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A VOLTAGE-SENSITIVE PERSISTENT CALCIUM CONDUCTANCE IN NEURONAL SOMATA OF *HELIX*

BY R. ECKERT* AND H. D. LUX

From the Neurophysiology Laboratory, Max Planck Institute for Psychiatry, 8 Munich 40, Federal Republic of Germany

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SUMMARY

1. An intracellular voltage clamp in conjunction with a patch pipette utilizing feed-back to monitor local current from the soma membrane were used to analyse transient and stationary currents in bursting pacemaker neurones in *Helix pomatia* and *H. levantina*.

2. A weak, net inward current flows during small ($\leqslant$ 20 mV) depolarizations. This current exhibits slow activation kinetics, persistence during prolonged depolarization, and slow turning off at end of depolarization. Consequently, the steady-state current–voltage curve exhibits a region of negative resistance from about -55 to -35 mV.

3. The slow inward current and the negative resistance characteristic are rapidly and completely abolished by substitution of Co^{2+} or La^{3+} for Ca^{2+} and are partially blocked by the Ca-blocking drug D-600. Substitution of Tris or glucose for Na^+ significantly reduces the inward current only after 15–20 min exposure, recovery being equally slow.

4. The inward current and the negative resistance characteristic of the I–V curve are greatly enhanced by Ba^{2+} substitution for Ca^{2+}. This is ascribed in part to Ba^{2+} carrying current through the slow inward current channels and in part to a suppression of the late K^+ current by Ba^{2+}.

5. The inward current is also present in many non-bursting neurones but fails to appear as a net inward current due to short circuiting by a leakage current or by the delayed potassium current. In these cells the slow inward current contributes to inward going rectification. Replacement of Ca^{2+} with Ba^{2+} enhances the current so as to produce a net inward current during small depolarizations in these neurones.

6. It is concluded that the slow inward current is carried primarily by

* On leave of absence from the Department of Biology, University of California, Los Angeles, and Senior Awardee of the Alexander von Humboldt Foundation, Bonn-Bad Godesberg, Federal Republic of Germany.

Ca^{2+} in the soma membrane of bursting pace-maker neurones and a number of non-bursting cells examined in the parietal ganglion of *Helix*.

7. The sensitivity to small depolarizations and persistence during prolonged depolarization suggests two roles for the Ca system in the generation of slow pace-maker oscillations. In this model the Ca system contributes to the slow depolarization which constitutes the onset of the pace-maker wave, and also contributes to the increment in $[Ca]_{in}$ which activates the Ca-sensitive K^+ conductance responsible for repolarization. The inhibition of spontaneous bursting by Ca-blocking agents supports this model.

INTRODUCTION

A weak persistent inward current with slow activation and inactivation kinetics was recently reported in bursting pace-maker neurones in the snail *Helix pomatia*. This inward current was first inferred from the observation that the net charge efflux during the second of two depolarizing voltage-clamp pulses is proportionally smaller than the measured increment in the concomitant extracellular K activity monitored at the soma surface. The charge carried by the K efflux appears to be partially short-circuited by a slowly developing component of inward membrane current, with the result that the net outward current can be smaller than the partial current carried by K^+ (Lux & Eckert, 1974). In subsequent experiments a late (i.e. steady-state) net inward current was recorded directly, and the late current–voltage curve was found to exhibit a negative resistance slope in the voltage range of the pace-maker oscillations (Eckert & Lux, 1975). A similar negative resistance slope has recently been reported in pace-maker neurones of *Aplysia* by Wilson & Wachtel (1974) and Gola (1974*b*) and by another group in *Aplysia* and *Otala* pace-maker neurones (Smith, Barker & Gainer, 1975).

We present evidence here that Ca^{2+} carries most of the inward current responsible for the negative resistance characteristic of the stationary I–V relations seen in the soma membranes of snail pace-maker neurones. The results also indicate that this slow Ca system contributes to the inward going rectification exhibited by many non-bursting neurones in these snails by producing an increase in slope resistance between -50 and -25 mV of the stationary I–V curve. We propose that the persistent inward Ca current (or $I_{in\ slow}$) serves two major functions in the generation of pace-maker waves in molluscan bursting pace-maker neurones: (1) it first produces a regenerative depolarization which contributes to the positive-going component of the pace-maker wave; and (2) it subsequently activates a Ca-sensitive component of the K conductance, possibly by

producing a rise in $[Ca]_{in}$ (Meech & Strumwasser, 1970; Meech, 1974*a*, *b*). A rise in the potassium conductance has already been shown to cause the repolarization phase of pace-maker wave (Junge & Stevens, 1973).

METHODS

Experiments were performed in late spring and summer on cell A (Kerkut & Meech, 1966) of the right parietal ganglion of *Helix pomatia*. During the winter months, some experiments were carried out on the homologous cell in *H. levantina*. Specimens were fed lettuce, carrots, and bone meal and were kept on a 24 hr cycle of lights 'on' at midnight and 'off' at 16.00 h. Experimental saline solutions listed in Table 1 were based on Gainer's (1972) snail saline. HEPES (*N*-2-hydroxyethylpiperizine-2-ethane sulphonic acid, Servo, Inc., Heidelberg) buffer was substituted for Tris except as noted. Experiments were performed with the preparation at $21 \pm 1°$ C.

TABLE 1. Compositions of control and experimental salines in m-mole/l.

	NaCl	KCl	$CaCl_2$	$MgCl_2$	Glucose	Hepes	
Control, Hepes	80	4	10	5	10	5	—
Control, Tris	80	4	10	5	10	—	5 Tris
Na-free Tris	—	4	10	5	10	—	85 Tris
Na-free glucose	—	4	10	5	160	—	5 Tris
Mg-EGTA	55	4	10	30	10	5	25 EGTA
Ca-free Ba	80	4	—	5	10	5	10 $BaCl_2$
Ca-free Co	60	4	—	5	10	5	30 $CoCl_2$
Ca-free Mg	65	4	—	30	10	5	—
Ca-free La	80	4	—	5	10	14	5 $LaCl_2$

Some cells were prepared for recording by 10 sec exposure of the intimate connective tissue sheath to high local concentrations of the proteolytic enzyme pronase. The enzyme was quickly washed away with liberal quantities of saline, and the connective tissue sheath was removed with fine glass needles and forceps. The use of the enzyme facilitated exposure of the cells and insertion of the electrodes. Since pronase perfused through squid axons is known to interfere with Na inactivation (Rojas & Armstrong, 1971) some experiments (Figs. 4, 6 and 7) were performed on cells prepared without the enzyme. The use of pronase produced no evident difference in membrane behaviour. Membrane potentials were recorded with 3 M-KCl-filled glass pipettes with resistances of 2–20 MΩ. Current was passed through similar electrodes with maximum resistances of 10 MΩ, shielded to within 0·2 mm of their tips with lacquer-insulated silver paint. The voltage-clamp circuit had a time resolution better than 100 μsec and was similar to that previously described (Neher & Lux, 1969; Lux, 1975). The reference electrode was a 0·1 M-KCl agar bridge in contact with an Ag-AgCl pellet separated from the bath ground and near the current-measuring electrode. Clamp currents from a limited patch of soma membrane were measured by applying a conventional clamp using intracellular current and voltage micro-electrodes and additionally placing a current-measuring pipette of 50–120 μm diameter, filled with Ringer, directly on the exposed soma. The pipette interior was held close to the potential of the reference ground by feed-back control. Through adjustment of the feed-back amplification of the pipette clamp, usually by a factor of 20–50, the effective internal resistance of the pipette was reduced to an

