OPENTIME HETEROGENEITY DURING BURSTING OF SODIUM CHANNELS IN FROG SKELETAL MUSCLE

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ABSTRACT Single voltage-activated Na^+ channel currents were obtained from membrane patches on internally dialyzed skeletal muscle segments of adult frogs. The high channel density in these membranes permitted frequent observation of the "bursting mode" of individual Na^+ channels during 400-ms records. We examined the opentimes within and between bursts on individual membrane patches. We used a new nonparametric statistical procedure to test for heterogeneity in the opentime distributions. We found that although 80% of all bursts consisted of opentimes drawn from a single distribution, the opentime distribution varied significantly from burst to burst. Significant heterogeneity was also detected within the remaining 20% of individual bursts. Our results indicate that the gating kinetics of individual Na^+ channels are heterogeneous, and that they may occasionally change in a single channel.

INTRODUCTION

The simplest way to model the functional behavior of specific membrane channels is to make the assumption that each channel behaves as one member of a homogeneous population. Electrical recording of the currents through single channels, however, has indicated that this may be an oversimplification. Multiple populations of channels have been demonstrated in single membrane patches (Hamill and Sakmann, 1981), as have shifts in the kinetic modes of one channel's function (Patlak et al., 1979; Hess et al., 1984; Horn et al., 1984). Nevertheless, the hypothesis of homogeneity is still usually applied to the subpopulations or individual modes in order to allow simple models to be developed. Our recent measurements on the Na^+ channels in adult mammalian cardiac myocytes and in frog skeletal muscle have shown that the channels can function in several different modes, one of which is prolonged bursting (Patlak and Ortiz, 1985*a*, *b*, 1986). We applied several statistical techniques to test the hypothesis that all channels in the bursting mode have the same opentime distribution. Our analysis indicated that individual bursts differed in their kinetics, and thus the hypothesis of homogeneity may be untenable.

METHODS

Sartorius muscles from *Rana pipiens* were used for these studies. Frogs were sacrificed and the muscles dissected using standard procedures. The muscles were soaked in a low-Na^+ saline (in millimoles per liter: TMA Cl, 90; NaCl, 10; KCl, 3; $CaCl_2$, 0.2; $MgCl_2$, 1; HEPES, 5; Glucose, 5; pH 7.4) containing 3 mg/ml collagenase (type 1A, Sigma Chemical Company St. Louis, MO) for 2–3 h at room temperature, then bathed in low-Na^+ saline containing 0.1 mg/ml protease (Type VII, Sigma Chemical Co.) for 20 min. The cells were transferred to a "relaxing" solution (in mM: Cs-Aspartate, 95; $MgCl_2$, 3; Na_2ATP, 5; HEPES, 5; EGTA, 0.1; pH 7.4) and soaked for 30 min. The fiber bundles were cut repeatedly in the transverse direction to produce many short segments of cut-open, internally dialyzed, cell segments, which were then dispersed by trituration.

Patch recordings were made with the cells bathed in fresh relaxing solution at 10°C. The patch pipette was filled with frog saline solution (in mM: NaCl, 100; KCl, 3; $CaCl_2$, 0.2; $MgCl_2$, 1; HEPES, 5; Glucose, 5; pH 7.4). Electrodes were placed ~500 μm from the cut end of the fiber where no signs of the progressive, slow disorganization of the muscle ends could be detected. Gigaohm seals could be readily obtained on these cells and the patches generally had between 50 and 500 channels.

Patch electronics were standard (Hamill et al., 1981). The analog current signal was filtered at 2 KHz with an eight-pole Bessel filter, then sampled at 10 KHz with a microcomputer. Since the cells were cut open, their resting potential was very close to zero, and all potentials reported are those applied with the patch pipette. 400-ms-long test pulses were delivered sequentially to −50, −40, −30, and −20 mV at 0.1 Hz from a holding potential of −120 mV. The interval between pulses to the same potential was thus 40 s.

