Inactivation of Cloned Na Channels Expressed in *Xenopus* Oocytes

DOUGLAS S. KRAFTE, ALAN L. GOLDIN, VANESSA J. AULD, ROBERT J. DUNN, NORMAN DAVIDSON, and HENRY A. LESTER

From the Division of Biology, California Institute of Technology, Pasadena, California 91125; Department of Microbiology and Molecular Genetics, University of California at Irvine, Irvine, California 92717; and Department of Medical Genetics, University of Toronto, Toronto, Ontario M5S 1A8, Canada

ABSTRACT This study investigates the inactivation properties of Na channels expressed in *Xenopus* oocytes from two rat IIA Na channel cDNA clones differing by a single amino acid residue. Although the two cDNAs encode Na channels with substantially different activation properties (Auld, V. J., A. L. Goldin, D. S. Krafte, J. Marshall, J. M. Dunn, W. A. Catterall, H. A. Lester, N. Davidson, and R. J. Dunn. 1988. *Neuron.* 1:449–461), their inactivation properties resemble each other strongly but differ markedly from channels induced by poly(A+) rat brain RNA. Rat IIA currents inactivate more slowly, recover from inactivation more slowly, and display a steady-state voltage dependence that is shifted to more positive potentials. The macroscopic inactivation process for poly(A+) Na channels is defined by a single exponential time course; that for rat IIA channels displays two exponential components. At the single-channel level these differences in inactivation occur because rat IIA channels reopen several times during a depolarizing pulse; poly(A+) channels do not. Repetitive stimulation (>1 Hz) produces a marked decrement in the rat IIA peak current and changes the waveform of the currents. When low molecular weight RNA is coinjected with rat IIA RNA, these inactivation properties are restored to those that characterize poly(A+) channels. Slow inactivation is similar for rat IIA and poly(A+) channels, however. The data suggest that activation and inactivation involve at least partially distinct regions of the channel protein.

INTRODUCTION

Voltage-dependent Na channels are found in a wide variety of excitable cell types and play a major role in the function of neurons, skeletal muscle, and cardiac tissue. In these cells, encoding of spike train signals depends partially on the gating kinetics of these channels. According to present concepts, the Na channel protein can

Address reprint requests to Dr. Henry A. Lester, Division of Biology 156–29, California Institute of Technology, Pasadena, CA 91125.

Dr. Krafte's current address is Sterling Research Group, 81 Columbia Turnpike, Rensselaer, NY 12114. Dr. Dunn's current address is Department of Neurology, Montreal General Hospital, McGill University, 1650 Cedar Ave., Montreal, Quebec H3G 1A4, Canada.

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occupy three groups of functional states: resting or closed, open or activated, and inactivated. This paper is concerned primarily with the transitions that lead (*a*) to inactivated states, either from the open or the resting states, and (*b*) from the inactivated to the resting states. These transitions are favored, respectively, by depolarized and hyperpolarized membrane potentials.

Recent advances in the application of molecular biological techniques have given the latest insights into the nature of these membrane proteins. Primary sequence data have been reported for four distinct Na channels from rat brain, two from *Drosophila,* and one each from *Electrophorus electricus* electroplaques, rat skeletal muscle, and rat heart (Noda et al., 1984; Noda et al., 1986*a*; Salkoff et al., 1987; Auld et al., 1988; Kayano et al., 1988; Loughney et al., 1989; Ramaswami and Tanouye, 1989; Rogart et al., 1989; Trimmer et al., 1989). In the cases where full-length cDNAs have been obtained, in vitro transcripts usually give rise to functional Na channels when injected into *Xenopus* oocytes (Noda et al., 1986*b*; Auld et al., 1988; Suzuki et al., 1988; Trimmer et al., 1989).

We previously reported comparative studies of inactivation for Na channels induced by injection of (*a*) a high molecular weight (MW) RNA or (*b*) unfractionated poly(A+) RNA from rat brain in *Xenopus* oocytes (Krafte et al., 1988*b*). There were marked differences in the properties of the Na channels induced by the two types of RNA. The high MW fraction induced channels that inactivated more slowly and at more positive potentials. Quantitative studies were limited by the small and variable signals obtained with fractionated tissue-derived mRNA.

We recently reported the sequence and functional expression of a cDNA clone, termed VA200, from rat brain RNA; it encodes a Na channel α subunit that we term rat IIA. The amino acid sequence of VA200 differs at seven positions from the cDNAs termed rat II (Noda et al., 1986*a*; Auld et al., 1988, 1990). At least two of these differences between the VA200 and rat II sequences have been confirmed by polymerase chain reaction amplification and RNase protection (Auld et al., 1990). However, phe at residue 860 is not found in naturally occurring IIA channels and was introduced by a cloning artifact; the actual residue encoded at this position is leu (Auld et al., 1990). It is not known whether the II and IIA proteins arise from different genes, from alternative RNA processing of a single gene, or from some other mechanism.

First results from the injection of in vitro transcripts of VA200 cDNA into oocytes showed that VA200 channels differ from those induced by poly(A+) RNA in two respects: (*a*) the VA200 channels display a current–voltage relation that is shifted ~20 mV more positive than the poly(A+) channel; and (*b*) like the high MW channels, the VA200 channels display slower inactivation. Difference (*a*) arises from one and only one of the seven differences in primary amino acid sequence between VA200 and rat II channels; the directed mutant M860 cDNA, which changes the artifactual phe to the naturally occurring leu, eliminates the difference (Auld et al., 1990). On the other hand, difference (*b*) can be eliminated by coinjection of low MW RNA and therefore presumably involves the action of an additional protein (Auld et al., 1988; Krafte et al., 1988*b*).

