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CONTROL OF THE DELAYED OUTWARD POTASSIUM CURRENTS IN BURSTING PACE-MAKER NEURONES OF THE SNAIL, *HELIX POMATIA*

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SUMMARY

1. The net outward current in bursting pace-maker neurones of the snail (*Helix pomatia*) during sustained and repeated voltage clamp pulses was studied. The properties of currents remaining in cobalt-Ringer or after TEA injection were compared with those in untreated cells.

2. With sustained voltage clamp depolarizations the net outward current first increases to a maximum at 150 msec and then declines to 60 % or less of its peak intensity. This depression, which is greater during repetition of short pulses (e.g. 100 msec pulses at 0·5 sec intervals), represents a true decrease in the outward flow of K (designated I_K) and is not due to a decreased driving force resulting from extracellular K accumulation. The steady-state current–voltage (I–V) relationship for I_K is N-shaped (Heyer & Lux, 1976).

3. A component of I_K persists when Ca and Mg in the medium are replaced by Co ($I_{Co\text{-}res}$). With voltage clamp depolarizations $I_{Co\text{-}res}$ increases rapidly to a maximum and then partially inactivates with voltage dependent time constants of hundredths or tenths of seconds. Repolarization removes the inactivation. Thus, repeated stimulation with short pulses does not increase the depression of $I_{Co\text{-}res}$. $I_{Co\text{-}res}$ (e.g. measured during voltage steps from holding potentials of -50 to near 0 mV) is smaller in test pulses preceded by depolarization and larger in pulses preceded by hyperpolarization. The steady state I–V relationship is not N-shaped. $I_{Co\text{-}res}$ is blocked by intracellular injection of tetraethylammonium (TEA).

4. Repeated voltage clamp depolarization to near 0 mV with 100 msec pulses for neurones with large Ca currents in normal Ringer produces a long-term depression which is maximal with 300–400 msec repolarizations (to -50 mV) between pulses. This corresponds with stimulus parameters for the maximum Ca current (Heyer & Lux, 1976). Intracellular injection

of Ca^{2+} (also Ba^{2+} and Co^{2+}) likewise reduces the total net outward current and especially the delayed outward current under voltage clamp.

5. The component of I_K which is removed by Co is identified as Ca dependent and designated $I_{K(Ca)}$. With single voltage clamp pulses $I_{K(Ca)}$ follows the approximate time course and voltage dependence of the slow inward Ca current ($I_{in\ slow}$; Heyer & Lux, 1976). Several lines of evidence suggest that Ca ions moving through the membrane activate $I_{K(Ca)}$.

6. Part of I_K cannot be blocked by intracellular TEA injection. In different neurones the magnitude of the I_K component resistant to TEA ($I_{TEA\text{-}res}$) is approximately proportional to the relative magnitudes of $I_{in\ slow}$. $I_{TEA\text{-}res}$ does not inactivate with sustained depolarization and shows pronounced long-term depression with repetitive stimulation at intermediate intervals and an increased outward current at the onset of the second and subsequent pulses following short repolarizations. The steady-state I–V relationship is N-shaped. $I_{TEA\text{-}res}$ is abolished by extracellular Co.

7. A net inward current with low depolarizations can be measured after TEA injection. Evidence is presented suggesting that TEA slows the onset of $I_{in\ slow}$ and delays its inactivation.

8. The K current is discussed as consisting of two components. One component is Ca independent and is suggested to have the voltage and time dependent properties of $I_{Co\text{-}res}$. It is compared with K conductances in such preparations as the squid giant axon. The second component proposed is dependent on Ca both for its activation (a transient effect of Ca ions moving through the membrane) and inactivation (a long lasting effect of increased concentrations of Ca at the inner surface of the membrane). It is considered to have many properties similar to $I_{TEA\text{-}res}$ and may be related to other TEA resistant K currents. Its properties are contrasted with those of previously described membrane conductances.

INTRODUCTION

Long depolarizing voltage clamp pulses produce net outward currents which in many neurones decay gradually. In bursting pacemaker cells of *Helix pomatia*, there is an additional long lasting depression of the net outward current which occurs with repetitive voltage clamp stimulation (Lux & Neher, 1972; Lux & Eckert, 1974). Such decreases could be due to a number of factors. They can reflect a true inactivation of the K current. In addition, accumulation of K near the membrane during the pulse could result in a decreased driving force on K^+ ions, reducing the net outward current. Such a mechanism has been proposed for other

molluscan neurones (Alving, 1969; Eaton, 1972). Finally an opposing current produced by an inward movement of positive charges may cancel part of the outward K current, reducing the measured current.

The short-circuiting effect of the inward current (Lux & Eckert, 1974; Eckert & Lux, 1975, 1976; Heyer & Lux, 1976) is not sufficient to account for the decrease in outward current in bursting pacemaker cells of *Helix pomatia*. In addition recordings with K-sensitive electrodes demonstrate that there is a decrease in K^+ efflux, confirming that the decrease in delayed outward current during maintained or repetitive depolarizations does, in fact, represent a depression of the outward charge transfer of K^+ ions. Under the conditions of these experiments we show that the decline in net outward current is not explicable in terms of the depressant effect of K accumulation during depolarization, suggesting that inactivation of K channels occurs.

In this paper we report that the net outward current remaining when the inward Ca current is blocked by Co shows a time and voltage dependent activation and inactivation which is similar to that previously reported for other currents in excitable membranes (e.g. see Cole, 1968, for references). The additional long lasting depression of the outward K current which develops during the interstimulus interval with repetitive depolarizations is abolished by extracellular Co. It is maximal for stimulus paradigms producing the greatest influx of Ca (Heyer & Lux, 1976). Depression of the net outward K current is similarly produced by the intracellular injection of Ca^{2+} (Ba^{2+} and Co^{2+}). We therefore attribute the long lasting depression to the effects of increased intracellular Ca.

These results suggested that the Ca dependence of the K current which is blocked by Co needed re-evaluation. Ca appears to have a transient facilitating effect on a component of the K current. Our evidence indicates that this component of the delayed outward current depends on the Ca moving through the membrane and therefore on the persistence of an inward Ca current ($I_{\text{in slow}}$; Eckert & Lux, 1975, 1976). It is not due to an increase in $[Ca]_i$ as suggested by Meech & Standen (1975).

Thus we describe the K current in bursting pace-maker neurones of *Helix pomatia* in terms of two components. One, resembling the typical ionic conductances of squid giant axons and nodes of Ranvier, is independent of specific effects of Ca ions. The second component of the K current is dependent on Ca for both its activation and inactivation. This second component is particularly interesting since many of its properties differ significantly from characteristics of conductances previously described for fibre membranes (Hodgkin & Huxley, 1952*a–d*).

A preliminary account of this work has appeared elsewhere (Lux & Heyer, 1976*a*).

METHODS

The preparation and recording techniques, the criteria for differentiating fast and slow bursters and the relationship between the current trajectories with repetitive simulation and the deficit current calculated from comparisons of voltage clamp currents with K electrode recordings are all described in the preceding paper (Heyer & Lux, 1976). Intracellular concentrations of ions were increased by two methods. Ca^{2+}, Ba^{2+}, Co^{2+} and tetraethylammonium (TEA) were all pressure injected into neurones from micropipettes (tip diameter 1·5 – 3 μm; walls parallel for 50 – 150 μm) at pressures from 0·5 to 6·0 atmospheres. Outflow of the solution was checked under microscopic control and pressure adjusted to inject a volume comparable with that of the cell within several minutes. The injection caused no visible swelling of the neurones. Electrodes were filled with 1 M solutions of $CaCl_2$, $CoCl_2$ or TEA-Cl in 66 mM-KCl. The latter was calculated as equivalent to the intracellular K^+ activity on the basis of ion sensitive electrode measurements. A solution of 100 mM-$BaCl_2$, 66 mM-KCl was also pressure injected. In addition TEA and Ca were electrophoresed into the neurones from pipettes containing 1 M TEA-Cl or $CaCl_2$ and 66 mM-KCl, at currents up to 750 nA for 0·25 to 5 min under voltage clamp.

Definitions

TEA tetraethylammonium ions

Co-Ringer normal snail Ringer (Eckert & Lux, 1976) in which all of the $CaCl_2$ and $MgCl_2$ have been replaced by 10 mM-$CoCl_2$

$I_{\text{in slow}}$ the slow inward Ca current (Eckert & Lux, 1976)

I_K the total delayed outward K current

$I_{\text{Co-res}}$ the Co resistant K current; i.e. I_K remaining in Co Ringer

$I_{\text{TEA-res}}$ the TEA resistant K current; i.e. I_K remaining after maximum effectiveness of intracellular TEA injections

$I_{K(Ca)}$ the K current which depends on the presence of Ca in the Ringer solution; similar but not entirely equal to $I_{\text{TEA-res}}$.

RESULTS

Relationship of extracellular K to the depression of net outward current

Voltage clamp records alone and compared with simultaneous K efflux data suggest that under the conditions reported here, the extracellular accumulation of K is not the major cause of the decline in net outward current.

The response of these neurones to different stimulus paradigms were tested to determine if a relationship existed between the depression of the net outward current and the total outward current. For example, in one such experiment the total duration of voltage clamp depolarization was fixed at 1000 msec and presented either as a continuous pulse or as ten 100 msec pulses. The response to these two patterns is seen in Fig. 9, upper trace, of the preceding paper (Heyer & Lux, 1976). Although the

100 msec pulses were spaced at 400 msec intervals, the depression of the net outward current at the end of 1000 msec of interrupted depolarization was greater than at the end of a prolonged pulse (i.e. 83 % for the interrupted pulses compared with 64 % for the continuous long pulse). At the same time the total time integral of the outward current during the ten 100 msec pulses was reduced to 45 % of that during the long pulse. The inward current alone was of insufficient magnitude to account for this difference and a true decrease in K efflux was measured by the K sensitive electrode (Fig. 9, lower trace, Heyer & Lux, 1976).

