Modification of Inactivation in Cardiac Sodium Channels: Ionic Current Studies with Anthopleurin-A Toxin

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ABSTRACT   The site 3 toxin, Anthopleurin-A (Ap-A), was used to modify inactivation of sodium channels in voltage-clamped single canine cardiac Purkinje cells at $\sim$12°C. Although Ap-A toxin markedly prolonged decay of sodium current (I_{Na}) in response to step depolarizations, there was only a minor hyperpolarizing shift by 2.5 ± 1.7 mV ($n = 13$) of the half-point of the peak conductance-voltage relationship with a slight steepening of the relationship from −8.2 ± 0.8 mV to −7.2 ± 0.8 mV ($n = 13$). Increases in G_{max} were dependent on the choice of cation used as a Na substitute intracellularly and ranged between 26 ± 15% (Cs, $n = 5$) to 77 ± 19% (TMA, $n = 8$). Associated with Ap-A toxin modification time to peak I_{Na} occurred later, but analysis of the time course I_{Na} at multiple potentials showed that the largest effects were on inactivation with only a small effect on activation. Consistent with little change in Na channel activation by Ap-A toxin, I_{Na} tail current relaxations at very negative potentials, where the dominant process of current relaxation is deactivation, were similar in control and after toxin modification. The time course of the development of inactivation after Ap-A toxin modification was dramatically prolonged at positive potentials where Na channels open. However, it was not prolonged after Ap-A toxin at negative potentials, where channels predominately inactivate directly from closed states. Steady state voltage-dependent availability (h_∞ or steady state inactivation), which predominately reflects the voltage dependence of closed–closed transitions equilibrating with closed-inactivated transitions was shifted in the depolarizing direction by only 1.9 ± 0.8 mV ($n = 8$) after toxin modification. The slope factor changed from 7.2 ± 0.8 to 9.9 ± 0.9 mV ($n = 8$), consistent with a prolongation of inactivation from the open state of Ap-A toxin modified channels at more depolarized potentials. We conclude that Ap-A selectively modifies Na channel inactivation from

the open state with little effect on channel activation or on inactivation from closed state(s).

INTRODUCTION

Anthopleurin A (Ap-A) toxin, which was isolated from the sea anenome *Anemone xanthogrammica*, is a member of the class of site 3 toxins, which bind to the extracellular surface of voltage-gated Na channels and cause a marked slowing of I_{Na} in response to step depolarizations (Catterall, 1980). Site 3 toxins also include the 60–70 amino-acid residue α-scorpion toxins from *Leiurus quinquestriatus, Centruroides sculpturatus, Tityus serulatus,* and *Androctonus australis* (Rochat, Bernard, and Couraud, 1979; Meves, Simard, and Watt, 1986) and other peptide toxins isolated from sea anenome, including Anthopleurin B (Ap-B [49 animo-acids]) also from *Anemone xanthogrammica,* and the *Anemone sulcata* toxins (ATXI [46 amino acids], ATXII [47 amino acids], and ATXIII [27 amino acids]) (Norton, 1991). While each of these toxins is thought to produce a similar effect on Na channels, there are striking differences in affinity for various isoforms of Na channels. Some site 3 toxins have a high affinity for insect and crustacean Na channels (Salgado and Kem, 1992), whereas others, like Ap-A toxin, have a 20–40-fold higher affinity for cardiac Na channels than for mammalian neuronal channels (Norton, 1991).

Classic experiments that "remove inactivation" by the use of proteolytic enzymes such as pronase or other chemical agents have suggested an intracellular locus controlling inactivation of Na channels. Armstrong and Bezanilla (1977) investigated the effects of internal pronase on the squid giant axon and proposed a model of inactivation as a particle or "ball" that would pivot on the end of a chain and bind to a receptor on the intracellular surface of the activated Na channel. Recent experiments using antibodies (Vasselev, Schueuer, and Catterall, 1988), sequence cuts (Stühmer, Conti, Suzuki, Wand, Noda, Yahagi, Kubo, and Numa, 1989), and site-directed mutagenesis (Moorman, Kirsch, Brown, and Joho, 1990; Patton, West, Catterall, and Goldin, 1992; West, Patton, Scheuer, Wang, Goldin, and Catterall, 1992; Hartmann, Tiedeman, Chen, Brown, and Kirsch, 1994) have identified intracellular residues in the linker sequence between domain III and IV that may compose such a ball or "hinged lid." However, site 3 toxins bind extracellularly and modify Na channel inactivation. They are not the only agents that affect inactivation via an extracellular binding site; triiodothyronine (Dudley and Baumgarten, 1993) and forskolin (Ono, Fozzard, and Hanck, 1995) are also thought to bind extracellularly and prolong I_{Na}. In addition, some of the spontaneous mutations in human skeletal muscle Na channels associated with skeletal muscle myotonia disorders involve extracellular residues and produce a phenotype similar to Na channels that have inactivation "removed" (Cannon and Corey, 1993; Chahine, George, Zhou, Ji, Sun, Barchi, and Horn, 1994). We postulated that investigation of the kinetic effects of Ap-A toxin, a site 3 toxin with a high affinity for cardiac Na channels, would provide information about inactivation. The experiments presented here provide evidence that for the cardiac Na channel the site 3 toxin Ap-A selectively and reversibly inhibits inactivation from the open state while producing little effect on either activation or inactivation from closed states.

METHODS

Cell Preparation

Cells were isolated from canine Purkinje fibers using the procedure of Sheets, January, and Foz-zard (1983). Purkinje fibers were cut into 2–3 mm segments and incubated in a shaking water bath (37°C) for 1.5–2 h in a modified Eagle's minimal essential medium (MEM) with 5 mg/ml Worthington type I or Boehringer Mannhein type B collagenase. Fibers were washed in 150 mM K-glutamate, 5.7 mM $MgCl_2$, 0.12 mM EGTA, and 5 mM HEPES (pH 6.2) at 37°C, and then were mechanically separated into single cells by application of shear force. Cells were stored at room temperature in MEM (pH 7.3), which contained 1.8 mM Ca, and were studied within 18 h.