The present study continues to examine the second difference. We exploited (*a*) the large signals obtained by injection of in vitro synthesized RNA and (*b*) the

increased temporal resolution of the two-electrode voltage clamp applied to smaller (stage II–III) oocytes (Krafte and Lester, 1989). We compare the properties of rat IIA– and rat poly(A+)–induced channels with regard to inactivation and single-channel gating. Our results show that Na channels arising from in vitro transcripts of either rat IIA cDNA differ from those arising from poly(A+) RNA in several characteristics of inactivation: onset rate, recovery rate, and voltage dependence. At the single-channel level, rat IIA–induced channels display more reopenings than poly(A+) RNA–induced channels. Our results with the rat IIA clones, which have been presented in abstract form (Goldin et al., 1988; Krafte et al., 1988*a*), thus confirm and extend data obtained with high MW fractions of rat brain mRNA (Krafte et al., 1988*b*).

METHODS

This study used two cDNA clones. The VA200 (Auld et al., 1988) and M860 (Auld et al., 1990) cDNAS differ in their coding region by a single nucleotide at position 2580. RNA transcripts for the VA200 cDNA were synthesized from the plasmid pVA200 as described by Auld et al. (1988) or from another plasmid, pVA200R, which contained the VA200 cDNA in the reverse orientation and used T7 RNA as opposed to SP6 RNA polymerase (both from New England Biolabs, Beverly, MA) to obtain sense transcripts. There were no differences between Na currents from these two plasmids. The M860 plasmid was linearized by digestion with Cla I and transcribed in vitro with T7 polymerase. Transcripts from the pVA200 clone were studied both in stage II–III and stage V–VI oocytes, but the M860 cDNA became available only after the experiments with stage II–III oocytes were completed and was therefore studied only with stage V–VI oocytes.

RNA was extracted from 10–14-d postnatal rats and fractionated on sucrose density gradients to obtain low molecular weight RNA as previously described (Goldin et al., 1986; Krafte et al., 1988*b*). Preparation and injection of stage V–VI oocytes was according to standard procedures (Krafte et al., 1988*b*). Where stage II–III oocytes were used, injections were done with the aid of a Nanopump (World Precision Instruments, Inc., New Haven, CT; Krafte and Lester, 1989). Oocytes were incubated for 2–4 d at 22°C in P/S solution (see Krafte et al., 1988*b*) or in a similar solution with 50 μg/ml gentamicin replacing the penicillin and streptomycin.

Two-microelectrode and patch-clamp recordings were done as described by Krafte et al. (1988*b*). The bath solution in all cases was (in mM): 96 NaCl, 2.0 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, and 5.0 HEPES, pH 7.5. Recording was done at 20–22°C.

Both macroscopic and single-channel data were analyzed using the pCLAMP software series (Axon Instruments, Inc., Foster City, CA). In general, analysis was as described by Krafte et al. (1988*b*). Time constants for inactivation were determined by fitting a single or double exponential plus a constant value to the decaying phase of the Na current records. For studies of steady-state inactivation, a 12-ms test pulse was given to the voltage eliciting peak currents, after a prepulse to various potentials. Current during the test pulse was normalized to the largest response and plotted vs. prepulse potential. A two-state Boltzmann distribution,

$$I = [1 + \exp[(V_m - V_{1/2})/k]]^{-1},$$

was then fit to the data to determine $V_{1/2}$, the half maximal voltage, and k, the slope factor, where V_m is prepulse potential. To assay recovery from inactivation, twin test pulses were given to voltages eliciting peak currents. The interval between the pulses was varied and current amplitude during the second pulse was plotted as a fraction of that during the first.

RESULTS

VA200- vs. Poly(A+) RNA-induced Currents

Fig. 1 compares recordings of Na currents of stage III oocytes injected with poly(A+) rat brain RNA (*A*) or rat IIA RNA from the VA200 clone (*B*). It is clear that the VA200 currents display a longer waveform, denoting slower macroscopic inactivation. This phenomenon is studied in this paper. Because of the improved settling time of the voltage clamp in the stage III oocytes (Krafte and Lester, 1989), a more complete analysis of the inactivation kinetics was possible than with the previously used stage V–VI oocytes.

Voltage Dependence of Inactivation

For experiments with VA200 currents, the waveforms of decay phases were described as the sum of two exponential components. Fig. 2 shows these time constants (τ_{fast}, τ_{slow}) of macroscopic inactivation. The currents arising from poly(A+) could be

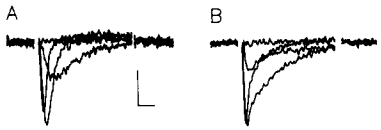

FIGURE 1. Sodium currents elicited by voltage steps in rat brain poly(A+) (*A*) and VA200 (*B*) RNA-injected stage III oocytes. Membrane potential is held at −100 mV and stepped to test potentials in 20-mV increments between −50 mV and +10 mV (*A*) or between −30 mV and + 30 mV (*B*). In *A*, the most negative potential that elicits Na currents is −40; in *B*, −20 mV. This difference reflects the shift in activation parameters between VA200 channels and poly(A+) channels, resulting from the phenylalanine for leucine substitution at residue 860 (see text). Records shown were obtained by subtracting currents in the presence of 500 nM TTX from those in the absence of TTX. Calibration bars: 2 ms, 100 nA.

fit to a single exponential. A slower component, when present, constituted <1% of the total current. Fig. 2 illustrates that the τ_{fast} vs. voltage curve for VA200 currents is shifted in the depolarizing direction relative to the single component for poly(A+). The second, slower exponential in rat IIA currents (note the differences in scale for τ_{fast} and τ_{slow}) contributes a significant fraction of the total current at potentials more positive than −20 mV. The presence of the slow component rendered it possible to record outward currents at potentials more positive than E_{Na} in VA200-injected oocytes, but not in those injected with poly(A+) RNA (data not shown).

Steady-state Inactivation

The voltage dependence of steady-state inactivation was assessed using the prepulse protocol (see Methods) and the results are plotted in Fig. 3. Several points arise from this analysis: (*a*) With 50-ms prepulses, inactivation occurs at consistently more positive potentials for rat IIA currents than for poly(A+) currents. (*b*) As the prepulse duration is increased, the steady-state inactivation is shifted in the hyperpolarizing direction. This effect is much more prominent for rat IIA currents than for

poly(A+) currents. (*c*) When 2-s prepulses are used, the difference between the points of half-maximal inactivation for rat IIA vs. poly(A+) is greatly reduced.

