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POTASSIUM CONDUCTANCE AND INTERNAL CALCIUM ACCUMULATION IN A MOLLUSCAN NEURONE

BY A. L. F. GORMAN AND M. V. THOMAS*

From the Department of Physiology, Boston University School of Medicine, Boston, Massachusetts 02118 U.S.A.

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SUMMARY

1. The *Aplysia* neurone R-15 was injected with the Ca^{2+} sensitive dye arsenazo III. Changes in dye absorbance were measured with a differential spectrophotometer to monitor changes in the free internal Ca^{2+} concentration, $[Ca]_i$, during membrane depolarization and during intracellular Ca^{2+} ion injection under voltage clamp conditions.

2. The absorbance change, and thus $[Ca]_i$, increases linearly with Ca^{2+} injection intensity at constant duration. The absorbance change produced by a constant intensity Ca^{2+} injection also increases with injection duration, but this increase is asymptotic.

3. The Ca^{2+} activated K^+ current, $I_{K,Ca}$, increases linearly with the increase in $[Ca]_i$ and its rise and decay follows closely the time course of the absorbance change produced by internal Ca^{2+} injection.

4. The Ca^{2+} activated K^+ conductance increases exponentially with membrane depolarization. The increase in K^+ conductance activated by a constant intensity and duration Ca^{2+} injection is on average e-fold for a 25·3 mV change in membrane potential.

5. The difference in net outward K^+ current measured during depolarizing pulses to different membrane potentials in normal and in Ca^{2+} free ASW was used as an index of $I_{K,Ca}$. Its time course was approximately linear for the first 50–100 msec of depolarization, but for longer times the relation approached a maximum. Simultaneous measurements of the arsenazo III absorbance changes were broadly consistent with the activation of $I_{K,Ca}$ being brought about by the rise in $[Ca]_i$ during a pulse.

6. The relation between Ca^{2+} activated K^+ conductance and membrane potential is bell shaped and resembles the absorbance *vs.* potential curve, but its maximum is displaced to more positive membrane potentials. The shift in the two curves on the voltage axis can be explained by the potential dependence of $G_{K,Ca}$.

7. The net outward K^+ current measured with depolarizing voltage pulses in normal and in Ca^{2+} free ASW is increased when $[Ca]_i$ is elevated by internal Ca^{2+} injection. With large and prolonged Ca^{2+} injections the net outward current is depressed following the decline of $[Ca]_i$.

8. The time and frequency dependent depression of the net outward K^+ current

* Present address: Shell Biosciences Laboratory, Sittingbourne, Kent ME9 8AG, England.

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which occurs during repetitive stimulation is shown to have no obvious temporal relation to the increase in $[Ca]_i$. The depression is relieved by an increase in $[Ca]_i$ caused by internal Ca^{2+} injection.

9. The net outward K^+ current measured with brief depolarizing pulses which approach the estimated Ca^{2+} equilibrium potential and therefore do not cause Ca^{2+} influx and accumulation is facilitated by a previous depolarizing pulse which causes a rise in $[Ca]_i$..

10. The facilitation experiments also suggest that the activation of $I_{K,Ca}$ by $[Ca]_i$ has a significant time constant. During a depolarizing pulse, the rise in $[Ca]_i$ next to the membrane, and hence $I_{K,Ca}$ is expected to follow the square root of time, but a delay in the activation of $I_{K,Ca}$ by $[Ca]_i$ could explain why the observed time course of $I_{K,Ca}$ is initially almost linear.

11. The potential dependence of the Ca^{2+} activated K^+ conductance can be explained if the internal Ca^{2+} binding site is about half way through the membrane.

INTRODUCTION

In molluscan neurones a component of the outward K^+ current which is produced by depolarization of the cell membrane is suppressed by procedures that prevent Ca^{2+} influx (Meech & Standen, 1975; Heyer & Lux, 1976*a*, *b*). Although the mechanism by which such changes occur is not well understood, several lines of evidence suggest that it is the rise in the internal Ca^{2+} concentration during depolarization which is responsible for membrane K^+ conductance activation. First, it has been shown that direct injection of Ca^{2+} ions into these cells causes an outward current to flow across the membrane that is caused primarily by a change in K^+ conductance (Meech, 1972, 1974; Brown & Brown, 1973; Gorman & Hermann, 1979) and we showed elsewhere that the outward current change produced by internal Ca^{2+} injection correlated well with the change in intracellular Ca^{2+} concentration as measured by the Ca^{2+} sensitive dye arsenazo III (Thomas & Gorman, 1977; Gorman & Thomas, 1978). Secondly, injection of the Ca^{2+} chelating agent EGTA into the cell to prevent a rise in intracellular Ca^{2+} during membrane depolarization abolishes a component of the total outward current which has been referred to as the Ca^{2+} activated K^+ current (Meech, 1978). Lastly, a correlation has been shown between the change in intracellular Ca^{2+} concentration produced by membrane depolarization as measured by the Ca^{2+} sensitive photoprotein aequorin and the change in the Ca^{2+} activated K^+ current (Eckert & Tillotson, 1978). We have investigated the relationship between the rise in free, intracellular Ca^{2+} concentration (resulting either from Ca^{2+} entry during membrane depolarization or from direct internal Ca^{2+} injection) and the change in the Ca^{2+} activated K^+ current over a range of membrane potentials under voltage clamp conditions. The free, intracellular Ca^{2+} concentration was measured using the metallochromic indicator dye arsenazo III, the absorbance change of which is linearly related to the change in free Ca^{2+} concentration (DiPolo, Requena, Brinley, Mullins, Scarpa & Tiffert, 1976; Gorman & Thomas, 1978; Thomas, 1979). Our results indicate that the activation of K^+ channels by an increase in intracellular Ca^{2+} concentration depends on the membrane potential. Aspects of this work have been presented previously in a preliminary form (Thomas & Gorman, 1978, 1979).

METHODS

All experiments were done on the neurone R-15 (Frazier, Kandel, Kupferman, Waziri & Coggeshall, 1967) in the abdominal ganglion of *Aplysia californica*. The experimental procedures, artificial sea-water (ASW) solutions, recording and voltage clamp circuitry and pulsed wavelength differential spectrophotometer were identical to those reported in the previous paper (Gorman & Thomas, 1980). When EGTA (2 mM) was used it was added directly to Ca^{2+} free ASW. In some experiments a separate Ca^{2+} micro-electrode containing 0·5 M-$CaCl_2$ was inserted into the neurone.

It was found that injection of Ca^{2+} ions through micro-electrodes under pressure was considerably less reliable than by electrophoretic injection. Despite a number of procedures which might have been expected to improve reliability, e.g. breaking the micro-electrode tip to increase its diameter, filtering the $CaCl_2$ solution, cleaning the glass, etc., Ca^{2+} injection under pressure often failed, particularly after repeated injections. In contrast, Ca^{2+} ions could be injected reliably and repeatedly by nanoampere currents from micro-electrodes with an outside tip diameter of about 1·5 μm. In voltage clamped cells, Ca^{2+} ions were injected from an intracellular micro-electrode by passing a current from it to ground. The clamp system automatically passed an equal and opposite current so that there was no net flow of current across the cell membrane associated with the electrophoretic injection. When the voltage clamp was switched off, a reed relay disconnected the current-passing electrode from it, and the electrophoretic current was then passed between this electrode and the Ca^{2+} containing electrode so that there was again no net current flow. Current through the Ca^{2+} containing electrode was monitored continuously and was maintained constant by using an electrophoretic unit capable of passing 10^{-6} A through 10^8 Ω (model 160, WP Instruments Inc., New Haven, Conn.).

The passage of an ion from a micro-electrode is determined by its transport number. The transport number for the movement of Ca^{2+} ions in aqueous solution in the presence of an electric field is about 0·40 at 15 °C (Parsons, 1956), but this is likely to be only a rough approximation of the transport number for the type of glass micro-electrodes used in the present study. To determine an experimental value for the transport number, individual micro-electrodes filled with 0·5 M-$CaCl_2$ were inserted into a small droplet of Ca^{2+}-free ASW under oil. Current of the correct polarity to eject Ca^{2+} ions was passed between this electrode and a second micro-electrode. The change in the Ca^{2+} concentration of the droplet was monitored continuously using a miniature calibrated Ca^{2+} sensitive electrode capable of detecting Ca^{2+} changes in the μM range (Brown, Pemberton & Owen, 1976). The transport number, $n_{Ca^{2+}}$ for each microelectrode was determined from the relation

$$n_{Ca^{2+}} = \frac{zF\Delta C}{it},$$

where ΔC is the amount of Ca^{2+} injected (g ions), i is the current, t is the time (sec), z is the valence and F is the Faraday constant. The average value for $n_{Ca^{2+}}$ was 0·30 with an upper limit of 0·40. This value is in good agreement with the average value of 0·31 and the upper limit of 0·42 determined for similar micro-electrodes filled with 0·5 M-$CaCl_2$ in a separate set of studies using plasma emission spectroscopy (Gorman & Hermann, 1979).

RESULTS

The effects of Ca^{2+} injection on dye absorbance

The change in differential absorbance (ΔA) caused by injection of Ca^{2+} ions into the cytoplasm depends on the intensity and duration of the injection current. Fig. 1*A* shows the changes in dye absorbance produced by 30 sec Ca^{2+} injections at various intensities. At each intensity the absorbance change rose to a maximum which occurred several seconds after termination of the injection and thereafter decayed to the original level. The cell shown in Fig. 1*A* was tested with injection intensities of 10–500 nA. Although there were changes in the time course of the

absorbance decay with large injections of Ca^{2+}, the increase in intracellular Ca^{2+} during injection, as measured by the change in dye absorbance, usually declined to the original base line suggesting that the neurone is capable of handling large Ca^{2+} loads. The plot in Fig. 1*A* shows the linearity between the charge applied to the electrode and the change in dye absorbance. Similar results were obtained in two

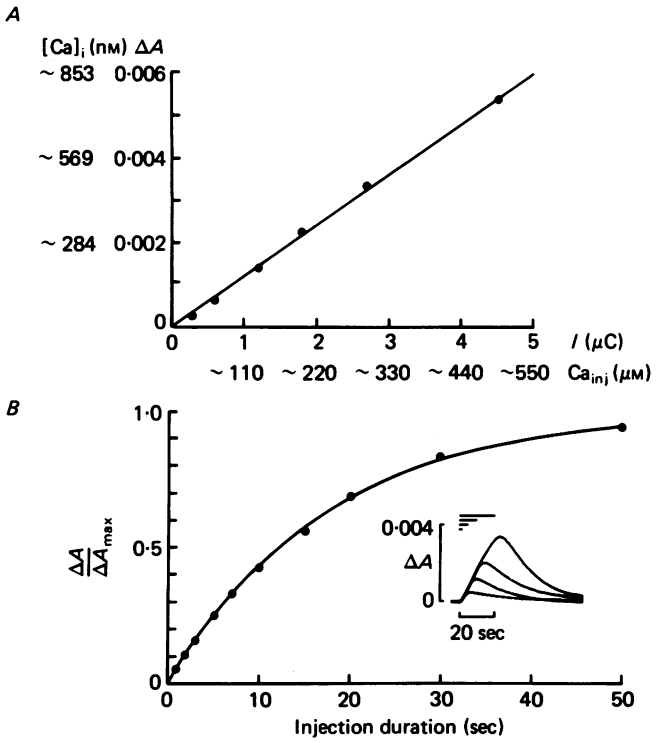

Fig. 1. Differential absorbance changes in response to intracellular Ca^{2+} injections at different intensities and durations. *A*, plot of the absorbance change *vs.* Ca^{2+} injection intensity. A constant injection duration of 30 sec was used. An approximate calibration for the change in intracellular Ca^{2+} concentration is shown next to the absorbance calibration and an approximate calibration for the amount of Ca^{2+} injected (based on a cell with a radius of 300 μm) is shown below the intensity calibration. A change in absorbance of 0·003 ΔA corresponds to a change in internal Ca^{2+} of about 430 nM. *B*, plot of the normalized absorbance change ($\Delta A/\Delta A_{max}$) *vs.* Ca^{2+} injection duration. A constant injection intensity of 100 nA was used. The experimental points are fitted with a single exponential curve with a time constant of 17·5 sec. The insert records shows responses to 2, 5, 10 and 20 sec duration Ca^{2+} injections at 100 nA. A change in absorbance of 0·004 ΔA corresponds to a change in internal Ca^{2+} of about 480 nM.

