Effect of *N*-Bromoacetamide on Single Sodium Channel Currents in Excised Membrane Patches

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ABSTRACT We have studied the effect of *N*-bromoacetamide (NBA) on the behavior of single sodium channel currents in excised patches of rat myotube membrane at 10°C. Inward sodium currents were activated by voltage steps from holding potentials of about −100 mV to test potentials of −40 mV. The cytoplasmic-face solution was isotonic CsF. Application of NBA or pronase to the cytoplasmic face of the membrane irreversibly removed sodium channel inactivation, as determined by averaged single-channel records. The lifetime of the open channel at −40 mV was increased about 10-fold by NBA treatment without affecting the amplitude of single-channel currents. A binomial analysis was used both before and after treatment to determine the number of channels within the excised patch. NBA was shown to have little effect on activation kinetics, as determined by an examination of both the rising phase of averaged currents and measurements of the delay between the start of the pulse and the first channel opening. Our data support a kinetic model of sodium channel activation in which the rate constant leading back from the open state to the last closed state is slower than expected from a strict Hodgkin-Huxley model. The data also suggest that the normal open-channel lifetime is primarily determined by the inactivation process in the voltage range we have examined.

INTRODUCTION

A depolarization of muscle or nerve membrane causes a rapid increase, i.e., activation, of sodium conductance, which is responsible for the rising phase of the action potential. If the depolarization is maintained, the sodium conductance decreases, or inactivates. The interaction between these two processes has been the subject of considerable investigation (Hodgkin and Huxley, 1952; Goldman and Schauf, 1972; Armstrong and Bezanilla, 1977; Bezanilla and Armstrong, 1977; Gillespie and Meves, 1980; Bean, 1981). One approach to this problem has involved the use of protein-specific reagents such as pronase or *N*-bromoacetamide (NBA), which modify the inactivation process

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(Armstrong et al., 1973; Bezanilla and Armstrong, 1977; Oxford et al., 1978; Nonner et al., 1980; Oxford, 1981). When applied to the cytoplasmic surface of a squid axon, these compounds abolish inactivation completely, and do so apparently without affecting the activation process. They thereby permit the study of the activation process alone.

The modifications caused by pronase or NBA have previously been described from records of the summed currents from $>10^4$ individual sodium channels. Some kinetic details are obscured by this averaging process. However, two recent technical advances now permit this subject to be studied at the level of individual sodium channels. The gigohm seal technique of Neher (1981), by increasing the adherence between the cell membrane and a glass pipette, simultaneously increases the resolution available for patch recording of single-channel currents, and permits the small patch of membrane under the pipette to be voltage-clamped directly by the current recording amplifier. This technique led to the first report of discrete sodium channel currents by Sigworth and Neher (1980), who used tissue-cultured rat myoballs. The other technical advance, the excised path technique (Horn and Patlak, 1980), permits the removal of the membrane patch from the cell with the patch electrode, thereby exposing the cytoplasmic face of the patch to the bathing solution, which can then be changed at will.

We have studied the behavior of individual sodium channels in excised patches (Horn et al., 1981*b* and *c*). The application of NBA or pronase to the cytoplasmic face of the membrane abolished inactivation irreversibly and had little effect on the activation kinetics. The treatment caused a marked increase in the open-channel lifetime, which suggests that the kinetics of the inactivation gates, rather than those of the activation gates, are the primary determinant of the channel's open time at the potentials we have studied. A preliminary report has appeared (Horn et al., 1981*a*).

MATERIALS AND METHODS

All experiments were performed on tissue-cultured myotubes that were prepared from the myoblasts of neonatal rats using previously described techniques (Horn and Brodwick, 1980). The growth medium consisted of Dulbecco's medium enriched with 10% horse serum, 6 g/liter glucose, 100 U/liter penicillin, 100 μg/ml streptomycin, 1% glutamine, and 1% chick embryo extract. The myoblasts were plated onto coverslips in sterile tissue-culture dishes, and were used for experiments from 5 to 8 d after plating.

Coverslips carrying myotubes were transferred to the experimental chamber just before the start of recording. All cells were pretreated with 10^{-6} g/ml α-bungarotoxin for 10 min to remove any contribution of acetylcholine-activated channels from the records. The initial bathing solution was a rat Ringer's solution consisting of 150 mM NaCl, 5 mM KCl, 5 mM glucose, 1.5 mM $CaCl_2$, and 5 mM HEPES buffer titrated to pH 7.4. The patch electrode contained this solution for all experiments. All solutions were passed through a filter with a pore size of 0.2 μm. All experiments were done at ~10°C.

Patch electrodes were made on a vertical micropipette puller in a two-step process. The electrodes were then coated with insulating varnish and lightly fire-polished to give a final tip diameter in the range of 0.5 μm. The resistance of the electrode filled

with and immersed in Ringer was 4–8 MΩ. Gigohm seals between the electrode and the cell's membrane were produced by application of gentle suction after the establishment of contact between the electrode and cell (Sigworth and Neher, 1980; Horn and Patlak, 1980; Neher, 1981).

A modified patch current recording circuit described previously (Horn and Patlak, 1980) was used. The bath potential was measured using a 7-M CsCl electrode. This signal was used as a reference for the command potential applied to the patch pipette. Holding potentials (−90 to −110 mV) and voltage-step command signals were generated by a computer, which was also used to sample the data at 100–400-μs intervals. Pulses were typically generated at a rate of 1/s. At this pulse rate we detected no evidence of decrease in the averaged currents (run down) during a set of several hundred pulses. In most records shown the currents were filtered before sampling with an eight-pole Bessel low-pass filter using a corner frequency of 1 kHz. The uncompensated current transients that resulted from steps of the command potential after establishment of gigohm seals were often large and had several components of decay. These currents were cancelled at several stages of the recording process. The fastest component was compensated with an analog circuit. For digital leak subtraction, negative-going pulses were sampled, averaged, and scaled for subsequent addition to the depolarizing test pulses. Finally, the records during a pulse series that contained no channel openings were averaged and subtracted from the other records of that set to produce the currents illustrated here.

