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CHARACTERISTICS OF SODIUM AND CALCIUM CONDUCTANCE CHANGES PRODUCED BY MEMBRANE DEPOLARIZATION IN AN *APLYSIA* NEURONE

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SUMMARY

1. The time course and voltage dependence of Na and Ca conductance changes produced by depolarization of the soma of the neurone R_{15} in the abdominal ganglion of *Aplysia juliana* were examined at temperatures of 10–14 °C.

2. During a maintained depolarization, Na currents turned on then decayed (inactivated). Inactivation was exponential with time constant τ_h. Activation (after correction for inactivation) was reasonably well described by the expression $G'_{Na}(t) = G'_{Na}(\infty)\,(1-\exp[-t/\tau_m])^3$ over a wide range of potentials.

3. τ_m and τ_h were both voltage dependent. In the range -20 to $+40$ mV, τ_m varied from 5 to 0·5 msec and τ_h from 25 to 8 msec (13·5 °C). Steady-state Na conductance (corrected for inactivation) was voltage dependent also, increasing sigmoidally with depolarization to a maximum of 25–30 μS at $+10$ to $+20$ mV. Half-maximal Na conductance occurred at a membrane potential of -8 mV and from -15 to -5 mV, a 5 mV change in membrane potential produced an *e*-fold change in steady-state Na conductance.

4. Steady-state inactivation of Na conductance ($h_{Na}(\infty)$) was voltage dependent with half-inactivation occurring at a membrance potential of -32 mV. Recovery from Na inactivation followed an exponential time course with a voltage-dependent time constant.

5. During a maintained depolarization Ca currents activated then decayed (inactivated) more slowly than Na currents. The decay was exponential with time constant τ_H. The decay of Ca current was not an artifact produced by an outward current. The amplitude of calcium tail currents, produced by voltage steps back to ϵ_K at different times during the decay of I_{Ca}, decayed also with a time constant close to τ_H.

6. Ca conductance (after correction for inactivation) could be described approximately by the expression $G'_{Ca}(t) = G'_{Ca}(\infty)\,(1-\exp[-t/\tau_M])^p$ but it was necessary to vary p from 1 to 2 at different potentials. No value of p gave as good a fit to this model as that obtained for Na currents.

7. τ_M and τ_H were voltage dependent. In the range of potentials from 0 to $+60$ mV, τ_M varied from 9 to 5 msec and τ_H from 300 to 50 msec (13·5 °C). Steady-state

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Ca conductance (corrected for inactivation) was voltage dependent also, increasing sigmoidally with depolarization to a maximum of 10–15 μS at +30 to +40 mV. Half-maximal Ca conductance occurred at a membrane potential of +12 mV, and from +10 to +20 mV a 6 mV change in membrane potential produced an e-fold change in Ca conductance.

8. Steady-state inactivation of Ca conductance ($h_{Ca}(\infty)$) varied with holding potential (V_H). Half-inactivation occurred with depolarization to −20 mV. At potentials more negative than −40 mV, $h_{Ca}(\infty)$ was less than at −40 mV, i.e. hyperpolarization produced Ca 'inactivation'.

9. Recovery from Ca inactivation did not follow an exponential time course with a single time constant but appeared to consist of two phases, the first with a time constant in the order of milliseconds and the second with a time constant of seconds.

INTRODUCTION

The inward sodium and calcium currents produced by membrane depolarization in R_{15} (Adams & Gage, 1979) are transient currents, like the Na currents recorded in squid axons (Hodgkin & Huxley, 1952*a*). Hodgkin & Huxley (1952*d*) successfully devised a mathematical model to describe Na conductance (G_{Na}) changes in squid axons. Na conductance could be described by $G_{Na} = \bar{G}_{Na}\, m^3h$, where $\bar{G}_{Na}$ is the maximum steady-state conductance that can be produced by the Na permeability system, all the other variables are functions of voltage and time, and m^3h represents the fraction of maximum Na conductance activated. Following a step depolarization from a resting potential at which Na conductance is not activated ($m_\infty = 0$), sodium conductance can be described by $G_{Na}(t) = G'_{Na}(\infty)\,(1-\exp[-t/\tau_m])^3 \exp(-t/\tau_h)$, where $G'_{Na}(\infty)$ would be the steady-state Na conductance if inactivation remained at its resting level. The first order rate constants τ_m^{-1} and τ_h^{-1} are functions of voltage and describe that rate of change of m and h.

The aim in this paper was to attempt to describe in a similar way the Na and Ca conductance changes in the *Aplysia* neurone, R_{15}. Our main interest was in the Ca conductance system about which less is known. Although voltage-dependent Ca conductances insensitive to TTX have been reported in a wide variety of excitable membranes (see Baker & Glitsch, 1975) full analyses of the characteristics of Ca conductance have been confined to voltage clamp studies in crustacean muscle fibres (Keynes, Rojas, Taylor & Vergera, 1973; Hencek & Zachar, 1977), in the tunicate egg-cell membrane (Okamoto, Takahashi & Yoshii, 1976) and in the *Helix* neurone (Kostyuk & Krishtal, 1977; see also appendix by Yu Shakhovalov). The present study of Na and Ca conductances in an *Aplysia* nerve cell membrane provides more information about both conductance systems and their functional independence. This information is necessary for associating the voltage dependent, non-linear, capacitive currents, described by Adams & Gage (1979, in the press) with specific ion conductance mechanisms.

METHODS

The methods used were essentially those of the preceding paper (Adams & Gage, 1979).

An attempt was made to fit Na and Ca currents to an equation of the form (Hodgkin & Huxley, 1952*d*): $I(t) = I'(\infty)\,(1-\exp[-t/\tau_m])^p \exp(-t/\tau_h)$, where $I(t)$ is the current amplitude

at time t. Records were fitted by computer to this model in a series of steps which will be described as applied to Na currents. The same procedure was used for analysis of Ca currents.

Capacitive and leakage currents were subtracted from records assuming linear leakage and capacitive current. τ_h was obtained from the slope of a linear regression line fitted to ln $I(t)$ against t during the later part of the decay phase of a Na current. Recorded $I(t)$ was multiplied by exp (t/τ_h) to give $I'(t)$, the current with inactivation unchanged. $I'(\infty)$ was the maximum,