other experiments using currents of 10–100 nA at 10 sec duration. The straight line fitted to the experimental points has a slope of 0·0012 $\Delta A/\mu C$. An approximate calibration for the change in internal Ca^{2+} can be obtained from the change in dye absorbance. For the cell shown in Fig. 1*A* a change in ΔA of 0·0012 units corresponds to an increase in internal Ca^{2+} concentration of about 170 nM. The R-15 neurones used in these experiments have a diameter of about 300 μm and, therefore, if we assume spherical geometry, a volume of $1{\cdot}4 \times 10^{-8}$ l. The average experimental

transport number for our Ca^{2+} injection electrodes was 0·3, thus a 1 μC injection of Ca^{2+} would have increased the *total* intracellular Ca^{2+} concentration by about 110 μM. The straight line fitted to the experimental points thus has a slope of 170 nM increase in intracellular Ca^{2+} per 100 μM injected Ca^{2+} which would correspond to a rise in free intracellular Ca^{2+} of about 1·6 nM per μM injected load at the end of a 30 sec injection. This measurement, although subject to at least two kinds of errors (see below), compares favourably with similar measurements in squid axon (Brinley, 1978).

Although the rise in $[Ca]_i$ after a constant injection period is linearly related to the rate of Ca^{2+} injection (Fig. 1*A*), its rise with time at a constant rate of injection is asymptotic, as illustrated in Fig. 1*B*. This implies that estimates of the intracellular Ca^{2+} buffering capacity will be higher if they are estimated from longer injections. It can be seen, however, that the departure from linearity of the free Ca^{2+}/time relation is relatively small for injections lasting 10 sec or less (Fig. 1*B*), so measurements made within this period will give a reasonable estimate of the 'instantaneous' buffering capacity for Ca^{2+} ions. We estimate that at very early times (about 300–500 msec) about 1/50 of the Ca^{2+} ions injected into the cytoplasm are free.

The data points in Fig. 1*B* were taken from the peak absorbance changes which occurred in response to 100 nA Ca^{2+} injection currents as a function of the injection duration, rather than from the time course of a single long injection. The inset in the Figure shows several of the injections from which the graph was compiled. Injections lasting from 1 to 50 sec were fitted to a relation of the form

$$\Delta A/\Delta A_{max} = 1-\exp(-t/\tau) \quad (1)$$

The parameters which best described this result were $\Delta A_{max} = 0{\cdot}0057$ (corresponding to $[Ca]_i = 750$ nM) and $\tau = 17{\cdot}5$ sec. For injections lasting 100 sec or longer, the increase in $[Ca]_i$ was greater than predicted by a pure asymptotic relation, but this phenomenon was not investigated further.

The optical probe ends were slightly smaller than the diameter of the typical cell body (about 300 μm) to preclude measurements from outside the cell. The injection experiments were designed primarily to determine the effects of changes in intracellular Ca^{2+} concentration on membrane K^+ conductance and, therefore, the injection electrode was located typically in close proximity to cell membrane, but in some experiments may have been slightly out of the light path. This will cause an underestimation of the free intracellular Ca^{2+} and an overestimation of the injected Ca^{2+} *vs.* free Ca^{2+} concentration ratio.

Ca^{2+} injection and the Ca^{2+} activated K^+ current

Injection of Ca^{2+} ions into molluscan neurones activates a current carried primarily by K^+ ions (Meech, 1978). It is of interest to compare directly the change in K^+ current to the change in absorbance produced by Ca^{2+} injection. In the experiments described below the comparison between the amplitude and time course of the K^+ current and absorbance change was made at a membrane potential (-40 mV) which was positive to the K^+ current reversal potential (about -70 mV). Fig. 2*A* shows a plot of the normalized change in absorbance ($\Delta A/\Delta A_{max}$) and the normalized Ca^{2+} activated K^+ outward current ($I_{K,Ca}/I_{K,Ca\,max}$) *vs.* time for a cell injected with Ca^{2+} for 10 sec by a current of 200 nA. The similarity between the time course of the two

responses is apparent. The K^+ outward current, however, rose and decayed later than the absorbance change, e.g. in Fig. 2*A*, the peak of $I_{K,Ca}$ occurred about 2·5 sec after the peak of the absorbance change. The distance between the injection electrode and the inner membrane surface, however, was not known with any certainty in this experiment. Recent data (Gorman & Hermann, 1979) show that the amplitude of $I_{K,Ca}$ at any membrane potential depends on the position of the injection electrode

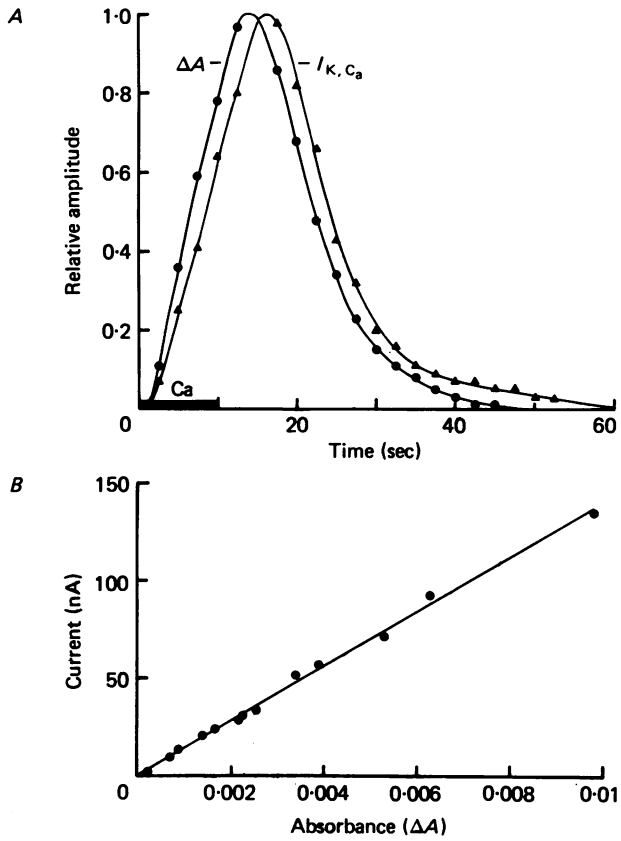

Fig. 2. Effects of intracellular Ca^{2+} injection on membrane outward current. *A*, plot of the normalized absorbance change ($\Delta A/\Delta A_{max}$) and of the change in outward K^+ current ($I_{K,Ca}/I_{K,Ca\ max}$) produced by a 10 sec, 200 nA internal Ca^{2+} injection *vs.* time. *B*, plot of the outward K^+ current response *vs.* the change in absorbance produced by constant duration (30 sec) Ca^{2+} injection at different intensities. An absorbance change of 0·01 units corresponds to an increase in internal Ca^{2+} of about 1·4 μM. The holding potential was −40 mV in *A* and *B*.

as well as on the injection parameters. When the injection electrode was located within about 20 μm of the membrane, the rise and decay of the absorbance and K^+ current signals were much closer in time and their peak values occurred within 0·8 sec of each other.

The decay of the absorbance signal at the end of a Ca^{2+} injection occurred in two phases (see Fig. 2*A*). The absorbance decay (after the first few seconds) could be fitted by an exponential decay curve consisting of two time constants. For six cells

which were analysed in detail, the initial phase had a time constant of decay of $6{\cdot}49 \pm 0{\cdot}82$ sec (S.E. of the mean) and the final decay phase a time constant of $28{\cdot}25 \pm 1{\cdot}75$ sec (S.E. of the mean). The decay of $I_{K,Ca}$ also occurred in two phases of similar time course (see Fig. 2*A*), but typically the time constant for the final decay phase was slightly longer than that for the decay of the absorbance signal. The dual exponential decay of $I_{K,Ca}$ has been shown previously (Gorman & Hermann, 1979) and is also a characteristic feature of the decay of the light induced K^+ current in some molluscan neurones which is caused by release of Ca^{2+} from internal stores (Andresen, Brown & Yasui, 1979).

The relation between the peak $I_{K,Ca}$ and the maximum change in absorbance produced by Ca^{2+} injection at various intensities, but constant duration (30 sec), is shown in Fig. 2*B*. The excellent linearity of the relation is apparent. A similar relation was found in other cells where an injection duration of 10 sec was used. The straight line fitted to the experimental points has a slope of 50 nA per $0{\cdot}0035\ \Delta A$. This corresponds to an increase in the outward current at a holding potential of -40 mV of 10·6 nA per 100 nM change in free intracellular Ca^{2+}. The results in Fig. 2*B* when taken together with those presented in Fig. 1*A* explain the previous findings (Gorman & Hermann, 1979) that the Ca^{2+} activated outward current is a linear function of Ca^{2+} injection intensity. The average slope of the straight line relating the peak Ca^{2+} activated outward current to the Ca^{2+} injection intensity for five cells held at -40 mV was 16·7 nA per μC of charge.

