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INDUCTION AND REMOVAL OF INWARD-GOING RECTIFICATION IN SHEEP CARDIAC PURKINJE FIBRES

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SUMMARY

1. In sheep cardiac Purkinje fibres superfused with K-free, Na-free medium, the membrane potential can be stable either at a low negative level (−50 mV) or at a high negative level (−100 mV). The mechanism underlying the existence of these two stable potential levels was investigated using the two-micro-electrode voltage-clamp technique.

2. By applying a voltage clamp of a certain duration at an appropriate level the membrane potential could be shifted from one stable level to the other. The shift was observed in Cl-free medium, excluding a redistribution of Cl as a possible explanation.

3. Currents during and following a voltage step and their change with amplitude and duration of the voltage step could not be explained on the basis of depletion or accumulation of K ions in the narrow extracellular clefts.

4. Instantaneous currents determined from the high negative resting level showed a high conductance and a pronounced inward rectification, while measurements from the low negative resting level indicated a low conductance and absence of inward rectification. The steady-state current–voltage relation was dependent on the holding potential and showed memory or hysteresis.

5. Estimation of the conductance by superimposed short voltage-clamp pulses showed an increase in conductance during a hyperpolarizing clamp from the low negative level and a decrease in conductance during a depolarizing clamp from the high negative level. The time-dependent current during a hyperpolarizing clamp from the low negative level reversed direction at a potential level corresponding to E_K, assuming a cleft K concentration of about 1 mM. In the presence of 0·1 mM-Ba the time-dependent current was abolished.

6. The results suggest that the shift between the two stable levels is due to a time-dependent conductance change in the K inward rectifier channel, i_{K_1}. The existence of memory excludes activation or de-activation only depending on the voltage gradient. Interaction of extracellular K ions with a site in the membrane is proposed as the activating mechanism.

INTRODUCTION

Inward rectification plays an important role in the genesis of the plateau and the long duration of the cardiac action potential. The K conductance which is high at the resting potential falls to a low value upon depolarization. According to an analysis by McAllister & Noble (1966) the conductance of the inward rectifier is dependent on the electrochemical gradient for K ions ($E_m - E_K$) and the extracellular K concentration, $[K]_o$. The conductance increases for negative values of $E_m - E_K$ and decreases for positive values; the conductance is higher the greater $[K]_o$. The change in conductance with potential is assumed to occur instantaneously; in reality this means that the mechanism responsible for the opening and closing of the channels is considered to be so fast that its evolution in time can be neglected when compared to the time-dependent changes of other conductance channels. The experiments to be reported will show, however, that (1) slow time-dependent conductance changes occur in the K inward rectifier and (2) the mechanism responsible for the opening and closing of the channels is different from a simple voltage-dependent gating mechanism. Preliminary results have been published in communication form (Carmeliet, 1979).

METHODS

Purkinje preparations of sheep hearts 1 mm in length were prepared according to the procedure described by Aronson, Gelles & Hoffman (1973). The two-micro-electrode voltage clamp technique of Deck, Kern & Trautwein (1964) was employed. The membrane potential was amplified and led to the input of the voltage clamp amplifier which also received rectangular command pulses. The voltage clamp amplifier had an output voltage range of ± 150 V and its output was passed through the current micro-electrode inserted midway in the preparation. In order to prevent damage to the preparation by excessively high currents the clamp circuit was automatically interrupted, using an overload detector (New & Trautwein, 1972). Transmembrane current was measured by an operational amplifier circuit that held the bath potential at virtual ground. Signals were displayed on an oscilloscope and on a pen writer (Gold Brush 2400). The composition of normal Tyrode in mM was NaCl, 150; KCl, 5·4; $CaCl_2$, 3·6; $MgCl_2$, 0·5; Tris Cl, 10; the solution was gassed with 100% O_2. Na-free, K-free solution was made by substituting 150 mM-Tris Cl or choline Cl for NaCl and omitting the KCl. Solutions containing a variable amount of K were made by substituting KCl for Tris Cl.

RESULTS

Two stable potential levels in K-free, Na-free solution

The starting point of this investigation was was an unpublished observation by Hecht & Weidmann (cited by Carmeliet, 1961*b*) that sheep cardiac Purkinje fibres show two stable resting potentials in K-free, Na-free solution. A resting potential of about -100 mV is obtained when the solution is first made Na-free and afterwards K-free; a low level of about -50 mV is obtained when the reverse sequence is followed. A shift from one level to the other can be brought about by clamping the potential at an appropriate level for a given duration. The phenomenon and the accompanying current changes are illustrated in Fig. 1. The preparation was first superfused with Na-free medium, followed by a K-free, Na-free solution, and the potential stabilized at -100 mV. From this resting level the potential was clamped

at −60 mV during 11 sec (Fig. 1*A*). During the clamp an outward current is generated, which declines with time. After 11 sec the clamp is released; the potential does not return to the −100 mV level but slowly declines to −50 mV where it stabilizes. Following this shift in resting potential to the lower level the membrane was clamped at −90 mV during 11 sec (Fig. 1*B*). Instantaneously an inward current flows through the membrane and declines with time to become slightly net outward at the end of the clamp. When the clamp is released the membrane does not return to the initial −50 mV but slowly hyperpolarizes and reaches −100 mV after about 20 sec where it stabilizes.

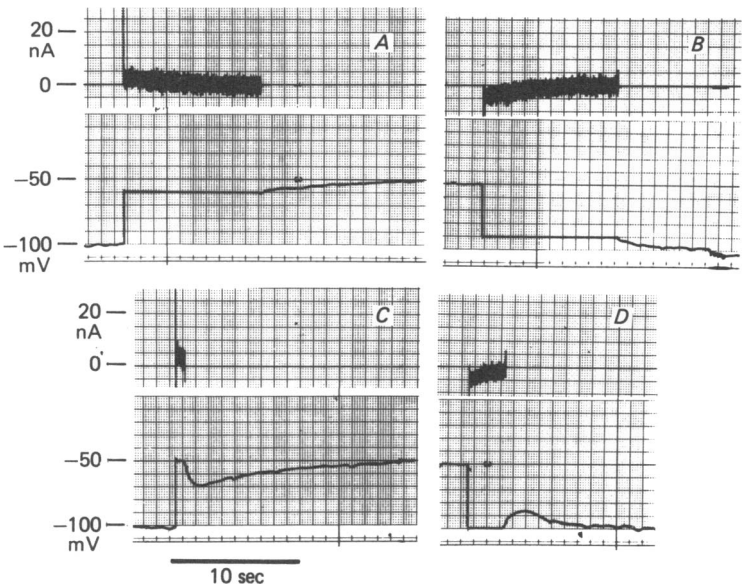

Fig. 1. Demonstration of the existence of two stable potential levels and the possibility of shifting the potential from one level to the other by appropriate clamps in a sheep cardiac Purkinje fibre superfused with a K-free, Na-free medium. *A*, depolarizing clamp to −60 mV during 11 sec; *B*, hyperpolarizing clamp to −90 mV during 11 sec; *C*, depolarizing clamp to −50 mV during 1 sec; *D*, hyperpolarizing clamp to −100 mV during 3 sec.

A shift from one level to the other can also be brought about by other clamp programmes. This is illustrated for a depolarizing clamp to −50 mV during 1 sec in Fig. 1*C* and back to −100 mV for 3 sec in Fig. 1*D*. It is interesting to note that the membrane potential following the release of the depolarizing clamp initially hyperpolarizes before returning to the −50 mV level (Fig. 1*C*), while the release of the hyperpolarizing clamp results in an initial depolarization followed by hyperpolarization to −100 mV.