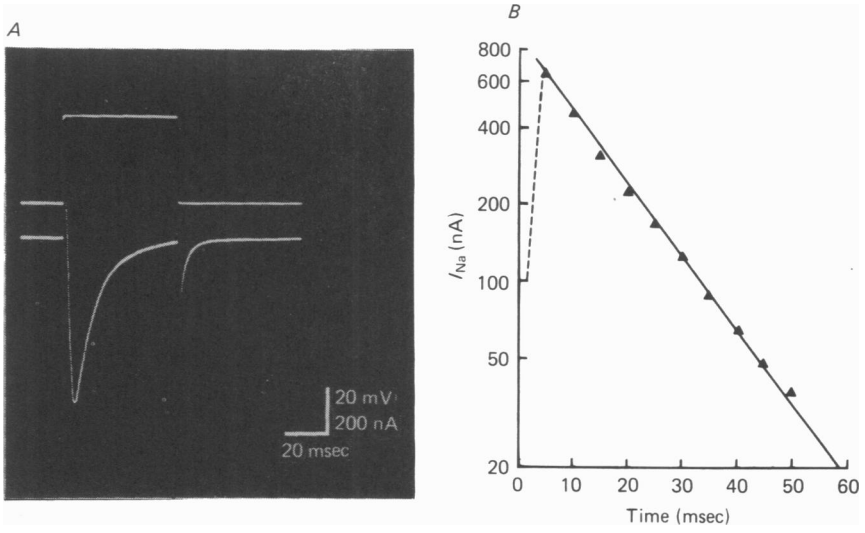

Fig. 1. Time course of a Na current (I_{Na}). *A*, inward Na current (lower trace) generated by a depolarizing voltage step of 42 mV (upper trace) from a holding potential of −48 mV. Temp. 13·5 °C. *B*, semilogarithmic plot of the decay of I_{Na} against time illustrating the exponential decay of the current (time constant 18 msec).

steady-state ($t = \infty$) value of $I'(t)$. A value for τ_m was obtained from the slopes of linear regression lines fitted to ln $(1-[I'(t)/I(\infty)]^{1/p})$ against t for a series of p's from 1 to 5. $I'(t)$ was computed for corresponding values of p and τ_m and compared with the experimental $I'(t)$ to obtain the best fit using a least squares fitting procedure.

To avoid confusion between Na and Ca kinetic rate constants, τ_m and τ_h were used when referring to Na and τ_M and τ_H when referring to Ca.

RESULTS

Sodium conductance

Decay of Na current

The time course of Na currents was generally determined from records obtained in solutions containing high tetraethylammonium (TEA) concentrations (50–100) mM and Mn^{2+} ions (10–15 mM), so that K and Ca currents were inhibited (Adams & Gage, 1979). The Na current shown in Fig. 1*A* was generated by a 42 mV depolarizing step to −6 mV from a holding potential of −48 mV. The time constant of decay of the sodium current (τ_h) was obtained from a semilogarithmic plot of I_{Na} against time, as illustrated in Fig. 1*B*. In this experiment, τ_h was 18 msec (temp. 13·5 °C). The decay (inactivation) of Na currents was generally exponential with a single time constant.

Activation of Na conductance

When the current in Fig. 1*A* was multiplied by exp (t/τ_h) (see Methods) $I'(t)$ was obtained. This was converted to $G'(t)$ by dividing by $V_c - \epsilon_{Na}$, where V_c is the clamp potential and ϵ_{Na} is the experimentally determined Na equilibrium potential. Experimental values for $G'(t)$ are shown in Fig. 2 (circles). By substituting $p = 1, 2, 3$ and 4 in the eq. $G'(t) = G'(\infty)$. (1-exp $[-t/\tau_m])^p$, values of τ_m of 2·3, 2·0, 1·8 and 1·6 msec

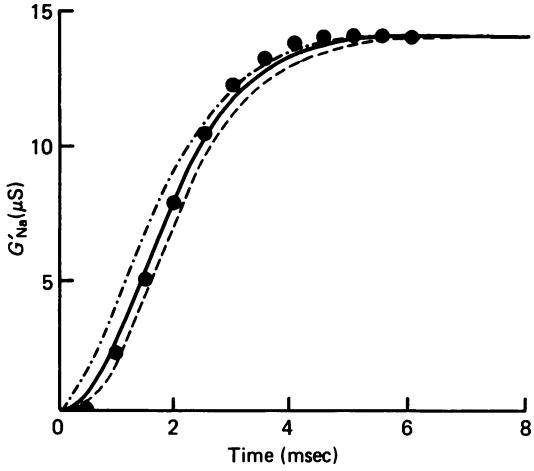

Fig. 2. The time-dependent increase in Na conductance. The current shown in Fig 1*A* was multiplied by exp (t/τ_h) and converted to conductance by dividing by $V_c - \epsilon_{Na}$ (circles). The lines give $G'_{Na}(t)$ from the equation, $G'_{Na}(t) = G'_{Na}(\infty)$ $(1 - \exp[-t/\tau_m])^p$ (see text). The lines show theoretical curves when p was set at 2 (dash–dot line), 3 (uninterrupted line) and 4 (dashed line) with corresponding τ_m values of 2·0, 1·8 and 1·6, respectively. $p = 3$ gave the best fit.

respectively were obtained. Predicted values for $G'(t)$ from corresponding values for τ_m and p are shown in Fig. 2 for comparison with the experimental $G'(t)$. It can be seen that the best fit was obtained with $\tau_m = 1{\cdot}8$ msec and $p = 3$. In general, $p = 3$ gave the best fit to experimental data. However, as can be seen in Fig. 2, the fit was often less than perfect.

Voltage dependence of τ_m and τ_h

τ_m and τ_h were both affected by clamp potential, becoming smaller with increasing depolarization. This effect was particularly marked with τ_m. Results obtained in two experiments are shown in Fig. 3. τ_m decreased by a factor of 4 in the range of clamp potentials from -10 to $+10$ mV (Fig. 3*A*). In contrast, τ_h changed by a factor of less than 2 over the same potential range (Fig. 3*B*).

Voltage dependence of Na conductance

Sodium conductance was calculated for any membrane potential form the steady-state Na current $I'(\infty)$ by dividing by $V_c - \epsilon_{Na}$. For convenience this conductance will be simply denoted as G'_{Na}, a function of membrane potential but not time. To

allow comparison between cells, G'_{Na} was normalized to the maximum steady-state Na conductance obtained between + 15 and + 20 mV ($\bar{G}_{Na}$). Results obtained in two experiments in which $\bar{G}_{Na}$ values were 25 and 29 μS, are shown in Fig. 4. The relation-

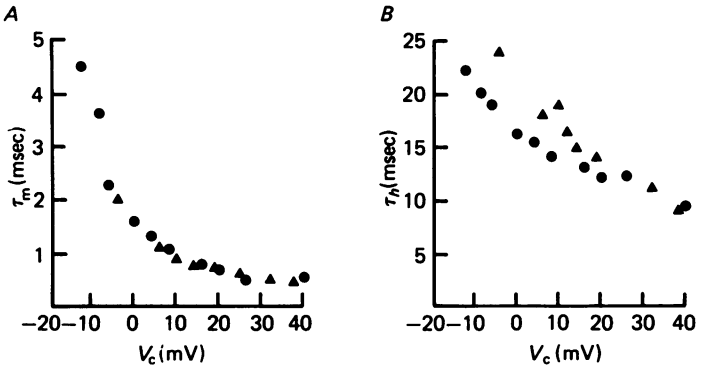

Fig. 3. Voltage dependence of the time constants of activation (τ_m) and inactivation (τ_h) of Na conductance. Results are shown for two neurones with holding potentials of − 48 mV (circles) and − 46 mV (triangles). Temp. 13·5 °C.