Effects of membrane potential on the Ca^{2+} activated K^+ conductance

In four cells a stimulus of constant intensity and duration was used to inject Ca^{2+} by ionophoresis at different membrane holding potentials between -100 and $+50$ mV. In three experiments, Ca^{2+} free ASW was used to eliminate changes in internal Ca^{2+} during membrane depolarization and in one experiment normal ASW was used as a control against other possible effects of Ca^{2+} free medium. The change in absorbance produced by repeated Ca^{2+} injection was unaffected by changes in holding potential and was constant within about 5% in the cells tested. The difference between the holding current and the peak current response to Ca^{2+} injection was used as an index of $I_{K,Ca}$. The difference current from one of these cells in Ca^{2+} free ASW is shown in Fig. 3 and is non-linear. The insert records show that the absorbance change produced by Ca^{2+} injection was constant at different holding potentials whereas the current increased substantially at more positive potentials. The reversal potential for the Ca^{2+} activated K^+ current was -69 mV in this cell. The current was inward and small at more negative potentials and outward and large at more positive holding potentials. The instantaneous current–voltage relation for the K^+ current activated by a Ca^{2+} injection is reasonably linear (unpublished results, A. Hermann & A. L. F. Gorman), therefore the conductance estimated from the relation $G_{K,Ca} = \frac{I_{K,Ca}}{V - E_K}$ can be used as a measure of the Ca^{2+} activated K^+ conductance and is plotted in Fig. 4*A* on a logarithmic scale versus membrane holding potential. The conductance ($G_{K,Ca}$) increased e-fold for a 24 mV change in potential. The changes in the other two cells studied in Ca^{2+} free ASW were similar. Fig. 4*B* shows a plot of $G_{K,Ca}$ *vs.* membrane potential from a cell studied in normal ASW. In this case each change in

holding potential at potentials greater than about −40 mV produced a sizable increase in dye absorbance. When the membrane is held at positive potentials for long periods (> 3 min), however, the Ca^{2+} channel is almost completely inactivated (Gorman & Thomas, 1980). The cell was studied under these conditions. The results are similar to those shown in part *A* of the same Figure. The Ca^{2+} activated K^+ conductance increased e-fold for a 25·6 mV change in holding potential.

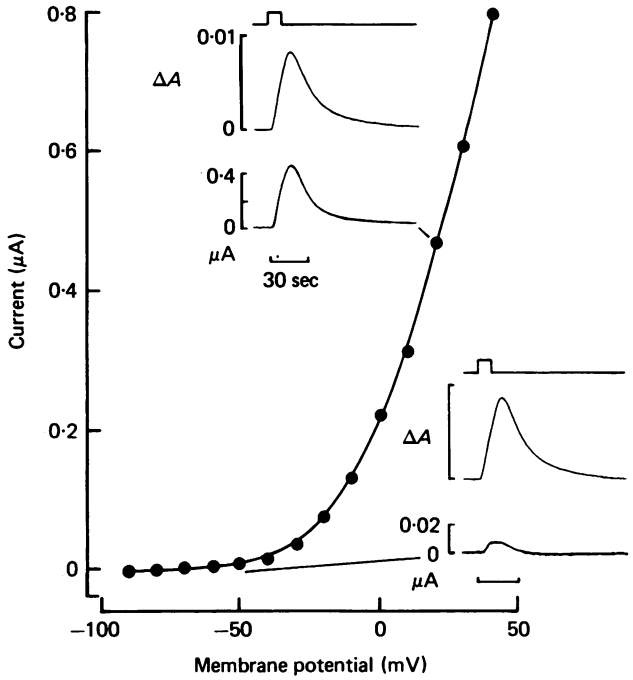

Fig. 3. Effects of membrane potential on outward K^+ currents in Ca^{2+} free ASW activated by a constant intracellular Ca^{2+} injection. The insets show the differential absorbance change and outward K^+ current produced by two 10 sec, 100 nA intracellular Ca^{2+} injections at a holding potential of −50 mV and +20 mV, respectively. A change of 0·01 absorbance units corresponds to an average increase in intracellular Ca^{2+} concentration of about 1·6 μM. The plot shows the K^+ current produced by a constant Ca^{2+} injection at the indicated membrane holding potentials. The points represent the difference between the peak of the K^+ current and the holding potential current. The reversal potential for the K^+ current was −69 mV; at more negative holding potentials the current was inward.

One mechanism by which the voltage dependence might occur is that the affinity of the Ca^{2+} binding site for the Ca^{2+} activated K^+ channel could be greater at more positive membrane potentials. The physical basis for such an effect would be that the Ca^{2+} binding site is (in terms of the electrical field) part way through the membrane rather than at its surface. As the membrane is depolarized, the potential at the Ca^{2+} binding site would become more *negative* with respect to the intracellular environment, thus making the binding of Ca^{2+} energetically more favourable. It follows directly from the Nernst equation for Ca^{2+} ions that a change in potential of 12·5 mV (at 16 °C) between the Ca^{2+} binding site and the intracellular environment will have

the same effect on the Ca^{2+} binding as would an e-fold change in intracellular Ca^{2+} concentration.

We assume that the reaction between Ca^{2+} and the binding site (S) is

$$Ca + S \rightleftharpoons CaS,$$

where CaS is a membrane complex corresponding to a conducting (or open) state of the K^+ channel. It was shown above that $I_{K,Ca}$ (and hence $G_{K,Ca}$) varies linearly

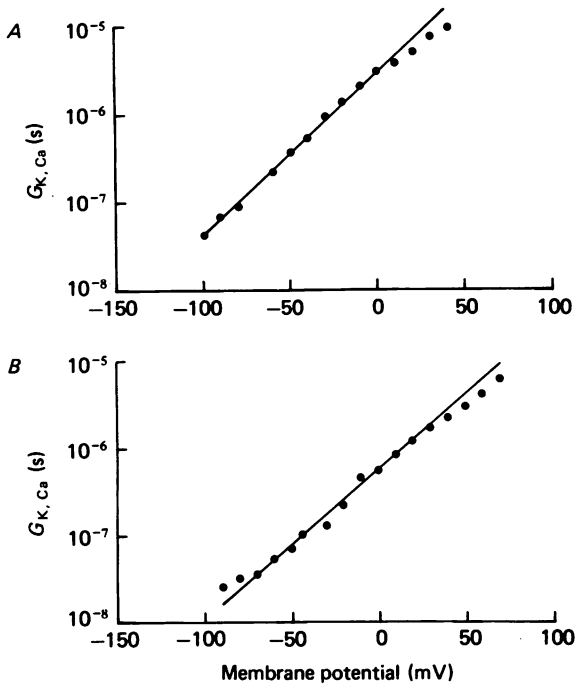

Fig. 4. Logarithmic variation of Ca^{2+} activated K^+ conductance with membrane potential, *A*, logarithmic plot of membrane K^+ conductance activated by a constant intracellular Ca^{2+} injection *vs.* membrane holding potential in Ca^{2+} free ASW. *B*, logarithmic plot of membrane K^+ conductance activated by a constant intracellular Ca^{2+} injection *vs.* membrane holding potential in a different cell in normal ASW. In *A* and *B* the K^+ conductance was activated by a constant 10 sec, 100 nA electrophoretic injection of Ca^{2+} ions into the cell at each membrane potential.

with $[Ca]_i$ which suggests that each K^+ channel is opened by binding of a *single* Ca^{2+} ion. We also suppose that the concentration of Ca^{2+} at the site is related to $[Ca]_i$ by the expression

$$[Ca]_s = [Ca]_i . \exp(zF\delta V/RT), \tag{2}$$

where $[Ca]_s$ represent the Ca^{2+} concentration (or more strictly, the electrochemical activity) as 'seen' by the binding site, $[Ca]_i$ is the free intracellular Ca^{2+} concentration (measured by arsenazo III), V is the transmembrane potential, δ is the fraction of V (measured with respect to the inside) which is experienced at the binding site, i.e. the electrical distance of the site through the membrane from the inside, z is the charge of the Ca^{2+} ion ($z = 2$) and R, T and F have their usual

meanings. If we further assume that the K^+ conductance, $G_{K,Ca}$, is proportional to the concentration $[Ca]_s$, then $G_{K,Ca} = \gamma_K [Ca]_s$ where γ_K is a constant having the dimension of an elementary or unit conductance in S/mole. Equation (2), after substitution, can be expressed in logarithmic form

$$\ln G_{K,Ca} = zF\delta V/RT + \ln \Delta A \quad (3)$$

A constant Ca^{2+} injection was used in all experiments so that the change in $[Ca]_i$ (as measured by the change in dye absorbance) was always a constant, or $\Delta A = [Ca]_i = C$, and therefore can be neglected. For all four experiments, $G_{K,Ca}$ increased on average e-fold for a 25·3 mV change in potential. The expected change at 16 °C would be e-fold for a $RT/zF\delta$ (= 12·5 mV/δ) change in potential giving a value for δ of 0·49. This is consistent with the Ca^{2+} binding site being about half way through the membrane from the inside surface.

Although this interpretation is a very attractive one, it should be pointed out that the same dependence of $G_{K,Ca}$ on membrane voltage can be predicted without having to assume that Ca^{2+} must increase in concentration at some site in the membrane, if instead the dissociation between Ca and S is voltage dependent.

Kinetic properties of the Ca^{2+} activated K^+ current

The effects of depolarization of R-15 from a holding potential of −50 to +30 mV for 300 msec on dye absorbance and on membrane current before and after the addition of 1 mM-Cd^{2+} to normal ASW (10 mM-Ca^{2+}) are shown in Fig. 5*A*. The addition of Cd^{2+} to reduce Ca^{2+} influx during membrane depolarization (Kostyuk & Krishtal, 1977; Gorman & Thomas, 1980) produced substantial changes in both the absorbance and current records. Cd^{2+}, at this concentration, reduced the absorbance change and, therefore, the rise in intracellular Ca^{2+} concentration, by about 95% and the outward current at the end of the 300 msec depolarization by about 78%. The reduction of Ca^{2+} influx also changed the shape and time course of the outward current. In the presence of Cd^{2+} the current rose to a maximum which occurred within the initial 100 msec and thereafter declined to a minimum. The shape of the current can be accounted for by the movement of ions through channels with gates that are activated and inactivated by voltage as a function of time (Hodgkin & Huxley, 1952). The voltage dependent delayed K^+ current of myelinated nerve (Frankenhaeuser, 1963), squid axon (Ehrenstein & Gilbert, 1966) and of other molluscan neurones (Heyer & Lux, 1976*b*) exhibits similar properties. In normal ASW, however, the outward current was not only substantially larger, but rose non-linearly toward a maximum which occurred at the *end* of the voltage pulse. The increase in the outward current in normal ASW has been ascribed to the activation of a K^+ current by a change in internal Ca^{2+} concentration (Meech & Standen, 1975) or by the movement of Ca^{2+} ions through the membrane (Heyer & Lux, 1976*b*).