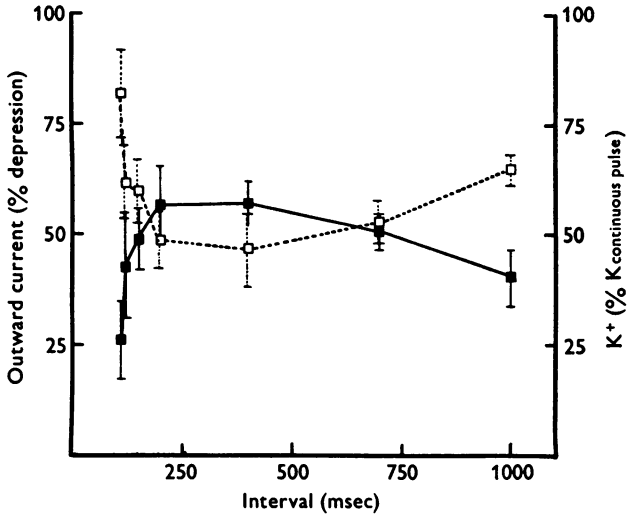

Fig. 1. The depression of the net outward current and the K efflux as functions of the frequency of stimulation (100 msec voltage clamp depolarizations; intervals measured between the onset of pulses). The depression in the sum of ten 100 msec pulses (from −50 to +7 mV) is presented as % total current during a single 1000 msec depolarization (■ left scale). The K efflux is presented as the ratio of K efflux from ten 100 msec pulses to the K efflux from a single 1000 msec. pulse (– – □ – –, right scale). Vertical bars indicate standard deviations for two to four trials on each of three neurones in which the same stimulus intervals were tested.

If the depression of the outward current were due to K accumulation, it should be positively correlated with the amount of K which flows out of the cell. We did not find this to be true. For example, when intervals between the ten 100 msec pulses were varied, both the depression of the outward current and the total K efflux were affected. In Fig. 1 the total K efflux and depression of the net outward current are presented as functions of the interval between the voltage pulses. The relationship clearly shows that depression is inversely correlated with the efflux.

Although the depression of the net outward current is not a function of the total K efflux, it still may be related to the concentration of extracellular K at the end of stimulation. However, our records do not indicate that this is likely. In Fig. 2 the responses of a neurone to a single long pulse and short pulses repeated at 200 and 800 msec intervals are presented. The depression of net outward current is the greatest with repetition at 200 msec intervals (71 %, triangle, Fig. 2*B*). In this neurone the

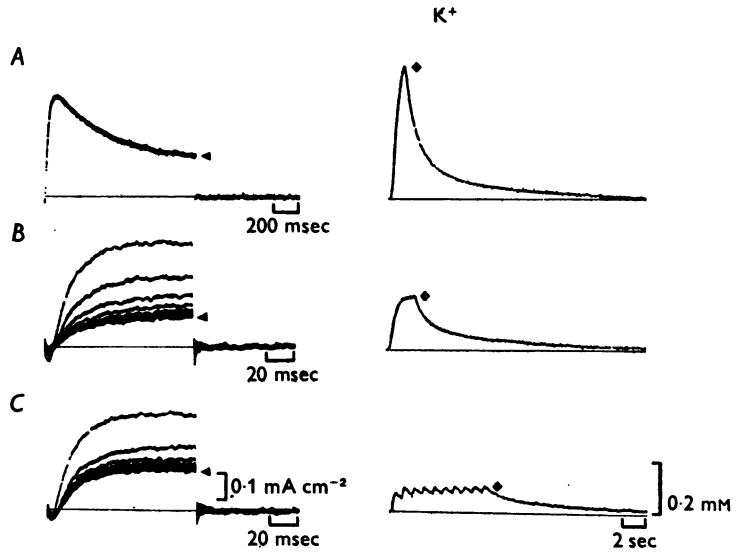

Fig. 2. The net outward current (column *I*) recorded during sustained (*A*) and repeated voltage clamp pulses (superimposed traces from ten 100 ms pulses at 200 msec intervals in *B* and at 800 ms intervals in *C*), compared with the concomitant K activity at the cell soma surface during and following these stimulus paradigms (column K^+). The magnitude of the outward current after 1000 msec of continuous (*A*) or interrupted (*B*, *C*) depolarizations (triangles, column *I*) and the K activity at the same time (squares, column K^+) are indicated for each trial. Data are typical of those from other fast bursters.

depression resulting from the two other stimulation paradigms (Fig. 2*A*, *C*) is the same (60 %). In contrast, simultaneous records of the external K activity show the peak concentration after the long pulse (square, Fig. 2*A*) and the least during the repetition with long interpulse intervals (Fig. 2*C*). Furthermore, with repetition at 800 msec intervals, the net outward current continues to decline over the least the first five pulses, whereas extracellular K accumulation changes little between the second and the fifth pulse.

Thus, much of the decline in outward current is due to depression of the K current not solely attributable to a decreased driving force on K ions.

Components of I_K

The Co resistant K current ($I_{Co\text{-}res}$)

The net outward current is depressed when the Ca^{2+} and Mg^{2+} in the Ringer solution are replaced by Co^{2+}. In slow bursters this usually amounts to less than 50% while in fast bursters up to 85% of the net outward

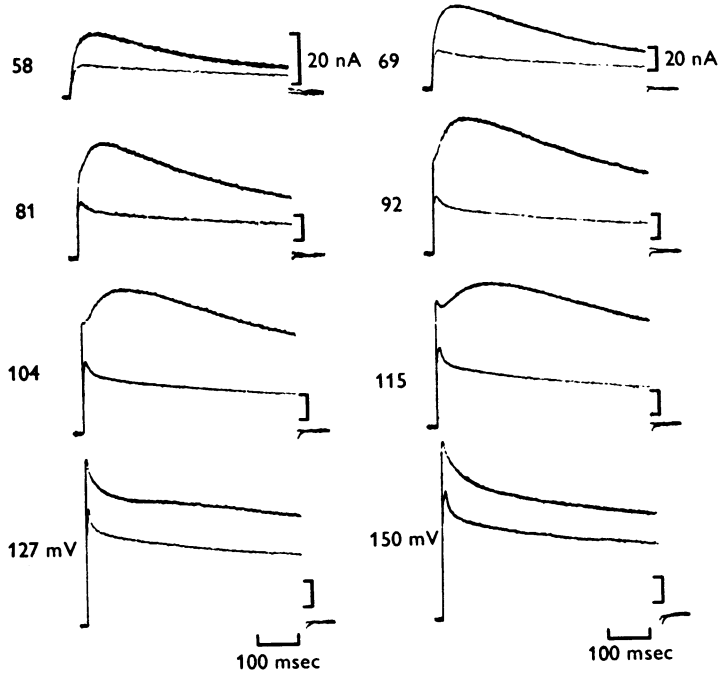

Fig. 3. Typical current trajectories from the responses of a fast burster to 500 msec voltage clamp pulses of increasing size (values to the left of each pair of trajectories indicate the height of the pulse in mV from −50 mV holding potential). The upper trace of each pair of responses is the current trajectory from the neurone in normal Ringer; the lower trace of each pair indicates the response in Co-Ringer and thus represents the trajectory of $I_{Co\text{-}res}$ (see text).

current with depolarization to near 0 mV is abolished by Co^{2+}. The component of the outward current which is Co resistant will be designated $I_{Co\text{-}res}$. It has strongly time and voltage dependent activation and inactivation characteristics. $I_{Co\text{-}res}$ includes components of the fast outward current (I_{fast}, see Neher, 1971; and also Connor & Stevens, 1971) which is

nearly inactivated at the holding potential of −50 mV and the delayed outward K currents.

Voltage dependence of $I_{\text{Co-res}}$

The records in Fig. 3 show the current trajectories of $I_{\text{Co-res}}$ (lower trace in each pair of trajectories) during 500 ms depolarizing pulses of increasing magnitude. The current–voltage (I–V) relationships (based on current values after 100 ms depolarization) obtained from one neurone are given in Fig. 4. As evident from the current trajectories, $I_{\text{Co-res}}$ increases monotonically as a function of depolarization.

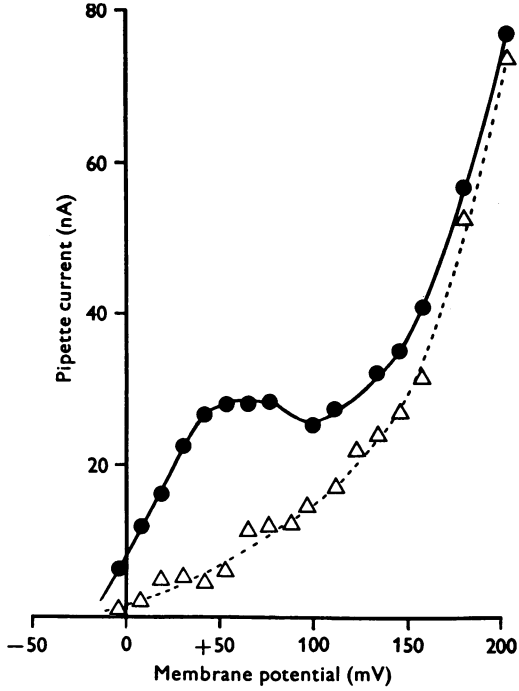

Fig. 4. The amplitude of the net outward current after 100 msec of voltage clamp depolarization as a function of the membrane potential (for pulses from −50 mV holding potential). Responses of a typical fast burster in normal Ringer (–●–) and currents recorded from that neurone in Co-Ringer (- - - △ - - -) are shown.

Activation and inactivation of $I_{\text{Co-res}}$

With maintained depolarization the net outward current in Co-Ringer declines (see Fig. 3). The depression of $I_{\text{Co-res}}$ ranges up to 45% with time constants of tenths or hundredths of seconds. At depolarizations greater than about +150 mV the depression of $I_{\text{Co-res}}$ with prolonged

depolarizations is abolished or even reversed (not shown in Fig. 3). This is sometimes associated with apparent damage to the neurone (e.g. an increased leakage conductance during subsequent pulses).

The activation of $I_{\text{Co-res}}$ is voltage dependent; the rates of $I_{\text{Co-res}}$ onset increase with increasing depolarization (see Fig. 3). Increases in holding potential from -50 mV to -60 mV and 100 ms prepulses of -70 to -95 mV preceding 100 msec test pulses increase the magnitude and rate of rise of $I_{\text{Co-res}}$. Likewise decreasing the holding potential from -50 mV to -40 mV and prepulses of -50 to -15 mV decrease the available $I_{\text{Co-res}}$ and decrease its rate of rise.

