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THE DEPENDENCE OF SLOW INWARD CURRENT IN PURKINJE FIBRES ON THE EXTRACELLULAR CALCIUM-CONCENTRATION

BY H. REUTER*

From the Department of Physiology, University of Berne, Switzerland

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SUMMARY

1. In sodium-free solution electrical constants of short Purkinje fibres were similar to those in Tyrode solution. Alterations in the extracellular calcium concentration ($[Ca]_o$ = 0; 1·8; 7·2 mM) had no appreciable effect on these constants, unless the fibres were soaked in calcium-free solution for more than 40 min.

2. In sodium-free solution without calcium there was constant or increasing outward current in response to sudden depolarizations (voltage-clamp technique) over the whole voltage range −85 to +40 mV. In calcium-containing solution initial outward current was followed by a slow change in current towards zero which was sometimes large enough to produce a net inward current. This current had a threshold in the voltage range −60 to −40 mV. It was not affected by alterations in the extracellular chloride or magnesium concentrations. The dependence on $[Ca]_o$ suggests that the slow inward current is carried by calcium ions.

3. Negative slopes in the steady-state current–voltage relations were obtained in fibres soaked in calcium-containing solutions but were never observed in calcium-free solution.

4. The calcium equilibrium potential (E_{Ca}) was estimated to be about 150 mV, inside positive.

5. In Tyrode solution the slow inward current was smaller than in sodium-free solution and its threshold was shifted to about −20 to −10 mV. It was dependent on $[Ca]_o$ as in sodium-free solution. It was increased by adrenaline and not affected by tetrodotoxin.

6. It is concluded that calcium ions carry an appreciable membrane current in the inward direction when the membrane of the Purkinje fibre is depolarized. This calcium current may be involved in excitation–contraction coupling.

* Present address: Department of Pharmacology, University of Mainz, Langenbeckstr. 1, Mainz, Germany.

INTRODUCTION

There is much evidence from tracer experiments that calcium exchange in cardiac muscle is more rapid during contraction than at rest (e.g. Winegrad & Shanes, 1962; Langer & Brady, 1963; Niedergerke, 1963). Recent experiments have presented indirect evidence that in Purkinje fibres (Reuter, 1966) or in frog ventricles (Niedergerke & Orkand, 1966; Hagiwara & Nakajima, 1966) a part of the membrane current during depolarization may be carried by calcium ions. By the extension of the voltage-clamp technique to cardiac Purkinje fibres (Deck, Kern & Trautwein, 1964) new and better methods for analysing ionic currents in heart have become available. The present report describes voltage-clamp data obtained with the aim of giving information about Ca movement. The results suggest that there is an appreciable calcium inward current as a consequence of fairly strong membrane depolarization.

METHODS

Sheep and calf hearts were obtained at the slaughterhouse and carried to the laboratory in cool (about 4° C) Tyrode solution. Purkinje fibres were removed from the left ventricles. Two ligatures were set at a distance of 1–2 mm by means of fine silk threads, as suggested by Deck *et al.* (1964). The preparations were kept in oxygenated Tyrode solution at 37° C.

Membrane potentials were measured in the short fibre sections as potential differences between an intra- and an extracellular micro-electrode. The micro-electrodes were held by agar-Tyrode half-cells, the Ag–AgCl electrodes of the half-cells were connected to the grids of cathode follower tubes. The potential difference between the two electrodes was recorded with a Tektronix 502 oscilloscope. A second intracellular micro-electrode was used for passing current. The micro-electrodes were filled with 3 M-KCl (Nastuk & Hodgkin, 1950). They had resistances between 10 and 20 MΩ and tip potentials of less than 5 mV.

For voltage-clamp measurements a feed-back system similar to that used by Dudel, Peper, Rüdel & Trautwein (1966*b*) with a negative feed-back operational amplifier (Philbrick U.S.A. 4JX) was employed. Current was measured by the voltage drop across a 22 KΩ resistor between the bath and ground. The limitations of this kind of voltage-clamp technique have been described by Deck *et al.* (1964) and by McAllister & Noble (1966) and apply equally to the present experiments.

