Analysis of Sodium Current Fluctuations
in the Cut-Open Squid Giant Axon

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ABSTRACT Patch pipettes were used to record the current arising from small populations of sodium channels in voltage-clamped cut-open squid axons. The current fluctuations associated with the time-variant sodium conductance were analyzed with nonstationary statistical techniques in order to obtain an estimate for the conductance of a single sodium channel. The results presented support the notion that the open sodium channel in the squid axon has only one value of conductance, 3.5 pS.

INTRODUCTION

Direct determinations of the conductance of a sodium channel in the squid giant axon from single-channel recordings are not available. Although this parameter has been obtained in a variety of other preparations using patch-recording techniques (Hamill et al., 1981), the squid giant axon has not yet yielded the resolution necessary to measure the current through a single sodium channel.

We have used the voltage-clamped cut-open axon preparation (Llano and Bezanilla, 1980) in conjunction with patch-recording techniques to record current fluctuations associated with the time-variant sodium conductance of the squid axon membrane. The reduction in recording area obtained through the use of patch pipettes has allowed us to study small populations of channels. In this situation, the channel-induced fluctuations comprise a significant fraction (on the order of 5%) of the mean current. These fluctuations have been analyzed using the nonstationary statistical techniques introduced by Sigworth (1980) in his study of the sodium channels from the node of Ranvier. This paper presents the application of this type of analysis to the determination of the conductance of the single sodium channel in the cut-open squid giant axon. A preliminary report of this work has appeared previously (Llano and Bezanilla, 1982).

METHODS

Preparation and Voltage Clamp

Segments of the giant axon from the squid Loligo pealei and Loligo opalescens were used to obtain sheets of axonal membrane as previously described (Llano and Bezanilla, 1980).
The sheet of membrane was bathed in an external solution containing artificial seawater (ASW) (440 mM NaCl, 50 mM MgCl2, 10 mM CaCl2, 10 mM Tris Cl); the internal side of the membrane was perfused with CsF (200 mM CsF, 10 mM Na glutamate, 530 mM sucrose, 10 mM Tris Cl). All solutions were buffered to pH 7.3. The temperature in the chamber was controlled to 3–6°C.

Fig. 1 presents a schematic diagram of the voltage-clamp and current-recording arrangement used in these experiments. The potential on either side of the membrane was measured by means of glass pipettes of internal diameter 30–40 μm (Pext and Pint) positioned within 30 μm of the membrane and connected through Ag-AgCl pellets to voltage followers; a floating platinum wire was inserted in the pipettes to reduce the high-frequency impedance of the electrodes. The resulting transmembrane potential (Vm) was fed to a conventional voltage-clamp circuit which compared Vm with a command signal (Vc) and injected current through the axonal sheet by means of an external platinum plate (Ee) and an internal current electrode (Ei). The internal bath was kept at ground by means of an auxiliary feedback circuit (Caux), which compared the internal voltage signal with ground and injected current through the internal current electrode, a procedure similar
to that used by López-Barneo et al. (1981) in the perfused squid axon. The internal current electrode was also used to measure the total current flowing through the axonal sheet.

To record the current from a small patch of membrane, fire-polished pipettes of internal diameter 1.0–2.0 μm were used. The pipettes were filled with the same solution that bathed the internal surface of the axonal sheet and were connected to a high-gain current-to-voltage converter through an Ag-AgCl wire. A Narishige (Tokyo, Japan) hydraulic micromanipulator was used to position the pipettes against the internal surface of the axonal sheet.

The current-to-voltage converter used to measure the current from the pipette was similar in design to the one described by Hamill et al. (1981). The feedback resistance was 1 GΩ. The positive input was connected to a circuit that provided DC shifts used to vary the potential of the patch under the pipette and small voltage pulses used for checking the pipette resistance. The output of the current-to-voltage converter was passed through a variable-gain amplifier and subsequently filtered through a six-pole Bessel filter with a corner frequency of 1.5 kHz.

