J. Physiol. (1976), **260**, *pp.* 75–103
With 16 *text-figures*
Printed in Great Britain

AN ANALYSIS OF THE ACTIONS OF LOW CONCENTRATIONS OF OUABAIN ON MEMBRANE CURRENTS IN PURKINJE FIBRES

BY IRA COHEN, JÜRGEN DAUT AND DENIS NOBLE
From the University Laboratory of Physiology, Parks Road, Oxford

(*Received 26 November 1975*)

SUMMARY

1. The influence of low concentrations (5×10^{-8} to 5×10^{-7} M) of ouabain on the K gradient in sheep cardiac Purkinje fibres was observed by measuring changes in the reversal potential for a K specific current, i_{K_2}, and by measuring total steady-state current–voltage relations.

2. Provided that the bathing solution K concentration, $[K]_o$ was not too low, these doses of ouabain were often observed to increase the K gradient, i.e. the reversal potential was shifted in a negative direction.

3. The change in the reversal potential and in the current–voltage relation could be mimicked by reducing the value of $[K]_o$ in the absence of ouabain. It is therefore suggested that ouabain may stimulate the $Na^+ - K^+$ exchange pump and so reduce the K concentration, $[K]_e$, in the clefts of the preparation.

4. At sufficiently low values of $[K]_o$, a dose of ouabain that was stimulatory may become inhibitory. The reversal potential for i_{K_2} then shifts in a positive direction.

5. During either stimulation or inhibition, the speed of change of reversal potential is consistent with a change in $[K]_e$, which may change fairly rapidly. It is not possible to account for the results solely by changes in intracellular concentration, $[K]_i$.

6. Low concentrations of ouabain were found to have no effect on the activation curve, s_∞ (E_m), controlling i_{K_2}. It is concluded that the changes in i_{K_2} are solely attributable to changes in reversal potential.

7. Since net stimulation of the Na^+–K^+ exchange pump was observed to occur at doses of ouabain that exert a strong positive inotropic action on Purkinje fibres (Blood, 1975), it is not likely that the inotropic action is causally related to net pump inhibition.

INTRODUCTION

The cardiac glycosides are used in a variety of clinical conditions to improve the performance of the heart (see, for example, Smith & Haber, 1974). The desired therapeutic actions are to increase the strength of the beat and to suppress certain disturbances of rhythm. However, at dose levels that are not much larger than the therapeutic level, the glycosides may themselves induce arrhythmias (see, for example, Scherf & Schott, 1973, chapter 12). The therapeutic and toxic doses vary between individuals and depend so greatly on a variety of factors, such as plasma Ca^{2+} or K^+ concentration, plasma acidity and anoxia, that a dose that is therapeutic under some conditions may easily prove toxic under other conditions. It is therefore important to study the influence of these factors on glycoside action. One of the aims of this paper is to investigate the way in which ouabain action on membrane currents in Purkinje fibres is dependent on the extracellular K concentration.

The mechanism of the therapeutic action of ouabain is not yet known. One possibility that has been suggested recently (see Baker, Blaustein, Hodgkin & Steinhardt, 1969; Langer, 1970; Langer & Serena 1970) is that the therapeutic action may arise as a secondary consequence of inhibition of the sodium pump. According to this theory, enhanced intracellular Na following inhibition may allow Ca ions to accumulate intracellularly via an action on the Na^+–Ca^{2+} exchange mechanism. A positive inotropic action, attributable to greater availability of intracellular Ca, should therefore always be linked to a rise in intracellular Na and a fall in intracellular K.

In studies using radioactive tracers to measure changes in the distribution of ions following application of cardiac glycosides some workers have indeed found the results expected from inhibition of the Na–K exchange pump, namely an increase in intracellular Na (e.g. Langer & Serena, 1970) and a loss of intracellular potassium (e.g. Müller, 1965; for further references see Lee & Klaus, 1971). However, other work on the K content of cardiac muscle does not confirm these results and there are indications that a therapeutic effect may even be accompanied by an increase in intracellular K (Hagen, 1939; Boyer & Poindexter, 1940; Godfraind, 1973; Godfraind & Ghysel-Burton, 1976) or a retardation of K^+ loss (Holland, Grieg & Dunn, 1954; Tuttle, Wit & Farah, 1961; Brown, Acheson & Grupp, 1970; for further references see Lee & Klaus, 1971).

Another finding that suggested a link between inhibition of the pump and the positive inotropic effect was the parallelism found between the sensitivity of Na/K–ATPase preparations to cardiac glycosides and the magnitude of the positive inotropic effect in various animal species

(Repke, 1965; Portius & Repke, 1962). In addition, a parallelism between the degree of inhibition of ATPase and the magnitude of the positive inotropic effect produced by various doses of cardiac glycoside was found (Besch, Allen, Glick & Schwartz, 1970; Akera, Larsen & Brody, 1970). Once again, however, there is no consensus of opinion on this question since other experimenters have found a clear dissociation of the positive inotropic effect and inhibition of Na/K–ATPase (Okita, Richardson & Roth-Schechter, 1973; Peters, Raben & Wasserman, 1974). They found that the onset and the washout of the positive inotropic effect was much faster than the onset and especially the very slow washout of enzyme inhibition by cardiac glycosides. Moreover, stimulation rather than inhibition of the Na/K–ATPase by therapeutic concentrations of ouabain has been found by various groups (Okita *et al.* 1973; Peters *et al.* 1974) which argues against the view that blockage of the Na–K pump is causally related to the increase in strength of contraction.

The use of the voltage-clamp method to measure ionic currents in cardiac muscle has provided a new way of tackling the problem. Purkinje fibres are particularly useful in this case for there is evidence that the reversal potential for the pace-maker current, i_{K_2}, is at or close to the K equilibrium potential, E_K (Noble & Tsien, 1968; Peper & Trautwein, 1969). Changes in the value of E_K, and changes in the shape of the membrane current–voltage relations, may be used to detect changes in the K gradient across the cell membrane. Although in a preceding paper (Cohen, Daut & Noble, 1976) we have shown that the use of the i_{K_2} reversal potential to estimate E_K is more complex than previously suspected, we have found consistent changes under the influence of ouabain which depended on the K concentration in the medium and on the dose of ouabain used.