Analysis of the 'threshold' for the potential shift: a voltage- and time-dependent phenomenon

Voltage clamp of variable duration

The shift from one stable potential level to the other is not an instantaneous phenomenon but requires time. In order to estimate the importance of this parameter the membrane was clamped at a given potential for variable durations and the evolution of the membrane potential was followed on release of the clamp. Fig. 2 illustrates results obtained for hyperpolarizing (*A*) and depolarizing (*B*) clamps. The

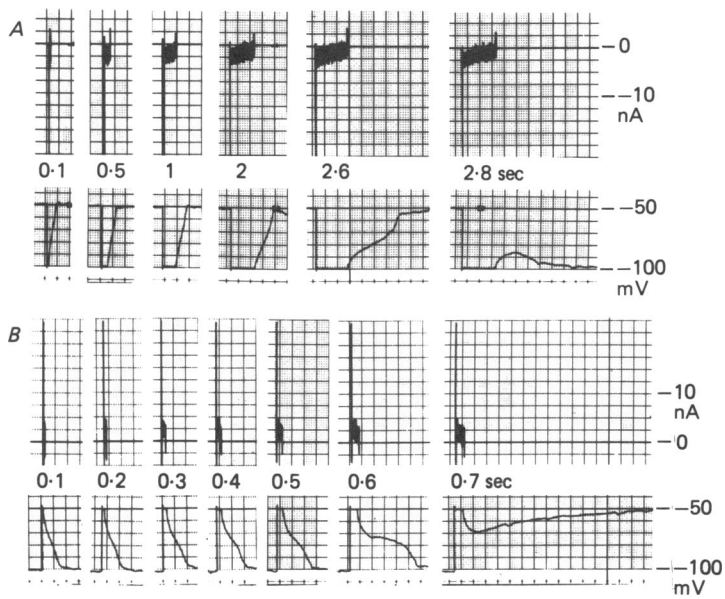

Fig. 2. Effect of increase in duration of hyperpolarizing (*A*) and depolarizing (*B*) clamps on the evolution of the membrane potential after release of the clamp. Figures between the current and potential records indicate duration of the clamp in seconds. With increase in clamp duration the return of the potential to the initial level is retarded. For a critical clamp duration different for depolarizing and hyperpolarizing clamps, the potential stabilizes at a new level. Sheep cardiac Purkinje fibre superfused with K-free, Na-free solution.

two stable potential levels are −50 and −100 mV. When the membrane was clamped from −50 to −100 mV for 100 msec (Fig. 2*A*) and the clamp released, the potential quickly returned to the initial low level (−50 mV). However, with increase in clamp duration the spontaneous depolarization on clamp release became slower and after a 2·8 sec clamp, the potential stabilized at the hyperpolarized level of −100 mV. For depolarizing clamps to −50 mV (Fig. 2*B*) a similar succession of events is recorded. With an increase in clamp duration the plateau-like repolarization to the high potential level is more and more retarded, and after a clamp of 0·7 sec, repolarization to the maximum negative potential fails and the potential stabilizes at −50 mV.

The actual value of the clamp duration required to cause the shift in potential was

variable from one preparation to the other. In six out of thirteen experiments in which this phenomenon was investigated, the clamp duration for threshold was shorter for depolarizing than for hyperpolarizing clamps (as in Fig. 2); in five the reverse was true and in two no difference was found. Most preparations were thus characterized by a preference for one of the two stable potentials. In some preparations, this preference was so marked that only one stable potential could be obtained; the results on these preparations (about 20% of the total) were usually not analysed. The actual value of the 'threshold' duration was also variable from one preparation to another and was dependent on the potential level of the clamp; extreme values of 0·5–60 sec were found (median 10 sec).

Voltage clamp to different potential levels

The possibility of obtaining a shift from one potential level to another was dependent on the clamp voltage (Fig. 3). In Fig. 3*A* the resting potential was −55 mV and hyperpolarizing clamps of about 5 sec were applied to levels between −90 and −130 mV. In all cases an inward current flows through the membrane during the clamp. This current decreases with time for clamp levels between −90 and −110 mV, is more or less constant at −120 mV and increases at −130 mV. Threshold for a shift to the high resting potential is obtained at −105 mV or more negative levels.

In Fig. 3*B* the resting potential was −110 mV and depolarizing clamps between −90 and −40 mV were applied. During the clamps to levels between −90 and −70 mV an outward current flows through the membrane; the current is constant or slightly increasing with time. On release of the clamp the potential returns to the initial high level. For clamps positive to −60 mV the outward current shows a pronounced decrease with time. On release of the clamp the potential shifts to the low level eventually after a transient oscillatory undershoot.

In general, hyperpolarization beyond a certain level results in a stable shift to the high resting potential and, on the other hand, depolarization beyond a given level ends in a shift to the low resting potential. The following exceptions, however, should be mentioned: very strong hyperpolarizations sometimes were not followed by stabilization at the high level and strong depolarizations did not result in a shift to the low level. Although in all these instances large currents flow through the membrane the results are not likely to be caused by damage but rather due to depletion–accumulation (see later).

Hypothetical considerations

At this stage of the description it seems worthwhile to introduce possible explanations for the existence of two stable states. At the resting potential(s) net membrane current should be zero and the slope of the current–voltage relation should be positive. Due to the particular experimental condition of Na-free, K-free solution the number of currents which determine the I–V relation is restricted. Passive inward as well as active outward currents carried by Na ions are excluded. After one hour perfusion with a Na-free medium the intracellular Na concentration may be considered negligible; this can be concluded from Na efflux studies (Bosteels & Carmeliet, 1972) and from direct intracellular Na activity measurements (Ellis, 1977). In Na-free medium also the pacemaker current is largely reduced (Deck *et al.* 1964;

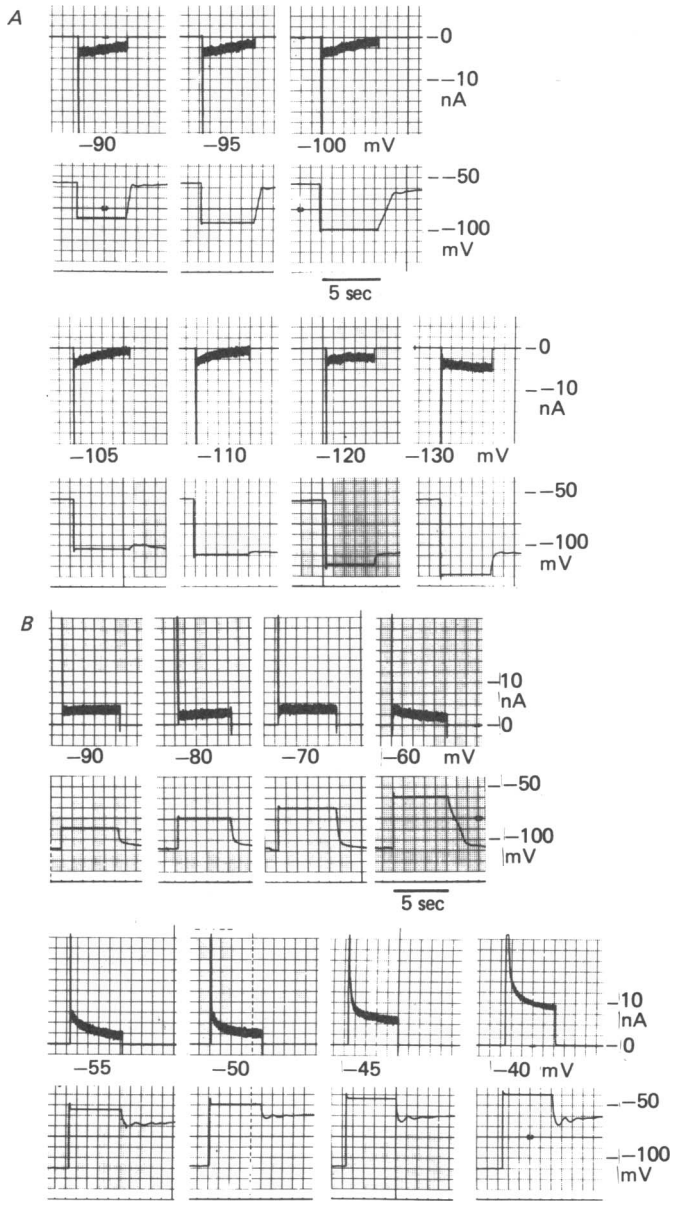

Fig. 3. *A*, effect of clamp level of hyperpolarizing clamps on the evolution of the membrane potential after release of the clamp. Level of the clamp in mV is indicated by figures between current and potential records. The duration of the clamp was 5 sec. 'Threshold' for steady repolarization is obtained for clamps of −105 mV and more negative. A decrease in inward current is observed in all records except for −120 and −130 mV where the direction of the current is reversed. Sheep cardiac Punkinje fibre superfused with K-free, Na-free medium. *B*, effect of level of depolarizing clamps on the evolution of the membrane potential after release of the clamp. Clamp level is indicated by figures between current and potential records. For clamps positive to −60 mV a decreasing outward current is observed. Threshold for steady depolarization is obtained for clamps to −55 mV and more positive. Sheep cardiac Purkinje fibre superfused with K-free, Na-free medium.