Solutions

In many experiments extracellular solution contained (millimolar) 15 Na^+, 185 TMA^+, 2 Ca^{2+}, 4 Mg^{2+}, 0.010 Cd^{2+}, 200 MES^-, 12 Cl^-, and 10 HEPES (pH 7.2). Intracellular solution contained (millimolar) 200 TMA^+, 200 F^-, 10 EGTA, and 10 HEPES (pH 7.2). The increased osmolarity of the solutions helped to decrease series resistance. Concentrations of Mg^{2+} and Cd^{2+} were chosen to maximize block of I_{Ca} while producing minimal effects on I_{Na} (Hanck and Sheets, 1992a; Sheets and Hanck, 1992). We did not observe any differences in modification based on the presence of additional divalent cations. For some experiments the control extracellular solution contained (millimolar) 15 Na^+, 135 Cs^+, 2 Ca^{2+}, 154 Cl^-, and 10 HEPES (pH 7.2). In these cases the intracellular solution contained (millimolar) 15 or 0 Na^+, 135 or 150 Cs^+, 150 F^-, 10 EGTA, and 10 HEPES (pH 7.2). Toxin was obtained from Sigma Chemical Co. (St. Louis, MO) and was added to the extracellular solution. Actual concentrations of Ap-A toxin were calculated from measurements of the purity of Ap-A toxin determined by optical density measurements, assuming the molecular weight to be 5133 gm M^{-1} and the $OD^{1\%}_{280}$ to be 35.4. Concentrations were 0.34 of that predicted from the mass of the sample. Preliminary experiments were carried out to determine the concentration range of toxin effect, and a concentration of 340 nM Ap-A toxin (nominal concentration 1 μM) was chosen for these studies because this concentration modified >90% of the Na channels, producing a uniform population of modified channels and yet minimize the cost of experiments.

Temperature was controlled using a Sensortek (Physiotemp Instruments, Inc., Clifton, NJ) TS-4 feedback-controlled thermoelectric stage placed adjacent to the bath chambers and typically varied less than 0.5°C during an experiment. Cells were studied between 9.8 and 13.0°C.

Recording Technique

Recordings were made using a large bore, double-barrelled glass suction pipette for both voltage clamp and internal perfusion as previously described (Makielski, Sheets, Hanck, January, and Foz-zard, 1987). Current responses were measured with a virtual ground amplifier (Burr-Brown OPA-101) with a 5 MΩ feedback resistor connected to the outflow channel of the bath via a 3-M KCl bridge and Ag/$AgCl_2$ pellet. Voltage protocols, using locally written control programs (D. Hanck), were imposed from a 12-bit DA converter (Masscomp 5450, Concurrent Computer, Tinton Falls, NJ) over a 30/1 voltage divider. Data were filtered by the inherent response of the voltage-clamp circuit (corner frequency near 125 kHz) and recorded with a 16-bit AD converter on a Masscomp 5450 at 300 kHz. Time constants of the capacity transients without capacity compensation were <30 μs, and a fraction of the current (~65%) was fed back to compensate for series resistance (Makielski et al., 1987).

Cells were placed in the aperture of the pipette in a low Ca^{2+} solution and transferred to one of four experimental chambers where they were allowed to form a high resistance seal to the pipette.

After a cell had sealed to the pipette the cell membrane inside the pipette was disrupted with a manipulator-controlled platinum wire. Voltage control was assessed by evaluating the time course of the ionic current in steps (V_t) to -40 mV, the time course of the capacitive current in steps between -150 and -190 mV, and the negative slope region of the peak current-voltage relationship (Makielski et al., 1987; Hanck and Sheets, 1992b). The holding membrane potential (V_h) was typically -150 mV, which insured full I_{Na} availability under our experimental conditions, and the cell membrane was depolarized once per second or once each 1.5 s. With these experimental protocols "slow inactivation" was not apparent either in control or in the presence of toxin.

Data Analysis

Data were capacity corrected using 16 scaled current responses to steps between -150 mV and -190 mV. Leak resistance (R_L) was taken as the reciprocal of the linear conductance between -190 and -110 mV. For the 17 cells included in this study, R_L values were 99 $\pm$ 62 MΩ. Cell capacitance was measured from the integral taken over 10 ms of the current responses to voltage steps between -150 and -190 mV and was 87 $\pm$ 24 pF. Peak I_{Na} was taken as the mean of four data samples (over 16.67 μs) enclosing the maximal value after digitally filtering at 5 kHz and after leak correcting by the amount of leak extrapolated from the time-independent linear leak currents elicited between -110 and -190 mV.

Data were analyzed and graphed on a Masscomp 5450 computer using locally written programs (D. Hanck) or on a SUN Sparcstation (IPX or Sparc10/40 or 55) using SAS (Statistical Analysis System, Cary, NC). Fitting programs on the Masscomp or on the SUN used algorithms from the Numerical Algorithms Group library (NAG, Oxford, UK) or from SAS. I_{Na} tail-current relaxations were fitted with a sum of exponentials with DISCRETE (Provencher, 1976) to determine time constants, amplitudes, baselines, and standard errors of the estimate for fitted parameters. This program provided a modified F-statistic that was used to evaluate the number of exponential components that best described the data. Unless otherwise specified, all summary statistics are expressed as means $\pm$ one standard deviation (SD). Regression parameters are reported as the estimate and the standard error of the estimate (SEE).

The following procedures were used to account for spontaneous time-dependent changes in kinetic parameters during recordings. We have previously shown that indices of cardiac Na channel kinetics (e.g., the half-point of peak conductance-voltage relationship) shift to more negative potentials as a linear function of time under our experimental conditions (Hanck and Sheets, 1992b). To account for time-dependent changes in channel kinetics, control data taken before and after the experimental intervention were averaged or kinetic indices were estimated by linear interpolation between the control and wash measurements before comparison with data from Ap-A toxin modified Na channels.

RESULTS

Ap-A Toxin Rapidly and Reversibly Modified I_{Na}

Within seconds of exposure to Ap-A toxin, I_{Na} decay showed evidence of slowing, and modification was complete within 2–3 min as evidenced by marked slowing of I_{Na} decay (Fig. 1, A and B). Modification of I_{Na} by Ap-A toxin was completely reversed by depolarizing the membrane potential to -10 mV for 5 min in the absence of Ap-A toxin (Fig. 1 C). If the holding potential was -150 mV, I_{Na} remained modified for many minutes (data not shown). Washout of Ap-A toxin during maintained depolarization most likely results because inactivated Na channels have a low affinity for site 3 toxins (Strichartz and Wang, 1986; Warashina, Jiang, and Ogura,
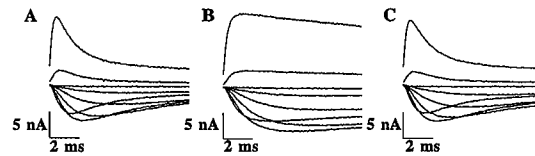
FIGURE 1. Family of I_{Na} responses during step depolarizations to -80, -65, -55, -45, -35, -20, 10, and 40 mV from a V_h of -150 mV in control (A), after exposure to 340 nM Ap-A toxin (B), and in wash (C). The cell was depolarized to -10 mV for 5 min after toxin was removed from the bath. Data are shown capacity corrected, but not leak corrected, and digitally filtered at 5 kHz. *Cell E8.03*: C_m 102 pF, R_L 40 MΩ, 12.4°C, $[Na^+]_o/[Na^+]_i$ (millimolar) 15/15. $[Cs^+]_o/[Cs^+]_i$ (millimolar) 135/135.

1988). G_{max} after washout of toxin decreased minimally (7.6 $\pm$ 7.6%, $n = 16$) consistent with a negligible loss of Na channels during exposure to toxin.