After establishment of a gigohm seal, a hyperpolarizing holding potential was applied to the pipette, and the bathing solution was changed to an "internal" (i.e., cytoplasmic face) solution consisting of 160 mM CsF, 5 mM Cs-HEPES, pH 7.3. When the old bathing solution had been exchanged with at least its volume of internal solution, the membrane patch was excised by sudden withdrawal of the electrode from the cell. Solution changes were considered complete after the chamber volume had been perfused at least 10 times. The electrode was then withdrawn so that only its tip was immersed in order to reduce capacitance between it and the bath.

Data were collected from 31 excised patches. In 10 preparations the bathing solution was changed to the "internal" solution plus between 10 and 300 μM *N*-bromoacetamide (Sigma Chemical Co., St. Louis, Mo.). Treatment times with this reagent ranged from 3 to 10 min, after which the patch was returned to standard internal solution for recording. Pronase (Sigma Chemical Co.) was similarly applied in eight experiments at a concentration of 0.17 mg/ml.

Binomial analysis was performed on sets of data stored in digitized form. Individual records were displayed, and a pointer was set to the zero current level. Another pointer was set to the level of current recorded when one channel was open. Pointers to the levels of mutliple channel openings were set as integral multiples of the first level. The values of the pointers were checked for each record, and then the data for each time point were sorted according to the level to which they were nearest. This information was subsequently used to calculate the probabilities that zero, one, two, or three channels were open at any time point, and to calculate the mean normalized current for use in the maximum likelihood estimates (see Results and Appendix).

RESULTS

NBA Removes Inactivation

Individual sodium currents can be clearly resolved in current records from excised membrane patches. The top half of Fig. 1 illustrates the current response to individual voltage pulses at two different potentials. A holding

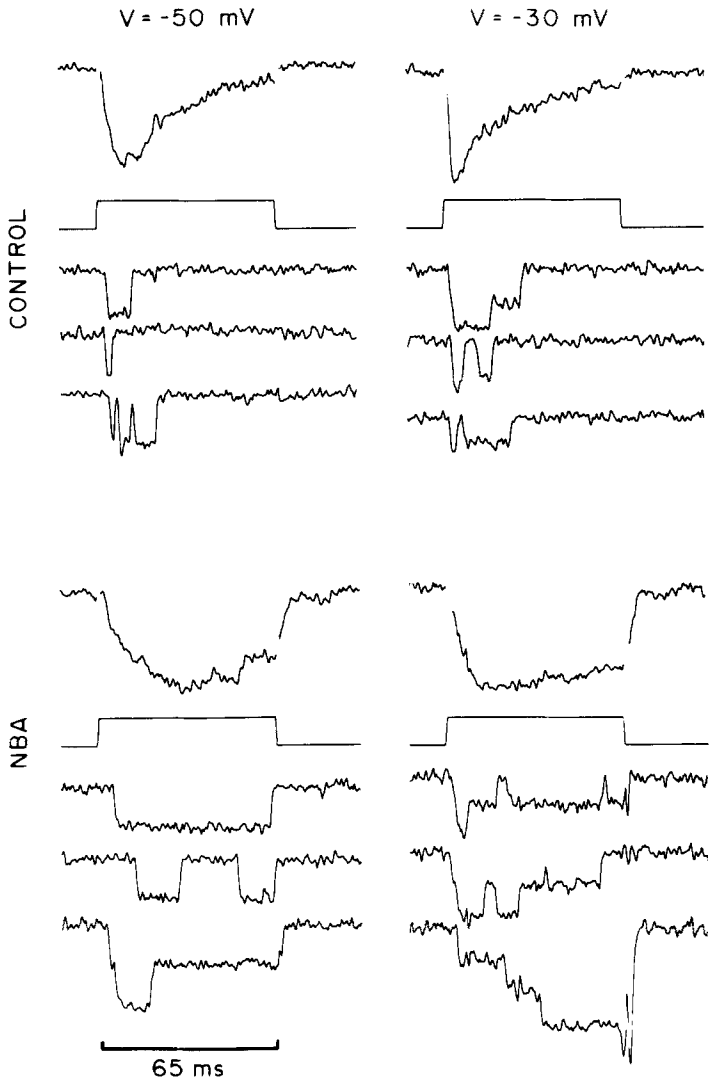


FIGURE 1. Effect of NBA on single sodium channels. The top and bottom halves of this figure show data from a control patch and from a different patch treated with 300 μM *N*-bromoacetamide. Each section of the figure shows, from top to bottom, the averaged current from single records, a schematic voltage pulse, and three individual records. A vertical calibration of 1 pA for the individual records is given by the amplitude of the schematic voltage pulse. The averaged currents are arbitrarily scaled to about the same amplitude. Note the presence of overlapping single-channel events in some individual records. The control averages were obtained from 144 individual records at each voltage. The averages after NBA treatment were obtained from 96 records at each voltage. Holding potential, −110 mV. The apparent effect of NBA on the activation kinetics is produced by the unequal scaling of the currents.

potential of −110 mV was applied to remove most of the resting inactivation of the channels under the patch. The amplitude of the current through the channels depended on the membrane potential, and at −50 mV it was ~1.4 pA. The conductance of these channels has been estimated to be 15 pS (Horn et al., 1981*c*). The random opening and closing of channels is apparent in the individual records and is consistent with previous reports (Sigworth and Neher, 1980; Horn et al., 1981*b*). The mean level of activity of sodium channels after a step of voltage can be summarized by averaging the patch response to many single pulses (Sigworth and Neher, 1980; Horn et al., 1981*b*). The top row of Fig. 1, which shows the average of >100 traces, has the typical appearance of whole-cell sodium currents that activate after a delay and later inactivate.

