EVIDENCE FOR INTERACTIONS BETWEEN BATRACHOTOXIN-MODIFIED CHANNELS IN HYBRID NEUROBLASTOMA CELLS

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ABSTRACT Current records from voltage-clamped membrane patches containing two batrachotoxin-modified sodium channels were analyzed to determine whether these channels are identical and independent. In most two-channel patches, the experimentally observed probabilities that zero, one, or two channels are open differ from the binomial distribution, demonstrating that the two channels are nonidentical or nonindependent or both. From the same current records, we also determined the rate for the transition from two open channels to one open channel and for the transition from one open channel to zero open channels. These data are consistent with closing rates for the two channels that are equal and independent. Both probability and closing rate data can be fit by a model wherein the channels are identical, the closing rates are independent, and the opening rate is greater when the other channel is closed than when it is open. The implications of this model for analyzing noise spectra and current variance are examined.

INTRODUCTION

Independence of ionic channels is an important assumption for analyzing single-channel current fluctuations in voltage clamped membrane patches and noise spectra of voltage-clamped cells (Neher and Stevens, 1977). Moreover, it is usually accompanied by the assumption that ionic channels of the same kind are identical (e.g. Colquhoun and Hawkes, 1983). We have tested these assumptions in a particularly simple case—patches that contain two batrachotoxin (BTX)-modified sodium channels. If the channels are identical and independent, then the probability that 2, 1, or 0 channels are open should follow a binomial distribution. A departure from this distribution would indicate that at least one of the assumptions is false.

BTX-modified sodium channels are particularly convenient for our purpose because they do not inactivate (Khodorov, 1978; Huang et al.,1982). Also, the number of channels present in a membrane patch can be unambiguously determined by examining the current record for a voltage where the channels are almost always open. A previous analysis of one-channel patches showed relatively simple kinetics involving two closed states and one open state (Huang et al., 1984).

One motivation for testing the independence hypothesis is that recent reports suggest that sodium channels form dimers or trimers (Almers et al., 1983; Almers and Stirling, 1984; Aldrich et al., 1983; Catterall and Morrow, 1978; Angelides and Nutter, 1984), structures that might lead to nonindependence.

METHODS

Cell Culture and Experimental Setup

Cell preparations and electrophysiological setups used are the same as those described by Huang et al. (1984). The hybrid neuroblastoma NG108-15 cells were a kind gift from Dr. Werner Klee of the National Institute of Mental Health, and batrachotoxin(BTX) was generously supplied by Dr. John W. Daly of the National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases. The external medium contained 130 mM NaCl, 30 mM tetraethylammonium (TEA) chloride, 0.5 mM $CaCl_2$, 0.8 mM $MgCl_2$, 5.4 mM KCl, and 20 mM HEPES-Tris buffer (pH 7.4). Experiments were carried out between 1 and 4 h after adding a saturation dose of 3.5 μM BTX to the external medium at 23°C. Under this condition the resting potential of the cell is close to 0 mV (Huang et al., 1984).

Patch pipettes were fabricated following Hamill et al. (1981), had resistance of 5 ± 1 M Ω, and were filled with the same solution as the external medium. The area of membrane patches formed is estimated to be between 1 and 4 μm^2 (Sackmann and Neher, 1983). After establishing a seal of ~10 G Ω between the pipette and cell membrane, the patch was voltage clamped in the cell attached configuration and current was recorded with an L/M-EPC5 amplifier (List-Electronic, Darmstadt, West Germany). The electrodes were Ag/AgCl half cells from E. W. Wright (New Haven, CT). The current was processed with an analog filter (Krohn-Hite, Cambridge , MA) operating in low-pass rc-mode with a cutoff frequency of 1.5 kHz. Its performance was similar to a four-pole Bessel filter, and the frequency at -3db level was 0.5 kHz. This filtering is adequate for the channels studied, because the relaxation times expected for the transitions of channels are more than ten times longer than the filtering time constant.