Similar results were obtained when Ca^{2+} free ASW was used to reduce Ca^{2+} influx and in all subsequent experiments Ca^{2+} free ASW was used to separate the Ca^{2+} dependent from the voltage dependent component of the total outward K^+ current. Fig. 5*B* shows an example of this type of separation. The outward current produced by voltage clamp pulses of various durations was measured in normal ASW and after equilibration in Ca^{2+} free ASW. The difference between the outward currents under

the two conditions (ΔI) represents the Ca^{2+} activated K^+ current $I_{K,Ca}$ and is shown in Fig. 5*B*. It can be seen that the time course of $I_{K,Ca}$ is similar but not identical to the time course of the absorbance change, which is shown in the same Figure. The absorbance change is expected to be a measure of the integral of the inward Ca^{2+}

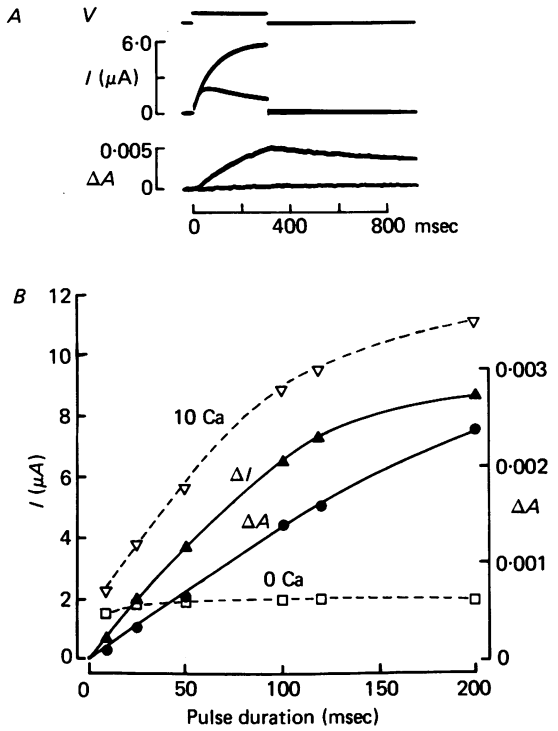

Fig. 5. Effects of Ca^{2+} influx reduction on outward K^+ currents. *A*, outward currents and differential absorbance changes at 660–690 nm before and after the addition of 1 mM-Cd^{2+} to normal ASW. The current (middle) and absorbance (bottom) records show superimposed oscilloscope traces produced by 300 msec depolarizing voltage clamp steps from −50 to +30 mV in normal ASW and after the addition of 1 mM-Cd^{2+}. A change in 0·005 absorbance units represents an increase in intracellular Ca^{2+} of about 580 nM. *B*, plot of the peak outward current and the peak differential absorbance change in response to step depolarization at different durations from −50 to +50 mV. The total outward current in normal ASW (open inverted triangles, 10 mM-Ca) and in Ca^{2+} free ASW (open squares, 0-Ca) as well as the difference between these two currents (filled triangles, ΔI) are shown for comparison to the change in absorbance (filled circles, ΔA).

current over the duration of the step depolarization, i.e. a linear increase in absorbance with time represents a constant Ca^{2+} current (Gorman & Thomas, 1980). Fig. 5 suggests that the inward Ca^{2+} current rises rapidly at the beginning of the pulse, and then stays constant for about 100 msec, declining slowly at longer times as judged by the lower rate of absorbance change. The decline could possibly be due to a partial saturation of the dye absorbance change as $[Ca]_i$ increased, but we would

expect such saturation only for very much larger absorbance changes, so the decline probably reflects a genuine reduction in the Ca^{2+} current. This conclusion is in agreement with the results of experiments where the Ca^{2+} current was measured directly when all other currents were eliminated (Kostyuk & Krishtal, 1977; Akaike, Lee & Brown, 1978; Tillotson & Horn, 1978; Tillotson, 1979).

It is clear from Fig. 5 that the activation of $I_{K,Ca}$ is not directly related to the inward Ca^{2+} current, as had been postulated by Heyer & Lux (1976*b*), but bears a much closer resemblance to the increase in $[Ca]_i$ as a result of the Ca^{2+} influx (see Fig. 1, Gorman & Thomas, 1980). Unfortunately, there are at least two complicating factors which must be taken into account in this type of analysis. Firstly, estimates of $I_{K,Ca}$ from current difference measurements will also contain a component representing the inward Ca^{2+} current, but under most conditions the Ca^{2+} inward current component is expected to be relatively small. Secondly, $I_{K,Ca}$ will be activated by the rise in $[Ca]_i$ at the inner surface of the membrane, which is expected to differ both in size and in time course from the average rise in $[Ca]_i$ throughout the cell as measured by the change in dye absorbance. This is expected to be a far more serious complication.

The effects of membrane potential on the Ca^{2+} activated K^+ current

When the R-15 neurone is depolarized beyond about -30 mV there is a net outward flow of current. The relationship between outward current and membrane potential in a number of molluscan neurones is *N* shaped at positive membrane potentials in normal saline, but the *N* shape is abolished in Ca^{2+} free solution, by external Co^{2+} and by intracellular EGTA (Meech & Standen, 1975; Heyer & Lux, 1976*a*, *b*; Thompson, 1977; Meech, 1978). For these cells, the total outward current is carried primarily by an efflux of K^+ ions from the cell (Heyer & Lux, 1976*a*, *b*). Fig. 6*A* shows a plot of the current–voltage relation of cell R-15 in normal and in Ca^{2+} free ASW. Ca^{2+} free ASW reduced substantially the *N* shape of the relation. The difference between the two curves (ΔI) has been used as an index of $I_{K,Ca}$ (Meech & Standen, 1975) and is plotted in the same Figure along with the change in dye absorbance (ΔA) in normal ASW. The absorbance change was completely abolished in Ca^{2+} free ASW. The absorbance and the current difference curves are bell shaped, but with maxima which are displaced on the voltage axis.

If a rise in intracellular Ca^{2+} activates K^+ channels, then it is the K^+ conductance rather than the K^+ current which should vary with the Ca^{2+} concentration at the inner membrane surface. The effect of plotting the current rather than the conductance will be to displace the peak of the curve to a more positive potential, so this might be responsible for the displacement of the absorbance and current curves in Fig. 6*A*. The reason for the displacement between current and conductance is that as the membrane potential is made more positive, the K^+ current will increase even if the K^+ conductance remains constant, as given by the relation $G_{K,Ca} = \frac{I_{K,Ca}}{V - E_K}$. The K^+ equilibrium potential, E_K, can be estimated from the reversal potential of the Ca^{2+} activated K^+ current which we find to be around -70 mV in R-15 (see also Gorman & Hermann, 1979). The relation will hold only if the conductance of an *open* K^+ channel is independent of the membrane potential, but this is apparently a

reasonable approximation to make for R-15, since its instantaneous current–voltage relation is linear (Eaton, 1972).

Fig. 6*B* shows a plot of the estimated K^+ conductance in normal ASW and in Ca^{2+} free ASW along with the difference in conductance (ΔG). Normalized values for the dye absorbance change ($\Delta A/\Delta A_{max}$) and for the conductance difference ($\Delta G/\Delta G_{max}$) are plotted *vs.* membrane potential in Fig. 6*C*. The conductance curve is closer to the

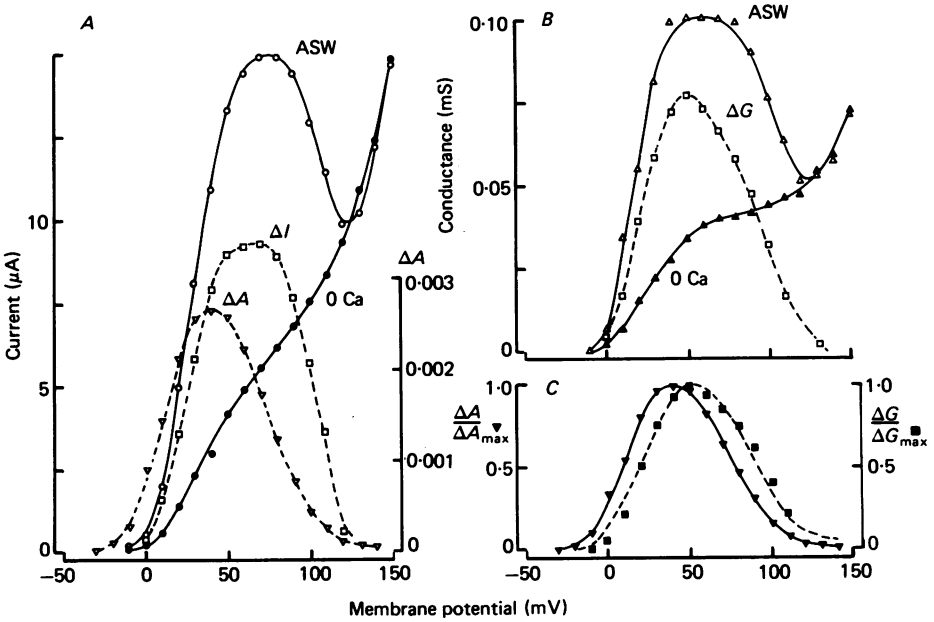

Fig. 6. Effects of Ca^{2+} removal on the outward current, membrane conductance and the differential absorbance change. *A*, plot of the outward current in normal ASW (open cirles) and in Ca^{2+} free ASW containing 2 mM-EGTA (filled circles) produced by 1 sec depolarizing steps to the indicated membrane potentials from a holding potential of −50 mV. The difference between the outward current curves is shown by the broken line connecting the open squares. The differential absorbance change is shown by the broken line connecting the open inverted triangles. A change of 0·003 absorbance units corresponds to an average increase in intracellular Ca^{2+} of about 350 nM. *B*, plot of the total membrane conductance in normal ASW (open triangles) and in Ca^{2+} free ASW (filled triangles) *vs.* membrane potential. The difference between the two curves (ΔG) is shown by the interrupted line connecting the open squares. *C*, plot of the normalized absorbance change ($\Delta A/\Delta A_{max}$) *vs.* membrane potential. The continuous curve through the absorbance values was drawn by eye. The broken curve through the conductance values represents a theoretical curve from eqn. (4) fitted to the peak of the maximum conductance change.

absorbance curve, but the displacement of the maxima is still significant. This difference suggests that the activation of the K^+ conductance also depends on voltage, i.e. changes in intracellular Ca^{2+} open more K^+ channels as the membrane is depolarized. The dashed curve fitted to the experimental conductance data in Fig. 6*C* was calculated from the empirical relation

$$\Delta G^* = \Delta A^* . a \exp(VF\delta/RT), \quad (4)$$

where $\Delta G^* = \Delta G/\Delta G_{max}$, $\Delta A^* = \Delta A/\Delta A_{max}$, V is the membrane potential during a

depolarizing pulse, a is a constant and δ is a factor which determines the voltage dependence. There is general agreement between the theoretical curve drawn from eqn. (4) and the experimental points shown in Fig. 6*C*, but in this and in the six other cells which were analysed, this agreement was never perfect and often worse particularly at potentials on either side of the maximum conductance change.

Much of the difficulty in determining the relationship between K^+ conductance, changes in intracellular Ca^{2+} and membrane potential revolves around the inherent limitations of the current subtraction technique, some of which are difficult to compensate for. One of the principal limitations of the technique is that subtraction of currents has to be done on records taken at very different times. Time is needed to complete a current–voltage relation, to change solutions and to await equilibration in Ca^{2+} free ASW before retesting the neuron, but membrane properties change slightly with time even in the absence of changes in the external medium, and these changes assume greater importance when current differences are used. A different type of problem involves the amount of current carried by Ca^{2+} across the membrane in normal ASW. It is possible that the inward Ca^{2+} current causes a substantial reduction in the net outward current in normal ASW, and it might account for some of the displacement of the rising phase of the current difference curve with respect to the absorbance curve but this seems unlikely on the basis of available data. The Ca^{2+} current of cell R-15 is less than 0·5 μA at its peak, and at the end of a 200 msec pulse is less than 0·2 μA (Tillotson, 1979 and personal communication). When brief pulses were used (about 100 msec) a correction for the Ca^{2+} current does shift the rising phase of the current difference closer to the absorbance curve, but when longer duration voltage clamp pulses were used (1000 msec, Fig. 6) a correction of about 0·2 μA is inconsequential.