Effects of Coinjecting Low Molecular Weight RNA on Rat IIA Na Channel Inactivation

Auld et al. (1988) have shown that coinjection of low MW RNA restores normal inactivation kinetics to VA200 currents. Coinjections also restore the steady-state voltage dependence of inactivation as shown in Fig. 4 (see figure legend for $V_{1/2}$ values). One clear result was that in these same oocytes the voltage dependence of *activation*, as estimated by the voltage giving peak currents, did not differ between VA200 and VA200 + low MW RNA currents. These results imply some uncoupling between the activation and inactivation processes. A possible mechanism for this phenomenon is described in the Discussion.

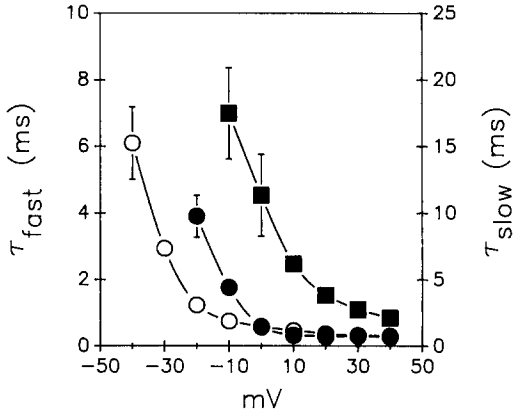

FIGURE 2. Time constant (τ) of inactivation vs. test potential for poly(A+)- and VA200 RNA–induced currents. Time constants were determined by fitting either a single or double exponential to the decay phase of the Na current. The open circles represent values obtained from poly(A+) RNA induced currents; a single exponential was sufficient to describe inactivation. The solid circles and squares are the result of fitting a double exponential plus a steady-state value to the VA200 Na current inactivation: $I = A_{fast} \exp(-t/\tau_{fast}) + A_{slow} \exp(-t/\tau_{slow}) + A_{\infty}$. Note the different time scales for τ_{fast} and τ_{slow}. The smooth lines are spline interpolations of the data. Data are averaged from 8–10 oocytes. For $t = 0$ at the start of the depolarizing pulse, the average ratio A_{slow}/A_{fast} was 0.18 at +10 mV. For $t = 0$ at the peak of the pulse, the average ratio was near unity.

Recovery from Inactivation

Na currents arising from rat IIA RNA also displayed longer times for recovery from inactivation than did those from poly(A+) RNA. At −100 mV, poly(A+) Na currents recovered completely in <100 ms (data not shown). Rat IIA currents, however, displayed only 50% recovery after 100 ms; complete recovery seemed to occur with an additional, much slower component (Fig. 5) Thus, complete recovery required >5 s at voltages equal to or more positive than −100 mV.

In another protocol used to examine recovery from inactivation, oocytes were subjected to a train of depolarizing pulses at varying frequencies. We studied the relative current amplitude vs. pulse number in the train (Fig. 6 *A*). For oocytes injected with poly(A+) RNA and studied at a pulse frequency of 10 Hz, there is no attenuation. For rat IIA currents, however, there was 40–50% attenuation of current

