# ACTIVATION OF CALCIUM CHANNELS

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Activation of Ca channels has been described as a Hodgkin-Huxley process in which the time constants have ratios of two or greater (1, 2, 3) or as a three-state linear sequential process with two very different time constants (4). We examined activation by measuring the turn-on and turn-off of macroscopic Ca currents and the discharge of single Ca channels. The results show that neither a literal Hodgkin-Huxley mechanism nor a three-state model applies. A minimum model of activation requires at least four states.

## METHODS

The experiments were done using isolated nerve cell bodies of *Helix*. A combined voltage clamp was used (5) in which potential was measured with a microelectrode and current was delivered by a suction pipette. The latter also allowed dialysis of the cell's interior. The clamp amplifier was a high frequency operational amplifier (op amp) (Teledyne 1030, Teledyne Philbrick, Dedham, MA) that allowed rapid clamping with the full op am gain ($\sim 10^5$) at direct current. Single Ca-channel currents were measured with the patch-clamp method in the cell-attached configuration. In these experiments the neurons were voltage-clamped using a conventional two microelectrode clamp (6) and the patch was held at the bath potential. K currents were suppressed by Cs substitution, TEA and 4-AP. Na currents were suppressed by Tris substitution and/or TTX. Linear components of leakage and capacitance were removed by subtraction of hyperpolarizing pulses. Asymmetry currents were corrected using currents after Co substitution for Ca. Extracellular Ca concentration was 10 or 40 mM. A nonringing zero-phase digital filter was used to reduce background noise as an aid in identifying single Ca channel openings.[1] This filter avoided the introduction of phase lag which would have seriously compromised waiting time measurements.

## RESULTS AND DISCUSSION

The capacitive current transient in the combined clamp experiments was 95% complete within $\sim 50$ $\mu$s and $R_s$ was found to be $<5$ K$\Omega$. The membrane was thus clamped within 1–2 mV during times when tail currents were measured (7). The tail currents following a voltage-clamp step to +30 mV for 5 ms had two time constants in a ratio of $\sim 8$ at a return potential of $-50$ mV (Fig. 1 *A*). At this potential channel activity is zero in the steady-state.[1] This result excludes a literal Hodgkin-Huxley model, which predicts only a single exponential in the tail currents at potentials where activation is zero. The slow component was clearly voltage-dependent whereas the fast component was only slightly voltage-dependent (Fig. 1 *B*). The ratio of the two time constants at potentials where activation is not zero was $\sim 8$, indicating discrepancies with both an $m^2$ model, which requires a ratio of two, or an $m^n$ model, which requires $n$ $\tau$'s with fixed ratios. These two-tail $\tau$'s could not account for the delay in the turn-on at similar potentials (Fig. 2 *A*) as would be required by a sequential three-state model. Turn-on was well-described by an $m^2$ model; parameter estimates are shown in Fig. 2 *B*. If we compare the $\tau$ measurements between $-50$ and zero mV in Figs. 1 *B* with those in Fig. 2 *B*, we find a $\tau_s$, which may be an approximation of a weighted sum of exponential functions with $\tau_m$ and $\tau_m/2$ and a $\tau_F$ that is much faster than

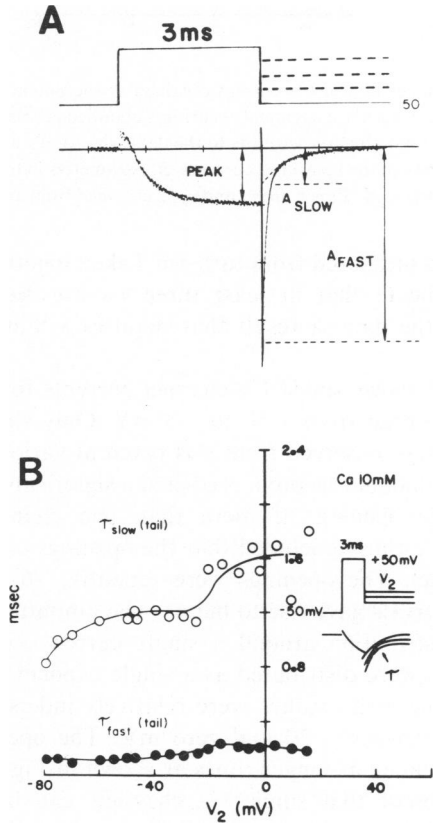


FIGURE 1 *A*, example of the Ca current obtained from stepping to +30 mV and returning to a holding potential of $-50$ mV. The tail current shown is fit with a sum of two exponentials with $\tau$'s of 0.15 and 1.2 ms; the model curve is indistinguishable from the data. The peak current during the step is indicated as well as the amplitudes associated with the fast and slow exponentials. *B*, time constants obtained from two exponential fits to tail currents obtained at various return potentials following a depolarization to +50 mV, which maximally activates the current.

