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CALCIUM-INDUCED INACTIVATION OF CALCIUM CURRENT CAUSES THE INTER-BURST HYPERPOLARIZATION OF *APLYSIA* BURSTING NEURONES

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SUMMARY

1. A triphasic series of tail currents which follow depolarizing voltage-clamp pulses in *Aplysia* neurones L2–L6 was described in the preceding paper (Kramer & Zucker, 1985). In this paper, we examine the nature of the late outward component of the tail current (phase III) which generates the inter-burst hyperpolarization in unclamped cells.

2. The phase III tail current does not reverse between −30 and −90 mV, and is relatively insensitive to the external K^+ concentration. In contrast, Ca^{2+}-dependent K^+ current ($I_{K(Ca)}$), elicited by intracellular Ca^{2+} injection, reverses near −65 mV, and the reversal potential is sensitive to the external K^+ concentration.

3. Addition of 50 mM-tetraethylammonium (TEA) to the bathing medium causes a small increase in the phase III tail current. In contrast, $I_{K(Ca)}$ is completely blocked by addition of 50 mM-TEA.

4. The phase III tail current is suppressed by depolarizing pulses which approach E_{Ca}, is blocked by Ca^{2+} current antagonists (Co^{2+} and Mn^{2+}), and is blocked by intracellular injection of EGTA. The phase III tail current is reduced by less than 10% after complete removal of extracellular Na^+.

5. These bursting neurones have a voltage-dependent Ca^{2+} conductance which exhibits steady-state activation at a membrane potential similar to the average resting potential of the unclamped cell (i.e. −40 mV).

6. The steady-state Ca^{2+} conductance can be inactivated by Ca^{2+} injection, or by depolarizing pre-pulses which generate a large influx of Ca^{2+}.

7. The steady-state Ca^{2+} conductance has a voltage dependence similar to that of the phase III tail current. The Ca^{2+}-dependent inactivation of the steady-state Ca^{2+} conductance occurs in parallel with the phase III tail current; both have a similar sensitivity to Ca^{2+} influx, and both processes decay with similar rates after a depolarizing pulse.

8. Hence, we propose that the phase III tail current is due to the Ca^{2+}-dependent inactivation of a steady-state Ca^{2+} conductance.

9. The decay of $I_{K(Ca)}$ following simulated spikes or bursts of spikes is rapid (less

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than 1 s) compared to the time course of the phase III tail current and the inter-burst hyperpolarization (tens of seconds). Thus, we conclude that $I_{K(Ca)}$ does not have a major role in terminating bursts or generating the inter-burst hyperpolarization in these cells.

10. We present a qualitative model of the ionic basis of the bursting pace-maker cycle. The central features of the model are the voltage-dependent activation and the Ca^{2+}-dependent inactivation of a Ca^{2+} current.

INTRODUCTION

Molluscan bursting pace-maker neurones fire bursts of action potentials separated by inter-burst periods during which the membrane potential is hyperpolarized. There has been a general consensus that the termination of the bursts, and the inter-burst hyperpolarization that follows, are the result of an increased K^+ conductance (Gainer, 1972; Junge & Stephens, 1973; Gola, 1974; Smith, Barker & Gainer, 1975; Thompson, 1976; Smith, 1978; Carnevale & Wachtel, 1980; Gorman, Hermann & Thomas, 1981). This increase is thought to be brought about by an elevation in the intracellular Ca^{2+} concentration during the burst of spikes (Johnston, 1976; Thompson, 1976; Meech, 1979; Gorman *et al.* 1981). Gorman & Thomas (1978) have demonstrated, with the use of Arsenazo III microspectrophotometry, that an increase in internal Ca^{2+} occurs during each burst of spikes in *Aplysia* bursting neurone R15. Moreover, a Ca^{2+}-dependent increase in K^+ conductance has been demonstrated in many molluscan neurones, including bursting pace-maker cells (Meech, 1974). Indeed, several investigators have shown that Ca^{2+} influx (Barker & Gainer, 1975*b*; Eckert & Lux, 1976) and changes in internal Ca^{2+} (Meech, 1974; Gorman & Hermann, 1982) are necessary for the maintenance of normal bursting pace-maker activity.

Recently, however, it has been found that elevating the internal Ca^{2+} concentration has an additional effect in molluscan neurones: it reduces the Ca^{2+} current by causing the inactivation of voltage-dependent Ca^{2+} channels. Depolarizing pulses which allow Ca^{2+} influx (Eckert & Tillotson, 1981) as well as iontophoretic injection of Ca^{2+} (Plant, Standen & Ward, 1983) reduce the Ca^{2+} current during subsequent voltage-clamp depolarizations, and injection of EGTA (ethyleneglycol-bis-(β-aminoethyl ether)-*N*, *N'*-tetra-acetic acid) reduces the inactivation of Ca^{2+} current (Eckert & Tillotson, 1981). The Ca^{2+}-dependent inactivation mechanism must operate well within the physiological concentration range of internal Ca^{2+}, since EGTA injection removes some pre-existing inactivation of the Ca^{2+} current (Eckert & Tillotson, 1981). Furthermore, there is a steady-state voltage-dependent Ca^{2+} current flowing into bursting pace-maker cells at rest (Eckert & Lux, 1976). With this in mind, it seemed that Ca^{2+}-dependent inactivation of the steady-state Ca^{2+} current could contribute to the inter-burst hyperpolarization of bursting cells. In this report we present evidence for such a mechanism, and show that the Ca^{2+}-dependent inactivation of the Ca^{2+} current is the predominant cause of the inter-burst hyperpolarization in certain *Aplysia* bursting pace-maker neurones.

METHODS

All of the experiments, unless otherwise noted, were performed on the left upper quadrant bursting (l.u.q.b.) neurones L2–L6 (Frazier, Kandel, Kupfermann, Waziri & Coggeshall, 1967) of

the abdominal ganglion of *Aplysia californica*. These cells were axotomized by circumscribing the left upper quadrant of the ganglion with a razor blade sliver. When bursting neurone R15 was used, the cell was not axotomized. The method of preparing the neurones for recording, and procedures for voltage clamp, intracellular iontophoresis, and recording of tail currents are described in the preceding paper (Kramer & Zucker, 1985).

All experiments were performed at 21–23 °C. The compositions of artificial sea water (ASW) solutions are given in the previous paper (Kramer & Zucker, 1985). All solutions were buffered to pH 7·5 at 21 °C with HEPES. The variability among measurements is expressed as mean ± S.E. of the mean.

RESULTS

In the preceding paper (Kramer & Zucker, 1985) we described a series of tail currents which flow following depolarizing voltage-clamp pulses in l.u.q.b. cells. These tail currents correspond in sequence and in time course to the slow potential changes which characterize the inter-burst period of bursting pace-maker activity. The most prolonged of the slow tail currents (phase III) is an outward current which relaxes very slowly (up to 30 s) following brief (< 100 ms) depolarizing pulses. Since the phase III tail current closely corresponds in time course to the inter-burst hyperpolarization, it is probably the current responsible for generating the hyperpolarization in the unclamped cell. It has been demonstrated recently that the inter-burst hyperpolarization in cell R15 is generated by a slow outward current which is apparently identical to the phase III tail current (Lewis, 1984; Adams, 1985). The following experiments were designed to determine the nature of the current flowing during phase III.

Effects of raising the external K^+ concentration

If the current flowing during phase III is primarily Ca^{2+}-dependent K^+ current ($I_{K(Ca)}$), then it should be reduced by raising external K^+, and it should reverse below E_K. Fig. 1*A* shows this characteristic of $I_{K(Ca)}$, elicited by intracellular iontophoretic injections of Ca^{2+}. In normal ASW, the Ca^{2+}-elicited current is outward at a membrane potential (V_m) more positive than −62 mV, and is inward at a V_m more negative than −62 mV. The reversal potential ranged from −53 to −70 mV in ten l.u.q.b. cells. These values are low compared to previously reported values of E_K in l.u.q.b. cells (i.e. −77 mV: Coyer, Halsey & Strong, 1983), probably because the $I_{K(Ca)}$ was contaminated with a Ca^{2+}-activated non-specific inward current, which has a reversal potential near −22 mV (Kramer & Zucker, 1985). The reversal potential of the Ca^{2+}-elicited current shifted to −42 mV when the cell was bathed in 40 mM-K^+ ASW; in four l.u.q.b. cells the reversal potential ranged from −36 to −44 mV. These values are in good agreement with the expected E_K in 40 mM-K^+ ASW (−42 mV, assuming an initial E_K of −77 mV).

The behaviour of the current elicited by Ca^{2+} injection stands in sharp contrast to the behaviour of the phase III tail current (Fig. 1*B*). Tail currents were elicited by 70 ms depolarizing pulses to a test potential (V_t) of 0 mV from a holding potential (V_h) between −80 and −30 mV. Inspection of Fig. 1*B*1 shows that the phase I tail currents reversed at more depolarized potentials in high-K^+ ASW, as expected for K^+ tail currents. In contrast, the phase III tail currents did not reverse between −35 and −90 mV, either in normal ASW (seven cells) or in 40 mM-K^+ ASW (four cells).

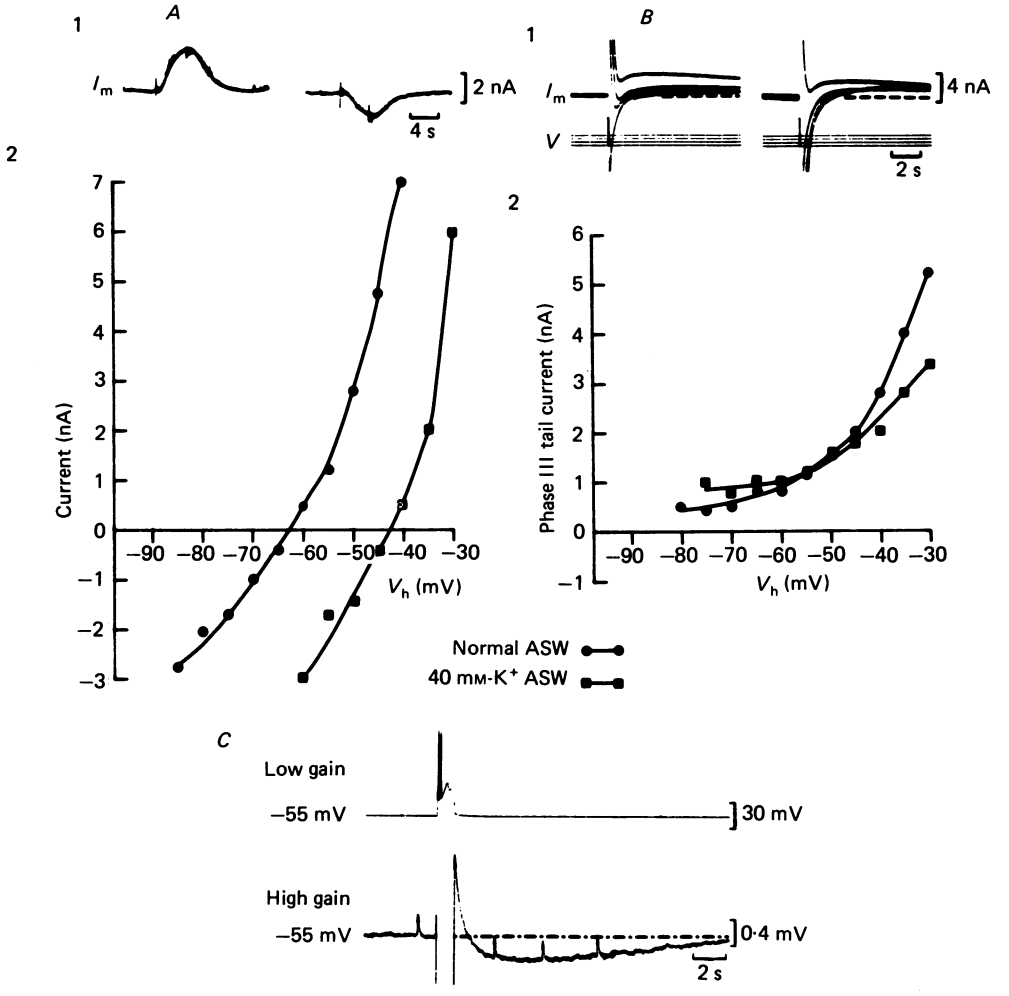

Fig. 1. The effects of external K^+ concentration on $I_{K(Ca)}$ and the phase III tail current. *A*1, $I_{K(Ca)}$ elicited by intracellular injections of Ca^{2+} (4 s, 200 nA) in normal ASW (left) and 40 mM-K^+ ASW (right) ($V_h = -50$ mV); *A*2, current–voltage relations of peak Ca^{2+}-activated current, elicited as above. *B*1, slow tail currents elicited by 70 ms pulses to 0 mV from V_h of −40, −50, −60 and −70 mV in normal ASW and 40 mM-K^+ ASW; *B*2, current–voltage relations of phase III tail current; the phase III current was measured 3 s after each pulse. Tail currents were elicited as in *B*1. *C*, low- and high-gain records of the response of a current-clamped l.u.q.b. cell in 40 mM-K^+ ASW to a 10 nA current pulse (same cell as in *A*2 and *B*2). Note post-burst hyperpolarization in high-gain recording (upward transients are spontaneous synaptic potentials).