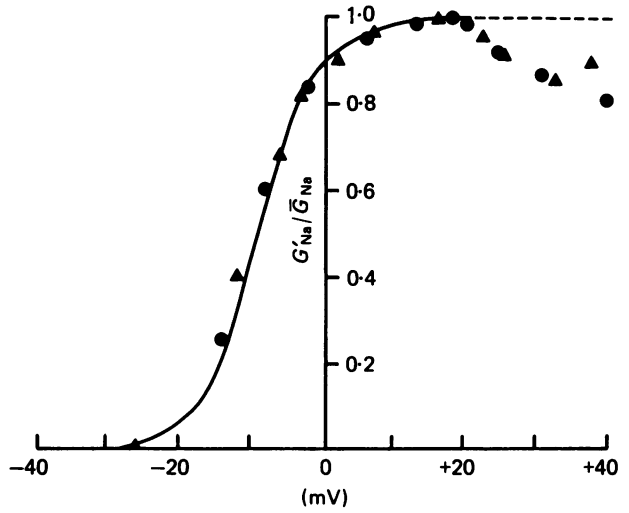

Fig. 4. Voltage dependence of steady-state Na conductance (G'_{Na}) expressed as a fraction of $\bar{G}_{Na}$, the steady-state Na conductance obtained at + 15 mV. Data were obtained in ASW containing 15 mM-Mn and 50 mM-TEA. Holding potentials were − 48 mV (circles) and − 46 mV (triangles). Temp. 13 °C. Note the sigmoidal increase in G'_{Na} as a function of membrane potential with half-maximum activation occurring at − 8 mV.

ship between normalized Na conductance ($G'_{Na}/\bar{G}_{Na}$) and membrane potential was S-shaped with a half-maximum at a membrane potential of − 8 mV. The maximum slope of the curve (at about − 10 mV) gives an e-fold increase in Na conductance for a depolarization of 5 mV. The gradual decrease in the Na conductance at membrane potentials more positive than + 15 mV may be due to rectification of the Na conductance as observed in myelinated nerve fibres (Dodge & Frankenhaeuser, 1959) and perfused squid giant axons (Keynes & Rojas, 1976).

Voltage dependent inactivation of Na current

In a variety of excitable tissues, the amplitude of Na currents is markedly affected by the level of the membrane potential (holding potential) from which a depolarizing pulse arises. A similar effect was seen in R_{15}. In the experiment in which the relationship shown in Fig. 5*A* was obtained the membrance was set at a holding potential

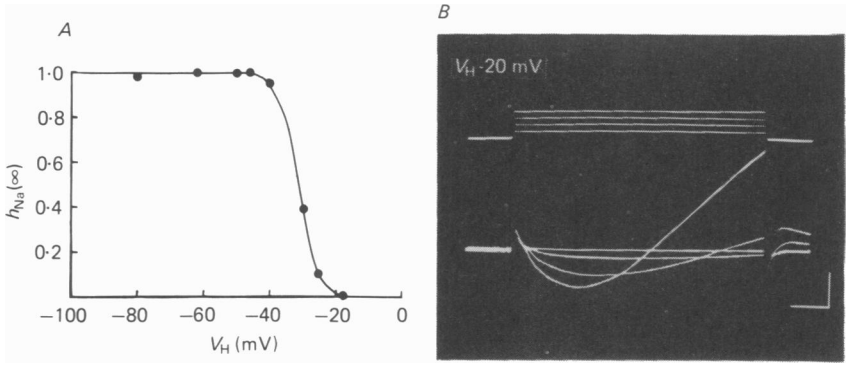

Fig. 5. *A*, voltage-dependent steady-state inactivation of Na current. Pulses to 0 mV were used to activate I_{Na} from a variety of holding potentials (V_H) held for 2 sec before a pulse. Temp. 12.5 °C. The potential dependence of Na channel inactivation is adequately described by the equation (uninterrupted line): $h_{Na}(\infty) = (1+\exp[(V_H+32)/3])^{-1}$ (see text). *B*, inactivation of I_{Na} in normal ASW at a holding potential (V_H) of −20 mV. Depolarizing clamp steps activated Ca currents but no Na currents. Temp. 12 °C. Calibration: vertical, 50 mV (voltage traces), 100 nA (current traces); horizontal, 20 msec.

(V_H) for 2 sec and Na current then generated by pulsing to 0 mV. V_H was varied over a wide range to evaluate its effect. The peak amplitude of the sodium current (I_{Na}) obtained at each V_H is expressed as a fraction of the Na current elicited by the same clamp pulse with very negative prepulse ($\bar{I}_{Na}$). This fraction is called $h_{Na}(\infty)$ which denotes the fraction of the maximum current or conductance that is generated from a holding potential applied until a steady-state of inactivation is attained. The 'steady-state inactivation' curve for Na obtained by plotting $h_{Na}(\infty)$ against V_H is very steep between −50 and −20 nV. At −20 mV the Na current was almost completely inactivated.

That Na current is completely inactivated at a holding potential of −20 mV can be seen in Fig. 5*B*. Here, when another cell was held at −20 mV in normal artificial sea water (ASW), superimposed depolarizing pulses generated only Ca currents: there were no Na currents. It is clear that the characteristics of the steady-state inactivation curve for Na current in R_{15} are quite similar to those described for Na current in a variety of nerve membranes.

Recovery from Na inactivation

The time course of the recovery from inactivation can be measured using the double pulse technique as described by Hodgkin & Huxley (1952*c*). The results of a double-pulse experiment in R_{15} are illustrated in Fig. 6. Sodium currents in response to two depolarizing pulses of the same amplitude at varying intervals were obtained

in normal ASW containing 50 mM-TEA and 15 mM-Mn. In Fig. 6*A* superimposed traces of Na currents are shown with the onset of the second pulse delayed after the first pulse by 5, 10, 20, 32 and 100 msec in turn. The time course of recovery from

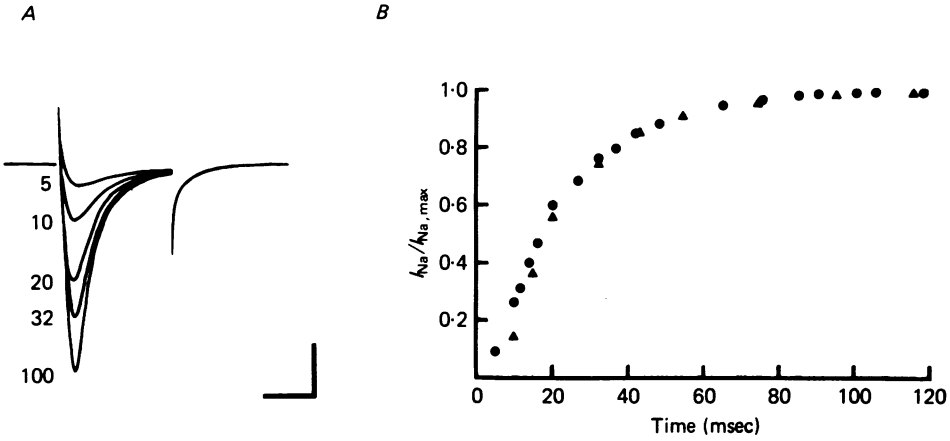

Fig. 6. Recovery of Na currents from inactivation monitored with the double-pulse technique. *A*, Na currents obtained in normal ASW containing 15 mM-Mn and 50 mM-TEA at varying intervals after a preceding (conditioning) pulse. Holding potential (V_H) −48 mV. Activating potential (V_c) −3 mV (40 msec duration). Temp. 13.5 °C. Calibration: vertical, 200 nA; horizontal 20 msec. *B*, plot of $I_{Na}/I_{Na,max}$ as a function of time between the two clamp pulses. Data obtained for two different holding potentials V_H −48 mV (filled circles) where V_c is −3 mV (40 msec duration), and V_H −60 mV (filled triangles) where V_c is 0 mV (40 msec duration). The time constants for recovery from inactivation were 20 msec (V_H −48 mV) and 18 msec (V_H −60 mV).