The composition of the bathing solutions is given in Table 1.

RESULTS

Electrical constants in sodium-free solution. Electrical constants of the Purkinje fibre membrane were obtained from four fibres soaked in sodium-free, calcium-containing (1·8 mM) solution. The data are listed in Table 2. Membrane capacity (C_m) was calculated from the initial capacitative current during small clamp steps. Input resistance (V_0/I_0) and membrane resistance (R_m) were determined from steady-state membrane currents flowing during small depolarizing clamp steps (5–10 mV). The present

TABLE 1. Composition of solutions (mM)

Solution	NaCl	Choline-Cl	Sucrose	KCl	$MgCl_2$	NaH_2PO_4	$NaHCO_3$	Tris-buffer (+HCl or maleic acid to pH 7·4)	Glucose
1 Tyrode	137	—	—	5·4	1·05	0·42	11·9	—	5
2 Na-free	—	137	—	5·4	1·05	—	—	5	5
3 Na-free	—	—	274	5·4	1·05	—	—	5	5

These solutions contained 0, 1·8, or 7·2 mM-$CaCl_2$. Solutions containing $NaHCO_3$ were equilibrated with 95 % O_2+5 % CO_2, solutions containing Tris-buffer were equilibrated with 100 % O_2.

values agree with those obtained from fibres soaked in Tyrode solution (Weidmann, 1952; Fozzard, 1966). Changes in external calcium-concentration ($[Ca]_o$) did not greatly affect membrane capacity and membrane resistance until the fibres deteriorated in calcium-free solution. The effect of calcium ions on the membrane resistance was small and non-significant. With ten fibres in sodium-free solution the values at extracellular calcium concentrations of 0, 1·8 and 7·2 mM were 1920 ± 265 (S.E.), 2370 ± 445 and $2240 \pm 475\ \Omega\ cm^2$, respectively. With six fibres in Tyrode solution the corresponding values were 1810 ± 310, 1820 ± 265 and $1500 \pm 160\ \Omega\ cm^2$.

TABLE 2. Electrical constants of Purkinje fibres

Expt. no.	Fibre diam. (μ)	l (mm)	RP (mV)	V_0/I_0 (KΩ)	R_m (Ω cm²)	C_m (μF/cm²)
1	80	2·1	86	140	2000	19
2	60	1·8	81	400	2700	8
3	100	1·8	83	267	2970	10
4	80	1·7	87	290	2300	9
Mean	80	1·85	84	275	2490	11·5

l = length of the fibre; RP = resting potential; V_0/I_0 = input resistance of the fibre; R_m = membrane d.c. resistance; C_m = membrane capacity. NaCl was replaced by choline-chloride in these experiments; $[Ca]_o$ = 1·8 mM.

When preparations were soaked in calcium-free solutions the resting potentials underwent only a small decrease (3–10 mV) during the first 20–40 min but dropped drastically thereafter (to values around −30 mV). As described by Dudel, Peper & Trautwein (1966*a*), a large drop in the resting potentials in calcium-free solution was always accompanied by a rise of membrane conductance and a loss of rectifier properties. The experiments to be reported in this paper were all performed with fibres which still had a resting potential close to normal.

Voltage control was not perfect during the first 1–5 msec of the clamps, especially if fibres soaked in Tyrode solution were depolarized (cf. Deck *et al.* 1964). The artifacts at the beginning of the clamps, however, did not affect slow currents which were of interest in the present paper.

Slow inward-current in sodium-free solutions with different $[Ca]_o$. In ten preparations soaked in sodium-free bathing fluids with or without calcium, constant outward current was observed during the whole duration (500 msec) of depolarizing clamp steps up to about −50 mV. During stronger depolarizations membrane currents were different in calcium-free and calcium-containing solution.