The currents from ~10 ms after the start of the pulse consisted of (*a*) a background Na^+ current, which had occasional isolated short openings, and (*b*) occasional prolonged bursts of openings with total mean duration >100 ms. We measured the current amplitude of each burst, then idealized the record using the half-amplitude crossing criterion (Colquhoun and Sigworth, 1983). Opentime arrays, from which the burst statistics were calculated, were derived from the idealized records. The calculation of the burst variability statistic, S, and the scrambling of opentimes to calculate the null hypothesis statistic, S^*, are described below and were programmed using Turbo Pascal (Borland International, Inc., Scotts Valley, CA) on an IBM PC computer. The results that we report here were derived from the study of fifteen separate patch recordings, although we concentrate on nine instances where three or more bursts were observed in a single patch at one potential.

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RESULTS

Fig. 1 shows 10 consecutive records at a pulse potential of −40 mV. The "fast" inward Na^+ current, which was large in this patch (more than 50 pA), inactivated within ~10 ms, and is truncated in these high resolution recordings. Subsequent "late" currents show the two modes of behavior that have been previously observed for Na^+ channels in muscle, background and burst modes (Patlak and Ortiz, 1986). Background currents are present in all ten traces; they are characterized by isolated short openings. Bursts are highly correlated series of openings, presumed to be due to the prolonged activity of one channel. Bursts can be seen in the second and seventh traces of the figure. Our studies have shown that Na^+ channels are responsible for both forms of the late currents: They occur in proportion to the number of channels in the patch, they have the same slope conductance and reversal potential as the fast currents, and both fast and late currents disappear when the membrane is depolarized for extended periods.

As can be seen in the figure, the opentimes for the burst in the second trace appear to be considerably longer than for the burst in the seventh trace. We see such differences in opentime kinetics in many of the bursts that we have recorded, although the differences are usually less obvious than in this example. Such differences might occur due to random association of openings drawn from a single open time distribution. Alternatively, they may indicate heterogeneity of the closing rate from burst to burst. We sought to discriminate between these hypotheses.

One established nonparametric method to determine if two samples come from the same distribution is the Kolmogorov-Smirnov test (Smirnov, 1948), where the maximum difference between the cumulative percentage distributions of two samples is compared with that expected for random samples from one distribution. When applied to the two bursts in Fig. 1, for example, the maximum difference between the two distributions is 35.7%, indicating that the distributions are significantly different ($P < 0.001$). The Kolmogorov-Smirnov test has limited value, however, because it only allows comparison of burst pairs.

Another established method to make such discriminations is the likelihood ratio test. If the number of openings in each burst is large, and if the opentimes are exponentially distributed, then a likelihood ratio test is well defined