Of more serious concern, the problem of changes in the leakage conductance in different ASW solutions remains unresolved, and causes some uncertainty about the falling phase of the current difference curve. The total outward current can be divided empirically into a Ca^{2+} dependent component, a voltage and time dependent component and a voltage dependent, but time independent, or leakage component. The leakage component estimated from the current response to hyperpolarizing pulses or from the instantaneous jump in current during depolarizing pulses is small (less than 2 % of the total outward current in normal ASW) and approximately linear between about -150 mV to about $+50$ mV, but increases non-linearly at more positive potentials. The current subtraction procedure should eliminate the leakage component if it remains constant, but the leakage component is increased in Ca^{2+} free ASW particularly when EGTA is present (unpublished results, A. Hermann & A. L. F. Gorman). For this reason, the outward current in R-15 at very positive membrane potentials in normal ASW is less than the comparable current in Ca^{2+} free ASW (see Fig. 6*A*). This suggests that we may underestimate the falling phase of the current difference curve.

In summary, it appears that the K^+ conductance activated by the influx and subsequent accumulation of Ca^{2+} ions during membrane depolarization is voltage dependent, which is in agreement with the results of the Ca^{2+} injection experiments. The difference current method is, however, insufficiently precise for the voltage dependent of the Ca^{2+} activated K^+ conductance to be quantified from these results with any precision, and in this respect Ca^{2+} injection is by far the better method.

Effects of Ca^{2+} injection on outward current responses

It has been reported that injection of Ca^{2+} ions into some molluscan neurones can depress their outward current response to membrane depolarization by as much as 60% (Heyer & Lux, 1976*b*). We have looked for a similar phenomenon in R-15 using sizable Ca^{2+} injections and several types of stimulus protocol. The simplest type of stimulus paradigm is shown in Fig. 7. The membrane was depolarized in normal

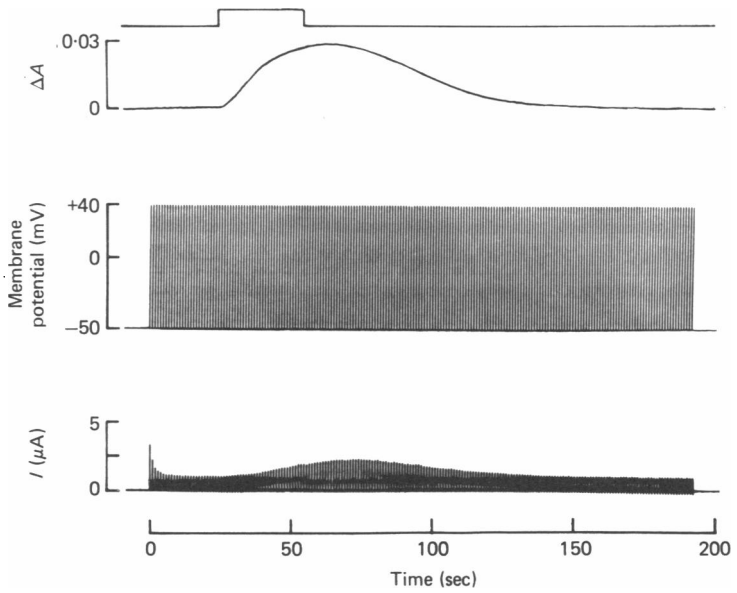

Fig. 7. Effect of Ca^{2+} injection on outward currents to step depolarizations. The top record indicates the time during which Ca^{2+} was iontophoretically injected into the cell and the second record (ΔA) shows the change in differential absorbance in response to this injection. The third record shows the repetitive (1 Hz) 100 msec voltage clamp pulse from −50 to +40 mV and the bottom record shows the outward currents produced by each voltage clamp pulse before, during and after Ca^{2+} injection.

ASW from −50 mV to +40 mV for 100 msec at a rate of 1 Hz and after the first 5 sec the net outward current became identical after each pulse. Ca^{2+} ions were then injected iontophoretically (400 nA) for 30 sec. The absorbance signal and the outward current response increased during the injection but returned slowly to their original levels following its termination. There was a reasonably good correlation between the change in absorbance and the increase in the net outward current response in this and in other experiments without any indication of a depression of the outward current *during* the injection.

The experiment shown in Fig. 7 also demonstrates a phenomenon which has been reported previously (Heyer & Lux, 1976*a*; Eckert & Lux, 1977), namely the depression of the net outward current by successive pulses in a train. Over the first few pulses before Ca^{2+} injection, the outward current decreased significantly, eventually stabilizing at about 40% of its value for the first pulse. A depression of the net outward current also occurred in Ca^{2+} free ASW, but was less pronounced. The depression of the outward current in normal and in Ca^{2+} free ASW is a function of the

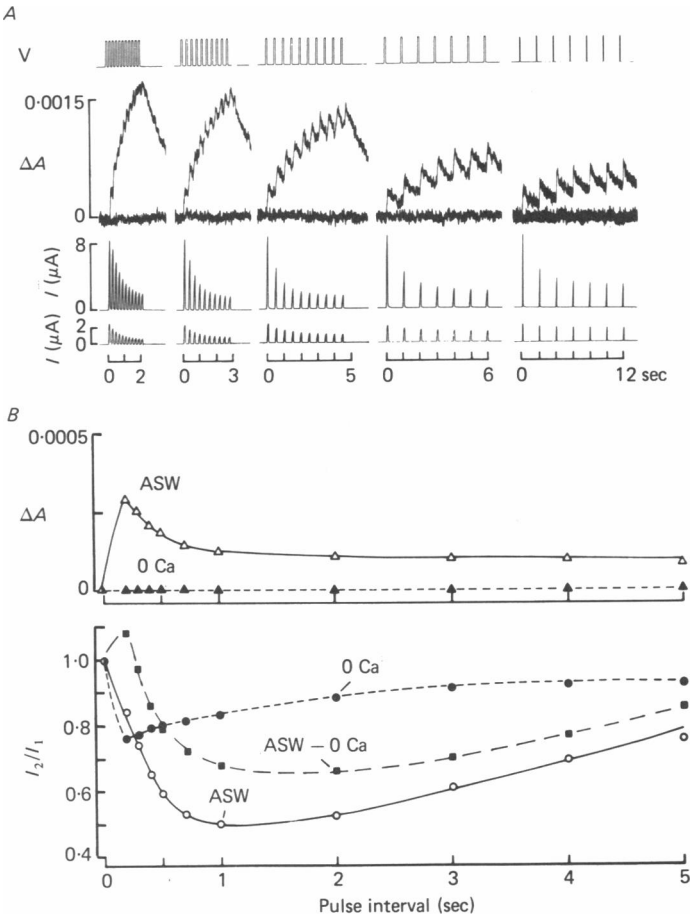

Fig. 8. Effects of stimulus interval on membrane outward current and differential absorbance. *A*, 100 msec step depolarization from −50 to +30 mV (top records) at frequencies of 5, 4, 3, 1 and 0·5 Hz. The second set of records (ΔA) shows the superimposed absorbance traces in normal ASW and in Ca^{2+} free ASW. The third set of records shows outward current responses in normal ASW and the bottom records outward current responses in Ca^{2+} free ASW. *B*, plot of absorbance changes and outward current responses *vs.* pulse interval. The top plot (ΔA) shows the absorbance change at the start of the second depolarizing step in normal ASW and in 0-Ca^{2+} ASW. The bottom plot (I_2/I_1) shows the ratio of the second current response (I_2) to the first current response (I_1) in normal ASW (ASW), in Ca^{2+} free ASW (0-Ca) and the ratio of the difference between the two sets of data (ASW - 0-Ca). The interval between the first and the second depolarizing voltage step at each frequency is indicated on the abscissa.

interval between successive pulses in a train (Eckert & Lux, 1977; Aldrich, Getting & Thompson, 1979). In normal ASW, the depression was maximum at pulse intervals of about 1–2 sec and was apparent for intervals up to at least 15 sec, whereas in Ca^{2+} free ASW maximal depression occurred at intervals of less than 200 msec (the smallest interval tested in our experiments), but was insignificant for intervals greater than about 3 sec (Fig. 8). It has been suggested that in normal ASW the intracellular

accumulation of Ca^{2+} ions during a depolarizing pulse interferes with the activation of $I_{K,Ca}$ by a succeeding pulse (Eckert & Lux, 1977). There is, however, no obvious relation between the time course of internal Ca^{2+} accumulation as measured by arsenazo III, which is maximum at pulse intervals of 200 msec or less (Fig. 8) and

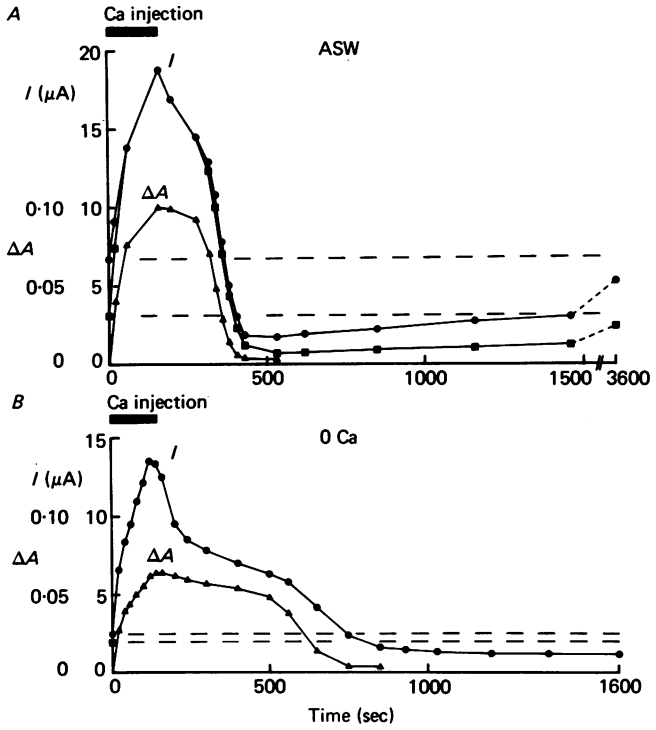

Fig. 9. Effects of intracellular Ca^{2+} injection on differential absorbance changes and on outward currents to step depolarization in normal and in Ca^{2+} free ASW. *A*, plot of the absorbance change (filled triangles) and the first outward current (filled circles) and the fifth outward current response (filled squares) produced by trains of five 100 msec depolarizing voltage clamp pulses from -50 to $+50$ mV at a frequency of 1 Hz in normal ASW during and after a 145 sec 500 nA electrophoretic injection of Ca^{2+} ions into the cell. The top dashed line indicates the peak amplitude of the first outward current response and the bottom dashed line the peak amplitude of the fifth outward current response before the injection. *B*, plot of the absorbance change and the first and fifth outward current response in the same cell produced by identical stimulus conditions in Ca^{2+} free ASW during and after a 160 sec, 500 nA electrophoretic injection of Ca^{2+} ions. The top and bottom dashed lines indicate respectively the peak amplitude of the first and fifth outward current responses before Ca^{2+} injection. An absorbance change of 0·1 units corresponds to a change in intracellular Ca^{2+} of about 8·5 μM.

the time course of the net outward current depression in ASW media containing normal Ca^{2+}. It is possible that the rise in internal Ca^{2+} becomes less effective in activating $G_{K,Ca}$ during a train of pulses, but other factors such as inactivation of the Ca^{2+} channel (Tillotson & Horn, 1978), a change in the K^+ equilibrium potential and thus in the driving force on K^+ ions caused by extracellular K^+ accumulation (Eaton, 1972) and inactivation of the K^+ channel (Aldrich *et al.* 1979), etc. need to be

considered before the basis of the net outward current depression can be fully understood. It is important, however, to make a clear distinction between the time and frequency dependent depression of the net outward current which our experiments confirm, and the depression of the net outward current by intracellular Ca^{2+} injection reported by Heyer & Lux (1976*b*) which is the opposite of the result shown in Fig. 7.

