MODAL GATING BEHAVIOR OF CARDIAC SODIUM CHANNELS IN CELL-FREE MEMBRANE PATCHES

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ABSTRACT Single voltage-activated Na^+ channel currents were obtained from membrane patches of isolated ventricular cells of guinea pig hearts. The currents were compared when measured from cell-attached patches and from the same patch but at least 20 minutes after manual excision. The averaged currents showed a distinctly delayed decay in the excised patches due to the appearance of long lasting openings or bursts of openings. In contrast to control patches, the open time distribution in excised patches requires at least two exponentials. A short mean open time was voltage independent for cell-attached patches (0.38 ms ± 0.07 ms between −60 and −20 mV, 6 cell-attached patches; and 0.41 ± 0.1 ms, 7 excised patches). The long mean open time found in excised patches was clearly voltage dependent and increased from 0.48 ± 0.14 ms (−80 mV) to 2.87 ± 0.35 ms (−20 mV, regression coefficeint +0.88, 7 patches). Sweeps with long openings appeared in clusters. The clustering of records with long openings, short openings, or without openings (nulls) was quantified by a runs analysis which showed a highly significant nonrandom ordering. The results show that in excised patches inactivation is temporally hibernating.

INTRODUCTION

The typical gating behavior of cardiac Na channels is characterized by a clustering of short openings at the very beginning of a depolarizing voltage step, indicating that the channel is quickly transfered into an inactivated or absorbing state (Kunze et al., 1985; Patlak and Ortiz, 1985; Wilson et al., 1985). However, in a small percentage of sweeps, long lasting or burstlike openings can be observed (Kohlhardt et al., 1985; Patlak and Ortiz, 1985; Nilius et al., 1986, 1987*a*). The coexistence of obviously different kinetic schemes within the same sample of trials challenges the assumption that Na channels can only operate in a certain kinetic scheme. It will be shown that the cardiac Na channel can undergo slow transitions between different sets of states in a distinct state model. Such transitions have already been proposed for Na channels (Patlak and Ortiz, 1986; Patlak et al, 1986; Nilius et al. 1987*b*) as well as for Ca channels (Hess et al, 1984) and Cl channels (Blatz aned Magleby, 1986). The slow transition between different sets of kinetic states ("modes") should be satisfied by at least three criteria: (*a*) the coexistence of kinetic schemes within the same sample that are distinctly different, (*b*) a slow shift between these different gating schemes, e.g., a clustering of sweeps showing only one type of gating, (*c*) the availability of a tool that distinctly changes the sojourns of the channel in a certain kinetic scheme. The present studies address the question of the existence of a modal gating behavior in cardiac Na channels. It will be shown that after manual excision of cardiac membrane patches the Na channel transiently loses its absorbing state (inactivation). An alternating shift between a mode with inactivation and one with defective inactivation can be observed, indicating a modal gating behavior of these channels.

METHODS

All experiments were performed on 200–300-g guinea pigs. Ventricular cells were dissociated as described elsewhere (Kao et al., 180; Nilius et al., 1987*a*). For dissociation 0.1% collagenase (Sigma Chemical Co., St. Louis, MO, type I) and 0.1% hyaluronidase were used. The cells were four times washed with Eagle's Minimum Essential Medium (Immuchemie, Berlin, GDR) and stored for some hours in the same medium. For patch clamp measurements the isolated and washed cells were transfered into a 0.1-ml chamber and were superfused with the following solution (mM): 140 K-aspartate, 10 EGTA, 1 $MgCl_2$, 5 Hepes, pH 7.4, titrated with KOH. In this solution the membrane potential is near zero. After washing and superfusion it seems unlikely that residual proteases from the cell isolation may influence the gating behavior of the Na channels. The patch clamp electrodes were filled with a Hepes-buffered solution (mM): 140 NaCl, 5.4 KH_2PO_4, 2.5 $CaCl_2$, 11 glucose, 5 Hepes, titrated with NaOH to pH 7.4. All experiments were performed at room temperature (20 ± 1°C). The patch pipette used was extremely small (resistance >20 M) and selected for a small number (~8% of the patches were single channel patches) of Na channels in the patch.

