STATISTICAL ANALYSIS OF SINGLE SODIUM CHANNELS

Effects of *N*-Bromoacetamide

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ABSTRACT Currents were obtained from single sodium channels in outside-out excised patches of membrane from the cell line GH_3. The currents were examined in control patches and in patches treated with *N*-bromoacetamide (NBA) to remove inactivation. The single-channel current-voltage relationship was linear over the range −60 to +10 mV, and was unaffected by NBA. The slope conductance at 9.3°C was 12 pS, and the Q_{10} for single channel currents was about 1.35. The currents in both control and NBA-treated patches showed evidence of a slow process similar to desensitization in acetylcholine-receptor channels. This process was especially apparent at rapid rates of stimulation (5 Hz), where openings occurred in clusters of records. The clustering of records with and without openings was analyzed by runs analysis, which showed a statistically significant trend toward nonrandom ordering in the reponses of channels to voltage pulses. NBA made this nonrandom pattern more apparent. The probability that an individual channel was "hibernating" during an activating depolarization was estimated by a maximum likelihood method. The lifetime of the open state was also estimated by a maximum likelihood method, and was examined as a function of voltage. In control patches the open time was mildly voltage-dependent, showing a maximum at about −50 mV. In NBA-treated patches the open time was greater than in the control case and increased monotonically with depolarization; it asymptotically approached that of the control patches at hyperpolarized potentials. By comparing channel open times in control and NBA-treated patches, we determined β_A and β_I, the rate constants for closing activation gates and fast inactivation gates. β_I was an exponential function of voltage, increasing *e*-fold for 34 mV. β_A had the opposite voltage dependence. The probability of an open channel closing its fast inactivation gate, rather than its activation gate, increased linearly with depolarization from −60 to −10 mV. These results indicate that inactivation is inherently voltage dependent.

INTRODUCTION

The mechanisms underlying the voltage-activated sodium conductance in nerve and muscle membrane have been a mystery for over three decades. A variety of experimental and theoretical approaches have been used to attack this problem, including voltage-clamp measurements, gating currents, biochemical analysis, pharmacology, noise analysis, single-channel recording, and reconstitution (for some recent reviews, see Armstrong, 1981; Brodwick and Eaton, 1982; Rogart, 1981; Barchi, 1982; French and Horn, 1983). One of the difficulties in understanding the underlying process is its complexity. This is apparent in a number of proposed kinetic schemes (e.g., see Armstrong and Bezanilla, 1977; Nonner, 1980). Macroscopic currents, the summed currents of thousands of individual channels, have two predominant phases in response to a brief depolarization: activation, an early phase in which channels are opening, and inactivation, in which channels later begin to close. There may be intricate interactions and considerable temporal overlap between these processes. It is not even clear whether the inactivation process is inherently voltage-dependent or gets its voltage dependence from being coupled to activation (see discussions in Armstrong, 1981; French and Horn, 1983). In addition, a slower process, usually called slow inactivation, reduces the macroscopic sodium currents when the membrane is held at depolarized potentials for seconds to minutes (Adelman and Palti, 1969; Chandler and Meves, 1970; Rudy, 1978; Bezanilla et al., 1982; Fernandez et al., 1982).

To simplify the analysis of the mechanisms underlying the gating of sodium channels, we decided to use chemically altered channels in which the fast inactivation was abolished (Armstrong et al., 1973; Oxford et al., 1978; Nonner et al., 1980; Oxford, 1981; Patlak and Horn, 1982). In this situation, we hope activation can be studied without the complications introduced by inactivation.

We have used excised-patch recording (Hamill et al., 1981) in GH_3 cells, a rat pituitary cell line. The patches were internally treated with *N*-bromoacetamide (NBA) to remove inactivation (Oxford et al., 1978; Oxford, 1981; Patlak and Horn, 1982). We examined the effect of membrane potential and temperature on the amplitude of sodium channel currents both in control patches and those treated with NBA. We also examined the effects of NBA on the relationship between open channel lifetime and

membrane potential which enabled us to estimate the separate effects of activation and fast inactivation on the open channel lifetime. Our experiments also revealed a slow inactivation-like process, which was more apparent after NBA treatment.

METHODS

All experiments were performed on tissue-cultured GH_3 cells, kindly provided to us by Drs. Aaron Fox and Julio Fernandez. The growth and subculturing of the cells are described by Fernandez et al.[1] The cells were plated onto coverslips which were then transferred to a glass-bottomed chamber for experiments.

The general procedure for recording has been described elsewhere (Hamill et al., 1981). We used outside-out patch recording for all experiments shown here. The head stage of the patch clamp contained a Siliconix U-430 dual FET amplifier (Siliconix, Santa Clara, CA; suggested to us by Dr. R. Levis) and a 10-GΩ feedback resistor. The frequency response was tuned up to 4 kHz with a high-frequency boost stage (Hamill et al., 1981). The patch electrodes were either pulled from Kovar sealing glass (Corning 7052, Corning Glass Works, Corning, NY; Rae and Levis, 1983) or from alumino-silicate glass (A-M Systems, Everett, WA). The platinum wire used in fire-polishing the patch electrodes was coated with Kovar sealing glass, allowing the tips of the pipettes to be coated through condensation of the volatile vapor products of the glass. This idea was suggested to us by Drs. G. Eisenman and J. Dani. All electrodes were Sylgard-coated to reduce input capacitance (Sylgard 184, Dow Corning, Midland, MI). The electrode resistances in the recording solutions were 3–15 MΩ.

The bathing solution was: 160 mM NaCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mMNa-HEPES (pH 7.4). The "internal" solution in the pipette contained 120 mM CsF, 11 mM EGTA, 2 mM$MgCl_2$, 1 mM $CaCl_2$, 10 mM Cs-HEPES (pH 7.4).[1] In most experiments 50–200 μM NBA (Sigma Chemical Company, St. Louis, MO) was added to the internal solution. We automatically corrected for a liquid junction potential of −10 mV in these solutions (cf. Fernandez et al., footnote 1). The bathing chamber was cooled for recording as indicated in the text. Voltages are given as inside minus outside.

Experimental Protocol

After obtaining a gigaohm seal, we disrupted the membrane under the pipette by gradual suction. Thereafter, we usually excised the patch within a few minutes. NBA began to remove fast inactivation within minutes (Patlak and Horn, 1982). Recordings were typically started about 20 min after excision. Excised patches were very hardy and often lasted >2 h.

Test pulses were applied from a holding potential in the range of −90 to −140 mV. In most experiments (as indicated in the text) the test pulse was preceded by a 20-ms prepulse to either −120 mV (for usual activating test pulses) or +50 mV (for tail-current measurements). The test pulse had a variable duration and voltage. The interpulse interval was also variable and ranged in our experiments from 200 to 1,500 ms.

In some experiments a leakage series was obtained by averaging and storing the currents from 16 pulse sequences using a P/-4 procedure starting at the holding potential (Bezanilla and Armstrong, 1976). This averaged record was scaled and added to each activating pulse sequence. The pulses were delivered by a programmable stimulator (PAGE-10, Page Electronics, Duarte, CA). The current was amplified, low-pass filtered by an eight-pole Bessel Filter (LPF 902, Frequency Devices, Haverhill, MA), and then sampled by a 12-bit analog-digital converter at intervals of 130–500 μs. The stimulating voltage pulses and current sampling were controlled by a PDP 11/34 computer (Digital Equipment Corp., Marlboro, MA) using Dan Brown BASIC modified by Gary Yellen. Each current trace was stored in a 256-word buffer on a floppy disk for later analysis.