**Experimental Procedure**

After initial observation of the total membrane current, a fire-polished pipette \( (R_p = 5–7 \text{ MΩ}) \) was brought close to the internal face of the axonal sheet while monitoring the pipette resistance \( (R_p) \). Establishment of contact between pipette and membrane resulted in an increase in pipette resistance to values on the order of 100 MΩ. Under these conditions, when the axonal sheet is depolarized through the voltage-clamp circuit, the current recorded through the pipette is the sum of the current arising from the patch under the pipette and the current from neighboring areas of the axonal sheet. The extent of the latter component depends on the exact value of the seal resistance. In order to record the current from the patch under the pipette without contamination from the rest of the axonal sheet, the holding potential of the axonal sheet was kept at −40 mV and the positive input of the current-to-voltage converter was set to the value necessary to keep the patch holding potential at −60 mV. This procedure allowed for activation of the channels under the pipette while maintaining the rest of the axonal sheet under conditions in which the sodium channels were inactivated.

While holding the potential of the patch hyperpolarized by 20 mV with respect to the rest of the axonal sheet, depolarizing steps were applied through the voltage-clamp circuit at intervals of 1/s. Typically, a set of 160 depolarizations was recorded at each potential, interspersed with hyperpolarizing pulses to −120 mV and measurements of the seal resistance. Each current record was digitized at intervals of 100 μs and stored on floppy disks in a North Star (San Leandro, CA) microcomputer for further analysis.

**Data Analysis**

Scaled records from hyperpolarizing pulses were used to subtract the leakage current and the capacitive transients from each of the records obtained at depolarizing potentials. The corrected records were divided into groups of 20, and the mean current was obtained as a function of time for each. The variance was calculated for each group from the residual fluctuations remaining in each record after subtraction of the mean current, in the manner described by Sigworth (1980). The mean current and variance for all groups of 20 records obtained at the same potential were averaged and stored for analysis.

Fig. 2 presents the mean current and corresponding variance as a function of time for depolarizations to −10 (Fig. 2a) and 60 mV (Fig. 2b). The variance calculated in this way contains contributions from the thermal noise in the preparation and the recording
system, in addition to the fluctuations caused by the voltage-dependent gating of the sodium channels. The former contributions were therefore subtracted before further analysis was performed. Theoretical predictions of the expected contributions to the total variance from the thermal noise associated with the recording pipette, shunt resistance, passive membrane impedance, and operational amplifiers yielded a value of 0.24 pA² for a recording bandwidth of 1.5 kHz. The typical range of background variance measured in these experiments at the holding potential was 0.5–0.6 pA². This discrepancy may be accounted for by extra noise sources associated with the stray capacitances introduced by the pipette and holder assembly (Hamil et al., 1980), which were not included in the calculations. Strong evidence that the variance obtained during a depolarizing pulse is associated with the gating of the channels rather than with a conductance-dependent increase in thermal noise is given by the experimental result presented in Fig. 2b. At a potential of 60 mV, which is close to the reversal potential of the sodium channel in these experiments, the fluctuations caused by the gating of the sodium channels should vanish; however, the total conductance is high and would result in an increase in variance if this were to be an important source of background noise. As seen in Fig. 2b, no significant increase in variance is present at this potential. This suggests that the background variance is independent of membrane potential. Therefore, in the succeeding analysis, the variance calculated from the first 100 points of each current record (taken at the holding potential) has been subtracted from the ensemble variance.

Values for the single-channel current (i) and the number of channels (N) were estimated by fitting the observed relationship between mean current (I) and variance (S²) to that predicted by a one-conducting-state model (Sigworth, 1980):

\[ S^2 = \frac{I}{N} - \frac{I^2}{N^2} \]  

(1)

The single-channel conductance, γ, was calculated as γ = i/(V - V_{Na}), where V_{Na},
corresponds to the reversal potential for Na ions and \( V \) to the membrane potential. This calculation assumes that the instantaneous current-to-voltage relation is linear, which is approximately the case for this preparation under the ionic conditions used.

RESULTS

The records presented in Fig. 2a illustrate how the current variance follows the time course of the mean current. The peak variance at this potential is 5.50 pA\(^2\); this is equivalent to a standard deviation of 2.35 pA, which corresponds to 5.5% of the value of peak mean current.

