985) proposed a direct interaction of internal Na^+ ions with a site intimately related to inactivation thus interfering with an otherwise normal process. In addition, preliminary observations by Shenkel and Bezanilla (unpublished results) indicate that single native eel Na^+ channels tend to open repeatedly along the depolarizing pulses when studied under inverse gradient conditions and at positive potentials. They have also observed long silent periods comparable to those described here. Taking this in consideration, it is possible that some of the channels are actually perfectly healthy and that the behavior reported here accounts for the mentioned previous observations.

The noninactivating macroscopic currents reconstructed from consecutive records of channels with long bursting epochs resemble those of channels in native membranes which have been treated with proteolytic or oxidizing reagents to remove inactivation [e.g., see Armstrong et al. (1973), Oxford et al. (1978), and Yeh and Oxford (1985)]. The intense and prolonged single-channel activity is somewhat reminiscent of that caused by certain neurotoxins which affect Na^+ inactivation [e.g., see Kirsch et al. (1989)]. Our data suggest that, in this mode, the inactivation mechanism of the purified channel is not functioning properly. Channels often open in bursts that reappear in two or more successive traces. The frequency at which the burstlike activity appears varies between experiments, as well as within the same experiment, sometimes even at the same pulse potential, and with the holding potential. Channels do not appear to stay open during repolarization but very frequently are open at the onset of the pulse. This suggests that gating processes which follow channel opening are perturbed. The most likely candidate for these alterations is fast inactivation. It is known from a variety of studies, mostly with macroscopic current measurements, that the part of the molecule responsible for inactivation is somewhat exposed and can be readily modified or damaged. The fact that there are multiple ways of preventing normal inactivation might account for the variations in gating observed.

In this respect, Shenkel and co-workers (Shenkel et al., 1989) have characterized the LFA-purified reconstituted eel channel in planar lipid bilayers, following tryptic modification. In those experiments, trypsin-modified channels exhibited activation gating kinetics and voltage dependence which were essentially identical with those expected if fast inactivation were removed. Although more precisely controlled experiments are called for, it seems quite possible that total removal of fast inactivation will result in uniform behavior of the channels, whereas selective, though variable, damage to inactivation could result in different levels of inactivation.

Could the Channels Switch between Gating Modes? Because of the low level of activity under regimens involving voltage alone (we never saw more than three channels open simultaneously, nor did we see large peak open probabilities), it is difficult to determine the real number of channels in each patch, and thus it is uncertain whether a given channel might shift its gating spontaneously. Because both types of gating often occurred in a patch during a 0.5–4-h experiment (e.g., Figures 1, 3, and 5), ensemble currents often exhibited two components which appear to be summations of "pure" slow and fast currents. The conductances and the mean open times were similar in both fast and slow channel gating within the same experiment. The principal difference among them was the number of openings per trace and, consequently, the probability of being open among traces with openings. We have not yet established rigorous criteria for separating records according to the type of gating. However, because overlaps of long intense gating activity were extremely rare occurrences, we believe that the channels may be able to gate alternatively in both ways.

Bimodal gating has been reported for Na^+ and other ion channels in their native environments (Hess et al., 1984; Patlak & Ortiz, 1985; Nowycky et al., 1985; Patlak & Ortiz, 1986; Yue et al., 1989). It is clearly seen in sodium channels from

cardiac (Patlak & Ortiz, 1985) and skeletal muscle (Patlak & Ortiz, 1986) cells. In those cases, however, long bursting activity of an individual channel in cell-attached patches was observed only occasionally. In addition, a change in the gating pattern from short-lived to long-lived inactivation has been documented for other Na^+ channels after patch excision [e.g., see Nilius (1988)]. It seems possible that excision might damage cytoskeletal associations, or might remove cytoplasmic factors, which are essential for normal inactivation (Auld et al., 1988; Zhou et al., 1989). It is, therefore, possible that the frequent bimodal gating we observe here is a reflection of a somewhat altered gating property of the intact channel.

Inactive Periods. Channels gating in either of the two general patterns described here often entered inactive periods. We initially surmised that these silent periods might be an artifactual property resulting from the biochemical manipulations. A low level of channel activity is not expected in a cell that exhibits a mean channel density of $500/\mu m^2$ as determined from [^{3}H]TTX binding studies (Agnew et al., 1983). These prolonged inactive periods reduce the chances of seeing a channel and may explain a recent observation by Shenkel and Sigworth (personal communication) that in the native eel Na^+ channel the overall probability of opening is low and that the peak probability is a shallow function of voltage. Long silent periods have also been observed in other preparations of muscle origin [e.g., see Kunze et al. (1985)]. The role of these quiescent periods in the normal physiology of the electroplax is uncertain.

The need of strong hyperpolarizing holding potentials, followed sometimes by even more hyperpolarized prepulses, to enhance channel opening has also been observed in patches excised from the native electrocyte. In addition, in some experiments, we observed a noticeable inverse relation between the length of the silent periods and the degree of hyperpolarization of the holding and prepulse potentials. Though not consistently seen, on some occasions there was a clear decrease in the occurrence of long-lived bursting activity after further hyperpolarization of the holding potential. It would follow then that the appearance of these inactive periods may be influenced by voltage. In this respect, this phenomenon would resemble slow inactivation in other Na^+ channels (Rudy, 1978).

In summary, we have described progress in the study of the gating behavior of the purified and reconstituted eel sodium channel in response to voltage alone. The combination of improved methods for rapid biochemical isolation and reconstitution has enabled routine study of the channel at a single-channel level and in the absence of neurotoxins. Depolarizing voltage steps revealed two patterns of gating behavior, a fast inactivating mode (exhibiting "normal" activation and inactivation) and a slow inactivating mode (with variable gating and altered inactivation). In addition, spontaneous long inactive periods (perhaps related to slow inactivation) were routinely observed. The voltage dependence and functional role of these are yet to be determined. The same channels under the influence of BTX behave in a manner essentially identical with toxin-treated channels in cell membranes in other preparations and in planar lipid bilayers. The gating processes observed with the present reconstituted preparation are not inconsistent with studies carried out with more nearly physiological preparations.

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An Integumentary Mucin (FIM-B.1) from *Xenopus laevis* Homologous with von Willebrand Factor†

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ABSTRACT: We present a new protein from *X. laevis* skin termed "frog integumentary mucin B.1" (FIM-B.1) with a general structure similar to FIM-A.1 (formerly "spasmolysin"). The central region consisting of tandem repeats of 11 amino acid residues is probably a target for extensive O-glycosylation, whereas the C-terminal cysteine-rich domain shows pronounced homology with the C1-C2 domains and the C-terminal end of von Willebrand factor. Furthermore, we describe homology with antistasin, an anticoagulant peptide from a leech. We also discuss some implications concerning the evolutionary origin of von Willebrand factor. In situ hybridization studies revealed the expression of FIM-B.1 exclusively in mucous glands of the skin. This is comparable with FIM-A.1 but is in contrast to all other physiologically active peptides, which are synthesized in granular glands.

In the past, secretions from frog skin turned out to be the source for biogenic amines and a variety of different physiologically active peptides (Erspamer, 1971), which probably act in defense mechanisms. Due to the embryonic relationships of the tissues (Alpert et al., 1988), many of these peptides have counterparts in the nervous system as well as in the gastrointestinal tract (Erspamer & Melchiorri, 1980; Pearse, 1979). *Xenopus laevis* skin contains two different types of glands—granular (or poison) and mucous glands (Engelmann, 1872). All of the active polypeptides investigated so far are

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