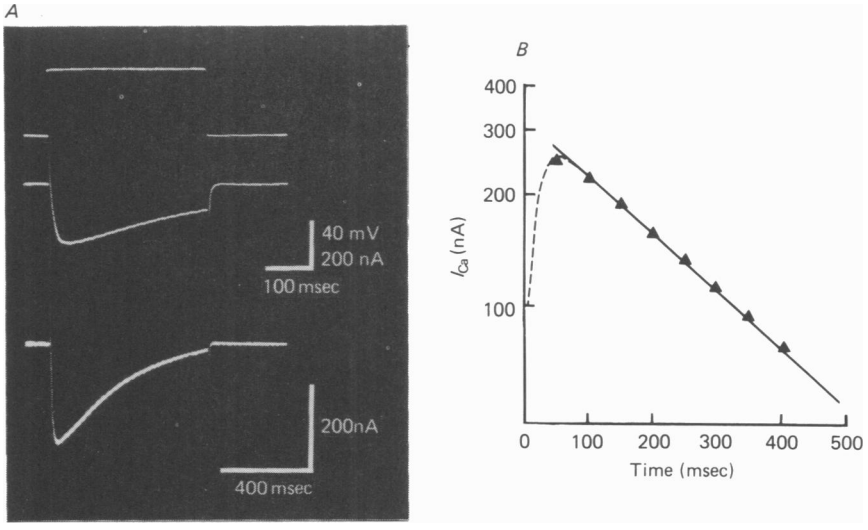

Fig. 7. Time course of a Ca current (I_{Ca}). *A*, I_{Ca} recorded at two different sweep speeds in response to a depolarizing pulse to +12 mV (top trace) in Na-free (choline substitution) ASW containing 10^{-5} M-TTX and 70 mM-TEA. Holding potential, −42 mV. Temp. 13·5 °C. *B*, semilogarithmic plot of I_{Ca} against time (msec). The decay of I_{Ca} is exponential with a time constant of 260 msec.

inactivation recorded in two such experiments is shown graphically in Fig. 6*B*. In these experiments, pulses to 0 mV from a holding potential of −60 mV (triangles) or to −3 mV from a holding potential of −48 mV (circles) were used to generate Na currents. The recovery from the inactivation in both cases followed an exponential time course with time constants of 18 msec (triangles) and 20 msec (circles) at 13·5 °C. The time constant for the recovery from inactivation may be compared to the time constant for the onset of inactivation of the Na conductance during maintained depolarization at more positive potentials (Fig. 3*B*).

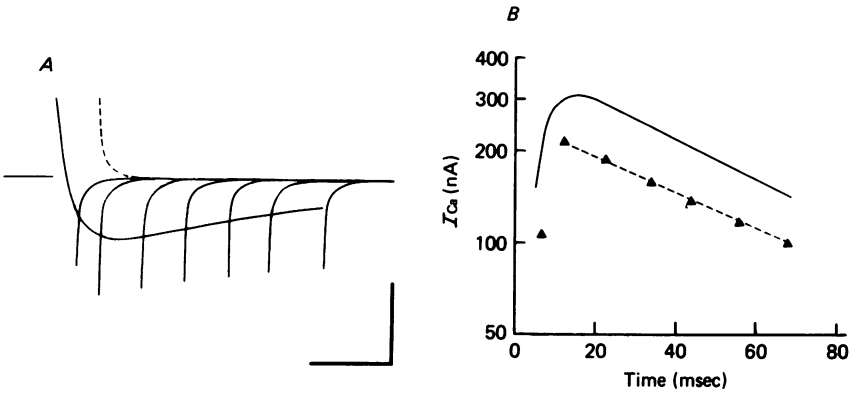

Fig. 8. Determination of τ_H from Ca tail currents. *A*, tail currents elicited at different times during successive Ca currents were obtained in a Na-free solution containing 70 mM-TEA and 22 mM-K^+ upon repolarization from +26 mV to a holding potential of −46 mV ($=e_K$). The dashed trace is the capacitive current in response to a voltage pulse of opposite polarity. Temp. 13 °C. Calibrations: vertical, 400 nA; horizontal, 20 msec. *B*, semilogarithmic plot of tail current amplitude after subtraction of the capacitive current (triangles) against time after activation of the Ca. The decay in the amplitude of the tail currents was exponential with a time constant of 75 msec which is close to the decay time constant of the Ca current (uninterrupted line) of 70 msec.

Calcium conductance

Decay of Ca current

In Na-free solutions containing high TEA concentrations the time course of Ca currents could be reasonably well defined. A Ca current recorded in a solution containing 10^{-5} M-TTX, no Na (choline substitution) and 70 mM-TEA is shown in Fig. 7*A*. The clamp pulse was to +12 mV from a holding potential of 42 mV (temp. 13·5 °C). The upper current trace shows the growth phase of the current and the lower trace on a slower sweep shows the decay phase more fully. As can be seen from the semilogarithmic plot of current amplitude against time in Fig. 7*B*, the decay of the Ca current was exponential with a single time constant of 260 msec. The decay of Ca currents was generally found to be exponential.

It could be argued that the apparent decay of Ca current as shown in Fig. 7 is contaminated by the onset of an outward K current in spite of the high TEA concentration. In order to test this hypothesis, the time course of inactivation of Ca was determined from the amplitude of the Ca tail current at the end of a command pulse during the decay phase (Hodgkin & Huxley, 1952*b*). In order to avoid any contribu-

tion from K currents, steps were made back to the K equilibrium potential (ϵ_K). The external K concentration was increased to 22 mM-K to make ϵ_K more positive and so avoid the inactivation of Ca conductance that occurs upon holding the cell at potentials more hyperpolarized than -40 to -50 mV (see Fig. 12). The capacitive