McAllister & Noble, 1966; DiFrancesco, 1981*a*, *b*). For potentials negative to −60 mV other time-dependent currents, such as the positive dynamic current (i_{qr}), the slow inward current (i_{si}) and the slow outward current (i_x) are outside the activation range of potentials (see Carmeliet & Vereecke, 1979) (in the section 'Interference of other currents' this problem is further analysed). With respect to K currents it is important to note that the K concentration in the extracellular clefts will not be zero due to the net loss of K from the cells in K-free solution. The equilibrium potential for K ions, E_K, will thus attain a finite value. Major changes in the intracellular K concentration, however, may be neglected: experiments with ^{42}K have shown that the half-time for K efflux is in the order of 200 min (Carmeliet, 1961*a*; Carmeliet & Verdonck, 1977). Therefore the equilibium potential for K will not change too largely when the duration of the experiment in K-free solution is limited to less than 1 hr.

Taking these restrictions into consideration we may consider the following four different possibilities to explain a current-induced shift from one stable state to the other (Fig. 4). In Fig. 4*A* the membrane is characterized by a single current–voltage relation which intersects the voltage axis at three different levels, of which two have a positive slope (stable levels) and one a negative slope (unstable level). According to this hypothesis a hyperpolarizing or depolarizing clamp of sufficient magnitude should result in a quasi-instantaneous shift from one level to the other. The results in Fig. 2 however, show that clamps of rather long duration are required to cause the shift.

A second possibility to explain the shift between both stable levels is presented in Fig. 4*B*. It is assumed that chloride ions are passively distributed and carry an appreciable current. The effect of hyperpolarizing or depolarizing current is supposed to result in a change of intracellular Cl concentration; when the current is interrupted the membrane potential stabilizes at the new equilibrium potential for Cl ions.

This hypothesis, however, cannot be held for the following reasons: the amount of charge flowing across the membrane during a clamp which causes a shift in resting potential of 50 mV is insufficient to change E_{Cl} by this amount. From the currents in Fig. 3*A* and *B* (total current of about 20 nA) and assuming a preparation of 50 μm diameter and 1 mm length, it can be calculated that the change in the intracellular Cl concentration is of the order of 0·1 mM while about 40 mM concentration is required for a 50 mV change in equilibrium potential.

Furthermore, no simple relation exists between total charge transfer during the clamp and the possibility of shifting the potential to a new stable state on release of the clamp. The examples given in Fig. 3*A* show that the total amount of charge transfer is practically the same at −90 and −120 mV but the threshold is only obtained in the latter case; at −105 mV less charge flows during the clamp when compared to the clamp at −120 mV, but threshold is obtained in both cases. A similar conclusion can be reached from a comparison of the currents during depolarizing clamps (Fig. 3*B*). In all cases, whatever the amount of charge transfer, the new resting level is the same.

Finally, the importance of an eventual shift in E_{Cl} was excluded by the observation that a shift from one potential level to the other was also possible in Cl-free media (Cl ion substituted by acetylglycinate).

The two remaining hypotheses concern either a change in extracellular K and thus of E_K or a change in conductance.

Sheep Purkinje fibres are composed of cells separated by narrow intercellular clefts (Sommer & Johnson, 1968; Hellam & Studt, 1974). Diffusion in these narrow extracellular spaces and exchange with the bulk solution is slow. Since the volume of the narrow extracellular space is small (less than 1 % of the intracellular volume),

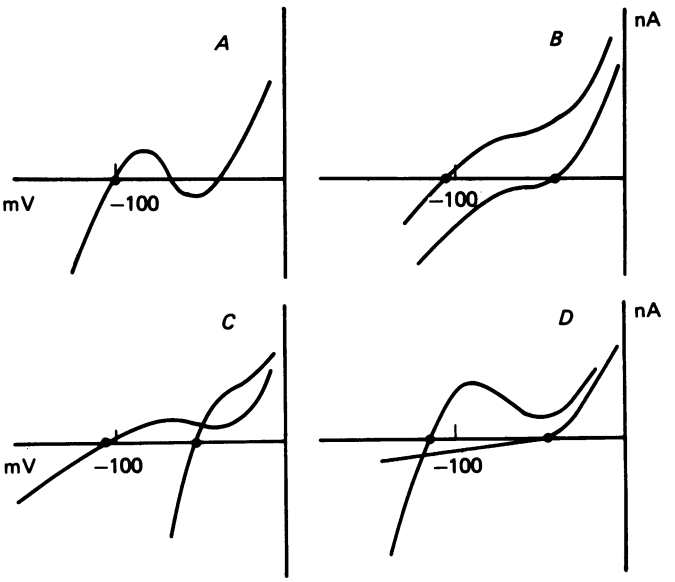

Fig. 4. Different hypothetical possibilities to explain the existence of two stable potential levels and the current-induced shift from one level to the other. *A*, the *I–V* relation intersects the voltage axis at three different levels, of which two have a positive slope (stable levels) and one a negative slope (unstable level). *B*, the shift between the two stable levels is due to a change in intracellular Cl concentration. The chloride *I–V* relation is assumed to be linear. *C*, the shift is caused by a current-induced K depletion or accumulation. *D*, the shift is due to induction or removal of inward rectifier properties in the i_{K_1} channel; E_K is assumed to remain constant.

accumulation or depletion occurs when the membrane potential deviates from the resting potential. During a depolarizing clamp from −100 to −50 mV outward current will result in K accumulation and a decrease in E_K. The reverse effect, i.e. depletion, is expected for a hyperpolarizing clamp. In each case the membrane potential is supposed to stabilize at the new E_K value.

According to the fourth hypothesis the shift in potential is not due to a change in E_K (E_K remains constant) but rather to an induction or the removal of inward rectifying properties. When the membrane potential is at the low level (−50 mV) the inward K rectifier is supposed to be closed and the current–voltage relation is mainly determined by the outward K rectifier. When the membrane is hyperpolarized, the conductance of the inward rectifier is activated in a time-dependent way. In contrast, when the membrane is depolarized the conductance is deactivated.

The experiments to be described in the following sections support the K conductance hypothesis. Evidence will be presented that activation of this K conductance is not a simple voltage-dependent gating mechanism.

The shift is not due to depletion or accumulation of K ions

(1) Depletion during a hyperpolarizing clamp will result in a negative shift of E_K. This shift will be variable with clamp level and clamp duration. In the absence of active K influx the change in the cleft K concentration, however, can never be such that E_K becomes negative to the voltage level of the clamp, because K ions would then move outwards, resulting in accumulation. This means that the potential level at which the membrane stabilizes after release of a hyperpolarizing clamp should always be positive to the clamp level but variable with clamp level and clamp duration. The examples in Figs. 1, 2 and 3, however, indicate that the membrane potential, once the threshold is attained, always stabilizes at the same level whatever the voltage or duration of the preceding clamp; the direction of the potential change was not invariably a depolarization, as expected, but either a hyperpolarization (Fig. 1*B*), a depolarization (Fig. 3*A*) or a depolarization followed by hyperpolarization (Figs. 1*D* and 2*A*). A similar argumentation can be held for depolarizing clamps. The level at which the membrane potential stabilized did not change with clamp level or clamp duration. It could be positive (Fig. 1*A*) as well as negative to the clamp level (Fig. 3*B*). In many cases the evolution was biphasic: after an initial hyperpolarization the potential showed a secondary depolarization (Figs. 1*C* and 2).