Treatment of the cytoplasmic face of the membrane with 300 μM NBA for several minutes removes the inactivation process, as can be seen in the averaged currents in the lower half of Fig. 1. The full extent of the rise in the averaged currents is realized by this treatment, and for the 65-ms duration pulses shown, there is little inactivation of the currents. The individual current traces also show that there is a dramatic increase in the lifetime of the channel's conducting state. NBA had no observable effect on the single-channel conductance at these potentials.

The average current traces after NBA treatment have some "sag" before the end of the pulse. The extent of sag is correlated with the extent of NBA treatment, with more heavily treated patches having little or none, as illustrated by the average currents in Fig. 2 (shown at a slower time base). When sag was present, its time constant was significantly slower than that of normal inactivation at the same potential. It is therefore unlikely that it represents the summation of the responses of treated and untreated channels, but rather may represent some intermediate level of inactivating response that is first exposed and then removed by NBA.

The effect of pronase is similar to that of NBA: inactivation is removed and the single-channel open duration is drastically lengthened, as illustrated in the right half of Fig. 2. Note the very slow time base at which these traces were recorded. There is no apparent sag in the averaged currents. However, pronase has the deleterious effect of removing treated channels from the pool of functional channels in the patch. In control records, ~80% of the voltage pulses to −40 mV elicited single-channel currents (see legend of Fig. 5). After the membrane was treated with pronase, however, only 5–10% of pulses caused channel events, even when the membrane potential was held at −140 mV for long periods between pulses. Often those records that had opening events occurred sequentially. This diminution of activity after pronase prevented us from collecting sufficient data to analyze quantitatively.

NBA Increases Channel Lifetimes

We measured the durations of individual channel events that did not overlap or terminate at the end of the pulse both before and after treatment with

NBA. The histograms of the channel open times for these two cases are shown in Fig. 3. The mean channel open time, calculated by averaging the durations of the single events, increases almost 10-fold after treatment with NBA.

Neither histogram can be well fitted by a single exponential. In each histogram the first several bins are too high to fit the nearly exponential decay of the subsequent bins. In the case of the NBA data, the fast component could represent a population of untreated channels, but average currents from these records did not show an inactivating component, which would be the expected

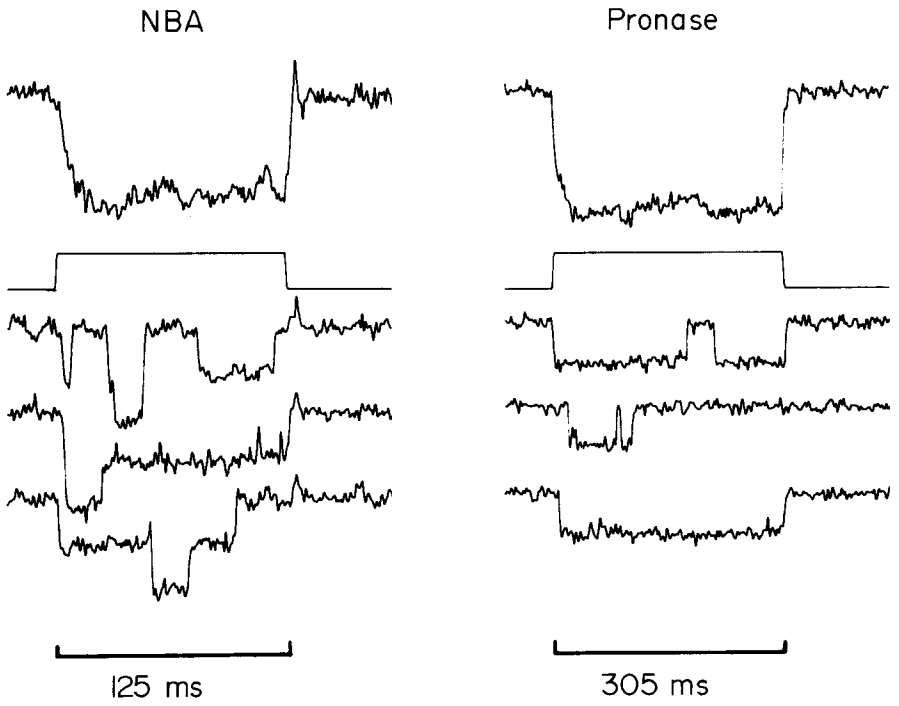


FIGURE 2. The effect of long-duration voltage pulses after treatment by NBA or pronase. Labels and vertical calibration as in Fig. 1. All voltage pulses to −40 mV. The patch on the left was treated with 300 μM NBA; that on the right was treated with pronase. Note the absence of sag in the averaged currents, which were obtained from 96 individual records (NBA) and 48 individual records (pronase).

result of this hypothesis. Furthermore, the control histogram also appears to have more than one component. Our data may support the conclusions of Sigworth (1981*b*), who used fluctuation analysis to conclude that Na channels have more than one open state.

Our procedure for measuring channel lifetimes introduces a sampling bias, since longer events have a higher probability of overlapping and are therefore less likely to be counted. We have not attempted to make a correction for this bias because the probabilities for channel transitions were not stationary

during the pulse, and because the open times appear to be nonexponential. Although this bias makes our estimates of channel open times only approximations, the differences between the data before and after NBA treatment are so large that the change in open time is unambiguous.

Channel Activation Is Minimally Affected by NBA

If sodium channel activation is to be studied with NBA-treated membranes, it is important to determine whether the sole effect of NBA is to remove inactivation or whether it has an additional effect on the activation process.