Fig. 8 also demonstrates a second aspect of the effects of successive pulses on the outward current. The curve drawn through the points labelled ASW–OCa in Fig. 8*B* represents the difference between the data obtained in normal and in Ca^{2+} free ASW and suggests that at short intervals (about 200 msec) the outward current may be facilitated by a preceding conditioning pulse. The results were somewhat variable when done under the conditions used to obtain the data shown in Fig. 8, i.e. repetitive, constant amplitude pulses. At short pulse intervals, the pattern of outward current responses in normal ASW shown in Fig. 8*A* was observed most commonly, but in some cases the outward current response to the second pulse in a repetitive train of pulses was the same or slightly larger than the response to the first pulse. This pattern never occurred at longer intervals in normal ASW or at any interval in Ca^{2+} free ASW.

One possible reason for the discrepancy between our results and those of Heyer & Lux (1976*b*) is that the depression of the net outward current may occur only for much larger injections of Ca^{2+}, and this possibility was investigated in the experiment illustrated in Fig. 9. In addition, we investigated the effect of Ca^{2+} injection on the time dependent depression of the net outward current for successive pulses in a train, by using a stimulus paradigm of five 100 msec pulses from -50 to $+50$ mV at a rate of one per sec, repeated with a minimum interval of 30 sec. Fig. 9*A* shows the effect of a 145 sec, 500 nA, Ca^{2+} injection on the peak amplitude of the outward current, for the first (circles) and fifth (squares) pulses in each train. Their values before the Ca^{2+} injection are shown by the dotted lines. As expected from the larger Ca^{2+} injection, this experiment shows a much greater increase in net outward current than was observed in Fig. 7, but it additionally demonstrated that the time dependent depression of the outward current during each pulse train is completely abolished. At the end of the Ca^{2+} injection, the dye absorbance rapidly returned to a value close to its initial base line, and there was a similarly rapid fall in the net outward current with a reappearance of the time dependent depression, but the fall continued until the currents for both the first and fifth pulses in each train were only 25 % of their initial values. Although there was a subsequent slow recovery, it was still incomplete after one hour.

Two hours after the Ca^{2+} injection, the experiment was repeated in Ca^{2+} free ASW (Fig. 9*B*). The outward currents at the end of the first and fifth pulses were almost identical prior to Ca^{2+} injection (dashed line), and became exactly so during the injection, so only the results for the first pulse are shown. There was again a huge increase in outward current during the Ca^{2+} injection, followed by a decline to significantly below its initial value when the absorbance returned towards its baseline. The smaller absorbance change in Fig. 9*B* compared with Fig. 9*A* probably reflected a partial loss of dye from the R-15 soma during the two hours separating these measurements, as judged by the steady decrease in absorbance which occurred at the 570 nm isosbestic wavelength during this time. In both experiments the

absorbance change during Ca^{2+} injection was sufficiently large that it was at or near saturation, i.e. no longer linear with respect to $[Ca]_i$, which could explain why the correspondence between absorbance change and the increase in outward current in Fig. 9*B* was not exact. In both experiments $[Ca]_i$ rose to at least 8·5 μM, and possibly much higher, as judged from the absorbance change during the Ca^{2+} injections, which is two orders of magnitude higher than the changes which occur during bursting pacemaker activity in this neurone (Thomas & Gorman, 1977; Gorman & Thomas, 1978). It is therefore not clear whether the depression in outward current *after* a massive Ca^{2+} injection has any physiological significance, although it could correspond to the depression of net outward current which was reported by Heyer & Lux (1976*b*).

Facilitation of the outward current

Measurements of the arsenazo III absorbance change show that the rate of Ca^{2+} removal is sufficiently low for there to be an overall rise in $[Ca]_i$ during successive pulses in a train (Fig. 8). The extent of the accumulation of Ca^{2+} obviously depends on the stimulus paradigm, but it might be possible to find conditions where the rise in $[Ca]_i$ for successive pulses increases $G_{K,Ca}$ by an amount greater than the decrease due to inactivation (which is maximum at pulse intervals of about 1–2 sec). The result would then be an over-all facilitation of $G_{K,Ca}$.

The results in Fig. 8 suggest that it is difficult to observe facilitation of the outward current when the successive pulses are of the same amplitude, a finding which is in agreement with the results of previous studies (Eckert & Lux, 1977; Aldrich *et al.* 1979). Any facilitation due to Ca^{2+} accumulation is likely to be partially masked by inactivation under these conditions. To remove this masking, we devised an alternative experimental protocol, in which two pulses of different amplitudes were compared. The first ('conditioning') pulse was to +30 mV, a potential at which the Ca^{2+} entry was relatively large (cf. Fig. 6; see also Gorman & Thomas, 1980, Fig. 4). The second ('test') pulse was to +110 mV, a potential at which relatively little Ca^{2+} entry was expected. When the test pulse was given alone, there could thus be relatively little activation of $G_{K,Ca}$, whereas if it was preceded by the conditioning pulse, $[Ca]_i$ at the inner surface of the membrane might be significantly elevated at the start of the test pulse, especially in comparison with the very small rise which would be expected to occur during the test pulse. Under these conditions, the resulting facilitation of $G_{K,Ca}$ might be greater than the inactivation, particularly if the inactivation occurs with some delay, causing an over-all increase in outward current.

The result, shown in Fig. 10, demonstrates that this is indeed the case. The brief (50 msec) test pulse to +110 mV caused no detectable absorbance change, when presented either in isolation or at varying times after a pulse to +30 mV, whereas the +30 mV pulse produced a relatively large and constant absorbance change. The right-hand half of Fig. 10 shows all the voltage, absorbance and current records superimposed, and it can be seen that the current during the test pulse to +110 mV in greatly increased from its normal value (left-hand side of Figure) by a previous pulse to +30 mV (right-hand side). The effect decays quite rapidly, however, and is negligible when the two pulses are separated by more than about 0·5 sec.

The decay presumably represents both the time course of the decline in $[Ca]_i$ at

the inner surface of the membrane following the pulse to + 30 mV (which is expected to be faster than the average decay measured by arsenazo III), and of the inactivation of $G_{K,Ca}$ which is observed during a train of depolarizing pulses. Unfortunately, this experiment provides no way of estimating the relative contributions of the two effects. The facilitation of the outward current was abolished by Ca^{2+} free ASW,

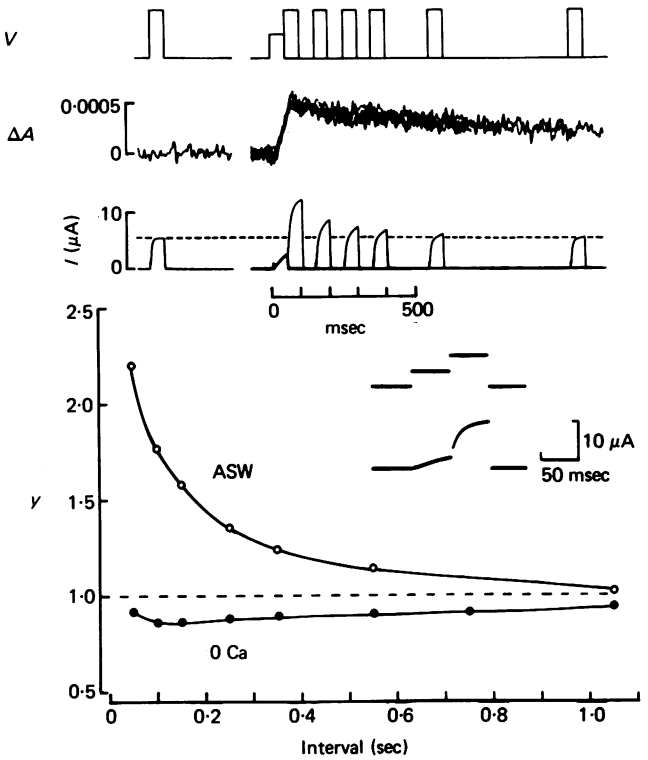

Fig. 10. Outward current responses to brief voltage clamp depolarizations following a preconditioning stimulus. Upper section, outward currents (pen recorder records) produced by a 50 msec depolarization from − 50 to + 110 mV, before and at various intervals after a 50 msec depolarization from − 50 to + 30 mV in normal ASW. The middle superimposed pen recorder records show the absorbance change produced by the + 30 mV pulse. A change in absorbance of 0·0005 units corresponds to an increase in intracellular Ca^{2+} of about 60 nM. The insert shows oscilloscope traces of the voltage (top trace) and current (bottom trace) from the same experiment. Lower section, plot of the ratio of the conditioned outward current to the unconditioned outward current ($y = I_c/I_{uc}$) at various intervals after the conditioned pulse in normal and in Ca^{2+} free ASW.

where the conditioning pulse caused about a 20 % reduction in the outward current response to the test pulse.

The results shown in Fig. 10 also provide further information on the *activation* of $G_{K,Ca}$ by a rise in $[Ca]_i$. The current elicited by a step depolarization to + 110 mV in the absence of a previous conditioning pulse (left-hand side of Figure) rose rapidly at the start of the pulse and then remained essentially constant. This suggests that the component of G_K which depends *only* on voltage (Ca-independent) shows little

if any time dependence under these conditions. If this steady value (dashed line) is subtracted from the current waveforms on the right-hand side of Fig. 10, then the remaining portion of each waveform should represent the Ca^{2+}-mediated component of G_K. Over this time $[Ca]_i$ at the inner surface of the membrane will be falling steadily, but the decline during *each* pulse is probably small enough to neglect. Therefore, the portion of each pulse which lies above the dotted line represents the time dependence of $I_{K,Ca}$ at some (almost) constant $[Ca]_i$.

Clearly, the activation of $I_{K,Ca}$ during each pulse is not immediate, but instead has a time constant of around 12 msec, (estimated from records such as the oscilloscope record shown in the insert in Fig. 10), which must reflect the opening of more channels, i.e. an increase in $G_{K,Ca}$ as a result of membrane depolarization. If $G_{K,Ca}$ had remained constant both before and during each step depolarization to +110 mV, the current waveforms would have been completely square. The result thus provides further evidence to show that $G_{K,Ca}$ is voltage dependent. If the voltage dependence arises because the Ca^{2+} binding site is part way through the membrane, as suggested previously, then changing the membrane potential will change the electrochemical activity of Ca^{2+} as seen by the binding site, so the time constant of approximately 12 msec for the activation of $G_{K,Ca}$ by a step depolarization to +110 mV would represent the rate constant for the Ca^{2+} binding site interaction plus the rate constant for the subsequent opening of the channel.