The patch clamp device was standard (Hamill et al., 1986). The currents were digitized at 10 kHz with an 8-bit A/D-converter and filtered with a low pass Bessel filter (48 dB/oct) set to 2 kHz. Mean currents were determined by averaging the signals each time over the ensemble of identical pulses consisting in 320 points each. Data were corrected for leakage and capacity transients by subtracting the averaged blank sweeps ("nulls"). A "half of unitary current amplitude" scheme was used for detection of state transitions of the channel. Histograms were described by sum of exponential fits (Marquardt-Levenberg algorithm, Brown and Dennis, 1972).

The mean open times in multichannel patches were estimated as $\tau_o = \Sigma_j n_j T_j/N$ where T_j is the duration of n_j open channels and N is the total number of channel closings (see also Horn et al., 1984). Alternatively, the

mean open time in multichannel patches was calculated from the histrograms obtained from traces with no overlapping events. Both types of analysis produced nearly the same values. If only traces with nonoverlapping single channel currents were analyzed in all patches two sets of openings could be detected: (*a*) short (S) openings with a mean open time of ~ 0.4 ms at potentials between −60 and −20 mV, and (*b*) long openings (L) defined by groups of openings longer than four times the mean open time of S openings separated by gaps shorter than four times the fast shut time (~0.2 ms). The pattern of openings was analyzed with a test for randomness (Wald and Wolfowitz, 1940; Swed and Eisenhart, 1943). Averaged data are expressed in mean ± SE of mean. The method used and the analyses have been described in detail elsewhere (Nilius et al., 1987*b*).

RESULTS

The gating behavior of single Na channels as shown in Fig. 1 is typical of most of the patches examined. Short openings (S) are seen in the first five sweeps that are followed by three nulls. The ninth and tenth sweep shows long openings (L). The averaged current exhibits a fast inactivation that is described by a time constant of 2.3 ms. The right panel in Fig. 1 shows 13 consecutive sweeps from the same patch but 22 min after manual excision of the patch. In the excised patch the appearance of long lasting or "bursty" openings is much more frequent than in the cell attached configuration. Two out of 76 sweeps showed L openings in the cell-attached patch, but 13 in 76 sweeps after excision were observed. The averaged current declined with a time constant of 3.4 ms. The mean probability of the channel opening at the time of the peak of the current was decreased from 0.41 to 0.39 by excision. The average number of openings per sweep was calculated from the distribution of openings per sweep from 1.4 (cell-attached) to 3.5 (cell-free) at −50 mV. The differences that were observed between excised and cell-attached patches also appeared but were quantitatively less accentuated when 20 mM ATP was added to the 140 mM K^+-aspartate solution. The patch shown in Fig. 1 was obviously a single channel patch (no overlapping events within 368 sweeps). Therefore, a transition of the individual channel between a slow and long duration ("bursty") opening mode seems to be most likely.

To quantify the obvious differences in the two types of openings the distribution of the open times was compared in cell-attached patches and after excision in inside-out patches. In cell-attached patches before excision (six patches, two single-channel patches, three patches with two channels, one patch with three channels) the distribution of the open times could be fit with a single exponential curve. If the data were taken from all controls obtained