With symmetrical intracellular and extracellular concentrations of Na^+ and Cs^+, peak G_{max} increased by 26 $\pm$ 15% ($n = 5$; $P < 0.02$). Data for a typical cell are shown in Fig. 2, A and B. Interestingly, in the presence of TMA^+ (Fig. 2, C and D) the change in G_{max} was much larger (77 $\pm$ 8%, $n = 8$), most likely as a result of a decrease in voltage-dependent block by intracellular TMA^+ (O'Leary and Horn,
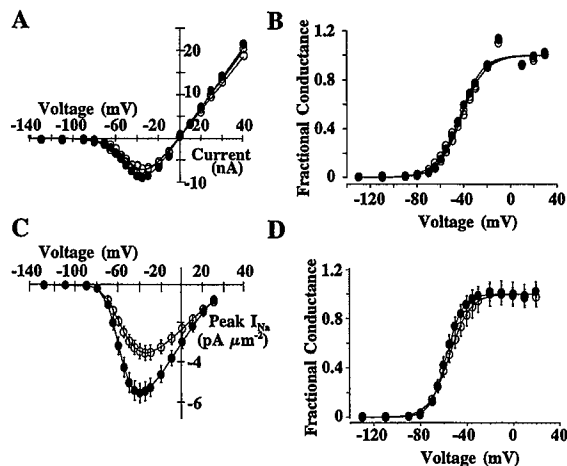

FIGURE 2. Effect of Ap-A toxin on the peak I_{Na}-voltage relationships (A and C) and peak G_{Na}-voltage relationships (B and D). (A and B) Data for a typical cell with symmetrical 15 mM Na^+ as the charge carrier and Cs^+ as the replacement cation. Control (○), in 340 nM Ap-A toxin (●) and wash (○). (C and D) Grouped data for eight cells with 15 mM Na_o and TMA^+ as the replacement cation. Control (○) and in Ap-A (●). In B and D, the solid lines represent the best fit of a transform of a Boltzmann distribution:

$$I_{Na} = \frac{(V_t - V_{rev}) G_{max}}{1 + e^{\frac{V_t - V_{1/2}}{s}}}$$

where I_{Na} is the peak current in the depolarizing step and V_t is the test potential. The parameters estimated by the fit were V_{rev}, the reversal potential, G_{max}, the maximum peak conductance, $V_{1/2}$, the half-point of the relationship, and s, the slope factor in millivolts. In symmetrical Na^+ and Cs^+, the $V_{1/2}$ for the cell shown in B changed from -42 mV in control to -43 mV after Ap-A toxin, and there was a small change in slope factor (-10.1 in control to -9.6 mV after Ap-A toxin). G_{max} increased for this cell from 3.1 nS/pF to 3.5 nS/pF. For the cells shown in D the solutions contained TMA^+, and the $V_{1/2}$ changed from -54 mV in control to -57 mV after Ap-A toxin, and there was a change in the slope factor from -7.6 ± 0.7 mV in control to -6.3 ± 0.7 mV ($n = 8$).

1994). Although the choice of substitute cation affected the magnitude of I_{Na}, the effects of Ap-A on the conductance-voltage relationships were similar. The voltage at one-half peak G_{max}, $V_{1/2}$, after Ap-A toxin modification was minimally shifted in 16 cells by -2.5 ± 1.7 mV from -53.7 ± 5.7 mV to -56.2 ± 5.4 mV. The slope factor of the Boltzmann relationship was also only modestly affected, becoming slightly steeper (by 1.1 ± 0.3 mV), increasing from -8.2 ± 0.8 mV to -7.2 ± 0.8 mV ($n = 16$). Although the changes in $V_{1/2}$ and slope factor were small, in all cases they returned toward control values after washout of Ap-A toxin. The minimal change in $V_{1/2}$ after modification by Ap-A toxin contrasts with that for mammalian neuronal Na channels (Gonoi and Hille, 1987; Cota and Armstrong, 1989), where the $V_{1/2}$ of the peak conductance-voltage relationship shifted by a greater magnitude after modification of inactivation. However, they are quite similar to data reported for frog node of Ranvier with other site 3 toxins (Mozhayeva, Naumov, Nosyreva, and Grishin, 1980; Warashina and Fujita, 1983; Neumcke, Schwarz, and Stampfli, 1985; Wang and Strichartz, 1985).

Ap-A Toxin Modification Had Little Effect on Na Channel Activation

Examples of leak and capacitance-corrected I_{Na} traces in response to step depolarizations to -50, -10, and 20 mV in control solutions and after exposure to Ap-A toxin are shown in Fig. 3 A. While the most dramatic effect of toxin on the time course of current was the slowing of current decay, the time-to-peak I_{Na} also was delayed as expected if inactivation were delayed (Fig. 3 B). To compare the time course of I_{Na} before and after modification by Ap-A toxin, I_{Na} recorded during 50-ms depolarizations were fit with a modified Hodgkin-Huxley equation as follows:

$$I_{Na} = A\left(e^{\left(-t/\tau_m \right)^{2.5}} + e^{\left(-t/\tau_h \right)} \right) + c \tag{1}$$

where I_{Na} is described as the sum of two scaled (A) exponential terms, (τ_m) raised to 2.5th power and τ_h, and a constant (c). The equation described the data well as can be seen in Fig. 3 A where the fits are shown superimposed on the current recordings. Summary data for τ_m and τ_h for 11 cells are shown in Fig. 3, C and D. The onset of I_{Na}, as reflected in τ_m, was affected by Ap-A toxin much less than was current decay (τ_h). The onset of I_{Na} was prolonged near threshold potentials while at more positive potentials the onset was unchanged; by paired t test τ_m was significantly larger than control only at test potentials less than -40 mV ($P < 0.01$).