Data Acquisition and Reduction

Data acquisition was performed on an LSI-11/23 computer with a hard disk drive (Plessey, Irvine, CA) under RT-11 operating system (Digital

Equipment Corp., Maynard, MA). Sampling time was 0.1 ms for each data point and a record usually consists of 2.3×10^5 points.

Two types of data analysis programs and a simulation data generator program were written in Pascal. One of the analysis programs provides a histogram of current amplitudes. From the positions of individual peaks in this histogram, we can calculate the conductance of the channels and from the area of each peak we can calculate the open and closed probability distribution. The other analysis program provides a histogram of time spent in each of three levels: level 0 (both channels closed), level 1 (one of the channels open), and level 2 (both channels open). The operating principle of this program is as follows: If two or more consecutive points are closest to one of the levels, then these points are regarded as belonging to that level. If not, then the point is regarded as belonging to the same level as the immediately preceding point. This algorithm does not operate properly when data have drifts. To avoid this problem, the variance was calculated to evaluate the fit for each 512 consecutive points, and the parts of the record for which the variance exceeded a preset value were rejected. Most of the data obtained, however, did not show significant drifting, and therefore our results were unaffected by this procedure. Our program is different from other automatic analysis programs (e.g. Sachs et al., 1982), which deal with two levels and are adequate in analyzing one-channel patches.

These programs were examined with simulated data that have known rates, signal-to-noise ratios, and time constants of filtering, and were found to perform effectively. The simulated data were also used to determine the statistical significance of the fit to the binomial distribution.

RESULTS

Fig. 1 *A* shows part of a current record from a voltage clamped on-cell patch that contains two BTX-modified sodium channels. The current-voltage relationship for the same patch (Fig. 1 *B*) shows that the conductance of each channel is 13 pS, a value typical of BTX-modified sodium channels. In Fig. 1 *A* there are three discrete current levels. The lowest level corresponds to two closed channels, the middle level to one open and one closed channel, and the highest level to two open channels. For brevity we call these levels 0, 1, and 2, respectively. An amplitude histogram for the entire record is shown in Fig. 1 *C*.

One way of testing whether the two channels are equal and independent is to determine whether the probabilities of the three levels follow a binomial distribution (Ehrenstein et al., 1970). To perform this test, we first determined from the experimental data the best-fit value for the probability that a channel is open. This was done by minimizing a sum F

$$F = \sum_{n=0}^{2} ([n]_{obs} - [n]_{calc})^2/[n]_{calc}, \quad (1)$$

where $[n]$ represents the relative weight of the level n in the entire record. The quantity F is similar to, but not the same as, the chi-square. Since the data points are correlated, the chi-square distribution cannot be used directly to estimate the statistical significance of the fit.

We have simulated two identical and independent channels for −80 mV, −60 mV, and −40 mV, using the transition rates for channels determined from one-channel patches. From the distribution of the quantity F obtained by simulation, we have estimated the probability that the experimental data are attributable to the binomial distribution. The probabilities that the records obtained from the membrane patch shown in Fig. 1 are compatible with binomial distribution are <1% at all values of the membrane potential recorded. The record shown in Fig. 1 *C* showed particularly large deviation from the binomial distribution, and the level of significance is <0.1%. In Fig. 1 *D*, the distribution obtained from Fig. 1 *C* is compared with the binomial distribution that minimizes F.

Overall, for seven of the nine two-channel patches, the likelihood that the distribution of conductance levels for a patch is consistent with the binomial distribution is <1%. For only two patches are the experimental data consistent with the binomial distribution. Some of the data are listed in Table I.

Next, we will try to address the question as to whether the channels that do not fit the binomial distribution are nonidentical or nonindependent. The approach we use is to assume that the channels are independent, determine the open probabilities of each channel based on this assumption (using the above equations and the measured values of [0], [1], and [2]), and examine whether these open probabilities are consistent with other information.