A plot of the mean current (\( I \)) vs. variance (\( S^2 \)) for a depolarization to 0 mV is shown in Fig. 3a. A fit of the experimental data to Eq. 1 allows determination of the parameters \( i \) and \( N \), which are used to generate the predicted (solid) curve. As can be seen in Fig. 3a, most of the experimental points are well fit by the quadratic equation. However, a few points, which correspond to the initial part of the rising phase of the current and associated variance, lie below the predicted curve and deviate significantly from the theoretical values. This deviation, which was evident in all experiments over the membrane potential range from -10 to +10 mV, suggests the presence of channel-related gating fluctuations with a time constant of 100 \( \mu \)s or less; these fluctuations would occur during the activation phase of the current and would be altered by their passage through the low-pass Bessel filter, whose corner frequency was set at 1.5 kHz. The variance arising from these fluctuations would therefore have been reduced, resulting in values smaller than those predicted from the rest of the data.

In order to correct for the effects of this bandwidth limitation, a digital frequency-boosting algorithm was designed and applied to each of the filtered current records. The steps followed in this procedure are schematized below:

\[ x(i) \rightarrow \text{High-pass filter} \rightarrow y(i) \rightarrow y(i)*A \rightarrow z(i) \rightarrow X(i) \]

In the first step, each current record, \( x(i) \), was passed through a digital Sine Butterworth six-pole high-pass filter, with a corner frequency setting of 4.0 kHz.
The transfer function of this filter, as well as specific details for its implementation in a digital form, are given by Otnes and Enochson (1978).

The output of the high-pass filter, \( y(i) \) in the scheme presented above, is multiplied by a scaling factor, \( A \), in order to account for the roll-off of the filters. This factor is given by:

\[
A = 0.3 \left[ \frac{F_c(HP)}{F_c(LP)} \right]^M,
\]

where \( F_c(HP) \) is the corner frequency of the high-pass filter; \( F_c(LP) \) is the corner frequency of the low-pass filter used in the original data acquisition; and \( M \) is the number of poles of the filters, which was the same for both high- and low-pass filters.

The scaled output of the high-pass filter, \( z(i) \) in the scheme above, was then added to the original record, \( x(i) \), to yield the final output, \( X(i) \), of the frequency-boosting procedure. This output was then passed through a six-pole Sine Butterworth digital low-pass filter set at a corner frequency of 2.5 kHz. This procedure resulted in an effective increase in bandwidth from the original value of 1.5 to 2.5 kHz.

Fig. 3b presents the plot of mean current vs. variance for the same set of data used in Fig. 3a after the frequency-boosting procedure was applied. All the experimental points are close to the predicted curve, including those which previously showed significant deviations from the theoretical values. Therefore, the frequency-boosting procedure was applied to all the data presented in this paper.

The functional relationship between mean current and variance was used to test predictions of the two-state model and to obtain estimates for the single-channel conductance. According to this model, both the single-channel conductance and the number of channels should be independent of membrane potential. The first prediction was tested by fitting the mean current-variance relationship at different membrane depolarizations with \( i \) and \( N \) as free parameters. Fig. 4a presents the mean current and current variance as a function of time for membrane depolarizations ranging from -30 to 20 mV. The peak variance increases when the membrane potential is changed from -30 to -10 mV, levels off at 0 mV, and decreases sharply at 20 mV. The latter decrease is expected because, although the mean current at this potential is large, most of the channels are activated and the channel fluctuations are therefore diminished. The corresponding plot of variance vs. mean current for each potential is shown in Fig. 4b. The solid line in each plot is given by the prediction of Eq. 1 with the parameters \( i \) and \( N \) shown in each panel. The functional relationship between mean current and variance is well fit by the theoretical prediction, which supports the two-state model. Neither the calculated values of \( \gamma \) nor the number of channels are significantly different for the various membrane potentials studied (see Table 1).

The independence of the number of channels with respect to the membrane potential was further tested by fixing the parameter \( N \) in the fit of the experi-
mental data to Eq. 1. As shown in Table I, the values of $\gamma$ obtained through this procedure are not significantly different from those obtained from the fitting in which both $i$ and $N$ were free parameters.

Estimates of $\gamma$ and $N$ for four different axons are given in Table I. The mean value of $\gamma$ obtained from this data is 3.5 pS (SD = 1.0 pS).