A possible problem with this analysis is that it ignores the activation of $G_{K,Ca}$ which must also occur at the *holding* potential, following the rise in $[Ca]_i$ as a result of the conditioning pulse to +30 mV. If the voltage dependence of 25·3 mV per e-fold change in $G_{K,Ca}$, calculated from the Ca^{2+} injection experiments, applies over the entire potential range from −50 mV (holding potential) to +110 mV, then the activation of $G_{K,Ca}$ just before the pulse would be only 1/600 as great as that which occurs during the pulse, which is clearly negligible (the ratio of activation at +110 mV to that at −50 mV is given by exp (110+50)/25·3).

DISCUSSION

The principal conclusion of our experiments is that the Ca^{2+} activated K^+ conductance is voltage dependent. This result can be demonstrated directly in experiments where a constant intracellular Ca^{2+} injection is used to activate the K^+ conductance at different membrane potentials, and can be inferred from those experiments where different intracellular Ca^{2+} concentrations, produced by Ca^{2+} influx during membrane depolarization to various membrane potentials, cause changes in the K^+ conductance. It has been suggested recently that there is a very good correlation between the change in the intracellular Ca^{2+} concentration as measured by aequorin and the difference between the net outward current produced by depolarizing pulses to various potentials in normal and in Ca^{2+} free ASW (Eckert & Tillotson, 1978) which has been identified as $I_{K,Ca}$. This correlation suggests that the Ca^{2+} activated K^+ conductance is not voltage dependent, but the independence of the aequorin light reaction at low internal Ca^{2+} concentrations in the presence of normal intracellular K^+ and Mg^{2+}, and its variable stochiometry at higher concentrations (Allen, Blinks & Pendergast, 1977) make it difficult to determine the relation between changes in internal Ca^{2+} and K^+ conductance changes. This criticism is not applicable to measurements made using the dye arsenazo III whose change in

absorbance is linearly related to the change in free ionized intracellular Ca^{2+} concentration (DiPolo *et al.* 1976; Gorman & Thomas, 1978; Thomas, 1979).

Activation of the K^+ channel appears to be produced by the binding of Ca^{2+} or other divalent cations to a membrane site (Meech, 1974; Meech & Standen, 1975; Gorman & Hermann, 1979). Our results suggest that a single Ca^{2+} ion binds to a negatively charged site which activates the K^+ channel. Ca^{2+} has access to this site from the inside surface, but presumably does not pass through the channel. The potential dependence of the Ca^{2+} activated K^+ conductance can be explained if the binding site is about half-way through the membrane from the inside surface. As the membrane is depolarized, the probability that a Ca^{2+} ion will react with the binding site which controls the channel is increased and, therefore, the number of K^+ channels in the open configuration is increased. The location of the binding site may explain the ability of different divalent cations to activate the K^+ channel. The order of affinity for activation of the K^+ conductance is

$$Ca^{2+} > Cd^{2+} > Hg^{2+} > Sr^{2+} > Mn^{2+} > Fe^{2+}$$

and suggests that the binding site can accommodate ionic radii between about 0·76 and 1·13 Å (Gorman & Hermann, 1979). The difference between the affinity of such similar size ions as Ca^{2+} (0·99 Å) and Cd^{2+} (0·97 Å) for the binding site, however, suggested that both the ionic and hydrated radius were important features in determining which ions activate the K^+ channel. If the binding site is within the membrane, then it is possible that a divalent ion has to be dehydrated, and therefore have the proper hydrated radius, before it can enter into a region of the membrane to act as a gating element.

An alternative possibility which could explain our findings is that there is a voltage dependent orientation of negatively charged molecules in the membrane during depolarization leading to the formation of conductive pores for K^+ ions which are activated or gated by Ca^{2+} ions at the inner membrane surface. The voltage dependent conductance changes induced in artificial lipid membranes by the negatively charged antibiotic alamethicin which is believed to form pores in the presence of a properly oriented electrical field across the membrane (see Mueller, 1976) could supply a suitable model for this possibility although typically the conductance change is much greater (e-fold per 6–8 mV) than the change in the Ca^{2+} activated K^+ conductance (e-fold per 25 mV). Such structural changes in orientation have been postulated to occur in starfish egg membranes (Ciani, Krasne, Miyazaki & Hagiwara, 1978) to explain the 'anomalous rectification' of these cells during hyperpolarization. The anomalous rectifier channels are selective for K^+ ions and there is evidence that their gating involves the binding of three K^+ ions to the external surface of the membrane (Hagiwara & Takahashi, 1974) following the voltage dependent formation of pores.

In modelling the reaction of Ca^{2+} with the presumed binding site in the membrane which controls the activation of the K^+ channel it is assumed implicitly that the K^+ conductance reaches a maximum when all the sites are filled with Ca^{2+} ions. We find that the increase in K^+ current, and therefore the K^+ conductance, is a linear function of the free intracellular Ca^{2+} concentration over a wide range of internal Ca^{2+} injection intensities without any evidence of saturation, but it is not clear that we can determine adequately such changes with our present techniques. First, our measurements of intracellular Ca^{2+} with arsenazo III are restricted to a small range of concentrations, e.g. at the maximum intracellular dye concentration typically used in these experiments (about 0·3 mM) the absorbance change saturates at intracellular Ca^{2+} concentration of about 14 μM (Gorman & Thomas, 1980). Second, most

of the Ca^{2+} injected into the cell is sequestered or removed from the cytoplasm and only a small fraction reaches the membrane as free ionized Ca^{2+} which restricts further our ability to test for saturation phenomena.

The rise in $I_{K,Ca}$ during depolarization resembles more closely the expected rise in intracellular Ca^{2+} next to the membrane (see Gorman & Thomas, 1980) than the kinetics of the inward Ca^{2+} current in these cells (see Adams & Gage, 1979). This finding tends to support the hypothesis that activation of the membrane K^+ conductance is initiated by the rise in $[Ca]_i$ (Meech & Standen, 1975) rather than by the inward movement of Ca^{2+} ions through the membrane (Heyer & Lux, 1976*b*), but our results are consistent with the previous conclusion (see Heyer & Lux, 1976*b*) that activation of the K^+ conductance occurs at a site within the membrane rather than at or near its inner surface. Moreover, our results show that activation of the K^+ conductance is not immediate. The intramembrane location of the site and the dependence of its activation on time make it difficult to choose fully, on the basis of simple kinetic arguments, between a system composed of K^+ channels which are opened by the movement of free, intracellular Ca^{2+} ions to a site either within or adjacent to the channel and a system composed of single channels which are transformed from a Ca^{2+} conductive state to a K^+ conductive state by the movement of Ca^{2+} ions into the cell. It is most unlikely, however, that a single channel is used for the simultaneous movement of Ca^{2+} and K^+ ions in opposite directions. If ionic movement in opposite directions occurs then it is more likely to be sequential, but we find no evidence from our measurements of the rise in $[Ca]_i$ at early times that Ca^{2+} influx stops or is diminished significantly under conditions where the Ca^{2+} activated K^+ current becomes appreciable.

One of the principal arguments against the hypothesis that the rise in $[Ca]_i$ is necessary for the activation of the K^+ conductance is that injection of Ca^{2+} ions intracellularly depresses $I_{K,Ca}$ (Heyer & Lux, 1976*b*). We find no evidence that a rise in $[Ca]_i$, produced either by injection of Ca^{2+} ions or by Ca^{2+} influx during membrane depolarization, inhibits or depresses directly the Ca^{2+} activated K^+ current as suggested by previous investigators (Heyer & Lux, 1976*b*; Eckert & Lux, 1977). To the contrary, the outward K^+ current measured by depolarizing pulses is increased *during* internal Ca^{2+} injection. The depression of this current reported by Heyer & Lux (1976*b*) occurs only during the subsequent decline in intracellular Ca^{2+} and is thus likely to be a secondary effect of Ca^{2+} accumulation. Similarly, the time and frequency dependent depression of the outward K^+ current which occurs in these cells during a train of depolarizing pulses bears no obvious temporal relation to the change in intracellular Ca^{2+} concentration and is relieved by intracellular Ca^{2+} injection. Our results, however, suggest that *both* the Ca^{2+} activated and the voltage dependent K^+ conductances of cell R-15 undergo a time and frequency dependent depression during repetitive stimulation. This is in contrast to results from *Helix* neurones (Eckert & Lux, 1977) which suggest that the depression is restricted to the Ca^{2+} activated K^+ conductance system and to results from *Tritonia* neurones where only the voltage dependent K^+ conductance system inactivates during repetitive voltage clamp pulses (Aldrich *et al.* 1979). In cell R-15, the time and frequency dependent inactivation of the Ca^{2+} activated K^+ current is greater and its time course of recovery is significantly longer than that for the voltage dependent K^+ current.

It is not possible to conclude from our findings that the K^+ channels which are

opened by Ca^{2+} ions represent a separate population from those which are opened by membrane depolarization, but there are important differences between the voltage and Ca^{2+} dependent components of the K^+ current of the molluscan neuronal membrane. In these cells, the voltage dependent K^+ current has been separated operationally into two underlying conductance systems (see Connor & Stevens, 1971; Neher, 1971), i.e. a fast K^+ conductance which is activated at more negative potentials (about -50 mV) and a delayed K^+ conductance which is activated at about -20 mV. The Ca^{2+} activated K^+ conductance differs from the voltage dependent K^+ conductances in that it is gated by Ca^{2+} ions, has no clear threshold for its activation and is affected to a lesser extent by changes in membrane voltage.

The data presented in this and in the preceding paper (Gorman & Thomas, 1980), suggest that the rise in $I_{K,Ca}$ during a depolarizing pulse results from the rise in $[Ca]_i$, but they also indicate that the relation between these parameters is quite complex. The Ca^{2+} injection experiments show that over fairly long times (about 10 sec) the activation of $G_{K,Ca}$ is linearly related to $[Ca]_i$, but at the much shorter times (10–100 msec), which are applicable for an action potential or a depolarizing pulse, some additional factors must be taken into account. First, the results in Figure 10 suggest that the activation of $I_{K,Ca}$ by a rise in $[Ca]_i$ has an overall time constant of about 12 msec at $+110$ mV, which is relatively long compared with a 10–100 msec event. Second, $G_{K,Ca}$ inactivates considerably over a 1 sec period (see Fig. 8), to the extent that this effect is also likely to be significant over the 10–100 msec range. A further complication is that when the rate of Ca^{2+} entry is constant, giving a linear rise in the average $[Ca]_i$ (as in Fig. 5), the rise of $[Ca]_i$ at the inner surface of the membrane is expected to follow the square root of time (Gorman & Thomas, 1980).