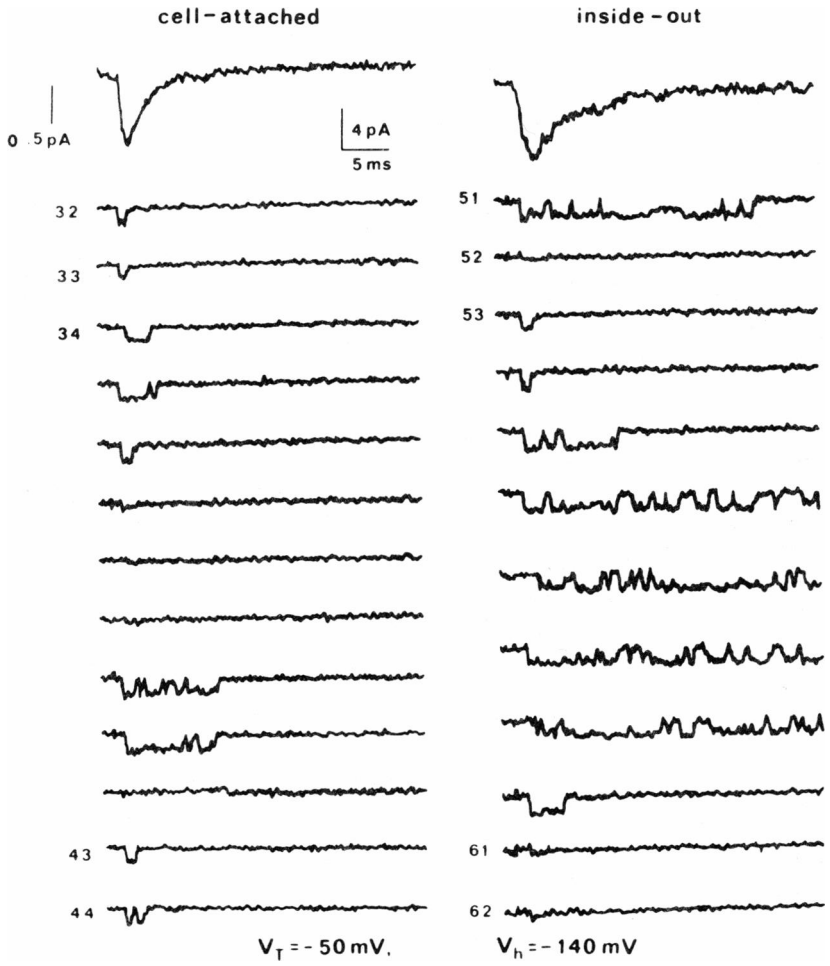

FIGURE 1 Changes in the gating behavior of cardiac sodium channels after manual excision. (*Left*) Cell-attached single-channel patch, 13 consecutive sweeps, holding potential −140 mV, 40 ms test stop to −50 mV. Note the long (or "bursty") openings in sweep 40 and 41. (*Right*) Same patch 22 min after manual excision, same voltage protocol. Sweep 51, and sweeps 55–60 show long-lasting openings. Note the slow decay of the averaged current. The averaged current at the top of the two panels was obtained from 76 sweeps (2 kHz filter, 10 kHz sampling rate, 1.2 Hz pacing rate, cell 070487-5).