To directly assess the effect of Ap-A toxin on the Na channel activation process, we measured tail current relaxations at negative potentials after Na channels had been opened by brief steps to very positive potentials. The tail relaxations represent the combination of channels deactivating (k_{OC}), inactivating (k_{OI}), and/or reopening (k_{CO}). At very negative potentials (≤ -100 mV), I_{Na} tail currents would be expected to represent predominately the rate of deactivation because k_{OC} should be much greater than either k_{OI} or k_{CO}. Data from a typical cell are shown in Fig. 4 A and B. I_{Na} tail currents were recorded both in control and in the presence of Ap-A toxin at test potentials between -150 and -70 mV for 25 ms after conditioning until peak I_{Na} at $+40$ or $+60$ mV. Tail currents were capacity corrected, trimmed by
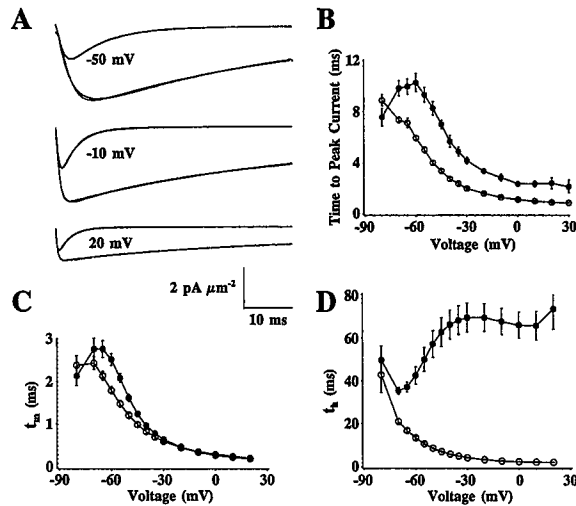
FIGURE 3. Effect of Ap-A toxin on the time course of I_{Na}. (*A*) Example of Na currents in response to step depolarizations of −50, −10, and +20 mV from a V_h of −150 mV. In each case lines are also drawn based on the fits of Eq. 1 to the data. Currents are shown leak and capacity corrected, digitally filtered at 20 kHz with only every 10th point graphed. *Cell L6.03*, 86 pF, R_L 210 MΩ. (*B*, *C*, and *D*) Means ± SEM for eight cells. In each figure control currents (○) were meaned from data taken in control and in wash and compared with data after exposure to Ap-A toxin (●). (*B*) Time to peak of I_{Na}. (*C*) Time constants of activation (τ_m) and (*D*) time constants of inactivation (τ_h) from fits of Eq. 1.

75–100 μs, and fitted with up to two exponentials with DISCRETE. I_{Na} tail-current relaxations to test potentials ≤ −100 mV were usually well fit by single exponentials, although sometimes a second, two- to fourfold slower time constant, accounting for <12% of the initial amplitude, could be detected. At potentials > −100 mV I_{Na} tail currents were better fit by two exponentials with the slower time constant accounting for a larger proportion of the initial amplitude at more depolarized potentials both in control and after Ap-A toxin. I_{Na} tail-current relaxation time constants returned to control after wash-out of the toxin with slightly longer time constants, as expected for the time-dependent shifts of kinetic parameters characteristic of this preparation (Hanck and Sheets, 1992*b*). Fig. 4 *C* shows a graph of the dominant time constants for the cell shown in Fig. 4, *A* and *B*, in control, in Ap-A toxin and after wash. At potentials ≥ −90 mV I_{Na} tail current time constants became significantly longer after Ap-A toxin. However, at potentials ≤ −100 mV I_{Na} time constants were little changed after modification by Ap-A toxin. Summary data of the time constants for six cells are shown in Fig. 4 *D*. The minimal change in I_{Na} tail current relaxations after modification by Ap-A toxin at very negative potentials is consistent with little effect of the toxin on the closing rate of Na channels.

Inactivation from Closed States Remained Intact in the Presence of Ap-A

Although decay of I_{Na} after Ap-A modification was markedly delayed (see Figs. 1 *B* and 3 *A*), during prolonged depolarizations it did fully decay, suggesting that a pathway to inactivation remained intact. To determine whether Na channel inactivation directly from closed states was slowed by Ap-A toxin, we measured the time
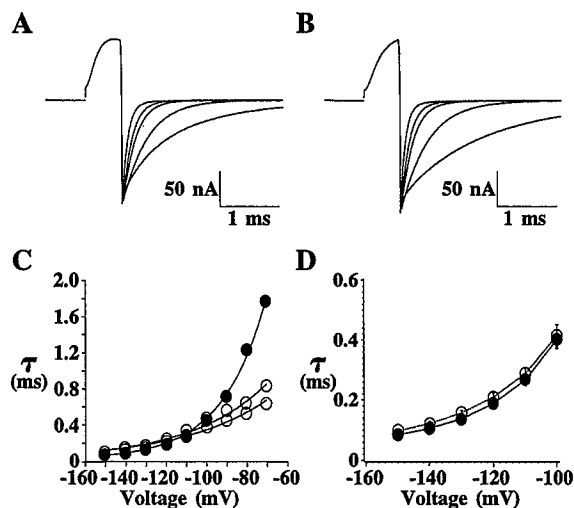
FIGURE 4. I_{Na} tail-current relaxations in control (A) and after modification by AP-A toxin (B). I_{Na} tail responses were recorded at test potentials between -150 and -70 mV after a conditioning step to $+40$ mV. (C). Plots of the dominant I_{Na} tail-current relaxation τ's in control (O), during exposure to AP-A toxin (●), and after washout of toxin (O). Cell E8.04: C_m 92 pF, R_L 38 MΩ, 12°C, $[Na^+]_o/[Na^+]_i$ (millimolar) 15: 15. (D) Means and SEM of the time constants of fits to I_{Na} tail-current relaxations for six cells in control (O) and after modification by AP-A toxin (●) for test potentials ≤ -100 mV. Data in control and after washout of toxin were meaned and fit as described in text. The dominant τ is plotted for both control (O) and after AP-A toxin (●).

course of the development of Na channel inactivation over a range of negative potentials where few, if any, channels open. From a holding potential of -150 mV Na channels were inactivated by stepping to conditioning potentials (V_c) between -120 and -70 mV for variable durations, and then Na channel availability was assessed in a test step to 0 mV after a short voltage step to -120 mV (or -110 mV) for 2 ms. Na channel inactivation was assayed from the ratio of peak I_{Na} in a step to 0 mV relative to that in the absence of a conditioning pulse. Fig. 5 shows data for a typical cell for three potentials (-80, -100, and -120 mV). Similar data were obtained from three cells. Both for control and Ap-A toxin modified I_{Na}, development of inactivation occurred with a complex time course, which was better described by two exponentials than by one. Over this voltage range Ap-A toxin did not slow the development of inactivation. In fact, at the most negative potentials inactivation developed slightly more rapidly in the presence of Ap-A, while at potentials near I_{Na} threshold development of inactivation was almost identical to control. These data indicate that Ap-A toxin does not delay Na channel inactivation from closed states and suggest that Ap-A toxin modifies Na channel kinetics by affecting inactivation from the open state.