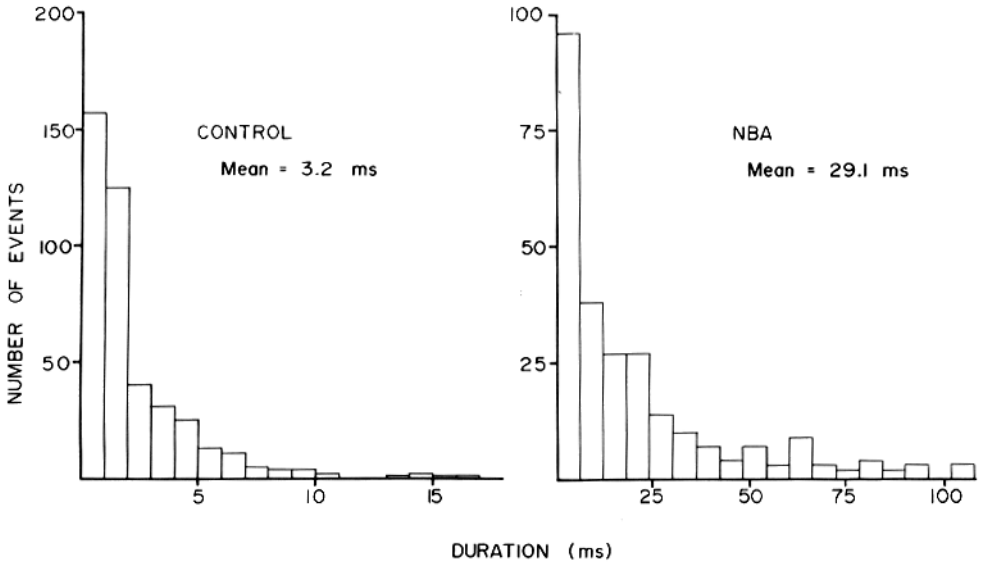
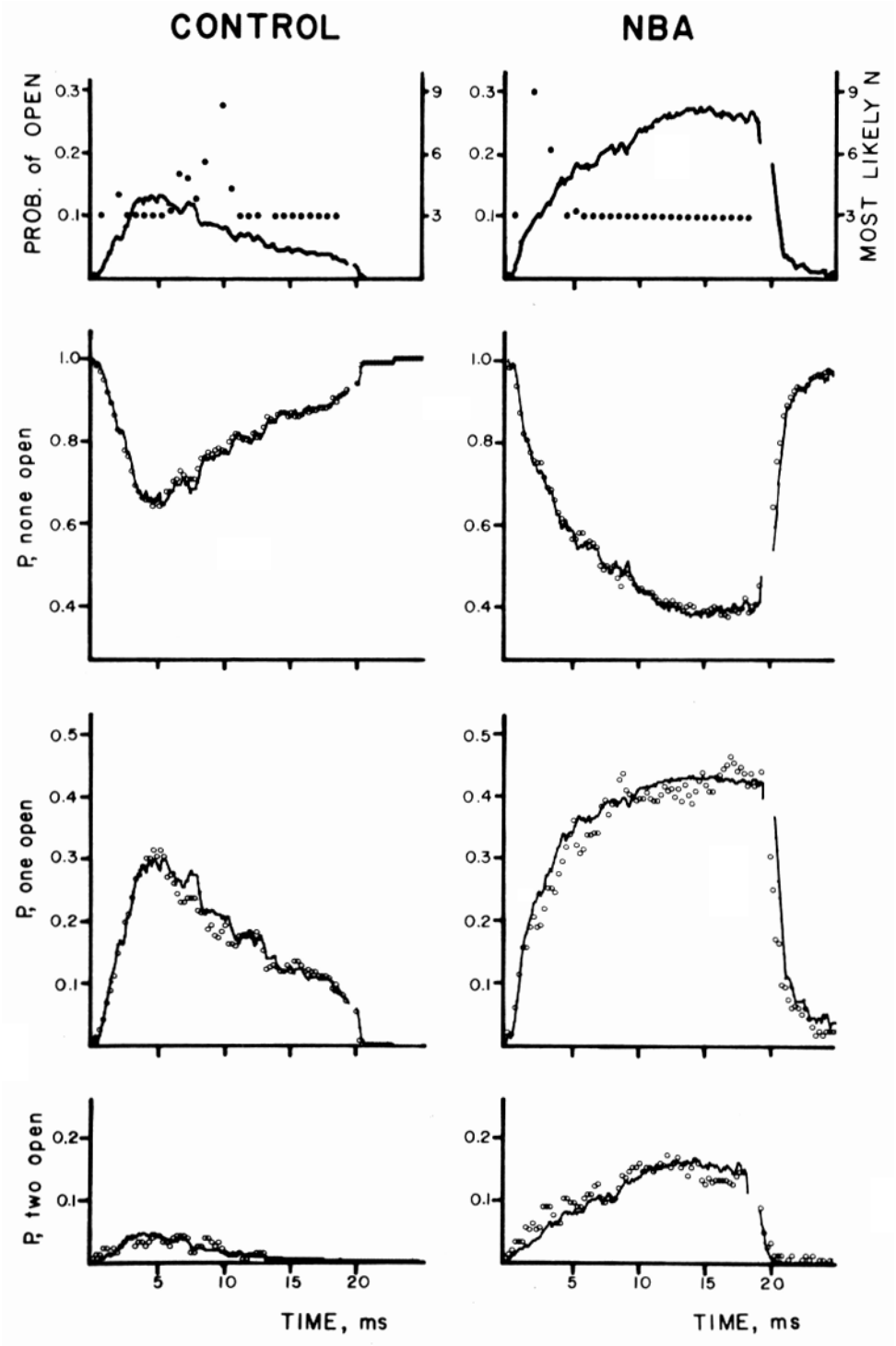


FIGURE 3. The effect of NBA on channel lifetime. Histograms of open-channel lifetime are plotted for control patches (left), and patches after NBA treatment (right). All voltage pulses were to −40 mV. The control data were measurements of 373 individual events from 4 different patches using 65-ms pulses. The NBA data were measurements of 268 individual events from 3 different patches, using 120-ms pulses. All patches had from three to five channels. The mean durations are shown.

For example, the increase in open-channel lifetime caused by NBA could be due exclusively to removal of inactivation; alternatively, NBA could slow the activation kinetics as well. We have therefore attempted to compare the activation kinetics before and after removal of inactivation with NBA.