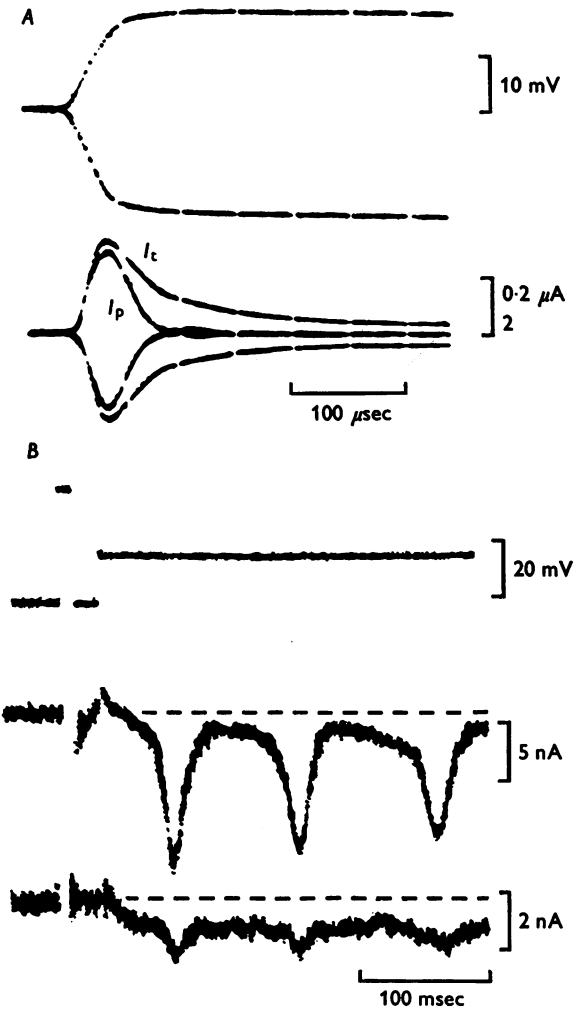

Fig. 1. *A*, capacitative currents at onset of positive and negative voltage steps of 18 mV which are simultaneously recorded within the ground loop of the bath (I_t) and by the patch pipette (I_p). The small overswing terminating the capacitative current I_p is not a property of the patch clamp. It stems from the phase shift which occurs by compensating for the input capacitance of the voltage recording micro-electrode. *B*, comparison of overall current recorded from bath (middle trace) with local current recorded from soma surface with patch electrode (lower trace). Upper trace shows potentials (brief prepulse and long test pulse) impressed on the soma membrane by the intracellular voltage clamp. Total current recorded at bath ground is greatly distorted by axon spike artifacts which are reduced by about 90% relative to the slow current in the patch pipette recording of the lower trace. Rationale for the brief prepulse in voltage trace is given in the legend to Fig. 2.

appropriate value at which the currents entering or passing the rim of the pipette became zero. Small radial dislocations ($< 20\ \mu$m) from the cell surface did not appreciably alter the current signal. Thus, during a voltage pulse to the cell interior, currents which crossed the patch of membrane covered by the pipette were measured in the lumen of the pipette and local current densities were estimated. Total membrane current was simultaneously recorded directly from the bath with an operational amplifier which held the bath at virtual ground. The current produced by the entire cell (and simultaneously measured at the virtual ground) was usually greatly disturbed by spike artifacts which obviously arose from unclamped regions of the axon. Only in the envelope of these transient changes was the current similar to that recorded in the patch pipette (Fig. 1).

In three experiments the capacitative current during a rectangular command step was investigated. It had a peak at about 25 μsec and declined somewhat slower than it rose (see Fig. 1*A*). The decay of the total current leaving the bath was considerably slower than that of the pipette, and showed in addition a tail which lasted some milliseconds. The declining slope of the pipette current was found to be determined by a time constant of 20–25 μsec by applying the method of Hodgkin, Huxley & Katz (1952). It was assumed that the time-consuming charging resulted from the presence of a resistance, r_s, in series with the membrane capacitance. These and r_s were determined from the charge displacement and from the time constant of decay. The capacitance under a patch pipette of 95 μm diameter was found to be 0·6–0·8 pF, so that r_s was estimated to be about 25–40 kΩ. For a soma of radius 120 μm an over-all series resistance of 6–10 kΩ obtains. A correction for the voltage drop across this resistance was not made. It was insignificant ($< 0{\cdot}2$ mV) for the small inward currents and produces at most an error of 4 mV for the largest recorded outward currents of about 100 nA.

Leakage currents were measured 0·5–1 msec after the onset of command steps, after the capacitative current of the pipette had ceased.

The stimulus programme was repeated at 20 or 30 sec intervals to provide for complete recovery from inactivation of membrane currents. Holding potentials were set between -45 and -50 mV as noted, which approximates the resting potential during quiescent periods and lies near or slightly positive to the average voltage during the hyperpolarized phase of the pace-maker waves during active bursting. While most of the experiments were performed with rectangular current pulses, triangular voltage ramps (Fig. 6*B*) were used in some of the ionic substitution and drug experiments. All signals were stored on magnetic tape for analysis and averaging with a Didac (Intertechnique, Paris) computer.

Both Tris-HCl and glucose were tested as replacements for NaCl in Na^+-substitution experiments. Glucose produced the lesser leakage current and was therefore chosen for use in most of the substitution experiments. During perfusion of the bath chamber, its contents were replaced with time constants between 5 and 7 sec as determined with ion selective (K^+ and Cl^-) electrodes. A 1 % level of the original solution was achieved within 35 sec. During changes of extracellular solution the fluid in the patch-clamp pipette was replaced with the new solution, and the pipette was carefully repositioned on the cell surface.

RESULTS

(1) *General properties of* $I_{\text{in slow}}$

Action currents associated with bursting pace-maker activity were detected in the undisturbed cell with the aid of the patch-pipette electrode.

