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THE EFFECTS OF CALCIUM ON OUTWARD MEMBRANE CURRENTS IN THE CARDIAC PURKINJE FIBRE

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SUMMARY

1. Properties of outward membrane currents in Purkinje fibres from sheep's hearts have been studied with particular reference to the effects of external Ca.

2. Altering Ca_o is found to shift the potential-dependence of channel activation. The effects are consistent with the idea that Ca ions bind to and neutralize negative charges at the external face of the membrane, but the different magnitudes of the effects of low Ca_o on the pace-maker and plateau currents suggest that the affinities of Ca-binding sites adjacent to each channel type are widely different.

3. Raising Ca_o causes a positive shift in the pace-maker current reversal potential, E_{K_2}, which may reflect a small elevation in the K concentration (K_c) in the restricted cleft space immediately outside the membrane. Other possible causes of the shift in E_{K_2} are also discussed.

4. Raising Ca_o has effects on the plateau and pace-maker current rectifier relations, and on the time-independent membrane current, which resemble those of a small increase in extracellular K concentration.

5. Possible mechanisms for an increase in K_c in elevated Ca_o are discussed. Positive shifts in E_{K_2} can be observed even when the membrane current becomes more inward, so it seems unlikely that the increase in K_c results from an activation of K channels by Ca ions. It is possible that increases in Ca partially inhibit the Na:K exchange pump.

6. The maximum transient outward current elicited by strong depolarizing steps is not affected by moderate reductions in Ca_o.

7. Reducing Na_o depresses the pace-maker current rectifier relation with little shift in the activation curve.

8. We conclude that some of the effects of Ca_o on outward currents are due to shifts in the potential-dependence of channel activation, while others result from a small increase in K_c. No evidence for a direct effect of Ca on K channels has been found in the present study.

INTRODUCTION

In many nerve cells some of the K channels in the plasma membrane seem to be controlled by the concentration of ionized Ca inside the cell (Meech & Strumwasser, 1970; Krnjević & Lisiewicz, 1972; Meech, 1974; Clusin, Spray & Bennett, 1975), while others are controlled by membrane potential (Meech & Standen, 1975) in a way similar to that first described by Hodgkin & Huxley (1952). Ca-dependent

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K channels might also be present in heart muscle since an increase in beat strength – caused for instance by increased stimulation rate, by adrenergic receptor stimulation, or by raised extracellular Ca – is often accompanied by an abbreviation of the action potential (Niedergerke, 1956; Otsuka, 1958; Reiter & Stickel, 1968). An increased K current underlying the action potential abbreviation could, therefore, be controlled by the raised intracellular Ca responsible for the positive inotropic effect. More direct evidence in support of this idea comes from experiments in which Ca was injected intracellularly into cardiac Purkinje fibres, and was found to cause a transient hyperpolarization and abbreviation of the action potential (Isenberg, 1975, 1977*a*).

The complexity of outward current systems in cardiac tissue makes it difficult to decide which might be dependent on membrane potential and which on intracellular Ca, or which arise from a third possible source, namely time-dependent changes in the driving force for K ions caused by changes in the K concentration immediately outside the membrane. There seem to be three distinct time-dependent outward currents in the Purkinje fibre:

(i) A slow K current, i_{K_2}, controls pace-maker activity (McAllister & Noble, 1967). The time and potential dependence of this current can be described in a Hodgkin–Huxley formalism:

$$i_{K_2} = s(V, t).\bar{i}_{K_2}(V), \tag{1}$$

where s is a first-order gating variable whose steady value increases sigmoidally from zero at -90 mV to unity at -60 mV, and where $\bar{i}_{K_2}$ is a time-independent rectifier relation (Noble & Tsien, 1968). $\bar{i}_{K_2}$ exhibits inward rectification, and extracellular K ions have the curious effect of increasing it at more positive potentials (cross-over effect – Noble & Tsien, 1968). Isenberg (1977*c*) has observed an increase in i_{K_2} during Ca injection, suggesting that this current may be Ca_i-dependent.

(ii) Between about -50 and 0 mV a separate plateau outward current with a more positive reversal potential is observed under voltage clamp. This plateau current has a non-exponential time course, but it can be split into a fast and a slow component, i_{x_1} and i_{x_2} respectively, each of which depends in a first-order manner on membrane potential (Noble & Tsien, 1969). Thus:

$$i_{\text{plateau}} = x_1(V, t).\bar{i}_{x_1}(V) + x_2(V, t).\bar{i}_{x_2}(V). \tag{2}$$

Increasing Ca_o depresses i_{x_1} (Kass & Tsien, 1975; see also Fig. 7 of the present paper).

(iii) Voltage-clamp steps positive to about -20 mV elicit a large transient outward current, i_{to} (Düdel, Peper, Rüdel & Trautwein, 1967) which is suppressed by chloride removal, and has consequently been referred to as the Cl current (Vitek & Trautwein, 1971; Fozzard & Hiraoka, 1973). However, Carmeliet & Verdonck (1977) have shown that Cl removal reduces the potassium permeability of the membrane, which raises the possibility that the transient outward current is carried by K (see also Kenyon & Gibbons, 1977). Siegelbaum, Tsien & Kass (1977) have recently shown that contraction and the transient outward current are related, and have proposed that this current is Ca_i-dependent.

In addition to these three time-dependent outward currents there is a time-

independent current, usually called i_{K_1}, which may be carried in part by K. This current is increased in some potential ranges by increases in Ca_o (Bassingthwaighte, Fry & McGuigan, 1976; Kass & Tsien, 1976) or by Ca injection (Isenberg, 1977*b*), so it too could be controlled by Ca_i.

The intention of the present work is to determine which (if any) of the outward currents in the Purkinje fibre are truly dependent on Ca_i, and to explore the reasons for the apparent Ca-dependence of others. Our procedure has been to examine the various current components while the extracellular Ca concentration is altered. This procedure is a less direct means of altering Ca_i than Ca injection, but it has the advantages of being reproducible and easier to carry out. The changes in Ca_i are also not localized to a single injection site. Extracellular Ca itself has effects on voltage-dependent gating mechanisms (Frankenhaeuser & Hodgkin, 1957), so these effects were investigated first, and allowed for in subsequent analysis. The remaining effects could be due either to changes in Ca_o or to concomitant changes in Ca_i, which should be in the same direction as, but not necessarily proportional to, the changes in Ca_o (see Allen & Blinks, 1978). Our general conclusion is that many of the effects of Ca, both extracellular and injected, which were outlined above can be explained by an increase in the K ion concentration immediately outside the membrane, and need not result from an interaction of Ca with K channels. Some of the results have been published in brief (DiFrancesco & McNaughton, 1977).

METHODS

Preparation

Hearts were removed from sheep within 5 min of death, and were transported from the slaughter-house to the laboratory in heparinized Tyrode solution at room temperature. Short lengths of Purkinje fibre (length 1·3–2·3 mm, diameter of the inner core of active tissue 0·2–0·4 mm) were excised from the left ventricle and stored at about 35 °C in continuously oxygenated Tyrode.

Apparatus

The experimental chamber, made from Perspex, was divided into an upper and a lower compartment by a thin perforated Perspex partition. Preparations were held onto this partition by a fine nylon net. Tyrode solution flowed into the upper compartment and out of the lower; thus the fibre was continuously exposed to fresh oxygenated solution. The volume of the upper chamber was 0·2 ml., and the solution flow rate 1·5 ml./min, so that solution changes were complete in less than 10 sec. The bath temperature was monitored continuously by a thermistor in the lower compartment.

Solutions flowed through a heat exchanger, mounted on top of a Cambion Peltier-effect heat pump, just before entering the bath. The heat pump was driven by a feed-back circuit capable of maintaining the solution temperature in the bath constant to within 0·1 °C. It was also possible to alter the solution temperature from one steady value to another within 30 sec. The bath temperature was normally set at about 37 °C.

Micro-electrodes were fabricated from 1·2 mm tubing containing a fused fibre (Clark Electromedical), and were filled by capillarity, usually immediately before use. The current-passing micro-electrode was filled with 2 M-K citrate at pH 7·0, and inserted near the centre of the fibre. The potential-measuring micro-electrode was filled with 3 M-KCl and inserted approximately one fifth of the fibre length away from the current-passing electrode (see below). Electrodes and holders were wrapped in earthed aluminium foil to minimize cross-talk.

The voltage-clamp circuit was conventional. Command pulses were supplied by a Digitimer, and were sometimes slowed to a rise time of about 10 msec to avoid oscillation at the threshold of the Na current. The clamp amplifier had a maximum output of $\pm$ 110 V and a variable gain.

In the range of gains usually employed the rise time of potential recorded from the fibre, in response to a step command pulse, was about 1 msec.

Membrane potential, membrane current and bath temperature were recorded on an SE ultra-violet recorder, and were also monitored during the experiment on a storage oscilloscope. The frequency response of the recording system was flat to 2 kHz, but sometimes (when the resistance of the voltage micro-electrode increased during an experiment) it became necessary to reduce the band width of the current recording channel to eliminate excessive noise and hum. If this was done, filtered and unfiltered records were compared on the oscilloscope to ensure that fast current components were preserved.

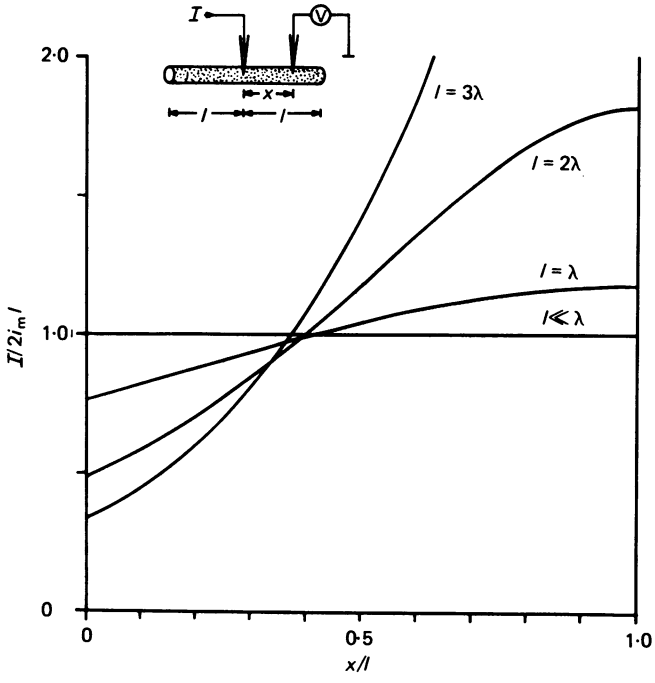

Fig. 1. The effect of position of the potential-recording electrode along the fibre (abscissa) on the ratio between the measured current (I) and the true membrane current ($2i_m l$) of a fibre uniformly clamped at the measured potential. The curves are for fibres of different overall half-length l. The point at which the curves cross the line labelled $l \ll \lambda$ (i.e. a short, well clamped fibre) gives the position at which the potential electrode should be placed to record membrane currents equal to those in a perfectly space-clamped fibre.