Ap-A Toxin Dramatically Slowed Inactivation from the Open State

To investigate the effect of Ap-A toxin on the development of Na channel inactivation from the open state, we measured the time course of the development of inactivation at positive conditioning potentials. A similar two-pulse development of
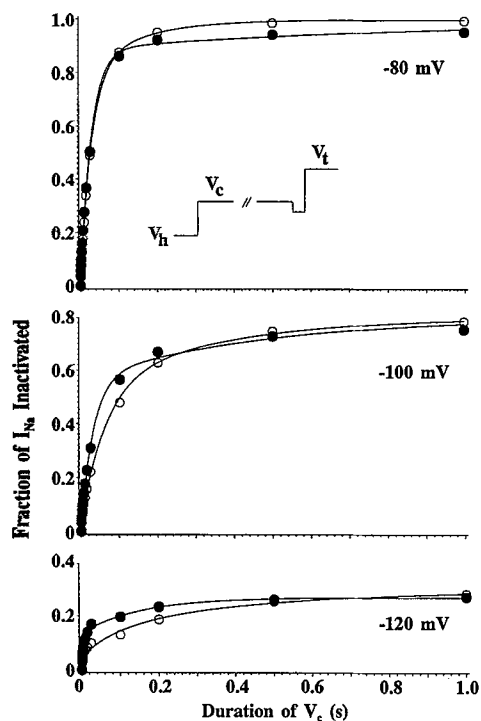
FIGURE 5. Two-pulse development of inactivation at -120, -100, and -80 mV in control (O) and after modification by Ap-A toxin (●). Voltage protocol is shown in the inset. Control data are the means of data obtained before and after wash of Ap-A toxin. Note that at the more negative potentials inactivation proceeded slightly more rapidly in the presence of toxin and was nearly identical at -80 mV. Cell M7.01, C_m 135 pF, R_L 65 MΩ.

inactivation protocol was used. Figs. 6, A and B, show the results for three cells at conditioning potentials of 60 and -20 mV in control and after modification by Ap-A toxin. At a conditioning potential of 60 mV inactivation developed rapidly and almost completely by 9 ms in control, but after modification by Ap-A toxin only 16 ± 3% of I_{Na} inactivated by 9 ms, which principally reflected residual inactivation of the channels expected to be unmodified by this concentration of Ap-A toxin in combination with a small number of channels that may have opened, closed, and then inactivated. As expected, inactivation under control conditions developed more slowly at the more negative conditioning potential of -20 mV. However, after Ap-A modification the fraction of channels that inactivated by 9 ms at -20 mV (24 ± 4%) was greater than the fraction that had inactivated at +60 mV. At a more negative conditioning potential of -40 mV inactivation of I_{Na} at 9 ms in Ap-A toxin was further increased to 28 ± 4%. Fig. 6 C illustrates the inverse voltage dependence of the fraction of Ap-A toxin modified channels that inactivated by 9 ms at conditioning potentials between -40 and $+100$ mV. The increase in inactivation of modified channels at more negative potentials suggests that as k_{OC} becomes larger at negative potentials a greater number of open channels return to a closed state and then inactivate.

Comparison of Steady State Voltage-dependent Availability before and after Ap-A Toxin

Because steady state voltage-dependent availability (h_∞ or steady state inactivation) is measured over a range of conditioning potentials where the probability of chan-
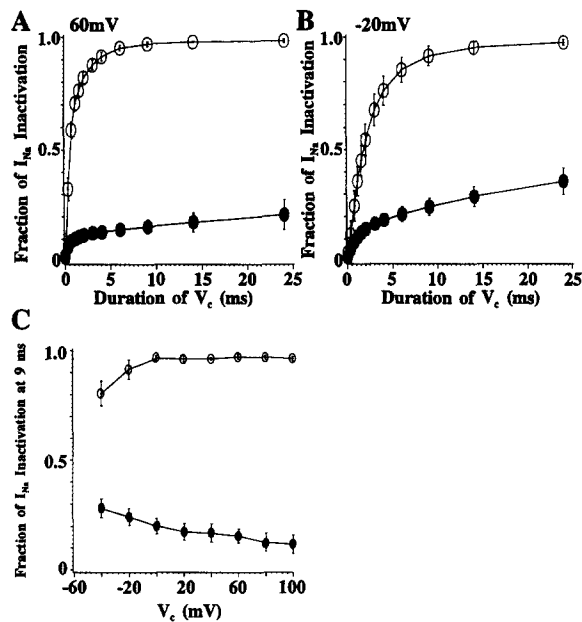
FIGURE 6. Two-pulse development of inactivation at more positive test potentials in control (O) and after modification by Ap-A toxin (●). Means and SD are plotted for three cells. The voltage protocol is similar to that shown in Fig. 5. Before stepping to the conditioning potential, there was a brief voltage step to -40 mV for 0.2 ms. Peak I_{Na} measurements were normalized to the peak I_{Na} measured in the absence of a conditioning step. (A) At a conditioning potential of 60 mV, $95 \pm 1\%$ of the peak I_{Na} had inactivated in control at 9 ms whereas only $16 \pm 3\%$ had inactivated after Ap-A toxin. (B) At a conditioning potential of -20 mV I_{Na} in control had inactivated by $92 \pm 4\%$ within 9 ms, but only by $24 \pm 4\%$ after Ap-A toxin. (C) Means $\pm$ SEM showing the fraction of I_{Na} inactivated by 9 ms as a function of potential for three cells in control (O) and in the presence of Ap-A toxin (●). Note that at positive potentials in control inactivation was virtually complete within 9 ms, while in the presence of Ap-A toxin, there was an inverse relationship of voltage to the fraction of I_{Na} that inactivated by 9 ms.

nel opening increases as the conditioning potential becomes more positive, the selective effect of Ap-A toxin on inactivation from the open state should produce a greater effect on availability at positive potentials. Steady state availability relationships were evaluated in steps to 0 mV after conditioning steps for 500 ms over a range of potentials. Fig. 7, A and B, shows current responses for a cell before and after Ap-A toxin, and Fig. 7 C plots the steady state voltage-dependent availability relationships for 11 cells. Channels could be fully voltage-dependently inactivated in the presence of toxin. As expected from the development data, there was virtually no effect of toxin on availability at the most negative potentials. The half-point of Na channel availability was shifted only slightly positive by 1.9 ± 0.8 mV. The slope factor became shallower (7.2 ± 0.8 mV to 9.9 ± 0.9 mV, $n = 8$) secondary to the relatively larger I_{Na} at more positive conditioning potentials where in control the contribution of Na channels inactivating from the open state would be expected to make the largest contribution to the relationship.

DISCUSSION

Modification of cardiac Na channels by Ap-A toxin resulted in dramatic slowing of I_{Na} decay consistent with modification of channel inactivation from the open state. The peak G_{Na}-V relationship after Ap-A toxin showed only a minor change of ~ -2-3 mV in its $V_{1/2}$ accompanied by slight steepening of the slope factor by
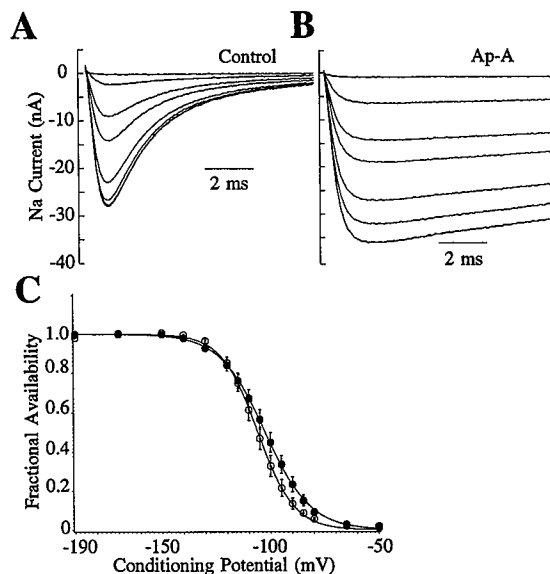
FIGURE 7. Na channel steady state voltage-dependent availability relationships. Cells were depolarized for 500 ms at each conditioning potential then depolarized to 0 mV and I_{Na} recorded. Peak I_{Na} was normalized to the peak I_{Na} in the absence of a conditioning step. Family of Na currents at 0 mV in control (A) and after Ap-A toxin (B). (C) Means and SEM for 11 cells in control (O) and after Ap-A toxin (●). Na_o 15 mM/Na_i 0 mM with either Cs^+ ($n = 3$) or TMA^+ ($n = 8$) as the cation substitute.