[1]Brown, A. M., and H. D. Lux. In preparation.

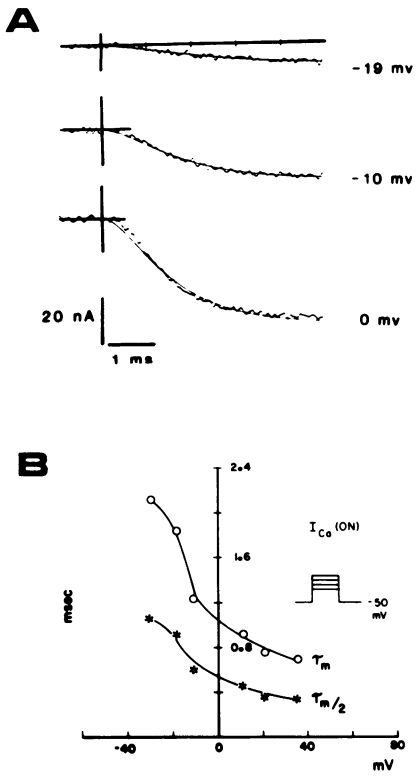


FIGURE 2 *A*, turn-on of Ca current obtained at the potentials shown. Currents are corrected for asymmetry currents obtained after substitution of Co for Ca. Smooth curves are fits to the data with an $m^2$ model. Note the greater delay in the measured currents. *B*, parameters extracted from data such as that in *A*. Shown are $\tau_m$ and $\tau_m/2$ obtained from an $m^2$ model.

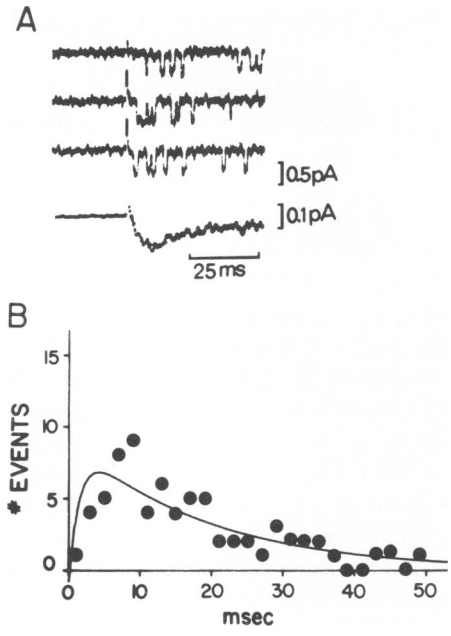


FIGURE 3 *A*, example of some Ca single channel records following a step from −50 to −5 mV at 28°C. The averaged current at the bottom came from summing 68 records. *B*, waiting time distribution for records such as those in *A* obtained at −20 mV at room temperature (RT). Data points are the number of points in a 2 ms bin. The smooth curve is a theoretical curve computed with parameters from a three-state model which were obtained from a stationary analysis of single-channel data (parameter values are $k_{+1} = 0.19$, $k_{-1} = 0.36$ and $k_{+2} = 0.2$). Note that the model predicts a "faster" waiting time distribution than is measured; this is interpreted as evidence that a higher-order model involving more closed states is required to describe turn-on from rest.

parameters measured from turn-on. Taken together, these results indicate that at least three $\tau$'s are required to described the data: a result that requires a minimum of four states.