Pulse protocols

Successive voltage-clamp pulses were applied with sufficient time between them to allow full return of the preparation to steady state at the holding potential. This return was normally judged by checking that the membrane current had returned to the steady level before the pulse, but in experiments with the transient outward current, which recovers slowly from inactivation, the current during successive pulses was carefully compared with the current in similar single pulses applied after a long period of rest. The actual pulse protocol used is noted in each Figure legend.

Action potentials were not routinely elicited, although the constancy of action potentials was occasionally checked during experiments. Fibres which were depolarized, and consequently did not have action potentials, were sometimes used, and the validity of results from these fibres were compared with similar experiments on normal fibres.

During changes of Ca_o voltage-clamp pulses exceeding the contractile threshold were normally applied, but we do not know if this procedure speeded changes in intracellular Ca concentration.

Solutions

Normal Tyrode contained (mM): NaCl, 137; KCl, 4; $CaCl_2$, 2; $MgCl_2$, 1; $NaHCO_3$, 12; NaH_2PO_4, 0·4; D-glucose, 5. Increasing Ca^{2+}, for instance, involved adding both Ca^{2+} and Cl^-, and allowance for the added Cl^- has been made in calculating expected surface-charge effects (see p. 352). All solutions were held at about 35 °C and bubbled with 95 % O_2: 5 % CO_2 during the experiment. Differences in junction potentials between the various solutions were found to be negligible.

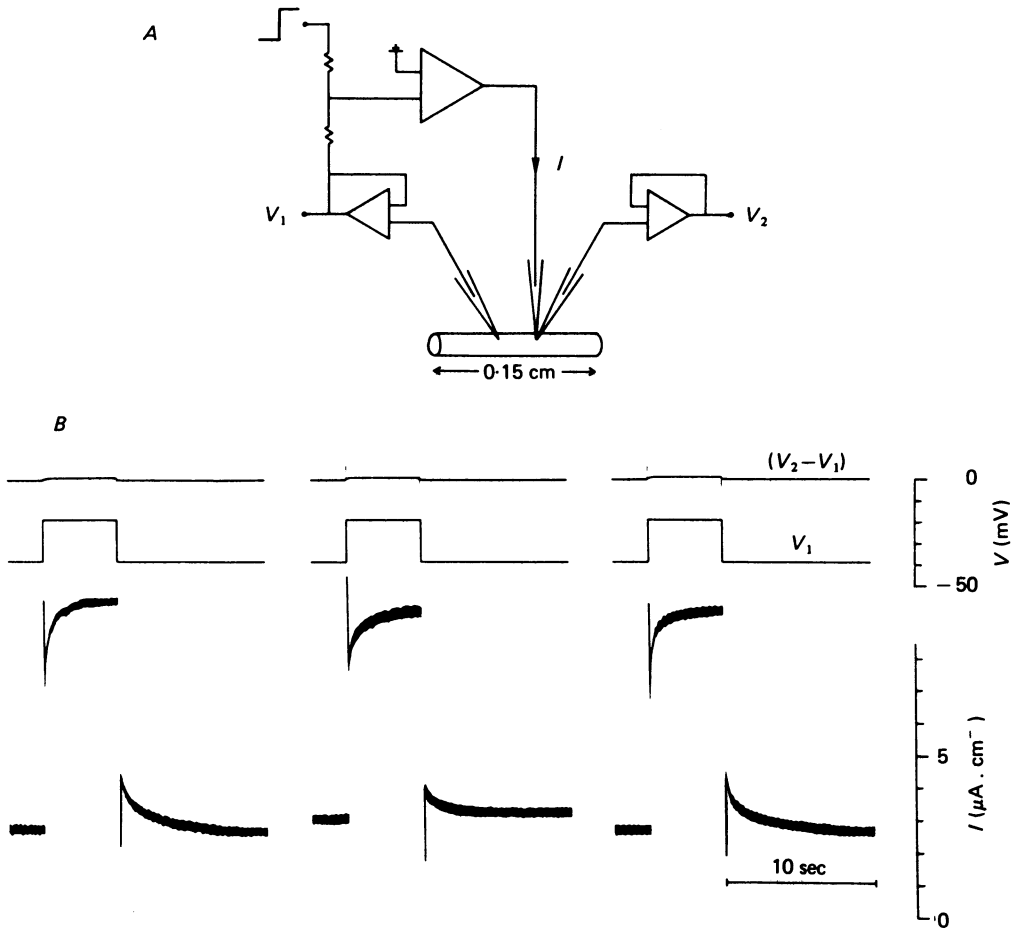

Fig. 2. An experiment to test the degree of potential inhomogeneity along a fibre. *A*, the placement of micro-electrodes. V_1 is the clamped voltage, V_2 tests inhomogeneity. The distance between V_1 and V_2 was 0·4 mm. Temperature 36 °C. Interval between successive pulses (cycle time) was 20 sec. *B*, potential inhomogeneity (upper trace), clamp potential (middle trace) and currents recorded in normal Tyrode, in 0·1 Ca 2·9 Mg Tyrode, and after return to normal Tyrode.

Optimal placement of micro-electrodes along the fibre

When a small current is injected into the centre of a short Purkinje fibre the potential decrement as a function of distance is given by (Weidmann, 1952):

$$V(x) = \frac{Ir_m}{2\lambda} \cdot \frac{\cosh[(|x|-l)/\lambda]}{\sinh(l/\lambda)}, \qquad (3)$$

where V is the deviation of membrane potential from its resting level, I is the injected current, x is distance from the current injection site, l is distance between current injection site and open-circuit termination of the fibre, r_m is membrane resistance of unit length, and λ is the space constant.

Fig. 1 shows the ratio between measured current, I, and the current, $2i_m l$ (where $i_m = V/r_m$ is the membrane current per unit length), which would be needed to maintain a perfectly space-clamped fibre of the same length at the measured potential V, as a function of the position of the potential-measuring electrode along the fibre. Placing the electrode near the current injection site gives an underestimate of true membrane current at the potential V, while near the cut end it is over-estimated. Between these two extremes there is an optimal position at which $I = 2i_m l$. This position is given by x^*, the solution of the equation:

$$\cosh\left(\frac{x^*-l}{\lambda}\right) = \frac{\lambda}{l}\sinh\frac{l}{\lambda}. \tag{4}$$

Fortunately x^* has a value, of close to $0{\cdot}4\,l$, which is practically independent of the degree of potential inhomogeneity (see Fig. 1). Potential decrements in the radial direction may cause additional non-uniformities, but in the absence of any knowledge of the radial space constant we are unable to estimate the extent of this possible source of error.

As a further test of the degree of potential inhomogeneity along the fibre a second potential electrode was inserted close to the current electrode in one experiment (see Fig. 2). The potential difference between the two electrodes did not exceed 2 mV in either normal or low Ca.

Nomenclature

When an activation curve shifts to a more positive potential, it is often said to have undergone a 'depolarizing shift'. This term may be confusing, as the shift is not related to depolarization of the cell membrane. We shall therefore refer to 'positive shifts' (and 'negative shifts') in activation curves, etc.

RESULTS

Surface-charge effects of Ca

The well known 'stabilizing' effect of Ca on an excitable cell has been attributed by Frankenhaeuser & Hodgkin (1957) to an increase in the electric field across the membrane when Ca ions partially neutralize fixed negative charges at the external surface of the membrane. This idea can be put on a quantitative basis by supposing the effects of adding Ca to be twofold:

(i) to increase the ionic strength of the extracellular solution, thus reducing the Debye length and consequently the surface potential (Chandler, Hodgkin & Meves, 1965); (ii) to neutralize surface negative charges by direct binding (Gilbert & Ehrenstein, 1969).

With these assumptions, the surface potential can be determined by fitting the parameters σ and K_m^{Ca} in the following equation (Gilbert & Ehrenstein, 1969) to the experimental results:

$$\frac{\sigma}{(1+[\mathrm{Ca}]_o/K_m^{Ca})} = \frac{1}{G}\left(\Sigma c_i\left[\exp\left(-\frac{z_i F\psi_o}{RT}\right)-1\right]\right)^{\frac{1}{2}}, \tag{5}$$

where K_m^{Ca} = Michaelis constant of binding of Ca to divalent negative sites, c_i = concentration of ionic species i, z_i = charge of ionic species i, σ = the surface charge density in the absence of bound Ca, ψ_o = the surface potential, $G = (2\pi F^2/N^2 RT\epsilon_w)^{\frac{1}{2}}$, ϵ_w = permittivity of water, and R, T, F and N have their usual meanings.

Eqn. (5) involves several assumptions which are not likely to be realized in practice, the major ones being that the surface charge is uniformly smeared over the membrane, and that all the charge is in the form of divalent sites which all have a common affinity for Ca. Nonetheless, more complex models do not seem worth investigating in view of the limited data available.