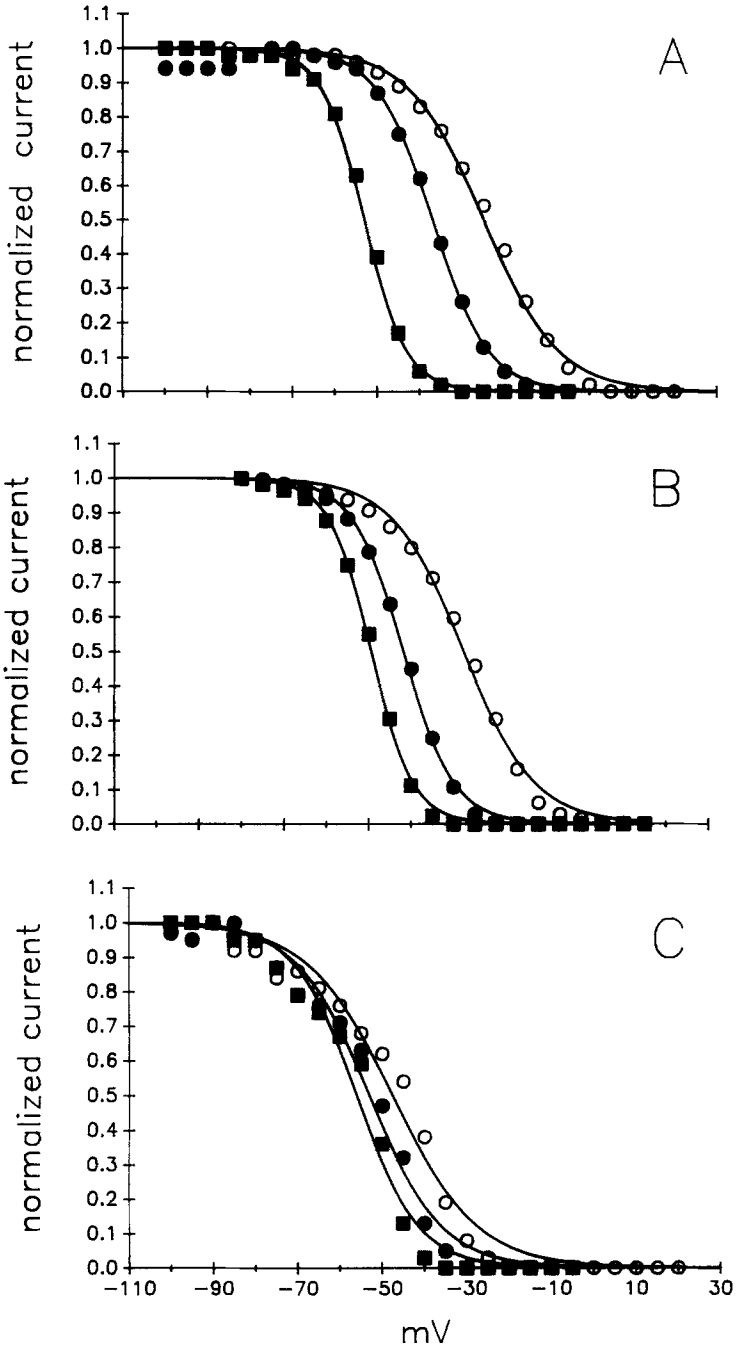

FIGURE 3. Steady-state inactivation of Na currents in oocytes injected with VA200 (*A*), M860 (*B*) or poly(A+) mRNA (*C*). Normalized peak currents are plotted vs. prepulse potential; holding potential was −100 mV. The prepulse durations were 50 ms (○), 500 ms (●), and 2 s (■). For *A*, $V_{1/2}$ and *k* (mV) were −25 and 8.8, −37 and 6.6, and −53 and 5 with increasing prepulse duration, respectively; for *B*, −27.6 and 8.8, −41.8 and 6.0, and −49.4 and 5.0; for *C*, −47.5 and 10.1, −53 and 8.6, and −56 and 7.4.

amplitude during a 10-Hz train of pulses. Even at 1 Hz there was a slight reduction in amplitude.

The waveforms of the rat IIA currents also changed during such a train. In Fig. 6 *C*, panels 1–3 show VA200 currents from the first and twentieth pulses in the train at various frequencies. The traces have been scaled so that the peak amplitudes are identical in all cases. The twentieth pulse in the train shows a faster macroscopic inactivation than the first pulse when the stimulation frequency is ≥ 1 Hz.

Single-channel Recordings

Fig. 7 shows single Na channel records from VA200- (*A–C*) and poly(A+) RNA–injected oocytes (*D–F*) at the same set of membrane potentials. Poly(A+)-induced channels show reopenings during the voltage step at −30 mV, but reopen much less frequently at −20 and −10 mV. VA200 channels, on the other hand, still tend to reopen quite frequently at −10 mV as well as at −30 mV. The number of openings per sweep was 2.5, 3.1, and 5.6 at −30, −20, and −10 mV, respectively, for VA200 channels. In comparison, poly(A+) channels opened an average of 1.8 times per

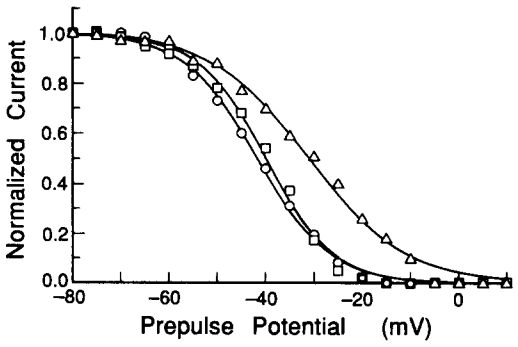

FIGURE 4. Coinjecting low MW RNA reconstitutes normal inactivation. Normalized current during a test pulse to +10 mV is plotted vs. prepulse potential. $V_{1/2}$ values are −42, −30.5, and −40 mV for poly(A+) (□), VA200 (△), and VA200 + low MW RNA (○), respectively.

sweep at −20 mV. In a patch where the Na gradient was reversed so that single-channel currents were outward, reopenings still occurred at +40 mV for VA200 RNA–induced channels (data not shown).

In addition to the differences in reopening frequency, the mean open time was shorter for VA200 compared with poly(A+) channels. Fig. 8 shows representative open-time histograms for VA200 (*A*) and poly(A+) (*B*) events at −20 mV. *C* gives the open time vs. test potential for poly(A+)-, VA200-, and high MW RNA–induced currents. The open times are essentially the same over this voltage range for poly(A+)- and high MW RNA–induced channels, while VA200-induced channels have shorter open times at any given potential.

Less extensive single-channel recordings from oocytes injected with M860 RNA indicate that, like VA200 channels, M860 channels reopen several times during a depolarizing pulse and display briefer open times than channels induced by poly(A+) RNA.

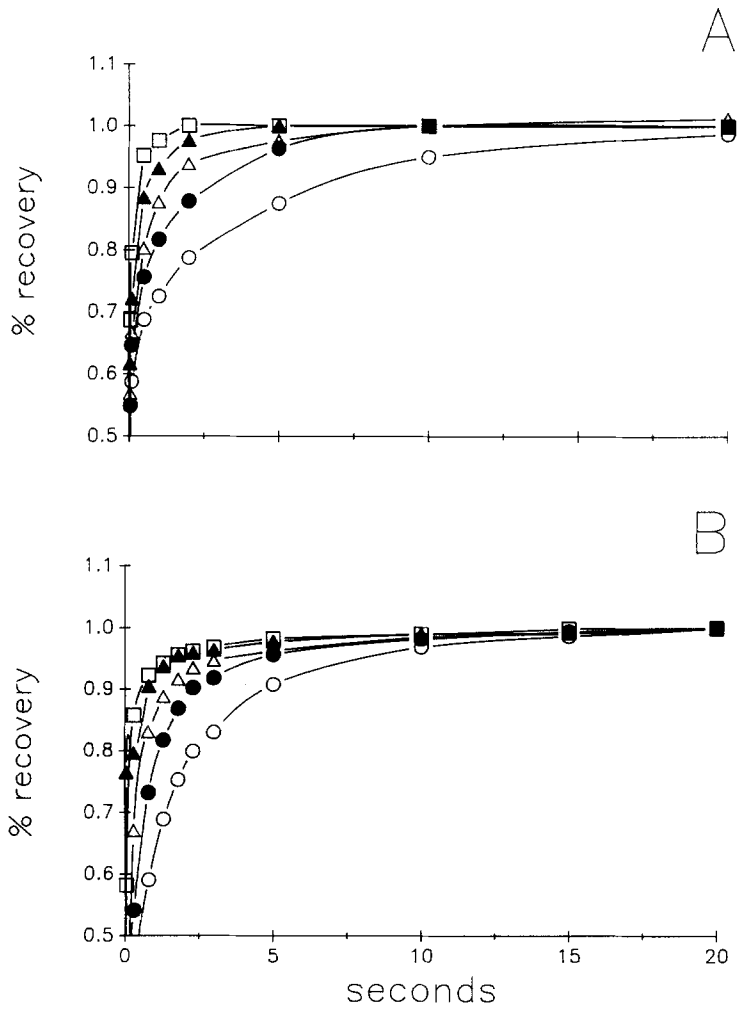

FIGURE 5. Recovery of rat IIA Na currents from inactivation. *A*, VA200 mRNA; *B*, M860 RNA. Recovery was assayed using a twin pulse protocol where the time between two 12-ms pulses was varied. The fraction of recovery of current during the second pulse is plotted vs. the interval between the pulses. Recovery was studied at five holding potentials: −80 mV (○), −90 mV (●), −100 mV (△), −110 mV (▲), and −120 mV (□).