In calcium-free solution steady outward current increased with increasing magnitude of depolarization. In several preparations the outward current increased slowly with time, especially during strong de-

polarization (Fig. 1). This is consistent with the experiments of McAllister & Noble (1966) and Vassalle (1966) indicating a slow increase in potassium conductance (g_K).

In calcium-containing solutions (1·8 or 7·2 mM) there was initial outward current of the same shape and size as in calcium-free solutions during the first 20–50 msec of depolarization. The initial outward current, however, decreased and reached a steady-state value within 100–300 msec. In several preparations after the current minimum there was again a very slow rise in outward current (see Fig. 6) which may signify either a delayed increase of potassium current (McAllister & Noble, 1966) or a slow decrease of calcium current. The strength of the initial outward current as well as the decline of current depended on the magnitude of depolarization. During

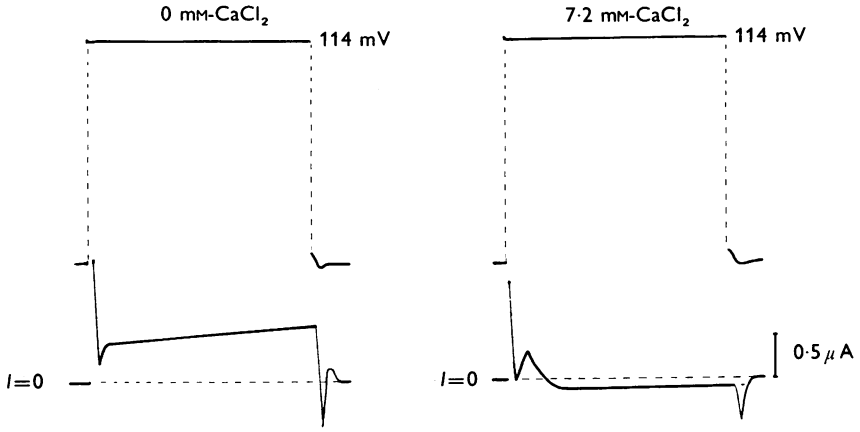

Fig. 1. Tracings of membrane currents recorded in a short Purkinje fibre (sheep) in sodium-free solution containing 0 mM (left) or 7·2 mM (right) $CaCl_2$; NaCl was substituted by choline-Cl. Top: change in membrane potential during voltage clamp from −80 mV (resting potential) to +34 mV. Bottom: membrane currents during voltage clamp; upward deflexion from $I = 0$ indicates outward current; pulse duration 500 msec. Note net inward current in calcium-containing solution.

potential clamps near the threshold range for the time-dependent changes in membrane current (−60 to −40 mV) the current decline was only small. With stronger depolarizations the initial outward current increased to higher peaks and afterwards declined to a larger extent.

In Fig. 1 membrane currents in calcium-free and calcium-rich bathing fluids are compared. In calcium-free solution there was outward current for the whole duration of the depolarizing pulse from −80 to +34 mV. In calcium-rich (7·2 mM) solution the initial outward current decreased and turned into inward current, indicating inward movement of positive charge or outward movement of negative charge. Since the inward current

in sodium-free solution is largely affected by alterations in $[Ca]_o$ and since it is not much affected by replacing choline-chloride by sucrose, it seems to be carried by inward movement of calcium ions rather than by outward movement of chloride ions. Alterations of $MgCl_2$ concentration in the bathing fluid between 0 and 10 mM had no influence on the inward current. In 8 of 10 experiments there was net outward current left in fibres soaked in calcium-containing solution, but this was always lower than the current recorded in the absence of calcium (Fig. 2). The time-dependent change in membrane current observed in calcium-containing solutions was always abolished or greatly reduced in calcium-free solutions before the mem-