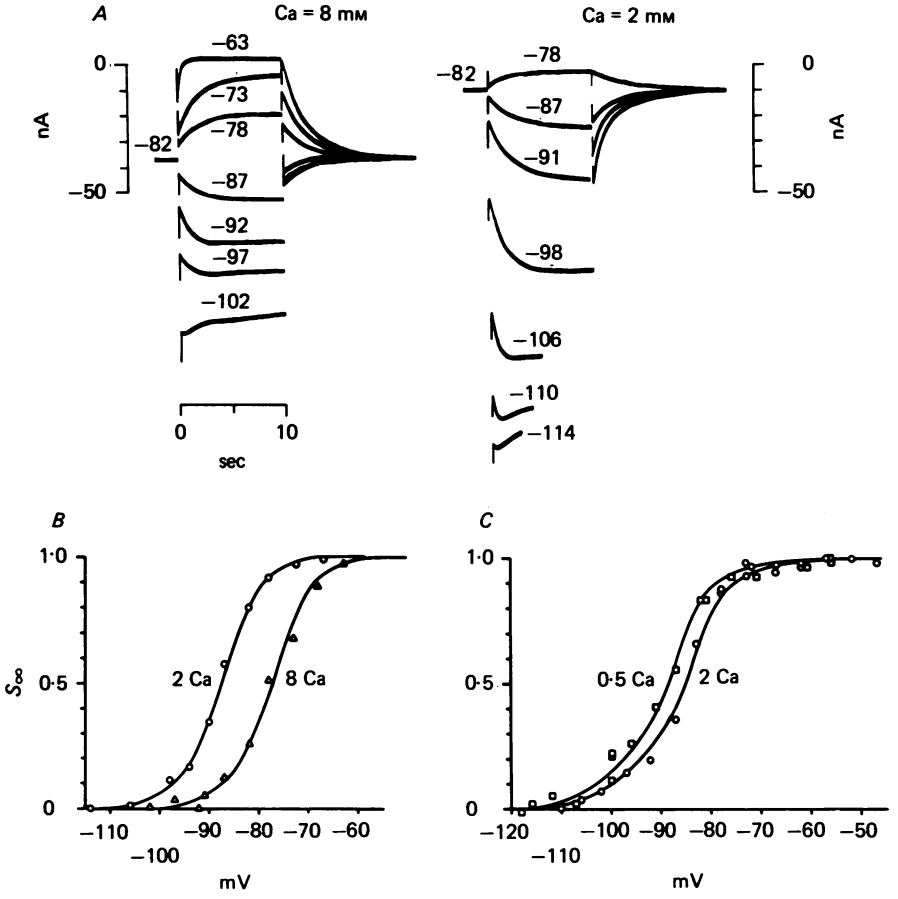

Fig. 3. The effect of raised and lowered Ca_o on the pace-maker current, i_{K_2}. *A*, currents recorded in response to step clamp pulses to various potentials (shown in mV), in 8 mM-Ca_o (left-hand panel) and in 2 mM-Ca_o. The current trace at −114 mV has been shifted up to fit on the chart. Tails on return to the holding potential from the three most negative clamp pulses in 2 mM-Ca_o were similar to the tail from −98 mV. Fibre length 1·35 mm, diameter 0·25 mm (diameters in this and subsequent Figures refer to the inner core of active tissue); temperature 37 °C; cycle time about 25 sec. *B*, normalized s_∞ activation curves in 2 mM-Ca (○) and 8 mM-Ca_o (△), from the experiment shown in *A*. Some pulses from a holding potential of −87 mV have also been included. The points show the dependence on pulse potential of the tail amplitudes recorded on return to the holding potential, normalized by the sum of the maximum positive and negative tail amplitudes. The curves through the points are identical except for a shift of 10 mV, and have been drawn by eye. *C*, normalized s_∞ curves in 0·5 mM-Ca_o (□) and 2 mM-Ca_o (○), from another experiment. The curves through the points are 3·5 mV apart. Holding potential −83 mV; fibre length 1·4 mm, diameter 0·25 mm; temperature 37 °C; cycle time 30–40 sec.

Typical shifts in the s_∞ activation curve when Ca is altered from its normal value of 2 mM are shown in Fig. 3. The shifts took place with a half-time of about 60–90 sec, and were well reversible if the preparation had not substantially deteriorated during

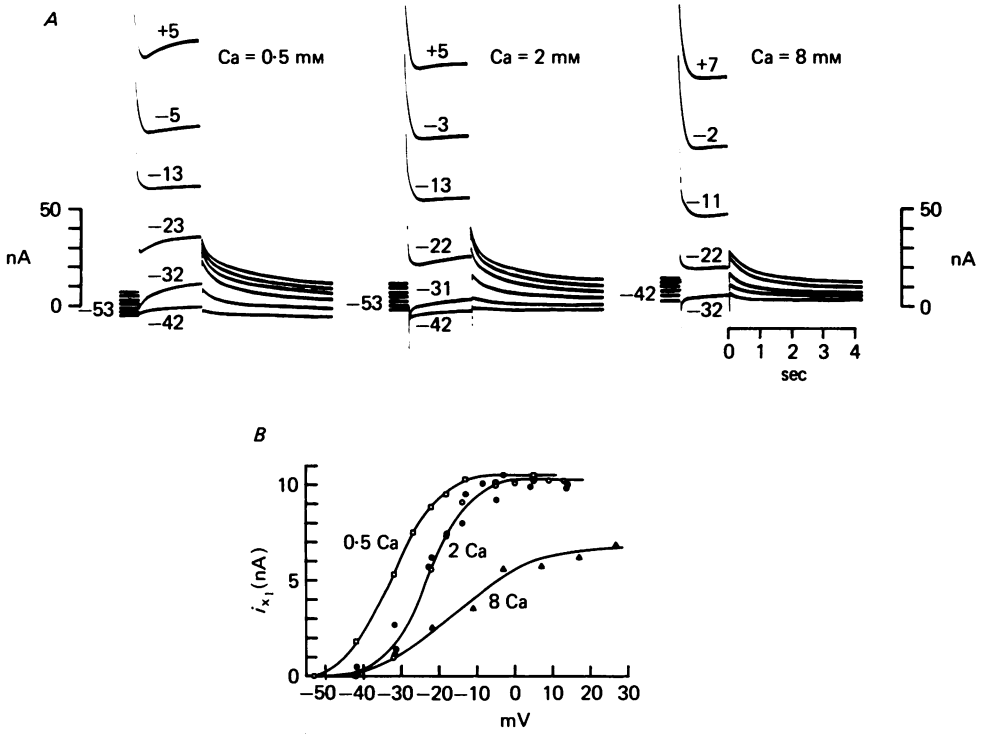

Fig. 4. The effect of raised and lowered Ca_o on the potential-dependence of activation of the fast component, i_{x_1}, of the plateau outward current. *A*, currents recorded in response to step clamp pulses from a holding potential at which i_{x_1} is fully deactivated. Ca concentration 0·5 mM (left-hand panel); 2 mM (centre panel) and 8 mM (right-hand panel). The current recorded during the pulse is a mixture of Ca current (seen as an inward transient at small depolarizations), transient outward current (the large outward 'spike' at stronger depolarizations) and the developing plateau outward current. The pulses were long enough to activate fully the fast component of plateau outward current. Only the plateau outward current contributes significantly to the tail amplitude, as the first two are deactivated very rapidly at the holding potential, and are therefore lost in the capacitative transient (see Fig. 10). The tails can be separated into a fast and a slow exponential component, i_{x_1} and i_{x_2} respectively, by plotting on log paper (cf. Noble & Tsien, 1969). The current zero (ordinate) is correct for the lowest trace of each set; other traces have been shifted up by increments of 2 nA. Same experiment as Fig. 3*A*. Cycle time 8–10 sec. *B*, the dependence of the i_{x_1} tail amplitude, determined as described in A, on pulse potential. The points were measured in the order 2 mM-Ca_o (○); 0·5 mM-Ca_o (□); 2 mM-Ca_o (◑); 8 mM-Ca_o (△); 2 mM-Ca_o (●). Curves drawn by eye; shift in the half-activation point is −9·5 mV in 0·5 mM-Ca_o, and +8 mV in 8 mM-Ca_o.

the experiment. Fig. 4 shows a similar experiment on the $x_{1\infty}$ curve. Raising Ca_o from 2 to 8 mM causes similar shifts in both $x_{1\infty}$ and s_∞ curves. Low Ca_o, however, has a much more pronounced effect on the $x_{1\infty}$ curve. A second difference is that high Ca_o depresses the maximum i_{x_1} activated by large depolarizing pulses (see below).

The different shifting effects of low Ca_o on the $x_{1\infty}$ and s_∞ curves can be better illustrated if Mg replaces the Ca removed. Preparations are then stable for some time, while in low Ca alone they tend to deteriorate. In a Tyrode containing 2·9 mM-Mg

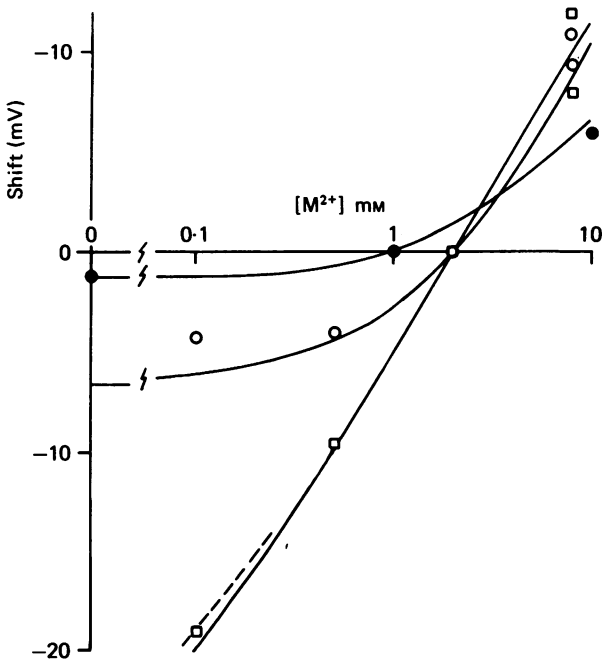

Fig. 5. Shifts in the $x_{1\infty}$ activation curve (□) and in the s_∞ activation curve (○, ●) as a function of divalent ion concentration, M^{2+}, where M^{2+} is either Mg (filled symbols) or Ca (open symbols). Collected results from several experiments. The $x_{1\infty}$ shift in 0·1 mM-Ca (lower left-hand point) was measured with Mg replacing Ca. The curves are calculated from eqn. (5) by varying the parameters K_m^{Ca} and σ to obtain a least-squares fit to the points; separate tests with an equation which included the binding of Mg had shown that best fits were obtained in all cases by assuming the binding of Mg to be negligible. The curves near the points for s_∞ shifts in both Ca (○) and Mg (●) were calculated with $K_m^{Ca} = 2{\cdot}72$ M and $\sigma = 0{\cdot}0051$ e$^-$/Å^2, corresponding to a charge separation of $d = 14$ Å. The continuous curve near the points for $x_{1\infty}$ assumes $K_m^{Ca} = 10{\cdot}3$ mM, $\sigma = 0{\cdot}0038$e$^-$/Å^2, corresponding to $d = 16{\cdot}2$ Å; the interrupted line is calculated with the same parameters, but assuming Mg replaces Ca in the Tyrode solution.

and 0·1 mM-Ca the s_∞ curve shifted by less than -1 mV (two experiments) while the $x_{1\infty}$ curve shifted by -19 mV (one experiment) and by -15 mV in a second less complete experiment (see Fig. 2).

Fig. 5 summarizes the shifts in s_∞ and $x_{1\infty}$ curves in various $[Ca]_o$ and $[Mg]_o$. Only experiments which resulted in reversible shifts are plotted; in two other experiments in 0·1 mM-Ca similar s_∞ shifts were observed, but the fibre deteriorated before return to normal Tyrode. The lines in Fig. 5 are best-fits of eqn. (5) to the shifts of both s_∞ and $x_{1\infty}$ curves in Ca and Mg. The lines near the points for the s_∞ curve are the shifts predicted, in Ca and in Mg, by taking $K_m^{Ca} = 2{\cdot}72$ M and $\sigma = 0{\cdot}0051$ e$^-$/Å^2. With these assumptions the surface potential in normal Tyrode is $\psi_o = -63{\cdot}7$ mV. The continuous line near the points for $x_{1\infty}$ shifts assumes $K_m^{Ca} = 10{\cdot}3$ mM, $\sigma = 0{\cdot}0038$ e$^-$/Å^2, with $\psi_o = -26{\cdot}9$ mV in normal Tyrode. These latter parameters are

similar to those calculated by Brown (1974) for shifts in the excitation threshold in the Purkinje fibre.