In three of four cells, the phase III current elicited in 40 mM-K^+ ASW was reduced when elicited from a V_h more positive than −50 mV, and was increased when illicited from a V_h more negative than −60 mV. In the remaining cell, the phase III tail current was reduced throughout the range of V_h values tested, although this effect was greatest above −60 mV (see Discussion for an interpretation of these results).

L.u.q.b. neurones continue to exhibit post-burst hyperpolarizations, even when the

membrane potential is more negative than the reversal potential of $I_{K(Ca)}$. Fig. 1*C* shows a voltage recording from an l.u.q.b. cell in 40 mM-K^+ ASW, which was current clamped at a V_m of −55 mV by injecting hyperpolarizing current through a second electrode. The reversal potential of $I_{K(Ca)}$, determined by Ca^{2+} injection at different potentials, was −42 mV. A short depolarizing current pulse was given to elicit a short burst of spikes. At the end of the current pulse, the cell hyperpolarized below the initial V_m, and the decay of the post-burst hyperpolarization had a time course that was similar to the inter-burst hyperpolarization which occurred in the cell under normal conditions. The post-burst hyperpolarization did not reverse at any V_m tested (from −40 to −90 mV).

Effects of external tetraethylammonium

$I_{K(Ca)}$ can be blocked with extracellular tetraethylammonium (TEA) (Meech & Standen, 1975; Thompson, 1977; Hermann & Gorman, 1979). Fig. 2*A* shows $I_{K(Ca)}$ elicited by Ca^{2+} injection at different V_h values. After addition of 50 mM-TEA to the bathing medium, the outward component of the Ca^{2+}-elicited current was blocked, revealing an inward current whose extrapolated reversal potential was about −24 mV. This current is a Ca^{2+}-activated non-specific cation current, and is discussed more fully in the preceding paper (Kramer & Zucker, 1985). The component of the Ca^{2+}-elicited current which was blocked by TEA decreases at hyperpolarized levels, and has a null potential near −75 mV, close to the value of E_K. Fig. 2*B* shows the effect of TEA on the phase III tail current. In four l.u.q.b. cells tested, rather than causing a block of the phase III current, addition of 50 mM-TEA actually caused a slight increase in the peak outward current, especially when depolarized holding potentials were used. Addition of TEA caused the phase III tail current elicited at a V_h of −40 mV to increase by 25 ± 11 % ($n = 4$).

Action potentials elicited in l.u.q.b. neurones exposed to 50 mM-TEA have a much longer duration than normal, as has been observed in other molluscan neurones (e.g. cell R15; Hermann & Gorman, 1981). The Ca^{2+} influx during each TEA-broadened spike is much greater than normal (Gorman & Thomas, 1978). In some l.u.q.b. neurones, there is a steady-state activation of an outward current (probably $I_{K(Ca)}$) at the resting potential, which is removed by 50 mM-TEA (see Fig. 5). Thus, normal bursting pace-maker activity often does not occur in TEA-treated cells. Instead, the membrane potential remains depolarized and the cells fire periodic, broad spikes. Fig. 2*C* shows, however, that a post-spike hyperpolarization with a time course and amplitude similar to that of the normal inter-burst hyperpolarization is observed in TEA-treated l.u.q.b. cells which have been current clamped to a hyperpolarized V_m at which the spontaneous firing frequency is low.

There has been some disagreement about the concentration of external TEA required to block $I_{K(Ca)}$ in molluscan bursting pace-maker neurones. Hermann & Gorman (1979) measured $I_{K(Ca)}$ elicited by intracellular iontophoretic injection of Ca^{2+} and found a K_D of 0·4 mM for external TEA. Thompson (1977) and Deitmer & Eckert (1984) found that a slow outward tail current which was attributed to the relaxation of $I_{K(Ca)}$ was reduced only about 20 % by 100 mM-TEA. We have also measured the external TEA sensitivity of $I_{K(Ca)}$, elicited both by influx and injection of Ca^{2+}.

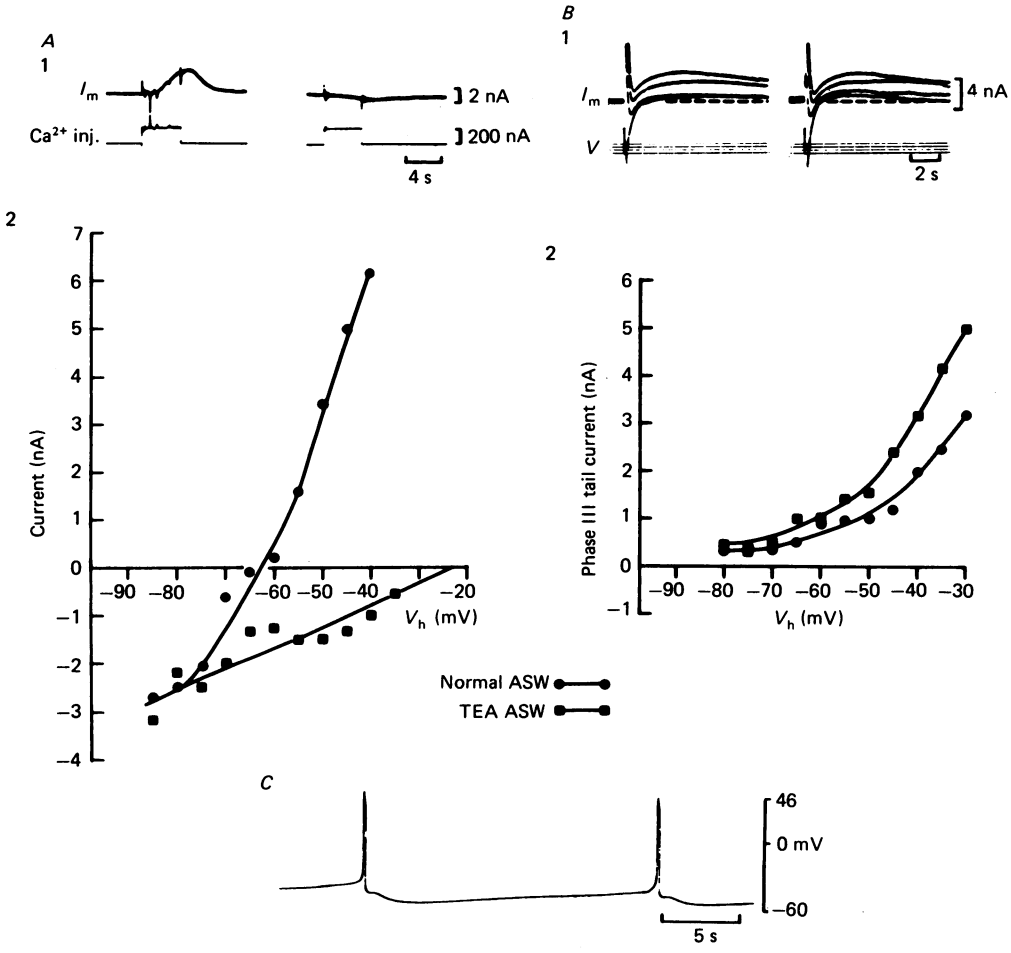

Fig. 2. The effect of external TEA on $I_{K(Ca)}$ and the phase III tail current. *A*1, $I_{K(Ca)}$ elicited by 4 s Ca^{2+} injections in normal ASW (left) and 50 mM-TEA ASW (right) ($V_h = -50$ mV); *A*2, current–voltage relations of the peak Ca^{2+}-activated current, elicited as above. *B*1, slow tail currents elicited by 70 ms pulses to 0 mV from V_h of −40, −50, −60 and −70 mV in normal ASW and 50 mM-TEA ASW; *B*2, current–voltage relations of phase III tail current; the phase III tail current was measured 3 s after each pulse. Tail currents elicited as in *B*1. *C*, spontaneous firing in a current-clamped l.u.q.b. cell in 50 mM-TEA ASW. Note prolonged hyperpolarizations following broadened spikes.

We obtained an indication of $I_{K(Ca)}$ elicited by Ca^{2+} influx by measuring the late outward current during depolarizing pulses. The total late outward current during the pulse represents the sum of two major ionic currents, in addition to smaller leak and Ca^{2+} currents: the delayed rectifier K^+ current, which increases continuously with voltage, and $I_{K(Ca)}$, which is suppressed as the pulse approaches E_{Ca}. Thus, the outward current at the end of a pulse has an N-shaped relationship with pulse potential (Meech & Standen, 1975; Hermann & Gorman, 1979). We measured the sensitivity of this N shape to external TEA and compared it to the TEA sensitivity of $I_{K(Ca)}$ elicited by Ca^{2+} injection. In each of three cells, external TEA was equally

effective at blocking both the Ca^{2+}-injection-elicited $I_{K(Ca)}$ and the $I_{K(Ca)}$ component of outward current during depolarizing pulses; each was reduced by half at an external TEA concentration of 1–5 mM, close to the K_D determined by Hermann & Gorman (1979). 50 mM-TEA completely blocked $I_{K(Ca)}$ elicited by either method. The discrepancy between the effectiveness of TEA in blocking these processes, and the ineffectiveness of TEA in blocking slow tail currents attributed to $I_{K(Ca)}$ (Thompson, 1977; Deitmer & Eckert, 1984), can be explained by contamination of the presumed $I_{K(Ca)}$ tail current with another slow outward tail current found in bursting pace-maker neurones (see below).

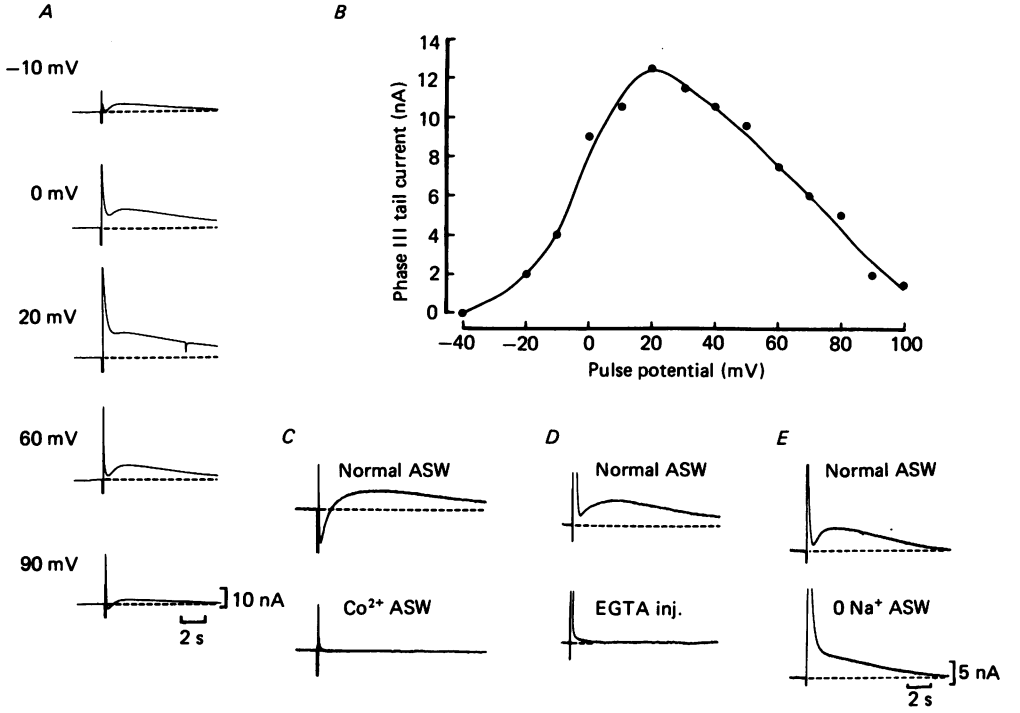

Fig. 3. Dependence of the phase III tail current on Ca^{2+} influx and accumulation. *A*, slow tail currents elicited by 70 ms pulses from a V_h of −40 mV, up to the pulse potential indicated. *B*, relation between pulse potential and peak amplitude of the phase III tail current. *A* and *B* were done in 50 mM-TEA ASW. *C*, the effect of replacement of external Ca^{2+} with Co^{2+} on the tail currents. *D*, the effect of intracellular injection of EGTA (300 nA for 4 min) on the tail currents. *E*, the effect of replacement of external Na^+ with TMA^+ on the tail currents. Tail current records were obtained from different l.u.q.b. cells in *C*, *D* and *E*, and were elicited by 70 ms pulses to 0 mV from a V_h of −40 mV.