Voltage and current electrodes were then carefully inserted. Cells in which bursting activity (Fig. 2*A*) persisted after electrode penetration always exhibited a minimal leakage current. This was ascertained from the small, instantaneous current with linear positive voltage dependence, which

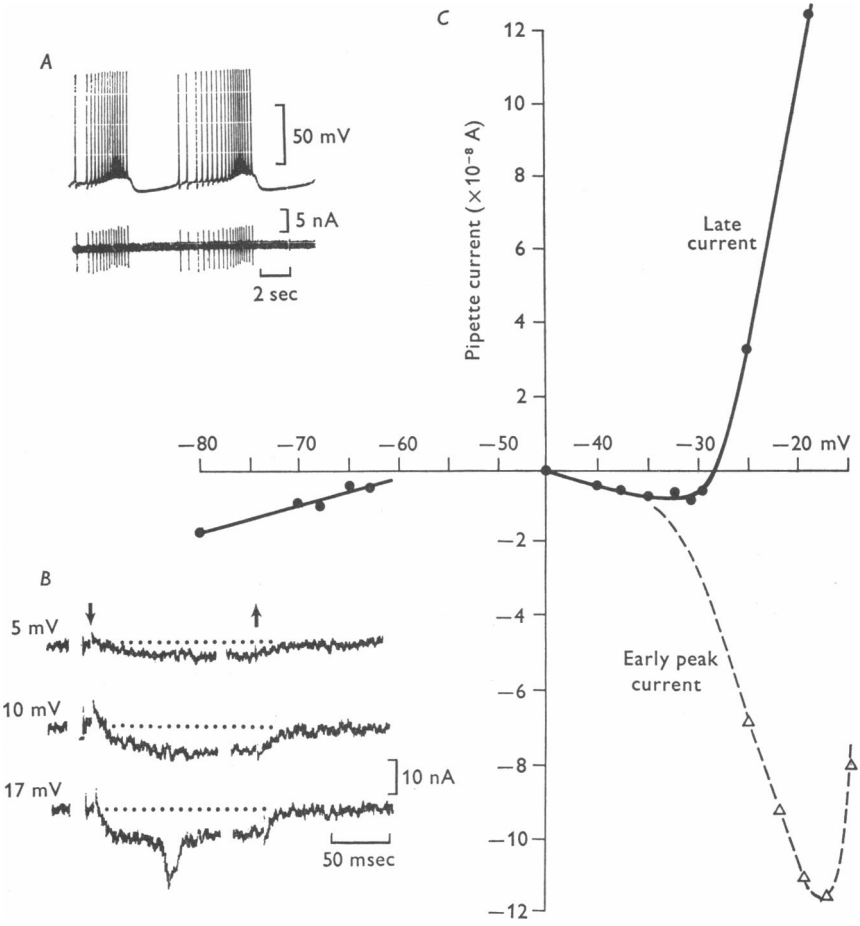

Fig. 2. Steady inward current in a bursting pace-maker cell of *Helix*. *A*, typical trains of impulses riding on pace-maker waves. Lower trace shows current recorded from soma membrane with patch pipette electrode; downward designates inward current. *B*, current recorded with patch electrode during *ca.* 1 sec clamps of depolarizing amplitudes indicated at left; holding potential −45 mV. Arrows show 'on' and 'off' of voltage pulse. Command pulse preceded by 10 msec, 35 mV depolarizing conditioning pulse to inactivate fast early current transient. Trace interrupted for 250 msec during gap. Early current not displayed. *C*, current–voltage curves of maximal late steady and early transient currents. Control Ringer throughout.

appeared during step voltage dislocations. These cells characteristically produced a small maintained net inward current during clamp depolarizations of up to 20 mV, starting from a holding potential of -45 mV to -55 mV (Fig. 2*B*). Cells without bursting pace-maker activity, which were either silent or showed regular spike activity, seldom exhibited the maintained inward current unless calcium was replaced by Ba^{2+} in the Ringer fluid. However, a decrease of the chord conductance in the same voltage region, also termed inward going rectification, was normally observed in these cells. The leakage current in response to a depolarizing pulse was assumed at low pulse potentials to approximate the instantaneous (ohmic) currents produced by hyperpolarizing pulses of the same amplitude (< 5 mV). When the inward ohmic current evoked by the depolarization was added to the time dependent current evoked by the depolarization, an inward current was also evident in cells showing greater leakage. The leakage current increased gradually with time after electrode impalement, and so the best recordings of slow inward current were obtained during the first hour or so after impalement. In the voltage region of -35 to -30 mV, before the appearance of the late outward (K^+) current, the slow inward current reached an amplitude of up to 12% of the maximum early inward current recorded at larger depolarizing pulses (Fig. 3*A*, *B*).

The stationary (i.e. steady-state, late) *I–V* curve shows a negative resistance slope between holding potential and about -35 mV (Fig. 2*C*). At greater depolarizations the slope becomes positive and then shows a sharp increase with further depolarization. The strong outward current which generates the steep region of the stationary *I–V* curve is produced by a K^+ efflux (Hodgkin & Keynes, 1955; Neher & Lux, 1973). The strong activation of the K^+ system above -30 m*V* obscures the current–voltage relations of the slow inward current produced in response to large depolarizations. Inhibitor of the inward current (e.g. Co^{2+} and D-600) partially suppress the late outward current as well, preventing their use to determine the kinetics and amplitude of the unidirectional outward current. Alternatively, tetraethylammonium (TEA) only partially blocks the outward current (85 and 50% suppression respectively for injected and externally applied TEA). Thus the inward current at positive voltage steps of more than 20 mV remained obscured by the late outward current even at high concentrations of TEA (see also Neher & Lux, 1972).

The small net inward current evoked by low positive voltage steps ($\leqslant 20$ mV) could be due to either a correspondingly small unidirectional ion flux through the slow system or to the masking of a much larger inward current by a nearly equal outward K^+ current simultaneously activated at low voltages. To test the latter possibility a K^+-sensitive

electrode (Lux, 1975) was used to monitor increments of K^+ activity at the soma surface during depolarizing pulses of up to 20 mV. No significant increments in K^+ activity were seen, although K^+ effluxes corresponding to a pipette current of 5 nA are resolved by this technique