All three effects must be taken into account in any description of the time course of the activation of $I_{K,Ca}$ during a depolarizing pulse. Unfortunately, we do not know enough about the inactivation mechanism to be able to propose a complete theoretical model. Nevertheless, the problem can be explored in a more qualitative way, i.e. to ask whether it is possible for the three effects to result in a time course of $I_{K,Ca}$ similar to that actually observed (as in Fig. 5). The model used is of the form

$$X + Ca \underset{-k_1}{\overset{k_1}{\rightleftharpoons}} CaX \underset{-k_2}{\overset{k_2}{\rightleftharpoons}} CaY,$$

where X represents a closed channel, CaX represents an open channel and CaY represents an inactivated channel (of course, we do not know whether this model is a valid description of the inactivation mechanism, but it was chosen as being the simplest possible one). For $[Ca]_i$ increasing with the square root of time, the appropriate differential equations for X, CaX and CaY with respect to time were solved graphically on a Hewlett–Packard 9821 calculator/plotter, for various values of k_1, k_{-1}, k_2 and k_{-2}. Fig. 11 shows one such solution, for respective values of 30 M^{-1}, 3, 3 and 1 per second. Comparison with Fig. 5 shows that CaX follows the observed time course of $I_{K,Ca}$ quite well for times up to 200 msec, especially the near linearity over the first 50 msec. In qualitative terms, both the inactivation and the square-root-of-time dependence of Ca^{2+} accumulation tend to reduce the rate of increase of CaX with time (causing an overall decrease at long times which is also observed in

practice for $I_{K,Ca}$), but at short times this is offset by the effect of the finite rate constant for the formation of CaX, resulting in a more nearly linear increase of CaX with time over the first 50 msec. The model is highly speculative, but it does demonstrate that the conclusions drawn in this and in the preceding paper (Gorman & Thomas, 1980), concerning both the accumulation of $[Ca]_i$ and the resulting activation of $I_{K,Ca}$, are *compatible* with the observed time course of $I_{K,Ca}$ activation.

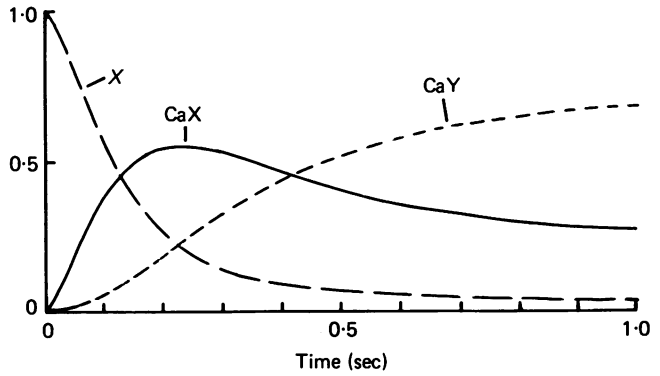

Fig. 11. Theoretical curves showing the changes in a population of Ca^{2+} activated K^+ channels with time following the influx and subsequent internal accumulation of Ca^{2+} ions where X represents a closed channel, CaX is an open channel and CaY is an inactivated channel (see text for further discussion).

In conclusion, our results show that the Ca^{2+} activated K^+ channel is surprisingly similar to more 'conventional' ion channels, in that it apparently shares with them the properties of voltage dependence, non-instantaneous activation, and a slower (partial) inactivation. The voltage dependence of $G_{K,Ca}$ is clearly of considerable physiological significance. It means that $G_{K,Ca}$ can play a much more major role in repolarizing the membrane from the peak of an action potential than might have been inferred from the very much smaller Ca^{2+} activated 'tail' currents which can be recorded at membrane potentials near the resting potential after a depolarizing pulse or from the currents which are activated by Ca^{2+} injections of similar size to the Ca^{2+} entry during a burst of action potentials in R-15 (Gorman & Thomas, 1978). An additional factor is that although the average $[Ca]_i$ does not decline very fast compared with the falling phase of an action potential (see, e.g., Fig. 5*A*), the $[Ca]_i$ at the inner surface of the membrane is expected to fall much more rapidly as Ca^{2+} diffuses further into the cell (Gorman & Thomas, 1980), causing a corresponding fall in $G_{K,Ca}$. This effect too will cause a relatively rapid reduction in $G_{K,Ca}$ as the membrane repolarizes.

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REFERENCES

ADAMS, D. J. & GAGE, P. W. (1979). Ionic currents in response to membrane depolarization in an *Aplysia* neurone. *J. Physiol.* **289**, 115–141.

AKAIKE, N., LEE, K. S. & BROWN, A. M. (1978). The calcium current of *Helix* neuron. *J. gen. Physiol.* **71**, 509–531.

ALDRICH, R. W., GETTING, P. A. & THOMPSON, S. H. (1979). Inactivation of delayed outward current in molluscan neurone somata. *J. Physiol.* **291**, 507–530.

ALLEN, D. G., BLINKS, J. R. & PENDERGAST, F. G. (1977). Aequorin luminescence: relation of light emission to calcium concentration – a calcium-independent component. *Science, N.Y.* **196**, 996–998.

ANDRESEN, M. C., BROWN, A. M. & YASUI, S. (1979). The role of diffusion in the photo-response of an extraretinal photoreceptor of *Aplysia*. *J. Physiol.* **287**, 283–301.

BRINLEY, F. J., JR (1978). Calcium buffering in squid axons. *A. Rev. Biophys. Bioeng.* **7**, 363–392.

BROWN, A. M. & BROWN, H. M. (1973). Light response of a giant *Aplysia* neuron. *J. gen. Physiol.* **62**, 239–254.

BROWN, H. M., PEMBERTON, J. P. & OWEN, J. D. (1976). A calcium-sensitive microelectrode suitable for intracellular measurement of calcium (*II*) activity. *Analyt. chem.* **85**, 261–276.

CIANI, S., KRASNE, S., MIYAZAKI, S. & HAGIWARA, S. (1978). A model for anomalous rectification: electrochemical-potential-dependent gating of membrane channels. *J. membrane Biol.* **44**, 103–134.

CONNOR, J. A. & STEVENS, C. F. (1971). Voltage clamp studies of a transient outward membrane current in gastropod neural somata. *J. Physiol.* **213**, 21–30.

DIPOLO, R., REQUENA, J., BRINLEY, F. J. JR., MULLINS, L. J., SCARPA, A. & TIFFERT, T. (1976). Ionized calcium concentrations in squid axons. *J. gen. Physiol.* **67**, 443–467.

EATON, D. C. (1972). Potassium ion accumulation near a pacemaker cell of *Aplysia*. *J. Physiol.* **224**, 421–440.

ECKERT, R. & LUX, H. D. (1977). Calcium-dependent depression of a late outward current in snail neurons. *Science, N.Y.* **197**, 472–475.

ECKERT, R. & TILLOTSON, D. (1978). Potassium activation associated with intraneuronal free calcium. *Science, N.Y.* **200**, 273–439.

EHRENSTEIN, G. & GILBERT, D. L. (1966). Slow changes of potassium permeability in the squid giant axon. *Biophys. J.* **6**, 553–566.

FRANKENHAEUSER, B. (1963). A quantitative description of potassium currents in myelinated nerve fibres of *Xenopus laevis*. *J. Physiol.* **169**, 424–430.

FRAZIER, W. T., KANDEL, E. R., KUPFERMANN, I., WAZIRI, R. & COGGESHALL, R. E. (1967). Morphological and functional properties of identified cells in the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **30**, 1288–1351.

GORMAN, A. L. F. & HERMANN, A. (1979). Internal effects of divalent cations on potassium permeability in molluscan neurons. *J. Physiol.* **296**, 393–410.

GORMAN, A. L. F. & THOMAS, M. V. (1978). Changes in the intracellular concentration of free calcium ions in a pace-maker neurone, measured with the metallochromic indicator dye arsenazo III. *J. Physiol.* **275**, 357–376.

GORMAN, A. L. F. & THOMAS, M. V. (1980). Intracellular calcium accumulation during depolarization in a molluscan neurone. *J. Physiol.* **308**, 259–285.

HAGIWARA, S., MIUAZAKI, S., MOODY, W. & PATLAK, J. (1978). Blocking effects of barium and hydrogen ions on the potassium current during anomalous rectification in the starfish egg. *J. Physiol.* **279**, 167–185.

HAGIWARA, S. & TAKAHASHI, K. (1974). The anomalous rectification and cation selectivity of the membrane of a starfish egg cell. *J. membrane Biol.* **18**, 61–80.

HEYER, C. B. & LUX, H. D. (1976*a*). Properties of a facilitating calcium current in pace-maker neurones of the snail, *Helix pomatia*. *J. Physiol.* **262**, 319–348.

HEYER, C. B. & LUX, H. D. (1976*b*). Control of the delayed outward potassium currents in bursting pace-maker neurones of the snail, *Helix pomatia*. *J. Physiol.* **262**, 349–382.

HODGKIN, A. L. & HUXLEY, A. F. (1952). The components of membrane conductance in the giant axon of *Loligo*. *J. Physiol.* **116**, 473–496.

KOSTYUK, P. G. & KRISHTAL, O. A. (1977). Separation of sodium and calcium currents in the somatic membrane of mollusc neurones. *J. Physiol.* **270**, 545–568.

MEECH, R. W. (1972). Intracellular calcium injection causes increased potassium conductance in *Aplysia* nerve cells. *Comp. Biochem. Physiol.* **42**A, 493–499.

MEECH, R. W. (1974). The sensitivity of *Helix aspersa* neurones to injected calcium ions. *J. Physiol.* **237**, 259–277.

MEECH, R. W. (1978). Calcium-dependent potassium activation in nervous tissues. *A. Rev. Biophys. Bioeng.* **7**, 1–18.

MEECH, R. W. & STANDEN, N. B. (1975). Potassium activation in *Helix aspersa* neurones under voltage clamp; a component mediated by calcium influx. *J. Physiol.* **249**, 211–239.

MUELLER, P. (1975). Electrical excitability in lipid bilayers and cell membranes. In *International Review of Science Biochemistry*, series 1, vol. 3, ed. RACKER, E., pp. 75–120. London: Butterworth.

NEHER, E. (1971). Two fast transient current components during voltage clamp on snail neurons. *J. gen. Physiol.* **58**, 36–53.

PARSONS, R. (1959). *Handbook of Electrochemical Constants*. New York: Academic Press.

THOMAS, M. V. (1979). Arsenazo III forms 2:1 complexes with Ca and 1:1 complexes with Mg under physiological conditions. *Biophys. J.* **25**, 541–548.

THOMAS, M. V. & GORMAN, A. L. F. (1977). Internal calcium changes in a bursting pace-maker neuron measured with arsenazo III. *Science, N.Y.* **196**, 531–533.

THOMAS, M. V. & GORMAN, A. L. F. (1978). Voltage dependent Ca^{2+} entry in a molluscan neuron investigated with arsenazo III. *Biophys. J.* **21**, 178*a*.

THOMAS, M. V. & GORMAN, A. L. F. (1979). Voltage dependence of the Ca^{2+} activated outward current in molluscan neurons investigated with arsenazo III. *Biophys. J.* **25**, 266*a*.

THOMPSON, S. H. (1977). Three pharmacologically distinct potassium channels in molluscan neurones. *J. Physiol.* **265**, 465–488.

TILLOTSON, D. (1979). Inactivation of Ca conductance dependent on entry of Ca ions in molluscan neurons. *Proc. natn. Acad. Sci. U.S.A.* **76**, 1497–1500.

TILLOTSON, D. & HORN, R. (1978). Inactivation without facilitation of calcium conductance in caesium-loaded neurons of *Aplysia*. *Nature, Lond.* **273**, 312–314.