DISCUSSION

There are two major differences among the Na channel currents induced in *Xenopus* oocytes by in vitro transcripts from the VA200 cDNA clone, from the M860 clone, and by unfractionated poly(A+) RNA from rat brain. First, the current–voltage relation for the VA200 channels peaks at a potential roughly 20 mV more positive than for M860 or poly(A+) channels; and second, inactivation is very similar for the VA200 and M860 channels but is slower than for the poly(A+) channels. The molecular basis for the first difference has been traced to a single base change

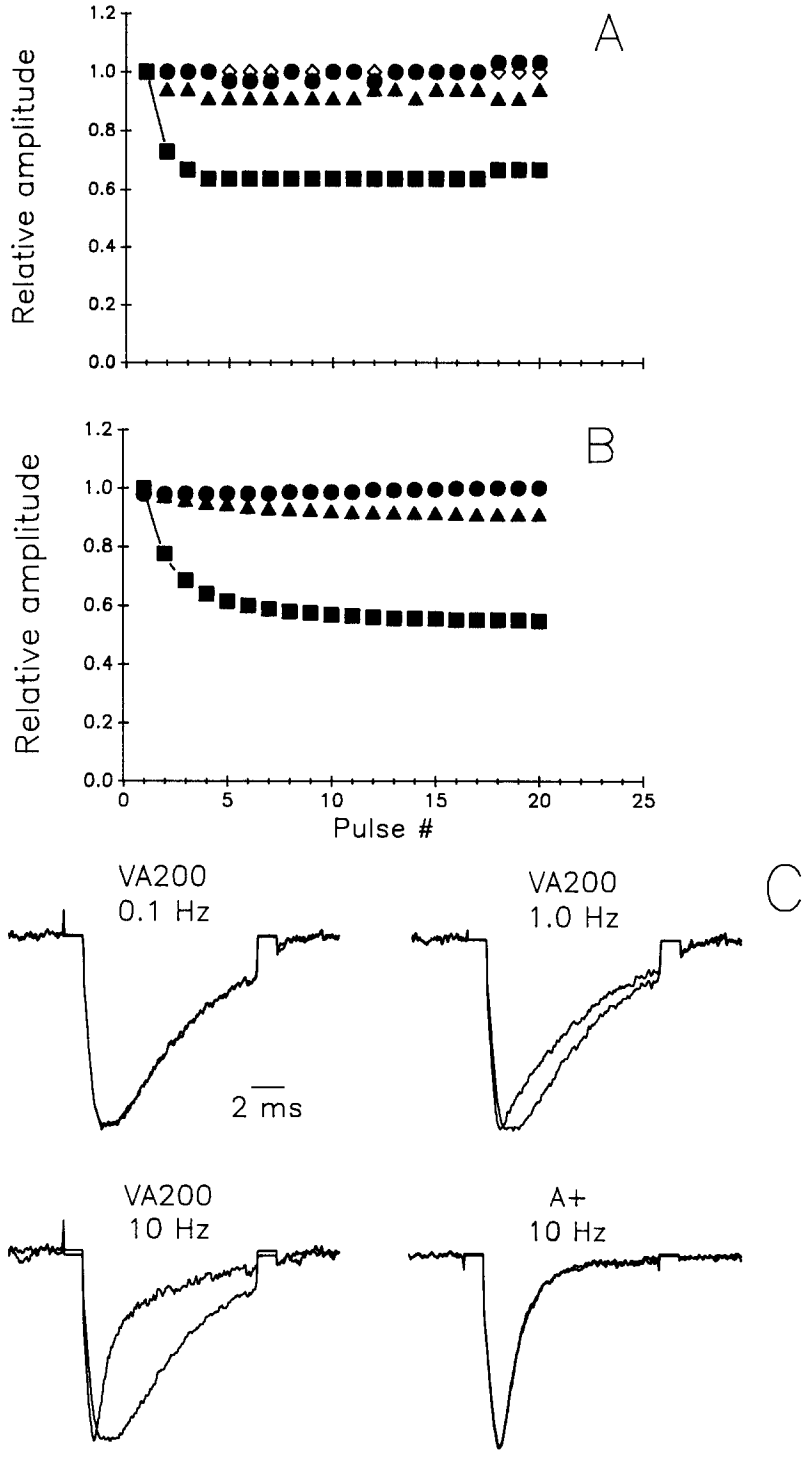

FIGURE 6. Effect of pulse frequency on current amplitude for rat IIA Na currents. A train of twenty 12-ms pulses to 0 mV was applied from a holding potential of −100 mV. Normalized amplitude relative to the first pulse in the train is plotted vs. pulse number. *A*, VA200; *B*, M860. The solid symbols show data for RNA from the cDNA clones: 0.1 Hz (●), 1 Hz (▲), and 10 Hz (■). The open symbols show data for poly(A+) RNA at 10 Hz. *C*, Current traces for the first and twentieth pulses in the train were scaled so that amplitudes were the same for VA200 at 0.1, 1.0, and 10 Hz as well as for poly(A+) currents at 10 Hz. Horizontal bar represents 2 ms. The rather broad peak of the control currents may be due to poor spatial control in these stage VI oocytes.

resulting from a cloning artifact, as reported elsewhere (Auld et al., 1990); this article analyzes the second of these two differences, which has a different and less certain origin.