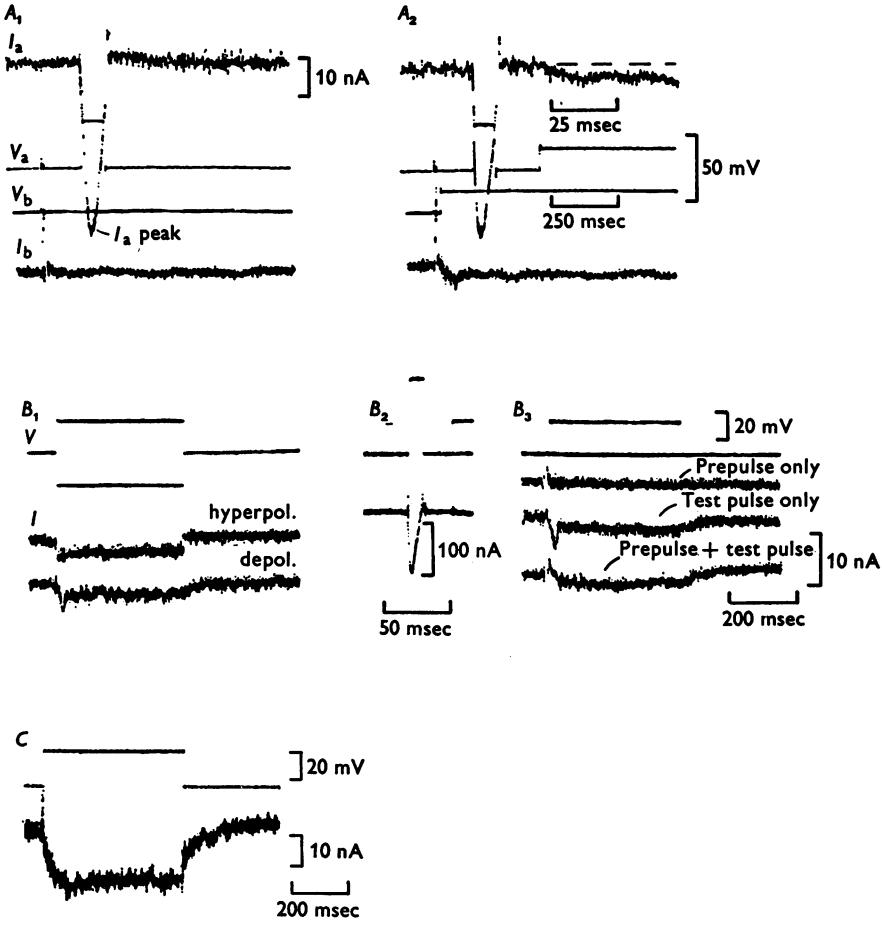

Fig. 3. Early phase of the steady inward current and voltage program. A_1, brief conditioning pulse activates fast current. A_2, steady inward current evoked by prolonged +20 mV step follows fast current. I_a and V_a at 10 × faster sweep than I_b and V_b. Fast transient current not registered on trace I_b. B_1, hyperpolarizing and depolarizing 20 mV pulses. Hyperpolarization shows leakage current. B_2, conditioning prepulse to activate fast inward current. B_3, three sweeps as labelled. Prepulse eliminates axon artifact. Time base × 10 slower and current gain × 10 higher than in B_2. Holding potentials −50 mV throughout. A and B, normal Ringer. C, axon artifact was sometimes absent in Ba (see Fig. 9); Ringer contained 5 mM-Ca^{2+} and 5 mM-Ba^{2+}. Temp. 20–21° C.

(Lux & Eckert, 1974). Furthermore, injected or extracellular TEA enhanced the net inward current at small potentials only slightly. These results suggest that the net inward current recorded at potentials more negative than -35 mV is, largely, representative of the unidirectional flux of the ion species carrying the inward current. This conclusion is consistent with instantaneous current measurements, during the development of the slow inward current. No appreciable increase in instantaneous leakage conductance was obvious in the voltage range of -73 to -30 mV.

Since $I_{\text{in slow}}$ does not inactivate completely as a function of time (Figs. 3, 10*A*; Fig. 3 in Eckert & Lux, 1975) the question arose whether it might be steadily activated at the resting potential in quiescent cells or near the hyperpolarized extremes of the pace-maker potentials. This was examined by step clamping from various holding potentials (Fig. 4).

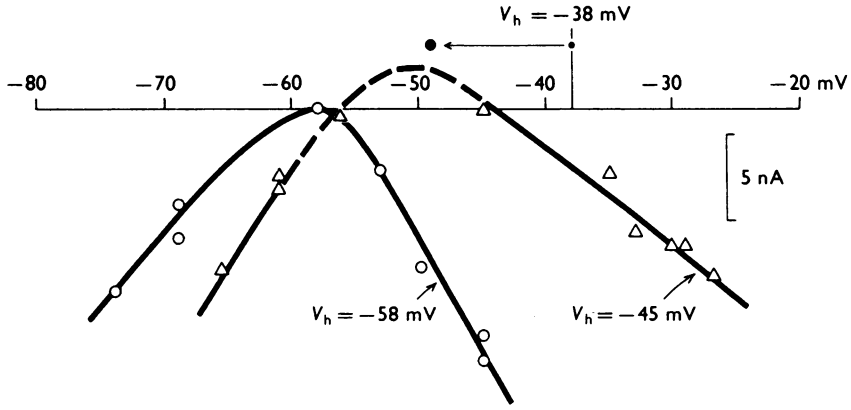

Fig. 4. Influence of holding potential on late currents. Three holding potentials (V_h): -58, -45, and -38 mV. Abscissa, membrane potential; ordinate, current 200 msec after start of command step relative to steady-state current at corresponding holding potential. During hyperpolarization (-70 mV) steady-state and initial leakage current are similar. Dashed line is an interpolated approximation inferred from other experiments (e.g. Fig. 8). Normal Ringer; Temp. 21° C.

Since the maximum inward current in response to each pulse was measured as the displacement from the steady current of the respective holding potential, any steady currents at the holding potential were thereby subtracted. It is evident that activation of the slow inward current can occur at potentials as high as about -55 mV when stepping from a holding potential of -58 mV or above. At lower holding potentials (e.g. -45 mV in Fig. 4) the negative resistance region of the I–V curve shows a shallower slope. This suggests that some inactivation does occur as a function of

time at lower holding potentials. With the holding potential adjusted to still lower values (e.g. −38 mV in Fig. 4) hyperpolarizations in the range of 10 mV produce an upward displacement in the current signal. A similar 'outward current' was reported to occur with hyperpolarization from low holding potentials in R15 of *Aplysia* by Wachtel & Wilson (1973), but was interpreted by those authors as a regenerative outward current with a postulated electrogenic pump as its source. It now appears to represent merely the cessation or reduction of the steady inward current which flows while the membrane is clamped to a low holding potential (see Fig. 3*A* of Eckert & Lux, 1975).

The time course of development of the slow current is normally obscured by the presence of an inward current transient soon after the onset of a low-amplitude depolarizing pulse (Fig. 3*B*). This transient and others that often follow must arise from active spike generation at low threshold which occurs in the unclamped axon when the clamped soma membrane does not develop an early inward current. Use of the patch-clamp pipette for current monitoring greatly reduced (by about 90 %) the amplitude of the invading axon-spike currents (Fig. 1*B*), but the residual axon currents none the less interfered with an analysis of the activation time course of the slow current. The first axon spike was generally delayed significantly by applying a short (7–10 msec) depolarizing conditioning pulse of 30–40 mV amplitude 10 msec before the test pulse (Fig. 3A_1 to B_3). This was just sufficient to inactivate the fast inward current without measurably affecting the delayed K system and to attain the base-line current again. Occasionally, a preparation showed little or no axon spike transients at low stimulus amplitudes. Spike generation was also suppressed to a large extent in solutions in which part or all of the Ca^{2+} was replaced by Ba^{2+} (Fig. 3*C*).

The kinetics of the turning on of the slow current in normal Ringer was examined with the aid of the conditioning prepulse to delay the initial axon spike as explained above. The rising phase (i.e. downward deflexion following onset of the voltage pulse) of $I_{\text{in slow}}$ evoked by small depolarizations is considerably slower than the large transient inward current which appears at higher potentials (Fig. 3). The activation kinetics are strongly voltage dependent (Fig. 5). As the pulse amplitude is increased, the rise time decreases from about 100 msec (80 % full deflexion) during 4 mV pulses to about 20 msec during 18 mV pulses. Because of the low signal level and low signal:noise ratio, the exact time course of the activation of the inward current is not clear in our recordings. The decay of the slow inward current at the termination of the pulse appears to have similar voltage-dependent kinetics (Fig. 5). Some observations suggest a secondary slow phase of the turning on and the decay of $I_{\text{in slow}}$.

(2) *Ionic mechanisms*

$I_{in\ slow}$ must be carried by a species of ion which is in electro-chemical equilibrium at membrane potentials more positive than the potentials which evoke the current. Na and Ca ions are therefore both candidates as current carriers. The results which follow indicate that it is primarily Ca^{2+} which carries the steady inward current in the soma membrane of pace-maker neurones.

