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Identification and kinetic properties of the current through a single Na^+ channel

(patch recording/open-close kinetics/tunicate egg)

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ABSTRACT **The kinetic properties of a single Na^+ channel in the tunicate egg cell membrane were studied by the patch-recording technique. A conventional micropipette filled with a solution of 600 mM NaCl and 1.5 mM $MnCl_2$ was used as the patch electrode. The seal resistance between patch electrode and egg surface was more than 1000 MΩ. In the patch recording, the current fluctuations at a given membrane potential consisted of pulse-like events of a uniform amplitude. The amplitude was 1 pA at −56 mV and decreased as the membrane potential was made more positive. It is suggested that the fluctuation in the patch current is the current through a single Na^+ channel for the following reasons. First, the reversal potential of the pulse-like fluctuation in the patch current was approximately equal to that of the total membrane Na^+ current in a solution equivalent to that in the patch electrode. Second, the charges transferred by the patch current and by the total membrane Na^+ current during a 170-msec command pulse showed parallel dependence on membrane potential. Third, the kinetic properties of the pulse-like fluctuations in the patch current were analyzed according to the Hodgkin–Huxley model, and it was shown that the time sequences of the pulse-like events were compatible with those for a single Na^+ channel.**

Na^+ channels identical with those found in other excitable membranes except for the lack of tetrodotoxin binding have been observed in the tunicate egg cell membrane (1). The Na^+ current density of the tunicate egg cell is much lower than the Na^+ current densities found in other excitable membranes; however, it has been suggested that the conductance for a single tunicate Na^+ channel is equal to that of other Na^+ channels (2). The amplitude of the current through a single Na^+ channel has been estimated indirectly by dividing the total membrane current by the number of channels estimated from the binding of tetrodotoxin (3) or by analyzing the fluctuation of the total membrane current (4). In order to analyze the kinetic properties of Na^+ channels, it is desirable to record the current through a single Na^+ channel directly. Conti and Neher (5) have directly recorded the current through a single K^+ channel in squid axons by applying the patch-recording technique developed by Neher *et al.* (6). In the present report, the patch-recording method was applied to the tunicate egg cell to observe a single Na^+ channel current. There are good correlations between the kinetic properties observed in a single Na^+ channel and those predicted from the Hodgkin–Huxley model (7).

MATERIALS AND METHODS

The denuded tunicate egg, which was prepared by enzymatic digestion of the chorion, was voltage clamped with two microelectrodes as described (1). Rectangular voltage pulses 170 msec in duration were applied from the holding level of −120 mV under the following conditions: the rise time to 90% level was 0.4 msec, the capacitative transients lasted for 1 msec, and large inward currents caused a potential error of 0.6 mV/10^{-8} A.

The patch current was recorded with the current–voltage converter designed by Neher *et al.* (6) (see Fig. 2*a*). The experimental bath was filled with 600 mM choline chloride/1.5 mM $MnCl_2$, pH 7.0, and the patch pipette was filled with 600 mM NaCl/1.5 mM $MnCl_2$, pH 7.0, to observe Na^+ current only in the patch region. The patch electrode was a conventional micropipette that had not been fire polished. The resistance of the patch electrode was 15–25 MΩ. When pushed against the egg surface, this micropipette developed an excellent contact after 5–15 min. The shunt resistance across the seal to ground was more than 1000 MΩ, and the background noise level became as low as 0.4 pA (peak-to-peak value), which was almost equivalent to that of the patch electrode in air. The low concentration of $MnCl_2$ was chosen to exclude contamination by Ca^{2+} current and yet to maintain sufficient negative surface charge, thereby facilitating the accumulation of Na^+ around the openings of Na^+ channels (8). Because the activation potentials for the delayed and anomalous K^+ currents of the tunicate egg cell were more positive than +25 mV and more negative than −70 mV, respectively, in the solution equivalent to that of the patch pipette, the superposition of K^+ currents on the observed current should be minimal. In order to examine the kinetic parameters of the total membrane Na^+ current, we performed a conventional voltage-clamp experiment in a solution of 300 mM or 600 mM NaCl and 1.5 mM $MnCl_2$ after recording from the patch current. The kinetic parameters did not depend upon the Na^+ concentration within the limit of measurement errors.