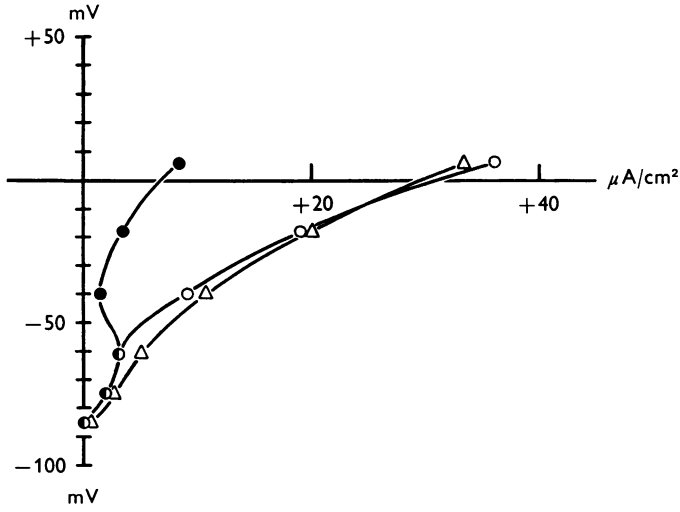

Fig. 2. Current–voltage relations of a Purkinje fibre (sheep) in sodium-free solution with and without $CaCl_2$. From values obtained in calcium-containing (1·8 mM) solution steady-state current strengths at the end of the depolarizing pulses (●) and current strengths of initial peak outward current (○) are plotted; from values obtained in calcium-free solution only steady-state current–voltage relation (△) is plotted. Further description in text.

brane resistance had appreciably decreased. This strongly suggests that the decline in current records during depolarizations, first observed by Deck & Trautwein (1964) and Hecht, Hutter & Lywood (1964), is due to an inward movement of calcium ions.

Figure 2 shows current–voltage relations of a Purkinje fibre soaked in sodium-free solution (solution 2 in Table 1) with and without calcium. In calcium-containing solution (1·8 mM) the steady-state current–voltage relation shows negative conductance in the range −60 to −40 mV, with a current minimum near −40 mV. At the threshold for the time-dependent current change, as discussed above, the steady-state membrane current was

less than at smaller depolarizing steps (cf. Dudel, Peper & Trautwein, 1966*a*). This negative slope of the current–voltage relation seems to be related to the inward movement of positive charge (cf. Figs. 1 and 3). If choline-chloride in sodium-free solutions was replaced by sucrose the negative conductance was not much affected. In calcium-free solution the negative slope of the current–voltage relation disappeared and the curve became more flat (Fig. 2). The initial peak outward current observed in calcium-containing solution and the steady-state outward current recorded in calcium-free solution, however, were similar in amplitude. This may indicate that the shift of the steady-state current–voltage relation along the current axis by an increase in $[Ca]_0$ is at least partly due to a