![Graph](image)

**Figure 4.** (a) Comparison of the time course of the variance (lower trace in each panel) and mean current (upper trace) for the indicated membrane potentials. The vertical calibration bar corresponds to 20 pA for the mean current traces and 2.5 pA$^2$ for the variance traces; the horizontal bar corresponds to 10 ms. (b) Plot of the variance ($S^2$) vs. mean current ($I$) from the data shown in a. Dots represent the experimental points; the solid curve was drawn from Eq. 1 with the values of $i$ and $N$ given on each panel.
DISCUSSION

The results presented support the notion that the open sodium channel in the squid giant axon possesses only one value of conductance, a conclusion also reached from previous studies of time-dependent current fluctuations of sodium channels in myelinated nerve (Sigworth, 1977, 1980). The value of 3.5 pS, estimated as the conductance of the open state of the channel, is similar to a previous estimate by Conti et al. (1975) from their study of the power density spectrum of currents recorded from voltage-clamped squid axons. Estimates of the single-channel conductance of sodium channels in other preparations obtained through nonstationary fluctuation analysis have yielded values that are higher than the one obtained in this work for squid axon; thus, single-channel conductances of 6.4 and 7.4 pS have been reported for the sodium channels from frog node of Ranvier (Sigworth, 1980) and egg cells from Halocynthia roretzi (Ohmori, 1981), respectively, and a similar value was obtained for rat myelinated nerve after corrections for the differences in temperature and extracellular sodium concentration (Neumcke, 1982). Single-channel conductances for the sodium channels from a variety of tissue-cultured preparations in which recordings of the current through a single channel have been obtained are slightly larger, even after appropriate corrections are made for the temperature dependence of the conductance (e.g., rat myoballs, Sigworth and Neher, 1980; neuroblastoma, Quandt and Narahashi, 1982; and chromaffin cells, Fenwick et al., 1982).

When comparing these various estimates of single-channel conductance with the lower value obtained in the squid axon, one may consider several explanations for the difference. One possibility is the presence of channels under the rim of the pipette, which would contribute attenuated current and consequently decrease the estimated value of single-channel conductance. Steps were taken in our experiments to minimize this problem by inactivating the channels that were not under the pipette and by keeping a high (>10-fold) ratio between shunt

| Experiment | $V_m$ (mV) | $N$ | $\gamma_1$ (pS) | $\gamma_2$ (pS) | $T$ (°C) |
|------------|------------|-----|----------------|----------------|---------|
| WH81AG04   | -40        | 21  | 6.3            | -              | 3       |
| WH81AG24   | -20        | 174 | 2.6            | -              | 3       |
|            | -40        | 90  | 3.3            | -              | -       |
| CT82JN18   | -10        | 131 | 3.6            | -              | 6       |
| CT82JN31   | -30        | 92  | 3.5            | 2.6            | 6       |
|            | -20        | 163 | 3.2            | 3.1            | -       |
|            | -10        | 235 | 2.9            | 2.7            | -       |
|            | 0          | 183 | 3.5            | 3.5            | -       |
|            | 20         | 183 | 2.7            | 2.7            | -       |

Values of single-channel conductance ($\gamma$) and number of channels ($N$) were obtained from four different axons at the membrane potentials ($V_m$) indicated. $\gamma_1$ corresponds to the values obtained when $i$ and $N$ were free parameters in the fit; $\gamma_2$ corresponds to the values obtained with $N$ held constant at 190. See text for details.
resistance and pipette resistance. We have no direct way to evaluate this contribution, but certainly this problem was not present in the experiments of Conti et al. (1975), which yielded similar values for the single-channel conductance in this preparation. An attractive possibility is the presence of very fast fluctuations associated with the gating of the sodium channel of the squid giant axon. Any gating-related fluctuations having time constants of 60 μs or less would be significantly reduced in the experiments presented in this work as a result of the bandwidth. In this case, the value of single-channel conductance obtained from the study of the relationship between mean current and variance would be artificially low. Another possible explanation for the low value obtained for the single-channel conductance is a blocking effect of external divalent cations. External divalent cations, in concentrations similar to those used in this work, have been shown to partially block the sodium channels in the squid giant axon (Taylor et al., 1976).

From the experimental evidence available to date, it is not possible to decide unequivocally whether the comparatively low value of single-channel conductance obtained from this work results from the existence of such fast kinetic processes or whether it represents a real species difference between the squid axon and the other preparations mentioned above. Additional studies of channel fluctuations or recordings of single-channel currents in this preparation, obtained with a significantly improved bandwidth, are necessary to answer this question.¹

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¹ Experiments now in progress suggest that with larger pipettes and an extended bandwidth the estimate of the single-channel conductance is larger than 3.5 pS (R. A. Levis, F. Bezanilla, and R. M. Torres, unpublished observations).
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