Dependence of phase III tail current on Ca^{2+} influx and accumulation

Since changes in the intracellular concentration of Ca^{2+} are critical for the maintenance of bursting pace-maker activity (Meech, 1979; Gorman & Hermann, 1982), we wondered whether the phase III tail current was Ca^{2+} dependent. If the phase III tail current is dependent upon Ca^{2+} influx during the preceding depolarizing pulse, then pulses which approach E_{Ca} should not generate any phase III tail current.

Fig. 3*A* shows tail currents elicited by 70 ms pulses to various pulse potentials. In order to produce rectangular voltage pulses to very large pulse potentials it was sometimes necessary to use 50 mM-TEA to reduce the large outward current activated during the depolarizing pulse. In two l.u.q.b. cells, however, no external TEA was necessary, and the results were qualitatively the same. Fig. 3*B* shows that the magnitude of the peak phase III tail current reaches a maximum following pulses to +20 mV, and declines following pulses which approach E_{Ca} (e.g. pulses to +100 mV). In each of seven l.u.q.b. cells tested, the amplitude of the phase III tail current exhibited an inverted U-shaped relationship with pulse potential. Thus, the amplitude of the phase III tail current seems to be sensitive to the amount of Ca^{2+} influx during the preceding pulse.

The phase III tail current is blocked by treatments which block voltage-dependent Ca^{2+} channels, or which prevent the build-up of Ca^{2+} inside the cell. In each of six l.u.q.b. cells tested the phase III tail current was reduced by more than 95% following replacement of external Ca^{2+} with Mn^{2+} (Fig. 3*C*) or Co^{2+}, both effective blockers of voltage-gated Ca^{2+} channels (for review, see Hagiwara & Byerly, 1981). In each of six l.u.q.b. cells, intracellular injection of the Ca^{2+} chelator, EGTA, completely suppressed the phase III tail current (Fig. 3*D*). The phase III tail current of R15 is also blocked following the intracellular injection of EGTA (Lewis, 1984). Replacement of external Na^+ with TMA^+ initially (within 10 min of removal of Na^+) had only a minor effect on the phase III tail current of l.u.q.b. cells (Fig. 3*E*), reducing the amplitude measured at 4 s after the pulse by only 8·6±2·3% ($n = 5$). In contrast, Na^+ removal greatly reduced the phase II tail current, as described in the preceding paper (Kramer & Zucker, 1985). In two of five l.u.q.b. cells tested, prolonged removal of external Na^+ (> 15 min) resulted in a gradual and largely irreversible decrease of the phase III tail current. This effect may be due to a build-up of intracellular Ca^{2+}, due to suppression of the Na^+–Ca^{2+} exchange pump (see Discussion). Replacement of 490 mM (79%) of the external Cl^- with methylsulphate ions had little effect on the phase III tail current; in three cells tested the amplitude of the phase III tail current changed by less than ±5% after partial removal of external Cl^-. These results suggest that the current flowing during phase III is specifically dependent upon Ca^{2+} influx which occurs during the depolarizing pulse, and is relatively insensitive to many other extracellular ions.

Voltage dependence of the phase III tail current

The phase III tail current is larger when elicited at more depolarized V_h values (Figs. 1*B* and 2*B*). In the experiments described above, the voltage dependence of the phase III tail current was ascertained by eliciting tail currents only after the membrane current was allowed to reach steady state at any given V_h value. We have also examined the voltage dependence of the phase III tail current in another way, as illustrated in Fig. 4. A 50 ms depolarizing pulse to +25 mV (V_1) was used to generate the phase III tail current. A hyperpolarizing post-pulse to V_2 was utilized in order to determine the voltage dependence of the phase III tail current. The currents generated by this pulse paradigm are the result of two factors. First, the hyperpolarizing pulse to V_2 elicits an instantaneous leak current, and time- and voltage-dependent ionic currents. Secondly, the depolarizing pulse to V_1 initiates the usual

series of slow tail currents. The slow tail currents can be observed independently from the other currents at each V_2 by subtracting the current elicited during a hyperpolarizing pulse to V_2 alone (dotted line in Fig. 4*A*), from the net current generated during a V_1, V_2 pulse sequence (continuous line in Fig. 4*A*). The difference between the currents generated by the two-pulse paradigms represents the voltage dependence of the phase III tail current. The relationship between phase III amplitude and V_2 is shown in Fig. 4*B*. The phase III difference current decreases at hyperpolarized potentials, but does not reverse at any V_2 value. The voltage dependence of the phase III difference current is very similar to the steady-state voltage dependence of the phase III tail current (Figs. 1*B* and 2*B*).

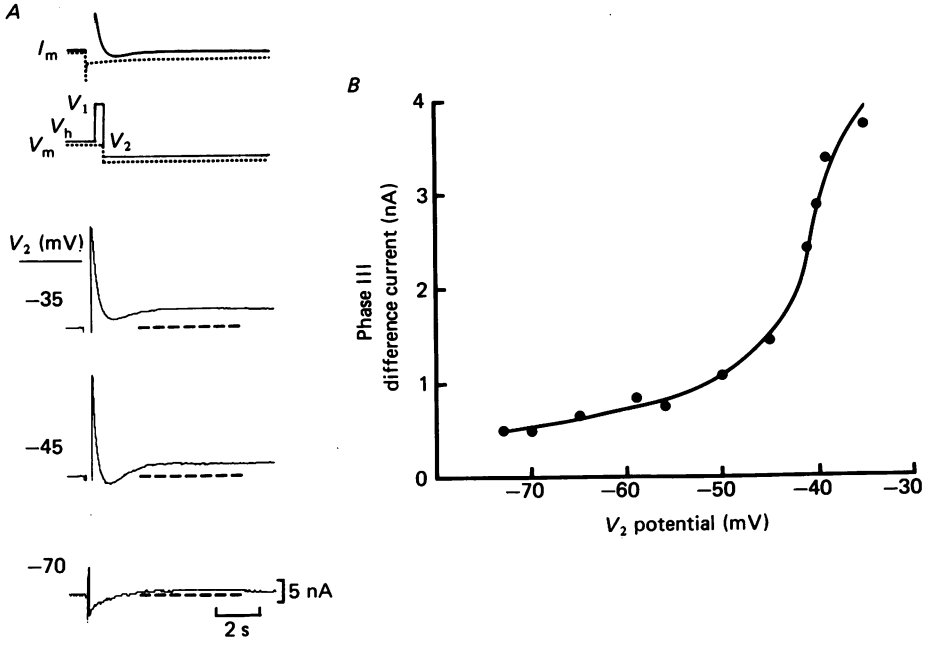

Fig. 4. Voltage dependence of the phase III tail current. *A*, pulse paradigm used to determine the voltage sensitivity of the phase III tail current. Currents elicited by hyperpolarizing pulses to V_2 (dotted line) were digitized and subtracted from currents elicited by a V_1, V_2 pulse sequence (continuous line). The depolarizing pulse (V_1) was a 50 ms pulse to +25 mV. Difference currents obtained at three values of V_2 are shown below. The dashed lines mark the holding current prior to the pulse. *B*, relation between V_2 potential and the phase III difference current, measured 4 s after the onset of the V_2 pulse.

Steady-state voltage-dependent Ca^{2+} current in l.u.q.b. cells

Eckert & Lux (1976) described a persistent, voltage-dependent inward current in *Helix* bursting cell somata. These cells produce a small maintained inward current during small depolarizations (< 20 mV) from V_h values between −55 and −45 mV. This current is evidently due to an increased Ca^{2+} conductance (Eckert & Lux, 1976; Kramer & Zucker, 1985), and contributes to the negative resistance characteristic seen in the pace-maker voltage range (i.e. −50 to −30 mV) of the steady-state *I–V* relationship of these bursting cells (Eckert & Lux, 1976). Small hyperpolarizing pulses

(< 10 mV) from −38 mV produce a net outward current, suggesting that some voltage-sensitive Ca^{2+} conductance is activated at this V_h.

In order to characterize the steady-state Ca^{2+} current flowing in l.u.q.b. cells, we generated steady-state *I–V* curves in bathing solutions containing substances which block specific components of the steady-state current (Fig. 5). Long (2 s) hyperpolarizing and depolarizing voltage-clamp pulses from −37 mV were used to obtain steady-state changes in current. In normal ASW, the steady-state *I–V* curve exhibits an inward rectification (negative resistance) which appears above about −40 mV. In

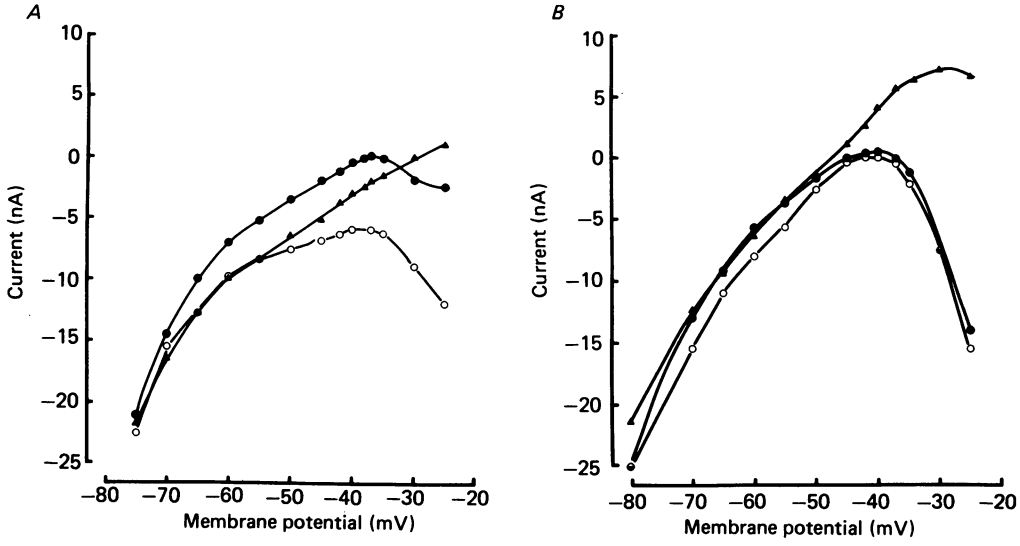

Fig. 5. Steady-state *I–V* relations of l.u.q.b. cells. *A*, *I–V* curves obtained from a weakly bursting cell in normal ASW (●), 50 mM-TEA ASW (○), and 50 mM-TEA ASW in which Mn^{2+} was substituted for Ca^{2+} (▲). Current values were measured at the end of 2 s pulses from a V_h of −37 mV. *B*, *I–V* curves obtained from a vigorously bursting cell. Symbols as in *A*.

a cell which had been weakly bursting before being voltage clamped (Fig. 5*A*), addition of TEA resulted in an inward shift in current, which was large within the pace-maker potential range, and which diminished with hyperpolarization, such that it was very small at −70 mV. Our explanation for this shift is that TEA blocked a steady-state K^+ conductance, probably $I_{K(Ca)}$ (see Hermann & Gorman, 1979). The TEA also enhanced the inward rectification above −40 mV, suggesting that activation of a K^+ current can suppress the appearance of the negative resistance characteristic of bursting neurones. Subsequent substitution of Mn^{2+} for Ca^{2+} in the bathing medium resulted in an outward, voltage-dependent shift in the *I–V* curve, eliminating the negative resistance region in the pace-maker potential range. The outward shift probably results from block of the voltage-dependent Ca^{2+} current.

Different results were obtained from a cell which had been bursting strongly before being clamped (Fig. 5*B*). The addition of TEA had little effect on the steady-state *I–V* curve, while Mn^{2+} substitution for Ca^{2+} resulted in a large voltage-dependent outward shift in the pace-maker range. The vigorously bursting cell apparently had

less steady-state $I_{K(Ca)}$ blocked by TEA than did the weakly bursting cell. The vigorously bursting cell, however, had more steady-state, voltage-dependent Ca^{2+} current blocked by Mn^{2+} substitution than did the weakly bursting cell. These results provide an estimate of the magnitude of the steady-state Ca^{2+} current flowing at the average resting potential in these bursting neurones (about -37 mV); it is at least 5–10 nA. These results also suggest that the variability in the strength of bursting activity may lie in the relative magnitudes of the steady-state $I_{K(Ca)}$ and the steady-state Ca^{2+} current.