Several other groups have used the voltage clamp technique to study the actions of cardiotonic steroids (Aronson, Gelles & Hoffman, 1973; Isenberg & Trautwein, 1974; Lederer & Tsien, 1975). With the exception of Aronson *et al.* (1973) these studies have used concentrations of cardiotonic drug that are known to produce marked inhibition of the pump, e.g. 10^{-6} M ouabain. Similar doses were also used in the radioactive tracer studies of Langer & Serena (1970) and in the biochemical studies of Besch *et al.* (1970). Such concentrations are invariably associated with toxic effects (Sanyal & Saunders, 1961; Tuttle *et al.* 1961; Farah & Wit, 1963). Therapeutic doses of ouabain, on the other hand, produce plasma concentrations in the range $2-10^{-8}$ to 5×10^{-7} M (Smith & Haber, 1974). We therefore decided to investigate the effects of low concentrations of ouabain with the voltage-clamp technique. We found that doses in the therapeutic range can produce changes in the K^+ gradient that reflect

stimulation of the Na^+–K^+ exchange pump, whereas 'toxic' effects such as membrane depolarization, potential oscillations and spontaneous activity were invariably associated with inhibition of the pump. We have not analysed the 'toxic' actions in this paper since we have concentrated primarily on understanding the effects of relatively low ouabain doses. Some of this work has appeared in abstract form (Cohen, Daut and Noble, 1975).

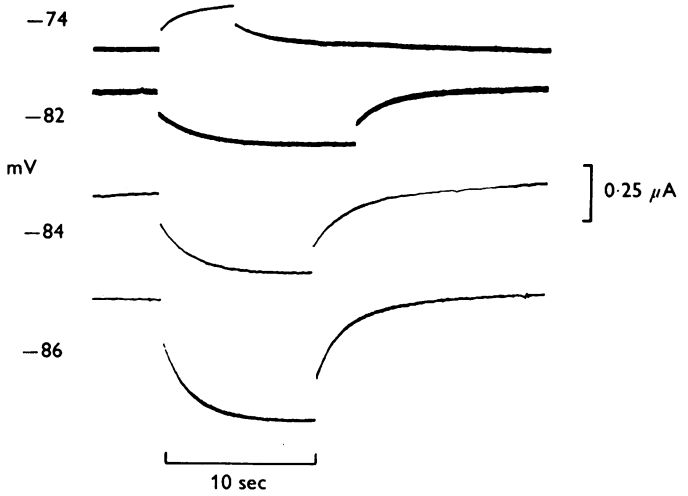

Fig. 1. Membrane currents recorded during voltage clamp pulses from the resting potential (−78 mV) to the potentials indicated. The fibre was placed in Tyrode solution containing 2·7 mM-K^+.

METHODS

The methods were the same as in the preceding paper (Cohen *et al.* 1976). Ouabain stock solutions were prepared by dissolving 250 mg ouabain (Sigma, London) in 3 ml. methanol and diluting it with 4 mM-K^+ Tyrode to 1×10^{-4} or 1×10^{-5} M ouabain. These stock solutions were then further diluted with the appropriate Tyrode solution to give final concentrations of ouabain between 5×10^{-8} and 5×10^{-7} M.

In most of the experiments described in this paper we have measured steady-state currents over a wide range of membrane potentials. Some records from one of our experiments are shown in Fig. 1. It can be seen that it may require up to 10 sec for steady-state currents to be achieved and that, since recovery is also quite slow, it is necessary to allow 20 sec or more between pulses. The time taken to construct a complete current–voltage diagram is therefore several minutes. It is nevertheless important to use steady-state conditions since experiments using much shorter pulses of constant duration may not be used to distinguish between effects on total membrane current and effects on the kinetics of current change.

Where it is necessary to observe effects on the reversal potential for i_{K_2} over a relatively short time scale, we have simply used short pulses near E_{rev} without

constructing full current–voltage diagrams in every case. For similar reasons, the estimates of E_{rev} are usually more accurate than the steady-state current–voltage diagrams suggest since we usually used a fine grid of short pulses to determine E_{rev} to within 1 or 2 mV (cf. Cohen *et al.* 1976, fig. 2). Such pulses were not used to plot points on the current–voltage diagram unless it was clear that a steady-state current level had been achieved.

RESULTS

Changes in the steady-state current–voltage diagram produced by ouabain may arise from several possible actions: (*a*) changes in electrogenic pump current; (*b*) changes in ion equilibrium potentials; (*c*) changes in ionic conductances; and (*d*) changes in conductance kinetics. The changes we have observed may involve (*a*), as described also by Isenberg & Trautwein (1974). We have also observed substantial changes in the K equilibrium potential and there may be consequential changes in ionic conductance. The over-all effects are therefore quite complex particularly since the direction of an effect may also vary as a function of ion and drug concentrations. Our initial results were therefore confusing and difficult to interpret. We found that a key factor in interpreting the results was to determine the effect of extracellular K^+ concentration on the i_{K_2} reversal potential and steady-state current–voltage diagrams in the *same* experiment as the effects of ouabain. This was found to be important for two major reasons. First, as we have shown in a preceding paper (Cohen *et al.* 1976) the influence of $[K]_0$ on the reversal potential for i_{K_2} is not necessarily well predicted by the Nernst equation since the immediate extracellular K^+ concentration, $[K]_e$, is rarely equal to $[K]_0$, on the bathing solution concentration. Secondly, the influence of extracellular K^+ on the current–voltage diagram is somewhat variable from a quantitative point of view. A decrease in $[K]_0$ reduces the K^+ conductance to depolarizing currents (Carmeliet, 1961; Hall & Noble, 1963). If this effect is large enough, the current–voltage diagrams may cross each other (see Noble, 1965). This is frequently, but not always, the case. Similar results have been obtained in the present experiments. In all cases, reduced $[K]_0$ decreases the conductance to depolarizing currents, sometimes giving rise to a cross-over effect (see Figs. 7 and 8) and sometimes just failing to do so (see Fig. 2). In order to interpret our results using ouabain in terms of changes in ionic gradients we therefore found it essential to first measure the effect of $[K]_0$ alone in each experiment.

Influence of extracellular K^+ in absence of ouabain

Fig. 2 shows the result of an experiment in which the effects of 4, 5·4 and 8 mM-$[K]_0$ were compared. The effects of ouabain at these various $[K]_0$ values in this experiment will be presented in Figs. 3–6.

As expected, the reversal potential for i_{K_2} changes substantially in a positive direction as $[K]_o$ is increased. It is also clear that, in this experiment, the cross-over effect is virtually absent. Instead, the steady-state curves approach a similar current value at about -40 mV.

The values of current at which E_{rev} for i_{K_2} occur are of some importance since they enable us to determine whether $[K]_o$ influences currents other than i_K. The total steady-state membrane current, i_i, is given by

$$i_i = i_K + i_{in_B} + i_p, \tag{1}$$

where i_{IN_B} is the background inward current responsible for maintaining the resting potential positive to E_K and i_p is any current carried by the Na–K exchange pump. At E_{rev}, i_K is zero and we have

$$i_i\,(E_{rev}) = (i_{in_B})_{E_{rev}} + (i_p)_{E_{rev}}. \tag{2}$$

Hence the current required to hyperpolarize the membrane to E_{rev} is the net current carried by i_{IN_B} and i_p at that potential.