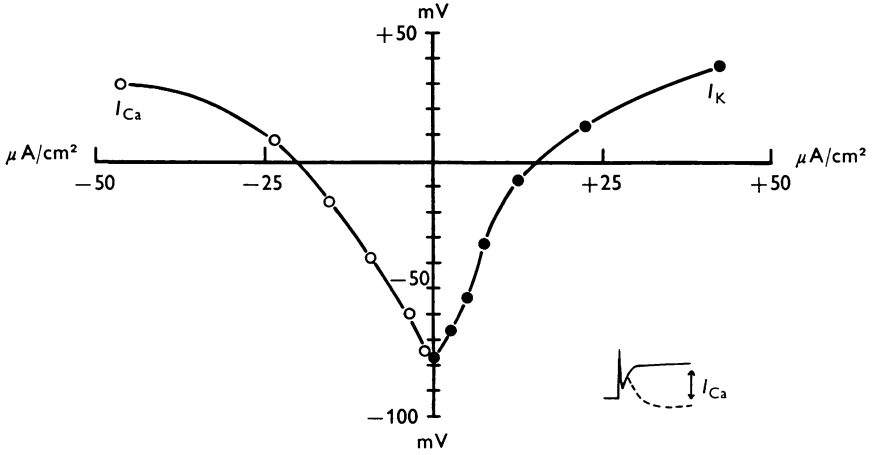

Fig. 3. Membrane current densities at different membrane potentials for inward and outward currents in sodium-free solution (same fibre as in Fig. 1). The inset shows typical traces of membrane currents in the absence (continuous line) and presence (interrupted line) of calcium (7·2 mM). The difference is considered to represent total inward current of calcium ions (I_{Ca}) and is plotted to the left (○). The curve to the right (●) represents outward current in calcium-free solution which is considered to be mainly carried by potassium ions (I_K). Note net inward current of calcium.

delayed increase in calcium current rather than to a change of membrane permeability for e.g. potassium or chloride ions or an increase in threshold for delayed rectification (cf. Reuter, 1966; McAllister & Noble, 1966).

Membrane current densities (μA/cm² membrane) for inward and outward current at different membrane potentials are plotted in Fig. 3. The values were calculated from the same experiment shown in Fig. 1. The curve to the right of the ordinate was obtained in calcium-free solution and represents outward current which is considered to be mainly carried by potassium ions (I_K). The inset in Fig. 3 shows typical traces of mem-

brane currents in the absence and presence of calcium, as shown in Fig. 1. A series of measurements at different potential levels was made and the differences between current strengths in calcium-free and calcium-

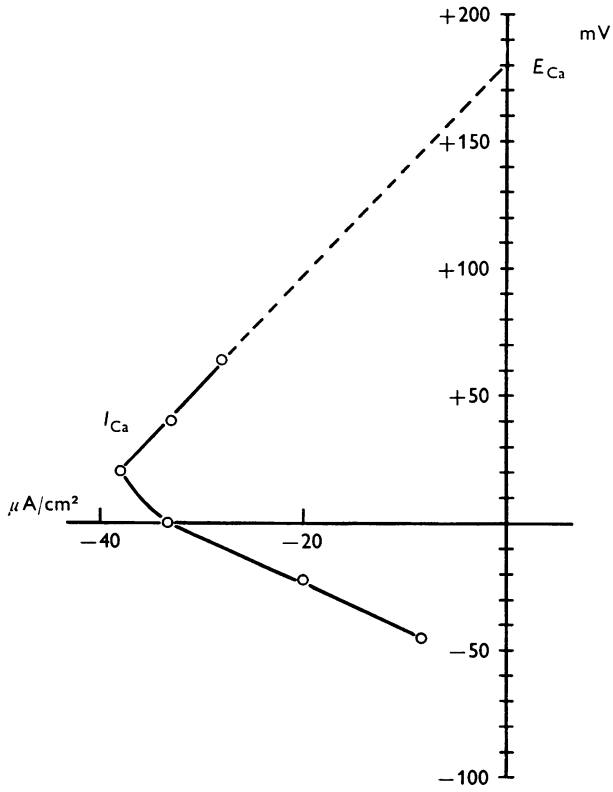

Fig. 4. Current–voltage relation for slow inward current in a Purkinje fibre in sodium-free, calcium-containing (7·2 mM) solution. The positive slope of the current–voltage relation between +20 and +65 mV, when extrapolated (interrupted line) intersected with the voltage axis at +180 mV. This value is considered to represent calcium equilibrium potential (E_{Ca}).

containing solutions were tentatively considered to represent total current carried by calcium ions (I_{Ca}). The values were plotted to the left of the ordinate since calcium-current is an inward current. In this instance inward current was even larger than outward current.

In a few experiments the membrane potential of the Purkinje fibres could be clamped up to +70 mV. It could then be observed that the slow inward current became smaller again in the range 20–40 mV, inside positive. The resulting positive slope of the current–voltage relation for inward current, when extrapolated, intersected with the voltage axis at

about +180 mV (Fig. 4). Although there may be several sources of error in the determination of the calcium equilibrium potential (E_{Ca}) by this method, it is expected from these experiments that E_{Ca} is in the range of +90 to +180 mV. This corresponds to an intracellular calcium-concentration (at various $[Ca]_o$) of about 10^{-5}–10^{-9} M as calculated from Nernst's equation ($E_{Ca} = RT/2F \ln [Ca]_o/[Ca]_i$). A net gain in intracellular calcium concentration of about 2×10^{-5} M is calculated for an impulse of 300 msec duration from the charge transfer during maximal calcium inward current.