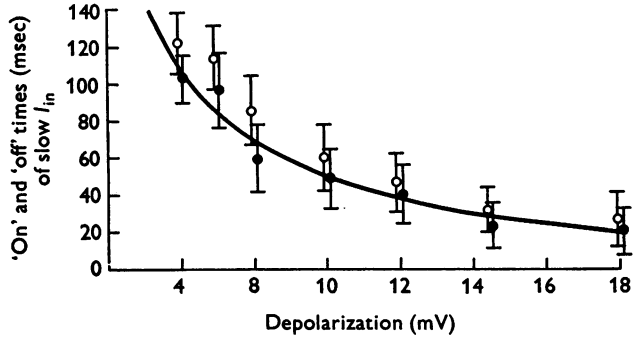

Fig. 5. Turning on and decay times as functions of depolarizing pulse amplitude. Holding potential −48 mV. Prepulse was used to inactivate fast system and delay axon artifacts (see Fig. $2B_3$). At depolarizing potentials above +18 mV the time course of current onset was obscured by axon spike artifacts. Means with S.D. of eight repetitions at each voltage. Normal Ringer. Curve drawn for activation times only. ●, time to turn on to ~ 80% max.; ○, time to turn off to base line.

Application of a freshly prepared Ringer containing tetrodotoxin 30μmole/l. (Sankyo) had no effect on $I_{in\ slow}$; however, this is not sufficient evidence to rule out a slow Na system even though tetrodotoxin is reported to block the Na component of the action potential in molluscan neurones (Geduldig & Junge, 1968). Substitution experiments depended on the appropriate choice of a sodium substitute and careful avoidance of the long-lasting inhibitory potentials which commonly occur in response to solution changes in the snail cells. Choline could not be used as a Na^+ substitute because it produced a massive rise in leakage conductance, due perhaps to a pharmacological action on membrane receptors. Tris produced only a small initial leak, or none. Glucose was also a good substitute and appeared to preserve the cells rather well. The cells continued bursting in Ringer in which the Na^+ was fully replaced with the equivalent amount of glucose (Fig. 6*B*). There was a very gradual slowing of the spike train, prolongation of spikes, and a decrease in amplitude modulation of the pace-maker waves. The gradual deterioration of the burst pattern with time in glucose was equally slowly reversed with introduction of normal

Ringer (Fig. 6A_3). The slow inward current could conveniently be produced by a slowly rising ramp. Its maximum during the voltage ramp was similar to that recorded during tests with rectangular voltages. Little difference is seen between current traces of the same cell during a rising and falling voltage ramp in normal Na and 0-Na Ringer (Fig. 6B). The

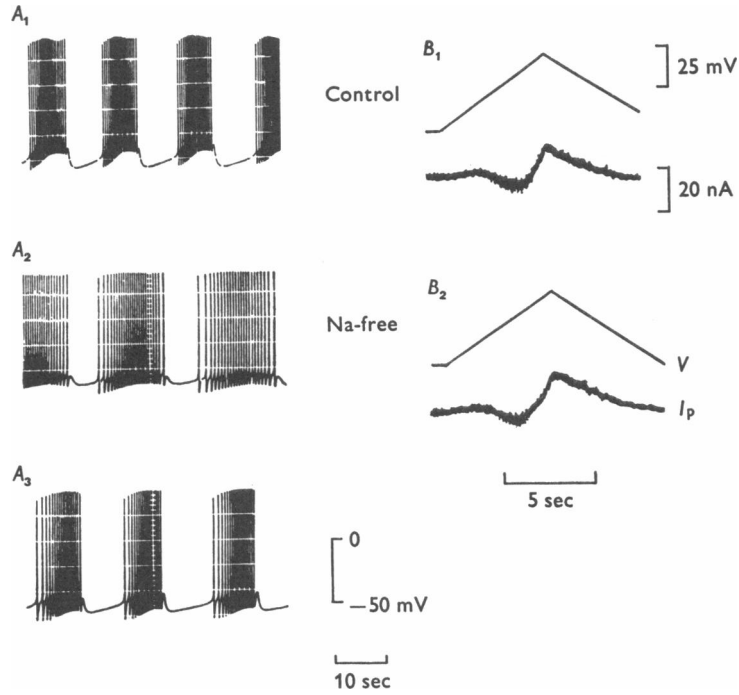

Fig. 6. Complete replacement of sodium chloride with glucose in Ringer. A_1, bursting activity in normal NaCl Ringer. A_2, 10 min after glucose Ringer replaces normal saline. Pace-maker waves shallower, but bursting persists. A_3, return to normal Ringer. B_1, same cell in control Ringer. Upper trace, ascending (depolarizing) and descending voltage-clamp ramp. Lower trace, simultaneous current in patch pipette. B_2, 10 min after NaCl substitution by glucose.

inward currents in the control and 0-Na experiments began when the ramp reached -54 mV and then exhibited a similar slope. When the ramp potential was sufficient to produce an outward current during the rise, the inward current failed to reappear during a subsequent falling limb of the ramp. This has also been noted by Gola (1974*b*), and can be attributed to a persistence of a K conductance. For ramps of lower amplitudes the inward current gradually reappeared on the falling limb.

A plot of inward current intensity *vs.* NaCl concentration with glucose

substitution is shown in Fig. 7. The net inward current was essentially unaffected until 95% or more of the NaCl was replaced by glucose. Even with full replacement (i.e. 1 mM-NaCl) the slow inward current was suppressed by only about 20% after 10 min. Although the cell continues to fire action potentials of nearly normal amplitude, the early inward current becomes considerably slower.

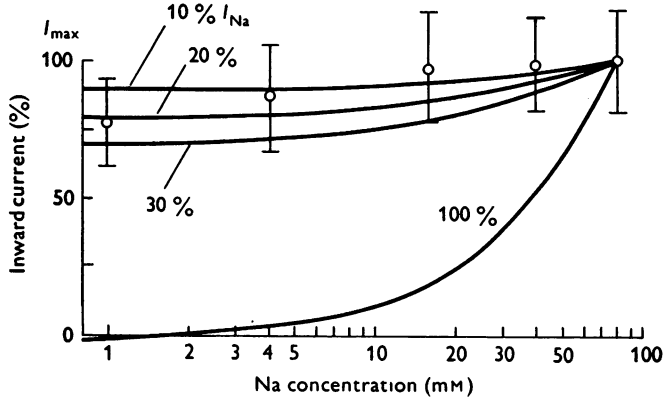

Fig. 7. Strength of steady inward current as a function of the extracellular Na concentration. NaCl replaced with glucose. Means with S.D. of six measurements at each point. 1 mM on abscissa designates 1 m-mole or less (i.e. 0-Na). Holding potential before ramp potential was −68. Values plotted are for maximum late inward currents obtained during depolarizing ramp (see Fig. 6*B*). The upper curves give relations for the inward current assuming three different % values for the proportion carried by Na, for the simple case in which Na and Ca fluxes are independent. Internal [Na^+] taken as 3·6 m-equiv. (Thomas, 1972).