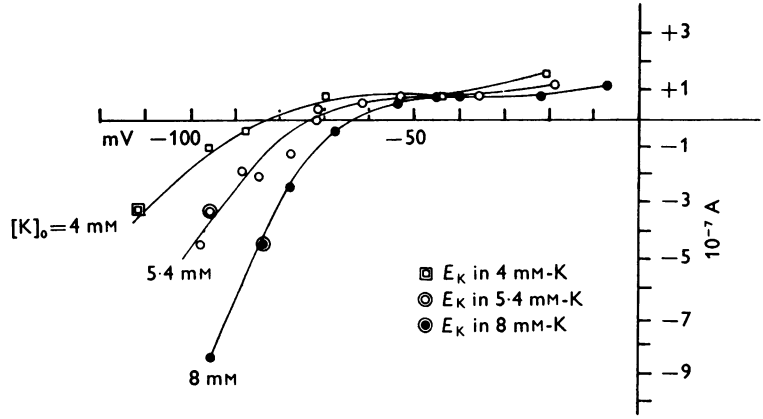

Fig. 2. Influence of $[K]_o$ on the steady-state current–voltage diagram. E_K is -111 mV in 4 mM-$[K]_o$, -95 mV in 5·4 $[K]_o$ and -82 mV in 8 mM-$[K]_o$.

From Fig. 2 we can see that at 8 mM-$[K]_o$ this current is $4{\cdot}5 \times 10^{-7}$A. At 5·4 mM-$[K]_o$ the current required is a little smaller, $3{\cdot}5 \times 10^{-7}$A. In 4 mM-$[K]_o$ a similar value is obtained. Thus, there is only a relatively small variation in i_i (E_{rev}) as $[K]_o$ is raised. This result was also obtained in other experiments (see e.g. Figs. 7 and 8) and it suggests that i_{IN_B} and i_p are relatively unaffected by changes in $[K]_o$. Though we cannot, of course, exclude compensating variations in i_{IN_B} and i_p, this seems unlikely.

Some reduction in i_p (leading to an increasing negative value of i_i (E_{rev})) would, of course, be expected when $[K]_o$ is reduced below the level

required to maintain the activity of the pump. Our results do not show this effect. This means *either* that the K^+ activation is already maximal at all the K^+ concentrations used *or* that the activity of the pump, although K^+ sensitive in this range, is self-regulating via its effect on the local K^+ concentration $[K]_e$. This possibility will be discussed further later (see Discussion).

The action of low concentrations of ouabain when $[K]_o$ *is large*

Fig. 3 shows the effect of exposing the fibre to 5×10^{-7} M ouabain while bathed in 8 mM-K. The result is quite unexpected. There is a moderate hyperpolarization: the resting potential changes from -64 to -72 mV and the current required to hyperpolarize the membrane is greatly reduced. These changes are in the opposite direction to those expected when ouabain inhibits the pump.

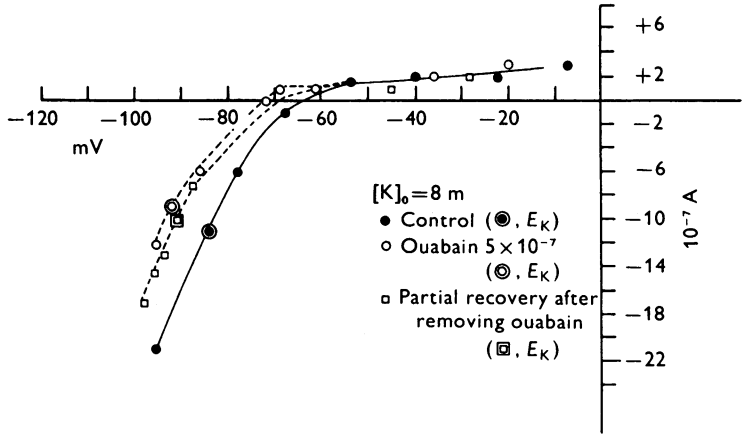

Fig. 3. Effect of ouabain on the steady-state current–voltage relation in 8 mM-K^+.

Moreover, E_{rev} changes substantially in a negative direction from -83 to -92 mV. The simplest interpretation of this result is that the pump is stimulated by this concentration of ouabain (see Discussion). The reversal potential would then shift in a negative direction either as a consequence of an increase in $[K]_i$, or of a decrease in $[K]_e$, or some combination of the two.

Can we distinguish between these possibilities? There are two observations that suggest that the change in E_{rev} is primarily due to a decrease in $[K]_e$. First, the increase required in $[K]_i$ is very large and may be inconsistent with the maintenance of osmotic equilibrium (see Discussion). Secondly, the changes in the current–voltage diagram that occur following

the negative shift in E_K closely mimic those produced by a decrease in $[K]_o$ in the absence of ouabain.

This is shown in Fig. 4 in which we have superimposed the 5·4 mM-$[K]_o$ control curve (Fig. 2) from the same experiment on to the results from

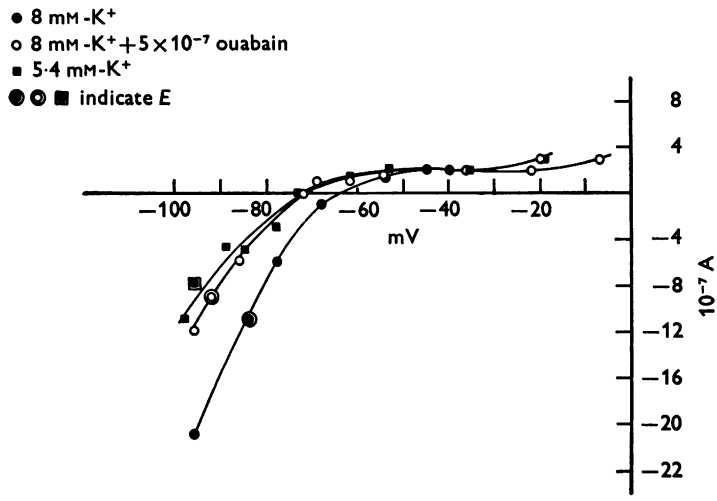

Fig. 4. Comparison between influence of ouabain (5×10^{-7} M) and reduced $[K]_o$ on the steady-state current–voltage relation.

Fig. 3. The 5·4 mM-$[K]_o$ curve in the absence of ouabain is clearly very similar to that in 8 mM-$[K]_o$ in the presence of ouabain. Our interpretation of this result is that, although the bathing solution $[K]_o$ is 8 mM, the value of $[K]_e$ immediately outside the cell membranes has decreased to a level nearer 5·4 mM. (It should be noted that the 5·4 mM-$[K]_o$ curve was obtained earlier in the experiment before any ouabain was administered.)