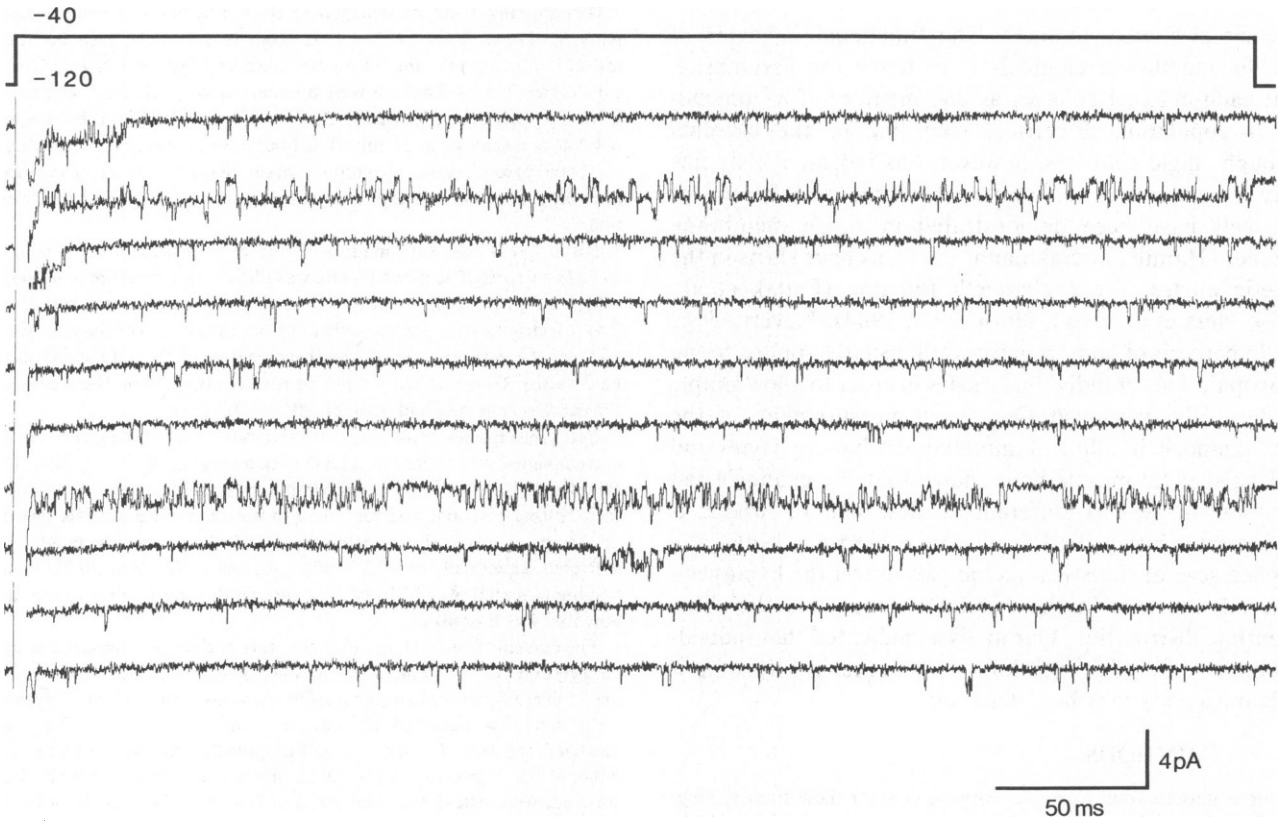

FIGURE 1 Patch recordings of late currents through Na^+ channels. The topmost trace represents the 400 ms voltage pulse from holding potential of −120 mV to a test potential of −40 mV. The subsequent traces are the currents recorded from one patch during 10 sequential test pulses. (Pulses to three other potentials were given between the illustrated traces, so the interval between each trace was 40 s). The initial surge of inward Na^+ current (truncated in these records) was >50 pA, corresponding to the activity of ~250 channels in this patch. The early currents inactivated within the first 10 ms, revealing late Na^+ currents that consisted of background and burst currents. Each burst appears to have been the prolonged activity of an individual channel. The second and seventh traces show such bursting channels, and they illustrate the variation in kinetics that are often seen within one patch.

(see Appendix). However, we found that two exponentials are sometimes needed to fit the distributions of opentimes within individual bursts. We therefore developed a more general nonparametric test for heterogeneity.

If we make the assumption that channels have a unique opentime distribution and that it is the same from one burst to another (i.e., channels are homogeneous), then the opentime within any burst will be sampled from a single parent population of opentimes. The mean opentime for a burst will be independent of the order of the sampled opentimes. We tested this null hypothesis in the following way. Suppose the opentimes for k bursts are listed in order of appearance, each burst, $j = 1,2,\ldots,k$, having n_j openings. Let $\bar{T}_j$ be the mean opentime of the jth burst. A weighted estimate of the variability of $\bar{T}_j$ is given by

$$S = \sum_{j=1}^{k} n_j(\bar{T}_j - \bar{T})^2,$$

where $\bar{T}$ is the grand mean of the $n = \Sigma_{j=1}^{k} n_j$ opentimes. If the mean opentimes in individual bursts are significantly different from those in other bursts, then the value of the statistic S will be larger than expected for a homogeneous population of opentimes.