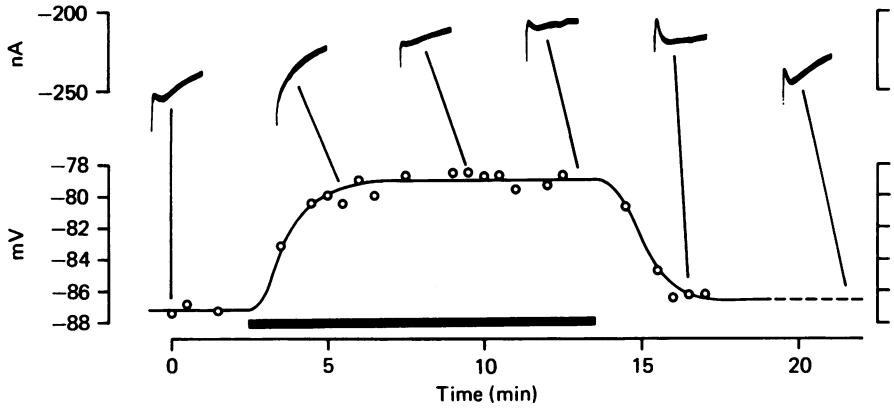

Fig. 6. The time course of the effect of 8 mM-Ca_o (duration of admission shown by the black bar) on the s_∞ activation curve (lower points, curve) and on E_{K_2} (upper traces). The lower curve and open circles show the potential at which i_{K_2} is half activated (i.e. $s_\infty = \frac{1}{2}$). These points were calculated by applying alternate positive and negative pulses, each sufficiently large to fully activate and deactivate i_{K_2} (to -51 mV for 4 sec and to -117 mV for 2 sec, respectively, from a holding potential of -81 mV). From the current tail amplitudes on return to the holding potential, the value of s_∞ at the holding potential can be calculated from:

$$s_\infty\ (-81\ \text{mV}) = \frac{\text{(negative tail amplitude)}}{\text{(positive tail)} + \text{(negative tail)}}.$$

From this one value of s_∞, and from the dependence of s_∞ on potential determined in a prior pulse series (cf. Fig. 3) the half-activation point can be calculated by assuming (as seems reasonable; see Fig. 3) that the activation curve does not change shape when it is translocated on the potential axis by altering Ca_o. This quick method of measuring s_∞ shifts is less accurate than plotting full activation curves, but has the advantage that rapid shifts can be followed. The upper traces show the currents recorded during the pulse to -117 mV, at various times during the admission of 8 mM-Ca_o. The early transient decay (occupying the first quarter of the 2 sec pulse) results mainly from the closure of i_{K_2} channels, since the i_{K_2} tails on return to the holding potential were observed to follow a time course similar to that of this early decay. The later current change was not correlated with a change in tail amplitude, and may reflect depletion of K ions in the clefts between cells (see text). Fibre length 1·75 mm, diameter 0·15 mm; temperature 37·4 °C; cycle time (between successive positive pulses) was 30 sec.

The striking difference in the behaviour of s_∞ and $x_{1\infty}$ curves in low Ca can be well accounted for simply by assuming, as above, that the negatively charged groups near the two channels have a different affinity for Ca. It is also possible (DiFrancesco & McNaughton, 1977) that low Ca acts to increase intracellular cyclic AMP (Harary, Renaud, Sato & Wallace, 1976) and therefore that the s_∞ shifts in low Ca are the sum of a large negative shift, caused by a surface-charge action, and an almost equal positive shift caused by a pseudo-adrenergic effect (Hauswirth, Noble & Tsien, 1968). Another effect of elevated intracellular cyclic AMP is to enhance the maximum amplitude of i_{x_1} (Tsien, Giles & Greengard, 1972; McNaughton & DiFrancesco, 1977). Fig. 4 shows that the amplitude of i_{x_1} is depressed in elevated Ca_o, so it is possible that changes in cyclic AMP are responsible for the apparent difference in Ca-binding properties of surface-charge groups near the i_{x_1} and i_{K_2} channels.

The amount of Ca bound to the membrane can be calculated from K_m^{Ca} and the surface potential. The relation between bound Ca and Ca_c is not Michaelis, since ψ_0 changes with Ca_c, and therefore the concentration of Ca immediately adjacent to the membrane is not constant. It is interesting to note that the amount of Ca bound approximately follows the empirical half-power relation between bound Ca and Ca_c which was observed by Baker & McNaughton (1978) in direct determinations of Ca binding to squid axons. This non-Michaelis relation might, therefore, be due to electrostatic interactions between binding sites, rather than to a population of binding sites with differing affinities as was suggested by Baker & McNaughton (1978).

The effects of Ca on the pace-maker current reversal potential, E_{K_2}

Alterations in the potential gradient across the membrane caused by binding of Ca ions to the external face of the membrane would not be expected to change the reversal potential of a channel permeable to a single ion, since it depends only on the ratio of ion activities in the extracellular and intracellular fluids some distance away from the membrane. However, raising Ca_o does in fact have an effect on the reversal potentials of both i_{K_2} and i_{x_1}. In Fig. 3*A* a pulse to -102 mV was approximately at the reversal potential of i_{K_2} in 8 mM-Ca_o, while in 2 mM-Ca_o a pulse to -114 mV was required to reach E_{K_2}.

In another experiment the time course of the shift in reversal potential was observed (Fig. 6). When Ca_o was changed from 2 to 8 mM, E_{K_2} shifted in the positive direction by about 5 mV. The time course of the shift roughly paralleled the arrival of Ca at the membrane, as measured by observing at the same time the time course of the shift in the s_∞ activation curve. The shift in E_{K_2} was not maintained in this experiment, and over the next 10 min E_{K_2} returned slowly to more negative values. Re-admitting 2 mM-Ca_o caused a similar but opposite transient shift in E_{K_2} of about 6 mV in magnitude.

The simplest explanation of these observations is that raising Ca_o causes an increase in the potassium concentration in the deep, narrow clefts between adjacent cells in the syncitial Purkinje fibre bundle. A 6 mV shift in E_{K_2} (Fig. 6) would reflect a 1·25-fold change in K_c (where K_c is the K concentration in the clefts), and a 12 mV shift (Fig. 3*A*) a 1·57-fold change, if the i_{K_2} channel is perfectly selective for K.

Other explanations for the shift in E_{K_2} must also be considered. A direct effect of Ca ions on E_{K_2} is not likely since i_{K_2} appears to be highly K-selective (Noble & Tsien, 1968; Peper & Trautwein, 1969). Changes in K_i of the order required to produce the observed shifts in E_{K_2} also seem unlikely in view of the rapidity of the effect (Fig. 6), since the ratio of intracellular to cleft volume in the Purkinje fibre is very high (Mobley & Page, 1972). A more serious difficulty is caused by the presence of the small, unstirred cleft space outside the membrane, since accumulation and depletion of potassium ions in this space can have effects on the measurement of reversal potentials (Baumgarten & Isenberg, 1977; DiFrancesco & Ohba, 1978). Depletion processes almost certainly affect E_{K_2} in the Purkinje fibre in normal solution, since E_{K_2} is surprisingly negative; from the values of $[K]_i = 150$ mM and $[K]_o = 4$ mM a maximum value of $E_{K_2} = -97$ mV is expected, while measured values are commonly up to 20 mV more negative than this (Cohen, Daut & Noble, 1976; see also Figs. 3*A* and 6). This discrepancy might be caused by a steady depletion of K ions in the clefts, perhaps as a result of Na pump hyperactivity in fibres recovering from

excision, or perhaps because the inward membrane currents required to maintain the holding potential (see Fig. 3*A*) causes a significant net flux of K into the cell. Both these processes would produce negative values of E_{K_2}, without greatly affecting the measurement of changes in E_{K_2}. Alternatively, rapid K_c depletion occurring actually during the decay of i_{K_2} may contribute an artifactual time-dependence and a consequent error in the estimation of E_{K_2} (DiFrancesco & Ohba, 1978), and this rapid depletion might also affect the measurement of changes in E_{K_2}.

Even if the measurement of E_{K_2} is affected by depletion, one would expect depletion to reduce K_c even further in high Ca, and consequently make E_{K_2} more negative, since the net current at the holding potential is more inward in high Ca (see Fig. 3*A*; a similar change in current at the holding potential was observed in the experiment of Fig. 6, but is not shown). This inward change in net membrane current is caused, at least in part, by a deactivation of i_{K_2} channels due to the positive shift in the s_∞ activation curve, and therefore reflects a decrease in the outward K current across the membrane. Such a decrease should, of itself, cause a negative shift in E_K, and might be responsible for the tendency of E_{K_2} to drift back towards more negative values after some time in high Ca (Fig. 6). The observation of an initial positive shift in E_{K_2} suggests that the net K ion efflux across the membrane has increased in spite of the decrease in outward current, and has produced an accumulation of K outside the membrane. A net increase in K efflux when the K current has decreased can only result from concomitant changes in other ion movements. For instance, elevated Ca might be reducing the activity of the Na : K exchange pump. A possibility not obviously consistent with these experiments, though, is that the positive shift in E_{K_2} might be caused by Ca opening K^+ channels, since such an action of Ca should be accompanied by an increase in outward current, while in fact the reverse is observed.

The effects of Ca on the pace-maker current rectifier relation, $\bar{\imath}_{K_2}$

In addition to its effects on the reversal potential of i_{K_2}, elevated Ca has effects on the $\bar{\imath}_{K_2}$ rectifier relation which accord with the idea that Ca causes a small elevation in the K ion concentration just outside the membrane. The potential dependence of $\bar{\imath}_{K_2}$ has been determined from the relation (cf. eqn. (1)):

$$\bar{\imath}_{K_2}(V) = \frac{\Delta i_{K_2}}{\Delta s}, \tag{6}$$

where Δi_{K_2} is the current change observed during a clamp pulse from the holding potential, V_H, to potential V, and where Δs is the associated change in the fractional degree of activation of channels. The traces of Fig. 3*A* were used to construct the $\bar{\imath}_{K_2}$ relation shown in Fig. 7*B* according to this formula. Elevated Ca_o causes a positive shift in E_{K_2}, consistent with a reduction in the driving force on K ions, but this reduction does not reduce the K current at all potentials; in fact, there is a small increase in $\bar{\imath}_{K_2}$ positive to about -100 mV. Small elevations in K_o cause a similar cross-over in $\bar{\imath}_{K_2}$ relations (Noble & Tsien, 1968).

In another experiment in which Ca_o was lowered from 2 to 0·5 mM the change in $\bar{\imath}_{K_2}$ was found to be consistent with the idea that reducing Ca_o causes a reduction in K_c, and in this experiment a cross-over of the $\bar{\imath}_{K_2}$ relations at -90 mV was observed. Thus the maximum available K current is *reduced* at potentials positive to -90 mV in low Ca_o, even though the driving force on K ions is increased.