It is also worth noting that ouabain has only a relatively small effect on i_i (E_{rev}), which is slightly reduced in Fig. 3. This is consistent with the view that i_p is increased, though the results are probably not sufficiently reliable to draw any stronger conclusions.

The influence of reduced $[K]_o$ on ouabain action

Fig. 5 shows the result of the same dose of ouabain when the bathing solution K concentration is reduced to 5·4 mM-$[K]_o$. The effect is similar to, though smaller than, that at 8 mM-$[K]_o$.

By contrast, Fig. 6 shows that when the bathing solution [K] is reduced to 4 mM the direction of action is reversed. The membrane current is changed in the inward direction at all potentials. This change may be consistent with the view that at this level of $[K]_o$ ouabain at a concentra-

tion of 5×10^{-7} M inhibits the pump, thus producing a reduction in i_p and a positive shift in E_{rev}. It is difficult to determine the relative contributions of these two factors to the total change in current at each potential.

The over-all conclusion of this experiment is that the direction of

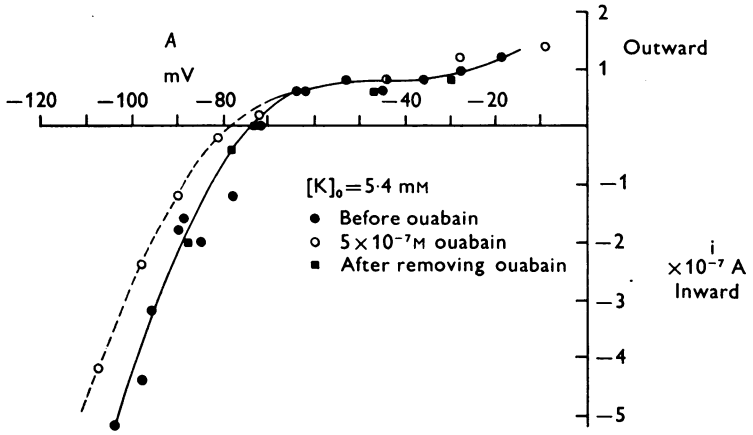

Fig. 5. Effect of ouabain on a fibre bathed in 5·4 mM-$[K]_o$. After application of ouabain at 5×10^{-7}M (○) there is a negative shift in E_K to 102 mV and less current is needed to hyperpolarize to any potential. There is a total reversal after ouabain removal (■).

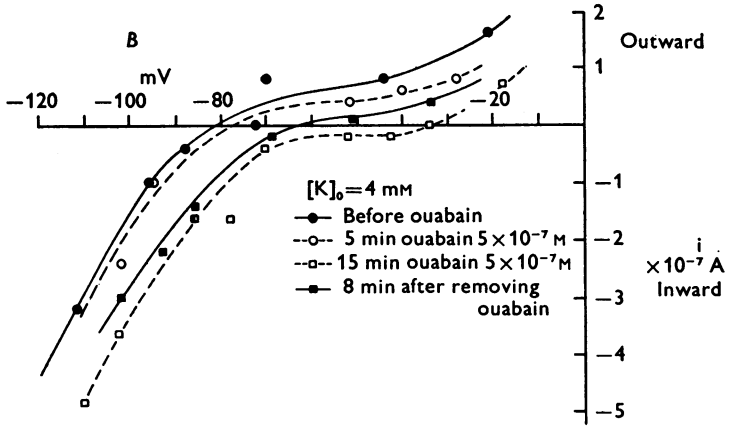

Fig. 6. Effect of ouabain on a fibre bathed in 4 mM-$[K]_o$. The control curve is the filled circles. Five minutes after 5×10^{-7} M ouabain is applied there is a small inward shift of current at all potentials (the open circles). Fifteen minutes after drug application the inward current shift becomes larger (the open squares) and is close to 10^{-7} A at all potentials. A parallel inward current shift at all potentials indicates pump blockage. There is a partial reversal of this effect 8 min after drug removal (the filled squares).

action of ouabain is dependent on the level of $[K^+]$ in the bathing solution. At high values of $[K]_o$, 5×10^{-7} M ouabain shifts E_{rev} in a negative direction, while at low values of $[K]_o$ ouabain shifts E_{rev} in a positive direction. This produces as a consequence the result that in the presence of ouabain E_{rev} for i_{K_2} varies much less with changes in $[K]_o$ than it does in the absence of ouabain. Thus the values of E_{rev} in 5·4 mM and 8 mM-$[K]_o$ differ by only 8 mV in the presence of ouabain compared to 13 mV before ouabain. The comparison between 4 and 8 mM-$[K]_o$ is even more striking.

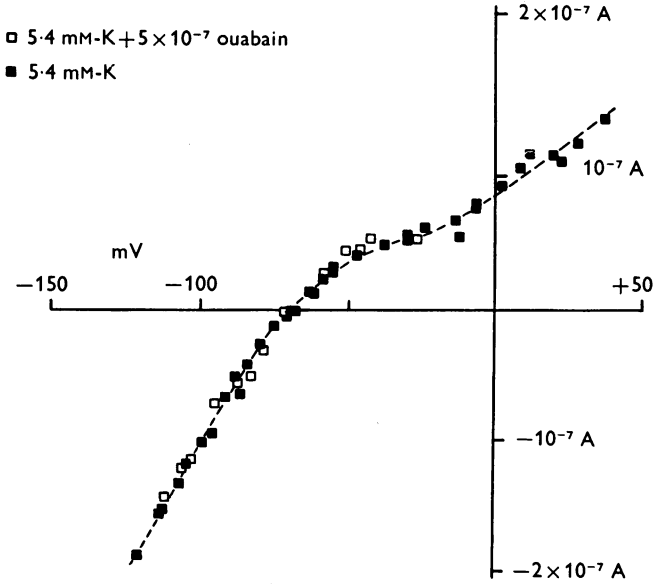

Fig. 7. Result of experiment in 5·4 mM-K in which ouabain (5×10^{-7} M) had no effect.

The difference before ouabain is 29 mV (see Fig. 2). After ouabain, E_{rev} in 8 mM-K^+ is -91 mV. Although the value in 4 mM-K^+ is a little uncertain (the potential grid used was not fine enough and the fibre was not in a steady-state condition in ouabain) we know that it must be significantly less than -111 mV since the effect of ouabain in this case was to block the pump. The difference between the values of E_{rev} in ouabain must therefore have been reduced to significantly less than 20 mV.