DATA ANALYSIS

Current records were collected in blocks of 16, and were later analyzed in blocks. A "consecutive sequence" of records included an interruption of about 800 ms between each 16-record block for writing to a disk. The stimulus train was not interrupted, although the currents from zero to three pulses were missed after each block, depending on the interpulse interval. Records without channel openings were usually averaged and subtracted from each record in the block to remove residual capacity transients. Each digitized record was converted into a vector of integers, corresponding to the number of open channels at each time point (Patlak and Horn, 1982). This was accomplished by setting horizontal cursors on the displayed records at levels corresponding to single channel current levels. The threshold for a transition between adjacent levels was taken as halfway between the levels. Each record was examined after fitting in this manner, and questionable fits were discarded.

The series of vectors (typically representing 150–500 current records) produced by pulses to a given voltage were then examined for stationarity by averaging groups of vectors at the beginning and end of the series. Experimental series with obvious drift or rundown were discarded at this stage.

Maximum Likelihood Analysis

We used the maximum likelihood method for several aspects of our data analysis. This method can be used for estimating parameters and testing hypotheses (Hoel et al., 1971; Rao, 1973). Briefly, the method involves the calculation of the likelihood (i.e., the probability) of observing the experimental data for a given model, and then maximizing the likelihood with respect to the parameters of the model, thus yielding the maximum likelihood estimates of these parameters. Details of the method are discussed in Rao (1973).

We used this method to estimate the number of channels, N, in each experimental series (Patlak and Horn, 1982; Sachs et al., 1982). N was estimated at each time point after the onset of a depolarization (Patlak and Horn, 1982). A single value of N was usually obtained for all time points during a test pulse.

The estimate of open state lifetime is a simple procedure if one assumes that sodium channels have only one open state (Horn and Standen, 1983; see also Fenwick et al., 1982). The procedure requires no information about the number of channels. The maximum likelihood estimate of lifetime, θ, is

$$\hat{\theta} = \frac{\sum_i n_i T_i}{n} \qquad (1)$$

[1]Fernandez, J. M., A. Fox, and S. Krasne 1983. Comparison of whole cell and patch recording in GH_3 cells. *J. Physiol. (Lond.)*. Submitted for publication.

where T_i is the duration of n_i open channels and n is the number of observed closing transitions (Horn and Lange, 1983; Horn and Standen, 1983). If $L^*(x|\theta)$ denotes the logarithm of the likelihood of the experimental data, x, the variance of $\hat{\theta}$ is approximately

$$\mathrm{Var}(\hat{\theta}) = -\frac{1}{\frac{d^2}{d\hat{\theta}^2} L^*(x|\hat{\theta})}$$

(Hoel et al., 1971). In this example

$$\frac{d\,L^*(x|\theta)}{d\theta} = -\frac{n}{\theta} + \frac{\sum_i n_i T_i}{\theta^2}.$$

A trivial calculation shows that $\mathrm{Var}(\hat{\theta}) = \hat{\theta}^2/n$. We used this relationship to determine standard errors of our lifetime estimates.

The method of obtaining maximum likelihood estimates of the kinetic parameters for Markovian gating models is described in detail in Horn and Lange (1983). We have, however, made a few additions and improvements. We have, for example, estimated initial conditions, as well as rate constants in our models. We have also used a variable metric method to maximize the likelihood with respect to the parameters of interest (Powell, 1978). This method produced about a threefold reduction in the number of calculations of the likelihood than was needed previously. Since a single calculation of the likelihood for a given experimental series of records can take 10 min, using the continuous-time algorithm, this improvement is substantial indeed. We also analyze our experiments on a VAX 11/730 computer (Digital Equipment Corp., Marlboro, MA), which increases computational speed by about one third over the PDP 11/34.

RESULTS

Individual sodium channel currents were well resolved in outside-out patches over a voltage range from −60 to −10 mV, as shown in Fig. 1. Each panel in Fig. 1 shows two current responses, as well as the averaged current from at least 140 voltage pulses. The averaged responses are scaled to the same amplitude for all voltages to facilitate a comparison of their time courses. Depolarization increases the rates of both the rising (i.e., activation) and falling (i.e., inactivation) phases of averaged records, as typically observed for macroscopic sodium currents. At the single channel level depolarization has several obvious effects. Both the amplitude and durations of the single channel currents depend on voltage. These points will be discussed below. Another effect is on the time when openings occur after a depolarizing voltage step. Fig. 2 shows histograms of latency between the onset of a depolarization and the time of the first channel opening (Fukushima, 1981; Horn et al., 1981 *a*; Patlak and Horn, 1982). These histograms

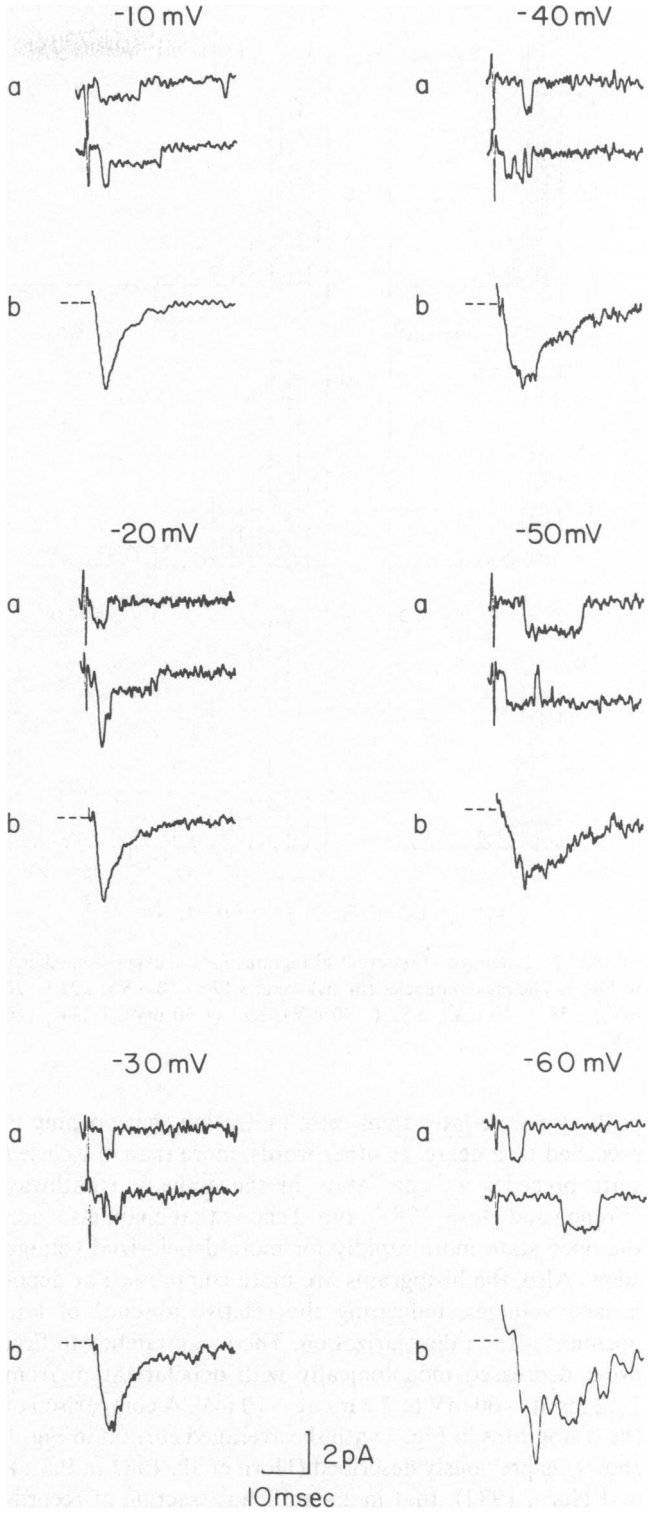

FIGURE 1 Examples of single channel currents (*a*) and averaged currents (*b*) from a control patch containing five channels. Averages of 140–446 records at each test potential were scaled to approximately the same peak amplitude. Holding potential = −120 mV, no prepulse; interpulse interval = 800–1,000 ms. Filter = 1.5 kHz for V = −20 to −50 mV; 1.0 kHz for V = −10 and −60 mV. The averaged records at −60 mV were additionally filtered with a digital gaussian filter at 490 Hz. Sampling interval = 190 μs for V = −10, 130 μs for V = −20 to −50, and 325 μs for V = −60 mV. Temperature = 9.1°C.