Two different measures of the activation process have been used: the rising phase of the mean currents, and the delay between the start of the pulse and the first channel opening. However, in order to estimate kinetic parameters, or to make comparisons between treated and untreated patches, it is necessary to know the number of channels within the patch, since both mean currents


CONTROL
NBA
PROB. of OPEN
MOST LIKELY N
P, none open
P, one open
P, two open
TIME, ms
TIME, ms

and latency-to-first-event measurements are influenced by the number of channels.

The number of channels in a patch is not directly measurable, but can be estimated from a binomial analysis of many individual responses of a patch to a voltage pulse. At any given time, the distribution of instances in which zero, one, two, three, or N channels are open, will be a binomial distribution on two conditions: (*a*) the response of a channel is independent from that of others, and (*b*) the probability that a channel is open at any particular time after the start of the pulse is constant from pulse to pulse. The distributions of observed conductance levels can be directly measured. Similar distributions can also be calculated as a function of the observed mean current by using integral estimates for possible numbers of channels. Comparison of the observed and calculated distributions leads to a best estimate for the number of channels. (See Appendix.)

Fig. 4 shows two examples of this type of analysis. The open circles in the lower three panels are the observed probabilities that zero, one, or two channels were open simultaneously as a function of time after the start of the pulse. The filled circles in the topmost panels are the number of channels that gave the maximum value for the likelihood function:

$$L^{**}(N) = \sum_{x=0}^{N} \xi_x \ln\left[\binom{N}{x}\left(\bar{I}/N\right)^x\left(1-\bar{I}/N\right)^{N-x}\right] \qquad \text{(A7)}$$

where $L^{**}(N)$ is the likelihood function for N channels in the patch, ξ_x is the observed number of instances of level x in the data set, $\binom{N}{x}$ is the binomial series weighting factor $N!/(x!(N-x)!)$, and $\bar{I}$ is the mean current normalized to a single-channel current equal to 1. To condense the results of our analysis, the points shown are the average of the maximum likelihood values for five sequential time points. The omitted symbols had values >20.

The most frequent estimate for N is three for both of the patches shown. The solid lines in the top panels are the calculated probability that one

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FIGURE 4. *Opposite* Binomial analysis of two patches. The left-hand plots are from a control patch; the right-hand plots are from an NBA-treated patch. The filled circles in the top traces are the average values of five sequential maximum likelihood estimates for the number of channels in the patch. The best overall estimate was three for each patch. The solid lines are the calculated probabilities (from top to bottom) of a channel being open, and of zero, one, and two channels open simultaneously as a function of time after the start of the pulse. All curves were calculated from the average currents using the assumption that three channels were present. The open circles in the lower three plots are the observed probabilities that zero, one, or two channels were open at each time. The observed and predicted probabilities are close to each other for all times, which indicates that three is an adequate estimate of the number of channels in both of these patches. A total of 144 pulses were analyzed for both the control and the NBA-treated case. The pulse potential was −40 mV.

channel is open for $N = 3$. The solid lines in the lower panels are the calculated probabilities that zero, one, or two channels would be open if three channels were present. These curves show that a good fit can be obtained between observed and predicted values at all times. Note that at the onset of currents the values for N are variable, and often are >20. This deviation appears to be an artifact of the analysis procedure, however (see Discussion).

Given that the number of channels in two patches is known, the currents from the two patches studied under the same conditions can be compared. If the inactivation process is sufficiently slow compared with the activation process, then the rate of conductance increase after a voltage step will be minimally affected by procedures that simply abolish inactivation. The probability-of-being-open curves from the two patches analyzed above are superimposed in Fig. 5A for comparison of their rising phases. The fact that the rising phases of the currents are similar is an indication that NBA has a minimal effect on the activation kinetics of these channels. The slight increase in activation rate after NBA treatment might be expected from the fact that activation and inactivation seem to have overlapping time courses (see Discussion).

Another measure of activation kinetics, the time interval, or latency, from the voltage step to the time of the first channel opening (first latency), is shown in Fig. 5B. These data are from the same pulses as shown in Figs. 4 and 5A. The histogram of the first latencies has a maximum at a time later than zero, as expected for a process in which multiple closed states precede an open state. (The derivation for the first-latency histogram of a three-state channel is given in the appendix.) The histogram in the control case is qualitatively similar to that observed after NBA treatment. Note also that the control histogram has significantly less events at later times. This observation is the expected result if Na channels can inactivate before opening (Horn et al., 1981*b*).

DISCUSSION

We have described here the behavior of single sodium channels using the excised patch technique. Our results show some of the versatility of this technique: it permits control of both the transmembrane potential and the intracellular, i.e., cytoplasmic face, solution. The technique allowed us to make high-resolution, single-channel recordings while applying protein reagents to the cytoplasmic face of the membrane. Our data can be summarized as follows: NBA removes inactivation without having sizable effects either on the activation kinetics or on single-channel conductance. The action of both NBA and pronase is to produce a marked increase in the lifetime of the open state, which suggests that the closing of inactivation gates is usually the principle determinant of channel lifetime in the voltage range we have studied.

Although single-channel recording provides new information about the sodium channel, it brings with it a host of limitations. For example, the gating can only be studied over a limited voltage range under usual ionic conditions. At potentials more negative than −50 mV, the channels had too low a probability of being open, and at potentials more positive than −10 mV, the

single-channel currents became too small in amplitude to study. Also, at positive potentials, channels tended to activate almost simultaneously and inactivate rapidly, thus obscuring single-channel behavior. The limited voltage range also hampered the characterization of open channel properties, such as the full current-voltage (I-V) relationship. Over our limited range the channels