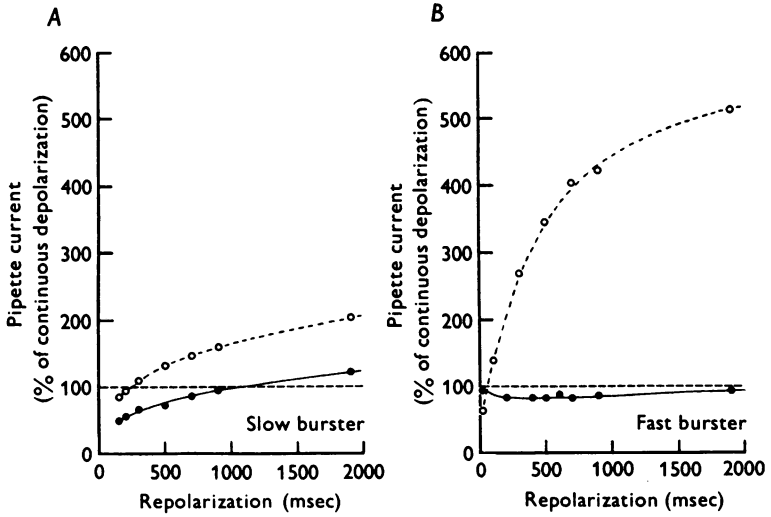

Fig. 5. The depression of net outward current as a function of the repolarization interval for a slow and a fast burster. The amplitude of the outward current at the end of ten 100 msec pulses (as a % magnitude of outward current after 1000 msec depolarization from -50 to $+7$ mV; i.e. 100 % indicates equal depression with interrupted and sustained depolarization) is plotted against the repolarization interval between the pulses in the presence of normal (–●–) and Co-Ringer (- - ○ - -) for these two neurones. The response pattern is similar for the slow burster and both neurones after Co treatment: depression decreases as the interpulse interval increases. In contrast, the depression of outward current in the normal fast burster increases with decreasing frequency until 500 msec intervals.

Long lasting Ca dependent depression of I_K

Characteristics of the long lasting depression

The delayed outward K current becomes depressed with sustained or repeated depolarizations. In Co Ringer this depression does not show any long lasting component but rather appears typical of other time and

voltage dependent inactivations (e.g. of the Na system; Hodgkin & Huxley, 1952*c*). Thus, the decline in $I_{\text{Co-res}}$ is greatest with sustained depolarization. When stimulation with ten 100 msec pulses is used, the depression (at all but the shortest intervals) is less than that produced by 1000 msec continuous depolarization and the depression decreases as a function of increasing interstimulus interval. Typical patterns for the dependence of depression on stimulus interval (Fig. 5) show the progressive removal of depression as a function of increasing repolarization intervals for Co treated neurones. The depression of I_{K} is partially removed by increasing the holding potential or preceding each test pulse with a hyperpolarizing prepulse.

Application of the same stimulus paradigms to neurones in normal Ringer solution does not produce the same relationship between depression and frequency of stimulus repetition. The depression of I_{K} in normal Ringer with repetitive stimulation is much greater than that seen in Co. Two examples of this depression in normal and Co Ringer are seen in Fig. 5 (see also Fig. 9). In *A* are data from a slow burster which has little Ca current even in normal Ringer. The forms of the two curves before and after Co are similar. As shown in *B*, the form of the frequency–depression relationship of the fast burster is clearly altered by Co. Before Co the depression increases with increasing repolarization intervals up to 400 msec and then declines with increasing intervals. In both fast and slow bursters the depression at short and intermediate stimulus intervals is greater at the end of ten 100 msec pulses than it is at the end of a long pulse.

Relationship of Ca to I_{K} depression

Thus, in addition to the time and voltage dependent depression revealed during Co treatment there exists a long lasting depression of I_{K} which appears to develop during repolarization of the membrane and is greatest at intermediate stimulus intervals. The failure of neurones to show this long lasting depression in Co Ringer implies that it is Ca dependent. The time course of the suppression does not, however, follow that of any known Ca current. This leads to the suggestion that it may be due to the effects of an increase in internal Ca. If this is true, then the magnitude of the long term depression should be dependent on the Ca influx. Our data clearly show this. The maximum long lasting depression of the outward current is greater in fast bursters (which have a larger Ca current) than in slow bursters (see Figs. 5 and 14). Further, the frequency dependence of the depression reveals that it is maximal for those frequencies at which the total Ca influx is the largest (compare Figs. 1, 5 and 14 of this paper with Figs. 10*B* and 13 of the preceding paper; Heyer & Lux, 1976). Recordings

with K-sensitive electrodes (e.g. see Fig. 1) reveal that the depression in net outward current represents a true decrease in K efflux and that with repetitive stimulation the frequency dependence of this decrease in I_K is similar to the frequency dependence of $I_{In\ slow}$ (compare Fig. 1 of this paper with Fig. 10*B* of the preceding paper, Heyer & Lux, 1976).

To directly test our hypothesis that increased $[Ca]_i$ depresses the efflux of K, we increased $[Ca]_i$ by injection. Typical responses are shown in Figs. 6*A*, *B* and 7. The time course of effects in one experiment with three

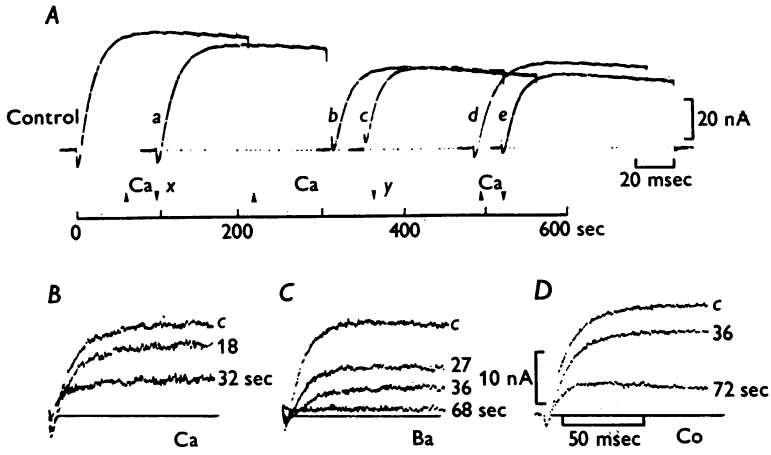

Fig. 6. Depression of the outward current by intracellular injection of divalent ions. *A*, time course of responses to 100 msec voltage clamp depolarizations of 70 mV during a period of 600 sec in one experiment. The beginning (upward arrows) and end (downward arrows) of each Ca injection by electrophoresis (at up to 250 nA under voltage clamp), times at which the neurone was released from voltage clamp to assay effects on spontaneous activity (*x* and *y*) and the onset of each voltage clamp depolarization (indicated by the start of the pulse) are all shown in positions relative to the lower time base. Currents are displayed on an expanded time scale. The control current at time 0 was measured after the insertion of the Ca electrode and was identical with that recorded before penetration with the Ca electrode. The responses reveal a progressive decline in the total net outward current and increase in instantaneous conductances during two periods of Ca injection (*a*–*c*), partial recovery (*d*) and further depression of the total outward current with renewed Ca injection (*e*). The spontaneous activity was essentially unaltered at *x*; when the neurone was released from clamp at *y* it hyperpolarized to −64 mV. *B*, *C* and *D*, responses to pressure injection of Ca (1 M-$CaCl_2$; 66 mM-KCl at 0·5–1·0 atm), Ba (100 mM-$BaCl_2$; 66 mM-KCl at 0·75–1·5 atm) and Co (1 M-$CoCl_2$; 66 mM-KCl at 0·5–1·5 atm). Responses to single 100 ms pulses (from −50 to +19 mV) are superimposed to show the progressive depression from the control pulse levels (*c*, before injection) during continued injection (duration of injection in seconds indicated for subsequent pulses). Changes in the instantaneous conductances have been subtracted.

pulses of intracellular Ca electrophoresis are presented in Fig. 6*A*. The onset of each current trajectory indicates the timing of the voltage clamp depolarization relative to Ca injections on the slower time scale. The individual currents themselves are shown on an expanded time scale. The first pulse of intracellular Ca produced a 11·3 % decrease in net outward current and very little increase in the instantaneous conductance. Spiking

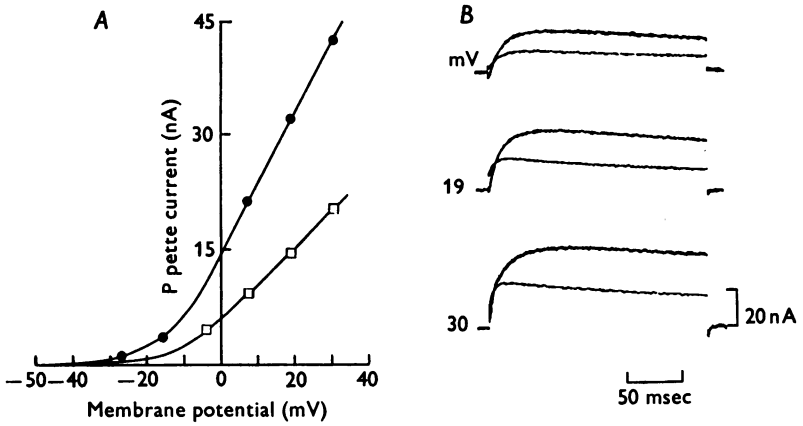

Fig. 7. *A*, voltage dependence of the total net outward current (measured after 100 msec of depolarization) before (-●-) and after (-□-) intracellular injection of Ca by electrophoresis under voltage clamp. The outward current after Ca injection is about 50 % of control values at all potentials. *B*, current trajectories during pulses to three different membrane potentials before (upper trace of each pair) and after Ca injection (lower trace of each pair) show the change in the time course of the net outward current following Ca injection. The total net outward current is decreased despite an apparent increase in the instantaneous conductance.

behaviour of the neurone (tested by releasing it from clamp at point *x*) was essentially unchanged. The second pulse of Ca caused a further reduction in the net outward current (*b*) and then a marked increase in the instantaneous conductance and rate of decay after the peak amplitude during the pulse with a further decrease in the time and voltage sensitive outward current (*c*). When the neurone was released from clamp at *y* it spontaneously hyperpolarized. These effects are partially reversible (*d*). Further reduction of the outward current with the third pulse of Ca (*e*) brought the total depression of the net outward current to 41·2 % of the control pulse amplitude. In our experiments the first and most long lasting effect of increased $[Ca]_i$ was the depression of the delayed outward current. Ca injection by pressure or electrophoresis in other experiments produced up to 60 % depressions in the total net outward current and 70 % decreases in the time and voltage depending component.