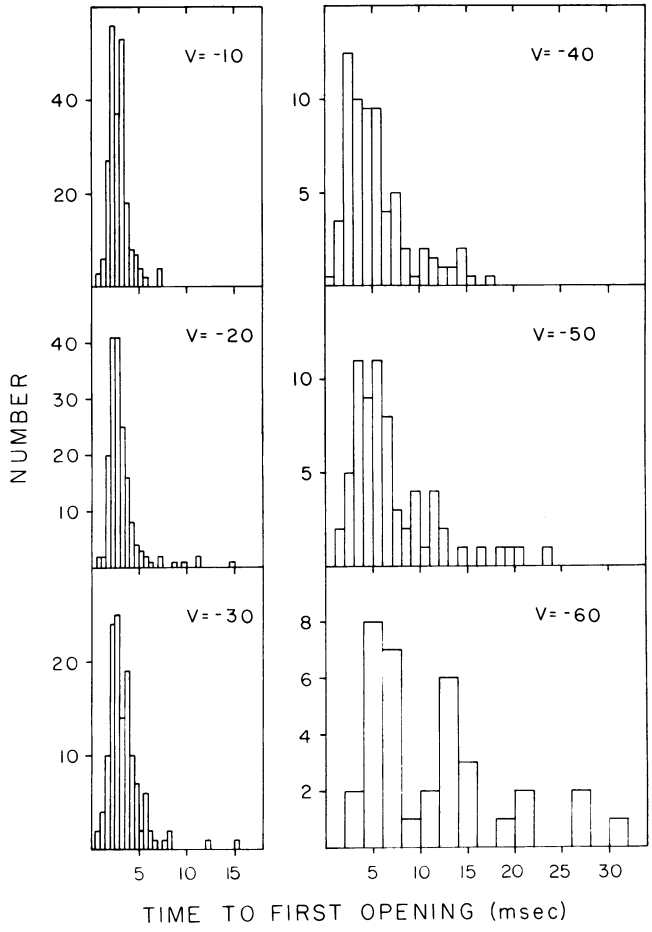

FIGURE 2 Latency-to-first-event histograms for the experiment shown in Fig. 1. The mean latencies (in ms) were: 3.17 (−10 mV), 3.25 (−20 mV), 3.58 (−30 mV), 5.83 (−40 mV), 7.27 (−50 mV), 11.24 (−60 mV).

peak at a time later than zero, indicating that opening is preceded by a delay. In other words, more than one closed state precedes an open state in the activation pathway (French and Horn, 1983). Fig. 2 shows that channels reach the open state more rapidly for more depolarized voltage steps. Also, the histograms are more compressed at depolarized voltages, indicating the relative absence of late openings after a depolarization. The mean latency to first event decreased monotonically with depolarization from 11.2 ms at −60 mV to 3.2 ms at −10 mV. A comparison of the histograms in Fig. 2 with the averaged current in Fig. 1 shows, as previously described (Horn et al., 1981 *a*; Patlak and Horn, 1982), that in a significant fraction of records channels are opening for the first time late in the record, when macroscopic inactivation is well underway. It is also clear from Fig. 1 that the open channel lifetime is shorter than the time course of the decay of averaged currents. These results support the idea that the time courses of activation and inactivation overlap to a great extent in this preparation, as in sodium channels of rat myotube (see discussion in French and Horn, 1983).

Evidence for a Slow Process

We found evidence for nonrandom clustering of records that exhibited channel opening in consecutive pulses. Fig. 3 shows a series of 28 consecutive responses to 40 ms pulses to −20 mV from a holding potential of −110 mV. The interpulse interval was 1 s. Eight of the 28 records were blanks, i.e., did not produce channel openings. The series shows a clear trend in that the blanks tend to be clustered together rather than randomly scattered among the records with openings. One gets the impression from this series that a hibernating channel wakes up every few seconds and can be opened by depolarization. Then it goes back into hibernation. This pattern is reminiscent of the

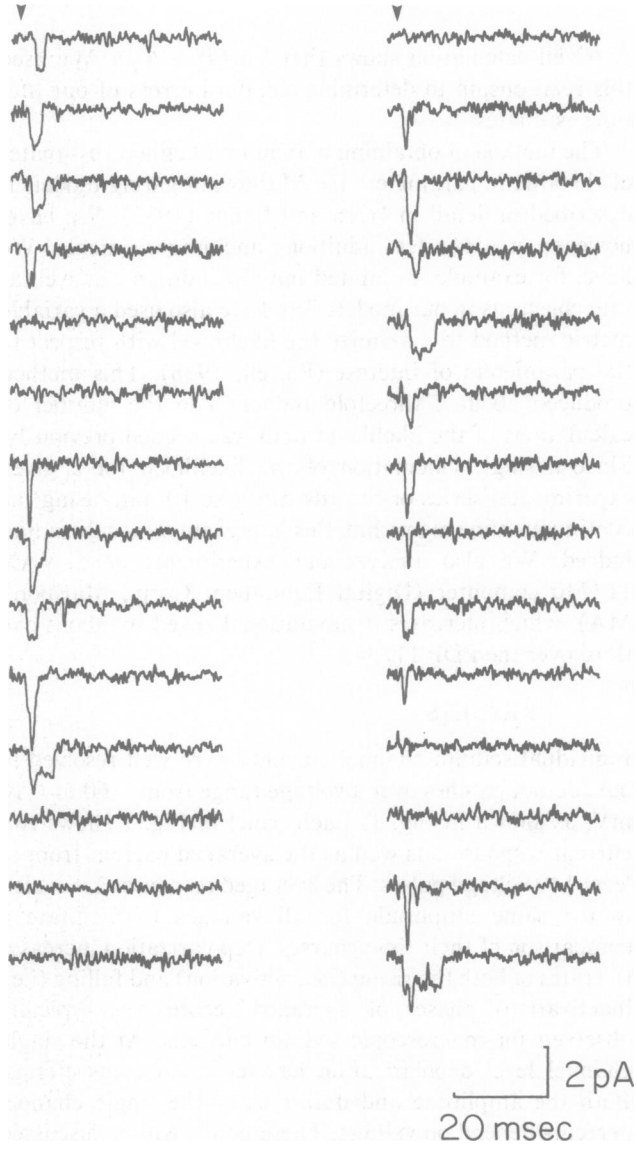

FIGURE 3 Twenty-eight consecutive current records (from top to bottom, beginning on the left column) showing clustering of records with and without openings. Test pulse to −20 mV (arrow) from a holding potential of −110 mV, no prepulse. The interpulse interval was 1 s. Temperature = 11.0°C.

behavior of acetylcholine-receptor channels at high agonist concentrations. Such channels slowly cycle into and out of a desensitized state (Sakmann et al., 1980).