(2) Conductance measurements at the two stable resting potentials also are contrary to expectations based on a change in E_K. It is well known that the membrane conductance depends on the extracellular K concentration and is higher at elevated $[K]_o$ (Mc Allister & Noble, 1966). If both stable levels are due to a difference in E_K the membrane conductance at the high negative level (low $[K]_o$) should thus be smaller than at the low negative level (high $[K]_o$). Measurements of the conductance by the instantaneous *I–V* relation reveal, however, a greater conductance over the whole voltage range when the membrane is stabilized at the hyperpolarized level (see next section and Fig. 8).

(3) The time-dependent current changes during clamps were contrary to those expected for depletion. Depletion currents are characterized by a decreasing inward current, the time-dependent component being greater the greater or longer the applied potential gradient (Baumgarten, Isenberg, McDonald & TenEick, 1977). The examples in Fig. 3*A* on the contrary show that a decreasing inward current at potential levels between -90 and -110 mV is replaced by an increasing inward current at -130 mV. A similar reversal of the current is also observed in the examples of Fig. 5 (compare clamps to -100 and -130 mV, E_h -60 mV). The tail currents following depolarizing clamps also cannot be explained by depletion. Fig. 6*A* shows that a 50 msec clamp from -95 to -40 mV is followed by an inward tail on return to the holding potential. However, as the clamp duration is increased to 100 and 200 msec (Fig. 6*B* and *C*) the inward tail decreases in amplitude. For depletion currents following a depolarization the contrary result would be expected: depletion should be more pronounced the longer the previous depolarization.

Finally, a depletion current during a hyperpolarizing clamp can never become net

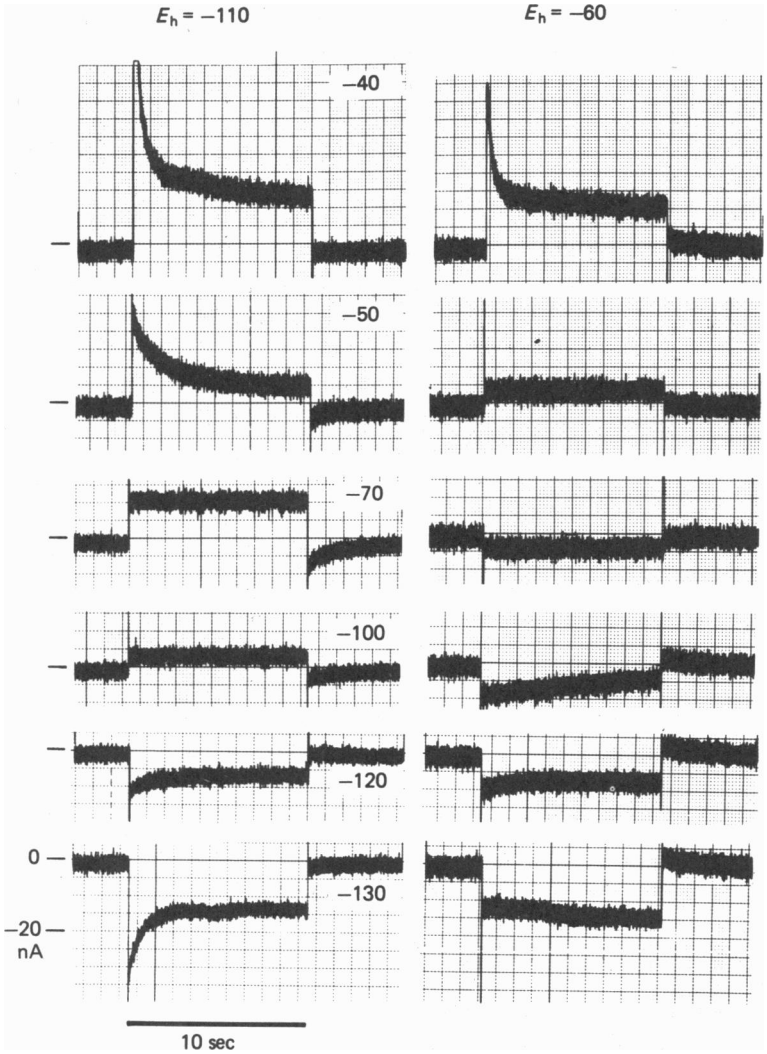

Fig. 5. Examples of currents obtained for 10 sec depolarizing and hyperpolarizing clamps from the high negative resting potential (−110 mV) and low negative resting potential (−60 mV) as holding potential. Sheep Purkinje fibre in K-free, Na-free solution.

outward. The examples in Figs. 1 *B* and 11, however, show that a net outward current can be present; this has been frequently observed for clamps from −50 mV to levels slightly positive to the high negative resting potential.

(4) Conductance measurements during clamps reveal an increase in conductance for hyperpolarizing clamps and a decrease for depolarizing clamps, while the reverse is expected for depletion and accumulation respectively (see next section).

From the preceding description it may not be concluded that depletion–accumulation phenomena are not present. Hyperpolarizing clamps from the high

negative resting potential result in a decreasing inward current, which is greater the more negative the clamp level (Fig. 5; compare clamps to −120 and −130 mV). In the same experiment the inward tail current following a depolarizing clamp is greater for a clamp to −70 mV than to −100 mV (Fig. 5). These tail currents were more pronounced the longer the depolarizing clamp. In the experiment illustrated in Fig. 6*D* a short (100 msec) depolarization from −95 to −75 mV is not followed by a tail current, but a definite inward tail is seen for a depolarization to the same level

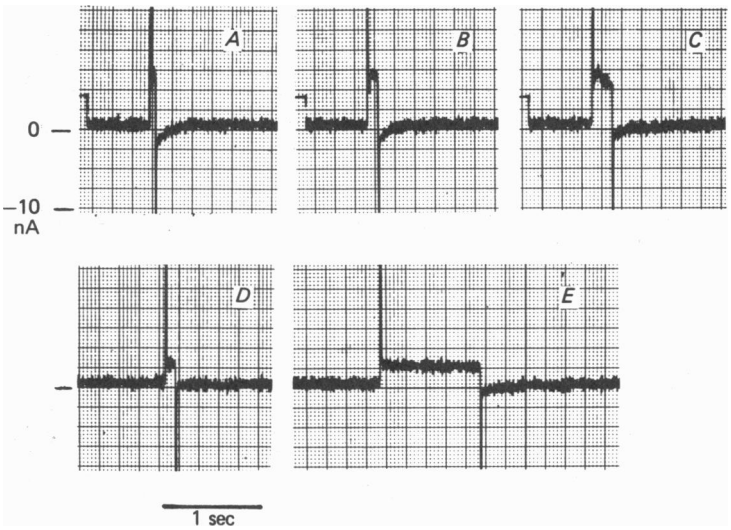

Fig. 6. Effect of clamp duration on tail currents on return to the holding potential. *A*, *B* and *C*, clamp from −95 to −40 mV during 50 msec (*A*), 100 msec (*B*) and 200 msec (*C*). *D* and *E*, clamp from −95 to −75 mV for 100 msec (*D*) and 1 sec (*E*).

which lasts 1 sec (Fig. 6*E*). The reason why depletion is seen in some experiments and not in others will become clear after the description of the results in the next section. Suffice it here to say that one of the conditions for observing K depletion, namely a conducting K channel, is not fulfilled in those experiments where depletion currents are absent.

The shift in potential is due to opening or closing of the K inward rectifier

From the previous section it is concluded that potassium depletion–accumulation, although present, cannot be made responsible for the shift from one stable level to the other. Another way to explain the observed phenomena is to assume that the two stable states are generated by changes in the conductance of the K inward rectifier channel. Arguments in favour of this hypothesis are found (1) in the results obtained by conductance measurements during clamps to different potentials and (2) in the comparison of the instantaneous current–voltage relation determined from the two stable potential levels as holding potential.

Conductance changes during hyperpolarizing and depolarizing clamps

Membrane conductance was estimated during hyperpolarizing and depolarizing clamps by superimposing 2–5 mV clamp steps of 50 msec duration at a frequency of 10/sec.