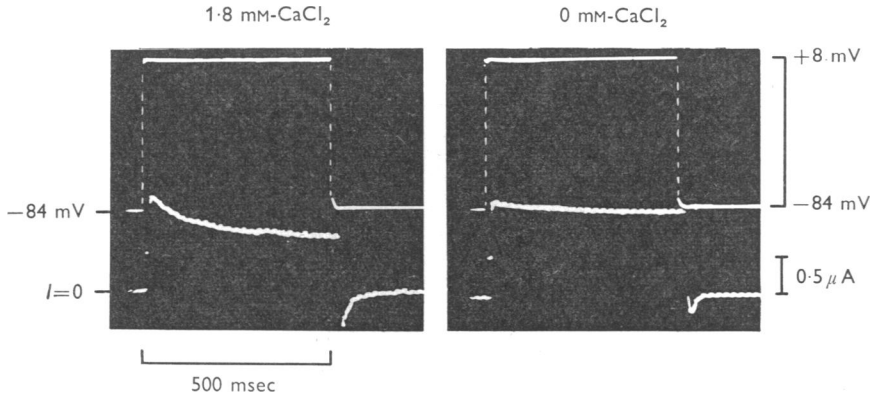

Fig. 5. Membrane currents (bottom) recorded in a short Purkinje fibre (sheep) in Tyrode solution containing 1·8 mM (left) or 0 mM (right) $CaCl_2$. Voltage clamp (top) from −84 mV (resting potential) to +8 mV.

Slow inward-current in Tyrode solution with different $[Ca]_o$. In principle the results concerning slow inward current in sodium-containing solution were similar to those obtained with fibres soaked in sodium-free solution. The slow inward current always observed in the presence of calcium was absent or greatly reduced in calcium-free solution (Fig. 5). In all experiments performed in Tyrode solution (thirteen fibres) outward current was larger than the slow inward current. The possibility that this inward current is due to a slow inactivation or reactivation of sodium current can be excluded, since it is even larger in sodium-free solution than in Tyrode solution. In addition, the equilibrium potential for this current has a much more positive value than the sodium equilibrium potential, as could be seen from clamp steps up to more than +50 mV. The dependence on $[Ca]_o$ again suggests that the slow current is carried by calcium ions. Sodium inward current is rapidly inactivated, whereas calcium current is well maintained. The threshold potential for the slow inward current in calcium-containing Tyrode solution was at about −20 mV as compared to −50 mV in sodium-free solution. In contrast, McAllister & Noble (1966)

found an opposite effect on the threshold for delayed potassium outward current.

There seems to be one point on which the present results in sodium-containing solutions differ from those obtained by Deck & Trautwein (1964) and by McAllister & Noble (1966). These authors recorded a 'tail' of outward current when the membrane was clamped back to its former resting potential. The present records do not show a slowly subsiding outward current. The discrepancy is most probably due to the relatively high potassium concentration of the present bathing solutions. For, 'tails' could be obtained when on one occasion $[K]_o$ was reduced from 5·4 to 2·7 mM.