Since the direction of action of ouabain is changed by varying $[K]_o$ it should be possible to find a value of $[K]_o$ at which no detectable change occurs. In some experiments we were able to achieve such a situation. Fig. 7 shows the influence of 5×10^{-7} M ouabain at a $[K]_o$ level of 5·4 mM. Unlike the situation in the previous fibre, where this dose of ouabain

produced a significant negative shift in E_{rev} at 5·4 mM-$[K]_o$, there is no change in this case. This result introduces a further observation that we have made, which is that there is a significant degree of variation in the response of individual fibres to the same concentrations of ouabain at any particular value of $[K]_o$.

Fig. 8 shows the response of the same fibre as in Fig. 7 to the same level of ouabain when the bathing solution $[K]_o$ is reduced to 2·7 mM-K. There is now a clear positive shift in E_{rev}, indicative of pump inhibition. In this

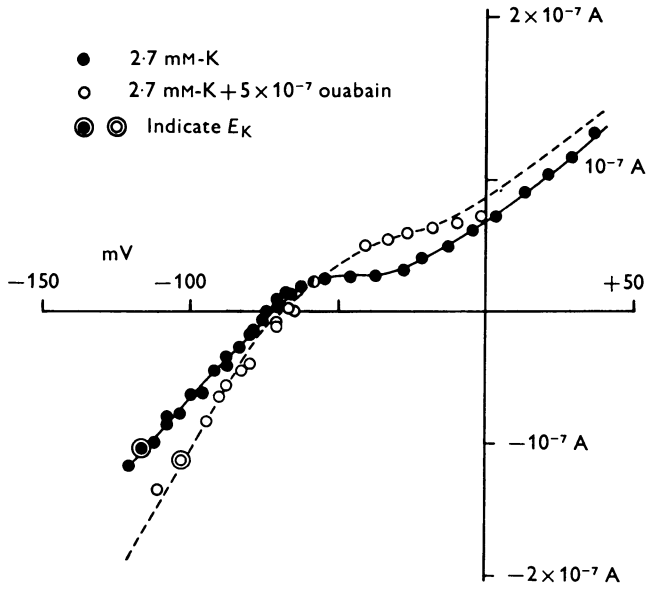

Fig. 8. Influence of ouabain (5×10^{-7} M) in 2·7 mM-K^+. E_K shifts from −118 to −103 mV. The interrupted line is taken from results in 5·4 mM-K^+ (see Fig. 7).

case, the ouabain curve crosses the control curve in the way expected when $[K]_o$ is increased. In fact the interrupted line plotted in Fig. 8 is the same as that drawn through the points in Fig. 7 obtained in 5·4 mM-$[K]_o$. We may therefore conclude that the result in Fig. 8 is consistent with the view that, as a result of pump inhibition, the value of [K] immediately outside the cells, i.e. $[K]_e$, has increased to about 5·4 mM-K. If this is the case then the ouabain curves in 5·4 and 2·7 mM-K^+ should superimpose. This may already be evident from Figs. 7 and 8 since the interrupted curve is common to both plots. Fig. 9 shows the result more clearly by plotting only the points from the two ouabain relations. It is particularly striking to note that the values of E_{rev} are virtually equal. This is a

further and even more striking example of the insensitivity of E_{rev} to bathing solution $[K]_o$ in the presence of ouabain.

As in the previous experiment, the conclusion we draw from this experiment is that the changes in E_{rev} produced by ouabain are primarily

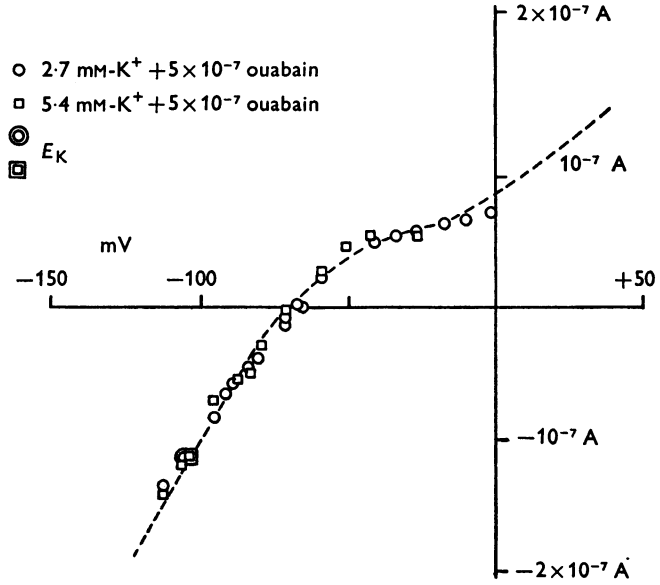

Fig. 9. Comparison between current–voltage relations in ouabain (5×10^{-7} M) at two different K^+ concentrations, 2·7 and 5·4 mM.

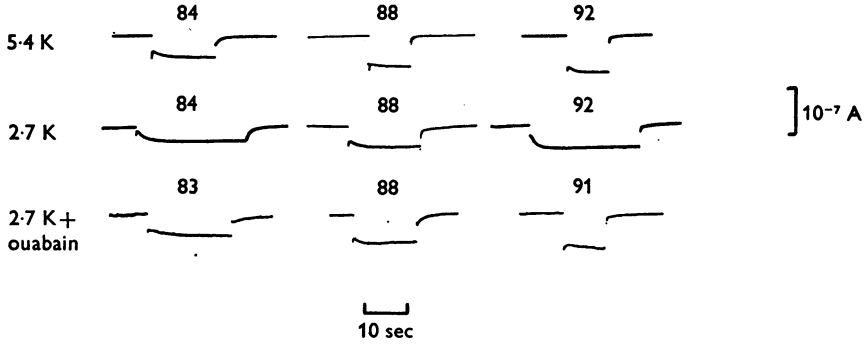

Fig. 10. Voltage-clamp currents in response to hyperpolarizing steps used in experiment illustrated in Figs. 9 and 11.

produced by changes in extracellular [K]. This conclusion is further strengthened by the finding that the relative magnitudes of i_{K_1} and i_{K_2} change in the manner expected for changes in $[K]_o$. Fig. 10 shows examples

of current records in response to hyperpolarizing pulses in the presence of 2·7 mM-K, 5·4 mM-K and 2·7 mM-K + 5×10^{-7} M ouabain. As expected, the ratio of time-dependent (i_{K_2}) current change to instantaneous current change (largely i_{K_1}) is considerably larger in 2·7 mM-$[K]_o$ than in 5·4 mM-$[K]_o$. When ouabain is added to the 2·7 mM-$[K]_o$ solution, however, the records change and resemble those obtained in 5·4 mM-$[K]_o$. This is confirmed in a quantitative way by plotting the instantaneous current as a function of potential in the three solutions. This has been done in Fig. 11.