Fig. 7*A* illustrates the change in inward current and the accompanying change in conductance during a hyperpolarizing clamp. At the start of the clamp the current

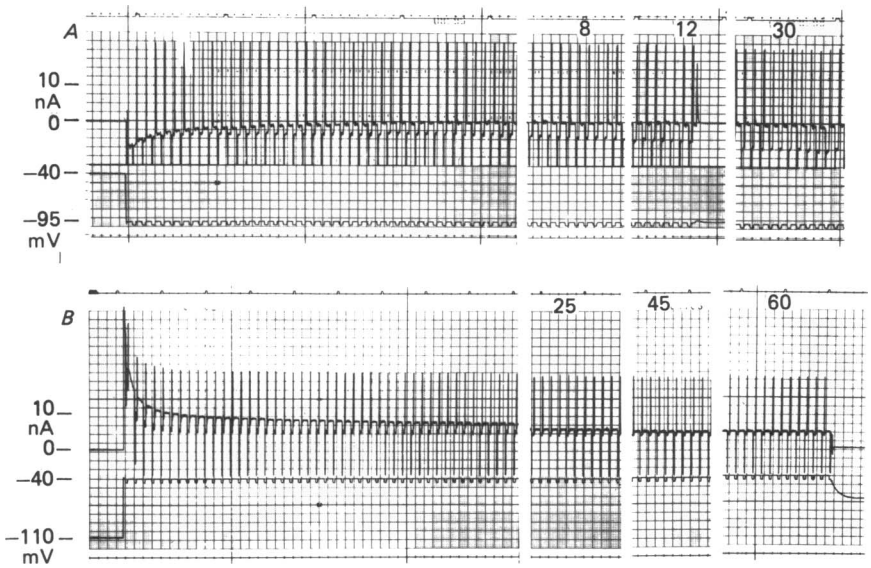

Fig. 7. *A*, estimation of membrane conductance during a hyperpolarizing clamp from −40 to −95 mV by superimposing 5 mV hyperpolarizing clamp steps of 50 msec duration at a frequency of 10/sec. Upper trace, time marks at 1 sec intervals; middle trace, current; lower trace, membrane potential. The Figure shows records obtained at the beginning of the hyperpolarizing clamp and after 8, 12 and 30 sec. After 12 sec the clamp was released and the potential was stable at −95 mV. Sheep cardiac Purkinje fibre superfused with K-free, Na-free solution. *B*, estimation of membrane conductance during depolarizing clamps from −95 to −40 mV by superimposing 5 mV hyperpolarizing clamp step of 50 msec duration at a frequency of 10/sec. Records shown were taken at the beginning and at different time intervals, given in seconds by figures. When the clamp was released the potential stabilized at the low level of −60 mV. Sheep cardiac Purkinje fibre superfused with K-free, Na-free solution.

changes due to the superimposed pulses are very small, indicating a small membrane conductance. During the course of the clamp the current pulses steadily increase, a result consistent with an increase in conductance. The relative increase was largest during the first two seconds; after 30 sec a stable value was obtained, at which time the conductance was about 10-fold larger than at the beginning of the hyperpolarizing clamp. This result suggests that the current pattern during a hyperpolarizing clamp is due to a rise in outward current. Fig. 7*B* illustrates the change in conductance for a depolarizing clamp to −40 mV in another preparation.

The membrane conductance decreases during the clamp and a steady state is obtained after 25–35 sec. The result suggests a decrease in outward current as responsible for the current pattern.

Dependence of the instantaneous I–V relation on the holding potential

The experiment consisted in clampling the membrane at different potential levels for 5 sec, either from a holding potential corresponding to the low level resting potential (−60 mV) or from a holding potential corresponding to the high level

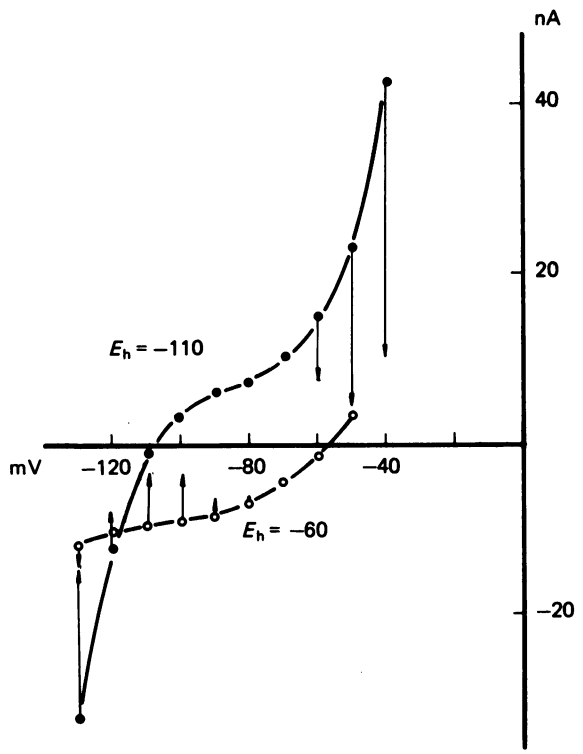

Fig. 8. Instantaneous current–voltage relations obtained from two different holding potentials: −110 mV (●) and −60 mV (○). Arrows indicate time-dependent changes during a 10 sec clamp. Sheep cardiac Purkinje fibre superfused with K-free, Na-free solutions. Same preparation as Fig. 5.

resting potential (−110 mV). In Fig. 8 instantaneous currents (open and filled circles) and currents obtained at the end of a 10 sec pulse (arrows) are plotted as a function of clamp potential. For the same experiment the current configuration and its evolution in time are illustrated in Fig. 5. The instantaneous *I–V* relation obtained from a holding potential of −60 mV is concave upwards with a slope which is fairly linear for potentials negative to −80 mV. During clamps to −70 and −80 mV the current was inward and constant. Negative to −80 mV and up to −120 mV inward current decreased with time, the rate of change being greater the more negative the

potential. At levels negative to −120 mV inward current increased, suggesting the existence of a reversal potential.

A totally different picture is obtained when the same potential levels are imposed from a holding potential corresponding to the high negative resting potential (−110 mV). The instantaneous *I–V* relation is now concave downwards. The slope conductance at the holding potential is fairly large; it increases at more negative potentials while it decreases at depolarized levels. The instantaneous *I–V* relation shows inward-going rectification properties, in the range of potentials negative to −60 mV, and outward rectification properties at potentials positive to −60 mV. A time-dependent decrease in outward current is observed for potentials at −60 mV and positive to this level.

The instantaneous current–voltage relations obtained from both holding potentials intersect at −115 mV. This voltage is close to the potential level at which the time-dependent current during a clamp from −60 mV reverses direction and is likely to correspond to the equilibrium potential for K ions, assuming the K concentration in the clefts to be about 1 mM. The difference between both instantaneous current–voltage relations also clearly shows inward-going characteristics. These results strongly suggest that a K current is implied.

The time-dependent changes during the clamps at different levels can be interpreted as a closing or an opening of the inward rectifier. For depolarizing clamps to −60 mV from a holding potential of −110 mV the instantaneous current is outward (K channel is open); during the clamp outward current decreases (K channel closes); when the inward rectifier is sufficiently closed, the potential will stabilize at the low level on release of the clamp. For hyperpolarizing clamps to −110 mV from a holding potential of −60 mV, the inward rectifier, which is closed at the start, now opens and carries more and more current with time: the time-dependent decrease in inward current is thus due to an increasing outward K current. When the inward rectifier is sufficiently open the potential will stabilize at the higher level. A strong argument in favour of this explanation is the finding of a reversal potential for the time-dependent current. For hyperpolarizing clamps to potentials negative to the intersection of the instantaneous *I–V* relation (e.g. −130 mV) inward current increases with time instead of the decrease observed at −110 mV. This is understandable since an increase in conductance of the inward rectifier will result in an increasing inward K current for potentials negative to E_K.

The preceding argumentation offers a sufficient explanation for the existence of two stable potential levels and for the time-dependent changes in current during the clamps.

The above description may suggest that instantaneous rectification was completely absent for hyperpolarizing clamps from a low resting potential. In some experiments, however, the instantaneous *I–V* relation was not linear and showed some concave behaviour. The amount of instantaneous rectification seems to depend on the actual K concentration in the clefts; when $[K]_0$ is increased to 0·5 mM in the bathing solution (see later) the instantaneous current–voltage relation determined from the low resting potential loses its linear behaviour.

Interference of other currents

All time-dependent current changes are not due to the opening and closing of the inward rectifier and other currents interfere in certain conditions.