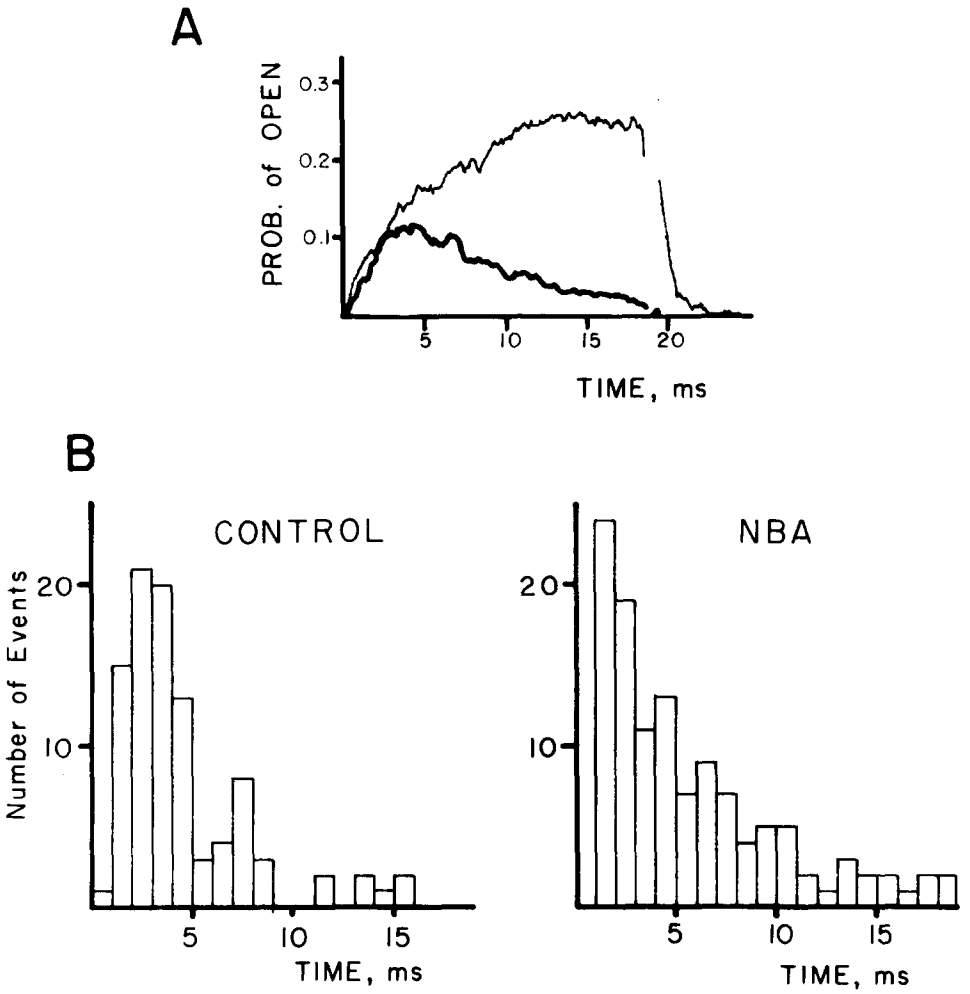

FIGURE 5. Comparison of the rising phase of sodium currents. (A) The probability-of-being-open curves from Fig. 4 are superimposed to compare their rising phases. Both currents have been scaled from the mean currents under the assumption that three channels were present. The dark line is the control record, and the light line is the record after treatment with NBA. (B) Latency-to-first-event histograms from the pulse sets in Fig. 4 and 5A. The left- and right-hand histograms show the interval between the onset of the voltage pulse and the first channel opening for control and NBA-treated patches. It was not possible to measure latencies greater than the duration of the pulses, 18 ms. In the control case, 49 out of 144 records had no channel events, whereas in the NBA case 24 of 144 records had no events.

were nearly ohmic, with a conductance of ~15 pS (Horn et al., 1981*c*). We could not detect subtle changes in the *I-V* relationship that might be caused by NBA.

An even greater problem in single-channel experiments is the difficulty in characterizing macroscopic kinetics. In usual membrane preparations, containing $>10^4$ channels, detailed macroscopic kinetics can be obtained in a rapid and repeatable manner over a wide voltage range. However, the averaging procedure that we used required hundreds of repeated pulses at one potential to produce only a ragged version of whole-cell sodium currents. In the future, such preparations as the cut-open squid axon (Llano and Bezanilla, 1981) or whole cell/excised membrane patch recordings from small spherical cells (Sigworth, 1981*a*) may overcome these limitations, because it may be possible to study macroscopic currents, gating currents, and single-channel behavior in the same preparation.

Determination of the Number of Channels

We used the maximum likelihood method to estimate the total number, N, of channels in a patch. This method produced consistent estimates of N at most time epochs, but apparently produced erroneous values during the initial rising phase of the averaged currents (Fig. 4). The erroneous values of N could be due to a sampling error. Only 144 records were analyzed for each patch shown in Fig. 4. Because the averaged probability of a channel being open is low during the rising phase of the currents, the frequency of overlapping events is also quite low during this period. Therefore the estimates of the rapidly changing probabilities could be inadequate during activation. We tested this possibility by simulating single-channel currents using a Hodgkin-Huxley model in which several channels functioned independently of one another. Ensembles of these simulated currents were then analyzed as above. The maximum likelihood estimates of N deviated from the known value in a fashion similar to our actual data when the number of records analyzed was <150, which suggests that the erroneous estimates of N were due to a sampling error.

When we use our best estimates for N, the probabilities for observing zero, one, or two channels open simultaneously at any time are very well fit by a binomial distribution, which supports the notion that channels are acting independently, as previously concluded by Sigworth (1980*b*) using fluctuation analysis. Using estimates of membrane area of a patch (Sigworth and Neher, 1980), the density of sodium channels in our experiments is less than ~4 channels/μm^2, which is at least two orders of magnitude less than that in squid axon (Levinson and Meves, 1975).

One difficulty with the binomial analysis is that the end product is the total number of channels in a patch and the overall probability of a channel being open. This probability lumps together all the factors that affect the channel's function, such as activation, inactivation, and resting inactivation (see Appendix). Another uncertainty in the binomial analysis is the assumption that all channels are reset on the average to the same state at the start of each pulse

in an ensemble. This assumption will be violated, for instance, by a gradual process like slow inactivation, or rundown of the preparation.