On some occasions the depression and change in kinetics of the net outward current remained stable for some minutes following prolonged intracellular Ca electrophoresis. This allowed us to look at the voltage dependence of the $[Ca]_i$ depressed current. An example of one such experiment is shown in Fig. 7. The I–V plot for pulses from the -50 mV holding potential does not reveal a significant shift of the curve under increased $[Ca]_i$ relative to the voltage axis; the total net outward current was reduced by about 50%. Current trajectories in Fig. 7*B* reveal the time course of this outward current at three voltages before (upper traces of each pair) and after (lower traces) Ca injection. The comparison suggests that $[Ca]_i$ selectively decreases a slowly inactivating component of I_K; the effects resemble those of decreasing $I_{K(Ca)}$ by extracellular Co (see Fig. 3 and Discussion below).

Effects of intracellular injection of Ba^{2+} and Co^{2+}

To compare the effects of $[Ca]_i$ with those of other divalent ions we pressure injected $BaCl_2$ and $CoCl_2$ (at 100 mM and 1 M concentrations, respectively, each with 66 mM-KCl). Co^{2+} caused some suppression of the net outward current but the effect was frequently small. During the injection of Ba^{2+} ions the delayed outward current was gradually diminished, showing no marked changes in kinetics. In two instances the time and voltage dependent I_K was completely abolished by Ba injection. The accompanying increases in leak conductance was by far too small to account for the loss of the voltage and time depending current. The effects of Ba^{2+} and Co^{2+} were only slightly reversible with time. Typical time courses of the currents are shown in Fig. 6 *C, D*.

Components of I_K: Ca dependent K current ($I_{K(Ca)}$)

In the preceding section we have presented data showing that the net outward current is decreased when $[Ca]_i$ is increased either by repetitive stimulation or by direct injection. Our data and those of Meech & Standen (1975) also show that Ca has a second, quite different effect on the K conductance of these neurones. From the I–V plot in Fig. 4 it is apparent that blocking the inward Ca current with Co eliminates a portion of the net outward current. This implies that there is a Ca dependent component of I_K, which will be designated $I_{K(Ca)}$.

The voltage dependence of $I_{K(Ca)}$ is approximated by the difference between the plots in normal and Co Ringer (Fig. 8). The curve reaches a maximum at about $+50$ mV and declines with higher voltages. In this example the current difference does not decrease to zero with membrane potentials of up to $+200$ mV. Similar plots for other neurones often did become zero at high membrane potentials ranging from $+135$ to $+185$ mV.

We attribute this variation to possible non-specific effects of Co-Ringer on the Ca independent component of I_K. The curve in Fig. 8 was calculated from voltage clamp currents and therefore actually represents the behaviour of $I_K - I_{in\ slow}$. However, this probably does not lead to a significant misrepresentation of the voltage dependence of this component of I_K. $I_{in\ slow}$, which is calculated to be much smaller than this component of I_K, has a voltage dependence which is similar to the curve in Fig. 8 (see the inset to Fig. 5, Heyer & Lux, 1976).

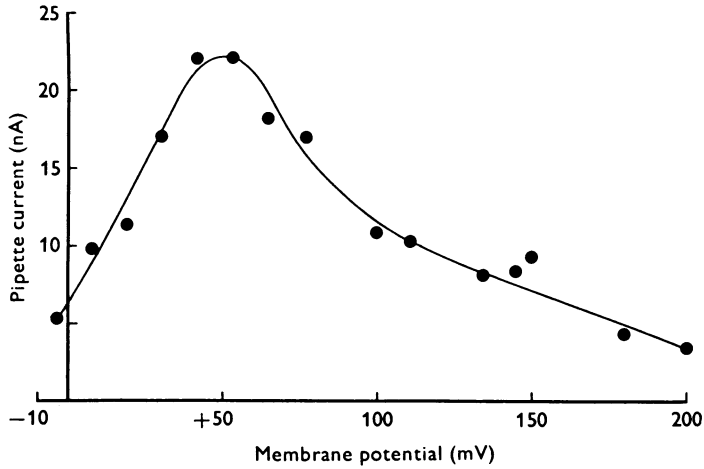

Fig. 8. The magnitude of $I_{K(Ca)}$ as a function of membrane potential. $I_{K(Ca)}$ is calculated as the difference between the net outward currents at 100 msec before and after substitution with Co in the Ringer for a typical fast burster (same neurone as in Fig. 4).

The comparison of current trajectories in Ca and Co containing Ringer solutions in Fig. 3 may indicate the approximate magnitude and time course of $I_{K(Ca)}$. In this burster $I_{K(Ca)}$ reached a maximum of 65 % of the total net outward current. The total Ca current is usually much smaller in slow bursters and, accordingly, the difference between outward currents in Ca and Co Ringer is less than that shown in Fig. 3.

The true time course of $I_{K(Ca)}$ is difficult to determine from these data due to the short-circuiting effect of $I_{in\ slow}$. However, the peak $I_{K(Ca)}$ probably occurs less than 80–100 msec after the onset of the pulse. $I_{K(Ca)}$ decays slowly after the peak.

Relationship of $I_{K(Ca)}$ to $I_{in\ slow}$

The blocking of $I_{K(Ca)}$ by external Co (in Ca- and Mg-free Ringer strongly implies that this component of I_K depends on Ca. Since increased

$[Ca]_i$ decreases I_K, it is unlikely that $I_{K(Ca)}$ depends directly on $[Ca]_i$ However, the time course for $I_{K(Ca)}$, taken as the difference between trajectories for normal and Co-treated neurones in Fig. 3, resembles that of $I_{in\ slow}$ (Figs. 2 and 8, Heyer & Lux, 1976). This suggests that the increase in K current results from a transient effect of Ca, such as the movement of ions through the membrane. It might therefore depend on the persistence of the inward Ca current, $I_{in\ slow}$. This latter hypothesis is supported by several lines of evidence. For example, $I_{K(Ca)}$ and $I_{in\ slow}$ have a similar voltage dependency (compare Fig. 8 with the inset to Fig. 5 of Heyer & Lux, 1976). Secondly, repetitive depolarizations at short intervals produce

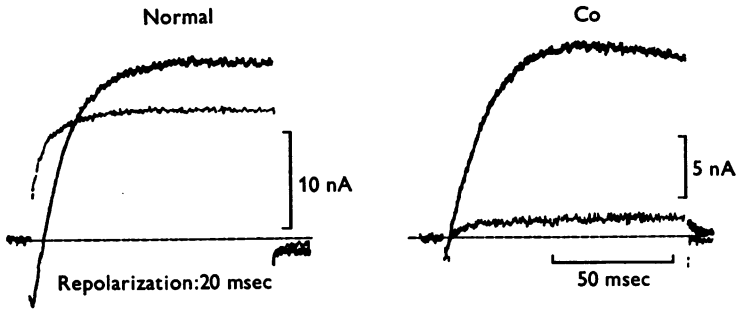

Fig. 9. Responses of a neurone to a series of ten 100 msec pulses (to +7 mV) with 20 msec repolarizations (to −50 mV). Trajectories for the first pulse (upper trace of each pair) and the tenth pulse (lower trace of each pair) are shown for the neurone in normal Ringer and in Co-Ringer. Note the rapid rise of the outward current at the onset of the tenth pulse in normal Ringer is abolished by Co. The greater depression and failure of the tail currents to become inward with repetitive stimulation in Co Ringer is also obvious. (Note: trajectories for the tenth pulse currents have been reset to start from the same base line as pulse 1 currents to facilitate comparisons of current amplitudes.)

an increase in net outward current at the onset of the second and subsequent pulses. Characteristics of this increase suggest it is related to the persistence of $I_{in\ slow}$. An example of this response is given in Fig. 9. The first and tenth current trajectories from a ten pulse series (100 msec pulses to +7 mV and 20 msec repolarizations to −50 mV) are presented. The amplitude of the net outward current during the first 20 msec of the tenth pulse is greater than that of the first pulse despite the depression of the outward current due to repetitive stimulation. The second and subsequent trajectories are all similar to the tenth (see also Fig. 13). As discussed in the previous paper some of the changes in kinetics with repetitive stimulation are due to partial inactivation of $I_{in\ slow}$. However, the decreased short-circuiting by an inward current is not of sufficient

magnitude to completely account for the rapid onset of outward current and the marked increase in outward current at the onset of the second and subsequent pulses. Recordings with K-sensitive electrodes show that there is an increase in K efflux at short interpulse intervals compared with intermediate stimulus intervals (see Fig. 1), confirming that this stimulus paradigm does result in a true increase in I_K. We will refer to this response as an 'augmentation' of I_K with short repolarization intervals. The augmentation follows the approximate time course for $I_{in\ slow}$. Since $I_{in\ slow}$ turns on rather slowly (Heyer & Lux, 1976) and appears to have time constants of tenths of seconds at membrane potentials near -50 mV (Eckert & Lux, 1976), the magnitude of $I_{in\ slow}$ at the onset of a subsequent pulse should be much greater following very short repolarization intervals (e.g. 20 msec) that it is following longer repolarizations (e.g. 100 msec). If the increase in K conductance is due to $I_{in\ slow}$, then I_K also should turn on faster with reactivation of $I_{in\ slow}$ at short intervals, before it has returned to base line levels. Responses of neurones such as those shown in Fig. 9 suggest that just such a phenomenon does occur in these cells. This augmentation can be detected at intervals of up to 80 msec between depolarizations for a holding potential of -50 mV.

The augmentation of I_K at the onset of pulse 2 and subsequent pulses with short stimulus intervals is entirely abolished by the substitution of Co^{2+} for Ca^{2+} and Mg^{2+} in the Ringer solution, as shown in Fig. 9 Co, supporting our hypothesis that it is Ca dependent.