Let P_1 and P_2 represent open probabilities of the first and second channels, respectively. In accordance with the approach described above, we assume that the channels are independent. Therefore,

$$[0] = (1 - P_1)(1 - P_2), \quad (2)$$

$$[1] = P_1(1 - P_2) + (1 - P_1)P_2, \quad (3)$$

$$[2] = P_1P_2. \quad (4)$$

The values of P_1 and P_2 thus determined are shown as a function of membrane potential in Fig. 2. In accordance with our approach, the points in Fig. 2 for two-channel patches do not represent actual probabilities. Rather they are hypothetical probabilities that are calculated to test our assumptions.

In Fig. 2 we compare the open probabilities for each of the two channels in the patch under the independence assumption (filled and unfilled symbols) with the open probability for a single channel in a patch (error bars). Circles, triangles, and squares represent three different patches. The error bars represent mean values ± standard deviations for nine channels from one-channel patches. For potentials more hyperpolarized than about -60 mV, the calculated differences between the open probabilities of two channels in the same patch are within the standard deviation differences between the open probabilities of channels in different cells. For these potentials, therefore, the departure from a binomial distribution could be explained by either nonidentity or nonindependence of channels in the same patch. For more positive potentials,

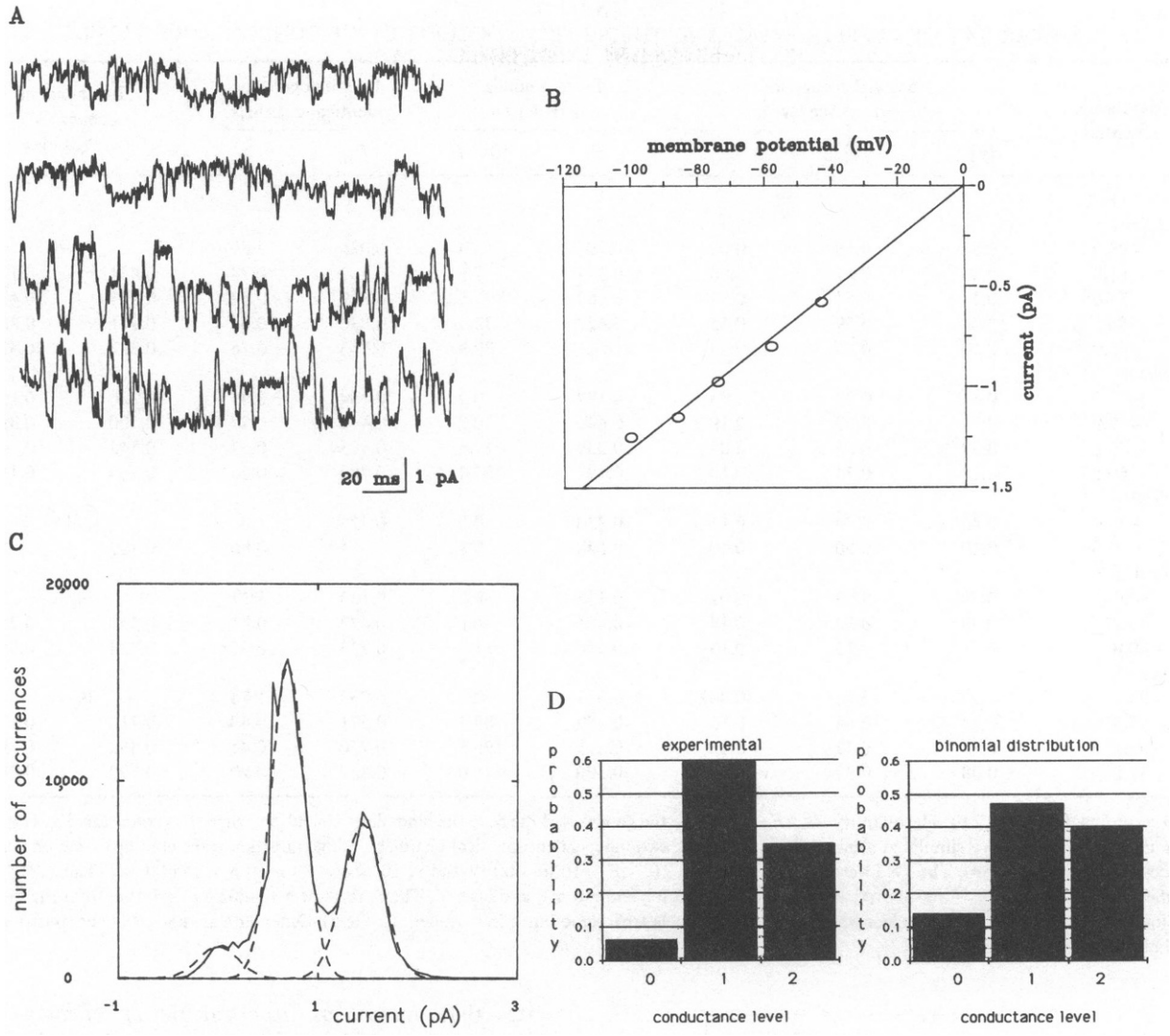

FIGURE 1 Example of data and estimation of open probabilities. (*A*) Current fluctuations in three discrete levels. Membrane potential clamped at −58 mV (*upper* two traces) and at −86 mV (*lower* two traces). Opening of channel corresponds to upward deflection. Highest level corresponds to two open channels (level 2), middle level corresponds to one open channel and one closed channel (level 1), and lowest level corresponds to two closed channels (level 0). (*B*) Single channel current-voltage relationship. Conductance of each channel is 13 pS. (*C*) Current level histogram for