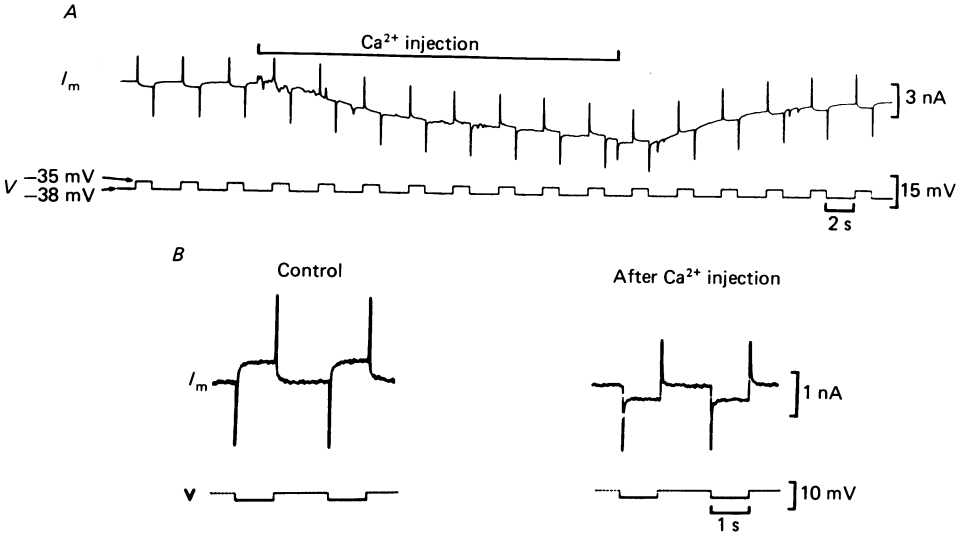

Fig. 6. Effect of Ca^{2+} injection on the negative resistance. *A*, outward currents resulting from the deactivation of a steady-state Ca^{2+} conductance were elicited by hyperpolarizing pulses to -38 mV from a V_h of -35 mV. Note the disappearance of the outward currents during the long injection of Ca^{2+}, and the slow recovery following the injection. *B*, elimination of the negative resistance characteristic following a 60 s injection of Ca^{2+}. Hyperpolarizing pulses as in *A*. ASW containing 50 mM-TEA was used both in *A* and *B* to block $I_{K(Ca)}$. Ca^{2+} injection intensity was 200 nA.

Inactivation of steady-state Ca^{2+} current by intracellular Ca^{2+}

Elimination of the negative resistance characteristic by Ca^{2+} injection. If the Ca^{2+} current active in the pace-maker range is similar to Ca^{2+} current activated by large depolarizing pulses, then it may exhibit Ca^{2+}-dependent inactivation. We attempted to test this possibility by measuring the amplitude of the steady-state Ca^{2+} current which could be deactivated by small hyperpolarizing pulses during Ca^{2+} injections. Since $I_{K(Ca)}$ is co-activated by both intracellular Ca^{2+} and membrane depolarization (Gorman & Thomas, 1980), its presence could interfere with the correct measurement of the steady-state Ca^{2+} current. Therefore $I_{K(Ca)}$ was blocked with 50 mM-TEA in all experiments in which the steady-state Ca^{2+} current was measured.

In Fig. 6*A*, outward currents due to deactivation of the steady-state inward Ca^{2+} current were elicited by repetitive hyperpolarizing pulses from a V_h of -35 mV to a V_t of -38 mV. Injection of Ca^{2+} caused a reduction of the outward current

generated during each hyperpolarizing pulse. The injected Ca^{2+} also caused the gradual activation of a non-specific inward current (see Kramer & Zucker, 1985). By the end of the prolonged injection, the negative resistance had nominally disappeared such that hyperpolarizing pulses began to evoke an inward current. In each of four l.u.q.b. cells, Ca^{2+} injection resulted in elimination of the negative resistance. The disappearance of the outward current at the end of a prolonged (60 s) Ca^{2+} injection is seen more clearly in Fig. 6*B*. The reduction of the outward current could result from an inactivation of the steady-state Ca^{2+} conductance, an increase in the total membrane conductance due to activation of the non-specific inward current, or a combination of both effects. The outward current gradually returned after the Ca^{2+} injections were terminated. Gorman, Hermann & Thomas (1982) also found that injecting Ca^{2+} into cell R15 reduced the negative resistance of the steady-state *I–V* curve.

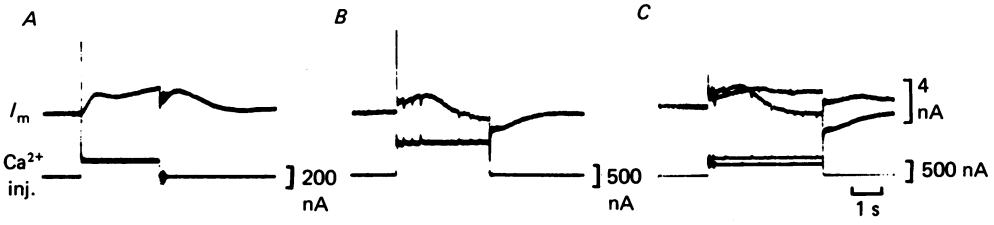

Fig. 7. Early outward Ca^{2+}-elicited current. *A*, Ca^{2+} injection results in a triphasic (outward–inward–outward) current in an l.u.q.b. cell held at −50 mV. Cell bathed in normal ASW. *B*, Ca^{2+} injection into the same cell in 50 mM-TEA ASW (V_h = −35 mV). Note block of the late outward current ($I_{K(Ca)}$). *C*, differential sensitivity of the early outward current, and the Ca^{2+}-elicited inward current, to injected Ca^{2+}. A small Ca^{2+} injection (250 nA, 10 s) elicits the outward current alone (top trace). A larger injection (450 nA, 10 s) elicits the outward current, followed by the inward current.

Injection of Ca^{2+} into either R15 or the l.u.q.b. neurones sometimes elicited an early outward current, in addition to $I_{K(Ca)}$ and the non-specific cation current described in the preceding paper (Kramer & Zucker, 1985). Fig. 7*A* shows a triphasic series of currents elicited by the injection of Ca^{2+} into an l.u.q.b. neurone held at −50 mV. At more depolarized V_h the early outward current component was obscured by a large $I_{K(Ca)}$ component. At more hyperpolarized V_h the early outward component was smaller and partly obscured by the inward component. The position of the Ca^{2+}-injection electrode in the cell and the amplitude of the Ca^{2+}-injection pulse were critical for the appearance of a clear early outward current component. The relative magnitudes of the three Ca^{2+}-elicited current components in l.u.q.b. cells were quite variable; the early outward component was clearly discerned in only three of the seven l.u.q.b. and R15 cells tested.

The late outward current can be effectively blocked with 50 mM-external TEA. Fig. 7*B* shows the remaining Ca^{2+}-elicited currents in an l.u.q.b. neurone held at −35 mV. The early outward component elicited in 50 mM-TEA ASW was reduced at more hyperpolarized V_h. In addition, the early outward current seen in 50 mM-TEA ASW was eliminated by replacement of external Ca^{2+} with Co^{2+}. These results suggest

that the early outward current is the result of Ca^{2+}-dependent inactivation of the steady-state inward Ca^{2+} current, mediated by the injected Ca^{2+}. The Ca^{2+} sensitivity of the early outward current seems to be greater than that of the non-specific cation current. Fig. 7*C* shows currents elicited by Ca^{2+} injections of two different injection magnitudes. The small injection elicits the early outward current component alone, whereas a larger injection elicits the early outward current, followed by the inward component. It was difficult to characterize the Ca^{2+}-elicited early outward current thoroughly, because its amplitude declined irreversibly during repeated Ca^{2+} injections.

Inactivation by pre-pulses. The steady-state Ca^{2+} current can be inactivated by pre-pulses which generate a large influx of Ca^{2+}. In Fig. 8*A*, a 100 ms depolarizing pre-pulse (P_1) to various potentials was used to generate a variable influx of Ca^{2+}. The outward current elicited by a hyperpolarizing test pulse (P_2) was eliminated if the P_1 pulse generated a large Ca^{2+} influx (e.g. a P_1 potential of +40 mV) revealing an inward leak current during P_2. The outward current during P_2 was only slightly reduced if the P_1 pulse potential approached E_{Ca} (e.g. a P_1 potenital of +100 mV). The relationship between the inactivation of the steady-state Ca^{2+} current and the P_1 potential is shown in Fig. 8*B*. The relationship plotted for this cell, as well as for three other l.u.q.b. cells tested, has a U shape, similar to curves relating Ca^{2+} current generated during large depolarizing pulses to pre-pulse potential (Eckert & Tillotson, 1981). Small pre-pulse potentials (−40 to 0 mV) and very large pre-pulse potentials (+70 to +100 mV) produce little Ca^{2+} current inactivation in a second pulse, whereas intermediate pre-pulse potentials (+10 to +60 mV) produce much more inactivation.

The depolarizing pre-pulses which were used to inactivate the steady-state Ca^{2+} current also elicit the series of slow tail currents described above, including the phase III tail current. If the amplitude of the phase III tail current measured immediately before P_2 is compared with the inactivation of the steady-state Ca^{2+} current, it is apparent that the two processes have a strikingly similar relationship with respect to P_1 potential (Fig. 8*B*). The degree of inactivation of the steady-state Ca^{2+} current, and the magnitude of the phase III tail current, seem to depend identically upon the magnitude of the Ca^{2+} influx during the preceding pulse.

The time courses of these two processes were compared by varying the interval between P_1 and P_2 while keeping the P_1 potential constant at +30 mV (Fig. 9). Both the steady-state Ca^{2+} current inactivation, and the phase III current, take several seconds to decay. The recovery of the negative resistance current is approximately exponential, with a time constant of about 6·0 s. The kinetics of the phase III tail current are difficult to ascertain, especially during the first 5 s after the pulse, when the phase II tail current is superimposed upon the phase III tail current. The late portion of the phase III tail current (more than 5 s after the pulse) decays with an approximately exponential time course with a time constant of about 10 s.

We have considered an alternative explanation for the decrease in the negative resistance caused by elevated intracellular Ca^{2+}. The preceding paper (Kramer & Zucker, 1985) described a Ca^{2+}-activated inward current due to an increase in membrane permeability to a number of cations. If this conductance increase is large and prolonged enough to contribute significantly to the total membrane conductance

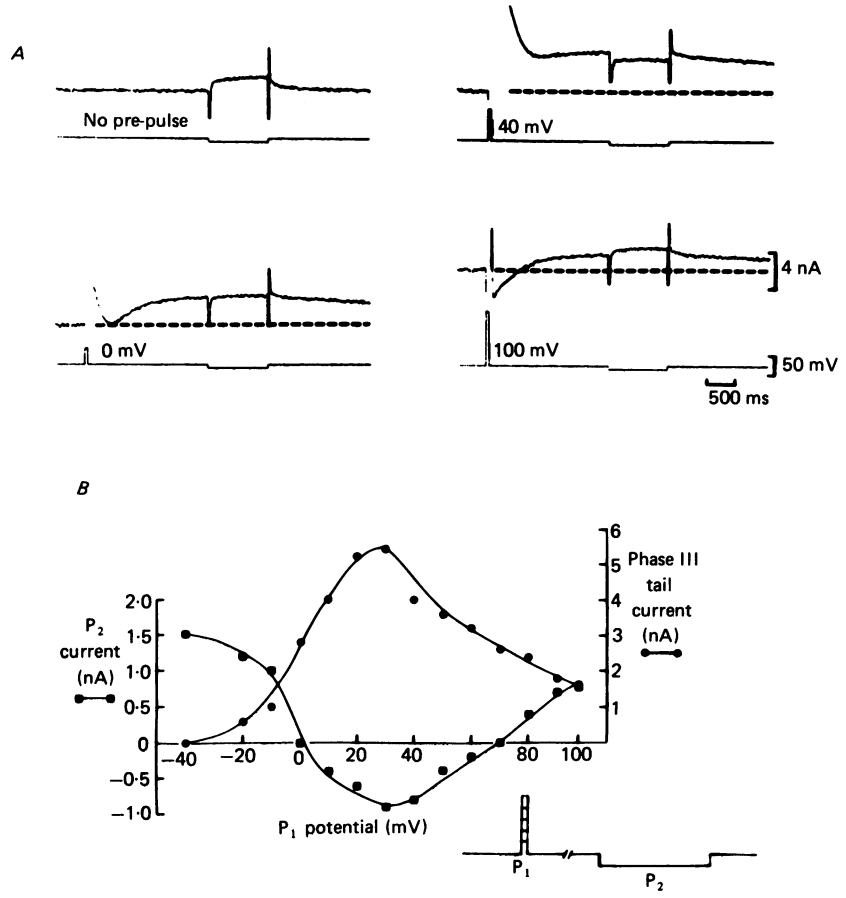

Fig. 8. Ca^{2+}-dependent inactivation of steady-state Ca^{2+} current by depolarizing pre-pulses. *A*, the current generated by a 1 s hyperpolarizing test pulse alone (P_2) to −43 mV from a V_h of −38 mV is shown at upper left. In subsequent records a 100 ms depolarizing pre-pulse (P_1) preceded P_2 by 2 s. The P_1 pulse has two effects which vary with the P_1 amplitude: the P_1 pulse generates a phase III tail current of variable amplitude, and the P_1 pulse reduces the outward current elicited by the P_2 pulse by a variable amount. The outward current during P_2 results from the deactivation of a steady-state Ca^{2+} current, and is thus an indication of the relative magnitude of the steady-state Ca^{2+} conductance. Dashed line denotes current level before the P_1 pulse. *B*, relation between the pre-pulse (P_1) potential and the current elicited by the P_2 test pulse. The phase III tail current amplitude, measured immediately before the onset of the P_2 pulse, is plotted with respect to a different ordinate. 50 mM-TEA ASW was used in both *A* and *B*.