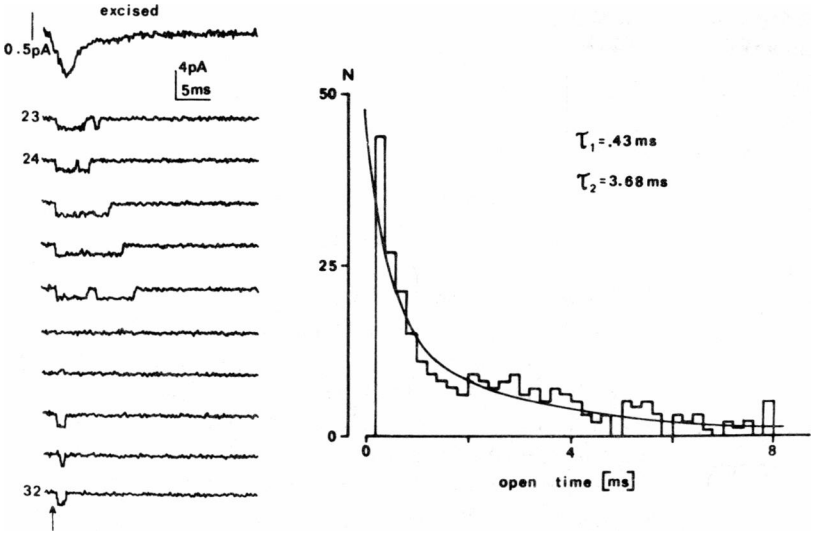

FIGURE 2 Biexponential distribution of the open times of a Na channel in an excised patch. The records shown in the left panel were obtained 35 min after excision. The 10 consecutive sweeps show five long-lasting (L) openings followed by three nulls that are followed by three S openings. The mean open times as calculated from the biexponential fit of the open-time distribution were 0.43 ms for S, 3.68 for L openings, respectively. The histrogram was fitted by $N = 36.3. \exp(-t/0.43) + 11.3 \exp(-t/3.68)$ (holding potential −120 mV, 40 ms step to −50 ms, sampling rate 10 kHz, filter 2 kHz, cell 070487-21). Note again the clustering of S, L openings, and nulls.

from cell-attached patches, a voltage-independent mean open time of 0.41 ± 0.06 ms (21 patches, 47 histograms) was measured at voltages between −60 and −20 mV. In the patches selected here for comparison between cell-attached and inside-out configuration the same behavior is shown (Fig. 3, left-hand panel).

The mean open time τ_o was voltage-independent between −60 and −20 mV (τ_o = 0.38 ± 0.07 ms, n = 10). For −80 and −70 mV the mean open time was significantly shorter than between −60 and −20 mV (τ_o = 0.21 ± 0.05 ms, n = 4). In contrast, the distribution of the open times in patches excised for >20 min could not be described with only one exponential. At least two time constants were necessary to fit the open time distributions (Fig. 2). The short mean open time was nonsignificantly changed compared with the cell-attached control patches (0.41 ± 0.1 ms, obtained from 11 histrograms, seven patches) between −50 and −20 mV. L openings have a mean open time of 3.7 ms in the example shown in Fig. 2 that is more than nine times the mean open time of S openings in the same experiments. A striking difference between S and L openings in excised patches, however, concerned their voltage dependence. In seven patches (one single channel patch was obviously spontaneously excised, same patches as in the cell-attached case) the mean open time was increased from 0.48 ± 0.14 ms (two patches) at −80 mV to 2.87 ± 0.35 ms at −20 mV (three patches). The correlation coefficient for linear correlation was 0.88 (14 points, seven patches). This clear voltage dependence of the mean open time of L openings contrasts with the behavior of the majority of openings (S openings) in cell attached patches (r = 0.16 between −60 and −20 mV). The dependence of the mean open time on the test potential is summarized in Fig. 3.

These results support the hypothesis that the cardiac Na channel, at least after excision, can fluctuate between different kinetic schemes. However, it should be tested to see whether the channel can really stay in one of the