The possible contribution of sodium ions to the slow inward current can be estimated by comparing amplitudes of the current at a given membrane potential when the cell is exposed to solutions with different external sodium concentrations. By employing the principles that the inward and outward fluxes M_1 and M_2 of an ion species are independent of those of other ions and only proportional to the individual ionic concentrations at each site of the membrane, Hodgkin & Huxley (1952) derived the relation between Na currents I_{Na} in normal ($[Na]_o$) and currents I'_{Na} in low ($[Na]'_o$)

$$\frac{I'_{Na}}{I_{Na}} = \frac{M'_{Na_1} - M'_{Na_2}}{M_{Na_1} - M_{Na_2}} = \frac{[Na]'_o/[Na]_o - \exp(E\text{-}E_{Na})F/RT}{1 - \exp(E\text{-}E_{Na})F/RT} \qquad (1)$$

in present sign convention (membrane potential referenced outside). E_{Na} is the equilibrium potential for Na in normal saline (+75 mV). This derivation can be extended to a mixed current $I_{in} = I_{Ca} + I_{Na}$ which may change to $I'_{in} = I_{Ca} + I'_{Na}$ in low Na saline. We denote the ratio of net fluxes of Ca and Na ions by

$$k = \frac{M_{Ca_1} - M_{Ca_2}}{M_{Na_1} - M_{Na_2}}$$

and assume the outward flux (M_{Ca_2}) of Ca ions to be negligibly small compared with the inward flux and the flux M_{Na_2} of sodium ions which are internally present at concentrations of about 3·6 m-equiv/l. (Thomas, 1972).

Since $$M_{Ca_1}/M_{Na_1} = k(1 - \exp(E\text{-}E_{Na})\, F/RT)$$

$$\frac{I'_{in}}{I_{in}} = 1 - \frac{1 - [Na]'_o/[Na]_o}{(k+1)(1 - \exp(E\text{-}E_{Na})\, F/RT)}. \qquad (2)$$

If I_{in} would be solely Na dependent, $k = 0$ and (1) results from (2) (see '100 % Na' slope in Fig. 7). $I'_{in}/I_{in} = 1$ for a pure Ca dependence of I_{in}. (2) is used to determine the slopes of I'_{in}/I_{in} in dependence of the external Na concentration for different contributions of I_{Na} to I_{in} in normal saline (10, 20 and 30 % of the total, see Fig. 7).

In order to avoid slowly developing secondary and tertiary effects of low $[Na^+]_o$, the Na-free experiments reported here were performed at relatively short times (i.e. < 20 min) after exchange of solutions. After 15 min in zero Na^+ a small decrease in I_{in} was seen. Since recovery after return to normal Ringer was slow (> 20 min) the time-dependent decline of $I_{in\,slow}$ is interpreted as possibly resulting from a rise in $[Ca]_{in}$ under reduced $[Na]_o$ (Baker *et al.* 1969) which would cause a rise in g_K (Meech, 1974*a*). These time dependencies were not due to poor access of the exchanged solutions to the cell membrane. Ba^{2+} or Co^{2+} substitutions (see below) under similar conditions produced maximal effects within 2–5 min of exchange.

Replacement of Ca^{2+} with Ba^{2+} in the test solution greatly enhanced the net inward slow current (Fig. 9*A*, *B*) and the negative resistance segment of the steady-state current–voltage plot (Fig. 8). The slow inward current grew quickly with time after Ba^{2+} exchange for Ca^{2+} until the size of the net inward current had increased 5–10 times that in the control saline. At the same time, the outward-current limb of the steady-state I–V curve undergoes a positive shift along the voltage axis (Figs. 8, 10*B*). In the unclamped condition the membrane showed progressive instability and a tendency toward action potentials with prolonged plateaus (Fig. 9*C*). All these effects of Ba^{2+} are consistent with enhanced conductance of the

Ca^{2+} (Ba^{2+}) channels and a reduction of the late K^+ conductance of the depolarized membrane (see Discussion).

Co^{2+} and La^{3+} when substituted for Ca^{2+} inhibited spontaneous bursting pace-maker activity, as they also do in R15 of *Aplysia* (Barker & Gainer,

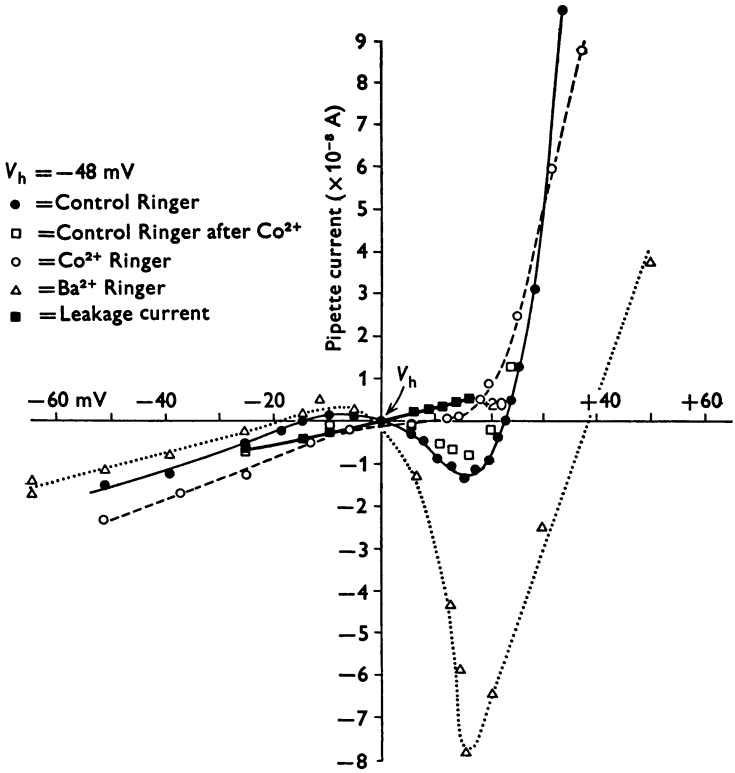

Fig. 8. Steady-state current–voltage relations in normal, Ba^{2+} and Co^{2+} Ringer. Maximum amplitudes of late currents during 500 msec pulses plotted against pulse amplitude from −48 mV holding potential (V_h). See Table 1 for concentrations. Leakage currents (■) plotted from instantaneous (< 3 msec) displacements of current trace in response to corresponding voltage steps at end of experiment utilizing an averaging computer (Lux & Eckert, 1974) to increase signal:noise ratio. All other currents were measured from 200 to 500 msec after beginning of command step. The upward deflexion of plots ● and △ with small hyperpolarization result from reduction of slow inward current (see Fig. 4).