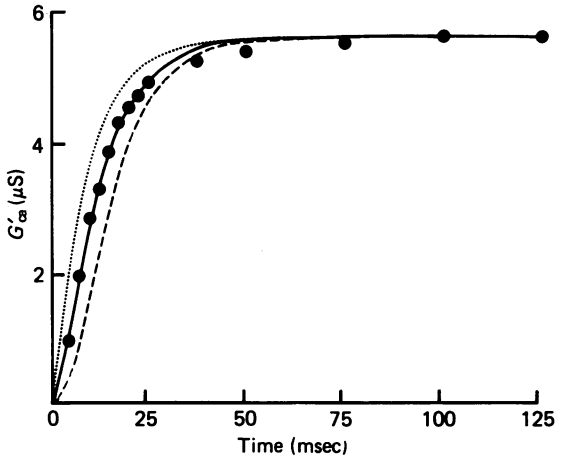

Fig. 9. Time course of the increase in Ca conductance corrected for inactivation (G'_{Ca}) produced by a voltage step to $+12$ mV from a holding potential of -42 mV in a Na-free solution containing 70 mM-TEA. Experimental points are shown as circles. The lines show $G'_{Ca}(t) = G'_{Ca}(\infty).(1-\exp[-t/\tau_M])^p$ with $p = 1$ (dotted line), 2 (uninterrupted line), 3 (dashed line), and τ_M values of 8·6, 8·2 and 7·9 msec respectively. Temp. 13·5 °C.

current (obtained from a hyperpolarizing step of equal magnitude from the holding potential) was subtracted from the total current to obtain the Ca tail current. In a Na-free, 22 mM-K ASW containing 70 mM-TEA, repolarization to -46 mV (ϵ_K in this experiment, determined from the reversal potential of potassium tail currents) at various times during the Ca current gave inward tail currents (Fig. 8*A*). The Ca current generated by a depolarizing clamp pulse to $+26$ mV ($V_H = -46$ mV) decayed exponentially with $\tau = 70$ msec as shown by the uninterrupted line in Fig. 8*B* (note logarithmic ordinate). The amplitudes of the Ca tail currents produced by repolarization to -46 mV at various times during the Ca current when plotted semilogarithmically against time (Fig. 8*B*), can be seen to decay exponentially with a similar time constant of 75 msec. These results indicate that the Ca current does not merely appear to decay because of the onset of outward K current and that the Ca current does indeed inactivate.

Activation of Ca conductance

In solutions containing 11 mM-Ca^{2+}, the average Ca equilibrium potential (ϵ_{Ca}) obtained from the reversal potential of Ca currents (Adams & Gage, 1979) was $+64 \pm 4$ mV (mean ± S.E. of mean, six cells). Calcium conductance (G_{Ca}) was calculated from $I_{Ca} = G_{Ca}.(V_c - \epsilon_{Ca})$, where I_{Ca} is Ca current and V_c is the pulse potential.

It it is assumed further that Ca conductance 'inactivates' with rate constant τ_H^{-1}

then it is possible to calculate what the Ca conductance ($G'_{Ca}(t)$) would be at any time t if depolarization caused no change in inactivation from $G'_{Ca}(t) = G_{Ca}(t).\exp(t/\tau_H)$.

A plot of $G'_{Ca}(t)$ (circles) aginst time obtained from a Ca current generated by a clamp pulse to +12 mV from a holding potential of −42 mV in a Na-free solution containing 70 mM-TEA, is shown in Fig. 9. It can be seen that Ca conductance increased along a sigmoid curve to a plateau level ($G'_{Ca}(\infty)$).

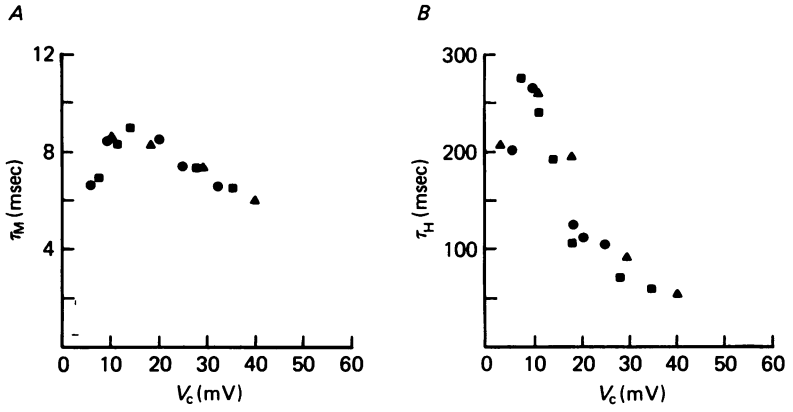

Fig. 10. Time constants of activation (τ_M) and inactivation (τ_H) of the Ca conductance as a function of membrane potential (V_c). Data were obtained in Na-free ASW containing 10^{-5} M-TTX and 70 mM-TEA at holding potentials of −40 mV (circles and squares) and −42 mV (triangles). Temp. 13·5 °C.

An attempt was made to describe $G'_{Ca}(t)$ in terms of the Hodgkin & Huxley model for Na conductance. If it is assumed that $G'_{Ca}(t)$ is given by an expression of the form $G'_{Ca}(t) = G'_{Ca}(\infty).\{1-\exp(-t/\tau_M)\}^p$, values for τ_M can be obtained by plotting $\ln(1-[G'_{Ca}(t)/G'_{Ca}(\infty)]^{1/p})$ against t. Fortunately, varying p from 1 to 4 did not have a pronounced effect on τ_M. It was found from a least squares fitting method, that usually $p = 1$ gave the best fits although in some experiments, generally for small depolarizations, a slightly better fit was obtained with $p = 2$. Predicted curves of $G'_{Ca}(t)$ obtained in this way are shown in Fig. 9 for $p = 1$ (dotted line), 2 (uninterrupted line) and 3 (dashed line). In this experiment, $p = 2$ provided the best fit to the experimental points (circles) but there is a clear disparity between experimental results and the model from 25 to 75 msec.

Voltage dependence of τ_M and τ_H

Values obtained for both τ_M and τ_H depended on membrane potential. The results shown in Fig. 10 were obtained in three experiments in which Ca currents were well defined in a Na-free solution (choline substitution) containing 10^{-5} M-TTX and 70 mM-TEA (temp. 12–14 °C). In one cell the holding potential was −42 mV, in the other two, −40 mV. It should be noted that τ_H could only be determined with confidence up to clamp potentials of about +40 mV beyond which, even in the presence of 70 mM-TEA, the onset of small K currents affected the decay of the late inward current.

At clamp potentials more positive than +15 mV, τ_M decreased with increasing

depolarization (Fig. 10*A*). This can be compared with the decrease in time-to-peak of Ca current with increasing depolarization described in the previous paper (Adams and Gage, 1979): both τ_M and τ_H would affect time-to-peak. τ_H was even more voltage-dependent than τ_M, decreasing by a factor of about 5 as depolarization was increased from +10 to +40 mV (Fig. 10*B*).

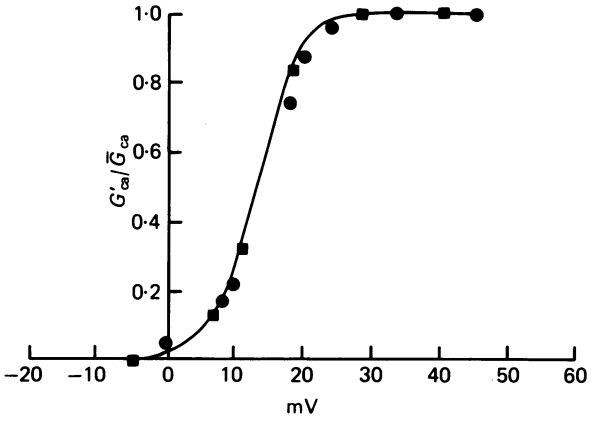

Fig. 11. Voltage dependence of steady-state Ca conductance (G'_{Ca}). Plot of $G'_{Ca}/\bar{G}_{Ca}$ against potential. Results were obtained in two experiments (squares and circles) in Na-free (choline substituted) ASW containing 10^{-5} M-TTX and 70 mM-TEA. Holding potential, −40 mV. Temp. 13·5 °C. The growth of the curve is sigmoidal with a mid-point at +12 mV and with an *e*-fold increase in $G'_{Ca}/\bar{G}_{Ca}$ for a 6 mV increase in membrane potential at +12 mV.