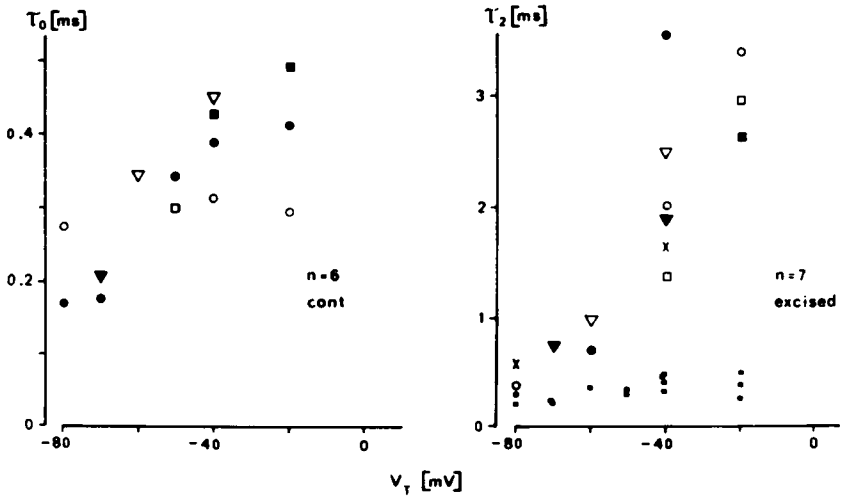

FIGURE 3 Voltage dependence of the mean open times in cell-attached patches (*left*) and from the same patches more than 20 min after manual excision (*right*). The values were obtained from the analysis shown in Fig. 2 (two single channel patches (o), three patches with two channels, one patch with three channels (o); one record was obtained from a spontaneously excised patch (×). Different symbols represent different patches. For controls only the mean open time from monoexponential fits was plotted against the test potential. No voltage dependence could be observed between −60 and −20 mV (τ_o = 0.38 ± 0.07 ms, n = 10, left side). The long mean open time, τ_2, in excised patches was clearly voltage dependent (r = 0.88, for details see text). No open time histrogram was calculated for the two-channel patch at −40 mV. The small symbols at the right panel reflect the voltage dependence of τ_o from cell-attached patches but in the same scaling as for cell-free patches, τ_2 (same values as in the left diagram).

proposed kinetic schemes for a long time. The previously discussed series of consecutive records show a clear trend in that the different types of openings and nulls tend to appear in groups rather than being randomly scattered. To check the randomness of the sweeps with different patterns of gating a "runs analysis" was performed. A "run" is defined as a sequence of like elements. Two different sets of elements were defined: element A, sweeps with no openings ("nulls", 0); element B, sweeps with at least one opening (short or long openings, SvL); or, element A*, sweeps being a "null" or showing short openings (OvS), and element B*, sweeps showing at least one long opening (L).

If there are m objects of one kind of element and n objects of another kind, the possible number of arrangements of the $m + n$ elements is

$$C_m^{m+n} = \frac{(m+n)!}{m!n!} = C_n^{m+n}.$$

If r is the number of groups of like elements (runs) then the probability to find R or less runs in a randomly ordered population is

$$P(r \le R) = (C_n^{m+n})^{-1} \cdot \sum_{r=2}^{R} f_r,$$

where

$$f_r = 2\, C_{k-1}^{m-1} \cdot C_{k-1}^{n-1}, \text{ where } r = 2k,$$

$$f_r = C_{k-1}^{m-1} \cdot C_{k-2}^{n-1} + C_{k-2}^{m-1} \cdot C_{k-1}^{n-1}, \text{ when } r = 2k - 1$$

(Wald and Wolfowitz, 1940).

For example, the three-channel patch in Fig. 4, left panel, shows three A elements (sweeps being "nulls," O), eight B* elements (sweeps with at least one L opening), and six sweeps that only show S openings. If the appearance of A elements is tested against B elements, one will count three runs. The probability of finding such a sequence is 0.025. If A* elements are tested against B* elements four runs can be counted, e.g., $P(r \le 4) = 0.0053$.

For larger samples, where the number of sweeps is greater than 40, the exact distribution of the number of runs can be approximated by an asymptotic distribution. Z