The effects of Ca on the plateau outward current, i_{x_1}

In Fig. 4. elevated Ca_o had the effect of depressing the maximum i_{x_1} decay tail activated by a strong depolarizing pulse, showing that the $\bar{i}_{x_1}$ rectifier relation was depressed by high Ca_o at potentials positive to the i_{x_1} reversal potential. In Fig. 7

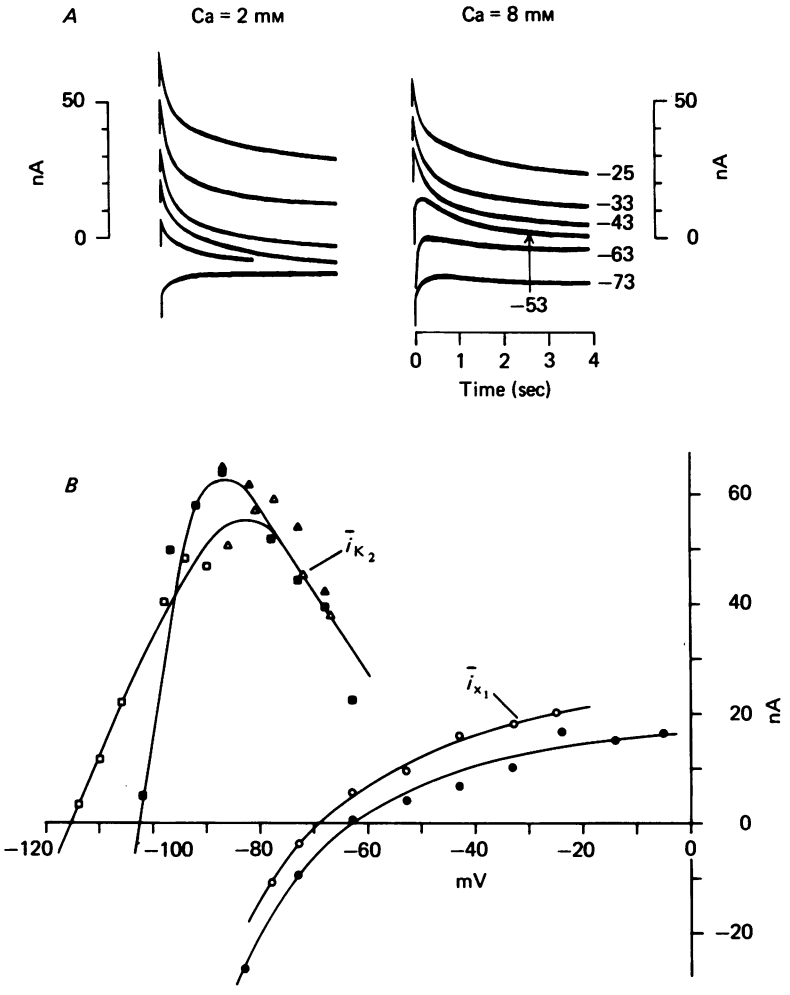

Fig. 7. The effect of increasing Ca_o from 2 to 8 mM on the pace-maker current rectifier relation, $\bar{i}_{K_2}$, and on the plateau outward current rectifier relation, $\bar{i}_{x_1}$. *A*, current records used to determine $\bar{i}_{x_1}$. The traces of Fig. 4 cannot be used because of the presence of other current components overlapping the plateau outward current. The i_{x_1} component was activated fully by a strong positive pulse (to +13 mV for 2 sec), and its decay observed during a clamp pulse to various more negative potentials, noted alongside each trace in 8 mM-Ca_o. Same experiment as in Fig. 3*A*. The current zero is correct for the upper traces in each panel; the lower four traces in 2 mM-Ca_o, and the lower two in 8 mM-Ca_o, have been shifted down by 10 nA for clarity. Cycle time 12 sec. *B*, left-hand points are $\bar{i}_{K_2}$ rectifier relations in 2 mM-Ca_o(□, △) and 8 mM-Ca_o (■, ▲), calculated using eq. 6. Pulses from a holding potential of −82 mV (□, ■) are shown in Fig. 3*A*; other pulses from holding potentials of −86 mV (▲) and −90 (△) were also used. Curves drawn by eye. Right-hand curves are $\bar{i}_{x_1}$ rectifier relations in 2 mM-Ca_o (○) and 8 mM-Ca_o (●) calculated by decomposing the traces in *A* into a fast (i_{x_1}) and a slow (i_{x_2}) component and applying eqn. (7) to the i_{x_1} component.

the $\bar{i}_{K_1}$ rectifier relation has been measured over an extended potential range by activating the current fully with a strong depolarizing pulse, then observing its decay during clamp pulses to more negative potentials. $\bar{i}_{x_1}$ was calculated from the relation

$$\bar{i}_{x_1}(V) = \frac{\Delta i_{x_1}}{\Delta x_1}. \tag{7}$$

Elevated Ca_o shifts the reversal potential, E_{x_1}, by about 6 mV in the positive direction (compare the traces at -63 mV in Fig. 7*A*) and shifts the $\bar{i}_{x_1}$ rectifier relation downwards over the whole potential range. If i_{x_1} is partly carried by K ions the positive shift in E_{x_1} would be expected if high Ca_o reduces the K gradient across the membrane, and the depression in $\bar{i}_{x_1}$ might also result from a small elevation in K_c. To see if this was a possible explanation the effects of K_o on $\bar{i}_{x_1}$ were observed in a separate experiment described in the next section.

The time course of the change in E_{x_1} (half-time ~ 300 sec) is found to be appreciably slower than the shift in the $x_{1\infty}$ activation curve (half-time ~ 100 sec, approximately the same as the half-time of the s_∞ shift). By contrast, the time course of the shift in E_{K_2} roughly parallels the shift in s_∞ (Fig. 6). We do not know whether this discrepancy reflects a different distribution of i_{x_1} and i_{K_2} channels, or whether it results simply from the different holding potentials and pulse protocols used in each type of experiment.

The dependence of the plateau outward current on K_o

In Fig. 8 the changes in $\bar{i}_{x_1}$ when K_o was increased from 4 to 8 mM are shown. The twofold change in K_o causes a shift in E_{x_1} of about 8·6 mV, slightly less than half that expected for a perfectly K-selective channel, and depresses the $\bar{i}_{x_1}$ rectifier relation over the whole potential range investigated in this experiment.

On the basis of the 8·6 mV shift in E_{x_1} for a twofold change in K_o observed in Fig. 8, the 6 mV shift in E_{x_1} observed in 8 mM-Ca_o (Fig. 7) might reflect an increase of K_c by a factor of 1·6. In the same experiment 8 mM-Ca caused a shift in E_{x_2} consistent with a 1·57-fold increase in K_c (see Fig. 3*A* and discussion on p. 357), in good agreement with the increase predicted from the shift in E_{x_1}.

The shape of the $\bar{i}_{x_1}$ relation and its dependence on K_o can be accounted for by assuming that ions crossing the membrane encounter a single energy barrier close to the inner surface of the membrane (cf. Jack, Noble & Tsien, 1975, p. 232). The slope of the relation between log K_o and E_{x_1} can be reproduced in this model by assuming that the channel is permeable both to K and to an ion with an equilibrium potential near 0 mV. The other ionic species involved does not seem to be Na, though, because when all the Na is replaced by choline we have observed no shift in E_{x_1}, although the $\bar{i}_{x_1}$ rectifier relation was increased by a factor of about 2 at all potentials, with little change in shape. In view of the positive shift in E_{x_1} when Ca_o is elevated (Fig. 7) it is possible that Ca flows through the i_{x_1} channels, but the observation that the shift in E_{x_1} in elevated Ca_o is quantitatively accounted for by the increase in K_c predicted from measurements of E_{K_2} argues against this possibility.

The plateau outward currents might not arise from ionic channels gated in the Hodgkin–Huxley manner at all; it is equally plausible to account for both i_{x_1} and the slower i_{x_2} component in terms of potassium accumulation in the cleft space during the strong depolarizing pulses required to activate these currents. Such

accumulation would cause an increase in outward current positive to about −70 mV and a decrease negative to this potential (see Fig. 13). Changes in K concentration in the clefts would therefore produce currents with a potential-dependence similar to that of $\bar{i}_{x_1}$. The only evidence against this idea is:

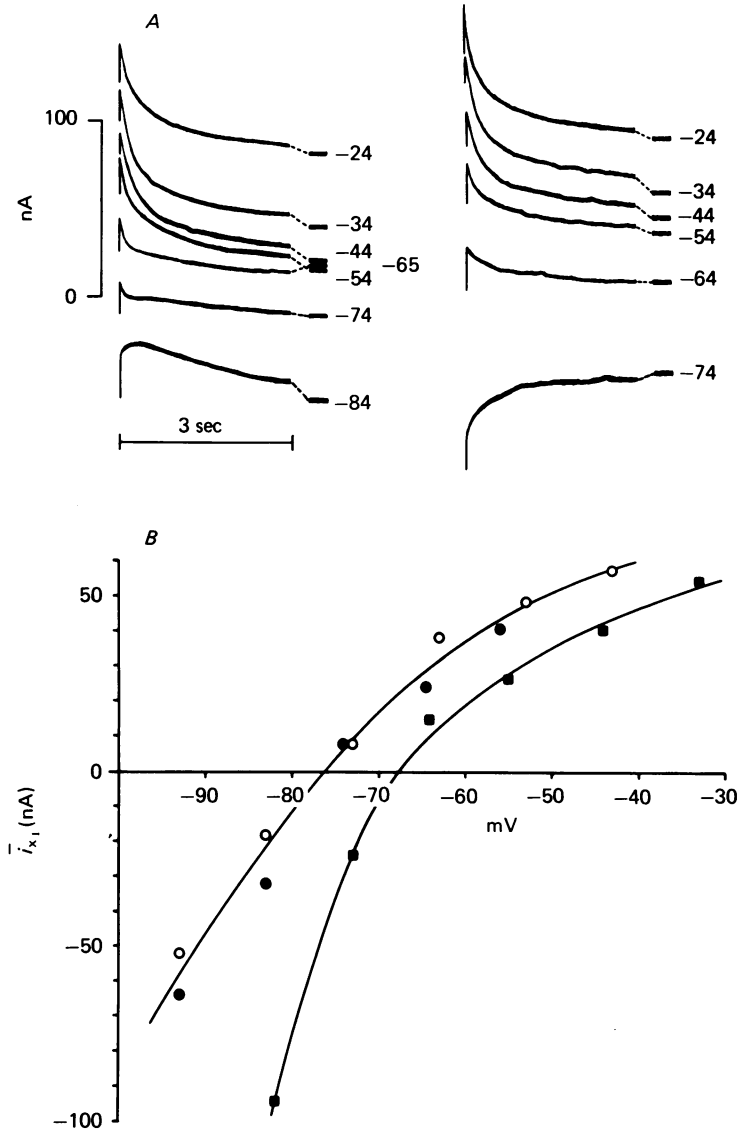

Fig. 8. The dependence of the plateau current rectifier relation, $\bar{i}_{x_1}$, on K_o. *A*, currents recorded when the preparation was repolarized to a variable potential (noted to the right of each trace) after a depolarizing pulse (to +7 mV for 1·5 sec) sufficiently strong to activate $\bar{i}_{x_1}$ fully. The left-hand panel shows currents in 4 mM-K_c, and the right-hand panel currents in 8 mM-K_c. The first 3 sec and the steady level of each trace are shown. Traces are shifted vertically for clarity; the steady currents are replotted in Fig. 13. Temperature 37·5 °C; fibre diameter 0·2 mm, length 1·75 mm; cycle time 35 sec. B, $\bar{i}_{x_1}$ rectifier relations in 4 mM-K (○); in 8 mM-K (■); and on return to 4 mM-K (●). Curves drawn by eye.