Voltage dependence of Ca conductance

Calcium conductance increased steeply with increasing depolarization in the range 0 to +25 mV. Results obtained in two experiments (temp. 13 °C) are shown in Fig. 11. Calcium conductance was calculated from the steady-state Ca current $I'_{Ca}(\infty)$ obtained after compensating for the exponential decay of conductance with time constant τ_H. For purposes of comparison Ca conductance is plotted as a fraction of the maximum steady-state Ca conductance ($\bar{G}_{Ca}$) recorded at clamp potentials more positive than +30 mV. In these two experiments $\bar{G}_{Ca}$ was 11·5 and 14 μS. From the line fitted to the points by eye, it can be seen that there was a sigmoidal increase in Ca conductance with membrane potential. The maximal slope of the curve gives an *e*-fold increase in Ca conductance for a depolarization of 6 mV. It can be seen that 50% of the maximum conductance was obtained at a membrane potential of +12 to +13 mV. This curve is very different from that obtained for Na conductance in R_{15} (Fig. 4).

Voltage dependent inactivation of Ca currents

Experiments aimed at determining the voltage dependence of inactivation of Ca currents were done as for the Na currents, i.e. a 'conditioning' holding potential was applied to the cell before a Ca current was generated with a depolarizing step to a standard potential. One complication in these experiments was that a small, transient, outward K current could have been generated by depolarizing steps from hyper-

polarized holding potentials (Hagiwara, Kusano & Saito, 1961; Connor & Stevens, 1971; Neher, 1971; Neher & Lux, 1972; Thompson, 1977) in R_{15}. This could slightly affect the amplitude of Ca currents generated from hyperpolarized holding potentials but such a K current would have occurred only with depolarizing steps from hyperpolarized (more negative than -50 mV) holding potentials and would have been

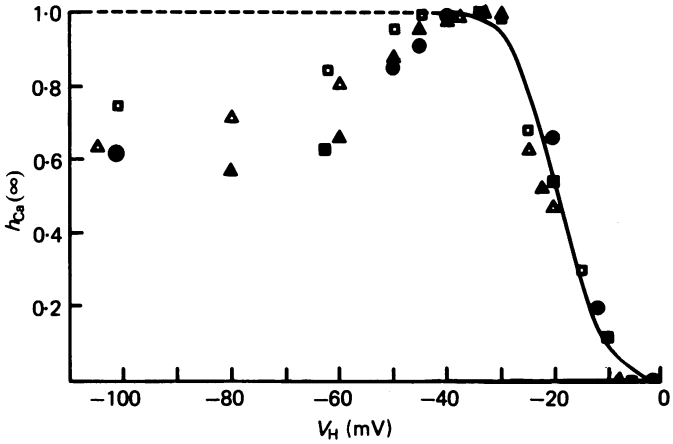

Fig. 12. The effect of holding potential (V_H) on the steady-state inactivation ($h_{Ca}(\infty)$) of Ca currents. Ca current amplitude at any holding potential is expressed as a fraction of Ca current amplitude at a V_H of -35 mV to give $h_{Ca}(\infty)$ which is plotted as a function of V_H. Data were obtained in Na-free (choline substituted) ASW containing 10 mM-4-aminopyridine and 70 mM-TEA (open symbols: triangles, depolarizing step (V_c) to $+15$ mV, temp. 12 °C; squares, V_c $+12$ mV, 12·5 °C) and in Na-free (Tris or choline substituted) ASW containing 70 mM-TEA (closed symbols: squares, V_c $+10$ mV, 10·5 °C; triangles, V_c $+12$ mV, 8 °C; filled circles, V_c $+15$ mV, 9 °C). The uninterrupted line shows $h_{Ca}(\infty) = [1+\exp([V_H+20]/4)]^{-1}$. On hyperpolarization beyond the resting membrane potential (-40 mV) the steady-state inactivation of I_{Ca} deviated from that predicted by the above eqn. (dashed line).

relatively small especially when depressed by high external concentrations of TEA as has been observed previously in other molluscan neurones (Connor & Stevens, 1971; Neher & Lux, 1972; Standen, 1974; Kostyuk, Krishtal & Doroshenko, 1975*b*; Thompson, 1977). In two experiments, the effect of hyperpolization upon Ca steady-state inactivation was examined in the presence of 4-aminopyridine which has been shown to block selectively the transient outward K current (Thompson, 1977). The delayed outward K currents were not affected by the 4-aminopyridine although this drug selectively inhibits the delayed K current in squid axon (Yeh, Oxford, Wu & Narahashi, 1976) and amphibian myelinated nerve (Ulbricht & Wagner, 1976). Consequently, TEA was added to solutions containing 4-aminopyridine in order to suppress the delayed K current. Therefore in all experiments in which the voltage dependence of Ca inactivation was to be evaluated, solutions contained high concentrations (50–100 mM) of TEA, or a mixture of 4-aminopyridine (10 mM) and TEA.

The steady-state inactivation curve for Ca current (Fig. 12) shows results obtained in five cells. Peak Ca current is plotted as a fraction of the peak Ca current generated from a holding potential of -35 mV, and the fraction is called $h_{Ca}(\infty)$. The holding

potential was varied in the range -100 to 0 mV and held for 2 sec so that a steady level of inactivation could be reached before applying a test pulse. The solid line shows $h_{Ca}(\infty) = \{1+\exp([V_H+20]/4)\}^{-1}$ which fitted the experimental point reasonably well at holding potentials more positive than -40 mV. However, with holding potentials more negative than -40 mV (the resting membrane potential) the experimental results clearly deviated from the curve (dashed line) predicted by the above equation.