"Runs analysis" can be used to test data such as these for randomness (Wald and Wolfowitz, 1940; Swed and Eisenhart, 1943; Gibbons, 1971). A "run" is a sequence of like elements. For example, our data can be seen as a sequence of seven runs that alternate between runs of blanks and runs with openings. If blanks tend to cluster together, the number of runs will tend to be smaller than expected for random ordering. By contrast if blanks never occur together, the number of runs will be larger than expected by chance. The exact probabilities for number of runs in ordered sequences of two types of elements are given in Swed and Eisenhart (1943). For the example in Fig. 3 the probability of observing 7 or fewer runs is 0.011. The most likely number of runs is 12, and the probability of observing 12 or fewer runs is 0.49. Note that this analysis underestimates the nonrandomness of sequential trials, because the patches contain more than one channel (see Discussion).

For larger samples, where the number of trials is >40, the exact distribution of the number of runs can be approximated by an asymptotic distribution, forming a standardized random variable, Z, with a mean of zero and variance of one (Gibbons, 1971). For our purposes

$$Z = -\frac{R - 2np(1-p)}{2\sqrt{n}\,p(1-p)} \qquad (2)$$

where R is the number of runs, n is the total number of trials, and p is the probability of at least one channel opening during a trial, i.e., the number of records containing at least one opening divided by n. The expected number of runs is $2np(1-p)$. When R equals this value, Z is 0. Positive values of Z correspond to clustering of records with openings. Negative values indicate a tendency to alternate between blanks and records with openings. Since our data show trends of a low number of runs, we utilized a one-tailed test. The associated P-value is simply the probability that a standard normal deviate is less than or equal to the observed Z.

We presumed that a channel can slowly cycle into and out of a nonactivatable state, from which it cannot be opened. When not in this state, a channel has some finite probability of being opened by a voltage pulse. If this is true, then randomness of a sequence of trials, as reflected by the variable Z, will depend on the stimulation rate. This is shown in Fig. 4 *A*.

Fig. 4 *A* plots Z vs. interpulse interval for the current responses to 45 ms pulses to −10 mV. The data show a trend toward nonrandom behavior for the shorter intervals, as expected. Dashed lines show the value of Z delimiting the critical regions, i.e., the $(1-\alpha)$th quantile point of the standard normal distribution, for the one-tailed test at α = 0.10 and 0.05. For the shortest interval, 200 ms, there were 61 runs in 157 trials, which contained 79 blanks. The expected number of runs for a random series is 79. Thus, the blanks show a significant tendency to be clustered together. In this experiment the clustering was not systematically related to p, the probability of channel opening.

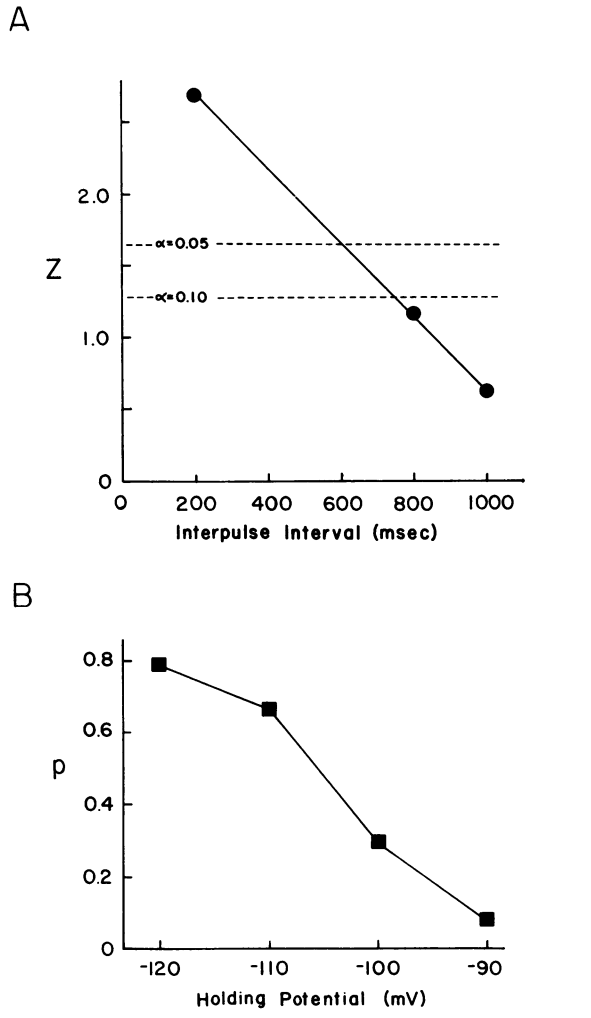

FIGURE 4 *A*, relationship between randomness of ordering of current records with and without openings. Z, (Eq. 2) plotted against interpulse interval. The critical region for Z is above the dotted lines at the 5% and 10% significance level. Each point shows data for 140–160 consecutive 45 ms pulses to −10 mV. Temperature = 9.1°C. *B*, effect of holding potential on p, the probability of a voltage pulse eliciting an opening. Test pulses to −20 mV applied directly from a holding potential of −90 to −120 mV. p was estimated from 95–144 records for each data point. Interpulse interval = 300 ms. Temperature = 11.0°C.

The above results show that nonrandom ordering of channel openings during sequences of pulses occurs when the stimulation rate is high. The clustering of events is probably due to the slow cycling of channels into and out of a nonactivatable, i.e., hibernating, configuration. This process cannot be observed if it is examined at intervals which are long by comparison with the cycle time of the process. In addition the depolarizations themselves might affect the cycle time of the process.

Although this slow process may be related in some way

to the slow inactivation of sodium channels, we have not tested this directly. Fig. 4 *B* shows that the probability of eliciting channel opening decreases as the holding potential is made more positive, as expected for slow inactivation. The midpoint of the curve varied between experiments in the range −85 to −110 mV. The relationship between Z and holding potential was complex in preliminary experiments. This may be due to the combined effects of holding potential on both p and the cycle time of slow inactivation.

Effects of NBA

We applied NBA to the cytoplasmic membrane surface of outside-out patches by adding it to the intracellular solution in the pipette. A concentration of 50–200 μM NBA began to slow the time course of fast inactivation within a few minutes, as in a previous report using inside-out patches (Patlak and Horn, 1982). Two obvious effects on the single-channel currents were an increased open time and a tendency for channels to continue opening late in the pulse. Extensive treatment, after about 20 min, usually removed all evidence of fast inactivation from averaged records (Fig. 8). Figs. 5 and 8 show examples of single-channel currents from an NBA-treated patch, illustrating the prolonged lifetime of open channels. Single channel current records typically showed long duration open times that were sometimes interrupted with brief closings (Figs. 5 and 8 *A*). This tendency towards bursting behavior of individual channels indicates the presence of at least 2 closed states, one with a brief dwell time corresponding to the occasional flickerings during a burst, and at least one other with a longer dwell time to account for the intervals between bursts.

Typically, the action of NBA proceeded in stages. First it increased open channel lifetime and slowed the inactivation process, but did not abolish it. This produced averaged currents which peaked and then slowly decreased to a steady state level (Patlak and Horn, 1982). After further treatment, NBA completely abolished fast inactivation in averaged records. This was usually accompanied by a slight, but consistent, change in the time course of the rising phase of averaged currents. As in NBA-treated patches of myotube membrane (Patlak and Horn, 1982), the averaged currents became somewhat less sigmoidal (i.e., more "exponential" than in control patches. A final effect of NBA in our experiments was a gradual reduction in the probability of a voltage pulse eliciting a channel opening. This effect, possibly related to slow inactivation, will be discussed below.