We obtained an estimate of the expected value of S under the null hypothesis by scrambling the order of the n opentimes and recalculating S. The process of scrambling, i.e., randomizing the order of the original opentimes, leads to the creation of an artificial data set. This was done 1,000 times to generate 1,000 scambled data sets. For each we calculated the value of the above statistic, denoted S_i^*, $i = 1,2,\ldots,1,000$. The empirical distribution of S_i^* can be compared with the original value of S obtained from the ordered data to see if the latter is unusually large, leading to rejection of the null hypothesis. This analysis is a type of randomization test. Such tests have been shown to be most powerful for a large class of alternative hypotheses (Lehmann, 1959).

We saw nine instances where three or more bursts occurred in a single patch at one membrane potential. For example, the bursts shown in Fig. 1 were second and third of the four bursts that were observed during 50 pulses to −40 mV. These bursts had 80, 128, 212, and 56 openings each. The mean opentime for all observations was 1.75 ms, while the means for each of the bursts were 1.8, 2.54, 1.05, and 2.56 ms, respectively. The value of S for these originally ordered data was 217.4.

Fig. 2 *A* shows the distribution of S^* for these bursts, plotted in the form of a histogram. The original value of S, indicated by the arrow, is larger than the largest value of S^*. The probability of this occurring is <.005 (confidence coefficient = 0.993, Lawless, 1982) under the null hypothesis. The mean value of S^*, 11.3, and its standard deviation, 9.1, also suggest, even though S^* is not Gaussian, that the original S did not arise as a random sample from this distribution. Further samples of this analysis are summarized in Table I. The analysis indicated marked heterogeneity in seven out of the nine instances of three or more bursts in a single patch.

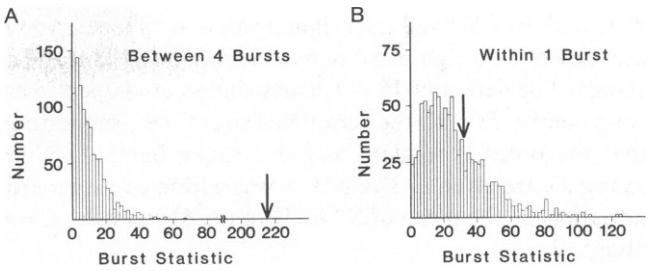

FIGURE 2 Statistical analysis of the variability between and within bursts. (*A*) The variability statistic, S, was measured between the four bursts seen at −40 mV in the patch illustrated in Fig. 1 (I.D. No. 18/−40). The magnitude of S is indicated by the arrow. The individual bars are a histogram of 1,000 values of the null hypothesis statistic, S^*, obtained by repeatedly scrambling the opentimes from the four bursts and recalculating the variability statistic. The value of S, 217.4, is much greater than the mean value of S^*, 11.3, indicating that the variability between bursts is greater than expected for a homogeneous population of channels. (*B*) The variability statistic, S, measured during the burst shown in the second trace of Fig. 1 (I.D. No. 18/−40, Burst 2) was calculated by dividing the burst into five subsections, each with an approximately equal number of openings. The value of S, 31.3, is indicated by the arrow. The null statistic, S^*, was calculated as above by scrambling the opentimes 1,000 times. The histogram shows that the resulting distribution, which has a mean value, 26.7, is not significantly different than S, indicating that the five subsections of the burst were drawn from the same opentime distribution.