Effect of NBA on Activation Kinetics

We have presented evidence that NBA has little effect on activation kinetics. Previous work on squid axon and frog nerve has produced similar results (Oxford et al., 1978; Nonner et al., 1980; Oxford, 1981). It is nevertheless worthwhile here to examine our evidence critically.

The most direct way to compare quantitatively the activation kinetics of single-channel signals is to compare the time course of the rising phases of the probability-of-being-open curves. These data can be determined for an individual channel and are normalized to eliminate the influence of the number of channels in the patch. We have shown that the activation phase of the sodium currents is minimally affected by NBA treatment (Fig. 5A). However, Fig. 5A suggests that activation is still proceeding when inactivation has already commenced. This can also be seen by comparing Figs. 5A and B. In a significant fraction of the control records the first channel opened at a time when the mean current was decaying. If the time course of activation and inactivation overlap and if closed channels can inactivate (Horn et al., 1981*b*), then we would expect that the removal of inactivation would make the activation rate faster. A slight increase in this rate is noticeable after NBA treatment (Fig. 5A), but it is not clear whether this effect is statistically significant. Therefore our results cannot make a definitive statement about the magnitude of influence of NBA on activation kinetics. However, this influence is small when compared with the dramatic increase in the open-channel lifetime caused by NBA.

The second evidence used to show that NBA has little influence on activation kinetics is that the first-latency histogram after NBA treatment is similar to that of the control case. The test is literally correct if channels must open before they inactivate, but because this is not necessary (Horn et al., 1981*b*), an increase the average latency to first opening would be expected. Fig. 5B shows some evidence of this slowing. In the control case the mean latency was 5.0 ms, whereas after NBA treatment the mean latency was 6.3 ms. However, our data are again insufficient to definitively resolve this effect.

Kinetic Models of the Na Channel

The Hodgkin-Huxley model (1952) gives specific predictions about the effect of the inactivation process on the channel lifetime, T. The model predicts that the mean lifetime of the open state is $T = 1/(3\beta_m + \beta_h)$, where $3\beta_m$ and β_h are the rate constants for closing the activation and inactivation gates, respectively. When inactivation is removed, T increases to $1/3\beta_m$. The values of β_m and β_h at -40 mV were estimated from our data as follows. Single exponentials were fit to the slowest activation process (see Reuter and Stevens, 1980) in averaged records from NBA-treated patches, and to the inactivating phase of control records, to provide estimates for τ_m and τ_h, respectively. The values of τ_m and τ_h for the patches analyzed in Figs. 4 and 5 are 5 and 9 ms.

The equilibrium probability of a channel being open for the Hodgkin-Huxley model is m^3_∞ when the inactivation process is removed. We can measure this quantity directly from Fig. 4 as 0.28. The value of β_m is obtained from the relationship $\beta_m = (1 - m_\infty)/\tau_m = 69\ s^{-1}$. Because the h_∞ curve in muscle has a very negative midpoint (see Pappone, 1980), $\beta_h \cong 1/\tau_h = 111\ s^{-1}$. The open time predicted for the control case from this type of analysis is 3 ms, quite close to the observed value (Fig. 3). However, removal of inactivation in this model increases this open time only to ~5 ms. This is clearly contrary to our observations, which indicate a 10-fold increase in mean channel open time. Thus, in contrast to the predictions of the Hodgkin-Huxley model, channels are far more likely to close by inactivating than by returning to a previous closed state. Although this conclusion can be made firmly only for the potential −40 mV, we have observed a marked increased in channel lifetime at several more positive potentials (unpublished observations).

The activation process has been modeled as a linear sequence of first-order reactions leading to the open state (Bezanilla and Armstrong, 1977; Armstrong and Bezanilla, 1977; Armstrong and Gilly, 1979; Oxford, 1981). Our analysis of sodium activation after removal of inactivation suggests that in such a linear reaction scheme, the rate constant from the open state to the preceeding closed state is slower than that which would be expected from a model such as that proposed by Hodgkin and Huxley (1952), in which several independent gates all had an equal chance of closing the channel. Our observation is consistent with data obtained from squid axon, which also suggest that the rates for both activation and "deactivation" (i.e., closing via the activation pathway) are limited by the last transition preceding the open state (Armstrong and Gilly, 1979; Oxford, 1981).

APPENDIX

Determination of the Number of Channels in a Patch

If we assume that the channels in a patch function independently from one another, then

$$\bar{I}(t) = Np(t) \tag{A1}$$

where $\bar{I}(t)$ is the normalized mean current (taking the single-channel current equal to one), N is the number of channels, and $p(t)$ is the probability that a channel is open at time t.

For any one time t, the likelihood that a given ensemble of independent trials is a subset of the binomial distribution with $\tilde{N}$ channels and $\tilde{p}(t)$ probability of opening is (Binet, 1953):

$$L(N, p) = \prod_{i=1}^{Q} \binom{N}{x_i} p^{x_i} q^{N-x_i} \tag{A2}$$

where $q = 1 - p$, $\binom{N}{x_i}$ is the standard binomial weighting factor $N!/(x_i!\,(N - x_i)!)$, Q is the total number of observations, and x_i is the observed level (i.e.,

0, 1, 2 . . .) for any one observation. The likelihood function is maximum for those values of N and p such that

$$\frac{\partial \ln(L)}{\partial p} = 0, \tag{A3}$$

$$\frac{\partial \ln(L)}{\partial N} = 0. \tag{A4}$$

Eq. A3 is straightforward to solve and is satisfied when

$$p = \bar{I}/N. \tag{A5}$$

The solution to Eq. A4 may be numerically estimated and solved simultaneously with Eq. A5. Alternatively, the maximum of the likelihood function in N can be found (Dahiya, 1981):

$$L^*(N) = \prod_{i=1}^{Q} \binom{N}{x_i} (\bar{I}/N)^{x_i}(1 - \bar{I}/N)^{N-x_i}; \tag{A6}$$

or, alternatively,

$$L^{**}(N) \equiv \ln(L^*(N)) = \sum_{x=0}^{N} \xi_x \ln\left[\binom{N}{x}(\bar{I}/N)^x(1 - \bar{I}/N)^{N-x}\right] \tag{A7}$$

where ξ_x equals the total number of observations at level x. The maximum value for $L^{**}(N)$ is determined for integer values of $N \geq R$, where R is the largest observed level in all records.