~1 mV compared to control. Although decay of I_{Na} was prolonged by Ap-A toxin there was little change in the channel activation or deactivation as measured by analysis of the time course of onset of I_{Na} and tail current relaxations. In addition, there was only a small change in the development of inactivation at negative potentials where Na channels do not open, suggesting that inactivation directly from closed states is not substantially affected by Ap-A toxin. Steady state voltage-dependent Na channel availability had a similar half-point but a more shallow slope factor after modification by Ap-A toxin. Presumably the low affinity of Ap-A toxin for inactivated Na channels (Meves, Rubly, and Watt, 1984; Strichartz and Wang, 1986) permitted recording of I_{Na} after washing out the toxin by membrane depolarization to 0 mV for 5 min.

Associated with the marked slowing of I_{Na} decay following modification G_{max} increased by 26% after modification by Ap-A toxin when the cell was bathed in symmetrical intracellular and extracellular concentrations of Na^+ and Cs^+. The small change in G_{max} after washout of Ap-A toxin suggested that "drop-out" or damage to Na channels was not a significant problem. The 26% increase in G_{max} are similar to values observed for cardiac I_{Na} modified by either Ap-A toxin (Wasserstrom, Kelly and Liberty, 1993) or α-chymotrypsin (Clarkson, 1990; Sheets and Hanck, 1993). The increase in conductance is expected to result from a modest increase in the number of channels open at peak I_{Na} because single Na channel conductance has been shown not to increase after modification by α-chymotrypsin (Vandenberg and Horn, 1984; Zilberter and Motin, 1991) or after modification by site 3 toxins (Nagy, 1988; El-Sherif, Fozzard, and Hanck, 1992). Interestingly, the presence of intracellular and extracellular TMA^+ resulted in a larger increase in G_{max} after Ap-A toxin modification. This may have occurred because intracellular TMA^+ blocks I_{Na} in a voltage-dependent manner (Oxford and Yeh, 1985; O'Leary and Horn, 1994), and

Ap-A toxin may relieve this block. Because Cs^+ is not thought to have such a blocking effect on I_{Na}, the increase in G_{max} of 26% measured with Cs^+ as a replacement cation with equimolar intracellular and extracellular Na^+ should more accurately represent the increase that can be attributed to the modification of Na channel kinetics by Ap-A toxin.

Comparison of Site 3 Toxin Effects on Na Channel Activation

Our analysis of the time course of the onset of I_{Na} in response to step depolarizations (Fig. 3) showed no significant change at test potentials ≥ -40 mV consistent with little or no effect of Ap-A toxin on channel activation. At potentials < -40 mV, the onset of I_{Na} was delayed consistent with Ap-A toxin modified Na channels returning to a closed state and then reopening instead of inactivating from the open state. In addition, the I_{Na} tail current relaxations at very negative potentials (≤ -100 mV) after Ap-A toxin also suggest that the toxin has little effect on channel closing rate. The lack of effect on channel activation/deactivation is similar to studies in other preparations, which uniformly have described the effect of site 3 toxins to remove or slow inactivation without affecting activation (for review see Catterall, 1988; Ulbricht, 1990). Specific evidence that activation is unaffected by site 3 toxins includes little or no effect on an activation (m) variable in Hodgkin-Huxley fits to I_{Na} in step depolarizations (Bergman, Dubois, Rojas, and Rathmayer, 1976; Mozhayeva et al., 1980; Neumcke, Schwarz, and Stampfli, 1985) or in noise analysis (Conti, Hille, Neumcke, Nonner, and Stampfli, 1976), and little or no change in slope factor or half-point of conductance (Mozhayeva et al., 1980; Warashina and Fujita, 1983; Wang and Strichartz, 1985). Although studies in mammalian neuroblastoma cells have shown that site 3 toxins produce a hyperpolarized shift in conductance of 6–10 mV (Gonoi et al., 1984; Gonoi and Hille, 1987), this was interpreted as consequence of removal of fast, voltage-independent inactivation rather than a change in activation. In single-channel studies, Kirsch, Skattebol, Possani, and Brown(1989) found the major component of latency to first channel opening was unaffected by α-scorpion toxin in N18 neuroblastoma cells. Similar data have been reported for cardiac channels (Schreibmayer, Kazerani, and Tritthart, 1987; El-Sherif et al., 1992).

Effects of Site Toxins on Cardiac Na Channel Inactivation

In other preparations investigators have suggested that site 3 toxins slowed rather than removed inactivation because I_{Na} decays albeit over a much longer time. In frog nerve persistent current has usually been observed at more positive potentials (Mozhayeva et al., 1980; Wang and Strichartz, 1985), which has been interpreted to favor the idea of two inactivated states, where the first becomes poorly absorbing in the presence of toxin (Wang and Strichartz, 1985). We never observed noninactivating currents in the presence of Ap-A; I_{Na} fully inactivated over a similar voltage range in control and after toxin modification (Fig. 7). Other investigators, who have studied the effects of site 3 toxins on mammalian neuronal Na channels have also reported full inactivation of the current. Similar to what we observed for car-

diac I_{Na} steady state voltage-dependent availability had a similar half-point and a reduced slope factor in control and after α-scorpion toxin was applied to N18 cells (Gonoi et al., 1984; Kirsch et al., 1989).

We have shown that Ap-A toxin does not slow channel inactivation directly from closed states but slightly accelerates inactivation at very negative potentials. No other data on effects of site 3 toxins on the development of inactivation are available, although in a patch clamp study of single cardiac Na channels that were modified by ATXII, another site 3 toxin, El-Sherif et al. (1992), concluded that $C{\rightarrow}I$ transitions were unaffected by toxin because they observed no change in the probability that channels would fail to open. Two reports include information about recovery from inactivation. Wang and Strichartz (1985) reported that recovery at −100 mV occurred at the same rate after exposure to *Leirus* toxin but that fractional recovery at any given time was greater because of removal of the delay (≈ 5 ms) in recovery. For heart I_{Na} Wasserstrom et al. (1993) reported a prolongation of a long time constant of recovery, although in those experiments some control cells also showed an increase in the contribution of a slow time constant of recovery over a similar time period of recording.