The data consist of measurements on the inactivation and single-channel properties of Na channels induced by either of the two rat IIA in vitro transcripts or by tissue-derived RNA (the single-channel data are preliminary for the M860 clone). We find differences between rat IIA and poly(A+) channels in the rate and voltage dependence of inactivation, in the rate of recovery from inactivation, and in the response to repetitive stimulation. The properties of rat IIA channels can be transformed to those of poly(A+) channels by coinjection of low MW RNA. These results imply that, in addition to the high MW RNA encoding the α subunit of the

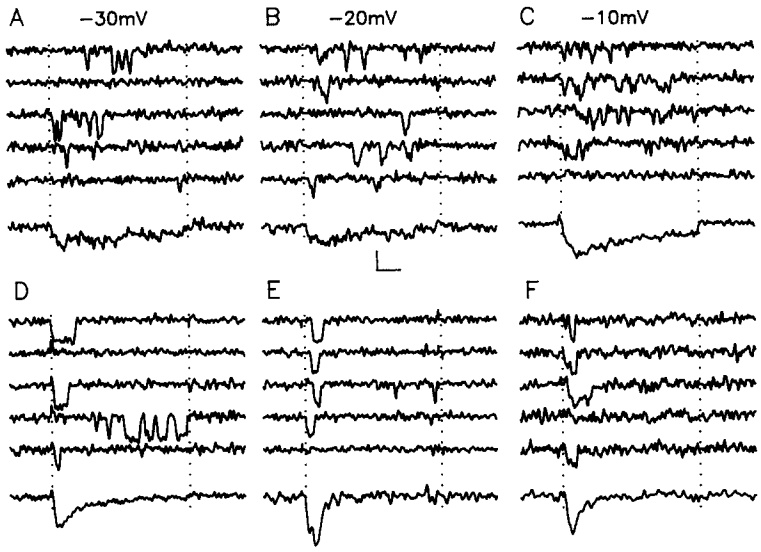

FIGURE 7. Single-channel records from outside-out patches for VA200 (*A–C*) and poly(A+) (*D–E*) at the voltages noted at the top of each column. Holding potential was −100 mV in all cases. The last trace in each group is an average of many sweeps. The dotted lines indicate the beginning and end of the voltage step. Calibration bars: 2 ms, 1 pA.

channel complex, one or more low MW mRNAs must be translated in order to confer normal inactivation properties on rat brain Na channels expressed in *Xenopus* oocytes. This protein might interact directly, for instance if it were one of the low MW β subunits, or indirectly, for instance if it were a processing protein. The choice between these two possibilities is at present unclear although we favor the latter explanation for reasons discussed below.

Specific Differences Between the Inactivation Properties of Rat IIA– and Poly(A+) RNA–induced Channels

We have previously noted that VA200 and M860 Na currents inactivated more slowly than poly(A+) RNA–induced currents (Auld et al., 1988, 1990). Due to the

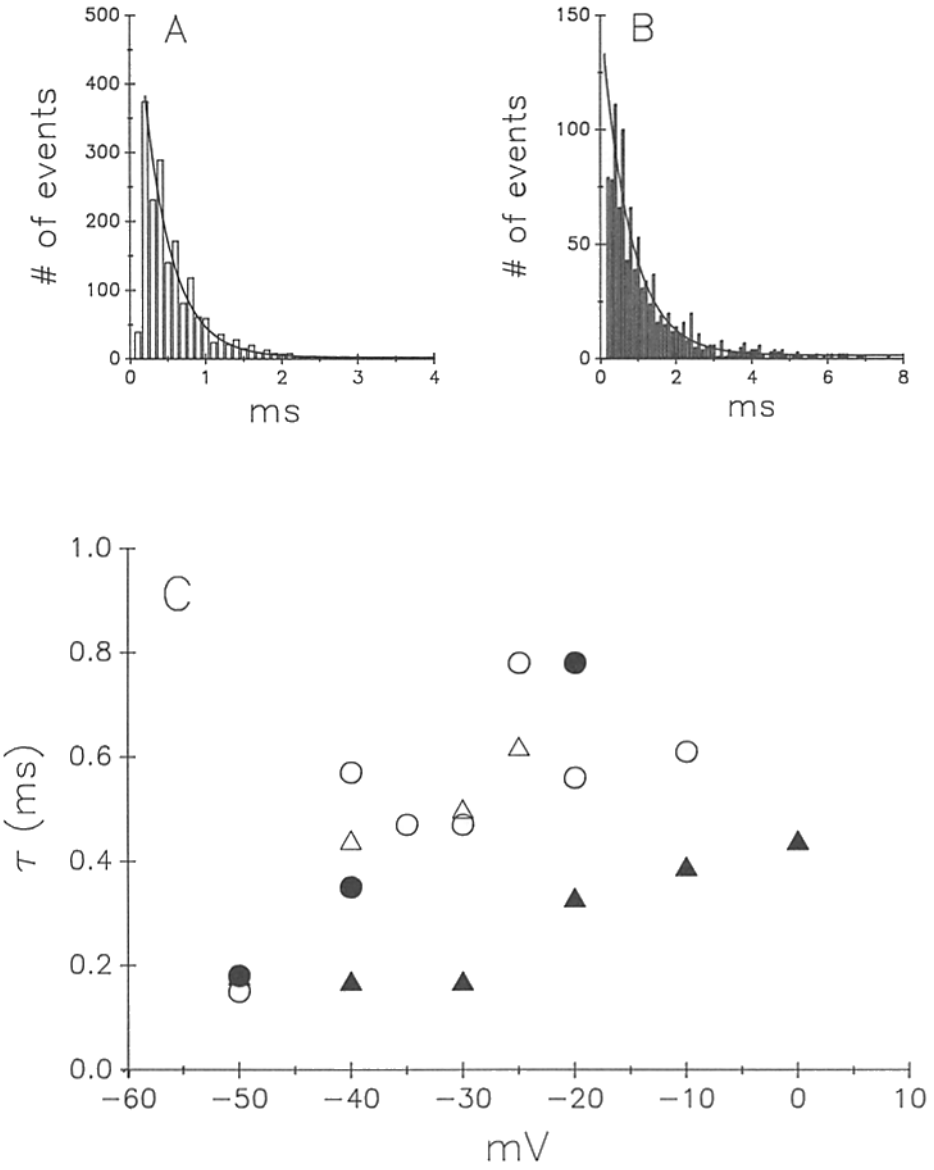

FIGURE 8. Mean single-channel open time vs. test potential for VA200 and poly(A+) RNA. *A* and *B* illustrate open time histograms for VA200 and poly(A+) events at −20 mV, respectively. Mean open time was determined by fitting a single exponential to these histograms at each voltage. The values obtained by these fits are plotted vs. test potential in *C*. The symbols represent poly(A+) RNA (this study [●]; from Krafte et al., 1988*b* [○]), VA200 (▲), and high MW RNA (△) from Krafte et al. (1988) for comparison. All data are from outside-out patches.