membrane potential of −58 mV. *Left* peak represents level 0, *central* peak represents level 1, and *right* peak represents level 2. Dashed curves represent an attempt to fit the level histogram as the sum of three Gaussians. Probabilities of occurrence are estimated from the fit, and are 6% for level 0, 60% for level 1, and 34% for level 2. A good fit to the Gaussian distribution indicates the filtering effect is not significant in estimating the level probabilities. (*D*) Comparison with the binomial distribution of level population. Membrane potential is −58 mV. The fit to the binomial distribution was performed in a manner similar to the chi-square minimization (see text). The best fit gave $P = 0.627$. The statistical significance that the observed distribution is attributable to binomial distribution was found to be far less than 10^{-3}, based on the distribution of F generated by simulation of two identical, independent channels (see text).

however, the calculated open probabilities of the two channels in the same patch in Fig. 2 differ from each other by much more than the differences among the nine single channels in different cells. Thus, for these potentials, explaining the departure from a binomial distribution on the basis of independent, nonidentical channels leads to the unreasonable conclusion that the open probabilities of the two channels in the same patch differ much more than the open probabilities for single channels in different cells. We conclude, instead, that for these cases the independence assumption is incorrect. Thus, in order to explain our data, it is necessary to conclude that there are interactions between some channel pairs—presumably those pairs that are closely spaced (perhaps dimers).

The next question we want to address is whether these interactions between channel pairs are related to the opening process or to the closing process. Analysis of one-channel patches has shown that there are at least two closed states in BTX-modified sodium channels (Huang et al., 1984). This results in ambiguities in identifying closed