Two additional types of experiments provide evidence to support our hypothesis that the increase in I_K is due to Ca moving through the membrane and not to an increase in $[Ca]_i$. In one set of experiments we used a hyperpolarizing test pulse, reasoning that if the augmentation were dependent upon $[Ca]_i$, then the response to a hyperpolarizing pulse should also be increased (see Meech, 1974, for effects of $[Ca]_i$ on responses to hyperpolarizing pulses). Alternatively, if the increase in I_K were due to an inward Ca current, then removal of that current with hyperpolarization (Eckert & Lux, 1976) should prevent augmentation of the response to hyperpolarizing pulses and the magnitude of the conductance should be unaffected by preceding pulses even at short repolarization intervals. Our experiments showed the latter to be true. The voltage clamp current amplitude in response to a hyperpolarizing pulse is the same whether that pulse is presented separately or in place of the tenth pulse in a series of pulses with short (e.g. 20 msec) repolarizations, when the outward current during depolarization is maximally augmented.

The second set of experiments to test this point involved the effects of changes in holding potential on the duration of the augmentation. Again we reasoned that if the augmenting of I_K at short intervals were due to

an increased $[Ca]_i$ then increasing the holding potential should not diminish the response since hyperpolarization would not be expected to remove Ca from the inner surface of the membrane. Alternatively, if the augmenting of I_K at the onset of the second pulse were due to the influence of an incompletely turned off $I_{in\ slow}$ from the first pulse, then increasing the holding potential could reduce the available $I_{in\ slow}$ and thereby decrease the period of repolarization following which the augmenting is apparent. The latter effect of increasing the holding potential was found. For example, in one neurone at -50 mV holding potential the augmentation was evident with 80 msec intervals between depolarizations to $+7$ mV but not with 100 msec repolarizations. When the holding potential was increased to -70 mV only at the shortest repolarization intervals (20 msec) did repetitive stimulation lead to any augmentation of I_K. With a decreased holding potential (to -30 or -40 mV) and repetitive activation (to $+7$ mV), augmentation during the second pulse was still evident after 300 msec repolarization.

The results of these experiments support our hypothesis that it is Ca moving through the membrane rather than an increased $[Ca]_i$ which is responsible for increasing I_K in these neurones.

Effects of intracellular tetraethylammonium ions (TEA)

Results presented in the preceding sections suggest that there are Ca dependent and Ca independent components of the K current. In an attempt to further isolate the calcium dependent effects on I_K we have used intracellular injection of tetraethylammonium ions (TEA) to block the Ca independent I_K. The component of I_K which is resistant to intracellular TEA will be designated as TEA resistant, $I_{TEA\text{-}res}$, and TEA treatment will refer to the intracellular injection of TEA except where noted otherwise.

TEA does not block $I_{in\ slow}$

As in other preparations (see Discussion) Ca currents in these neurones are not blocked by intracellular TEA. In Fig. 10 the response of a fast burster to small depolarizations before and after intracellular electrophoresis of TEA are shown. The slow inward current is clearly visible in Fig. 10*B*, with a time course apparently prolonged by TEA. The effects of TEA on $I_{in\ slow}$ are discussed in more detail below. Characteristics of the net outward current at higher levels of depolarization provide further evidence that TEA does not block $I_{in\ slow}$.

Characteristics of $I_{\text{TEA-res}}$

Some of the effects of intracellular TEA injection on potassium currents in *Helix pomatia* have been described (Neher & Lux, 1972; Kostyuk, Krishtal & Doroshenko, 1975*a*). The fast outward K current was abolished at maximally effective intracellular TEA concentrations.

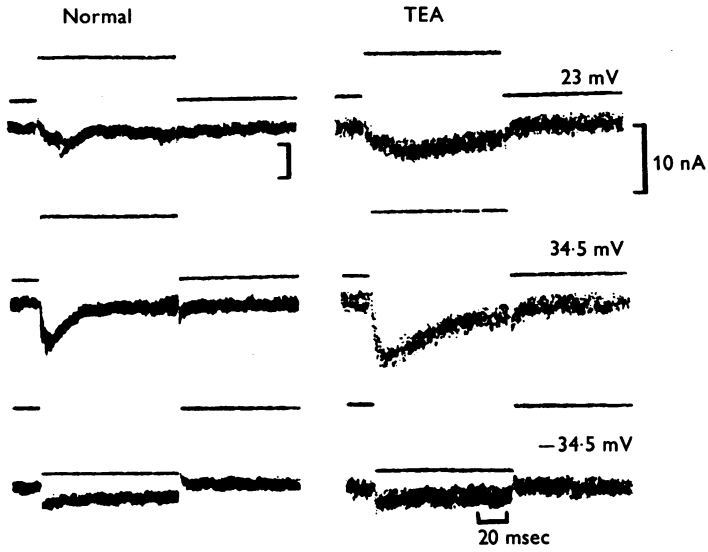

Fig. 10. Net inward current before and after TEA injection in a fast burster. Responses to low levels of depolarization (upper two sets of traces) and hyperpolarization (lower set of traces) before ('Normal') and after electrophoresis from a 1 M TEA-Cl-filled electrode at 350 nA for 3·5 min. under voltage clamp; i.e. injection was continued for about 1 min after maximal depression of the net outward current had been achieved. Voltage records (upper traces, holding potential −50 mV) and current records (lower traces) are shown. Current trajectories for responses to depolarizations have been corrected for the instantaneous leak conductance with hyperpolarizations of equal magnitudes. The net inward current of the normal neurone is more prolonged after TEA treatment.

Magnitude of the delayed outward current, $I_{\text{TEA-res}}$

The delayed outward current is depressed by TEA. In slow bursters up to 85 % of I_K could be blocked by intracellular TEA. When TEA was electrophoresed from an electrode containing a 1 M solution (at 350 nA) into a fast burster, however, the depression of I_K proceeded rapidly for only 2–3 min to 50 % or more of the original magnitude (measured at the end of a 1 sec depolarization to +6 mV). Continued injection did not further decrease the net outward current. For each neurone there was a maximum

depression of I_K beyond which further TEA injection was ineffective. All neurones reported here were studied after the maximal effect of TEA had been reached. There was no obvious recovery of the outward current over the period of 30–60 min during which the cell was studied. The component of I_K remaining under maximal TEA depression is designated as TEA resistant ($I_{TEA\text{-}res}$).

Since we anticipated artifactual readings of the K^+ electrodes even in the presence of small extracellularly appearing amounts of TEA (Neher & Lux, 1973), no data on K efflux were taken on these neurones. $I_{in\ slow}$ was estimated from changes in the current trajectories with repetitive activation (see preceding paper). TEA was least effective in those neurones (all fast bursters) with apparently large Ca currents and caused greater

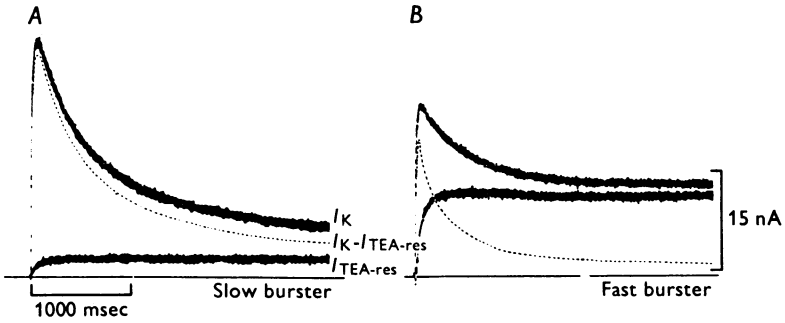

Fig. 11. Responses of slow and fast bursters to sustained (3 sec) voltage clamp depolarization (from -50 to $+19$ mV) in normal Ringer (I_K) and during the maximum effect of TEA in each cell ($I_{TEA\text{-}res}$). Note the nearly complete absence of time dependent inactivation of $I_{TEA\text{-}res}$ and its relative magnitudes in the slow and fast bursters (neurones with small and large amplitudes of $I_{in\ slow}$, respectively). The dashed lines indicate trajectories calculated by subtracting $I_{TEA\text{-}res}$ from I_K. This suggests that a rapidly rising, quickly declining component of I_K was blocked by TEA.

depression in those neurones where $I_{in\ slow}$ was smaller. The comparison of TEA effects on slow and fast bursters (which have small and large Ca currents, respectively) are shown in Fig. 11. This positive relationship between the magnitudes of $I_{in\ slow}$ and $I_{TEA\text{-}res}$ is consistent with the hypothesis that TEA primary blocks the Co resistant component of the delayed outward current, leaving the Ca dependent component ($I_{K(Ca)}$).

Time course of $I_{TEA\text{-}res}$. The time course of $I_{TEA\text{-}res}$ shown in Fig. 11 supports our hypothesis that TEA removes primarily the Co resistant component of I_K. During sustained depolarizations (up to 3 sec tested) $I_{TEA\text{-}res}$ shows no inactivation and the onset of the delayed outward current is greatly slowed. Therefore, a subtraction of $I_{TEA\text{-}res}$ from the

total I_K suggests that the current removed by TEA has a rapid rate of rise and a marked inactivation during sustained depolarization (see interrupted lines, Fig. 11). These are characteristics of $I_{Co\text{-}res}$.