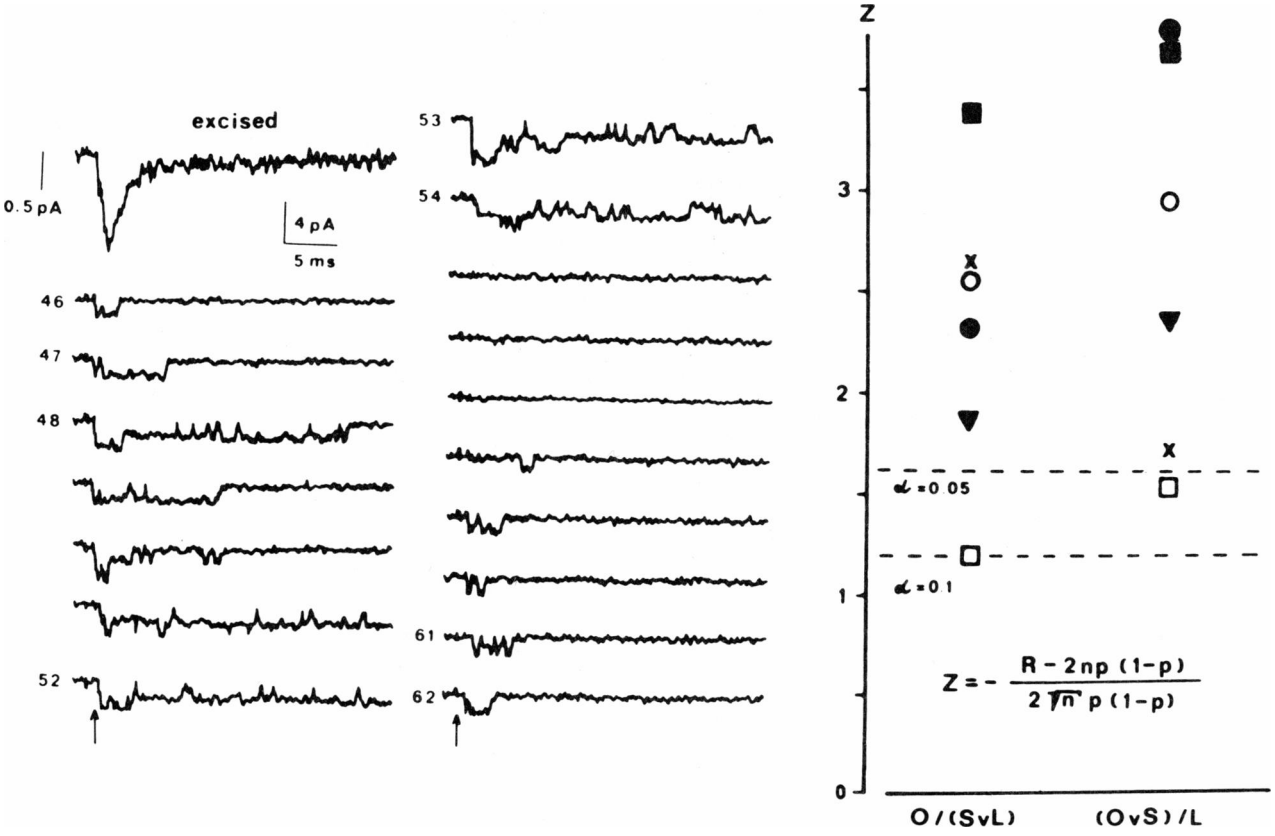

FIGURE 4 Run analysis of the gating behavior in an excised patch. At the left hand side 17 consecutive sweeps are plotted exhibiting three nulls, eight L, and six S openings. The number of runs for sweeps showing "nulls" vs. sweeps with S or L openings is three (O/SvL). The number of runs for (OvS/L) is four. The probability of observing three or fewer runs in the first case is 0.025. In the second case, the probability of observing four runs is 0.0053. Because three channels are in the patch this analysis underestimates, however, the nonrandomness of the sequential trials. The right panel summarizes the results from six patches. 76 sweeps were analyzed to calculate the random variable Z (inset, formula 4). The dotted lines mark the 5% and 105 significance level. Z above these lines indicate a nonrandom ordering, e.g., clustering, of runs (for further explanations see text, same symbols as in Fig. 3, holding potential −140 mV, test potential −40 mV).

is a standardized random variable with a mean of zero and a variance of one (Horn et al., 1984) and can be calculated by

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

where R is the number of runs (defined for A vs. B elements, or A* vs. B* elements), n the number of sweeps (76 in all tested patches). p is the probability to find an element A (test O/SvL) or to find an element B* (test OvS/L). The expected number of runs is $2np(1-p)$. If Z is above 1.64 or 1.21 then the probability that the runs are nonrandomly ordered is <0.05 and 0.1, respectively. For example, the consecutive 17 sweeps shown in Fig. 4 were taken from a sample of 76 sweeps with 18 A elements ("nulls"), 25 B* elements, and 37 sweeps with only S openings.

For the test (O/SvL) 20 runs were counted yielding a Z value of 2.37 (o) and $P(r \leq 20) = 0.0035$. For the test (OvS/L) 19 runs were observed. The corresponding Z value is 3.78 (o) and $P(r \leq 19) = 0.000047$. These data show that the runs tend to be grouped, e.g., it can be presumed that a channel will slowly alternate between kinetic schemes showing only L or S openings and those in which openings did not occur. The same result was obtained from six patches as shown in the right panel of Fig. 4.