(1) Depletion and accumulation are clearly present in the current records when clamps are applied from the high negative resting level (Figs. 5 and 6). The occurrence of depletion currents under these conditions is not unexpected; one of the factors

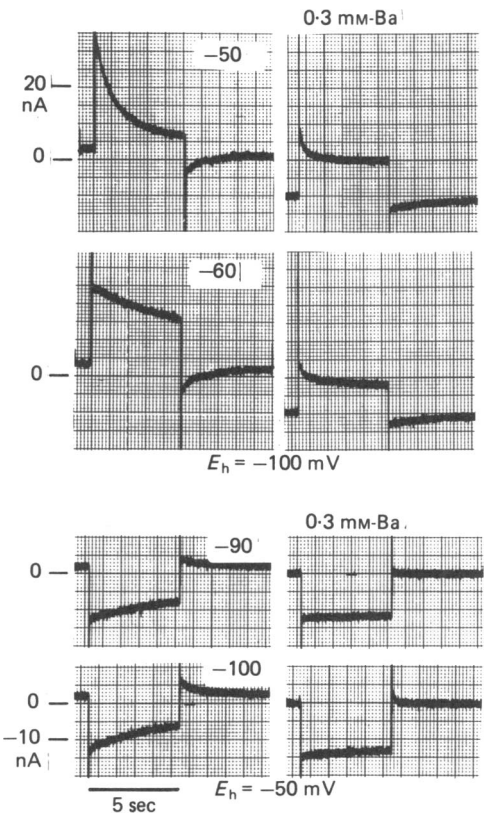

Fig. 9. Effect of 0·3 mM-Ba on holding current and time-dependent currents in K-free, Na-free solution, for depolarizing clamps from a holding potential of −100 mV (top), and for hyperpolarizing clamps from a holding potential of −50 mV (bottom).

which determines the extent of K depletion and accumulation is the conductance for K ions. The presence of a high K conductance explains why depletion and accumulation are pronounced when clamps are applied from the high negative resting level.

Important depletion or accumulation cannot occur, however, if the rectifier is closed, as for instance after a sufficiently long clamp to a depolarized level. This explains (i) why the inward tail following a clamp from −100 to −40 mV in Fig. 5 is smaller than after a clamp to −70 mV, (ii) why the inward tail after a clamp to −45 mV in Fig. 6 decreases as the clamp duration is prolonged, and (iii) why it is possible to record an inward current increasing with time for large hyperpolarizations

(e.g. to −130 mV), instead of a decreasing inward current expected from depletion (Fig. 5). However, as the clamp to a hyperpolarized level is prolonged and the rectifier opens, depletion will start to interfere. The simultaneous occurrence of an increase in K conductance and depletion have opposite effects on the time course of the current and depletion will limit the extent of the inward current. This explains why the steady-state current for a clamp to −130 mV (E_K: −60 mV) never attains the value for a fully open rectifier and remains less negative than the instantaneous current of a clamp to the same level starting from a holding potential of −110 mV (Fig. 8).

(2) Depolarizations to levels positive to −40 mV will activate the slow inward current (i_{si}) and the positive dynamic current (i_{qr}) and these currents will sum with the current change in the inward rectifier. Experiments with 4-aminopyridine (1 mM) added to the K-free, Na-free solution show that the time-dependent decrease in outward current was not changed for depolarizations up to −40 mV, but markedly reduced for clamps positive to this level (not illustrated). The time-dependent component also was not changed in the presence of 2 mM-Mn; this concentration of Mn ions, however, shifted the holding current (−90 mV) in the outward direction. This aspect was not further investigated.

(3) The voltage range in which the pacemaker in sheep Purkingje fibres activates is between −50 and −90 mV. The obvious question is therefore whether the time-dependent inward current on hyperpolarization in K-free, Na-free solution does not simply represent activation of the K current through the pacemaker channel (DiFrancesco, 1981*a*, *b*).

Since Ba ions block the i_{K_1} channel but exert no effect on the pacemaker current (DiFrancesco, 1981*a*, *b*) an answer to this question can be given by studying the behaviour of the time-dependent current in the presence of Ba ions. If the time dependency is due to the conductance change in the i_{K_1} channel it should disappear in the presence of Ba ions.

The examples illustrated in Fig. 9 show the effect of a moderate concentration of Ba ions (0·3 mM) on the currents for depolarizing clamps from a holding potential of −100 mV and for hyperpolarizing clamps from a holding potential of −50 mV. The effect of Ba ions is to shift the holding current in the negative direction especially at the holding potential of −100 mV and, more importantly, to reduce the time dependency for depolarizing as well as for hyperpolarizing clamps.

The effect of increasing Ba concentrations (10^{-6} to 10^{-4} M) is given in Fig. 10. The example shows an inward current which changes to a net outward current during a clamp step from −50 to −80 mV. At 10^{-6} mM-Ba the effect is barely visible but becomes quite pronounced at 10^{-5} mM; at 10^{-4} mM-Ba the time-dependent character completely disappeared. Fig. 11*A* further illustrates that Ba ions not only block the time-dependent currents for clamps positive to the reversal potential (−80 mV) but also for clamps negative to the reversal potential (−110 mV). Fig. 11*B* shows that the pacemaker is still activated in 3×10^{-3} M-Ba, if the K concentration is increased to 2·7 and 5·4 mM. The reason for not seeing this current in the K-free conditions is probably related to the low K concentration in the intercellular clefts. It is known that the conductance of the pacemaker channel is dependent on the $[K]_o$ concentration (Noble & Tsien, 1968).

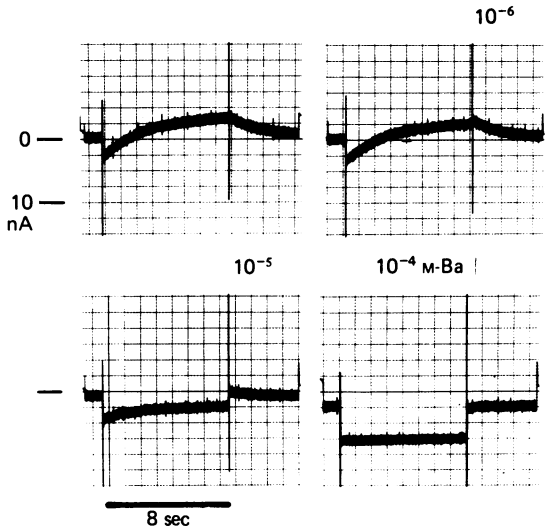

Fig. 10. Effect of an increase in the concentrations of Ba from 10^{-6} to 10^{-4} M. The examples show currents for an 8 sec clamp from -50 to -80 mV. Note net outward current in control conditions.

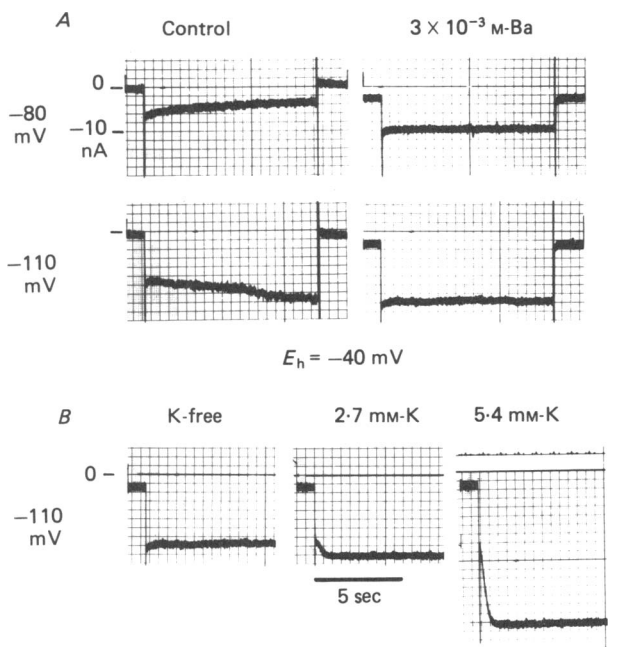

Fig. 11. *A*, effect of Ba 3×10^{-3} M on the time-dependent currents in K-free, Na-free solution for hyperpolarizing clamps from -40 (holding potential) to -80 (upper row) and -110 mV (lower row). In control conditions the current is inward and decreasing at -80 mV; at -110 mV the current is inward and increasing. This time-dependency disappears in the presence of Ba. *B*, addition of K ions 2·7 mM and 5·4 mM in 3×10^{-3} M-Ba results in the re-appearance of a time-dependent current (clamp level, -110 mV). This current is the pacemaker current, i_f, described by DiFrancesco (1981*a*, *b*) and is clearly different from the time-dependent current in *A*.