following a P_1 pulse, then the apparent inactivation of the negative resistance during P_2 could represent an increase in the current through this voltage-independent conductance pathway, instead of a true inactivation of the steady-state Ca^{2+} current. This possibility does not seem likely for the following reasons. First, the removal of the negative resistance by depolarizing pre-pulses is not affected by replacement of external Na^+ with TMA^+, whereas the Ca^{2+}-activated inward current is reduced

by Na^+ replacement. Secondly, the effect that the pre-pulse has on the current during P_2 depends upon the holding potential, whereas the Ca^{2+}-activated non-specific conductance does not (Kramer & Zucker, 1985). The pre-pulse causes a large and long-lasting (tens of seconds) apparent conductance increase (increased inward current upon hyperpolarization) when the holding potential is −37 mV (Fig. 9). We have repeated the two-pulse experiment at a more hyperpolarized holding potential (−60 mV) using the same P_1 and P_2 pulse amplitudes, and the same pulse durations (data not shown). At this V_h the pre-pulse causes only a brief (about 700 ms) apparent

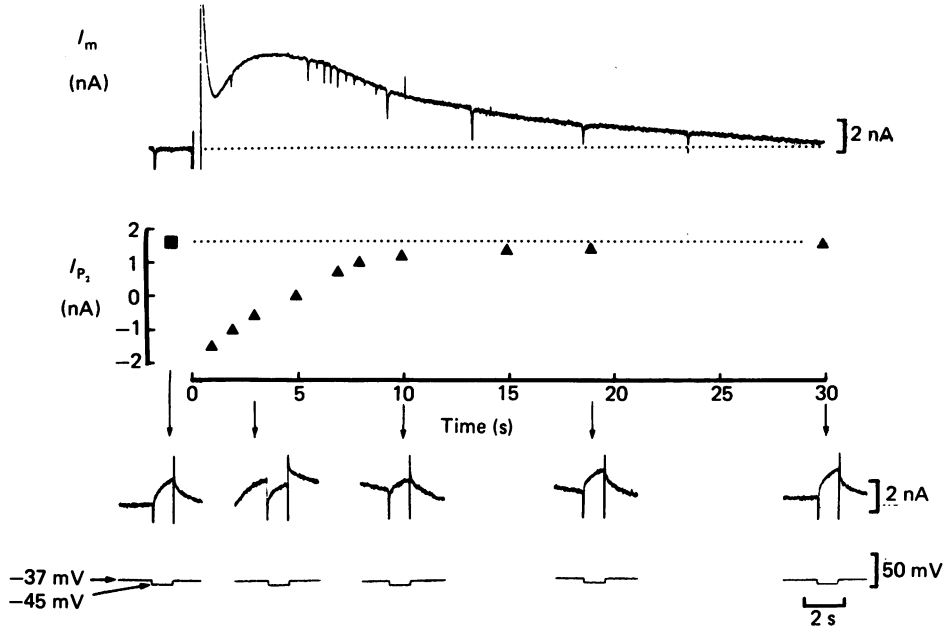

Fig. 9. Time course of inactivation of the steady-state Ca^{2+} current. Ca^{2+}-dependent inactivation of steady-state Ca^{2+} current was visualized by preceding a 1 s hyperpolarizing pulse to −45 mV (P_2) with a 70 ms depolarizing pre-pulse to +30 mV (V_h = −37 mV). The interval between the pre-pulse and P_2 was varied between 1 and 30 s. The top trace shows the series of tail currents resulting from the pre-pulse alone. Plotted below, on the same time scale, is the relationship between the current generated at the end of P_2, and the interval between the pre-pulse and P_2. The filled square and dotted line mark the P_2 current in the absence of a pre-pulse. Shown on the bottom are representative P_2 current traces elicited at various intervals after the pre-pulse, superimposed upon the slow tail currents. The P_2 current values were obtained by graphically subtracting such current traces from the slow tail currents elicited by the pre-pulse alone. The bathing solution was 50 mM-TEA ASW.

conductance increase. Thus, the apparent conductance increase caused by the pre-pulse is dependent upon the membrane potential, and is suppressed at hyperpolarized potentials. If a slow decline of the Ca^{2+}-activated inward current is responsible for the long-lasting apparent conductance increase which follows depolarizing pre-pulses, the conductance change responsible for this current must be voltage dependent. However, the conductance change which underlies the

Ca^{2+}-activated inward current appears to be independent of voltage (Kramer & Zucker, 1985). A more likely explanation for the long-lasting apparent conductance increase which follows depolarizing pre-pulses is that a steady-state voltage-dependent inward current is partly inactivated by the pre-pulse; thus there is less inward current available to be deactivated during the hyperpolarizing P_2 pulse. At a hyperpolarized V_h, there is less steady-state Ca^{2+} conductance, and hence there is less available to be inactivated by a P_1 pulse (see below).

Voltage dependence of the steady-state Ca^{2+} current

If the phase III tail current is due to the inactivation of a voltage-dependent, steady-state Ca^{2+} current, then the two currents should have similar voltage sensitivities. An indication of the voltage sensitivity of the steady-state Ca^{2+} current has already been presented in Fig. 5. In order to determine the voltage sensitivity of the steady-state Ca^{2+} current in a different way, 5 mV hyperpolarizing pulses from various V_h values were used to deactivate a portion of the current (Fig. 10*A*). Hyperpolarizing pulses resulted in an instantaneous inward current displacement, followed by the development of an outward current, due to the turn-off of the steady-state Ca^{2+} conductance. In cells that had been vigorously bursting before being voltage-clamped, pulses originating above −40 mV resulted in a current which was net outward by the end of the pulse. Pulses originating from progressively more hyperpolarized V_h resulted in progressively less outward current. The outward current developed with complex kinetics; the onset and offset of the current had both a fast phase, whose time course could not be resolved because of the low-pass filtering of the current signal (see Discussion), and a slow phase, with rise and decay times which were usually greater than 2 s.

The magnitude of the steady-state Ca^{2+} current deactivated by the pulse was estimated by measuring the amplitude of the outward current which develops upon hyperpolarization. Because of the complex kinetics of deactivation, the voltage sensitivity of the fast and slow phases of the outward current were analysed separately (Fig. 10*B*). The fast phase of the outward current could not be separated from the larger instantaneous inward leakage current. Thus an increase in the amplitude of the fast phase of deactivation appears as a decrease in the initial inward current triggered by the hyperpolarizing pulse (current at 200 ms after the onset of the pulse; left ordinate, Fig. 10*B*). The slow phase of deactivation (outward current which develops from 200 ms after the onset of the pulse to the end of the pulse) is plotted with respect to the right ordinate in Fig. 10*B*.

Both the rapid and slow phases of the deactivating inward current increase in parallel between −50 and −35 mV. Both phases are flat between −60 and −50 mV. This suggests that steady-state Ca^{2+} current activates above −50 mV, at which voltages it can be deactivated with fast and slow kinetics by hyperpolarizing pulses. The initial inward current shows an additional increase at voltages below −60 mV. This may reflect rapid hyperpolarizing activation of an 'anomalously rectifying' current (Tauc & Kandel, 1964), similar to that attributed to a K^+ conductance in neurone R15 (Benson & Levitan, 1983).

Because the rapidly developing current is contaminated with both the instantaneous current displacement and with the anomalous rectifier current, the voltage sensitivity

of only the slow phase of deactivation was compared to the voltage dependence of the phase III tail current (Fig. 10*C*). These two currents were both normalized, such that the outward current at −35 mV was set to a value of 1·0. The steady-state Ca^{2+} current, estimated by measuring its slow deactivation, has a voltage dependence which is nearly identical to that of the phase III tail current. Both currents show a sharp increase above −50 mV, which corresponds roughly to the hyperpolarized limit of the pace-maker potential range.

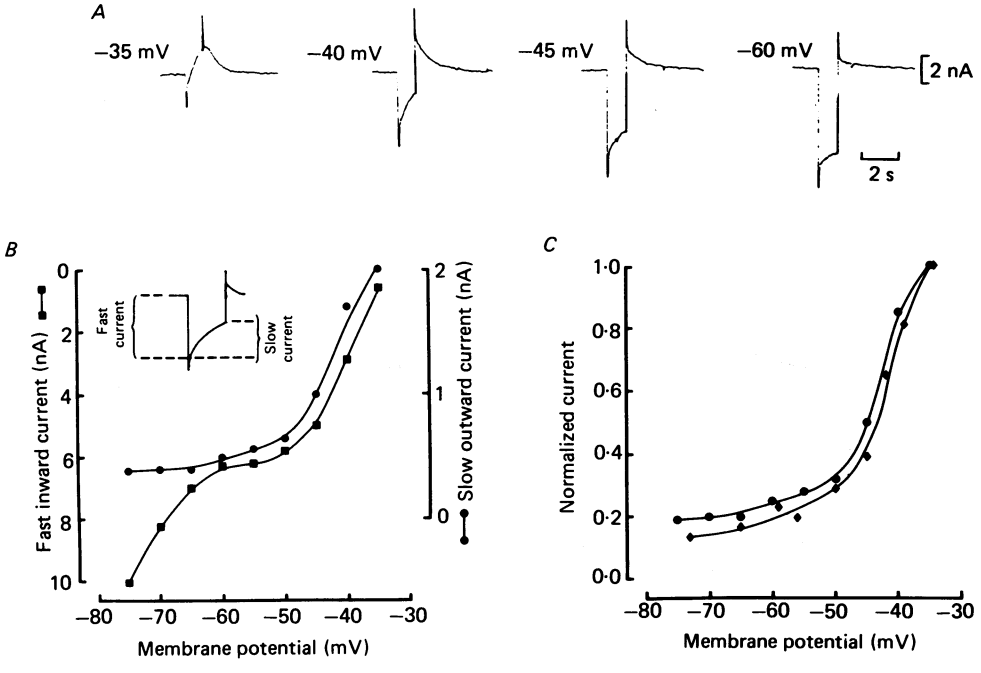

Fig. 10. Voltage dependence of the steady-state Ca^{2+} conductance. *A*, currents resulting from 5 mV hyperpolarizing pulses from the V_h indicated to the left of the traces. An outward current, due to the deactivation of a steady-state Ca^{2+} conductance develops with complex kinetics during the hyperpolarizing pulse. *B*, the relationship between V_h and the magnitude of the rapidly and slowly developing deactivation currents. The rapidly deactivating component includes inward leakage current; thus the two components are plotted with respect to different current ordinates. *C*, normalized values of the slow outward current from *B* (slow deactivation of steady-state Ca^{2+} current, ●) and the phase III difference current (◆, data from Fig. 4) plotted *vs.* membrane potential. For both parameters, the currents generated at a voltage of −35 mV were normalized to a value of 1·0.