(i) i_{x_1} exhibits a well defined reversal potential while a current resulting from accumulation should be biphasic near its apparent reversal potential;

(ii) we have observed that the $x_{1\infty}$ activation curve does not depend on holding potential between -50 and -70 mV, while the apparent activation curve of a current resulting from accumulation would be expected to depend on holding potential (Eisner, D. A., Cohen, I. & Attwell, D., personal communication);

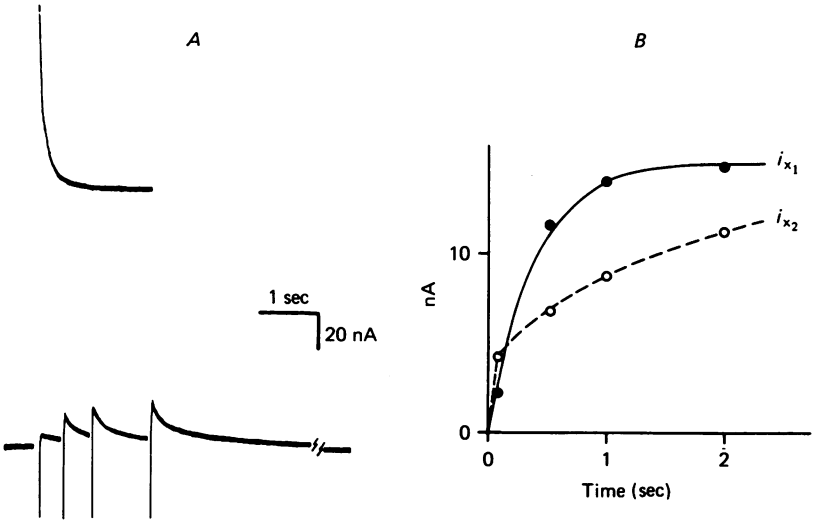

Fig. 9. The time course of activation of i_{x_1} and i_{x_2}. *A*, currents in response to pulses of various length to +9 mV from a holding potential of −43 mV. Fibre length 1·85 mm, diameter 0·15 mm; temperature 37·4 °C; cycle time 10–20 sec. *B*, the onset of i_{x_1} (●) and i_{x_2} (○) during the pulse, measured by separating the tail on return to the holding potential into fast and slow components. The activation of i_{x_1} is approximately exponential (solid line) with time constant 0·38 sec at +9 mV, but the activation of of i_{x_2} is not exponential, being composed of an early rapid rise, followed by a slower increase.

(iii) the time course of i_{x_1} during depolarizing clamp pulses, as measured by tail amplitude on return to holding potential, is a simple exponential (Fig. 9) in spite of its overlap with a large transient outward current.

Fig. 9 shows that i_{x_2}, in contrast to i_{x_1}, does not activate in a simple first-order manner; its time course has a very fast early onset followed by a slower rise. Such a time course resembles the integral of the current during the positive clamp step, which is consistent with the idea that i_{x_2} results from accumulation of K outside the membrane during the positive clamp step. Noble & Tsien (1969) observed a simple exponential onset of i_{x_2}, probably because the transient outward current had been suppressed in their experiments by the use of Cl media. Our tentative conclusion is that i_{x_1} may be a Hodgkin–Huxley current, but that i_{x_2} probably reflects changes in K concentration in the clefts.

If i_{x_2} is assumed to result from accumulation the extent of changes in K_c can be roughly estimated by comparing the size of i_{x_2} with the increase in outward current produced by increasing the K concentration in the bathing medium. For example, in the experiment of Fig. 8 a pulse to +7 mV for 1·5 sec in 4 mM-K_o would, from the effects of K_o on the same fibre (Fig. 12), have caused an increase in K_c of about 1·5 mM. This change in K_c would depress $\bar{i}_{x_1}$ by about 10 % at

– 43 mV (see Fig. 8). This change in $\bar{i}_{x_1}$ during the positive pulse will depress the i_{x_1} tail at longer times, but the size of the effect is probably not great enough to cause significant deviation from the exponential activation kinetics observed in Fig. 9.

Effects of Ca on the steady membrane current

Fig. 10 shows the effect of raising Ca_o, from 0·1 to 2 mM, on currents in the plateau range (cf. Kass & Tsien, 1976). The transient inward current component (Ca current, Reuter, 1967) at the step onset increases markedly, although the increase is not

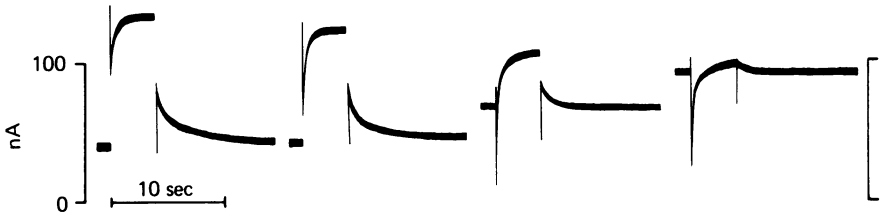

Fig. 10. Currents recorded in response to voltage-clamp steps in the plateau range (holding potential – 55 mV, step potential – 24 mV) during the change from Tyrode containing 0·1 mM-Ca and 2·9 mM-Mg to one containing 2 mM-Ca and 1 mM-Mg. The traces are (from left) before, and 20 sec, 3·5 min and 7·5 min after admission of normal Ca. The last trace is steady state. Temperature 37·3 °C; fibre diameter 0·2 mm, length 2·0 mm; cycle time 20 sec.

sustained. Raising Ca also causes a progressive decrease in both the outward current activated by the clamp step and the current tail (i_{x_1}) recorded on return to the holding potential, as expected when $x_{1\infty}$ shifts in the positive direction (Fig. 4). The increase in outward current at the holding potential is unexpected, though, since the positive shifts in $x_{1\infty}$ and s_∞ activation curves and the depression in $\bar{i}_{x_1}$ in high Ca should all tend to decrease outward current at all potentials, and the small increase in $\bar{i}_{K_2}$ occurs only at negative potentials (Fig. 7*B*). Clearly the time-independent ('background') current is also affected by Ca.

The effects of 0·5, 2 and 8 mM-Ca on the steady-state current–voltage relation, which represents the sum of time-independent current and the steady levels of the time-dependent current components, is shown in Fig. 11. A Ca_o-dependent increase in steady-state outward current is pronounced between about – 50 and – 75 mV. In two other experiments similar effects were seen in high Ca, while in low Ca (0·1 and 0·5 mM Ca) two experiments resembled Fig. 12 in showing only one cross-over point with the current–voltage relation in 2 mM-Ca, while two others had a second cross-over point at about – 80 mV.

Changes in the time-independent current alone can be determined from Fig. 11 by subtracting from the total steady-state current the changes, due both to shifts in activation curves and to changes in E_K, in the steady-state levels of time-dependent currents. For example, the change in the steady level of i_{K_2} is given by:

$$\Delta i_{K_2\infty} = \Delta(s_\infty . \bar{i}_{K_2}) = \bar{i}_{K_2} . \Delta s_\infty + s_\infty . \Delta \bar{i}_{K_2} + \Delta s_\infty . \Delta \bar{i}_{K_2}. \quad (8)$$

The dashed line in Fig. 11 shows the steady-state current in 8 mM-Ca with the effects of changes in time-dependent currents subtracted; thus the difference between this curve and that in 2 mM-Ca represents the Ca_o-dependence of the background current.

Positive to -55 mV, raising Ca_o reduces the background current, an effect which may be due to incomplete inactivation of Ca channels at voltages positive to their activation threshold (Reuter, 1968; Gibbons & Fozzard, 1975). Negative to -55 mV, Ca_o increases the current in the outward direction. Kass & Tsien (1976) have sug-

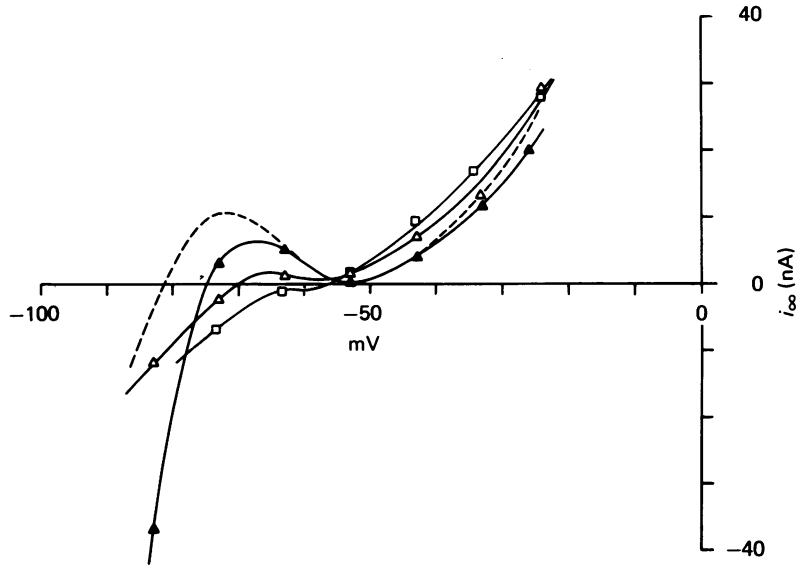

Fig. 11. The steady-state membrane current as a function of membrane potential in 8 mM-Ca (▲), 2 mM-Ca (△) and 0·5 mM-Ca (□). Continuous curves drawn through the points by eye. The dashed curve shows the current that would be observed in 8 mM-Ca if time-dependent currents had remained as they were in 2 mM-Ca (see text for details of this calculation). Thus the difference between the dashed curve and the curve in 2 mM-Ca shows the effect of raising Ca from 2 to 8 mM on the time-independent current. Same experiment as in Fig. 3*A*. Steady current recorded after 4 sec at each potential.

gested that this Ca_o-dependent outward current could result from a direct action of Ca on K channels. This apparent Ca-dependence of the time-independent current might, however, be due to an effect of the increased K concentration outside the membrane which seems to accompany increases in Ca_o, rather than reflecting a direct action of Ca on K channels. Increasing K has effects (Hall, Hutter & Noble, 1963) quite similar to the effects of increased Ca_o seen in Fig. 11. Fig. 12 shows the effects of small changes in K_o; evidently even quite small increments in K_o, of the order of those deduced from Figs. 3, 6 and 7 (1·3–1·6-fold) can have large effects on the steady membrane current. Lowering Ca_o also has similar effects, negative to -55 mV, to lowering K_o, although here an exact comparison between Figs. 11 and 12 is not possible in the absence of a detailed analysis of time-dependent currents in low Ca_o in the experiment of Fig. 11. It seems probable, therefore, that at least the major part of the Ca_o-dependence of steady outward current in the Purkinje fibre results not from a direct effect of Ca on K channels, but from an indirect effect of raised K_c on the membrane. While a small contribution of a Ca_o-dependent K^+ current cannot be ruled out, the effects of Ca are consistent with an indirect effect of K_c.