Voltage dependence of $I_{TEA\text{-}res}$. The voltage dependence of $I_{TEA\text{-}res}$ (see Fig. 12*B*) provides further evidence that $I_{in\ slow}$ and $I_{K(Ca)}$ are not blocked by TEA. Unlike the outward current which remains during Co treatment (see Fig. 4), a local minimum in net outward current between 90 and 100 mV is evident in $I_{TEA\text{-}res}$. This N-shape is associated with the voltage dependence of $I_{in\ slow}$. $I_{TEA\text{-}res}$ trajectories were analysed at two

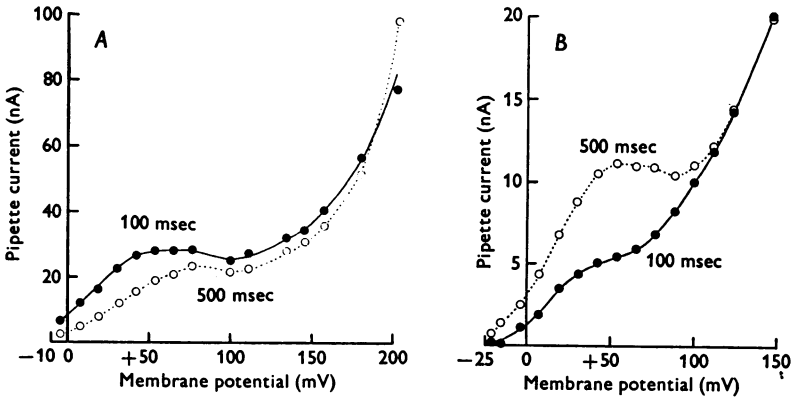

Fig. 12. Net outward currents as functions of membrane potential (for pulses from −50 mV) in a burster before (*A*) and after (*B*) TEA injection. The heights of the current trajectories (in nA) at 100 msec (-●-) and at 500 msec (- - ○ - -) after the onset of depolarization are shown. The N-shape of the plots for the normal neurone are similar for 100 and 500 msec values, whereas an N-shape becomes obvious only at 500 msec in the TEA-treated neurone.

times after the onset of the pulse. Measurements were taken at 100 msec for comparison with the maximal Ca dependent K currents in control neurones (see Fig. 12*A*). The magnitude of I_K was also determined at the end of the 500 ms pulse. In untreated neurones the shape of the I–V plot is little changed by this procedure (Fig. 12*A*), but in TEA the N-shape for outward currents at 500 msec was much more pronounced (Fig. 12*B*). There is evidence from the net inward currents with small depolarization as already presented and from repetitive stimulation to be discussed below that the time course of $I_{in\ slow}$ is prolonged with TEA.

Changes in $I_{TEA\text{-}res}$ *with repetitive activation*. The depression of I_K at long interstimulus intervals is attributed to effects of $[Ca]_i$ in untreated neurones. This long-term depression of the outward current is also obvious after TEA injection. Indeed, the maximum depression at intermediate and

long intervals is often greater during TEA treatment than before, especially in neurones with little long-term depression in the untreated state (see Fig. 14). In Fig. 13, lower traces, the records of responses to repetitive stimulation at 900 msec intervals before and after TEA injection show the marked depression after TEA. The time course of outward current is prolonged in TEA, but similar depressions appear with 300 msec pulses, when $I_{\text{TEA-res}}$ has reached its maximum.

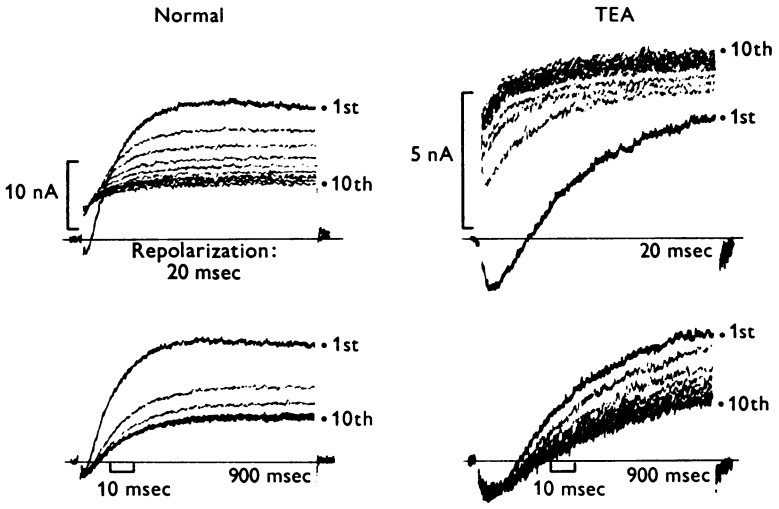

Fig. 13. The effects of TEA on current trajectories during repetitive stimulation with short and long repolarization intervals. Trajectories represent responses of a typical fast burster to trains of ten 100 ms pulses with 20 msec or 900 msec repolarization intervals (upper and lower sets of traces, respectively) before and after TEA injection. With short repolarization intervals the increase in instantaneous current and rate of rise of the second and subsequent pulses is obvious in the normal cell and even more pronounced after TEA injection. With long interpulse intervals there is a prolonged depression with repetitive stimulation and little or no increased instantaneous conductance at the onset of subsequent pulses. (Note: trajectories for the currents in second and subsequent pulses have been reset to start from the same base line as pulse 1 currents to facilitate comparisons of current amplitudes.)

The depression of $I_{\text{TEA-res}}$ is strongly frequency dependent. The graph in Fig. 14 shows that in this slow burster with little Ca current the maximum depression of I_{K} occurs with short interstimulus intervals or at the end of a sustained pulse for the untreated cell (see also Fig. 5). After TEA injection, the maximum depression occurs at 700 msec intervals and is still large at 2 sec intervals. Similar data from a fast burster, in a neurone in which the Ca-dependent current is already large (Fig. 14) show that the normal frequency dependence of I_{K} depression more closely resembles the

pattern of $I_{\text{TEA-res}}$, although maximum depression occurs at 300 msec intervals and the depression is much less at 2 sec intervals. With TEA injection a response of $I_{\text{TEA-res}}$ becomes very similar to that of $I_{\text{TEA-res}}$ from the slow burster.

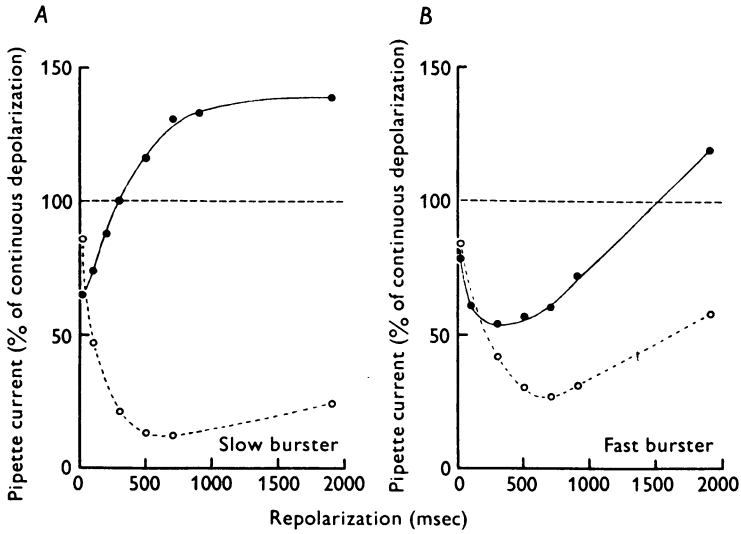

Fig. 14. The depression of net outward current during repetitive stimulation as a function of the repolarization interval for a slow (*A*) and a fast (*B*) burster. The amplitude of the outward current at the end of ten 100 msec pulses (as a % magnitude of outward current after 1000 msec sustained depolarization from −50 to +7 mV) plotted against the repolarization interval between pulses before (-●-) and after (- - ○ - -) TEA injection. The response pattern is similar for the fast burster before TEA and both neurones after TEA (cf. Fig. 7 for effects of Co on similar neurones).

As previously described the persistance of $I_{\text{in slow}}$ from the end of pulse 1 to the beginning of pulse 2 (following short repolarization intervals augment I_K at the onset of the second pulse. This effect is even more pronounced in TEA treated neurones. Current trajectories from a neurone before and after TEA injection are shown in Fig. 13 (upper traces). These represent responses to repeated alternations of 100 msec depolarizations to +7 mV and 20 msec repolarizations to −50 mV. The augmentation of I_K in second and subsequent trajectories is much more prominent after TEA injection. Indeed, at short intervals such as the one shown here, the outward current can be greater at the end of ten 100 msec pulses than it is at the end of a 1000 msec continuous pulse. These responses of TEA treated neurones contrast sharply with the behaviour of cells in Co Ringer (see Figs. 3, 4, 5).

Does TEA alter the characteristics of $I_{\text{in slow}}$? Current trajectories from neurones injected with TEA suggest that TEA alters the frequency dependence and the time course of $I_{\text{in slow}}$. A comparison of current trajectories before and after TEA injection in Fig. 15 shows the rate of rise during different levels of depolarizations. These records also reveal the kinetics of the outward current do not change appreciably with repetitive activation. The tenth pulse of a series of 100 msec pulses at 400 msec intervals is shown here normalized in height to the end of the first pulse (see Heyer & Lux, 1976). The comparison of current trajectories

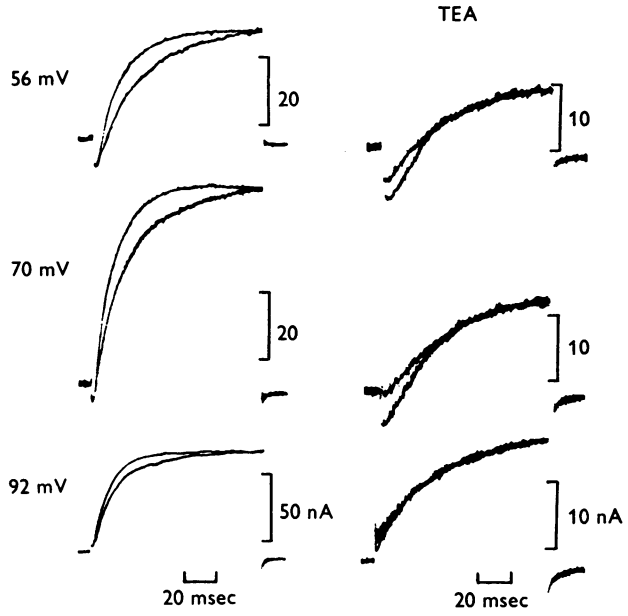

Fig. 15. Responses of a neurone to repetitive stimulation with different amplitudes of depolarization (values to the left of each set of traces indicate the magnitude of the pulses; from a holding potential of -50 mV) before and after TEA injection. Trajectories represent the response to the first pulse and to the tenth pulse normalized to the height of the first pulse at 100 msec. Current calibrations therefore refer only to the first pulse. An increase in inward current with repetitive stimulation is seen at all three levels of depolarization in the normal neurone. The current trajectories for TEA treated cells are slower than even tenth pulse trajectories from the normal neurone but do not indicate any increase in inward current with repetitive activation.

before and after TEA injection is complicated by possible effects of TEA on both K and Ca current kinetics. However, there is no apparent increase in inward Ca current during second and subsequent pulses, and the results

are consistent with the hypothesis that TEA treated neurones have a large Ca current which is maximally activated by the first pulse.