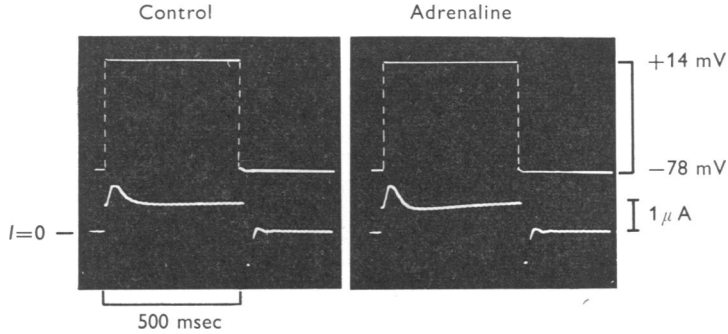

Fig. 6. Membrane currents (bottom) recorded in a short Purkinje fibre (calf) in Tyrode solution ($[Ca]_o$ = 1·8 mM) without (left) and with adrenaline (5×10^{-7} g/ml.; right). Voltage clamp (top) from −78 mV (resting potential) to +14 mV.

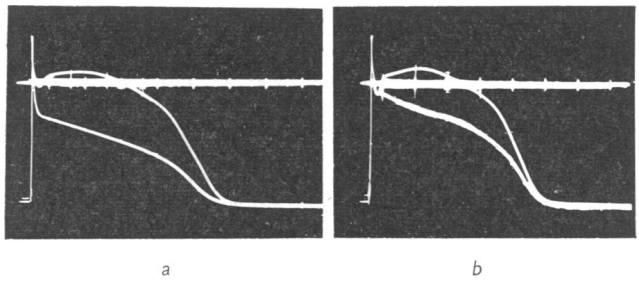

Fig. 7. Superimposed action potentials of two Purkinje fibres (sheep). (*a*) Effect of change in $[Ca]_o$ from 0·45 mM (lower trace) to 7·2 mM (upper trace); resting potential = −81 mV. (*b*) Control (lower trace) and effect of adrenaline 5×10^{-7} g/ml. (upper trace); resting potential = −85 mV. Upstrokes of action potentials have been retouched.

The calcium-current was not affected by large concentrations of tetrodotoxin (10^{-5} g/ml.) which greatly reduced or abolished sodium inward current in Purkinje fibres. On the other hand, adrenaline (5×10^{-7} g/ml.), which is known to increase ^{45}Ca-influx in contracting guinea-pig auricles

(Reuter, 1965), also increased slow inward current in four Purkinje fibres (Fig. 6). The plateau of the action potential of a Purkinje fibre was shifted to more positive potential levels either by increasing $[Ca]_o$ or by addition of adrenaline (Fig. 7). This observation is in keeping with the idea that calcium inward current flowing in the voltage range of the plateau is sensitive to $[Ca]_o$ and to adrenaline. The membrane resistance is relatively high during the plateau, and it would thus be expected that additional transfer of charge by calcium ions can shift the membrane potential by an appreciable amount.

DISCUSSION

Voltage-clamp data for short Purkinje fibres have recently been obtained by several groups. On certain points there is agreement, on others the experimenal results or the interpretations disagree.

All the results agree in showing that the steady-state current–voltage curve of preparations kept in sodium-free but calcium-containing solution has a negative slope conductance over the voltage range of about -60 to -30 mV, with a minimum of outward current near -30 mV (Dudel *et al.* 1966*a*; McAllister & Noble, 1966; this paper, Fig. 2). In my opinion the sensitivity of the current–voltage curve to alterations in $[Ca]_o$ indicates that calcium ions carry electrical charge. Inward calcium current would partly compensate for outward current (presumably mainly potassium current) and, according to this interpretation, give rise to a current minimum near -30 mV. Negative conductance was never observed in calcium-free solution, although the current–voltage relation was still non-linear. Furthermore, the membrane resistance as calculated from the initial outward current was not very different from that obtained in calcium-containing solution . The most powerful argument, however, in favour of a sizeable calcium influx is the occasional observation of a 'slow' *net* inward current in sodium-free solution together with its sensitivity to calcium removal. Such *net* inward current can hardly be carried by any ion other than calcium. A time-dependent decrease of outward potassium current (Deck & Trautwein, 1964) could only explain the findings if there were also inward current due to some other ions. Moreover, McAllister & Noble (1966) have shown that the time-dependent decrease in outward current recorded during strong depolarizations is not attributable to inactivation of the slow potassium conductance observed at weaker levels of depolarization.