The transient outward current

The transient outward current is an attractive candidate as a Ca_o-dependent outward current since it is most prominent while the fibre is contracting, and has a time course similar to that of the Ca current. Siegelbaum *et al.* (1977) have observed

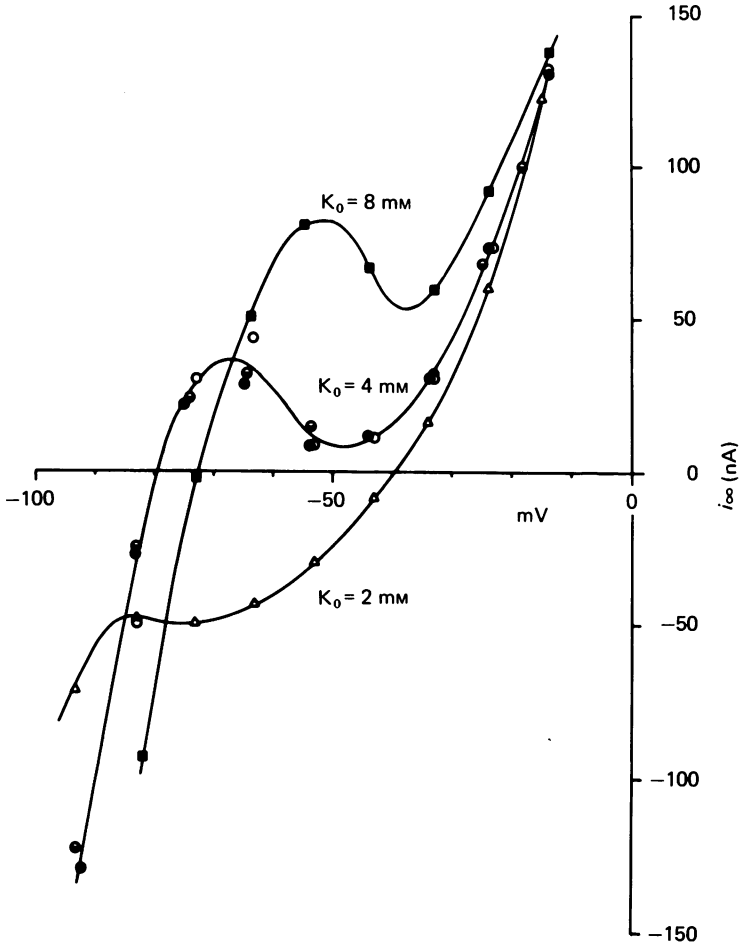

Fig. 12. The action of K ions on steady membrane currents. Curves were plotted in the order 4 mM-K (○); 8 mM-K (■); 4 mM-K (◒); 2 mM-K (△); 4 mM-K (●). Same experiment as in Fig. 8. Steady current recorded after 10 sec at each potential.

that the magnitude of the transient outward current parallels that of the contraction under a number of conditions, and have proposed that this current is Ca_i-dependent.

The effects of holding potential and 0·5 mM-Ca_o on the transient outward current have been investigated in the experiment of Fig. 13. From a holding potential of −79 mV (Fig. 13*A*, and open squares in Fig. 13*B*) the peak transient outward current (measured as the difference between the maximum and minimum values attained during the pulse) depends on the pulse potential in two phases: between −35 and −20 mV the peak current increases in sigmoidal fashion, then above

– 20 mV it rises more slowly. When the pulse sequence is repeated from more positive holding potentials (open circles, diamonds, triangles) the peak current at a given pulse potential is reduced, with little change in the shape of the dependence of peak current on potential. The inactivation curve obtained by plotting peak current at – 10 mV as a function of potential (Fig. 13*B*, filled circles) has a sigmoidal appearance (cf. Fozzard & Hiraoka, 1973).

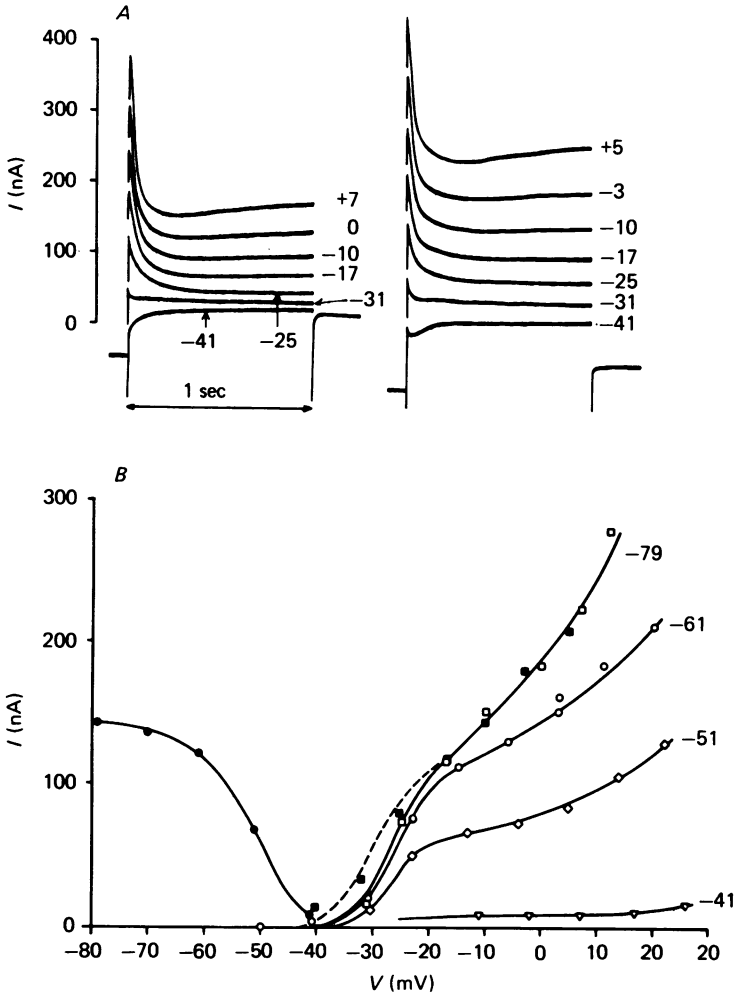

Fig. 13. *A*, transient outward currents in 2 mM-Ca (left-hand panel) and after 20 min in 0·5 mM-Ca (right-hand panel). The voltage-clamp step was from a holding potential of – 79 mV in 2 mM-Ca and – 77 mV in 0·5 mM-Ca to the potential noted alongside each trace. Temperature 36·9 °C; fibre diameter 0·3 mm, length 1·4 mm; cycle time 30 sec. *B*, right-hand curves: transient outward currents as a function of step potential (abscissa). The points show the difference between the peak height and the minimum value subsequently attained. Open symbols are currents in 2 mM-Ca, from holding potentials of – 79 mV (□), – 61 mV (○), – 51 mV (◇), and – 41 mV (▽). Filled squares (■) are currents in 0·5 mM-Ca, from a holding potential of – 77 mV. Left-hand curve, filled circles: the inactivation of the transient outward current recorded during a step to – 10 mV, as a function of holding potential.

This experiment is consistent with the idea that the transient outward current is controlled by an activation and an inactivation variable along the lines of the Hodgkin–Huxley model:

$$i_{to} = q.r.\bar{i}_{to}(V), \tag{9}$$

where q_∞ is an activation variable increasing sigmoidally between -40 and -20 mV; r_∞ is an inactivation variable decreasing sigmoidally between -65 and -40 mV; and $\bar{i}_{to}(V)$ is a channel exhibiting outward-going rectification, and with a reversal potential possibly as negative as E_K. Direct observation of the reversal potential is complicated by the speed of deactivation at negative potentials, which means that decay of the current overlaps the capacitative transient.

Reducing Ca_o has effects which are also consistent with a Hodgkin–Huxley type of model (filled squares, Fig. 13*B*). In 0·5 mM-Ca_o the threshold of activation is approximately 4 mV more negative (filled squares, dashed line) while the fully activated current positive to -20 mV is unchanged. In another experiment the inactivation curve was also found to shift in the negative direction (not shown).

Siegelbaum *et al.* (1977, Fig. 1*D*) have observed a reduction in the transient outward current in low Ca. Fig. 13*B* shows how this effect can be accommodated in the model of eqn. (9). From a holding potential in the range -65 to -45 mV, reducing Ca_o will produce a reduction in maximum transient outward current recorded during positive clamp steps because of a negative shift in the inactivation curve. This apparent Ca-sensitivity need not, therefore, reflect a true Ca-dependence of the transient outward current.

The effects of low Ca on the transient outward current in Fig. 13 are consistent with a Hodgkin–Huxley (1952) type of model, in which Ca_o shifts activation curves with no effect on channel conductances, but it is also possible that transient outward channels are gated directly by Ca_i. Changes in Ca_i have not been measured in the experiment of Fig. 13, and it is possible that even a long soak in low Ca_o has little effect on the release of intracellular stores of Ca with each depolarizing clamp pulse (S. Siegelbaum, personal communication). It is also possible that a reduction in the inward current in low Ca has partially obscured a concomitant reduction in i_{to}.

The effects of Na removal on outward currents

Removal of external Na is known to abolish the pace-maker current, i_{K_2} (McAllister & Noble, 1966). This phenomenon could be related to the increase in Ca_i consequent on Na removal, perhaps reflecting a dependence of i_{K_2} on Ca_i. The experiment of Fig. 14 explores the effect in more detail. Partial removal of Na (75% replacement of NaCl with choline Cl) is found to depress the rectifier relation, $\bar{i}_{K_2}$, with no change in E_{K_2}. The s_∞ activation curve is not significantly affected. Replacement of half of Na by choline caused a lesser reduction in $\bar{i}_{K_2}$, while complete replacement abolished i_{K_2}, with no apparent shift in s_∞ as time-dependence disappeared. These experiments rule out the possibility that time-dependence vanishes because of a large negative shift in s_∞. It is possible that the i_{K_2} channel is somehow activated by external Na.