1975*b*). A decrease of the instantaneous currents during hyperpolarizing pulses (leakage currents) was obvious with La^{3+} but was sometimes also observed with Co^{2+} and Ba^{2+}. Depolarization with injected current did not restore pace-maker bursting in these solutions. Mg^{2+} substitution had no pronounced effect, which may have been due to residual Ca^{2+} in the

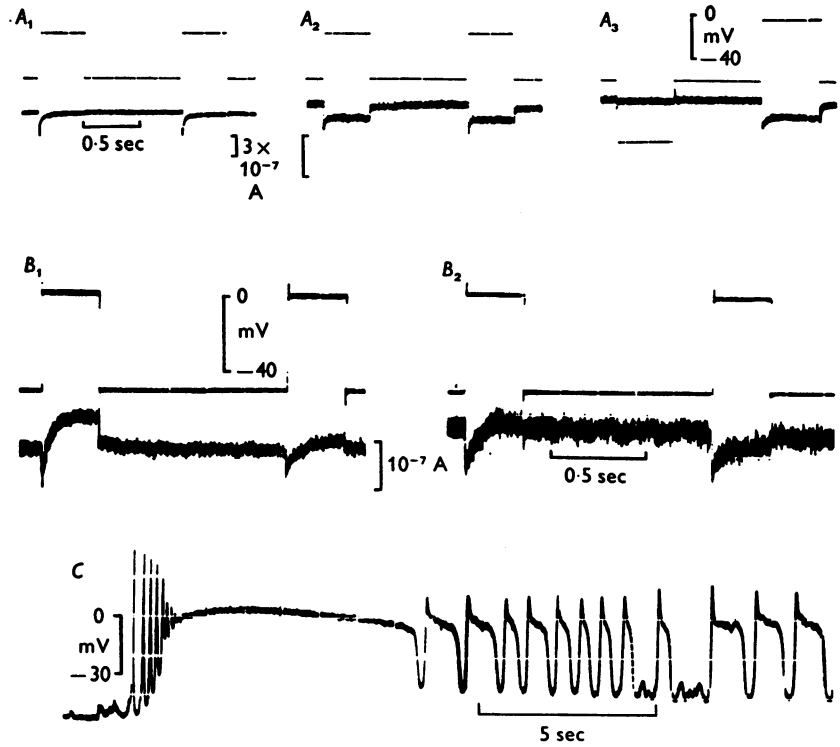

Fig. 9. Effects of Ba^{2+} on currents and potentials. A_1, normal Ringer. Small steady inward current. A_2, A_3, same cell 5 min after Ca^{2+} replaced with 10 mM-Ba^{2+}. B_1, different cell, normal Ringer. B_2, same cell as B_1, 1 min after Ca^{2+} replaced with Ba^{2+}. Note differences in amplitude and time course of currents evoked by first and second stimulus pulses (Lux & Eckert, 1974). The reason for increase in current pipette noise with Ba^{2+} appears to arise at Ag-AgCl electrodes. C, spontaneous potentials 30 sec after completion of Ba^{2+} exchange for Ca^{2+}.

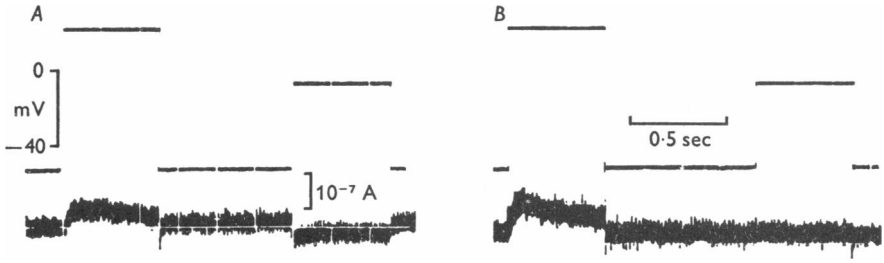

Fig. 10. Effect of Co^{2+} on $I_{in\ slow}$ A, Ba Ringer. Initial large pulse produces net outward current, smaller second pulse produces net inward current. B, 5 min after barium Ringer was replaced with cobalt Ringer (see Table 1). Pulses same as in A, but outward current was increased and inward current was eliminated.

solution. To remove most of the free Ca ions, 25 mM Mg-EGTA was added in place of an equimolar amount of NaCl (Table 1). This produced a large drop in input resistance, slow depolarization with repetitive uninterrupted firing, and failure to burst in response to injected repolarizing current. This is interpreted as the result of non-specific increase in membrane permeability with removal of Ca^{2+}.

The effect of $[Ca^{2+}]_o$ on pace-maker activity shows an optimum at mid-range concentrations, with bursting inhibited at very low and high concentrations (Barker & Gainer, 1973). This may be due to an effect of $[Ca^{2+}]$ in shifting the $I-V$ relations of the membrane (Frankenhaeuser & Hodgkin, 1957), and by an indirect action on a K conductance (Meech, 1974*a*). For this reason $I_{\text{in slow}}$ was not measured as a function of $[Ca^{2+}]_o$ in these experiments. Instead, Ca^{2+} or Ba^{2+} was replaced with Co^{2+}. This quickly and reversibly abolished the slow inward current (Fig. 10) and eliminated the negative-slope component of the steady-state $I-V$ curve (Fig. 8). The blocking effect of Ca currents by Co ions is well documented (Hagiwara & Takahashi, 1967; Geduldig & Junge, 1968; Hagiwara, 1973; Baker, Meves & Ridgway, 1973*a*). Since the leakage conductance of the cell remained unchanged or became slightly higher upon substitution of Co^{2+} for Ca (Fig. 8) it is evident that the loss of inward current was not due to a non-specific short-circuiting of the inward current by an increased membrane leak.

The drug D-600 (Tritthart, Fleckenstein, Herbst & Grün, 1969) blocks the Ca influx which accompanies depolarization in the squid axon (Baker *et al.* 1973*a*, *b*). In our experiments D-600 (Knoll, Ludwigshafen) applied in normal Ringer at a concentration of 10 m-mole/l. resulted in a decrease (but not complete blockage) of $I_{\text{in slow}}$ and also reduced the early inward current. The late outward current was also reduced somewhat by D-600. All three effects are consistent with partial blocking of an inward Ca^{2+} current.

Cells which did not burst seldom showed a late negative-resistance slope but nevertheless exhibited a reduced positive slope in that part of the voltage region corresponding to the negative slope region seen in bursting pacemaker cells. That is, those cells had $I-V$ curves which exhibited inward going (i.e. 'anomalous') rectification (Kandel & Tauc, 1966) between -50 and -25 mV. Such cells were clamped with slowly (*ca.* 10 mV/sec) rising and falling voltage ramps in order to generate dynamic $I-V$ curves as in Fig. 6*B*. Replacement of all the NaCl with glucose had no appreciable effect on the shape of the $I-V$ plot, but replacement of Ca^{2+} with Co^{2+} (Fig. 11) eliminated the inverted S-shape of the current–voltage curve. This suggests that $I_{\text{in slow}}$ plays a role in the inward going rectification exhibited by these cells.

It has already been reported that cooling of R15 in *Aplysia* converts the steady-state negative slope characteristic to a low-conductance positive slope, whereas warming has the converse effect on some non-bursting cells (Wilson & Wachtel, 1974).

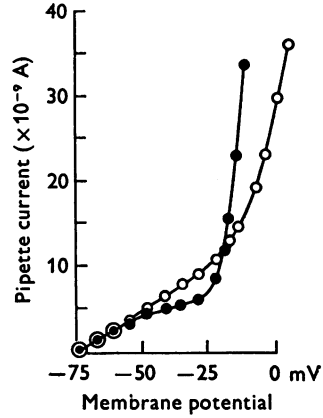

Fig. 11. Inward-going rectification in a non-burster cell. Resting potential was −52 mV. Voltage clamp was applied as a slow (8 mV/sec) depolarizing ramp. Ordinate shows current relative to current at holding potential (−75 mV). ●, Normal Ringer with Tris buffer; ○, Ca-free with 30 mM-$CoCl_2$.