The above results show that the order of appearance of opentimes in our original data is not random, i.e., that opentimes are different from one burst to another. It is also interesting to ask whether the opentime kinetics of a channel are constant within the 400 ms duration of one burst. We therefore determined the statistic S for a single burst by dividing the burst artificially into five subsections, each with approximately the same number of opentimes, and then tried our scrambling method. The result of this analysis on the first burst in Fig. 1 is shown in Fig. 2 *B*. For these data, S was 30.3. As above we created 1,000 scrambled data sets and calculated the statistic S_i^* for each. The

TABLE I
VARIABILITY BETWEEN BURSTS IN THE SAME PATCH

I.D.	Number of openings	Mean	S	Mean S^* ± SD	$\%S^* > S$
		ms			
1/−30	825	1.26	128.0‡	7.9 ± 5.8	0
1/−40	660	0.62	4.7‡	.9 ± .8	0.2
2/−20	272	2.51	23.5	21.7 ± 17.0	35
2/−40	406	0.91	14.2‡	1.7 ± 1.8	0
7/−40	429	1.08	5.3	3.3 ± 2.7	21
8/−20	515	2.11	168.3‡	34.6 ± 24.1	0
9/−40	336	0.91	29.4‡	2.2 ± 2.1	0
18/−20	707	2.19	387.6‡	40.2 ± 23.7	0
18/−40	476	1.75	217.4‡	11.3 ± 9.1	0

‡Greater than two standard deviations from the mean value of S*

distribution of S^* and the original value for S (see arrow) are shown in the figure. S^* had a mean value of 28.0 and a standard deviation of 19.4. Clearly the ordered opentimes were consistent with the scrambled opentimes, suggesting that the order is random within a single burst. Further examples are given in Table II. S was within two standard deviations of the mean of S^* in 41 of the 51 bursts that we observed.

The remaining 10 bursts (three of which are shown in Table II) were not homogeneous. In each case S is several standard deviations from the mean S^*. Furthermore, the fraction of S^* estimates that exceed S is very low. Both tests indicate that the heterogeneity is highly significant in these cases. For example, the second burst (trace 7) in Fig. 1 was heterogeneous. A period of increased opentimes, which can be seen in the latter half of the burst, probably gave rise to the heterogeneity that we detected.

DISCUSSION

The primary observation that we report here is that the opentime kinetics of bursting Na^+ channels are usually heterogeneous between different bursts, but are generally not so within a single burst. Although the underlying cause for the channel heterogeneity is not known, the interpretation of our results is compelling because of the nonparametric and general nature of the test that we used.

Various expressions of heterogeneity are already known, and might be possible explanations for the functional heterogeneity that we observe. In some cases, channels are expressed in two or more forms that are permanently different within the time course of a single experiment. For example, different genes may code for several types of channel that are mixed in the membrane. Alternatively, permanent post-transcriptional modification might result in different sub-populations of channels. Expressions of both TTX-sensitive and TTX-insensitive Na^+ channels in denervated or developing muscle is one example of such permanent heterogeneity (Sherman and Catterall, 1985). Another example could be enzymatic degradation or other experimentally induced channel alterations. These permanent channel differences would result in recordings in which kinetics could differ from one channel to the next.

Alternatively, channels may change their function reversibly during an experiment. If the change in function is limited to a few kinetic possibilities, and if the time spent in each is long enough to define its characteristics, then such alterations would be recognized as mode shifts (Hess et al., 1984). Ca, Na^+, and Glutamate-Activated channels have all been shown to undergo such shifts (Patlak et al., 1979; Hess et al., 1984; Horn et al., 1984; Moczydlowski et al., 1984; Weiss et al., 1984). Mode shifts in channels could be due to a reversible chemical modification (e.g., phosphorylation, methylation) or to changes in the protein's conformation on a slow time scale.

The appearance of bursting behavior in late Na^+ channel currents (Patlak and Ortiz, 1985*a*, *b*, 1986) probably represents such a mode shift. However, one might reasonably expect that channels functioning within a single mode would be homogeneous. Our data comparing different bursts indicate, however, that this is not the case. The heterogeneity that we observe may represent yet another reversible process affecting the channel. This additional kinetic variability might not be distinguishable as switching between several discrete modes if the number of such modes are great, and the modes are not very different from one another, or if the switching is very rapid. The statistical analysis of individual bursts is particularly useful because it shows: (*a*) that kinetics can shift during the 400 ms bursting of one channel, i.e., that the process appears to involve reversible shifts, not permanent differences between channels, (*b*) that such shifts are only seen in 20% of bursts, indicating that the dwell time for a kinetic behavior is of the order of seconds.