Fig. 6 shows that NBA has no effect on the amplitude of single-channel currents (filled symbols), by comparison with control patches (open symbols). The single-channel current-voltage (*I-V*) relationship was reasonably well fitted by a straight line over the range −60 to +10 mV. Fig. 6 also shows the effect of temperature on the *I-V* relationship in NBA-treated patches. The single-channel currents show only a modest temperature dependence (Q_{10}

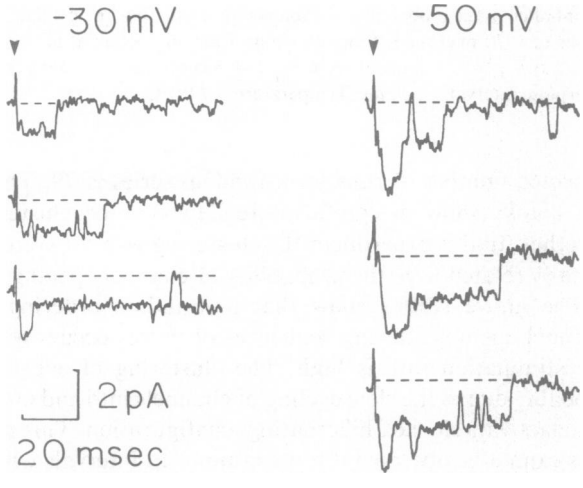

FIGURE 5 Single-channel tail currents after treatment with NBA. Currents recorded at −30 and −50 mV following a 30 ms prepulse to +50 mV from a holding potential of −110 mV. Interpulse interval = 1.5 s. Filtered at 1 kHz. Temperature = 9.2°C.

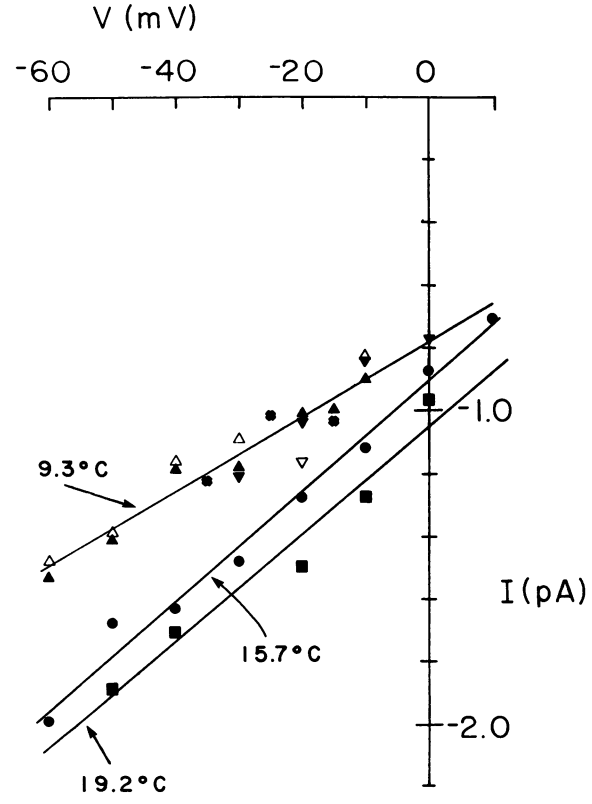

FIGURE 6 Single channel current vs. voltage relationship. Single channel current amplitudes measured at 19.2°C (■), 15.7°C (●), and 9.3°C (▲, ▼, x, △, ▽). Closed symbols from NBA-treated patches and open symbols from control patches. Standard errors are smaller than the size of the symbols. Lines are linear regression fits to the data giving correlation coefficients of 0.96 (9.3°C), 0.99 (15.7°C), and 0.98 (19.2°C). The slopes of the regression lines are 12 pmho at 9.3°C and 17 pmho at 15.7 and 19.2°C.

$\simeq$ 1.37 at -60 mV). The linear *I-V* relationship and the low Q_{10} both suggest the absence of rapid, unresolved gating over this voltage range (see Discussion). The single-channel slope conductance was 17 pmho at 15.7°C and 12 pmho at 9.3°C, which is similar to that found in sodium channels of tissue-cultured rat muscle (Sigworth and Neher, 1980; Horn et al., 1981 *b*).

We estimated lifetimes of channels in an open state using Eq 1. Fig. 7 shows the relationship between lifetime and voltage in both control and NBA-treated patches. In control records the lifetime is mildly voltage dependent, decreasing for voltages depolarized to -50 mV (see also Fig. 1). The decrease with depolarization is probably related to the inactivation process, since after NBA treatment the lifetime tends to increase nearly monotonically with depolarization. In one NBA-treated patch we estimated open time at -30 mV after either a hyperpolarizing prepulse of -120 mV (Fig. 8) or a depolarizing prepulse of $+50$ mV (Fig. 5). The estimates were similar, 12.8 ± 0.3 and 14.2 ± 0.9 ms for the two initial conditions. These are not significantly different at the 10% level, suggesting that an assumption of one open state is reasonable. The control results are similar to those reported by Fenwick et al. (1982) for bovine chromaffin cells, except that their lifetime-voltage relationship is shifted by about 20 mV in the depolarized direction.

NBA Causes Hibernation

After removing inactivation NBA slowly reduces the probability of channels opening during a pulse. The decreased probability could be caused by a destruction of channels. However, we found, in one patch analyzed in detail, that the number of channels estimated by the maximum likelihood method remained constant at four, throughout the 2.5 h period of observation, while the apparent probability of a channel being open slowly decreased. The decreased probability of opening was not measured systematically, but was not apparent during a typical experimental series of about 10 min. As in the case of pronase-treated patches (Patlak and Horn, 1982) the openings after prolonged NBA treatment appeared in cycles. For the experimental series that we analyzed in detail below, runs analysis showed a decidedly nonrandom pattern. We examined a sequence of 409 current responses to a 118 ms pulse to -30 mV from a holding potential of -90 mV (and preceded by a 30 ms hyperpolarization to -120 mV). The interpulse interval was 1 s. In this sequence there were 185 blanks and 177 runs. For this series, $Z = 2.06$, which is significantly nonrandom ($P \simeq 0.02$). For comparison, the expected number of runs for a series of 409 randomly ordered trials with 185 blanks is 204. Thus the data show a tendency for openings to occur in clusters. If this nonrandom behavior after treatment with NBA is due to the slow process we have called hibernation, it is possible that NBA is both shifting the channels into the hibernating state and slowing its cycle time so that nonrandomness is seen even for interpulse intervals of >1 s. We have seen this effect in every patch we have treated with NBA. However we have not compared these patches with control patches under comparable conditions (i.e., same voltages, temperatures, and interpulse intervals).

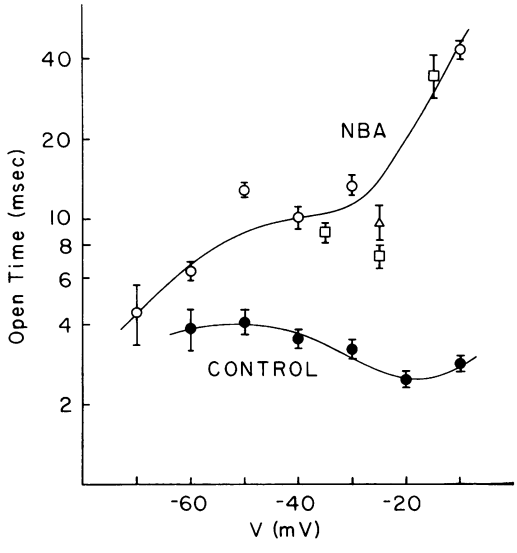

FIGURE 7 Open time plotted against membrane potential on a semilogarithmic scale. Control data (filled symbols) and NBA-treated data (open symbols) show mean and standard errors for lifetime of the open state. Each shape of open symbol represents a different experiment. Each symbol represents measurements containing 15–266 closing transitions. The smooth curves were drawn by eye. The NBA-treated data from -70 to -40 mV were obtained from tail currents after a prepulse to $+50$ mV (Fig. 5). All data were obtained at 9.0°–9.3°C.