RESULTS

Fig. 1 shows sample current traces in an egg recorded with the patch electrode; positive voltage pulses 170 msec in duration were applied to the membrane kept at −120 mV. The current showed pulse-like fluctuations in the inward direction at all membrane potential levels except at −75 mV. The activation level for the total membrane Na^+ current of the same egg cell was approximately −60 mV with the conventional voltage clamp. At −65 mV, which was just below the activation level, the pulse-like events appeared sporadically with durations shorter than 3 msec. Between −56 and −38 mV the current fluctuations showed a tendency to become a burst of activity, composed of pulse-like events with various durations. At higher membrane potential (−28 and −19 mV) the current usually showed a single pulse-like event of relatively longer duration and slightly smaller amplitude than at more negative potentials. The uniform amplitude of the current fluctuation at a given membrane potential suggests that the current is through a single Na^+ channel under the tip of the patch electrode. When the tip

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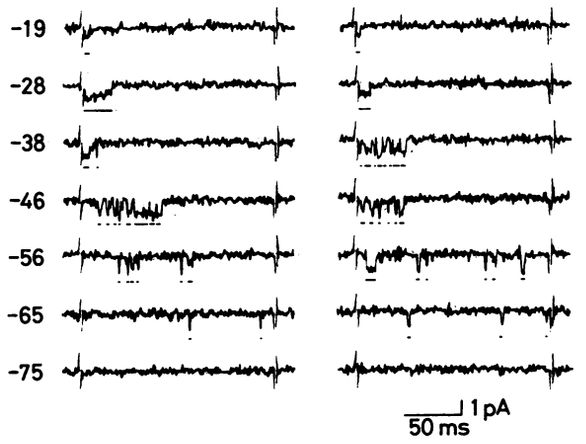

FIG. 1. Current traces recorded with the patch electrode at 13°C (egg EC-604). Membrane potential levels (in mV) during the command pulses 170 msec in duration are shown on the left. Two current traces are illustrated to show the stochastic variations at each potential level. The current fluctuation larger than a certain amplitude was identified as a single event. The duration corresponding to the width of the half-amplitude of each event is underlined.

of the patch electrode had a larger diameter such that its resistance before contact with the egg surface was less than 10 MΩ, multiples of the unitary amplitude of events were observed, especially at more positive membrane potentials, suggesting that the currents through a large number of Na^+ channels were recorded (not illustrated).

Current Amplitude and Membrane Potential. The mean amplitude of the pulse-like fluctuations in the patch current was linearly related to the membrane potential between −56 and −19 mV (Fig. 2*b*). At −56 mV, it was 1 pA, and it decreased with depolarization. The reversal of the direction for the pulse-like current was estimated to be +56 mV from a least-squares fit to the data points between −56 and −19 mV in four eggs. Below −65 mV, the mean amplitude deviated from the linear relationship, showing relatively smaller values. The reversal level estimated from all data points was more positive than +100 mV. The deviation of the mean amplitude from the linear relationship at more hyperpolarized levels may be due to the limited frequency response of the recording system, which made the apparent amplitude of the pulse-like current with short duration less than the real value. Considering the large standard deviations, the reversal level estimated from the data points between −56 and −19 mV agreed well with that for total membrane Na^+ current. The reversal levels for total Na^+ current were from +55 to +62 mV.

Charge Transfer. The charge transfer of the pulse-like fluctuations in the patch current and that of the inward Na^+ current through the total membrane of the same egg cell were plotted against the membrane potential in Fig. 2*c*. The charge transfer of the patch current was the product of the mean current amplitude multiplied by the summed duration of all pulse-like currents during a command pulse. The charge transfer of the total membrane current was calculated by integrating the Na^+ current traces. The charge transfer of the total membrane current showed a peak value of 2500 nA·msec at −46 mV and fell more sharply on the more negative side. The charge transfer of the patch current showed a similar dependence upon membrane potential, although there were larger variations due to the stochastic properties of the pulse-like events. The parallel voltage dependence of the patch and total membrane charge transfers and the apparent coincidence of the reversal potentials between