$I_{K(Ca)}$ *tail currents*

Since bursting pace-maker neurones possess a substantial $I_{K(Ca)}$ (Gorman & Hermann, 1982), it is important to know the role of $I_{K(Ca)}$ in bursting activity. Fig. 11*A* shows phase I tail currents following depolarizing pulses with a duration (15 ms) and amplitude (+30 mV) similar to that of an action potential in the unclamped cell. This current reverses below −65 mV, and the reversal potential is shifted to a more depolarized value in 40 mM-K^+ ASW (see Fig. 1*A*1 and *B*1). The

phase I tail current elicited at −38 mV is largely blocked by the addition of 50 mM-TEA. The phase I tail current elicited at more hyperpolarized V_h (i.e. −60 mV) is resistant to block by TEA, but is largely blocked by 4-aminopyridine (Kramer & Zucker, 1985), which is a blocker of the transient K^+ current, I_A (Thompson, 1977). Therefore the phase I tail current elicited by brief pulses from near −60 mV is due primarily to the relaxation of I_A. The phase I tail currents illustrated in Fig. 11 were all elicited from −38 mV.

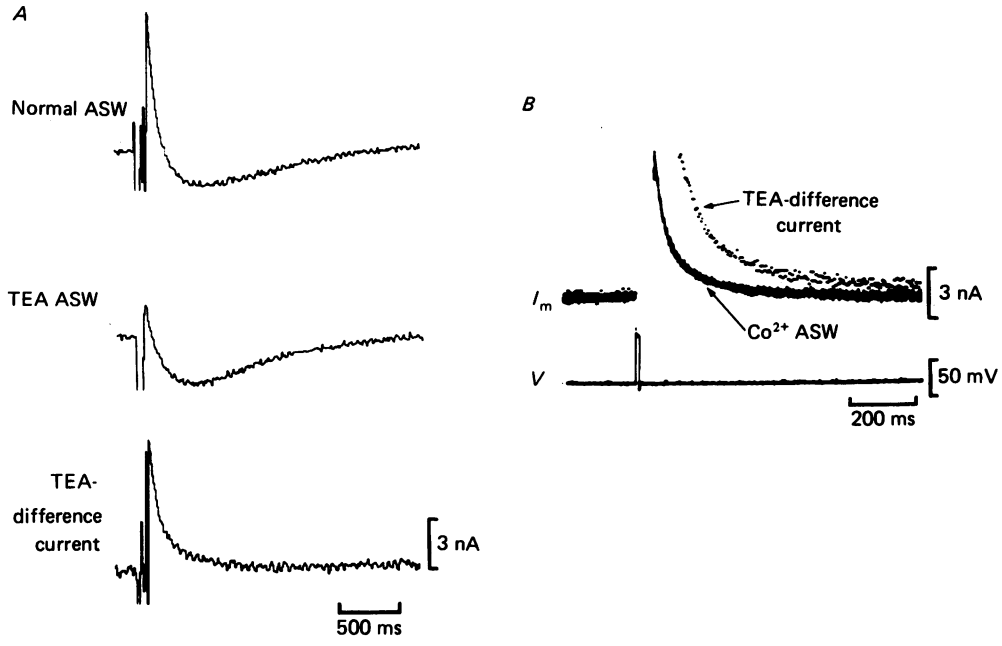

Fig. 11. $I_{K(Ca)}$ decay after a spike-like depolarization. *A*, digitized tail current records elicited by a spike-like depolarizing pulse (15 ms pulse to +30 mV from a V_h of −38 mV) in normal ASW and 50 mM-TEA ASW. The difference between the two records (the component of current blocked by 50 mM-TEA) is shown below. *B*, the TEA-difference current, shown on an expanded time scale, superimposed upon a tail current generated by the same pulse as in *A* in Co^{2+} ASW.

In order to visualize the time course of the $I_{K(Ca)}$ decay following a depolarizing pulse, the tail current elicited in 50 mM-TEA was subtracted from the tail current elicited in normal ASW (Fig. 11*A*). Addition of 50 mM-TEA results in an unequal block of two K^+ currents: $I_{K(Ca)}$ is totally blocked and the delayed rectifier K^+ current is only partially blocked (Hermann & Gorman, 1979); thus the TEA-difference current is composed of both $I_{K(Ca)}$ and delayed rectifier components. In Fig. 11*B*, the TEA-difference current is shown on an expanded time scale, superimposed upon a tail current elicited in ASW in which Co^{2+} was substituted for Ca^{2+}. The fast outward tail current elicited in Co^{2+} ASW decays much more rapidly than does the TEA-difference current because the slowly decaying $I_{K(Ca)}$ component of the phase I tail current is blocked in Co^{2+} ASW. The remaining outward tail current in Co^{2+} ASW reverses at −70 mV (data not shown); it is primarily due to relaxation of the

delayed rectifier K^+ current. Since most of the TEA-difference current is blocked in Co^{2+} ASW, most of the TEA-difference current must be due to the relaxation of $I_{K(Ca)}$. Thus, the time course of the decay of the TEA-difference current is primarily due to the slow decay of $I_{K(Ca)}$ after the depolarizing pulse.

The TEA-difference current decays much more rapidly after a depolarizing pulse than does the phase III tail current. The TEA-difference current illustrated in Fig. 11 decayed to half of its initial amplitude at 200 ms after the pulse, and was less

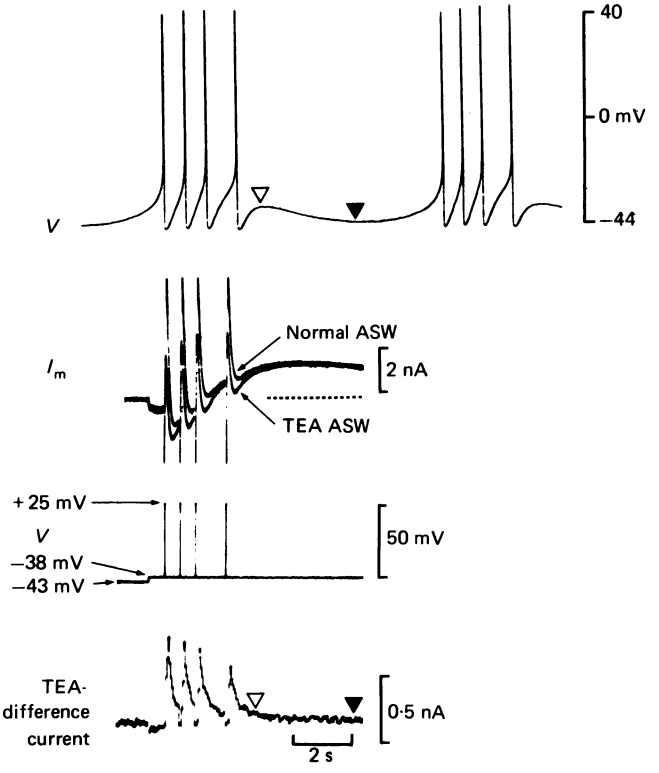

Fig. 12. $I_{K(Ca)}$ decay following a simulated burst in an l.u.q.b. cell. The simulated burst of spikes (third trace) consists of four spike-like depolarizing pulses (10 ms pulses up to +25 mV), riding upon a 5 mV depolarizing plateau. The pulse paradigm mimics changes in membrane potential which occurred during spontaneous bursts in the unclamped cell (top trace). Slow tail currents generated by the simulated burst in both normal ASW and 50 mM-TEA ASW are shown in the second trace. The dashed line represents the zero-current level. The difference between the slow currents elicited in normal ASW and 50 mM-TEA ASW is shown on the bottom trace. The open arrowhead denotes the time corresponding to the peak of the d.a.p.; the filled arrowhead denotes the time corresponding to the peak hyperpolarization during the inter-burst interval.

than 0·5 nA at 400 ms following the pulse. In each of three l.u.q.b. cells, the TEA-difference current was less than 0·4 nA by 1 s after the depolarizing pulse. In contrast, the phase III tail current decays over tens of seconds (see Fig. 9). The time course of the TEA-difference current resembles the time course of the after-hyperpolarization following single spikes; the hyperpolarization between an l.u.q.b. spike and the

depolarizing after-potenital (d.a.p.) which follows typically lasts 0·3–1·0 s. The time course of the TEA-difference current is quite different from the time course of the inter-burst hyperpolarization, which lasts for tens of seconds.

It is possible that the decay of $I_{K(Ca)}$ at the end of a burst of spikes is quite different than its decay at the end of a single spike. It has been suggested that $I_{K(Ca)}$ accumulates during a burst of spikes because of intracellular accumulation of Ca^{2+} (Gorman & Thomas, 1978; Gorman *et al.* 1982). Since the Ca^{2+}-activated K^+ conductance is voltage-sensitive, the depolarizing envelope of the burst may accentuate the $I_{K(Ca)}$ activated during the burst. This may be especially true at the time the burst is terminated: the peak of the d.a.p. In order to determine the dynamics of $I_{K(Ca)}$ during bursting activity, we examined the decay of $I_{K(Ca)}$ following a voltage-clamp pulse train which simulated a natural burst of action potentials (Fig. 12). The simulated burst consisted of four spike-like (10 ms) depolarizing pulses to +25 mV, superimposed on a prolonged 5 mV depolarizing plateau, which mimicked the depolarizing envelope of the burst including the d.a.p.

Slow membrane currents produced by the simulated burst obtained both in normal ASW and 50 mM-TEA ASW are shown superimposed, and the difference between these two currents is shown below. The TEA-blocked current is only about 0·1 nA at the time corresponding to the peak of the d.a.p. (800 ms after the last spike), and less than 0·05 nA at the time corresponding to the peak of the post-burst hyperpolarization (4 s after the last spike). In three other l.u.q.b. cells, the TEA-difference current was less than 0·3 nA at the time corresponding to the peak of the d.a.p. in each cell. In two of these cases, the outward current at the time of the d.a.p. was actually greater in 50 mM-TEA ASW than in normal ASW (see Fig. 2). The TEA-blocked current transients which follow each spike-like depolarization decay rapidly after each pulse and therefore do not summate a great deal. Moreover, the current transients do not grow, but decrease with each successive pulse. Our explanation for this behaviour is that Ca^{2+} influx during preceding depolarizing pulses causes a partial inactivation of the Ca^{2+} current during subsequent depolarizing pulses, thereby reducing the $I_{K(Ca)}$ activated by the Ca^{2+} transient of successive pulses (see Eckert & Ewald, 1982).

DISCUSSION

Contrary to the popular view that the inter-burst hyperpolarization of molluscan pace-maker neurones is due to an increase in the Ca^{2+}-dependent K^+ conductance, the evidence presented in this paper indicates that this is not the case, at least in the l.u.q.b. cells. The inter-burst hyperpolarization, as well as the phase III tail current, do not reverse when elicited below E_K, and are not blocked by TEA. A slow outward tail current which underlies the inter-burst hyperpolarization of neurone R15 behaves in a similar manner (Adams & Levitan, 1985). In contrast, membrane tail currents contributing to spike repolarization and fast after-hyperpolarizations have been shown to be Ca^{2+}-dependent and to reverse below E_K in snail neurones (Meech & Standen, 1975), vertebrate sympathetic neurones (Adams, Constanti, Brown & Clark, 1982) and hippocampal pyramidal neurones (Brown & Griffith, 1983). These currents are blocked by a low concentration (< 50 mM) of TEA and decay relatively rapidly (within 500 ms) following depolarizing pulses.

The phase III tail current is, nevertheless, dependent upon the influx and accumulation of intracellular Ca^{2+}. It is blocked by Ca^{2+}-channel antagonists, intracellular EGTA, and large depolarizing pulses which suppress Ca^{2+} entry. Other investigators have noted the Ca^{2+} dependence of slow outward tail currents in molluscan bursting pace-maker neurones (Johnston, 1976, 1980; Thompson, 1976; Smith, 1978; Carnevale & Wachtel, 1980; Lewis, 1984; Adams & Levitan, 1985).

The hypothesis most consistent with the properties of the *outward* phase III tail current is that it is actually generated by Ca^{2+}-dependent inactivation of a steady-state *inward* Ca^{2+} current. The phase III tail current and the inactivation of the steady-state Ca^{2+} current both exhibit the same sensitivity to Ca^{2+} influx during a depolarizing pulse (Fig. 8), and decay with similar time courses after the pulse (Fig. 9). The phase III tail current and the steady-state Ca^{2+} current have a strikingly similar voltage dependence (Fig. 11). Presumably, the phase III tail current is reduced at hyperpolarized potentials because the steady-state activation of the persistent Ca^{2+} current is small at hyperpolarized potentials; thus there is less voltage-dependent inward current available to be inactivated by a rise in intracellular Ca^{2+}.