Fig. 3 *A* shows single Ca-channel currents following a step in potential from −50 to −5 mV. Only single level openings were observed from this patch at various potentials. The binomial theorem predicted a significant number of multiple openings if more than one channel were present. We thus concluded that the openings came from one channel. The openings were repetitive (6) and the briefer intervals gave rise to bursts. The amplitudes had a normal distribution around a single current value. The open times were distributed as a single exponential function and the mean values were relatively independent of potential between −20 and zero mV. The opening frequency declined at longer times as shown in Fig. 3 *A* and this was proof that single Ca channels can inactivate. Moreover, they must reopen from the inactivated state.[1]

From analysis of the waiting times or the latencies until the first opening following the voltage step, we again found discrepancies with a three-state model of activation. For the following three-state sequential model

$$R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} A \underset{k_{-2}}{\overset{k_{+2}}{\rightleftharpoons}} O$$

the probability density function for the waiting time is given by

$$\omega(t) = \frac{\rho_1\rho_2}{\rho_2 - \rho_1} e^{-\rho_1 t} - \frac{\rho_1\rho_2}{\rho_2 - \rho_1} e^{-\rho_2 t} \qquad (1)$$

where

$$\rho_1, \rho_2 = 0.5\{(k_{+1} + k_{-1} + k_{+2}) \pm [(k_{+1} + k_{-1} + k_{+2}) - 4k_{+1}k_{+2}]^{0.5}\}.$$

The peak value of Eq. 1 as a function of time is found by setting the derivative to zero, solving for the time to peak, $t_p$, and reinserting the value into Eq. 1. After considerable rearrangement it is found that the maximum value of Eq. 1 is given by

$$\omega_{MAX} = \rho_1 e^{-t_p\rho_1} = \rho_2 e^{-t_p\rho_2} = f(\rho) \qquad (2)$$

Eq. 2 thus relates $\omega_{MAX}$, $t_p$, and the two $\rho$ values. When $f(\rho)$ is plotted it is found to be a bell-shaped curve with a maximum. The maximum value of this curve occurs at $\rho_p$ and is

$$f(\rho_p) = [t_p e^1]^{-1}. \qquad (3)$$

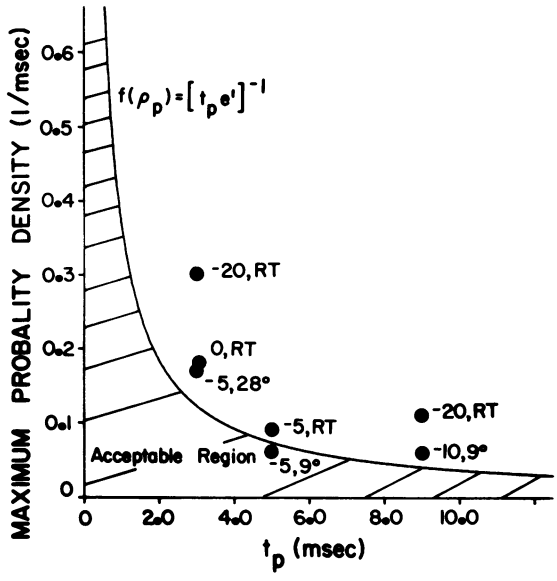


FIGURE 4 The smooth curve is the maximum allowable value of the peak of the waiting time probability density plotted as a function of $t_p$ as obtained in Eq. 3 for a three-state model. Measured values must lie in the "acceptable region" in order for the data to be consistent with a three-state model. Data obtained under various conditions are plotted with the potential and temperature indicated. Note that all but one of the data points lie outside this region, indicating an inconsistency with the model.

Note that this equation yields the maximum admissible values for $\omega_{MAX}$ in Eq. 2.

In Fig. 3 *B* we have plotted a waiting-time distribution, and the smooth curve is predicted from estimates of the parameters from a stationary analysis of the data using open- and closed-time histograms.[1] Measurements were done at $-20$ mV to avoid the complications caused by inactivation.[1] Note that many of the measured waiting times are "slower" than the predicted values, indicating that a higher order model involving more closed states may be required to describe turn-on from rest. When we attempted to fit the waiting time data with Eq. 1, we found that the two $\rho$ values often tended to a single value rather than the two values predicted by Eq. 2 and that the peak of the data was often not fit well. This is consistent with the results shown in Fig. 4 in which all but one of the measured maximum values of the waiting time distributions fall in the unacceptable region for a three-state model. This indicates that the measured responses are not realizable with a three-state sequential model (8). One source of error in the above analyses lends support to the argument. In Fig. 4, maximum values were taken from histogrammed data with finite bin widths, and these would tend to be smaller than a true peak value.

We conclude that a three-state model is not sufficient to describe activation of Ca channels. Single-channel data indicate a single open state from observations of the amplitude distributions as well as from the single exponential open time distribution. It appears that the structure of the closed states required to describe activation must be more complicated than that given by the three-state sequential model.

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