slow settling time of the voltage clamp in the large stage V–VI oocytes, however, Auld et al. (1988, 1990) did not present a detailed analysis of the inactivation kinetics. In this paper we have improved the measurements by obtaining kinetic data on the VA200 clone from stage II–III oocytes, where the membrane capacitance is much smaller and faster settling times can be obtained (Krafte and Lester, 1989).

The data presented in Fig. 2 show that the relationship between the time constant(s) of inactivation and membrane potential is altered in two ways for VA200 currents compared with poly(A+) RNA–induced currents: (*a*) VA200 currents inactivate with two exponential components, while poly(A+) currents inactivate with a single exponential component; and (*b*) there is a depolarizing shift in the τ_{fast} vs. voltage curves for VA200 compared with poly(A+) currents of ~20 mV. This probably reflects the phenomenon analyzed by Auld et al. (1990) with respect to the threshold of activation and the peak of the current-voltage relationship; both are shifted by ~20 mV in the depolarizing direction for VA200 currents, because of a single amino acid replacement at position 860. We have not performed measurements with M860 RNA on stage II–III oocytes to test this hypothesis directly.

Because we have injected only one mRNA species it is unlikely that separate translation products produce the source of the double exponential decay for VA200 records (e.g., a fast and a slow Na channel). It is of course formally possible that there are two sites for initiation or termination of translation. However, there are at least two other explanations for the presence of two time constants in the decay of VA200 currents. First, a single population of channels may have complex gating kinetics. Chiu (1977) has outlined a model with two closed states sequentially following the open state of the channel. This model gives rise to a double exponential decay of Na currents consistent with that found in frog myelinated nerve. Second, two functionally different channel populations might arise from posttranslational processing of the α subunit. The latter possibility is the more likely, given the results regarding recovery from inactivation outlined below.

A fraction of the rat IIA current recovers as rapidly as that for poly(A+) currents, while the remainder requires seconds to recover completely. In addition, the waveform for the twentieth pulse in a 10-Hz train shows faster inactivation than that of the first pulse in the train. One way to interpret these data is to assume that there are two populations of channels. The first inactivates rapidly ($\tau < 3$ ms) and recovers from inactivation rapidly (<100 ms), properties resembling those of poly(A+)-induced channels. The second population inactivates more slowly ($\tau > 5$ ms) and recovers from inactivation slowly (seconds) so that it is "fatigued" during a train of pulses. These results recall those reported by Gundersen et al. (1983) for Na channels induced in oocytes by mRNA from cat skeletal muscle. For this instance, Gundersen et al. (1983) postulated two different channels, one a T tubule channel and the other a surface membrane type.

Another point suggesting two channel populations is that the relative contribution of τ_{slow} to the waveform was variable (although always significant). While these data can most easily be explained by assuming two channel populations, one can still not rule out a complex gating scheme for a homogeneous channel population.

Voltage Dependence of Fast but Not "Slow Inactivation" Is Altered for Rat IIA Na Channels

In addition to the differences in kinetics of both onset and recovery from inactivation, we have also observed a depolarizing shift in the steady-state voltage dependence of inactivation for rat IIA currents relative to poly(A+) currents (Fig. 4). This difference is prominent when one assays inactivation for rat IIA currents relative to poly(A+) currents using 50-ms prepulses, but becomes less pronounced as prepulse duration is increased. When 2-s prepulses were used, we found no differences in the voltage dependence of steady-state inactivation. Prepulses of this length approach the time scale appropriate for the phenomenon termed "slow inactivation," an epithet that is certainly confusing in the context of the present study. After a depolarization of several seconds, Na channels seem to reach a long-lived inactivated state, so that a hyperpolarization for several seconds is required to return the channels to resting state. Judging from the results for prepulses of 2-s duration, rat IIA channels seem capable of reaching the "slow inactivation" state at a rate similar to that for normal channels. While the fast inactivation process is altered in rat IIA as compared with poly(A+) channels, the "slow inactivation" process is not. Perhaps "slow inactivation" is a property of the α subunit alone and does not require additional translation products from other mRNAs. These results recall those of Rudy (1978): internal pronase removed fast inactivation of squid axon Na channels, but left "slow inactivation" intact. Similar selective effects on fast inactivation are produced by a site-directed antibody (Vassilev et al., 1989).

Rat IIA Channels Reopen and Have Shorter Open Times Than Those for Poly(A+) RNA

The single-channel data confirm the two microelectrode results with respect to inactivation kinetics. Ensemble averages of rat IIA currents show slower inactivation than those from poly(A+) currents. In terms of microscopic kinetics, this difference results from a greater tendency for channels to reopen during a pulse (i.e., the inactivated state is no longer absorbing). These conclusions, with RNA synthesized from cDNA clones, are essentially the same as those previously reported for Na channels induced by a high MW RNA fraction (Krafte et al., 1988*b*).

In addition to a difference in the number of reopenings, the mean open time was shorter for rat IIA channels than for poly(A+) channels at any given voltage. This effect is opposite to the natural prediction from the slower macroscopic inactivation rates. The available data allowed us to analyze open times over only a limited voltage range (essentially -30–0 mV for VA200 events) due to the reduced current amplitudes, and therefore signal to noise ratio, at more depolarized levels. It is unclear whether the difference in open times can simply be explained by a depolarizing shift in the τ_{open} vs. voltage curve as was seen for τ_{fast} and steady-state inactivation.