Working at 13 °C, Deitmer & Eckert (1984) have observed in some l.u.q.b. neurones a slow Ca^{2+}-dependent outward current tail following long depolarizing pulses that is intermediate in duration between our phase I and phase III outward tail currents. Unlike our phase III current, their current reversed at E_K and was somewhat reduced by TEA, although the degree of TEA sensitivity varied among neurones. Both the low temperature and slowed Ca^{2+} diffusion away from the membrane after a long Ca^{2+} influx would prolong $I_{K(Ca)}$, which comprises part of our phase I tail current. Thus their slow $I_{K(Ca)}$ tail currents resemble prolonged phase I currents, and appear quite distinct from the phase III currents which terminate bursts at room temperature.

We found that increasing the external concentration of K^+ has a small, but significant effect on the phase III tail current (see also Johnston, 1980). The phase III amplitude is increased when elicited below -55 mV and decreased when elicited above -55 mV (Fig. 1*B*). The effects of elevated K^+ can be explained if it assumed that a portion of the phase III tail current is contributed by the axon 'stump' which remains after axotomy. It is likely that part of the axon stump is imperfectly space-clamped; thus in elevated K^+, this region depolarizes above the holding potential in the soma. When the soma is held more hyperpolarized than -55 mV, the axon stump is relatively depolarized and may contribute more phase III tail current than it does in normal ASW. When the soma is held more depolarized than -55 mV, the axon stump may become so highly depolarized that there is a large voltage-dependent increase in the steady-state Ca^{2+} influx, resulting in a local increase in the axonal intracellular Ca^{2+} concentration. The Ca^{2+} influx during a standard depolarizing pulse would therefore be depressed because of Ca^{2+}-dependent inactivation of some of the Ca^{2+} current which is normally activated by the pulse. The result would be a decrease in the amplitude of the phase III tail current at depolarized V_h potentials, because it is dependent upon the Ca^{2+} influx during the pulse.

The amplitude of the phase III tail current elicited at depolarized V_h is usually greater in 50 mM-TEA ASW than it is in normal ASW (Fig. 2). The effect of external TEA on the phase III tail current may also be due to contamination with currents originating in the axon stump. In the presence of TEA, subthreshold regenerative voltage responses in this poorly clamped region may be enhanced, similar to the broadening of spikes which occurs in TEA-containing ASW (Hermann & Gorman, 1981). Since longer depolarizing responses lead to a larger Ca^{2+} influx, the long Ca^{2+}-dependent hyperpolarizing currents which follow will be enhanced. Thus, the phase III tail current recorded in the soma will be enlarged. A similar contamination of the phase II tail current by axonal current may distort the correct measurement of its voltage sensitivity (Kramer & Zucker, 1985). Unfortunately, attempts to eliminate the possible contribution of current from the axon stump, by closely axotomizing the l.u.q.b. neurones (i.e. within 100 μm), eliminate both bursting and the slow tail currents, perhaps because the soma becomes loaded with Ca^{2+} due to leakage through the cut end of the axon.

Measurements of membrane conductance

If the phase III tail current and the inter-burst hyperpolarization of bursting neurones are caused by a decrease in the permeability of the membrane to Ca^{2+}, then it may be possible to measure a decreased *instantaneous* membrane conductance during the phase III tail current and during the inter-burst period. The *steady-state* membrane conductance during the inter-burst hyperpolarization has been measured by various investigators (Gainer, 1972; Junge & Stephens, 1973; Barker & Gainer, 1975*a*). In these studies, the membrane conductance was determined by measuring the *steady-state* voltage displacements which are produced by prolonged (i.e. 2 s by Junge & Stephens, 1973) current pulses. Such studies have established that the inter-burst hyperpolarization is accompanied by an increase in the *apparent* membrane conductance, which declines gradually during the inter-burst period. This apparent conductance increase has been instrumental in supporting the notion that the inter-burst hyperpolarization is generated by a slowly declining Ca^{2+}-dependent K^+ conductance. The apparent increase in the inter-burst conductance coincides with an increase in the internal Ca^{2+} concentration (Gorman & Thomas, 1978), and is blocked by the removal of external Ca^{2+} or by the intracellular injection of EGTA (Meech, 1974).

The voltage measured at the end of a long current pulse is sensitive to the voltage-gated conductances which change as a result of the pulse, as well as the instantaneous conductance present at the onset of the pulse. In order to obtain a valid estimate of the instantaneous conductance alone, the voltage displacement caused by a current pulse must be measured before voltage-gated conductances respond to the voltage change. This is not possible in *Aplysia* neurones, because the membrane time constant is very slow (typically 20–100 ms) due to the large membrane capacitance (typically 20 nF). Thus, during a current pulse, the voltage-gated channels (i.e. Ca^{2+} channels) have tens of milliseconds in which to respond before the membrane capacitance is fully charged.

The steady-state conductance of bursting neurones has also been measured under voltage-clamp conditions by observing current displacements produced by constant-amplitude voltage pulses. Smith (1978) measured the conductance during a slow outward tail current which followed a prolonged depolarizing pulse, by superimposing a small (3 mV peak-to-peak) square wave (5 Hz) upon the hold potential. The measured conductance appeared to decrease with a time course similar to that of the slow tail current. This measurement, appropriately, was considered to be the quasi-steady-state conductance, because voltage-gated ionic currents had a considerable time in which to activate (100 ms) during each half-cycle of the square wave.

In order to measure the instantaneous membrane conductance under voltage clamp, the current displacement caused by a voltage pulse must be measured before voltage-gated ionic currents are affected, and after the capacitive current transient has ended. We have maximized our ability to measure the instantaneous conductance by tuning the voltage clamp such that the voltage settles to its final value within 0·3 ms following a step change in voltage, and by signal-averaging the unfiltered output of the voltage-clamp current monitor. Despite our modifications, the capacitive current transient could not be distinguished from rapidly gated ionic currents. In

previous studies performed at 7–14 °C, the Ca^{2+} tail current was found to deactivate at the end of a pulse according to the sum of two exponentially decaying components; the fast component has a time constant of less than 0·5 ms (Smith & Zucker, 1980; Eckert & Ewald, 1983). At temperatures at which bursting pace-maker currents are large (i.e. 21 °C; Barker & Gainer, 1975*b*; Johnston, 1980) the Ca^{2+} current kinetics are even faster. The rapidly decaying ionic tail current blends smoothly with the capacitive current, precluding measurement of the instantaneous conductance. In fact, the ionic tail current can only be distinguished from the capacitive transient by subtraction of currents elicited by voltage pulses of equal amplitudes but opposite polarity (see, e.g. Zucker, 1981; Eckert & Ewald, 1983). Unfortunately, this procedure also subtracts out the current jump caused by the instantaneous conductance.

Why do bursting neurones exhibit an apparent increase in membrane conductance during the inter-burst hyperpolarization and during the slow outward tail current? Slow changes in the voltage-gated persistent inward current could account for the apparent changes in conductance, as illustrated in Fig. 13. In current-clamp mode (Fig. 13*A*), a hyperpolarizing current pulse results in a change in membrane potential (ΔV_m), which provides an indication of the membrane conductance (G_m). If the hyperpolarizing current pulse is applied in the presence of a voltage-sensitive inward current, then the pulse will result in the deactivation of some of the current, causing a regenerative hyperpolarization ($\Delta V_m'$). When measured in the presence of a voltage-sensitive inward current, the membrane conductance (G_m') will be underestimated because of the regenerative hyperpolarization. The underestimation will be largest immediately before a burst, for two reasons. First, the persistent inward current is highly voltage-sensitive, and the membrane potential gradually depolarizes during the inter-burst interval. Thus, the activation of the persistent inward current gradually increases during the inter-burst. Secondly, the persistent inward current is relatively inactivated immediately after a burst, because the burst results in an increase in the intracellular Ca^{2+} concentration, and the persistent inward current is subject to Ca^{2+}-dependent inactivation. Hence the membrane conductance appears to be maximum at the peak of the inter-burst hyperpolarization, and to decline during the inter-burst period.

Likewise, the apparent long-lasting conductance increase which follows depolarizing voltage-clamp pulses can be due to the presence of a voltage-dependent inward current which is partly activated at the hold potential, as illustrated in Fig. 13*B*. A hyperpolarizing voltage-clamp pulse elicits a change in membrane current (ΔI_m) which provides an indication of the membrane conductance (G_m). If there is any steady-state activation of a voltage-sensitive inward current, however, the hyperpolarizing pulse deactivates some of the inward current, leading to an outward-going current. Thus, the total change in current elicited by the pulse ($\Delta I_m'$) is reduced, and the membrane conductance (G_m') is underestimated. The underestimation is smallest immediately following a depolarizing pulse which causes an influx of Ca^{2+}, because the voltage-sensitive inward current is inactivated by intracellular Ca^{2+}. Hence, the membrane conductance appears to be largest immediately following the depolarizing pulse, and decays along a time course which reflects the decrease in intracellular Ca^{2+} concentration near the inside of the membrane.

Junge & Stephens (1973) also reported that when *Aplysia* bursting neurones were hyperpolarized by current injection below −67 mV immediately following a burst, the membrane potential relaxed to more hyperpolarized levels. They took this to represent a reversal of the inter-burst hyperpolarization, which would normally be relaxing to a more depolarized level. However, we believe this reflects the regenerative deactivation of voltage-sensitive Ca^{2+} current, which results in further hyperpolarization. This occurs at that 'threshold' potential at which the Ca^{2+} current is balanced by the outward currents. We note that −67 mV is more positive than the K^+

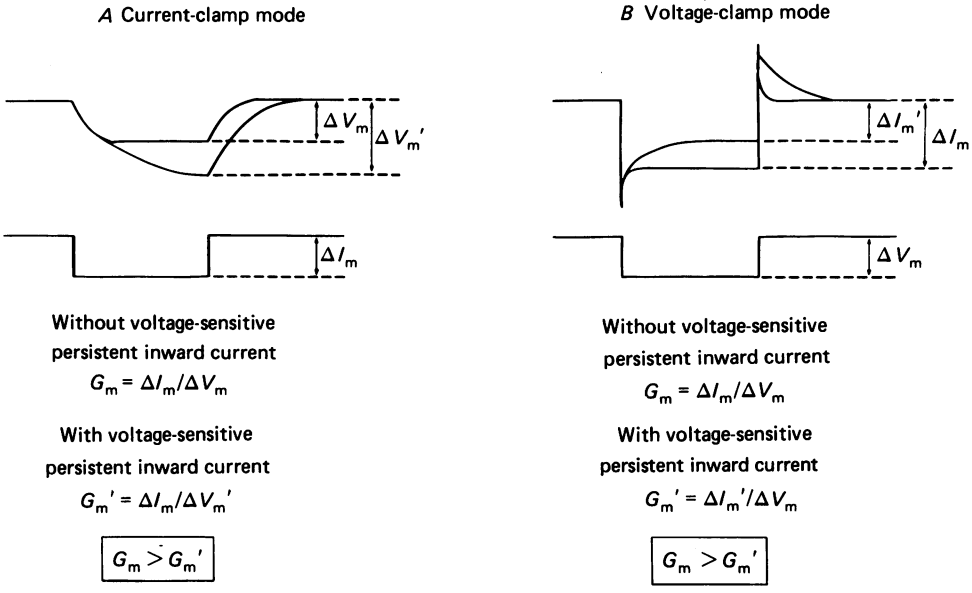

Fig. 13. Diagrammatic representation of conductance measurements with and without the presence of a voltage-sensitive persistent inward current. *A*, conductance measurement in current-clamp mode. *B*, conductance measurement in voltage-clamp mode. In both current-clamp and voltage-clamp modes, the presence of a voltage-sensitive persistent inward current leads to an underestimate of the true membrane conductance. The persistent inward current is depicted as having slow activation and deactivation kinetics, and transient capacitive currents have been included in voltage-clamp mode.

equilibrium potential. Junge & Stephens (1973) also found that this apparent reversal potential shifted in a less-than-Nernstian manner in a positive direction if external K^+ was raised. We propose that the depolarization in high K^+ loads the cells with Ca^{2+}, which inactivates the Ca^{2+} conductance, and so the 'threshold' for regenerative Ca^{2+} entry is more positive.

The evidence presented here suggests that much of the apparent conductance increase which follows bursts of spikes or depolarizing voltage-clamp pulses is due to a Ca^{2+}-dependent decrease in a voltage-sensitive Ca^{2+} conductance, rather than to a Ca^{2+}-dependent increase in a K^+ conductance. The apparent conductance increase, as noted above, is dependent upon the influx and accumulation of intracellular Ca^{2+} (Meech, 1974); the presence of $I_{K(Ca)}$, however, does not seem to

be necessary for its appearance. A long-lasting apparent conductance increase following depolarizing pulses is observed in l.u.q.b. cells, even after the addition of 50 mM-TEA to the bathing medium (Figs. 8 and 9), a concentration effective in blocking all of the $I_{K(Ca)}$ (Hermann & Gorman, 1979).