A comparison of current trajectories of I_K, $I_{TEA\text{-}res}$ and $I_{Co\text{-}res}$ reveals that the rate of rise of $I_{TEA\text{-}res}$ is slower than would be predicted by the simple subtraction of $I_{Co\text{-}res}$ from the total I_K. This could be due to an effect of TEA on the K conductance directly or on $I_{in\ slow}$, thereby indirectly altering I_K. Our experiments on TEA-treated neurones suggest a slowing of the onset of $I_{in\ slow}$ and, especially, an impairment of $I_{in\ slow}$ inactivation with TEA. Records already presented in Fig. 10 indicate that the time course of $I_{in\ slow}$ may be prolonged by TEA. The following results of repetitive stimulation at short intervals also imply that inactivation of $I_{in\ slow}$ takes longer in TEA. As previously described the augmenting effect on I_K attributed to $I_{in\ slow}$ can be measured for a maximum of 80–100 msec following a 100 msec pulse (from -50 to $+7$ mV) in typical untreated neurones. In TEA treated neurones, $I_{in\ slow}$ augmentation of I_K can be measured for up to 700 msec between pulses with the same stimulus paradigm. As in untreated cells, tests indicate that the increase in I_K reflects a persistent $I_{in\ slow}$. That is, the duration of this effect is dependent on the holding potential (i.e. the effects of $I_{in\ slow}$ can be seen for only 200–400 msec at -70 mV holding potential and up to the maximum test of 1900 msec at a holding potential of -30 mV), and responses to hyperpolarizing test pulses are not increased if the test is presented at the end of a train of rapid depolarizations (compared with presentation of the test in the absence of depolarizations; see Relationship of I_K to $I_{in\ slow}$).

The onset of $I_{in\ slow}$ may also be slowed by TEA. This is suggested by the large decrease in rate of rise for I_K under TEA (Fig. 15), the slowed time course of the net inward current after TEA injection (Fig. 10) and the shift of maximum N-shape in the I–V plot from 100 to at least 500 msec after TEA injection (Fig. 12). Additional evidence comes from increasing the pulse duration from 100 to 300 msec for repetitive stimulation.

Because $I_{in\ slow}$ reaches its maximum well before 100 msec in normal neurones, increasing the pulse duration from 100 to 300 msec does not greatly increase the Ca influx (see also Heyer & Lux, 1976). In contrast, increasing the pulse from 100 to 300 msec in TEA treated neurones appears to increase the total inward flow of Ca ions, suggesting that a considerable part or even the maximum $I_{in\ slow}$ occurs after 100 msec in these cells. An increased total Ca influx in TEA-treated neurones is inferred from a slight (8–17%) increase in the maximum depression achieved by trains of ten 300 msec pulses compared with ten 100 msec pulses. A similar increased depression with increasing pulse duration is not seen in normal neurones. The persistence of $I_{in\ slow}$ following repolarization (from $+7$ to -50 mV)

was also investigated using the augmentation of I_K at the onset of the second pulse as a measure. Prolonging the pulse from 100 to 300 msec caused $I_{in\ slow}$ to persist slightly longer (approximately 20 msec) in normal neurones. After TEA injection, $I_{in\ slow}$ persisting after repolarization could be prolonged up to 150% by increasing the duration of the pulse. This would also suggest that the peak of $I_{in\ slow}$ is delayed by TEA treatment.

Effects of intracellular TEA and extracellular Co

The results of the preceding sections suggest that the delayed outward potassium current in bursting pacemaker neurones of *Helix pomatia* consists of a Ca dependent component and a Ca independent component,

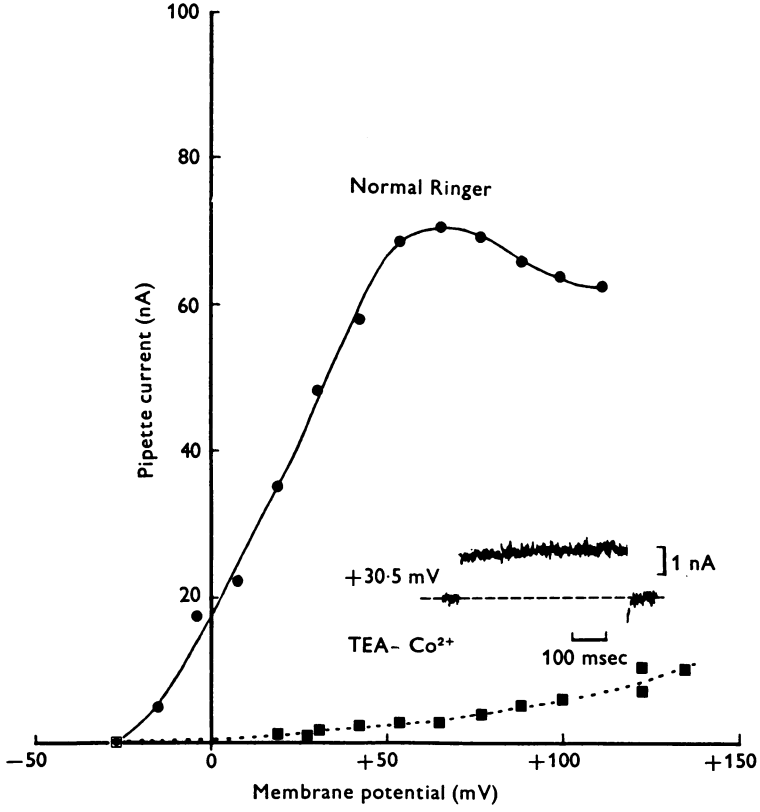

Fig. 16. The outward currents (measured at 100 msec) of fast burster (–●–) and the same neurone injected with TEA and bathed in Co-Ringer (- - ■ - -) as functions of membrane potential (with pulses from a −50 mV holding potential). Inset shows a typical current trajectory (a 500 msec pulse from −50 to +30·5 mV) for this Co, TEA treated neurone. Simultaneous intracellular TEA and extracellular Co virtually abolish the voltage and time dependent net outward current.

blocked by Co and TEA, respectively. If this is true, then the delayed outward K current should be completely blocked in a neurone injected with TEA and bathed in Co Ringer. We have tested the combined effects of these two substances on several neurones. When a neurone is treated with both extracellular Co-Ringer and intracellular TEA, the resulting current trajectories show virtually no time dependent variation but an increased instantaneous conductance (see inset to Fig. 16). The latter is only slightly voltage dependent (see Fig. 16), shows rectification as expected from constant field approximation (Cole, 1968) and can largely disappear when the Co is replaced by normal Ringer.

DISCUSSION

Depression of the delayed outward K current

During sustained and repetitive voltage clamp depolarization, the net outward current in neurones of *Helix pomatia* becomes depressed. Although the inward Ca current in part short-circuits the outward K current (Lux & Eckert, 1974; Heyer & Lux, 1976), several lines of evidence argue that this cannot directly account for the apparent depression of I_K. First, the Ca current is not large enough; it does not account for more than 20–30% of the total net outward current integrated over 1000 msec, whereas the time dependent total net outward current can be decreased more than 70%. In addition the depression of outward currents does not reflect the relative magnitudes of $I_{\text{in slow}}$ in different snail neurones. For example, the time dependent depression is greater in the slow burster, which has a smaller Ca current. Secondly, the time course of the Ca current is not compatible with the hypothesis. Although $I_{\text{in slow}}$ does not inactivate completely with time, it does reach a maximum before 50 msec (at voltages near 0 mV) and has declined substantially by 1000 msec depolarization at 0 mV. Finally, data from ion sensitive electrodes demonstrate that the actual outward flow of K decreases with time during a sustained pulse.

Thus, there is a true time dependent depression of I_K. The results of experiments reported here do not suggest that the depression is due in any large part to the accumulation of extracellular K. These experiments were done on neurones exposed as much as possible to the saline, with only a thin layer of small and partially torn glia covering the plasma membrane. The effective barrier to free diffusion appears as an extracellular space of about one hundred times the 200 Å space reported by Frankenhaeuser & Huxley (1957) and Adelmann, Palti & Senft (1973) for the squid giant axon (Neher & Lux, 1973). The K permeability of this barrier was estimated to

be about two orders of magnitude higher in the snail soma than in the squid axon.

Although there may be some local increase in K directly on the surface of the neurone not measured by this technique, the data from voltage clamp experiments also fail to demonstrate a positive relation between the depression of outward current and the total amount of net outward current (see also Lux & Heyer, 1975).

Effects of Ca on the K conductance in excitable membranes

The effects of extracellular Ca in controlling membrane excitability have been established: decreasing extracellular Ca shifts the steady-state I–V relationship for ionic conductances in a hyperpolarizing direction, thereby increasing neurone excitability (Frankenhaeuser & Hodgkin, 1956; Fishwan, Khodorov & Volkenstein, 1971). Much of this effect of external Ca is attributed to an unspecific interaction with negative membrane surface charges and is referred to as 'indirect effect' of Ca (Reuter, 1973).

Elucidation of the effects of internal Ca^{2+} ions has been more difficult. Some of the apparent problems may arise from the failure to differentiate between the effects of internal Ca on the passive permeability and effects on the time and voltage sensitive conductances. Increased $[Ca]_i$ can increase the passive K permeability of some membranes (e.g. red blood cell, Kreganov & Hoffman, 1972; and neurones, Krnjević & Lisiewiecz, 1972). Some authors have suggested that increased $[Ca]_i$ increases delayed K permeability in neurones (Meech & Standen, 1975), noting that Ca injection causes an increased membrane permeability, changes in spike wave form and often leads to spontaneous hyperpolarization (Meech, 1972, 1974). We tested this hypothesis directly by investigating the effects of Ca injection on voltage clamp currents and found that increased $[Ca]_i$ caused a *decrease* in the total net outward current and especially in the time and voltage dependent portion of that current. In addition, our results with repetitive stimulation suggest that the Ca which enters during depolarization can likewise lead to a depression of the net outward current.