The probability that the system is in some state x (corresponding to the number of channels open simultaneously) at time t is given by the binomial equation, assuming that the number of channels, N, remains constant during the entire period of observation:

$$p_x(t) = \binom{N}{x} p(t)^x(1 - p(t))^{N-x}. \tag{A8}$$

However, it is not possible to use a binomial analysis to determine what fraction of the channels present is already inactivated before the start of the pulse. This can be shown as follows.

Consider a total population of N channels. Assume that the number of channels that are not inactivated at the beginning of a voltage step is a random variable, M. First, let us examine the number of channels, S_M, open at a given time after the start of the voltage step:

$$\begin{aligned} S_M &= X_1 + X_2 + \cdots + X_M \qquad M \leq N. \\ X_i &= 1 \quad \text{with a probability } p \text{ of being open} \\ &= 0 \quad \text{with a probability } q = 1 - p \text{ of being closed.} \end{aligned} \tag{A9}$$

Let us also assume that X_i and M are distributed with binomial probability.

The random variables X_k, M, and S_M can be formally defined as follows:

$$\begin{aligned} \text{Prob}\{X_k = j\} &= f_j = p^j q^{1-j} \qquad j = 0, 1 \\ \text{Prob}\{M = m\} &= g_m = \binom{N}{m} a^m b^{N-m} \qquad m = 0, 1, \cdots N \\ \text{Prob}\{S_M = L\} &= h_L \end{aligned} \tag{A10}$$

where a is the probability of a channel being able to activate at the start of the voltage step, and $b = 1 - a$ is the probability that the channel is inactivated at this time. The distribution $\{h_L\}$ is a compound distribution of the sum S_M (Bailey, 1964).

The probability generating functions for these random variables are

$$\begin{aligned} F(s) &= \sum_{j=0}^{1} f_j s^j = ps + q \\ G(s) &= \sum_{m=0}^{N} g_m s^m = (as + b)^N \\ H(s) &= \sum_{L=0}^{N} h_L s^L. \end{aligned} \tag{A11}$$

We can write the probability distribution of S_M as

$$h_L = \sum_{m=0}^{N} g_m \,\text{Prob}\{S_m = L \mid M = m\}. \tag{A12}$$

Therefore,

$$\begin{aligned} H(s) &= \sum_{L=0}^{N} s^L \sum_{m=0}^{N} g_m \,\text{Prob}\{S_m = L \mid M = m\} \\ &= \sum_{m=0}^{N} g_m \sum_{L=0}^{N} \text{Prob}\{S_m = L \mid M = m\} s^L \\ &= \sum_{m=0}^{N} g_m \{F(s)\}^m. \end{aligned} \tag{A13}$$

where $\{F(s)\}^m$ is the m-fold convolution of $\{f_j\}$ with itself. Therefore $H(s)$ is given by

$$\begin{aligned} H(s) &= G(F(s)) = G(ps + q) \\ &= (aps + (1 - ap))^N. \end{aligned} \tag{A14}$$

$H(s)$ is the probability generating function of a binomial distribution, and

$$h_L = \binom{N}{L} (ap)^L (1 - ap)^{N-L}. \tag{A15}$$

For a nonstationary process, such as sodium activation, this analysis only holds for a fixed time after the voltage step, but can be applied by ensemble

averaging at other times, as used in Results. Notice that M, a, and p are either lost or inseparable in the derivation, which implies that a binomial analysis cannot itself give information about the amount of resting inactivation.

First Passage Time Distribution for a Three-State Channel

We assume that the sodium channel after removal of inactivation is described by a kinetic scheme with three states, two closed, and one open. We use this model for simplicity, and to illustrate the general approach of this method of analysis:

$$S_0 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} S_1 \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} S_2.$$

The k_i's are reaction rates. We furthermore assume that the reactions fit the restrictions of a true Markov process, i.e., that the time the channel remains in any one state is exponentially distributed, and that this time is independent of the channel's past history. In this model the channel is in state S_0 at time zero, and we wish to calculate the waiting time, W_{02}, for the first occurrence of state S_2, the open state. This is the first latency.

$W_{02}(t)$ can be solved by assuming that S_2 is an absorbing state (i.e., $k_{-2} = 0$), and then calculating the probability of being in S_2 as a function of time (see Feller, 1971). In this particular case,

$$\begin{aligned} W_{02}(t) &= 1 + \frac{R_2}{R_1 - R_2} e^{-R_1 t} - \frac{R_1}{R_1 - R_2} e^{-R_2 t} \\ R_1, R_2 &= [k_1 + k_{-1} + k_2 \pm \sqrt{(k_1 + k_{-1} + k_2)^2 - 4k_1k_2}]/2. \end{aligned} \tag{A16}$$

$W_{02}(t)$ estimates the probability that a channel enters the open state (S_2) by time t, given that it was in state S_0 at time zero. The histograms in Fig. 5B are in a form equivalent to the time derivative of $W_{02}(t)$.

The above equation is applicable for the behavior of a single channel. When the patch contains N channels, the following modification is necessary. The probability that any of N channels opens by time t, given that each channel was initially in state S_0 and is independent from all others is given by

$$P_N(t) = 1 - (1 - W_{02}(t))^N. \tag{A17}$$

For the three-state model, Eq. A17 is the sum of $N + 1$ exponentials, which in general is hard to evaluate. However, it may be possible to find a rate constant for the slowest exponential, which will have the value NR_2.

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