DISCUSSION

The evidence presented here indicates that the persistent inward current recorded from the soma membrane during prolonged depolarizing voltage clamp steps in *Helix* pace-maker neurones is carried primarily by Ca^{2+} ions. The current is largely independent of $[Na]_o$ during the initial 15 min of perfusion, is blocked completely by Co^{2+} and partially by D-600, and is strongly enhanced by Ba^{2+} substituted for Ca^{2+}. Ba^{2+} (and Sr^{2+}) ions are known to replace Ca^{2+} as the current carriers through electrically excited membrane channels in insect and crustacean muscle, and *Paramecium* (Werman & Grundfest, 1961; Werman, McCann & Grundfest, 1961; Naitoh & Eckert, 1968*a*, *b*). In those cases barium acts to increase the input resistance and depress the delayed K^+ current. This may be due to competition with Ca^{2+} at membrane sites which regulate the Ca-sensitive component of K^+ conductance. In addition Ba^{2+} appears to pass through the electrically excited Ca channels more readily than Ca^{2+}, for the increased inward current strength in Ba^{2+} Ringer exceeds the increment which can be attributed to the reduced leakage conductance (see Fig. 8).

In some membranes the current carried by Ca^{2+} is eliminated by replacing Ca^{2+} with Co^{2+} (Hagiwara & Takahashi, 1967; Geduldig & Junge,

1968), which is also true for the slow inward current reported here. These results provide strong evidence that in the soma membrane of the bursting pace-maker neurone most of the $I_{\text{in slow}}$ flows through a Ca^{2+}-selective channel.

The finding of Smith *et al.* (1975) that the amplitude of $I_{\text{in slow}}$ depends on Na concentration in *Aplysia* and *Otala* may have resulted from differences in their method of recording the clamp current. In the present experiments the recorded membrane current arose primarily from a patch of soma membrane, whereas Smith *et al.* recorded total current including that arising from an indeterminate area of axon hillock and axonal membrane. Differences between soma and axon fast inward currents have been reported by Kado (1973) and Junge & Miller (1974).

Whether the 'slow' Ca system described here is in fact separate from the 'fast' Ca system which participates with Na in producing the upstroke of the action potential recorded from the molluscan neurone soma (Geduldig & Junge, 1968; Meves, 1968; Krishtal & Magura, 1970) is not clear. The present data do not rule out the possibility that the soma membrane has only one voltage-dependent Ca conductance which exhibits simple time-exponential voltage-sensitive activation kinetics.

Activation of the delayed K system produces the steep positive limb of the steady-state I–V curve beginning at membrane potentials of about -35 mV and thereby prevents the slow Ca system from producing regenerative depolarizations beyond about -30 mV where the net current is zero (Fig. 2). While $I_{\text{in slow}}$ cannot be recorded directly during stimulus pulses which activate a strong outward K^+ current., indirect evidence points to a slowly developing inward current with repetitive depolarizations and during prolonged depolarizing pulses in molluscan neurons (Gola, 1974*a*; Lux & Eckert, 1974). Brodwick & Junge (1972) demonstrated that the post-tetanic K conductance increases as a function of the duration (up to 10 sec) of the depolarizing pulse, while Meech (1974*b*) has shown that the post-tetanic increase in K^+ conductance results from increased $[Ca^{2+}]_{in}$. The steady Ca current reported here may contribute to the increment in $[Ca^{2+}]_{in}$ responsible for the post-tetanic increase in potassium conductance.

The proposed role of the slow Ca conductance in inward going rectification is envisioned as follows, referring to Fig. 11: between -50 and -25 mV the inward Ca current in control Ringer causes an apparent decrease in conductance since it subtracts from the (i.e. flows against) depolarizing current. With further depolarization the concomitant rise in I_{Ca} enhances the K conductance (Meech & Standen, 1975), producing a steep rise in I_K. Both effects are suppressed in Ca^{2+}-free Co Ringer. Inward-going rectification has been seen in a number of cells, including neurones

(Kandel & Tauc, 1966; Nelson & Frank, 1967), vertebrate muscle (Katz, 1949; Nakajima, Iwasaki & Obata, 1962) and *Paramecium* (Naitoh & Eckert, 1968*a*). In cat spinal motoneurones it is eliminated by extracellular electrophoretic application of Co^{2+} and by administration of D-600 (Liebl & Lux, 1975).

Two features of the slow calcium conductance described here may be significant for regulatory functions: its persistence during a prolonged depolarization, and its activation at relatively high (up to -55 mV) membrane potentials. $I_{\text{in slow}}$ therefore exhibits properties which would be appropriate for a continuous modulation of Ca influx by steady or slowly changing membrane potentials. Ca influx has been implicated in certain metabolic phenomena (i.e. oxidation-reduction of NAD, axoplasmic transport) in nerves (Landowne & Ritchie, 1971; Hammerschlag, Dravid & Chiu, 1975). Other evidence suggests that voltage-sensitive slow calcium conductances exist in squid axon (Baker *et al.* 1973*b*), cardiac tissue (Trautwein, 1973), and *Paramecium* (Machemer & Eckert, 1975).

Several observations suggest that $I_{\text{in slow}}$ plays an important role in the generation of oscillatory waves in bursting pace-maker neurones. Co^{2+} and La^{3+}, which block I_{Ca} in various tissues and $I_{\text{in slow}}$ in pace-maker neurones, also block bursting in these cells. Similarly, those treatments (e.g. prolonged exposure to Na-free Ringer, illumination of R15 in *Aplysia*) which reduce input resistance, presumably by raising $[Ca]_{in}$ and thereby raising g_K, also inhibit pace-maker activity. Conversely, bursting is stimulated by those treatments which increase the net steady inward current; these include increased temperature (Carpenter, 1973; Wilson & Wachtel, 1974; Barker & Gainer, 1975*a*), certain convulsant drugs (Faber & Klee, 1972; David, Wilson & Escueta, 1974) or caffeine (unpublished observations).

We propose that in bursting pace-maker neurones $I_{\text{in slow}}$ contributes directly to the positive-going phase of the pace-maker wave and indirectly initiates the repolarizing phase which terminates the wave. In this model intracellular Ca which accumulates during the protracted flow of I_{Ca} during the depolarizing phase turns on a Ca sensitive K^+ conductance (Meech, 1974*a*), causing I_K to rise until it equals the inward current, at which time the wave peaks. During this period $[Ca^{2+}]_{in}$ and g_K continue to rise until the net current becomes outward and repolarization gets under way. The initial negative-going shift in potential which occurs as I_K exceeds $I_{\text{in slow}}$ presumably facilitates further repolarization by reducing $I_{\text{in slow}}$. The Ca-induced rise in g_K temporarily maintains the post-burst hyperpolarization (Junge & Stevens, 1973; Meech, 1974*a*). The lags inherent in accumulation and dispersal times for Ca near the inner

membrane surface can account, in principle, for the establishment of the oscillatory behaviour of the bursting pace-maker cell.

Dr I. S. Magura (personal communication) also reports a similar slow inward current in the presence of Ba^{2+} in non-bursting neurones of *H. pomatia* which exhibit inward-going rectification.

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