At more positive potentials, where the primary route to inactivation is expected to be from the open state, the development of inactivation was markedly slowed after modification by Ap-A toxin (Fig. 6), and a greater fraction of channels inactivated over the first 10 ms at −40 mV than at more positive potentials most likely because the magnitude of both k_{OC} and k_{CI} were large enough to allow channels to return to a closed state and then inactivate. This interpretation is supported by single-channel studies, where over the range Na channels activate, site 3 toxins produce an increase in the number of openings per sweep (Kirsch et al., 1989; El-Sherif et al., 1992).

At very positive potentials (i.e., >-20 mV), our data suggest that Ap-A toxin modified Na channels remained predominantly in the open state during the first 10 ms. El-Sherif et al. (1992) showed that after modification by the site 3 toxin ATXII mean channel open time increased monotonically as a function of potential, lengthening greater than fourfold at −20 mV. Long mean open times have also been demonstrated for Na channels modified by α-scorpion TsIV-5 toxin in neuroblastoma cells studied at 10°C (Kirsch et al., 1989), where mean open time was 5–6 ms at a test potential of −20 mV. Similar results for prolongation of mean channel open times were found in heart modified by α-scorpion TsIV-5 toxin (Kirsch et al., 1989) and in GH₃ cells modified by N-bromoacetamide (Horn et al., 1984). Because mean open times at any given potential are longer for cardiac Na channels than for neuronal Na channels (Kirsch and Brown, 1989), mean open times at very positive potentials of cardiac Na channels at 10–12.5°C would be expected to be >6 ms after modification by Ap-A.

Advantages of Ap-A Toxin for the Study of Inactivation

This toxin appears to have four important methodological advantages over other chemical or enzymatic agents that affect inactivation of cardiac Na channels: (*a*) Modification of Na channels by Ap-A toxin is both rapid and reversible. Slowing of the decay of I_{Na} was evident immediately upon exposure, and the modification

was complete within 2–3 min. Because modification by Ap-A toxin was rapid, spontaneous background shifts in kinetic indices were minimal, which is not the case for proteolytic enzymes where modification typically requires 20 min (Gonoi and Hille, 1987; Cota and Armstrong, 1989; Clarkson, 1990; Sheets and Hanck, 1993). The effects of the toxin were reversed by depolarization of the membrane potential to -10 mV and washing in control solution for 5 min. (b) Unlike enzymes such as α-chymotrypsin (Clarkson, 1990; Sheets and Hanck, 1993), Ap-A toxin did not augment or induce slow inactivation. (c) Modification occurred without significant loss of Na channels or damage to the experimental preparation as demonstrated by the return of G_{max} to values similar to those in control. In contrast, chemicals and enzymatic agents that modify inactivation may be difficult to apply to the experimental preparation without loss of Na channels (for review see Ulbricht, 1990; Hille, 1992). In such instances, it is difficult to assign observed changes solely to channel modification because of the possibility of damage to the preparation. (d) The predominate effect of Ap-A toxin was on the $O{\rightarrow}I$ transition. In addition, Ap-A toxin left intact $C{\rightarrow}I$ transitions.

Further investigation of site 3 toxins could contribute to our understanding of inactivation from the open state in cardiac Na channels. In the accompanying report we have used the toxin to investigate the voltage dependence of this transition (Sheets and Hanck, 1995).

REFERENCES

Armstrong, C. M., and F. Benzanilla. 1977. Inactivation of the sodium channel. II. Gating current experiments. *Journal of General Physiology.* 70:567–590.

Bergman, C., J. M. Dubois, E. Rojas, and W. Rathmayer. 1976. Decreased rate of sodium conductance inactivation in the node of Ranvier induced by a polypeptide toxin from sea anemone. *Biochimica et Biophysica Acta.* 455:173–184.

Cannon, S., and D. Corey. 1993. Loss of Na^+ channel inactivation by anemone toxin (ATX II) mimics the myotonic state in hyperkalaemic periodic paralysis. *Journal of Physiology.* 466:501–520.

Catterall, W. 1980. Neurotoxins that act on voltage-sensitive sodium channels in excitable membranes. *Annual Review of Pharmacology and Toxicology.* 20:15–43.

Catterall, W. A. 1988. Structure and function of voltage-sensitive ion channels. *Science.* 242:50–61.

Chahine, M., A. L. George Jr., M. Zhou, S. Ji, W. Sun, R. L. Barchi, and R. Horn. 1994. Sodium channel mutations in paramyotonia congenita uncouple inactivation from activation. *Neuron.* 12:281–294.

Clarkson, C. 1990. Modification of Na channel inactivation by alpha-chymotrypsin in single cardiac myocytes. *Pflügers Archiv.* 417:48–57.

Conti, F., B. Hille, B. Neumcke, W. Nonner, and R. Stampfli. 1976. Conductance of the sodium chan-

nel in myelinated nerve fibres with modified sodium inactivation. *Journal of Physiology.* 262:729–742.

Cota, G., and C. M. Armstrong. 1989. Sodium channel gating in clonal pituitary cells. *Journal of General Physiology.* 94:213–232.

Dudley, S. C., Jr., and C. M. Baumgarten. 1993. Bursting of cardiac sodium channels after acute exposure to 3,5,3'-triiodo-L-thyronine. *Circulation Research.* 73:301–313.

El-Sherif, N., H. A. Fozzard, and D. A. Hanck. 1992. Dose-dependent modulation of the cardiac sodium channel by sea anemone toxin-ATXII. *Circulation Research.* 70:285–301.

Gonoi, T., and B. Hille. 1987. Gating of Na channels: inactivation modifiers discriminate among models. *Journal of General Physiology.* 89:253–274.

Gonoi, T., B. Hille, and W. A. Catterall. 1984. Voltage clamp analysis of sodium channels in normal and scorpion toxin-resistant neuroblastoma cells. *The Journal of Neuroscience.* 4:2836–2842.

Hanck, D. A., and M. F. Sheets. 1992*a*. Effects of extracellular divalent and trivalent cations on the kinetics of the sodium current in single canine cardiac Purkinje cells. *Journal of Physiology.* 454:267–298.

Hanck, D. A., and M. F. Sheets. 1992*b*. Time-dependent changes in the kinetics of the sodium current in single canine cardiac Purkinje cells. *American Journal of Physiology.* 262:H1197–H1207.

Hartmann, H. A., A. A. Tiedeman, S. F. Chen, A. M. Brown, and G. E. Kirsch. 1994. Effects of III-IV linker mutations on human heart Na^+ channel inactivation gating. *Circulation Research.* 75:114–122.

Hille, B. 1992. Ionic Channels of Excitable Membranes. Second edition. Sinauer Associates, Inc., Sunderland, MA. 607 pp.

Horn, R., C. A. Vandenberg, and K. Lange. 1984. Statistical analysis of single sodium channels: effects of *n*-bromoacetamide. *Biophysical Journal.* 45:323–335.