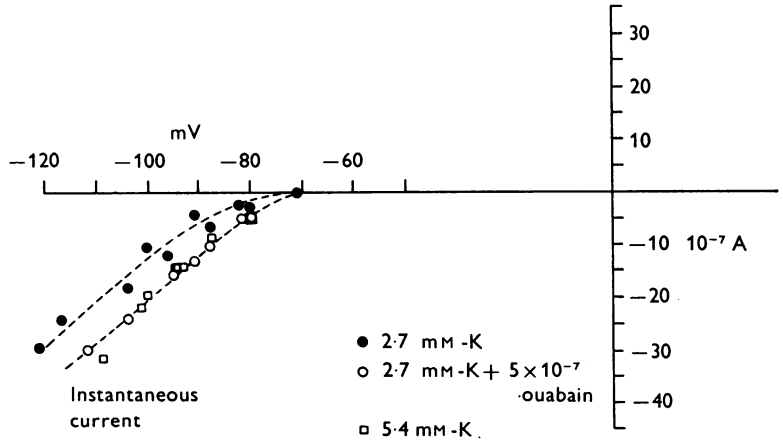

Fig. 11. Instantaneous current–voltage relations from the same experiment as Figs. 8–10.

The instantaneous current change in 5·4 mM-$[K^+]_o$ (□) is larger than in 2·7 mM-$[K^+]_o$ (●). The instantaneous current changes in 2·7 mM-$[K]_o$ plus ouabain (○) are equal to those in 5·4 mM-K^+. Since we have already shown that this result applies also to the total steady-state current change (Fig. 9) we may conclude that the voltage-dependent changes in i_{K_2} are also similar in magnitude. Thus, the changes in i_{K_2} produced by ouabain are fully accounted for by the change in $[K]_e$ produced by ouabain. There appears to be little direct action of ouabain on the i_{K_2} mechanism in the steady state (see Fig. 12*B*).

We have also found little or no effect of ouabain on the kinetics of i_{K_2}. Fig. 12*A* shows current records in 2·7 mM-$[K]_o$ before and after adding 1×10^{-7} M ouabain. The time course of i_{K_2} and the s activation curve are not significantly affected. Thus the location of s_∞ on the voltage axis is not significantly affected by ouabain. The results of Fig. 12*B*, also show no change in the amplitude of i_{K_2} at the holding potential (−75 mV). This result however is fortuitous since changes in amplitude of i_{K_2} do occur when there are significant changes in E_{rev}, as is clear from the

amplitudes of the time-keep dependent currents in Fig. 12. These changes are, however, fully accounted for by the change in $[K]_e$ and do not require any *direct* action of ouabain on the i_{K_2} mechanism.

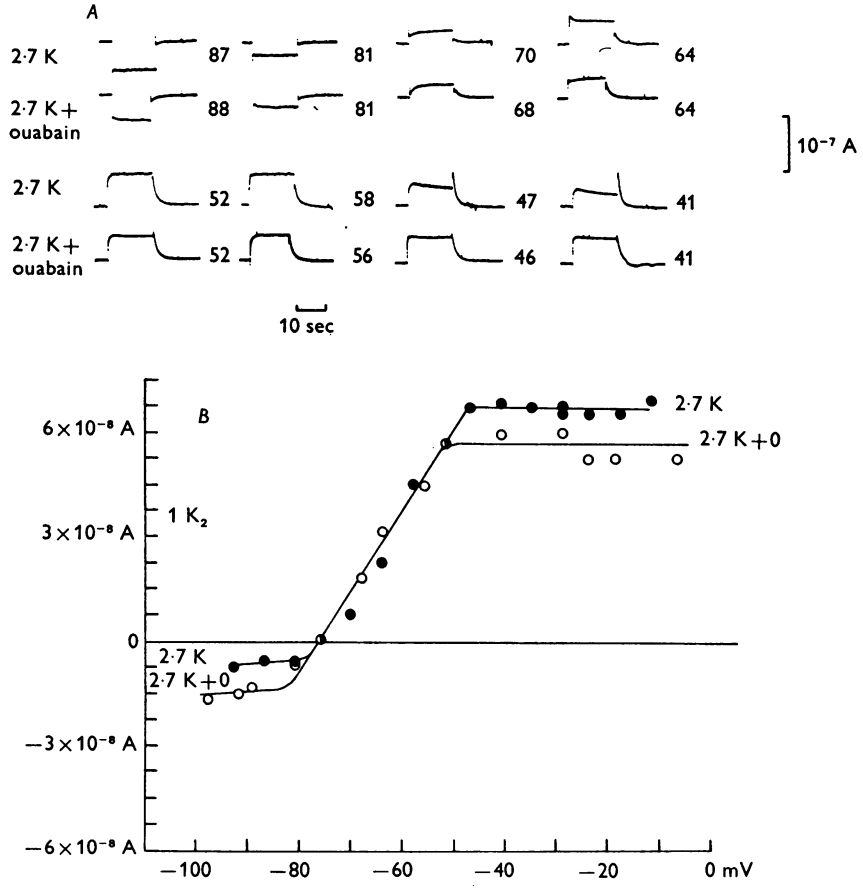

Fig. 12. Influence of ouabain (1×10^{-7} M) on kinetics of i_{K_2}.

A, currents in response to pulses from −75 mV to the potentials indicated. The magnitude and rate of decay of the current tails attributable to i_{K_2} are similar in both solutions (see *B*). The current responses during the pulses are similar when the pacemaker current, i_{K_2}, may be recorded. At potentials (e.g. −64, −47 and −41 mV) where other currents are present some differences are seen. These differences have not been investigated.

B, magnitudes of current tails as a function of pulse potential. Since i_{K_2} reaches steady-state values during each pulse these relations correspond to s activation curves. The half-amplitude point in ouabain shifts about 3 mV in negative direction. This shift is probably not significant since it is within the range of normal variation during an experiment.

The time course of E_K changes produced by ouabain

The results that we have described so far concern measurements of the influence of ouabain in steady-state, or near steady-state, conditions. Our overall conclusion is that the changes in E_{rev} produced are probably attributable primarily to changes in $[K]_e$. Another way of tackling this problem is to measure the time course of ouabain action since it should require very much less time to change $[K]_e$ in narrow cleft spaces in the preparation than to change $[K]_i$ in the large volume of intracellular space (see Discussion).