We analyzed the currents of one NBA-treated patch in detail to estimate the fraction of channels in the hibernating state. We estimated the number of channels in the patch and determined that the channels behaved independently of one another by using a procedure (Fig. 8) similar to that of Patlak and Horn (1982). First the number of channels was estimated by a maximum likelihood procedure. We examined 391 current responses of the 409 pulses mentioned above. The remaining records were too noisy to yield unambiguous transitions. Three representative traces are shown in Fig. 8 *A*. The maximum number of channels open at one time was two, and the estimated number of channels was four. The probability, $P(t)$, of a channel being open during the depolarization is plotted in Fig. 8 *B*. Figs. 8 *C* and *D* show $P_0(t)$ and $P_1(t)$, the probability of having exactly zero and exactly one channel open, respectively, at each time point of the record. The continuous lines in Figs. 8 *C* and *D* are the predicted probabilities, using the values in Fig. 8 *B* along with the binomial theorem, and the discrete points were obtained from the measured frequencies of having either zero or one channel open. The agreement is excellent, supporting the notion that the channels in the patch comprise a homogeneous population of independently gated members. The first latency histogram for this experiment is shown in Fig. 9.

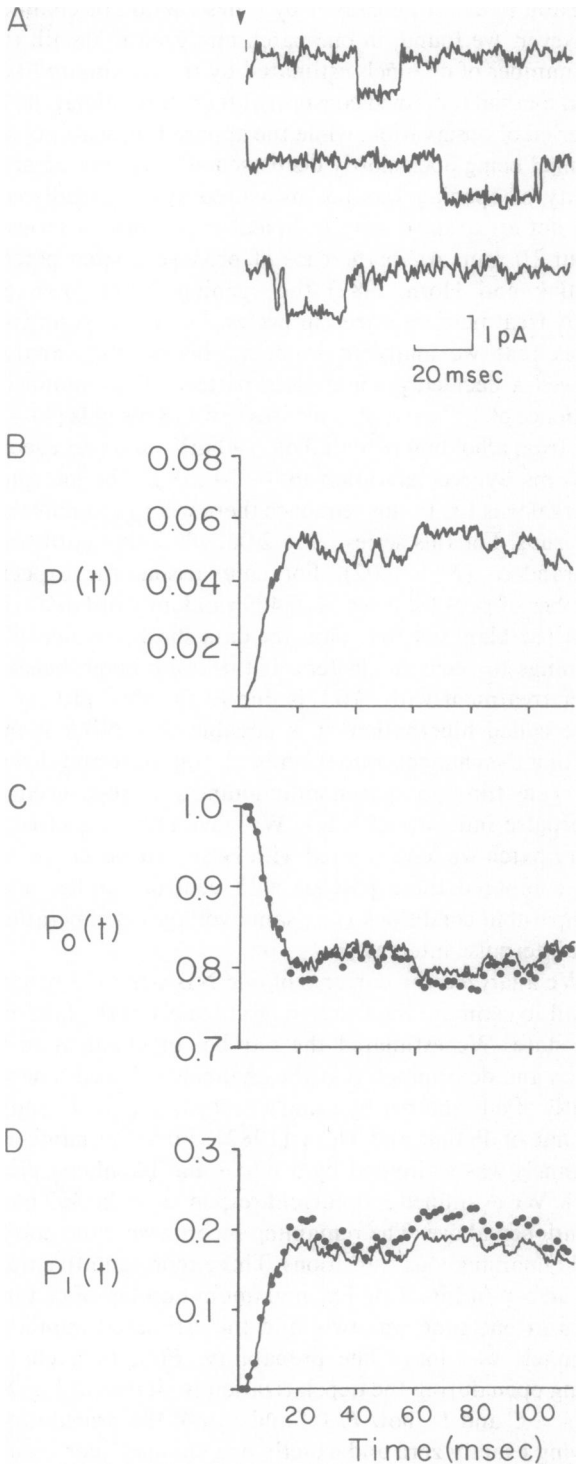

FIGURE 8 *A*, three current traces from an NBA-treated patch. Holding potential = −90 mV, prepulse = −120 mV, test potential = −30 mV. The patch contained four channels. Temperature = 9.5°C. *B*, probability of a channel being open, $P(t)$, obtained by summing the number of open channels at each time point for 391 records, and dividing by 4 × 391. *C*, probability of exactly zero channels being open, $P_o(t)$. The solid line (—) is the prediction of the binomial theorem, using $P(t)$ from *B*. The solid circles (•) were obtained from the relative frequency of no openings at each time point in the 391 records. *D*, the probability of exactly one channel being open, $P_1(t)$. The solid line (—) is the binomial prediction.

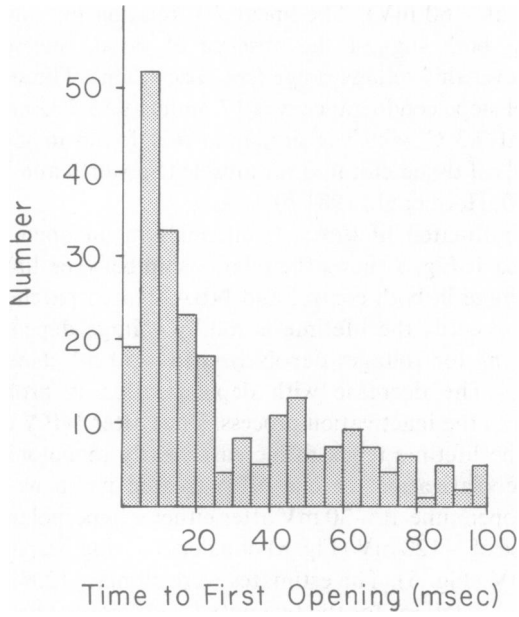

FIGURE 9 First latency histogram for an NBA-treated patch. Data from same experimental series as in Fig. 8. The mean latency was 29.4 ms.

Because the number of channels in this patch was not the same as that in the control (Figs. 1 and 2), they are not directly comparable. However, the long time to peak in the NBA-treated patch is indicative of either a decreased probability for channel opening, e.g., hibernation, compared to the control, a slower rate of activation for NBA-treated patches, or simply the effect of removing inactivation.