One obvious question is whether we have seen evidence for two populations at the single-channel level. In general, we have not. All records examined show similar τ_{open} vs. voltage relations. In addition, our stimulus frequency was always $\leq$1 Hz and

events as shown in Fig. 7 are characteristic of those we normally observed. We have not investigated frequency-dependent changes at the single-channel level. Thus the possibility of two populations at the single-channel level still exists and this area merits further investigation.

Molecular Nature of Inactivation and Activation

Of the two major differences among the Na channel currents induced in *Xenopus* oocytes by in vitro transcripts from the rat IIA cDNA clones and by unfractionated poly(A+) RNA from rat brain, one primarily involves activation (Auld et al., 1988, 1990) and the second, studied here, primarily involves inactivation. These two "abnormalities" have different sources. The first resides in the primary amino acid sequence of the channel and can be restored by correction of a cloning artifact (Auld et al., 1990). This article and that of Auld et al. (1988) do show, however, that coinjection of low MW RNA restores the second abnormality but not the first, as assessed by the peak of the current–voltage relation. These two abnormalities thus certainly involve different parts of the Na channel protein.

Hodgkin and Huxley (1952) initiated discussion of the degree to which activation and inactivation are coupled or independent processes. Further investigations have involved many subsequent experiments, in particular the immobilization of activation gating charge by an inactivating prepulse (Armstrong and Bezanilla, 1977; Nonner, 1980). The discussion has been rephrased in the context of recent models for Na channel structure (Noda et al., 1984; Greenblatt et al., 1985; Catterall, 1986; Guy and Seetharamulu, 1986) and of results from Stuhmer et al. (1989), raising the possibility that the voltage sensors reside in the S4 regions of the homologous domains. One now asks, "Where are the three m gates governing activation, and the single h gate governing inactivation; and how do they interact?" If the voltage sensor for inactivation can indeed be assigned to a single S4 region of a single domain, we would suggest that the region is either S4 III or S4 IV. This guess is motivated by the evidence showing that the putative cytoplasmic linker between domains III and IV participates in the transition from open to inactivated channels (Stuhmer et al., 1989; Vassilev et al., 1989) and has many protease sites consistent with earlier studies on proteolytic removal of inactivation.

The transitions from resting to open states would be governed primarily by the S4 regions of any or all of the four domains, as suggested by the data of Stuhmer et al. (1989) and Auld et al. (1990). Interestingly, most of the cAMP-dependent phosphorylation sites of the rat brain Na channel are found in the putative cytoplasmic linker between domains I and II (Rossie et al., 1987). This is potentially a region where activation could be altered by phosphorylation and dephosphorylation and inactivation could remain unchanged. Costa and Catterall (1984) found that conditions that promote phosphorylation of the Na channel in synaptosomes result in a decreased neurotoxin-stimulated Na flux. This reduction would be consistent with a depolarizing shift in the activation process, although Coombs et al. (1988) have found that a hyperpolarizing shift in the inactivation curve may account for the results.

At this point, the most important piece of data missing is the molecular nature of the modification produced by low MW RNA. More detailed models of inactivation, and of activation/inactivation coupling, must await such information.

Relationship to Previous Results

That RNA encoding Na channel α subunits alone produces abnormally slow macroscopic inactivation in *Xenopus* oocytes was first reported by Krafte et al. (1987), was confirmed by Auld et al. (1988, 1990), Trimmer et al. (1989), and Joho et al. (1990), and is also evident in the records of Suzuki et al. (1988). However, Stuhmer et al. (1987) have reported that rat II Na channel currents inactivate as rapidly as in normal neurons. Under the experimental conditions of this paper and our previous publications (Krafte et al., 1988*b*; Auld et al., 1988, 1990), VA200 currents inactivate more slowly than those from unfractionated poly(A+) brain RNA. To address the apparent discrepancy we injected RNA for rat II Na channels (generously supplied by M. Noda and S. Numa) and VA200 channels in the same batch of stage V–VI oocytes. Current amplitudes were adjusted by injecting various amounts of RNA. When currents on the order of 0.1–3 μA were recorded after injection of rat II RNA, inactivation rates were roughly equal to those described in our publications. The work of Stuhmer et al. (1987, 1989) typically involves signals that correspond to much larger whole-cell currents; indeed, for whole-cell currents larger than ~30 μA we found faster inactivation rates. More systematic comparisons are called for, but this phenomenon may explain the discrepancy between our results and those of Stuhmer et al. (1987, 1989). One attractive hypothesis is that there are limiting amounts of a protease or other processing protein in oocytes, such that with relatively low-level expression of α subunits alone, but not with higher levels, there is modification of the α subunit. This modification results in slower inactivation but decreased mean open time. The processing protein would be absent in mammalian cells such as the CHO line, where expression of the α subunit alone gives rapidly and completely inactivating currents (Sheuer et al., 1990).

Huguenard et al. (1988) reported a developmental change of Na channel properties in rat neocortical neurons. These cells develop a second, slower exponential component in the decay phase of the Na currents as the neuron matures. This developmental change may represent the appearance of a new set of channels or perhaps differential processing of a single population. The latter would be consistent with our observations of the VA200 channel. French and Gage (1985) have also reported a rat neuronal Na current that shows very slow rates of inactivation. These differences in Na channel inactivation between cell types could conceivably arise from differential expression of the low MW cofactor whose existence is suggested by our experiments. There is, however, no exact correlation between our results and in vivo phenomena. In the study of Huguenard et al. (1988), single-channel recording showed that the second exponential in the decay phase can be explained by long latency openings, not by bursts of openings as we have described here. The issue will be clarified by (*a*) additional quantitative electrophysiological data from different cell types, (*b*) identification of the low MW factor(s) involved, and (*c*) study of the tissue distribution of these factors.

Note added in proof. A recent publication describes inactivation properties of a cloned rat brain Na channel expressed in oocytes (Moorman et al., 1990).

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