DISCUSSION

The results presented above show that the gating of cardiac Na channels can be sensitively influenced by manual excision. A similar result has been reported by Horn and Vandenberg (1986) for sodium channels in tissue-cultured GH_3 cells from a rat pituitary cell line. In the case of GH_3 cells the changes induced by excision were dependent on the anion in the internal solution and also mainly appeared in a burstiness of openings that resulted in a delayed macroscopic inactivation. However, no detailed analysis has been reported. In cardiac Na^+ channels the following changes occurred about 20 min after excisio in a K^+-aspartate solution and were stable for nearly 1 h: (*a*) the appearance of long openings resulting in a second time constant in the fits of the open time distribution, (*b*) the voltage dependence of the long mean open time, (*c*) a grouping of traces showing only nulls, S or L openings. These changes are probably not due to the procedure of excision because a slowing down of the inactivation of macroscopic sodium currents can also be observed in whole cell measurements with intracellular dialysis (Nilius and Benndorf for the same cells, unpublished, see also Vandenberg and Horn, 1984 for GH_3 cells). It was also excluded that proteases at the surface of the membrane are responsible for the changes (Horn and Vandenberg, 1986). ATP (20 mM) in the bath solution also did not change the obvious alterations in the process of inactivation. One can speculate that the observed changes could be due to phosphorylation reactions in the cell that may stabilize the inactivation process.

The results showed that in the case of cardiac Na channel there is (*a*) a gating pattern consisting of kinetically different schemes within the same sample that are distinctly different, (*b*) a clearcut grouping of traces with similar gating behavior, (*c*) a tool (manual excision) to favor the appearance of long ("bursty") openings. Another tool to induce similar changes is the compound DPI 201-106 (Nilius, 1987; Nilius et al., 1987*b*). For these reasons, a modal gating behaviour of cardiac Na^+ channels seems to be the case. It is argued that the changes in the gating of Na channels is limited to a few kinetic schemes. From runs analysis it is obvious that the channel spends a time in each kinetic scheme that is long enough to define its characteristics in accordance with the mode-concept (Hess et al., 1984). Long openings might be due to a defect in the channel inactivation. The prolongation of the mean open time of the L openings with increased depolarizations could be due to a decrease in the backward rate from the open to the closed state. Horn and Vandeberg (1986) showed that depolarization increases the rate of inactivation. The well-established property that the open times of the Na channels are not very voltage dependent could therefore be explained by an opposite voltage dependence of the backward rate from the open into the closed state and the rate of inactivation. An alternative interpretation is also possible (Aldrich et al., 1983). If the inactivation is fast and not very voltage dependent, when compared with the backward rate from the open state to the closed state, a defective inactivation would increase the mean open time and would unmask the voltage dependence of the backward rate coefficient from the open to the closed state. This mechanism would also fit both the increased number of openings per sweep ("burstiness") and the increase in the mean open time due to stronger depolarizations.

Furthermore, the distinct voltage dependence of the mean open time of DPI-modified Na channels could be also explained by stabilizing the Na channel in a mode with defective inactivation.

In generalizing the results, it is tempting to speculate that cardiac Na^+ channels can jump between three different modes of gating: (*a*) mode 1 is characterized by a fast entrance into an absorbing state, the channel is available to open; (*b*) mode 2 is characterized by long lasting openings (or bursts of openings, Patlak and Ortiz, 1985) due to a defect in the inactivation; (*c*) mode 3 represents the channel in a nonavailable state. The normal gating is a shift between mode 1 and 3 whereas the channel will enter mode 2 only exceptional by excision. DPI and also Aconitine (Nilius et al. 1986, 1987*b*) favor mode 2; however lidocaine shifts the channel into a mode 3 (Nilius et al., 1987*a*).

The most severe shortcoming of this idea of a modal gating behavior of cardiac Na^+ channels is, however, the

striking heterogeneity of the open time during a sojourn in a state with defective inactivation (Patlak et al., 1986).

Received for publication 17 July 1987 and in final form 14 January 1988.

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