Trautwein, Dudel & Peper (1965, Fig. 8) also report on net inward current in sodium-free solution at membrane potentials near -30 mV. Their current seems to be insensitive to calcium removal and the authors therefore conclude that it cannot be carried by calcium ions. In a later

report (Dudel *et al.* 1966*b*) it is shown that this current grows in amplitude when the rate of depolarization is increased; or, expressed in different terms, there is rapid 'inactivation'. It is felt, therefore, that no real contradiction exists if one clearly distinguishes between two components of non-sodium inward current, (i) an 'early' and transitory current which is insensitive to $[Ca]_o$ and for which, to quote Dudel *et al.* (1966*b*), no ionic candidate can be named, and (ii) a slowly rising and well maintained current which is sensitive to $[Ca]_o$ (Reuter, 1966; Dudel *et al.* 1966*a*, p. 266, and Figs. 2, 5; present results), and thus most probably carried by calcium ions. The major difference between the interpretation given in the present paper and that given by McAllister & Noble (1966) is that at least some of the slow current changes are attributed to a significant calcium current, whereas McAllister & Noble were inclined to interpret the effects of calcium changes in terms of shifts in the voltage dependency of non-calcium currents (cf. Weidmann, 1955). It may be, of course, that both effects of calcium are present but more experiments are needed to determine the relative importance of the two effects.

On theoretical grounds the cardiac action potential might be terminated either by a time-dependent increase in g_K, as suggested by Noble (Noble, 1962; McAllister & Noble, 1966), or mainly by a slow and time-dependent decrease of g_{Na}, as suggested by Deck & Trautwein (1964). In sodium-free and calcium-free solutions the present current records as a rule show a creep towards higher values, consistent with the assumption of a time-dependent increase in g_K upon depolarization.

In analysing the present data calcium influx is calculated by taking the difference between recorded membrane current in the presence and in the absence of extracellular calcium. A similar procedure has successfully been applied in sorting out sodium current and non-sodium current in squid nerve (Hodgkin & Huxley, 1952*a*, *b*). This method, however, is only applicable if the ion species in question cross the membrane independently from one another. In the case of Purkinje fibres, it has been noted by several investigators that changes of membrane current as a function of time become less pronounced when sodium ions are removed from the extracellular space, suggesting an influence of $[Na]_o$ on the voltage- and time-dependent changes of g_K (Deck & Trautwein, 1964; Vassalle, 1966; McAllister & Noble, 1966). In contrast, in the present investigation slow inward current became more pronounced in sodium-free solution. This would agree with the assumption that sodium and calcium ions compete for inward movement through the same channels in the membrane (Wilbrandt & Koller, 1948; Lüttgau & Niedergerke, 1958). Dudel *et al.* (1966*a*) argue that the subtraction principle may not hold true in the case of calcium current. This may well be so; but there is little one can do at present to find out to

what extent the subtraction principle is justified. On the other hand it is re-assuring (i) that the dependence of potassium current in Purkinje fibres on membrane voltage, as determined by radio-potassium (Haas & Kern, 1966), fails to show a negative slope conductance over the voltage range -60 to -30 mV, and (ii) that flux measurements by radio-calcium show an increase in calcium-influx during activity as well as a dependence of ^{45}Ca-influx on $[Ca]_0$ (Winegrad & Shanes, 1962; Niedergerke, 1963). It is felt that the *quantitative* aspect of the present analysis will possibly be in need of revision although the main conclusion should hold true, namely that an appreciable amount of current is carried into Purkinje fibres by calcium ions when depolarization beyond a certain threshold has been applied to the surface membrane for a certain length of time. Such inward movement of calcium ions, however, must clearly be distinguished from effects of extracellular calcium on the movement of other ions through the membrane of Purkinje fibres (Weidmann, 1955).

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