TABLE I
COMPARISON OF EXPERIMENTAL AND THEORETICAL OCCUPANCY OF CONDUCTANCE LEVELS

Membrane potential	Observed occupancy of conductance levels			Fit to binomial distribution		Non-independent identical channels		Independent channels	
	$[0]_{obs}$	$[1]_{obs}$	$[2]_{obs}$	P	$10^3 \cdot F$	P	a	P(diff)	
mV									
28jan2									
−99.0	0.82	0.16	0.02	0.102	13.6	0.086	2.39	N/A	
−86.0	0.45	0.47	0.08	0.317	7.9	0.343	0.74	0.454	0.176
−74.0	0.14	0.54	0.32	0.589	13.5	0.659	0.82	0.758	0.422
−58.1	0.06	0.59	0.35	0.627	83.9	0.830	0.65	0.901	0.383
−42.9	0.10	0.55	0.35	0.621	29.9	0.733	0.76	0.827	0.423
dec2a									
−90.2	0.67	0.30	0.03	0.182	0.2	0.184	0.93	0.226	0.137
−79.9	0.31	0.50	0.19	0.440	0.2	0.446	0.97	0.500	0.380
−70.6	0.39	0.53	0.08	0.350	29.6	0.405	0.57	0.543	0.147
−60.5	0.11	0.78	0.10	0.497	325.2	0.780	0.26	0.864	0.116
oct04a									
−70.0	0.45	0.44	0.11	0.330	0.0	0.328	1.02	N/A	
−60.0	0.10	0.50	0.40	0.648	9.8	0.714	0.86	0.800	0.500
oct11b									
−90.0	0.75	0.23	0.02	0.135	0.2	0.133	1.11	N/A	
−70.0	0.34	0.52	0.14	0.401	6.9	0.433	0.81	0.541	0.259
−60.0	0.14	0.73	0.13	0.496	211.7	0.723	0.36	0.834	0.156
apr30c									
−93.1	0.934	0.064	0.002	0.034	0.7	0.033	1.78	N/A	
−67.3	0.24	0.64	0.13	0.449	83.1	0.571	0.49	0.710	0.176
−61.1	0.12	0.72	0.16	0.515	195.5	0.750	0.41	0.852	0.188
−37.2	0.08	0.82	0.08	0.500	436.0	0.837	0.20	0.890	0.090

Each recording duration is 23 s. The quantity $10^3 \cdot F$ is related to the goodness of the fit to the binomial distribution, where F is defined by Eq. 1. A larger value implies poorer fit. We simulated a membrane patch with two independent identical channels, using transition rates obtained from one channel patches (Huang et al., 1984). The 1% level of significance is at $10^3 \cdot F = 10$ for 40 mV and at $10^3 \cdot F = 3$ for 60 mV and 80 mV. The 0.1% level of significance is at $10^3 \cdot F = 15$ for 40 mV and at $10^3 \cdot F = 4.5$ for both 60 mV and 80 mV. The data show a significant deviation from the binomial distribution at −60 mV, but it is not clear whether the two channels are interacting or independent (see text). Other data are unlikely to be consistent with two independent channels.

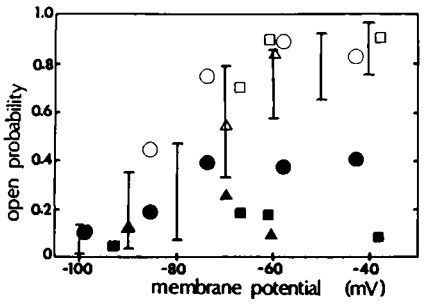

FIGURE 2 Comparison of open probabilities of channels from one-channel patches with hypothetical open probabilities of two channels from two-channel patches. The open probabilities of channels from two-channel patches are calculated assuming that these channels are independent. Bars: Mean open probabilities ±SD obtained from nine channels from one-channel patches. Circles: Hypothetical open probabilities of each of two channels from the patch displayed in Fig. 1 and Fig. 4. Squares and triangles: Hypothetical open probabilities of each of two channels from other patches. Filled data points represent smaller hypothetical open probabilities, and unfilled data points represent larger hypothetical open probabilities. The statistical significance that the pairs of open probabilities for two channel patches are attributable to channels from one-channel patches is 1.6×10^{-3} at −58.1 mV (circles) and $<10^{-4}$ at −42.9 mV (circles).

states that prevent us from calculating opening rates. Calculations of closing rates and open probabilities, however, can be performed without explicitly considering two closed states. By comparing the closing rates that occur when two channels are open with the closing rates that occur when one channel is open, we can determine whether the closing rates could be the cause of nonindependence of the open probabilities.

To determine the overall closing rates from level 2 to level 1, open-time histograms were obtained by measuring the durations in level 2. In order to determine the closing rates from level 1 to level 0, the appropriate durations in level 1 were obtained by starting at an instant of transition from level 2 to level 1 and then adding up all the time spent in level 1 until the next transition to level 0. This prescription can be understood intuitively by comparison with a two-level system where the transition rate is the inverse of the average dwell time. For the three-level system, a similar relation pertains, but it is only the time at risk for a given transition that is relevant. The time spent by the system in level 1 is at risk for a closing transition corresponding to one open channel, but the time spent in

level 2 is not, and should, therefore, not be regarded in calculating the closing rate that occurs when one channel is open.