Induction or removal of inward rectifying properties is not a simple voltage-dependent gating: existence of hysteresis

The experiment, described by Fig. 12, demonstrates that the system responsible for the time-dependent currents shows hysteresis or memory. The experiment was performed on a fibre, which showed two stable resting potentials at −97 and −60 mV, respectively. Instead of determining the instantaneous *I–V* relation from these two levels, a level of −80 mV (× in Fig. 12) was taken as the holding potential. In one

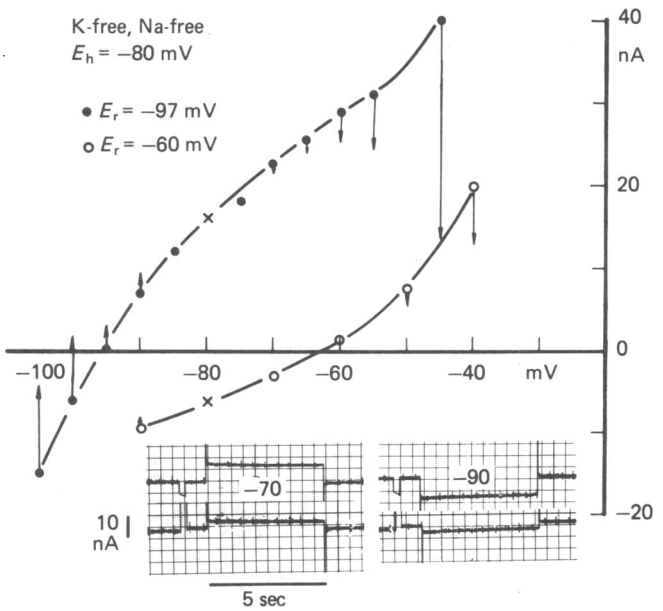

Fig. 12. Instantaneous current–voltage relations obtained from the same holding potential (−80 mV). Instantaneous inward rectification was present when the holding potential was reached from the high negative resting potential (−97 mV); rectification was absent when the same holding potential was reached from the low negative resting potential of −60 mV. Arrows indicate time-dependent changes during the 5 sec clamps at different levels. Inset: examples of currents obtained for depolarizing clamps to −70 mV and hyperpolarizing clamps to −90 mV. The upper row corresponds to (●) in the graph; the lower row to (○).

case the level of −80 mV was reached from a resting potential of −60 mV, in the other case from a resting potential of −97 mV. At −80 mV no time-dependent currents were present (the potential was held at this level for many minutes). Although the holding potential was the same in both conditions the *I–V* relation determined in this way was completely different (Fig. 12) and dependent on the pre-existing resting potential. For a resting potential of −97 mV the *I–V* relation showed inward rectification; rectification was absent when the pre-existing resting level was −60 mV.

These results not only eliminate depletion–accumulation phenomena and pacemaker activation–deactivation as the basic mechanism, but also show that the conductance changes are not due to a simple voltage-dependent gating.

The effect of changes in external K concentration

The addition of 0·54 mM-K (four experiments) to the Na-free medium resulted in stabilization of the resting potential at the high level, disappearance of the possibility to shift the potential to the low level, and important changes in the *I–V* relation (Fig. 13). The *I–V* relation determined from −40 mV (Fig. 13*B*, ○) is shifted in the outward direction and shows substantial inward rectification, when compared to the same *I–V* relation in the K-free solution (Fig. 13*A*). Instantaneous currents for

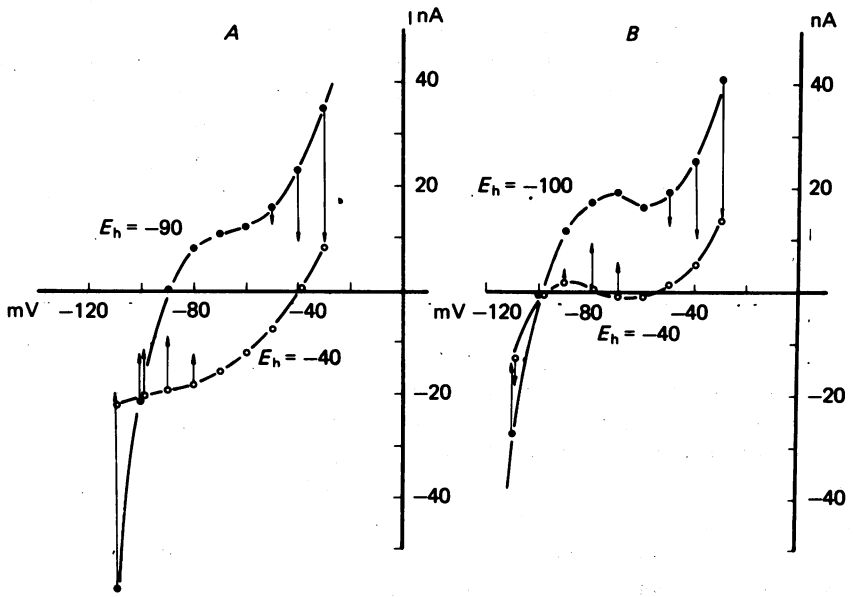

Fig. 13. Comparison of instantaneous current–voltage relations obtained from two different holding potentials in K-free, Na-free solution (*A*) and 0·54 mM-K, Na-free solution (*B*). Holding potential was −40 mV (○) and −90 mV or −100 mV (●). Time-dependent changes during the 5 sec clamps are given by the arrows.

potentials between −50 and −90 mV are all very close to zero and with time all current values become net outward (see arrows in Fig. 13*B*). The *I–V* relation obtained from a holding potential of −100 mV is also shifted in the outward direction in the presence of 0·54 mM-K (compare Fig. 13*B* with *A*, ●). In quantitative terms this effect is not so large as the shift of the *I–V* relation determined from a low potential level: the instantaneous *I–V* relation determined from the two holding potentials thus lie closer together when $[K]_o$ is increased. More important, however, is the fact that the decrease in outward current during a depolarizing clamp, whatever its duration, is never large enough to reduce net current sufficiently to stabilize the membrane potential at the low negative level. This explains why only one stable potential can be obtained. In the presence of a sufficient amount of $[K]_o$, total K conductance is thus increased, and, although depolarization still reduces this conductance, a sufficient proportion of K channels remain activated and in the open position. An

increase in $[K]_o$ does not eliminate time-dependent changes in conductance, but the relative contribution of the time-dependent component to the total conductance becomes smaller.

Addition of $[K]_o$ in a concentration below 0·54 mM resulted essentially in the same changes of $I-V$ relation, i.e. shift of the $I-V$ relation in the outward direction and appearance of inward rectification in the $I-V$ relation determined from an holding potential of -40 mV (one experiment with 0·1 mM and two experiments with 0·2 mM-K), the difference with 0·54 mM-$[K]_o$ being that there were still two potential levels where net current became zero.

Addition to $[K]_o$ to concentrations above 0·54 mM, e.g. 2·7 and 5·4 mM, further shifted the currents in the outward direction. Instantaneous currents appeared still dependent on the holding potential but the difference between $I-V$ relations determined from a high or low holding potential became smaller; time-dependent changes in current were still present for depolarizing as well as hyperpolarizing clamps. Interpretation of the current changes, however, becomes very difficult due to the pronounced depletion on hyperpolarization and accumulation on depolarization.