In some neurones, notably the squid giant axon, changes in $[Ca]_i$ do not appear to alter the delayed outward voltage clamp currents (Begenisich & Lynch, 1974). This does not represent a basic disagreement with the hypothesis that increased $[Ca]_i$ can depress K currents in the snail. As discussed below, the Ca dependent component of I_K probably represents only a very small portion of the K current in squid axons and the failure to detect changes in I_K with changes in $[Ca]_i$ is not unexpected.

Following our direct demonstration that increased $[Ca]_i$ decreases the net outward current, we reinvestigated the Ca dependent component of I_K.

Both $I_{\text{in slow}}$ and a component of I_K turn on slowly with depolarization and there is an 'augmentation' of I_K seen with depolarization following short periods of repolarization; this augmentation follows the approximate time course for the turning off of $I_{\text{in slow}}$. Results from tests with hyperpolarizing test pulses and different holding potentials also support our hypothesis that I_K is increased by Ca moving through the membrane (rather than increased $[Ca]_i$) and is therefore dependent on the persistence of $I_{\text{in slow}}$.

Effects of other intracellular divalent cations

In contrast with the variation in responses reported for injected Ca ions, raising the intracellular concentrations of other divalent cations produces a more consistent depression of outward currents in excitable membranes. Externally and internally applied Ba ions can depress both resting and active K conductances in many tissues (Sperelakis, Schneider & Harris, 1967; Hagiwara, Fukuda & Eaton, 1974). Other divalent cations have similar actions. In *Helix*, Ba injection can be far more effective than TEA or Co in blocking the outward K current. This suggests that Ba affects more than the Ca dependent I_K, although its effects on the inward Ca current have not been analysed.

It should be noted that despite its well known K blocking abilities, Ba can also lead to hyperpolarization of neuronal membranes (externally applied Ba, Nishi & Soeda, 1964; internally applied Ba, Meech, 1974). Nishi & Soeda (1964) attribute the effect of external Ba ions on frog sympathetic ganglion cells to an increased efficacy of an electrogenic pump.

Separation of the delayed outward current in snail neurones into two components

The results of our experiments suggest that the delayed outward current in bursting pace-maker neurones consists of two components. One component, revealed in Co Ringer and blocked by TEA, has time and voltage dependent activation and inactivation characteristics which are generally similar to those described for ionic conductances in other excitable membranes (e.g. the squid giant axon and the amphibian node of Ranvier; see Cole, 1968). In the latter preparations changing the extracellular Ca has an 'indirect effect' (Reuter, 1973) on currents; i.e. it shifts the I–V relationship along the voltage axis. The rapid rise and decay of a Ca independent outward current which includes at least part of the fast outward K current is also seen in verapimil-treated snail neurones (Kostyuk, Krishtal & Doroshenko, 1975*b*). In contrast Ca may be said to have a 'direct effect' on the second component of I_K; Ca mediates both the activation and inactivation of this component ($I_{K(Ca)}$). Activation of $I_{K(Ca)}$

depends on the persistence of an inward Ca current; inactivation of $I_{K(Ca)}$ is brought about by increased intracellular Ca concentrations. Both activating and inactivating effects of Ca are blocked by external Co but not by internal TEA. Indeed, these properties of the Ca control of K conductance, which are obvious in some snail neurones (e.g. those which have large slow inward Ca currents such as the fast burster), are revealed in other snail neurones by TEA treatment.

All our results are consistent with the hypothesis that the responses of $I_{TEA\text{-}res}$ reflect the properties of $I_{K(Ca)}$. It should be noted, however, that TEA and Co may not be entirely specific for the two components of I_K. Thus, the sum $I_{TEA\text{-}res} + I_{Co\text{-}res}$ ($= I_K'$; predicted from addition of current trajectories) can be less than the normal I_K by as much as 50 %, especially in slow bursters, although the time courses of I_K' and I_K are very similar. Some non-specificity is evident in the I–V relationships of both TEA-treated and Co-treated neurones. From the data presented in Fig. 4 it appears that Co treatment not only abolishes the N-shape but can also depress the net outward at higher voltages (e.g. up to +200 mV in this neurone). Clearly some of the apparent non-specificity of TEA could be due to its effects on $I_{in\ slow}$ or to TEA moving out of the neurone and acting externally. TEA treatment does not produce neurones with bell-shaped I–V plots (e.g. as in Fig. 8). Although such a plot might be expected from the relationship shown in Fig. 8, we cannot specify that $I_{K(Ca)}$ should produce a bell-shaped current–voltage curve because details of the voltage dependence and dependence on Ca movement at higher membrane potentials have not yet been evaluated. We suggested that the action of Ca on I_K is brought about by Ca currents moving through the membrane. It is probably only the inward Ca movement which mediates $I_{K(Ca)}$ and this component of the Ca flux may not have a defined zero level at high potentials. It may also be that at higher membrane potentials the TEA resistent current becomes progressively voltage dependent which would account for the failure of $I_{TEA\text{-}res}$ to reach zero at a supposed Ca equilibrium potential. In this respect it should be pointed out that the N-shape current–voltage relationship and the extracted 'bell-shaped' net current differences (see Meech & Standen, 1975, and Fig. 8 in Heyer & Lux, 1976) are not always reliable indicators of I_K behaviour. For example, with direct K flux determinations using ion sensitive electrodes it is obvious that the N-shaped K current–voltage relationship is preserved in the presence of a purely monotonic increase in net current with voltage following intracellular injections of EGTA buffers (Lux & Heyer, 1976*b*).

The effects of Co and TEA do appear to separate two components of I_K in the snail neurones. The behaviour of the conductance inferred from measurements of the TEA resistant current differs significantly from all previously described conductances, with the exception of those controlling $I_{in\ slow}$ as discussed in the preceding paper (Heyer & Lux, 1976). Indeed, we propose that movements of the $I_{in\ slow}$-Ca ions and the $I_{K(Ca)}$-K ions are coupled. In addition we report here the depression of this K current caused by increased $[Ca]_i$ resulting from the Ca influx due to activation of $I_{in\ slow}$. The inactivation of $I_{K(Ca)}$ develops comparatively slowly and is most obvious with moderate periods of repolarization (e.g. 400 msec at −50 mV) between test pulses. Indeed, depression of $I_{K(Ca)}$ may proceed

more rapidly at higher membrane potentials, for we see little evidence of this Ca-mediated depression during sustained depolarizations; during long depolarizations the Ca dependent component of I_K appears to follow the time course of $I_{in\ slow}$.

Relationship of K currents in other neurones to the two components of I_K in snail neurones

The relative strength of Ca dependent and Ca independent components of I_K is not a constant for all *Helix* neurones. In slow bursters, which have only a small $I_{in\ slow}$ (Heyer & Lux, 1976), the Ca independent I_K predominates. Other neurones have a very large $I_{in\ slow}$ (especially the fast bursters) and more clearly reflect characteristics of the Ca dependent I_K.

We propose that the relative proportions of Ca dependent and independent K conductances varies among the membranes of other excitable cells. Such a proposition was foreshadowed by Koketsu, Cerf & Nishi (1959) who suggested that the proportion of TEA resistant I_K may vary among neurones and even parts of neurones. Under this scheme, the behaviour of membranes with proportionally small Ca currents, such as that of the squid giant axon (Hodgkin & Keynes, 1957) should be dominated by a Ca independent I_K, which is time and voltage dependent, abolished by TEA (Armstrong & Binstock, 1965) and little affected by agents which block the inward Ca current or by changes in $[Ca]_i$ (Begenisich & Lynch, 1974; Baker & Glitsch, 1975). Other excitable membranes, notably synaptic terminals and muscle membranes, with proportionally larger Ca currents, would be expected to show a Ca dependent as well as a Ca independent K current. Although this has not been examined in detail in these preparations, the K conductance is frequently depressed by treatments which decrease the Ca current (Kao & Stanfield, 1970; Vassort, 1975; Kass & Tsien, 1975) and many investigators have noted that TEA does not block all of the delayed outward currents in these membranes (Keynes, Rojas, Taylor & Vergara, 1973; Ochi & Nishiye, 1974; Vassort, 1975; Mounier & Vassort, 1975 *a*, *b*). Clusin, Spray & Bennett (1975) suggested that the outward current of unknown composition in an electroreceptor organ is exclusively activated by intracellular Ca.

TEA and the inward Ca current

As reported here inward Ca currents or increases in intracellular Ca following stimulation have been found in a number of TEA-treated excitable membranes (Katz & Miledi, 1969; Baker, Hodgkin & Ridgway, 1971; Keynes *et al.* 1973; Stinnakre & Tauc, 1973; Llinás & Nicholson, 1975; Kleinhaus & Prichard, 1975; Vassort, 1975). It is difficult to deter-

mine whether TEA alters the magnitude of the Ca current. From our experiments it is not possible to determine whether the maximum amplitude of $I_{\text{in slow}}$ is altered by TEA and, due to the sensitivity of the K ions exchanger to TEA (Neher & Lux, 1973) and the probability that TEA can move across the membrane (Volle, Glisson & Henderson, 1972), experiments were not undertaken to measure the deficit current after TEA injection. Apparent increases in inward current would result from blocking a component of I_{K} normally obscuring the Ca current. This interpretation is favoured by Kleinhaus & Prichard (1975) for the appearance of a late Ca dependent spike component in leech Retzius cells, although their data do not preclude a real increase in Ca current. However, the increased excitability reported for TEA treated presynaptic terminals (Beaulieu, Frank & Inoue, 1967) and smooth muscle (Osa, 1974; Kirkpatrick, 1975) may well represent an increased Ca current. Likewise, the augmenting of post-tetanic hyperpolarization in TEA treated bull-frog ganglion cells appears to involve an increase in Ca influx (Minota, 1974). Our data on the effects of increasing the pulse duration on the net depression suggest that TEA might also increase the total influx of Ca under voltage clamp conditions.

An increase in total Ca influx could result from the effects of TEA on Ca current kinetics, especially from interference with inactivation of the Ca channels. In this report we have presented data suggesting that the inactivation of $I_{\text{in slow}}$ is slowed by TEA injection. Stinnakre & Tauc (1973) have demonstrated, using the Ca sensitive photoprotein aequorin, that a progressive increase in intracellular Ca (presumably due to an inward flow of Ca ions) continues throughout the action potential plateau found after TEA treatment of *Aplysia* neurones.

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