If the closing rates of the two channels are equal and independent, the overall closing rate that occurs when two channels are open should be exactly twice the closing rate that occurs when one channel is open. Experimental histograms of open times are shown in Fig. 3, and the relaxation times for a range of voltages are summarized in Fig. 4. The least-mean-square fits (solid lines) in Fig. 4 show that the ratio is very close to 2 and that the ratio is independent of the membrane potential. These observations were confirmed for several other two-channel patches, indicating that our data are consistent with closing rates for the two channels that are identical and independent. Thus, any nonindependence of open probabilities should be based on nonindependence of opening rates.

As previously indicated, our experimental data indicate

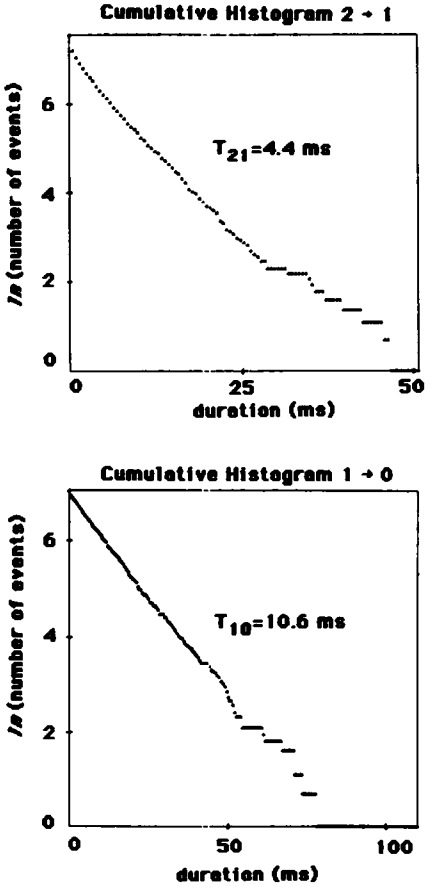

FIGURE 3 Duration histograms for transitions. The abscissas are time duration, ordinates are natural logarithms of cumulative number of events. The inverse of slopes represent relaxation times for transitions between observable levels. Levels 0, 1, and 2 are defined in Fig. 1. Membrane potential: 58.1 mV hyperpolarization. Duration for 1 to 0 transitions is measured from the time immediately following each 0 to 1 transition until the first occurrence of level 0, excluding the duration in level 2. Relaxation times are obtained by fitting to single exponential functions, and are shown in the figure.

that some channel pairs interact. We cannot determine the exact extent of this interaction because it is not possible to determine how much of the departure from the binomial distribution is caused by nonidentity of channels and how much is caused by assuming nonindependence. We can set an upper limit on the extent of the interactions assuming that all of the departure is caused by nonindependence. If we further assume that the open probability of one channel depends upon whether the other channel is open or closed, we can fit our experimental data as follows: Define P as the probability that a channel is open when the other channel is closed and aP as the probability that a channel is open when the other channel is open. States of the patch are represented by cc, co, oc and oo, where the first character represents the state of one channel and the second the state of the other channel. These states are related to the observable levels as follows: [0] = [cc], [1] = [oc] + [co], and [2] = [oo], where brackets represent the probability of state or level. Then,

$$P = [oc]/([cc] + [oc]) = ([1]/2)/([0] + [1]/2) \quad (5)$$

$$aP = [oo]/([co] + [oo]) = [2]/([1]/2 + [2]) \quad (6)$$

To fit the data of Fig. 1 *C*, with [0] = 0.06, [1] = 0.60, and [2] = 0.34, we obtain $P = 0.83$ and $a = 0.64$. The fact that a, the interaction parameter, is <1 indicates negative cooperativity, i.e., a channel is less likely to be open when the other channel is open than when the other channel is closed.