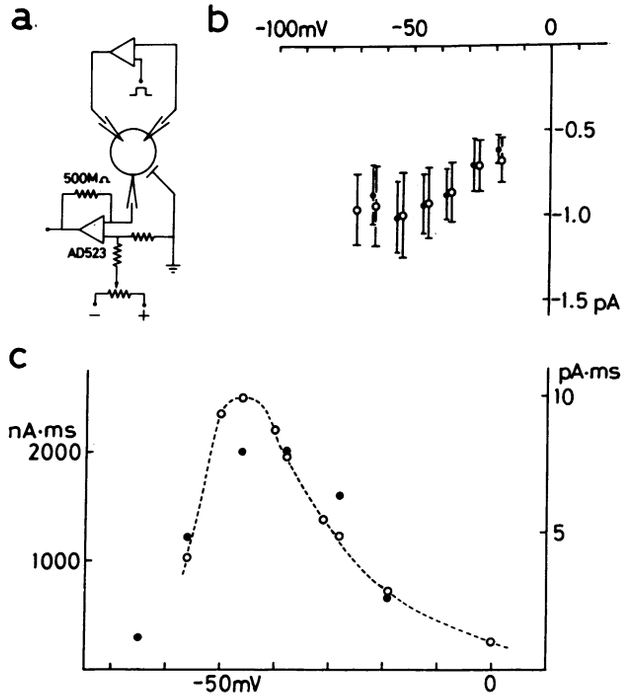

FIG. 2. (*a*) Diagram of the recording system. The output of the current–voltage converter was amplified and filtered at 660 Hz (18 dB/octave, low-pass filter). Then it was stored in a 8-bit 1000-word transient recorder (clock rate, 0.2 msec; Kawasaki Electronica TM 1510) for later illustration on a dc pen recorder. The amplitude of unitary events and the level of background noise were not reduced significantly even by filtering a signal at 170 Hz (18 dB/octave, low-pass filter), which is mostly due to the limited frequency response of the current–voltage converter which uses a 500-MΩ resistor in feedback circuit. (*b*) Amplitudes of the pulse-like currents (means ± SD). ○, Mean amplitudes of the pulse-like currents from four eggs (EC-430, EC-604, EC-709, and EC-718) in the range ±2 mV from the indicated potential. ●, Mean amplitudes from the egg (EC-604) illustrated in Fig. 1. The bath temperature was 13°C for egg EC-604 and 15°C for the others. (*c*) Charge transfers in egg EC-604. ○, Charge transfer of the total membrane Na^+ current; ●, charge transfer of the pulse-like fluctuations in the patch current. Left and right scales are for total membrane current and patch current, respectively.

these two currents strongly suggest that the pulse-like fluctuations with a uniform amplitude at a given membrane potential are a single Na^+ channel current. If this is the case, the ratio of the charge transfer of the total membrane current to that of the patch current at one membrane potential should give the total number of Na^+ channels in an egg. The mean value for five membrane potentials was $2.4 \pm 0.5 \times 10^5$ for the egg illustrated in Fig. 1.

Kinetic Properties. In order to confirm further the origin of the pulse-like events, we analyzed the kinetic properties of the fluctuations in the patch current quantitatively by measuring the latency, closed time, and open time (Fig. 3). The latency was defined as the time from the start of the command pulse until deflection of the first pulse-like event. The closed time means the interval between two successive pulse-like events, and the open time means the duration of each pulse-like event. The distribution of the latency showed a delayed peak from the start of the command pulse, at least at −46 mV, whereas at more positive membrane potentials the latency became obscured by capacitative transient. The closed time and the open time were distributed exponentially. When the membrane potential was more positive, both the latency and the closed time became shorter; the open time became longer when the membrane po-

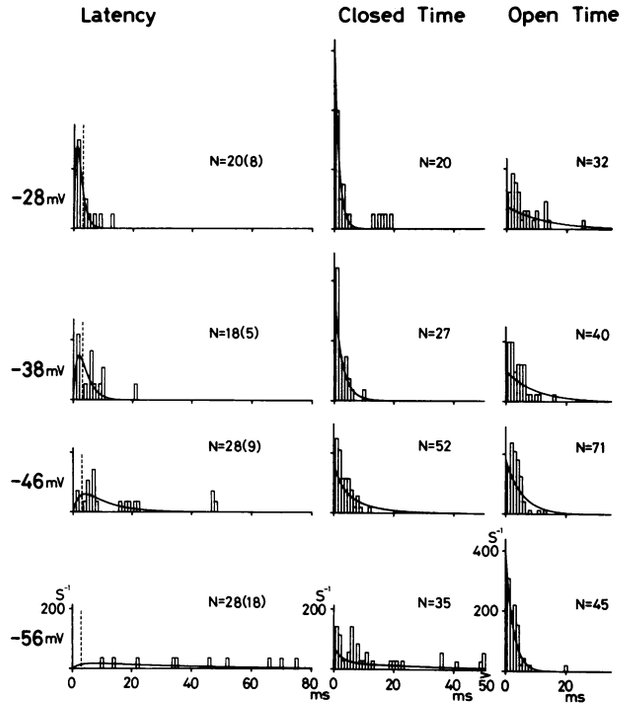

FIG. 3. Distributions of the latency, closed time, and open time with theoretical curves based on the Hodgkin–Huxley model (egg EC-604). All time points were measured from the half-levels of the amplitudes of the pulse-like events with a measurement accuracy of 1 msec. The transient induced by the start of the command pulses continued for 3 msec; this period is indicated by broken lines in the distributions of the latency. For those cases in which the inward deflection of the pulse-like event had begun before termination of the transient, the latency was plotted inside the broken lines and the open time was taken to be the sum of the time after the artifact and the expected time calculated from the theoretical distributions of the latency. The total sample number is given for each distribution. For comparison with the theoretical curve, the total sample number was normalized to 1. The numbers in parentheses in the latency distribution are the number of current traces without any pulse-like events during 170 msec. For the theoretical curves for the closed time it was necessary to normalize the probability density functions by the integrals from zero time to infinite time because, in the distributions of the closed time, the samples that could not reopen due to inactivation of Na^+ channels were excluded.