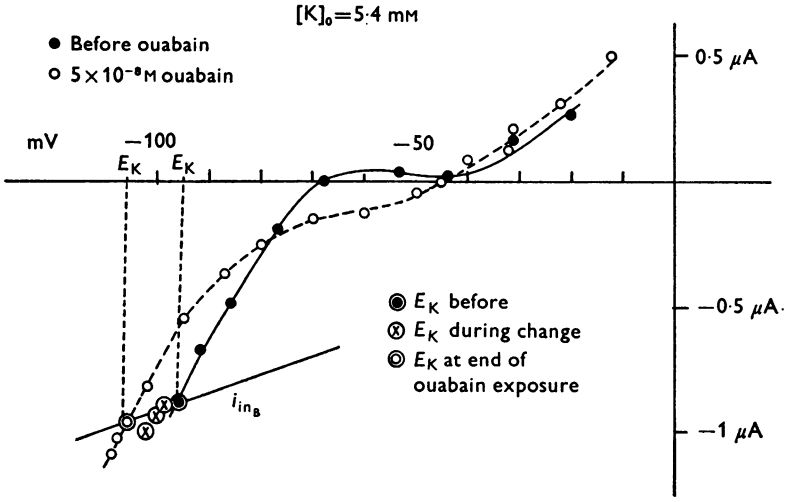

Fig. 13. Steady-state current voltage relation in 5·4 mM-$[K]_o$ before (●) and after (○) the application of ouabain at 5×10^{-8} M. The shift in E_K was followed as a function of time (the encircled' × 's), these time-dependent results show that the I_{E_K} (i_{in_B}) changes smoothly from the initial to final values. There is less than a 10% change in i_{in_B} for the 10 mV shift in E_K.

Fig. 13 shows the current–voltage relations obtained in a fibre exposed to 5·4 mM-$[K^+]_o$ Tyrode before and after adding 5×10^{-8} M ouabain. A negative shift in E_{rev} from −96 to −106 mV occurred. The current–voltage relation after ouabain action crosses the control curve in the way expected when $[K]_o$ is reduced. In this particular fibre the effect produced is sufficiently large to produce depolarization (from −68 to −45 mV) despite the hyperpolarizing shift in E_{rev}. This effect recalls the well known phenomenon that Purkinje fibres may depolarize when $[K]_o$ is made sufficiently small (Weidmann, 1956).

In this experiment we measured the value of E_{rev} at various times

during the onset of ouabain action using hyperpolarizing pulses to the region of E_{rev}. Samples of the current records obtained are shown in Fig. 14. It is quite clear that potentials that are negative to E_{rev} before the onset of ouabain action become positive to E_{rev} during the ouabain exposure. We have included three of the additional E_{rev} points obtained in Fig. 13. Note, once again, the relative constancy of i_i (E_{rev}). Only a very small increase in magnitude is observed as E_{rev} shifts.

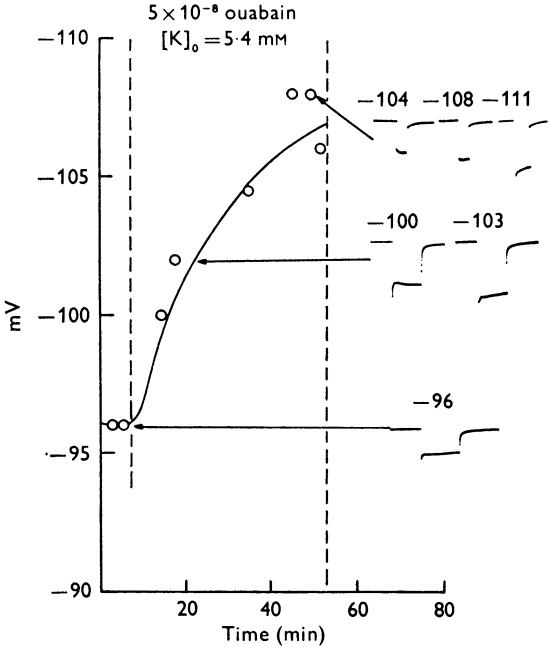

Fig. 14. Time course of shift in E_K during experiment shown in Fig. 13. The open circles show experimental measurements of E_K as a function of time, the continuous curve is fitted by eye to the experimental results. The dashed line to the left indicates the time of 5×10^{-7} M ouabain application. $[K]_o$ is 5·4 mM. The dashed line to the right indicates the end of the experiment. The current records at the right show the pulses on which the points arrowed were estimated.

The time course of the shift in E_{rev} is also plotted in Fig. 14. A significant shift (4 mV) in E_{rev} is recorded within 7 min, although about 50 min is required for the effect to approach a steady-state condition.

Fig. 15 shows the result of an experiment in which an inhibitory effect of ouabain (10^{-7} M) was recorded. It is interesting to note that during the first 5 min, there is a small, but probably significant, stimulatory effect. This is then followed by a fairly rapid shift in E_{rev} in the depolarizing

direction. E_{rev} changes from -92 to $-80{\cdot}5$ mV in 20 min. The rate of change in E_{rev} reaches 0·58 mV/min.

After 20 min exposure to ouabain a partial recovery was obtained on returning to a normal Tyrode solution. Partial recoveries are fairly typical when the action of ouabain is inhibitory. By contrast, fairly complete recovery is often obtained following stimulatory effects.

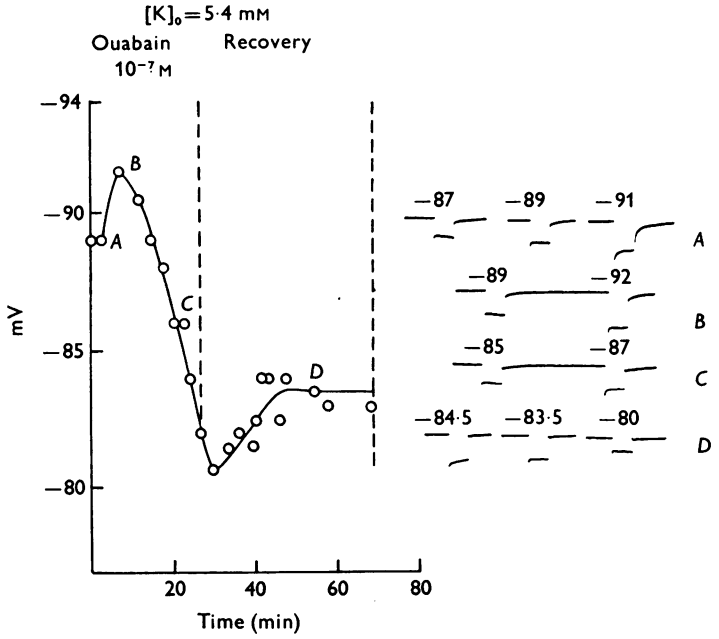

Fig. 15. Time course of E_K shift in the presence of 5·4 mM-$[K]_o$ and 1×10^{-7} M ouabain. Some of the current records on which points *A*, *B*, *C* and *D* are based are shown at the right.