DISCUSSION

Our results show a clear discrepancy with the binomial distribution and suggest that the explanation of this discrepancy is negative cooperativity between two channels in a patch. We do not expect that the negative cooperativity described above occurs between every pair of channels,

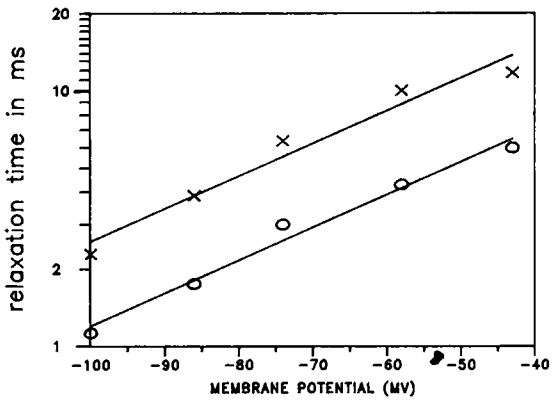

FIGURE 4 Voltage dependence of relaxation times. X, relaxation time for transition from level 1 to level 0. •, relaxation time for transition from level 2 to level 1. Solid lines, representing least mean square fits, are parallel.

particularly because we frequently obtain patches containing only one channel, indicating that these channels do not always occur in pairs. (It is difficult to imagine interactions between channels separated by large distances.)

We cannot estimate what fraction of channels do interact both because of the relatively small number of two-channel patches we have observed on NG108-15 cells and because of possible variability from cell to cell. Of the nine two-channel patches we have observed, five patches contained clearly interacting channels, two patches contained two independent, identical channels, and the other two patches showed a behavior consistent with either nonindependence or nonidentity. The interacting channels may not be in the majority when a larger number of two-channel patches are examined. They do not, however, appear to be exceptions.

We have examined whether or not the negative cooperativity observed in our experiment would be detectable with noise measurements by simulating two interacting channels and determining their noise spectrum. We found that changes in the transition rates corresponding to a two- to threefold decrease in cooperativity parameter a did not significantly alter the noise spectrum. Indeed, we did not detect a significant difference in noise spectrum even when all channel pairs were interacting. Since we expect a considerable number of noninteracting channels on the cell membrane, which would further dilute the effect, we do not expect membrane noise to provide an indication of interacting channels. Thus, for detecting interactions between channels, noise measurement is a rather insensitive method, and the single-channel recording method is uniquely sensitive and useful for the purpose. On the other hand, this also means that noise spectra can be used to determine parameters such as channel density or channel time constants without concern for whether or not the channels are independent.

The variance-measurement method, which was used by Sigworth (1980) and Neumcke and Stämpfli (1983) may be useful in examining whether the channels are independent or not. In this approach, the variance of the current is determined as a function of the mean current. If the variance is caused by the random opening and closing of identical channels, then the variance should be a parabolic function of the mean current and the data can be used to determine the number of channels and the single channel conductances. Thus, with this method, it is possible to test whether or not the channels are identical and independent. However, two identical channels with negatively cooperative interactions and two independent nonidentical channels appear indistinguishable in the variance-mean current plot.

The negative cooperativity we have discussed is unrelated to the negative cooperativity reported by Neumcke and Stämpfli (1983). The negative cooperativity they reported occurs only for high channel density, and is based on overlap of channel currents, i.e., the number of ions passing through the several channels is large enough to significantly deplete the number of ions available. The negative cooperativity described in this paper is based on a slower rate of channel opening when the neighboring channel is open than when it is closed. This negative cooperativity may be a useful property to consider in future attempts to relate channel structure to channel function.

In this paper, we have demonstrated that seven of the nine two-channel patches we have examined are not composed of identical, independent channels. For simplicity, this demonstration was based on the assumption that the channels have two states—one closed and one open. In fact, previous experiments have shown that these channels have two closed states (Huang et al., 1984). If it is assumed that the rate of channel opening depends only on whether the other channel is open or closed (not on which closed state it is in), then the same conclusion can be made on the basis of three-state channels.

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