A qualitative model of bursting pace-maker activity

A diagrammatic representation of the ionic mechanisms which are responsible for the bursting pace-maker cycle is shown in Fig. 14. The format of the diagram is similar to that of a diagram presented by Gorman *et al.* (1981); the outer circle shows changes

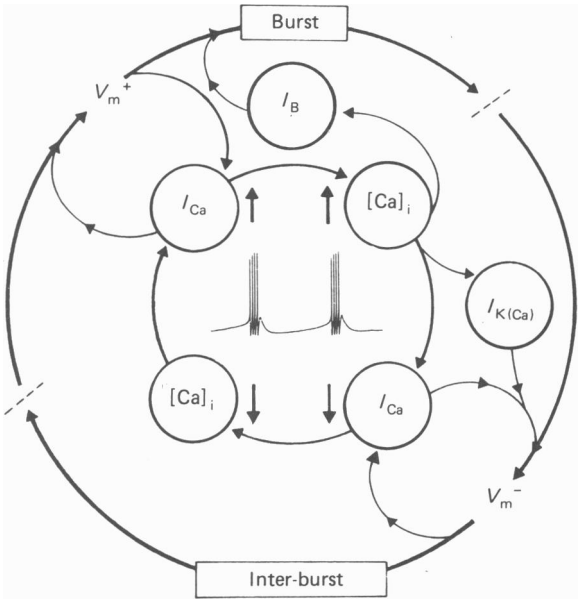

Fig. 14. A qualitative model of the ionic basis of bursting pace-maker activity. The currents represented are: I_{Ca}, voltage-dependent Ca^{2+} current; I_B, Ca^{2+}-activated inward current; and $I_{K(Ca)}$, Ca^{2+}-activated K^+ current. $[Ca]_i$ is the sub-membrane concentration of intracellular Ca^{2+}, and V_m is the membrane potential.

in membrane potential during the pace-maker cycle, and depicted inside are the processes which generate those changes in potential. Ionic currents which do not appear to be essential in generating the pace-maker cycle, such as the voltage-dependent Na^+ current, the delayed rectifier K^+ current, the transient K^+ current (I_A), and the anomalous rectifier K^+ current have not been included in the diagram (for a summary of these currents, see Adams, Smith & Thompson, 1980). The sequence of events during the pace-maker cycle is as follows: a voltage-dependent Ca^{2+} current (I_{Ca}), which exhibits some steady-state activation even at the most hyperpolarized potential within the pace-maker potential range, causes a slow regenerative depolarization. The cell eventually reaches threshold and begins spiking. During each spike there is a large influx of Ca^{2+} through voltage-dependent Ca^{2+} channels. The elevated concentration of intracellular Ca^{2+} ($[Ca]_i$) activates a Ca^{2+}-dependent

non-specific cation current (here denoted I_B). This current further depolarizes the cell, leads to the d.a.p. at the end of the burst, and along with the I_{Ca} current, maintains repetitive spiking during the burst. The elevated $[Ca]_i$ also results in the activation of $I_{K(Ca)}$. In l.u.q.b. neurones, this current decays rapidly after each spike and is mainly involved in repolarization following each spike in the burst. The elevated $[Ca]_i$ also causes the gradual inactivation of the voltage-dependent I_{Ca}. At the end of the burst (the peak of the d.a.p.) the inactivation of I_{Ca} is very large; thus the burst terminates because the total inward current (due to I_B and I_{Ca}) fails to exceed the total outward current (due to a leak through the resting membrane conductance, and to $I_{K(Ca)}$). Since the outward current prevails, the cell hyperpolarizes. I_{Ca} exhibits voltage-dependent deactivation, thus the hyperpolarization is regenerative. Various processes, such as diffusion away from the membrane, intracellular sequestration, and trans-membrane pumping lead to a gradual decrease in $[Ca]_i$ near the membrane. Consequently, the I_{Ca} gradually recovers from Ca^{2+}-dependent inactivation, and slowly begins to activate during the upstroke to the next burst.

The events shown between the two circles of the diagram represent those processes which support, but are not essential for, the oscillation of membrane potential. The $I_{K(Ca)}$ does not appear to be essential, because oscillatory behaviour can be observed even when the $I_{K(Ca)}$ is blocked with TEA (see Fig. 2*C*). The Ca^{2+}-activated inward current (I_B) may not be essential for maintaining the oscillation, because sufficient depolarizing drive may be provided by regenerative activation of the voltage-dependent I_{Ca}. The importance of I_B cannot be ascertained without some treatment which effectively and selectively blocks this current.

The processes occurring in the central circle of the diagram do appear to be essential for normal oscillatory activity. Blockade of I_{Ca} with Ca^{2+}-channel antagonists (Barker & Gainer, 1975*a*), or complete removal of external Ca^{2+} (with external EGTA; Gorman & Hermann, 1982), blocks bursting pace-maker activity. Prevention of intracellular Ca^{2+} accumulation, by injection of EGTA, results in abnormal bursts which 'hang up' in a depolarized plateau (Meech, 1974), presumably because the Ca^{2+}-dependent inactivation of I_{Ca} and the Ca^{2+}-dependent activation of $I_{K(Ca)}$ are both prevented. Treatments which cause a steady-state inactivation of most of the resting I_{Ca}, such as prolonged injection of Ca^{2+}, block bursting activity (R. H. Kramer & R. S. Zucker, unpublished observations). Oscillatory activity is blocked by treatments which interfere with the removal of intracellular Ca^{2+}, such as prolonged exposure to Na^+-free medium (Strumwasser, 1973; Barker & Gainer, 1975*a*; Carnevale & Wachtel, 1980), or reduced temperature (Wilson & Wachtel, 1974; Barker & Gainer 1975*b*).

Three Ca^{2+}-regulated ionic processes in bursting neurones

We have presented evidence that suggests that intracellular Ca^{2+} regulates three independent ionic currents in bursting neurones, as illustrated in Fig. 14, and that the three currents decay with different rates after a depolarizing voltage-clamp pulse which allows Ca^{2+} influx. $I_{K(Ca)}$ appears to decay relatively rapidly (within 1 s) following a brief pulse, and is predominant during the phase I tail current. The Ca^{2+}-activated inward current appears to decay at a slower rate (within 2 s) following a pulse, and is the predominant current during the phase II tail current. The

Ca^{2+}-dependent inactivation of the steady-state Ca^{2+} current decays slowly (up to 30 s) after a pulse, and is the predominant current during the phase III tail current.

Why do the three Ca^{2+}-regulated currents decay with different rates after a depolarizing pulse? We have considered two possible answers to this question: (1) the different ion channel species which are regulated by Ca^{2+} have different Ca^{2+} sensitivities (i.e. different Ca^{2+}-binding constants), and (2) the different Ca^{2+}-regulated channel species have different stoicheiometric requirements for the actions of Ca^{2+}. There are additional possibilities which are more difficult to investigate: for example, the various Ca^{2+}-regulated channel species may be distributed differentially in regions of the membrane which are exposed to different intracellular Ca^{2+} concentration transients during and after a pulse. Localized measurements of both membrane currents and the intracellular Ca^{2+} concentration are necessary for testing this third possibility.

There is suggestive evidence that both differential sensitivity to intracellular Ca^{2+} and different stoicheiometries of the Ca^{2+}-dependent effects are involved in governing the different time courses of the Ca^{2+}-regulated currents. At least two of the currents seem to have different sensitivities to intracellular Ca^{2+}. Smaller intracellular Ca^{2+} injections are required to elicit the early TEA-insensitive outward current than to elicit the non-specific inward current, as shown in Fig. 7*C*. The early, TEA-insensitive outward current seems to represent the Ca^{2+}-dependent inactivation of the steady-state Ca^{2+} current. Therefore, the inactivation of that current may be more sensitive to intracellular Ca^{2+} than is the activation of the non-specific inward current.

In contrast, we were unable to activate the non-specific inward current independently from $I_{K(Ca)}$, even with very small Ca^{2+} injections, despite the observation that the inward current component always preceded the $I_{K(Ca)}$ component. The activation kinetics of the inward current, however, appear to differ from those of $I_{K(Ca)}$ during a Ca^{2+} injection (Kramer & Zucker, 1985). The inward current rises steadily during a Ca^{2+} injection while $I_{K(Ca)}$ accelerates, suggesting that $I_{K(Ca)}$ channels require more Ca^{2+} than the inward current channels do for their activation. Evidence from single channel recordings of $I_{K(Ca)}$ channels from clonal anterior pituitary cells (Wong, Lecar & Adler, 1982), and cultured rat muscle (Barrett, Magleby & Pallotta, 1982) indicates that three Ca^{2+} ions are necessary to activate each $I_{K(Ca)}$ channel. Furthermore, in molluscan neurones, the dose–response curve relating membrane hyperpolarization to the intensity of Ca^{2+} injections displays a third-power relationship if large Ca^{2+} injections are used, suggesting that three Ca^{2+} ions are necessary for the activation of each K^+ channel (Meech, 1980; Meech & Thomas, 1980).

A scenario which accounts for the different time courses of the three Ca^{2+}-regulated currents is envisaged as follows. The Ca^{2+}-dependent inactivation of the persistent inward current has the greatest sensitivity to intracellular Ca^{2+}; therefore, the current which it generates (the phase III tail current) is the longest-lasting effect after a depolarizing pulse. Both the inward current and the $I_{K(Ca)}$ have similar (lower) sensitivities to intracellular Ca^{2+}; however, several Ca^{2+} ions are necessary for the activation of each $I_{K(Ca)}$ channel while only one is necessary for the activation of each inward current channel. Therefore, the inward (phase II) tail current outlasts the $I_{K(Ca)}$ current (predominant during the phase I tail current) after a depolarizing pulse.

If only the $I_{K(Ca)}$ is activated non-linearly by intracellular Ca^{2+}, any treatment which causes a steady-state increase in the cytoplasmic Ca^{2+} concentration will tend to enhance the $I_{K(Ca)}$ component with respect to the other Ca^{2+}-dependent components of the tail current series. The net amplitude of the $I_{K(Ca)}$ tail current may actually decrease, however, because the elevated *resting* Ca^{2+} concentration inactivates some of the Ca^{2+} current during the pulse, thus reducing the Ca^{2+} *transient* which elicits the $I_{K(Ca)}$ tail current (see Eckert & Ewald, 1982). Similarly, an elevated resting concentration of Ca^{2+} should cause a reduction of the phase II tail current due to Ca^{2+}-activated non-specific cation channels. Finally, an elevated intracellular Ca^{2+} concentration causes an increase in the steady-state inactivation of the persistent Ca^{2+} current and a decrease in the amount of persistent inward current available to be inactivated by the additional influx of Ca^{2+}. Consequently, there is a reduction of the phase III tail current.

There are a number of treatments which suppress bursting in bursting cells and reduce the phase II and phase III tail currents. We propose that some of these effects are due to the intracellular accumulation of Ca^{2+}. At reduced temperatures (i.e. < 15 °C), bursting activity is suppressed (Wilson & Wachtel, 1974; Barker & Gainer, 1975*b*), and the phase II and phase III tail currents are suppressed (Lewis, 1984). Removal of Ca^{2+} from molluscan neurones is temperature sensitive (Barish & Thompson, 1983), and thus cooling is likely to cause Ca^{2+} accumulation. Prolonged removal of external Na^+ results in the cessation of bursting activity (Strumwasser, 1973; Barker & Gainer, 1975*a*; Carnevale & Wachtel, 1980). Ca^{2+} efflux from molluscan neurones is sensitive to the external Na^+ concentration (Blaustein & Hodgkin, 1969; Satin, 1984). Both reduced temperature (Wilson & Wachtel, 1974) and the removal of external Na^+ (Smith *et al.* 1975; Carnevale & Wachtel, 1980; Lewis, 1984) cause the loss of the negative resistance in the pace-maker potential region of the steady-state I–V curve. Intracellular injection of EGTA, however, prevents the loss of the negative resistance (Gorman *et al.* 1982), suggesting that a build-up of intracellular Ca^{2+} mediates the effects of reduced temperature and Na^+ removal on bursting activity.

The pivotal roles of I_{Ca} and intracellular calcium suggest that they may be common targets for modulation by the variety of neurotransmitters and other substances which can alter the firing pattern of bursting pace-maker neurones (for reviews see Kupfermann, 1979; Levitan, Harmar & Adams, 1979).

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