tential was more positive than −28 mV. Following the Hodgkin–Huxley model, the time course of Na^+ current in the tunicate egg cell has been described as m^2h (1). The parameters m and h correspond to the activation and inactivation processes, respectively. The rate constants α_m, β_m, α_h, and β_h have the same meanings as in the Hodgkin–Huxley equations (7); the values measured for these constants at five membrane potentials (for the same egg cell illustrated in Fig. 3) are listed in Table 1. The opening rate constant for the activation process α_m was expected to have a finite value at −65 mV, and this might be the reason why sporadic pulse-like events were observed just below the activation level for total membrane Na^+ current. The opening rate constant for the inactivation process α_h was zero above −65 mV, whereas that for the activation process α_m had a nonzero value. Therefore, it was possible for a Na^+ channel to close and reopen several times before it became inactivated, producing a burst of pulse-like events (see Fig. 1). Furthermore, the expected probability density functions for the latency, the closed time, and the open time in a single Na^+ channel were calculated according to following equations with those rate constants measured from the total membrane Na^+ current (9, 10):

$$f_{\text{latency}}(t) = \frac{2\alpha_m^2}{\lambda_1 - \lambda_2}\left[\exp\{(\lambda_1 - \beta_h)t\} - \exp\{(\lambda_2 - \beta_h)t\}\right]$$

$$f_{\text{closed time}}(t) = \frac{2\alpha_m\beta_m}{(2\beta_m + \beta_h)(\lambda_1 - \lambda_2)}\left[(2\alpha_m + \lambda_1)\exp\{(\lambda_1 - \beta_h)t\} - (2\alpha_m + \lambda_2)\exp\{(\lambda_2 - \beta_h)t\}\right]$$

$$f_{\text{open time}}(t) = (2\beta_m + \beta_h)\exp\{-(2\beta_m + \beta_h)t\}$$

$$\lambda_1 = \tfrac{1}{2}\left(-3\alpha_m - \beta_m + \sqrt{\alpha_m^2 + 6\alpha_m\beta_m + \beta_m^2}\right),$$

$$\lambda_2 = \tfrac{1}{2}\left(-3\alpha_m - \beta_m - \sqrt{\alpha_m^2 + 6\alpha_m\beta_m + \beta_m^2}\right).$$

The calculated distributions are illustrated by thick solid lines in Fig. 3. The theoretical curves generally fit well with the actual distributions at all potential levels except for some random variations of the distributions due to the small numbers of samples. Thus, it was concluded that the time sequences of the pulse-like events were compatible with those expected for a single Na^+ channel.

Table 1. Kinetic parameters measured from total membrane Na^+ current according to the Hodgkin–Huxley model

Parameter	Membrane potential				
	−56 mV	−46 mV	−38 mV	−28 mV	−19 mV
α_m, $msec^{-1}$	0.062	0.147	0.337	0.606	0.980
β_m, $msec^{-1}$	0.196	0.082	0.028	0.0*	0.0*
α_h, $msec^{-1}$	0.0	0.0	0.0	0.0	0.0
β_h, $msec^{-1}$	0.0047	0.0198	0.0474	0.0775	0.1205
m_∞	0.241	0.641	0.924	1.0	1.0

Total membrane current of egg EC-604 was recorded in 300 mM NaCl/300 mM choline chloride/1.5 mM $MnCl_2$ after recording of the patch current. Low Na^+ concentration was used to improve accuracy of voltage clamp.

* These values were underestimated because the constant-field-type rectification was neglected for the Na^+ chord conductance in this calculation.

DISCUSSION

Ohmori (2) has estimated the unitary Na^+ current and the number of channels per egg in the tunicate egg cell by analyzing the ensemble means and variances of total membrane Na^+ currents for a constant command pulse. In his experiment, the egg cell was internally perfused with 400 mM KF and the bath solution was 400 mM NaCl/30 mM $MgCl_2$/10 mM $MnCl_2$. The unitary current estimated by this transient noise analysis was 0.75 pA at −49 mV, and the average number of channels was $1.5 \pm 0.8 \times 10^5$ per egg. After the corrections for the different external concentration of Na^+ and the different surface charges (8), the present experiment gives a value of 0.42 pA at −49 mV in the external solution equivalent to his experiment. Thus, the unitary current in the present experiment may be slightly underestimated due to the limited frequency response of the recording system, whereas that of the transient noise analysis may be overestimated due to superimposed variances other than that of open-close kinetics, such as transport noise variance (11) or insufficient subtraction of background noise (2).

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