DISCUSSION

In heart cells the current through the inward-going K rectifier is an important component of the total current at the resting potential. This component is usually regarded as a background current, i.e. time-independent. The results reported in this paper indicate that at least in K-free, Na-free solution the conductance through the inward rectifier does not change instantaneously with voltage but increases on hyperpolarization and decreases on depolarization in a time-dependent way; due to these conductance changes the membrane potential stabilizes at either a low or a high negative level. Time-dependent changes in the conductance of inward rectifier channels have also been described for other tissues: egg cell membrane of the starfish (Hagiwara, Miyazaki & Rosenthal, 1976); tunicate egg cell membrane (Ohmori, 1978) and frog sartorius (Standen & Stanfield, 1980). A more detailed analysis, however, reveals that in Purkinje fibres the underlying mechanism may not be the same. The time-dependent current change is not caused by a Hodgkin–Huxley type of activation–deactivation in which the state of the gate is fully determined by the potential gradient across the membrane. Activation occurs for potentials negative to -80 mV, while deactivation is seen in a different range of potentials, i.e. positive to -60 mV. The behaviour of the membrane at a given potential level as well as the steady state is dependent on the previous history; the system shows memory or hysteresis. This was best demonstrated by an experiment in which the holding potential was -80 mV and the two stable resting levels -97 and -60 mV (Fig. 12). Depending on whether the holding potential was reached from the high resting level or the low resting potential the instantaneous $I-V$ relation showed inward rectification or not. In this respect it is worthwhile to refer to results obtained by Dudel, Peper, Rüdel & Trautwein (1967) in Purkinje fibres, which are consistent with the existence of hysteresis. These authors determined $I-V$ relations by using voltage clamp ramps in Na-free medium, containing different concentrations of extracellular K, and found

that the *I–V* relation at slow ramp speeds (3 mV/sec) was steeper for depolarizing than for repolarizing ramps (their Fig. 1).

Since voltage clamps at levels different from the resting potential may induce K depletion or accumulation, it was necessary to exclude changes in E_K as the responsible factor for the shift of the membrane potential between the two stable levels and for the current pattern observed during the clamps. Important depletion and accumulation currents were seen when clamps were applied from the high negative resting level (Figs. 5 and 6). Such a result is quite normal, because one of the conditions for depletion–accumulation, a high K conductance, is fulfilled under these conditions. Evidence was presented that depletion or accumulation were absent in other conditions, i.e. low resting potential. (1) For hyperpolarizing clamps from −50 mV the current became net outward for moderate hyperpolarizations (Fig. 10) and changed direction for large hyperpolarizations (negative to −120 mV; Figs. 5 and 11). (2) The potential at which the membrane stabilized after release of the clamp could be positive or negative to the clamp level (Figs. 1–3). (3) The conductance at this new level was large at the high negative level and small at the depolarized level (Figs. 7 and 8). All these results exclude depletion–accumulation as the underlying mechanism.

Arguments were presented to exclude the pacemaker channel as the location of these time-dependent conductance changes. The existence of memory alone already rules out this possibility. This conclusion was further corroborated by experiments in the presence of Ba ions (Figs. 9–11); small concentrations of Ba ions blocked the time-dependent conductance change in the inward rectifier but did not eliminate the pacemaker current, which could be made visible by increasing $[K]_o$.

The existence of memory indicates that the changes in conductance are not solely conditioned by the movement of a gating molecule, only dependent on the change in electric field. A supplementary phenomenon, which influences the orientation of the gating moiety, is required. Such a phenomenon might be the binding or the access of external K ions to a certain site in the membrane: a hyperpolarizing pulse is thus thought to result in the movement of K ions towards this site. The conductance pathway is then opened and K ions can move in or out. The pathway through which K ions activate the conductance can be the same as or different from the permeation pathway (occupancy gating *versus* electrochemical gating; Ciani, Krasne, Miyazaki & Hagiwara, 1978). The reverse phenomenon, i.e. removal of K from the activating site, occurs during a depolarizing pulse.

This type of explanation is not fundamentally different from the assumptions made in different models proposed to explain inward rectification (Standen & Stanfield, 1978; Ciani *et al.* 1978; Cleeman & Morad, 1979; Hille & Schwarz, 1978). Inward rectification, according to these models, is due to the locking-in of a blocking particle on depolarization, and the unlocking on hyperpolarization; the removal of the block, requires the presence of extracellular K ions. Under the influence of the electrical gradient K ions from extracellular origin are supposed to interact directly or indirectly with the blocking particle. The probability that the channel is opened increases the higher the external K concentration and the greater the electrical gradient. In order to account for the instantaneous rectification, the interaction of K with the membrane site is assumed to occur very rapidly.

The only modification needed to explain our results is to assume that the interaction of external K with its sensitive site is not instantaneous as a whole, but instead occurs in two phases: a rapid, practically instantaneous one and a slow one; the contribution of both phases varies with the K concentration in the clefts: the higher the extracellular K the more important becomes the rapid phase. At elevated K concentrations in the bathing solution (> 2·7 mM) the sites may be saturated to such extent that only instantaneous rectification remains. Verification of this aspect, however, is impossible because of the existence of large depletion and accumulation currents under those conditions and must await isolation of single Purkinje cells.

When the resting potential is low (e.g. −50 mV) the concentration of K in the clefts and at the sensitive site may be assumed to be very low (net K outward movement is indeed small because the inward rectifier is closed) and instantaneous inward rectification is absent. When the resting potential level is high the K concentration in the clefts will be greater (the open state of the K inward rectifier results in a greater K leak from the cells) and instantaneous rectification will be present. During hyperpolarization from a low level K ions are moved to the activation site and the conductance pathway is activated in a slow time-dependent way. If the clamp is positive to E_K, K will move outwards, leading to an increase of K in the clefts, a process which in turn will facilitate further activation. This positive feed-back mechanism will affect the time course of the current during hyperpolarizing clamp and will continue to affect the evolution of the membrane potential after release of the clamp. It thus becomes understandable why the potential may continue to hyperpolarize, or may hyperpolarize following an initial small depolarization, after release of the clamp: during the clamp the inward K rectifier is sufficiently activated to keep the membrane potential at a level between the low and high negative level; since the inward rectifier is partly open, K ions will move out; more K ions thus become available to activate the conductance, resulting in further hyperpolarization. The same mechanism also explains why it was difficult to obtain a threshold for hyperpolarizing clamps to levels negative to E_K. The hyperpolarization still results in K movement from the clefts to the activating site and activates the conductance pathway, but now K ions are moving net inwards through the conductance pathway. This causes depletion and reduces the availability of activating K ions.

The existence of memory was the main basis for proposing the K-activation mechanism. A modulation of channel conductance by external K may not be the only mechanism, however. Injection of Ca ions into Purkinje fibres has been shown to increase the background i_{K_1} current (Isenberg, 1977). Recent patch clamp studies in cultured rat heart cells reveal the existence of a channel, with little selectivity between cations, activated by intracellular Ca ions, but not appreciably affected by voltage (Colquhoun, Neher, Reuter & Stevens, 1982). Further investigation will be needed to evaluate the eventual role of intracellular Ca.

The existence of two stable resting potentials in K-free, Na-free solution prompted the present investigation. Two stable potential levels in cardiac Purkinje fibres have been described in a number of experimental conditions: low temperature (Chang & Schmidt, 1960); low K, low Cl (Gadsby & Cranefield, 1977), Na-free, Ca-rich, TEA-containing solutions (Wiggins & Cranefield, 1976). In terms of changes in I–V relation the existence of two stable potentials in K-free, Na-free solution is different

from the situation described by Gadsby & Cranefield (1977). In low K, low Cl medium the potential can be changed from the low level to the high level by passing short hyperpolarizing pulses. According to Gadsby & Cranefield the existence of two stable resting potentials at a given value of $[K]_o$ requires that the steady-state net current–voltage relationship be 'N-shaped' with two zero-current intercepts in regions of positive slope conductance, the third unstable intersecting in a region of negative conductance. The present experiments demonstrate that time-dependent conductance changes in the i_{K1} channel offer another possible explanation. The situation in K-free, Na-free condition may be comparable with that described by Wiggins & Cranefield (1976) (16 mM-Ca, Na-free, 5–20 mM-TEA), in which long pulses are required to provoke the shift from one level to the other. Although $I-V$ relations were not measured by Wiggins & Cranefield it seems quite possible that the instantaneous $I-V$ relation in TEA also depends on the holding potential and that time-dependent changes in the K rectifier channel are responsible for the shift from one level to the other.

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