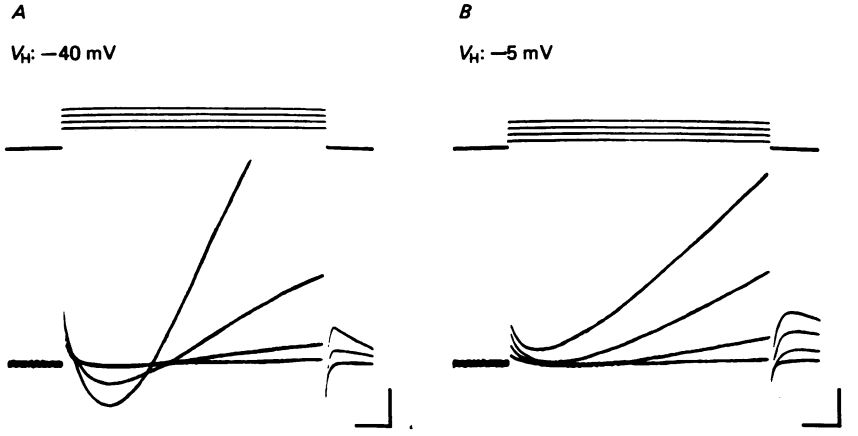

Fig. 13. Inactivation of Ca currents with conditioning depolarization. Membrane currents recorded in a cell in Na-free (Tris-substituted) ASW in response to depolarizing clamp steps (upper traces) from holding potentials of -40 mV (*A*) and -5 mV (*B*). Holding potentials were applied for 2 min before applying clamp steps. Ca currents were inactivated at the holding potential of -5 mV. Temp. 10 °C. Calibration: vertical, 50 mV (voltage traces), 100 nA (current traces); horizontal, 20 msec.

Whether solutions contained only TEA (filled symbols) or TEA plus 4-aminopyridine (open symbols) to suppress the early transient K current (Fig. 12), hyperpolarization caused depression of Ca currents. The phenomenon would seem to be real and to reflect inactivation of Ca conductance at hyperpolarized potentials. This conclusion is reinforced by the observation of inactivation of Ca gating currents at hyperpolarized potentials (Adams & Gage, 1979, in the press).

From the Ca steady-state inactivation curve (Fig. 12) it can be seen that Ca current is completely inactivated by holding the membrane potential at a level more positive than -5 to 0 mV. Membrane currents recorded in a Na free solution in response to depolarizing clamp steps shown in Fig. 13 illustrate the inactivation of Ca currents when the membrane potential of a cell was held at -5 mV. Calcium currents can be seen in Fig. 13*A* which shows the currents generated by depolarizing steps (top traces) from a holding potential of -40 mV. When the holding potential was changed to -5 mV in the same cell, Ca currents were no longer generated by depolarizing steps (Fig. 13*B*).

Recovery from Ca inactivation

In squid axons, recovery from Na inactivation follows an exponential time course with a single time constant (Hodgkin & Huxley, 1952*c*) and this was also seen in R_{15}

(Fig. 6). Similar experiments were done to determine the time course of recovery of Ca currents from inactivation using the double-pulse technique. Superimposed traces of Ca currents in response to a second clamp pulse delayed after the first by 50, 200 and 400 msec, and by 8 sec (holding potential, −42 mV) are shown in Fig. 14*A*. By varying the interval between the two pulses, it was possible to follow the time course

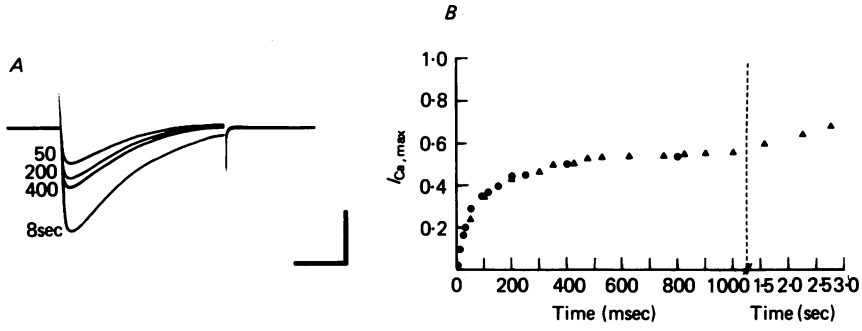

Fig. 14. Time course of recovery of Ca currents from inactivation determined using the double-pulse technique. *A*, inward Ca currents obtained in Na-free (choline substituted) ASW containing 50 mM-TEA, at varying intervals after a preceding command pulse. Holding potential −42 mV; command potential, +10 mV (550 msec duration). Temp. 13·5 °C. Calibration: vertical, 100 nA; horizontal 200 msec. *B*, plot of $I_{Ca}/I_{Ca,max}$. (where $I_{Ca,max}$ is the amplitude of Ca current in response to the first command pulse) as a function of time between the two command pulses of the same amplitude and duration. The data were obtained in two experiments. In the first (triangles), currents were generated by 550 msec pulses to +10 mV from a holding potential of −42 mV, in the second (circles), pulses (200 msec) were to +20 mV from a holding potential of −46 mV. Temp. 13·5 °C.

of recovery from the inactivation caused by the first pulse. Recovery from inactivation did not follow a single exponential time course but rather exhibited two phases. Recovery curves obtained in two experiments are shown in Fig.14*B*. Extracellular solutions contained no Na (choline substitution) and 50 mM-TEA. It can be seen that only 50–60% recovery of the amplitude of Ca currents had occurred in the first 1 sec after the conditioning activation. This early recovery phase could be fitted by an exponential curve with a time constant of 68 msec. It was followed by a slower recovery which appeared approximately exponential with a time constant of 2·5 sec. Even after 3·0 sec, only 60–70% recovery had occurred (Fig. 14*B*).

Deviation from a single exponential recovery from inactivation has also been reported for Na conductance in axonal membranes (Schauf, 1974; Chiu, 1977) and in frog atrial muscle (Haas, Kern, Einwächter & Tarr, 1971). The time course of recovery from inactivation of the Ca current is quite different from the single time constant of inactivation (τ_H) of the Ca current during maintained depolarizations (Figs. 7 and 10). Similar discrepancies between the rates of onset of, and recovery from Ca inactivation have been reported in mammalian cardiac muscle (Reuter & Scholz, 1977)

DISCUSSION

The central aim of this paper was to characterize sodium and calcium conductance changes by deriving kinetic constants within the framework of the Hodgkin &

Huxley (1952*d*) model. The Na conductance characteristics were in reasonable agreement with the formulation; $G_{Na} = \bar{G}_{Na} m^3 h$. However, the time course of Ca conductance could not be fitted by this model. In particular, no integral value for the power parameter gave a good fit to experimental results over the whole voltage range. In spite of this, the Ca results were presented in terms of τ_M and τ_H mainly for purposes of comparison with results obtained in other tissues. τ_H simply describes the rate of decay of Ca conductance during maintained depolarization and is a valid measurement independent of the model. It also turns out that the evaluated τ_M is rather insensitive to the value assigned to the power factor p and therefore serves as some index of the rate of activation of Ca conductance independent of the model.

One of our major concerns was that the decay of 'Ca' current might be due to summation of a steady Ca current with an increasing outward current, e.g. a K current. It has been found that Ca conductance does not inactivate rapidly in some excitable membranes (Keynes *et al.* 1973; Clusin & Bennett, 1977). On the other hand, fast inactivation of Ca conductance during prolonged depolarization has been reported in recent voltage-clamp studies of Ca currents in other molluscan neurones (Eckert, Tilloston & Ridgway, 1977; Kostyuk & Krishtal, 1977), unfertilized eggs of the tunicate *Halocynthia* (Okamoto *et al.* 1976), and in frog skeletal muscle fibres (Stanfield, 1977). Although it was thought that the high concentrations of TEA had effectively suppressed any outward K currents, the best evidence that the decay of Ca conductance during maintained depolarization is real and not contaminated by such a current was the demonstration of the parallel decay in the amplitude of Ca tail currents generated by stepping back to a potential (ϵ_K) at which there would have been no K current. It was concluded that τ_H is indeed a measure of the rate of inactivation of Ca conductance. Certainly the Ca conductance system must inactivate in R_{15}. Both preceding depolarizing pulses (Fig. 14) and depolarized holding potentials (Figs. 12 and 13) caused inactivation of Ca currents elicited by depolarizing pulses.