Kirsch, G. E., and A. M. Brown. 1989. Kinetic properties of single sodium channels in rat heart and rat brain. *Journal of General Physiology.* 93:85–99.

Kirsch, G. E., A. Skattebol, L. D. Possani, and A. M. Brown. 1989. Modification of Na channel gating by an alpha scorpion toxin from *Tityus serrulatus. Journal of General Physiology.* 93:67–83.

Makielski, J. C., M. F. Sheets, D. A. Hanck, C. T. January, and H. A. Fozzard. 1987. Sodium current in voltage clamped internally perfused canine cardiac Purkinje cells. *Biophysical Journal.* 52:1–11.

Meves, H., N. Rubly, and D. D. Watt. 1984. Voltage-dependent effect of a scorpion toxin on sodium current inactivation. *Pflügers Archiv.* 402:24–33.

Meves, H., J. M. Simard, and D. D. Watt. 1986. Interactions of scorpion toxins with the sodium channel. *Annals of the New York Academy of Sciences.* 479:113–132.

Moorman, J. R., G. E. Kirsch, A. M. Brown, and R. H. Joho. 1990. Changes in sodium channel gating produced by point mutations in a cytoplasmic linker. *Science.* 250:688–691.

Mozhayeva, G. N., A. P. Naumov, E. D. Nosyreva, and E. V. Grishin. 1980. Potential-dependent interaction of toxin from venom of the scorpion Buthus Eupeus with sodium channels in myelinated fibre. *Biochimica et Biophysica Acta.* 597:587–602.

Nagy, K. 1988. Mechanism of inactivation of single sodium channels after modification by chloramine-T, sea anemone toxin and scorpion toxin. *Journal of Membrane Biology.* 106:29–40.

Neumcke, B., W. Schwarz, and R. Stampfli. 1985. Comparison of the effects of Anemonia toxin II on sodium and gating currents in frog myelinated nerve. *Biochimica et Biophysica Acta.* 814:111–119.

Norton, R. 1991. Structure and structure-function relationships of sea anemone proteins that interact with the sodium channel. *Toxicon* 29:1051–1084.

O'Leary, M. E., and R. Horn. 1994. Internal block of human heart sodium channels by symmetrical tetra-alkylammoniums. *Journal of General Physiology.* 104:507–522.

Ono, K., H. A. Fozzard, and D. A. Hanck. 1995. A direct effect of forskolin sodium channel bursting. *Pflügers Archiv.* 429:561–569.

Oxford, G. S., and J. Z. Yeh. 1985. Interactions of monovalent cations with sodium channels in squid

axon. I. Modification of physiological inactivation gating. *Journal of General Physiology.* 85:583–602.

Patton, D. E., J. W. West, W. A. Catterall, and A. L. Goldin. 1992. Amino acid residues required for fast Na^+-channel inactivation: charge neutralizations and deletions in the III-IV linker. *Proceedings of the National Academy of Sciences, USA.* 89:10905–10909.

Provencher, S. W. 1976. A Fourier method for the analysis of exponential decay curves. *Biophysical Journal.* 16:27–41.

Rochat, H., P. Bernard, and F. Couraud. 1979. Scorpion toxins: chemistry and mode of action. *Advances in Cytopharmacology.* 3:325–333.

Salgado, V. L., and W. R. Kem. 1992. Actions of three structurally distinct sea anemone toxins on crustacean and insect sodium channels. *Toxicon.* 30:1365–1381.

Schreibmayer, W., H. Kazerani, and H. A. Tritthart. 1987. A mechanistic interpretation of the action of toxin II from *Anemonia sulcata* on the cardiac sodium channel. *Biochimica et Biophysica Acta.* 901: 273–282.

Sheets, M. F., and D. A. Hanck. 1992. Mechanisms of extracellular divalent and trivalent block of the sodium current in canine cardiac Purkinje cells. *Journal of Physiology.* 454:299–320.

Sheets, M. F., and D. A. Hanck. 1993. Modification of sodium channel inactivation by alpha-chymotrypsin in canine cardiac Purkinje cells. *Journal of Cardiovascular Electrophysiology.* 4:686–694.

Sheets, M. F., and D. A. Hanck. 1995. Voltage-dependent open-state inactivation of cardiac sodium channels: gating currents studies with Anthopleurin-A toxin. *Journal of General Physiology.* 106:617–640.

Sheets, M. F., C. T. January, and H. A. Fozzard. 1983. Isolation and characterization of single canine cardiac Purkinje cells. *Circulation Research.* 53:544–548.

Strichartz, G., and G. K. Wang. 1986. Rapid voltage-dependent dissociation of scorpion alpha-toxins coupled to Na channel inactivation in amphibian myelinated nerves. *Journal of General Physiology.* 88:413–435.

Stühmer, W., F. Conti, H. Suzuki, X. Wang, M. Noda, N. Yahagi, H. Kubo, and S. Numa. 1989. Structural parts involved in activation and inactivation of the sodium channel. *Nature.* 339:597–603.

Ulbricht, W. 1990. The inactivation of sodium channels in the node of Ranvier and its chemical modification. *In* Ion Channels. Vol. 2. T. Narahashi, editor. Plenum Publishing Corp., NY. 123–159.

Vandenberg, C. A., and R. Horn. 1984. Inactivation viewed through single sodium channels. *Journal of General Physiology.* 84:535–564.

Vasselev, P. M., T. Schueuer, and W. A. Catterall. 1988. Identification of an intracellular peptide segment involved in sodium channel inactivation. *Science.* 241:1658–1661.

Wang, G. K., and G. Strichartz. 1985. Kinetic analysis of the action of *Leiurus* alpha-toxin ionic currents in myelinated nerve. *Journal of General Physiology.* 86:739–762.

Warashina, A., and S. Fujita. 1983. Effects of sea anemone toxins on the sodium inactivation process in crayfish axons. *Journal of General Physiology.* 81:305–323.

Warashina, A., Z.-Y. Jiang, and T. Ogura. 1988. Potential-dependent action of *Anemonia sulcata* toxins III and IV on sodium channels in crayfish giant axons. *Pflügers Archiv.* 411:88–93.

Wasserstrom, J. A., J. E. Kelly, and K. N. Liberty. 1993. Modification of cardiac Na^+ channels by anthopleurin-A: effects on gating and kinetics. *Pflügers Archiv.* 424:15–24.

West, J. W., D. E. Patton, T. Scheuer, Y. Wang, A. L. Goldin, and W. A. Catterall. 1992. A cluster of hydrophobic amino acid residues required for fast Na^+-channel inactivation. *Proceedings of the National Academy of Sciences, USA.* 89:10910–10914.

Zilberter, Y. I., and L. G. Motin. 1991. Existence of two fast inactivation states in cardiac Na channels confirmed by two-stage action of proteolytic enzymes. *Biochimica et Biophysica Acta.* 1068:77–80.