In two out of nine cases we failed to detect heterogeneity between different bursts in a patch. This failure to reject the null hypothesis may have two causes. First, the channels in these two patches may have been homogeneous. Alternatively, the channels may have come from a heterogeneous population, but the four bursts that we observed in each case happened to have had opentimes very similar to one another. Larger numbers of bursts in future samples would help distinguish between these possibilities.

Although the mechanism of Na^+ channel bursting is not established, the kinetics of open and closed times during bursts are consistent with the hypothesis that bursts are due to transient dysfunction of the channel's inactivation gate (Patlak and Ortiz, 1986). If so, then the heterogeneity that we observe is in the channel's activation process. This is yet another indication that the gating of Na^+ channels is very complicated, apparently including sudden shifts between modes and/or heterogeneity in both activation and inactivation. It is our opinion that kinetic models of Na^+ channels must consider these possibilities.

TABLE II
VARIABILITY WITHIN INDIVIDUAL BURSTS

I.D.	Burst	Number of openings	Mean	S	$S^* \pm SD$	$\%S^* > S$
			ms			
18/−20	1	155	1.29	10.1	7.6 ± 5.1	25
	2	78	2.72	42.4	40.9 ± 25.6	38
	3	57	3.32	50.2	57.2 ± 37.6	52
	4	60	1.85	7.4	11.0 ± 7.5	64
	5	175	1.86	68.2‡	19.6 ± 13.3	1
	6	102	3.37	51.4	39.4 ± 27.3	26
	7	80	2.15	69.5‡	21.9 ± 13.5	1
18/−40	1	80	1.80	6.6	11.7 ± 7.9	71
	2	128	2.54	31.3	26.7 ± 17.7	34
	3	212	1.05	13.1‡	4.3 ± 2.9	0.5
	4	56	2.56	12.8	22.7 ± 14.6	73

‡Greater than two standard deviations from the mean value of S*

APPENDIX

One simple way of testing whether the opentimes differ between bursts is to start with the assumption that the opentimes in each burst are exponentially distributed. Under the null hypothesis that all opentimes are a sample from an exponential distribution with a parameter (i.e., rate constant) θ, the likelihood of all the data at one membrane potential is

$$L(\theta) = \prod_{i=1}^{n} [\theta \cdot \exp(-\theta \cdot T_i)],$$

where there are n openings of duration T_i, $i = 1,\ldots,n$. The maximum likelihood estimate of θ is the inverse of $\bar{T}$, the grand mean opentime.

The alternative hypothesis is that each of the k bursts has its own θ_i, $i = 1,\ldots k$, rate constant. The likelihood for this hypothesis is

$$L(\theta) = \prod_{i=1}^{k} \left\{ \prod_{j=1}^{n_i} [\theta_i \cdot \exp(-\theta_i \cdot T_j)] \right\},$$

where each burst has n_i, $i = 1,\ldots k$, openings, $n = \Sigma_{i=1}^{k} n_i$, and the maximum likelihood estimate of θ_i is the inverse of $\bar{T}_i$, the mean opentime of the ith burst.

The null hypothesis is a smoothly parameterized subset of the alternative hypothesis. Therefore a likelihood ratio test is appropriate here (Rao, 1973). In this case

$$2 \cdot \left[n \cdot \log(\bar{T}) - \sum_{i=1}^{k} n_i \cdot \log(\bar{T}_i) \right]$$

has a chi-squared distribution asymptotically, with $k - 1$ degrees of freedom, under the null hypothesis. We used this test (results not shown) on the above data, and found that it produced the same conclusions as our more general, nonparametric method.

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