Another concern was the possible contribution of an early transient K current to the early transient currents. This K current is generated only from hyperpolarized holding potentials and is sensitive to TEA (Connor & Stevens, 1971; Neher & Lux, 1972; Kostyuk, Krishtal & Doroshenko, 1975*a*, *b*; Thompson, 1977). It is unlikely therefore that such a current would have affected the results described here, first because solutions contained high concentrations of TEA and secondly, because many of the results were obtained with holding potentials close to the resting membrane potential. Furthermore, in several experiments in which 4-aminopyridine was added to solutions, there was no change in the time course or amplitude of the early currents over a wide range of holding potentials. As 4-aminopyridine is reported to be a potent blocker of the early transient K current (Thompson, 1977) these observations provide further evidence against the possibility that such a current might have affected measurements of Na and Ca currents.

The rate constants for activation and inactivation of both Na and Ca conductance were voltage sensitive, becoming larger with increasing depolarization. This is hardly unexpected for Na conductance mechanisms (Hodgkin & Huxley, 1952*d*). Voltage-dependent rates of inactivation of Ca conductance have been seen in other molluscan neurones (Eckert *et al.* 1977; Kostyuk & Krishtal, 1977). At any potential, the rates for both activation and inactivation of Na and Ca conductance were very different,

the rates for Ca conductance being lower by a factor of 5–10. In other words, the Ca conductance system is 'slower' than the Na conductance system.

Comparison of the voltage-dependent steady-state conductance curves for Na and Ca emphasizes the differences between the two systems. First there is a clear difference in the threshold potential for activation of Na and Ca conductance. Secondly, the mid point of the Na conductance–voltage curve occurs at about -8 mV whereas the mid point for Ca is at about $+12$ mV, approximately 20 mV more positive (depolarized) than for Na.

In addition to these differences in activation of Na and Ca conductance, there are equally marked differences in their voltage-dependent inactivation, strikingly illustrated in the selective and complete inactivation of the Na conductance on holding a cell at -20 mV at which potential the Ca conductance can still be activated by a depolarizing pulse (Fig. 5*B*). A similar selective inactivation of Na and Ca currents has been reported in the membrane of starfish eggs (Hagiwara, Ozawa & Sand, 1975). At potentials more positive than the resting membrane potential the voltage dependence of Na and Ca inactivation was similar in shape though the curve for Ca was displaced to the right (more positive potentials). However, Ca conductance appeared to 'inactivate' at hyperpolarized as well as depolarized potentials. Although a similar observation was originally reported in the *Aplysia* giant neurones, R_2, by Geduldig & Gruener (1970), in subsequent studies in *Helix* neurones (Neher, 1971; Standen, 1974, 1975; Kostyuk *et al.* 1975*b*) the hyperpolarizing 'inactivation' of Ca conductance was attributed to activation of an early outward K current. Evidence against the depression of the Ca conductance by hyperpolarization in the studies on *Helix* neurones was obtained by the use of TEA to selectively block the early outward K current. The results presented in this study, also obtained in the presence of TEA, strongly support a proposed difference in the inactivation mechanisms for Na and Ca conductances. Relatively high concentrations of TEA failed to reduce significantly the depression of the Ca current upon hyperpolarization beyond -50 mV. Furthermore, 4-aminopyridine which was also used to effectively block the transient outward current in R_{15}, did not significantly affect the Ca steady-state inactivation curve at hyperpolarized potentials. Finally, it will be shown in a later paper that 'calcium gating currents' are also depressed at hyperpolarized potentials. Evidence that the Ca conductance is depressed or 'inactivated' upon hyperpolarization has been obtained in a variety of other excitable cells, e.g. guinea-pig myometrium (Vassort, 1975) and uterine smooth muscle (Anderson & Ramon, 1976).

The rate of recovery from Na inactivation was similar to the rate of onset of inactivation in that the time course could be described by a single exponential. In contrast, the rate of recovery from Ca inactivation did not correspond to the rate of onset. The recovery from inactivation of the Ca conductance exhibited two-time constants while the development of Ca inactivation during maintained depolarization was exponential. A second-order process for inactivation itself may explain the behaviour of Ca conductance in R_{15} as has been proposed (Hille, 1976; Chiu, 1977) to account for the kinetics of inactivation of Na channels in axonal membranes.

The wide differences in the voltage-dependent activation and inactivation characteristics of the Na and Ca conductances strongly suggest that the voltage-sensitive 'gates' opening and closing these ionic channels respond separately and independently to electric fields within the membrane.

It is interesting to compare the characteristics of the voltage-activated Ca conductance described here with Ca conductance mechanisms elsewhere. Secretion of transmitter is thought to depend on Ca entry into nerve terminals through voltage-activated Ca channels (see Katz, 1969). This Ca current is squid nerve terminals like the Ca current in R_{15} (Adams & Gage, 1979) is blocked by Mn^{2+} and Co^{2+} ions (Katz & Miledi, 1969). Baker (1974) has estimated that the Ca permeability responsible for transmitter secretion increases *e*-fold for a 6 mV depolarization. This is the same as the voltage sensitivity of Ca conductance in R_{15}. The late Ca conductance in squid axon also increases *e*-fold for a very similar change (6·3 mV) in membrane potential, and inactivates during maintained depolarization (Baker, Hodgkin & Ridgway, 1971). It is interesting that some recent reports describe depression of transmitter release from hyperpolarized nerve terminals (Martin & Ringham, 1975; Shimahara & Tauc, 1975). Such an effect could be due to Ca 'inactivation' at hyperpolarized potentials as seen in R_{15} (Fig. 5*A*).

The rates of activation and inactivation of Na conductance in *Aplysia* are slower than in most other excitable membranes studied. The different rates of channel opening and closing in *Aplysia* may be due to structural differences in the channel proteins or to differences in the lipid environment. In studies on lipids bilayers, the nature of the membrane lipid has been shown to affect channel life time and conductance (Bean, 1973; Kolb & Bamberg, 1977). Moreover, it has been recently reported in *Aplysia* neurones (Stephens & Shinitzky, 1977) that the ionic conductance associated with the generation of the action potential is dependent on membrane fluidity determined by the cholesterol level. Enrichment of the membrane with cholesterol increases the membrane viscosity (Shinitzky & Inbar, 1976) which might slow the rate of conformational changes in membrane proteins and subsequently affect the time course of the depolarization-activated conductance change. It is interesting to speculate that differences in the rates and magnitudes of conductance changes in *Aplysia* and squid may be due to the high percentage of cholesterol in membranes of *Aplysia* neurones (Komai, Matsukawa & Satake, 1973).

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