Because linear kinetic schemes are both reasonable and mathematically tractable, we fitted our NBA-treated data with such models (Bezanilla and Armstrong, 1977; Armstrong and Bezanilla, 1977; Armstrong and Gilly, 1979; Armstrong, 1981; Oxford, 1981; French and Horn, 1983). Linear sequential models were utilized with one closed hibernating state, C_H, either one or two closed nonhibernating states, C_1 and C_2, and one open state, O.

$$C_H \rightleftharpoons C_1 \ (\rightleftharpoons C_2) \rightleftharpoons O$$

The rates of transition into and out of the hibernating state were made very small by comparison with 1/(pulse duration), so that no transitions between the hibernating state and the closed states occurred during the 118 ms pulses. This seemed reasonable because the clustering of records with openings was on a time scale of seconds. We placed C_H at the end of the scheme to simplify analysis, without implying that hibernation occurs only from state C_1. We estimated rate constants for the transitions between the closed nonhibernating states C_1, C_2 and the open state O, in addition to the probability of a channel residing in C_H, C_1, or C_2 at the onset of a depolarization. (A detailed analysis, presenting estimates of rate constants and possible kinetic

models of activation, will be the subject of a future paper.) The probability of a channel being in the hibernating state at the onset of the pulse, $P_H(0)$, is model dependent. For the scheme above, with one closed nonhibernating state, $P_H(0)$ was 0.79 ± 0.01; when the second nonhibernating closed state was included, $P_H(0)$ was 0.69 ± 0.03. These estimates indicate that a substantial fraction of the NBA-treated channels were in a hibernating state, in agreement with interpretations derived from runs analysis.

DISCUSSION

We have examined the behavior of individual sodium channels in outside-out patches of GH_3 cells. These experiments confirm and extend previous work on single sodium channels in a variety of cells (e.g. Tissue-cultured rat muscle: Sigworth and Neher, 1980; Horn et al., 1981 *a*, *b*; Patlak and Horn, 1982; Neuroblastoma: Quandt and Narahashi, 1982; Horn and Standen, 1983; Bovine chromaffin cells: Fenwick et al., 1982; Tunicate eggs: Fukushima, 1981). Our analysis has concentrated primarily on two issues. First we examined a slow process akin to desensitization in acetylcholine receptor channels (Sakmann et al., 1980). Second we studied the effect of voltage on single channel amplitude and open time in control and NBA-treated patches. We will discuss these issues in turn.

We began to use NBA with the idea that it simply removes inactivation, leaving the activation gates intact (Oxford et al., 1978; Oxford, 1981). NBA's effect is clearly more complicated than that. It has a progressive action, first slowing the fast inactivation and finally removing it. Also it seems to remove gradually the sigmoidal nature of the averaged probability of a channel being open. Lastly it seems to drive channels reversibly into a nonactivatable configuration. The first two of these effects are consistent with the simple removal of inactivation. For example, the change in the sigmoidal character of the averaged currents could be explained by the removal of inactivation from closed states. However, the progressive decrease in the probability of a channel being activatable is qualitatively different. This could be due to the effect of NBA at a different site. These effects should be considered in further studies using this, or other drugs, that remove inactivation. The use of inside-out patches, which allow brief application of drugs, may eliminate some of the effects of NBA caused by its continued presence in the outside-out patch configuration.

Hibernation

Slow inactivation, which has been examined in some detail in squid axon, is the best-known slow process in sodium channels (Adelman and Palti, 1969; Chandler and Meves, 1970; Rudy, 1978; Bezanilla et al., 1982; Fernandez et al., 1982). Recently, Matteson and Armstrong (1982) have also postulated a population of "sleepy" channels at low temperatures. In our experiments the apparent rates of transition between activatable and nonactivatable channels, as measured by nonrandom clustering of blank records and records with openings, are slow, on the order of 1 Hz or less. This is slower than the kinetics of sleepy channels, and closer to those of slow inactivation. We have no evidence, however, that the process we observe is identical to that of slow inactivation. A definitive statement will require a comparison of the relative rates and voltage dependences of each type of experimental measurement. In the interim we have chosen to call the nonrandom process we observed "hibernation."

The technique of runs analysis that we have used to examine nonrandom patterns of openings can be applied quite easily in other single-channel studies. For example, in continuous records of currents one can divide the records into equal time segments and examine the sequence of blank segments with those containing openings. The method has at least one drawback, however. It will tend to underestimate the nonrandomness of the data, depending on the number of channels in the population. For example, if a patch contains many independent channels, each with a low probability of opening, the transitions into and out of an activatable state will not be synchronized for the whole population. In this case even marked nonrandom behavior for a single channel will be obscured by that of others. We have not been able to derive a simple extension of runs analysis to handle this complexity, even when the number of channels is known.

This bias could explain why nonrandomness is so apparent in NBA-treated patches. After prolonged exposure, NBA reduces the probability of a channel being activatable, which effectively decreases the number of activatable channels. This would tend to unmask the nonrandom clustering of openings of individual channels.

Voltage Dependence of Open Time

A Hodgkin-Huxley (1952) model predicts a bell-shaped relationship between open time (i.e., the inverse of closing rate) and membrane potential (Sigworth and Neher, 1980), because at depolarized potentials the inactivation rate constant β_h dominates, and increases with voltage. At hyperpolarized potentials β_h is small, and channels close with rate 3 β_m, which increases with hyperpolarization. Our control data (Fig. 7) show such a moderate bell-shaped relationship between the inverse of the closing rate and voltage, having a maximum at about −50 mV. The shape is especially apparent on a linear plot. The Hodgkin-Huxley model also predicts that removal of the inactivation gate will cause the open lifetime to decrease monotonically with hyperpolarization, asymptotically approaching the control value. Our data also agree with this (Fig. 7).

The relationship of open time to voltage in NBA-treated patches is more complicated than expected for a simple reaction. If the rate of channel closing represents a first-order transition, one might expect an exponential depen-

dence of closing rate on membrane potential, i.e., a straight line on a semilogarithmic plot, as in Fig. 7. However, the relationship is more complex, and seems to show a "dip" at the same voltage as seen in the control data. This might indicate that some portion of the inactivation process has not been completely removed. Alternatively, the closing rate constant may have a more complicated relationship with voltage (Neher and Stevens, 1977).

The decreased lifetime at hyperpolarized voltages is indicative of rapid gating kinetics in this range. This is consistent with rapid macroscopic kinetics, even for activating depolarizations, at voltages < -50 mV (Fernandez et al.).[1] Unresolved rapid kinetics may be responsible for the apparent decrease in single channel conductance at hyperpolarized potentials, reported in some preparations such as myelinated nerve (Sigworth, 1980), *Myxicola* axon (Goldman and Hahin, 1978), squid axon (Fishman et al., 1977), and bovine chromaffin cells (Fenwick et al., 1982). In GH_3 cells, however, we found that the single-channel conductance was independent of membrane potential over the range from -60 to $+10$ mV (Fig. 6). The absence of unresolved rapid gating in our data is also apparent from the Q_{10} of the single-channel current amplitude. Unresolved rapid gating would be expected to increase the Q_{10} for the apparent single-channel currents to levels typically found for gating. However the value of Q_{10} was about 1.35 for voltages between -60 and 0 mV. This is within the range usually found for permeation (Schwarz, 1980).

Our analysis of open-channel lifetime presumes that sodium channels have a single open state. This assumption is difficult to reconcile with some gating models (e.g. Armstrong and Bezanilla, 1977) and with nonstationary covariance measurements in node of Ranvier (Sigworth, 1981). Nonexponential open-time histograms for single sodium channels also support the idea of multiple open states, but are subject to a bias due to the problem of overlapping openings (Patlak and Horn, 1982; Horn and Standen, 1983). Our assumption of one open state is based in part on open-time histogram measurements in neuroblastoma cells, where the probability of overlapping events was reduced by slow inactivation (Horn and Standen, 1983), and by our own analysis on GH_3 cells. We found that the estimates of lifetime in an NBA-treated patch (at -30 mV) derived from pulses preceded by a hyperpolarization agree with those preceded by a depolarization to $+50$ mV. In other words the lifetime is the same during activation as it is during "deactivation." This suggests that a strong depolarization is not driving the channels into another open state. One aspect of our data remains puzzling however. In control patches we consistently noticed a slight increase in lifetime at depolarized voltages (Figs. 1 and 7). This could indicate the presence of another open state, or else of another type of channel, that opens at depolarized voltages and has a longer lifetime.