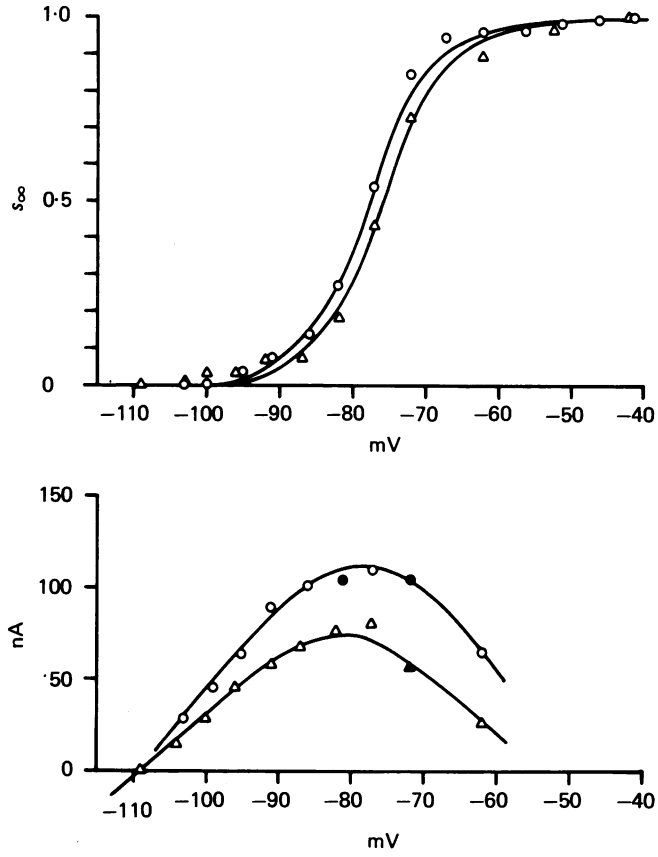

Fig. 14. The effects of sodium removal on the pacemaker current, i_{K_2}. *A*, s_∞ activation curves in normal Tyrode (○) and in a Tyrode in which 75% of the NaCl was replaced by choline Cl (△). The s_∞ curve in low Na is shifted positive by 1·5 mV. Holding potential −72 mV. *B*, i_{K_2} rectifier relations in normal Tyrode (○, ●) and in low-Na Tyrode (△, ▲). i_{K_2} was determined from full s_∞ curves (●, ▲) or from single voltage-clamp pulses (○, △). Temperature 36·9 °C; fibre diameter 0·35 mm, length 2·25 mm; cycle time about 20 sec.

DISCUSSION

Most of the effects of Ca_o on outward currents seem explicable in terms either of a shifting effect on the voltage dependence of activation parameters or of an increase in the K concentration (K_c) immediately external to the membrane. The first effect was expected, and the only unusual feature is the striking difference in the shifting effect of Ca_o on the $x_{1\infty}$ and s_∞ curves, a difference which may reflect differing affinities for Ca of binding groups in the membrane. An increase in K_c when Ca_o is elevated is more surprising, but the changes in the pacemaker current reversal potential, in the plateau outward current reversal potential and in the steady-state current are all consistent with a 1·3–1·6-fold increase in K_c when Ca_o is increased from 2 to 8 mM. Because of problems in interpreting reversal potentials when there is a small unstirred space outside the membrane (see DiFrancesco & Ohba, 1978) the idea that K_c increases in elevated Ca_o cannot be accepted categorically, but it

seems the most attractive explanation of our results. It would be interesting to measure K_c with a potassium electrode to obtain a more definite answer on this point.

The pace-maker current, i_{K_2}

The shifts in the s_∞ activation curve in altered Ca_o are satisfactorily explained by assuming that channel gating depends only on the field across the membrane, and that it is this field which is altered by the binding of Ca to the outside face of the membrane. The shifts are in the wrong direction for a direct dependence of channel gating on Ca, since raising Ca_o *reduces* the degree of activation at a given potential. The only way to rescue the idea that i_{K_2} is Ca_i-dependent would be to suppose that Ca_i decreases in raised Ca_o, which seems unlikely.

Reducing Na_o reduces $\bar{i}_{K_2}$, which could mean that raised Ca_i in the low-Na Tyrode locks some i_{K_2} channels in the open position, and therefore reduces the number available to be gated by membrane potential, but in view of the lack of a corresponding effect of altered Ca_o we are inclined to discount this possibility. The effect of low Na_o remains a puzzle, although it is clear from Fig. 14 that shifts in the s_∞ curve do not underlie the Na_o-sensitivity of i_{K_2}.

Direct injection of Ca transiently increases i_{K_2}, and injection of EGTA diminishes it (Isenberg, 1977*c*), observations which suggest a dependence of i_{K_2} on Ca_i. These observations are, however, also consistent with the idea that injections of Ca cause a small elevation in K ion concentration in the clefts. Elevated K increases the fully activated channel current ($\bar{i}_{K_2}$) at potentials positive to about -90 mV, while at more negative potentials $\bar{i}_{K_2}$ becomes more inward (cross-over effect, Noble & Tsien, 1968). In our experiments similar effects to those caused by elevated K_o have been seen in elevated Ca_o (Fig. 7*B*), while the effects of low Ca_o on $\bar{i}_{K_2}$ mimic those of low K_o. These effects are not consistent with the idea that Ca (whether Ca_i or Ca_o) is gating the pace-maker current, since $\bar{i}_{K_2}$ is neither elevated by high Ca nor depressed by low Ca at all potentials; the apparent Ca-dependent increase is confined to a potential range between about -100 and -70 mV.

The plateau current, i_{x_1}

There seems little reason to suspect i_{x_1} of being dependent on Ca, since the arguments advanced above for i_{K_2} apply equally to i_{x_1}. High Ca_o deactivates i_{x_1} because of the shifting effect on $x_{1\infty}$, and it also depresses $\bar{i}_{x_1}$. The latter effect is similar to that produced by elevated K_o, and here, in contrast to the case of $\bar{i}_{K_2}$, there is no potential range in which either high Ca_o or high K_o produces an enhancement of $\bar{i}_{x_1}$.

The transient outward current, i_{to}

A full characterization of this current is made difficult by the speed of its activation and deactivation and by the presence of overlapping current components. Within this limitation it is possible to express the current as the product of an activation variable, an inactivation variable, and a channel rectifier relation. The behaviour of the transient outward current in low Ca_o is also consistent with the idea that alterations

in Ca_o alter the potential-dependence of the gating variables, with no effect on the maximum current (Fig. 13).

It is equally possible, however, that the transient outward current depends on Ca_i rather than on membrane potential, since its maximum amplitude is correlated with the contraction of the fibre in a number of situations (Siegelbaum *et al.* 1977). This explanation of the control of the transient outward current would be consistent with the observations in the present paper if moderate changes in Ca_o do not have a significant effect on the Ca_i released with each depolarizing clamp pulse, which would explain why the transient outward current is little affected by changes in Ca_o.

The time-independent current

When the effects of changes in time-dependent currents are subtracted out, the time-independent current is found to exhibit a substantial dependence on Ca_o. The similarity between the effects of Ca_o and K_o on the time-independent current (Figs. 11, 12) suggests that the effects of Ca_o can result from an increase in K_c, as in the case of the time-dependent currents $\bar{i}_{K_2}$ and $\bar{i}_{x_1}$, rather than by means of a direct action of Ca on K^+ channels.

Isenberg (1977*b*) has observed effects of Ca injection on the time-independent current which closely parallel those of external application of Ca, both in general shape and in the position of the 'cross-over' of the steady-state curves (at about -90 mV). The only differences occur positive to -55 mV, where the relations of Fig. 11 show a second cross-over not seen either in the effect of K_o on the time-independent current (Fig. 12) or in Isenberg's experiments. The Ca_o-dependence of the steady current in this range may reflect a direct contribution of Ca ions to the membrane current, possibly as a result of an incomplete inactivation of Ca channels.

Isenberg (1977*b*) has also shown that the cross-over point of the steady current before and during Ca injection depends on K_o in an approximately Nernstian fashion, although the absolute values of the cross-over are about 7 mV positive to the usually accepted E_K of Purkinje fibres. A similar dependence on K_o of the cross-over in elevated Ca_o has been observed by Kass & Tsien (1976). These observations would appear to support the idea that Ca controls a K^+ channel with a Nernstian dependence on K_o, but they are in fact equally consistent with the hypothesis that the Ca-dependent cross-over results from an elevation in K_c. The K_o-dependent change in steady current exhibits a dependence on the average level of K_o similar to that of the Ca-dependent cross-over; thus, in Fig. 12, the cross-over point for the change 2 mM-K_o to 4 mM-K_o (average 3 mM-K_o) is at -85 mV, and that for the change 4 mM-K_o to 8 mM-K_o (average 6 mM-K_o) is at -67 mV. The difference between the two cross-over points (18 mV) exhibits a Nernstian dependence on the change in average K_o.

Mechanism of the increase in K_c

The present experiments suggest that Ca_o increases K_c in the restricted space immediately outside the membrane and the similar effects of Ca injection (Isenberg, 1977*a*, *b*, *c*) imply that Ca acts intracellularly. The apparent increase in K_c can be observed even when the net change in membrane current is inward (Fig. 3*A*), suggesting that an increase in net K efflux takes place even when the outward K

current is decreased. This observation makes it attractive to suppose that K movements affected by changes in Ca_i are coupled to the movements of other ions, and therefore do not appear as a direct contribution to the membrane current. Intracellular Ca might be inhibiting the Na : K exchange pump, or it could equally be activating a concurrent efflux of K and an anion, for instance Cl. Recently Ellis (1977) has shown a reduction in Na_i in Purkinje fibres when Ca_o is increased. Such a reduction in Na_i could be reducing the activity of the Na : K pump by decreasing the fractional activation of the internal Na-binding site. Two other possibilities are: (i) Ca_o might be reducing the negative surface potential at the outside of the membrane, and thereby reducing the K concentration near the Na pump activation site, even though the K ion concentration some distance away is increased; (ii) the increased K efflux in high Ca_o might be due to the operation of a Ca : K exchange. Both of these mechanisms might explain the effects of Ca_o described in the present paper, but would not account for the similar effects of Ca injection.

Ca-dependent K currents in other excitable cells

The Ca-dependence of both the pace-maker current rectifier relation ($\bar{i}_{K_2}$) and the time-independent current in the Purkinje fibre appears to result from the combination of two factors: Ca increases K outside the membrane, and the K current through the membrane is itself increased at potentials more than about 20 mV positive to E_{K_2} by this increase in K_c. Increasing K_c must, of course, decrease the K current near its reversal potential, so the membrane current–voltage relations are observed to cross; this K-sensitivity is therefore often called the cross-over effect (Hall *et al.* 1963; Noble & Tsien, 1968). A similar cross-over in the steady current–voltage relation is observed in ventricular tissue (McDonald & Trautwein, 1978), so it is possible that an apparent Ca-dependence of the outward current in this tissue (Bassingthwaighte *et al.* 1976) could also be explained by a Ca-dependent increase in K_c.

Clearly, an explanation such as we have proposed for the Purkinje fibre cannot be extended to Ca-dependent K currents in other excitable tissues if the cross-over effect is absent. Two other features may also help to distinguish an actual gating of outward currents by Ca_i from an indirect effect of K on the membrane. When a transient Ca-dependent K current is elicited, for instance by injecting Ca, a current produced by a combination of K accumulation and a cross-over effect should have a reversal potential equal to the cross-over point, which is normally 10–20 mV positive to E_K in the Purkinje fibre. Secondly, near its reversal potential such a current should be biphasic, since the position of the cross-over depends on the changing K concentration outside the membrane, and a sufficiently large increase in K concentration will cause the cross-over point to move positive to the holding potential.

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