Interaction Between Activation and Inactivation

The increased lifetime after NBA treatment supports the idea that an inactivation gate can close sodium channels in the control case at voltages more depolarized than -70 mV. From the data of Fig. 7, a rate constant for inactivation from the open state can be estimated directly. We assume that a channel leaves the open state either by closing its activation gate, with a rate constant β_A, or by closing its fast inactivation gate, with a rate constant β_I. β_A might also be called the rate constant for deactivation (Oxford, 1981). In the control case, the mean open-state lifetime is $1/(\beta_A + \beta_I)$. After NBA treatment it is $1/\beta_A$. Fig. 10 *A* plots β_I as a function of voltage. It has an

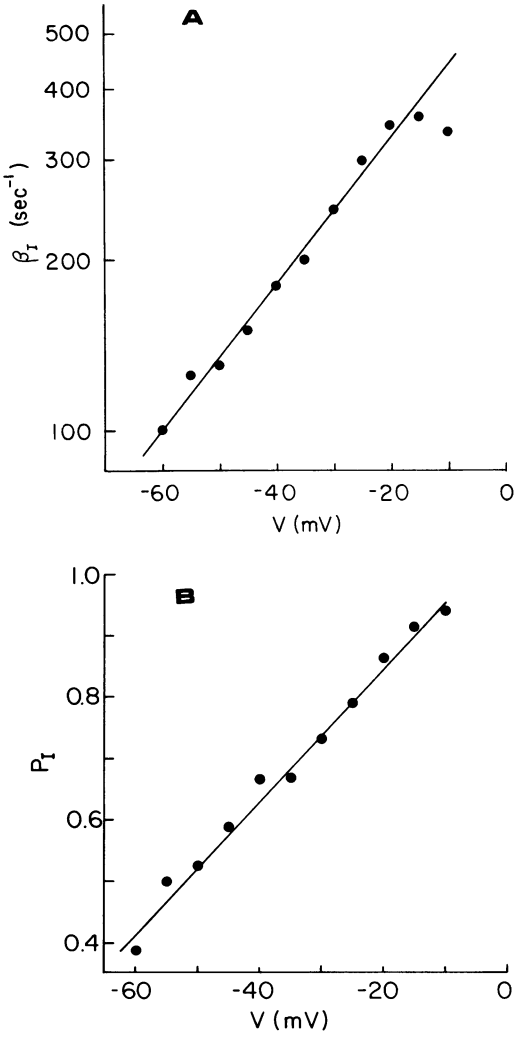

FIGURE 10 *A*, relationship between β_I and voltage, plotted on a semilogarithmic scale. The value at each voltage was obtained from the smooth lines in Fig. 7, corresponding to $1/\beta_A$ for the NBA case and $1/(\beta_A + \beta_I)$ in the control case. The straight line was drawn by eye. *B*, probability, P_I, as a function of voltage, that an open channel closes by inactivating, rather than deactivating. See text.

exponential dependence on membrane potential, increasing e-fold for 34 mV, suggesting a simple first-order reaction for inactivating from the open state. The voltage dependence is less steep than that derived for β_h by Hodgkin and Huxley (1952) for prepulse inactivation measurements, i.e., e-fold for 10 mV at negative voltages. This suggests that the voltage dependence of activation could play a role in the time course of the decay of macroscopic currents. Since the voltage dependences of β_A and β_I are in opposite directions, the open-state lifetime is only mildly voltage dependent.

Our analysis takes the position that an open channel can close in two ways, either by inactivating with probability $P_I = \beta_I/(\beta_A + \beta_I)$, or else by deactivating with probability $P_A = 1 - P_I$. These two probable destinations of an open channel are voltage dependent, as shown in Fig. 10 *B*, which plots P_I from -60 to -10 mV. Over this range the probability of an open channel closing its inactivation gate increases from 0.39 to 0.94. That is, open channels tend to deactivate at negative voltages and inactivate at positive voltages. Clearly the relationship is sigmoidal over a wider voltage range. The results of Fig. 10, to our knowledge, are the first demonstration that the inactivation process is inherently voltage dependent; it does not entirely depend on being coupled to activation for its voltage dependence.

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DISCUSSION

Session Chairman: Charles F. Stevens *Scribe*: Stephen Holloway

SPALDING: In view of the fact that you can see hibernation in both the untreated and the NBA-treated patch, what is the quantitative evidence that there is a particular hibernation associated with NBA?

HORN: In the presence of NBA the probability of a channel opening over a given voltage pulse decreases over a period of tens of minutes. Judging from the maximum likelihood estimates, the number of channels appeared to stay constant during our experiments. The rate of activation seen from the average records also stayed about the same. What that means is that the channels become nonactivable during the course of the experiment with NBA.

NBA is an irreversible reagent, but we don't remove it. The development of hibernation might be stopped if we used inside-out patches and washed out the NBA. We haven't investigated that. Recently, we have used trypsin to remove inactivation. Trypsin does not seem to have the effect of causing hibernation.

SPALDING: When I remove sodium activation by applying NBA in frog muscle, I find consistently that peak current decreases while inactivation is being removed. I wonder if that might be a macroscopic manifestation of hibernation.

HORN: That seems perfectly reasonable.

PATLAK: When we applied NBA to rat muscle cells (Patlak and Horn, 1982), we saw almost a 10-fold increase in single-channel lifetime at −40 mV. Here at the same potential you see only a twofold increase. Do you think that this difference is due to the species difference?

HORN: I'm not sure. We have not repeated these experiments on rat muscles.

STEVENS: Do you remember what the mean open time was before the NBA treatment?

HORN: It was 3 ms in both cases.

STEVENS: So the difference is not in the control.

PATLAK: Right. Another difference between the two experiments was that NBA was applied transiently to the muscle cells, while the GH_3 cells were constantly in NBA.

McCARTHY: Using neuroblastoma cells, I also see the behavior you describe where several runs with channels are followed by several runs without channels. The probability of seeing the data in Fig. 3 is 0.01, assuming it is a random process, but the corresponding probability in Fig. 4 *a* appears to be higher. What is the mean and variance of this probability among different patches?

HORN: The probability of nonrandom clustering was highly variable. The variability had a lot to do with the number of channels in the patch. The more channels, the more randomness, as you might expect from several independent channels hibernating but out of synchrony with one another.

McCARTHY: We still see clustering even when pulsing every 3 or 4 s.

HORN: If we pulse every 1.5 s, we usually don't see much clustering, but it varies from experiment to experiment.

STEVENS: What is the effect of holding potential on clustering?

HORN: We tried to look at that, but it ended up being very complicated. Initially we thought it would be slow inactivation. The problem is that if there is slow inactivation and one holds at a more negative potential, then you bring more channels into the activable pool and that makes it appear more random. As you depolarize, you remove the channels from the pool; this accentuates nonrandomness. The interaction between the number of channels and the apparent nonrandomness is a difficult problem. We could not think of a rigorous statistical way of dealing with it. So we stopped trying that analysis.

LECAR: Permitting independent measurements of rates, as opposed to extracting them from relaxation times, is one of the strengths of single-channel analysis over voltage-clamp. Your rate constants are well fit as exponential functions of voltage. How general is this satisfying observation?

HORN: There are several examples where rate constants are exponential functions of voltage from single-channel measurements. Chris Miller's sarcoplasmic reticulum channel and the Leibowitz and Dionne results presented at this meeting are examples.