Transient effects of ouabain action

We have demonstrated that when $[K]_o$ is relatively high or ouabain dosage is relatively low there is a stimulation of the Na^+–K^+ exchange pump resulting in a negative shift in E_{rev} which can be accounted for by depletion of $[K]_e$ in a restricted extracellular space.

We have also shown that when $[K]_o$ is relatively low or ouabain dosage relatively high, there is a block of the Na^+–K^+ exchange pump resulting in a positive shift in E_{rev} which can be accounted for by accumulation of $[K]_e$ in a restricted extracellular space.

It is also clear that in some of our experiments a transient negative shift in E_{rev} (low dose effect) was followed by a positive shift in E_{rev} (high dose effect). This can be seen in Fig. 15 which we interpret to indicate

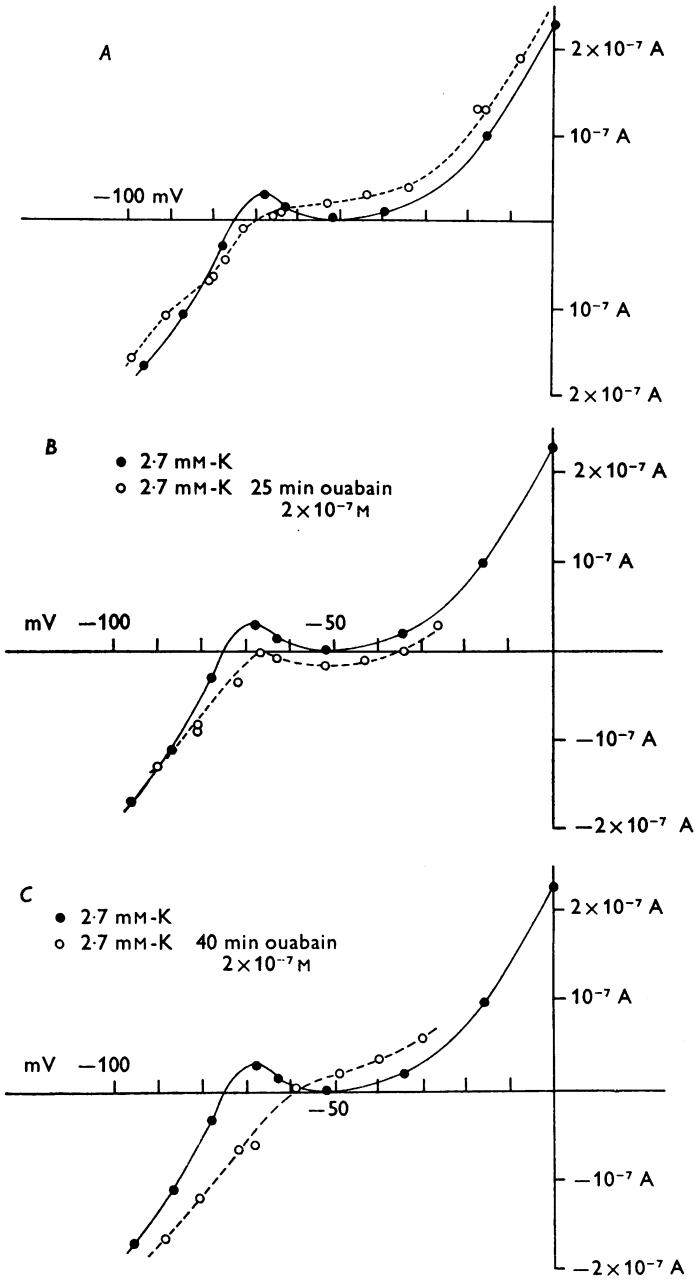

Fig. 16. Time course on the effects of 1×10^{-7} M ouabain on the steady-state current–voltage relations of a fibre bathed in 2·7 mM-$[K]_o$ (●). *A*, 10 min after drug application (○); *B*, 25 min after drug application; *C*, 40 min after drug application.

that at early times (during the build-up of ouabain concentration) there is stimulation and at later times there is inhibition of the Na^+–K^+ exchange pump. (See Discussion.)

The transient stimulation of the Na^+–K^+ exchange pump is also seen in steady-state current–voltage relations. Fig. 16 shows an experiment in 2·7 mM-K^+ and 2×10^{-7} M μ ouabain at various times after drug application.

At 10 min after drug application there is less current needed to hyperpolarize the cell to any potential beyond -80 mV (see Fig. 16*A*). This is the same result as described earlier (see *Influence of reduced* $[K]_o$ *on ouabain action*), and is suggestive of depletion of $[K]_e$ in a restricted extracellular space after Na^+–K^+ exchange pump stimulation.

At 25 min after application of the drug quite a different current–voltage relation is observed (see Fig. 16*B*). There is now less outward current at all potentials positive to -90 mV, and, at -90 mV the current–voltage relations of the control and drug curves cross, so that it takes less current negative to -90 mV to hyperpolarize the cell to any potential in the presence of drug than in its absence. This could represent a block of the Na^+–K^+ exchange pump as the dose builds up on the fibre after depletion of extracellular K^+ has already occurred.

The interpretation of the 25 min curve is supported by the current–voltage relation at 40 min (see Fig. 16*C*) in which there is a cross-over of the control and drug curves positive to the resting potential, and it now takes more current to hyperpolarize the cell to any potential. This is the same result as described earlier (*Influence of reduced* $[K]_o$ *on ouabain action*) and suggests blockage of the Na^+–K^+ exchange pump with accumulation of $[K]_e$ in a restricted extracellular space.

DISCUSSION

K accumulation and depletion during ouabain action

One of the main conclusions that we draw from our results is that the action of ouabain can be accounted for by accumulation and depletion of K^+ in a restricted extracellular space. In the preceding paper we have shown that in Purkinje fibres a restricted extracellular space (RES) exists, the K concentration of which ($[K]_e$) can differ substantially from the concentration ($[K]_o$) in the bathing Tyrode solution (Cohen *et al.* 1976). In this paper we describe the simultaneous and consistent change of two parameters during application of ouabain: (*a*) the shape of the steady-state current–voltage relation and (*b*) the reversal potential for the pace-maker current, i_{K_2}. Both of these parameters can be used as indicators of the value of $[K]_e$ in the RES. Although the changes in E_{rev}

observed might also indicate changes in $[K]_i$, there are quantitative arguments against this possibility (see Appendix).

The direction of the change in $[K]_e$ depends strongly on the dose of ouabain used and on the concentration $[K]_o$ in the bathing Tyrode solution. With low doses of ouabain and high $[K]_o$ the current–voltage relation and the value of E_{rev} changed in a way indicative of a reduction in $[K]_e$ (Figs. 3, 5). This effect was mimicked by reducing $[K]_o$ in the bathing Tyrode (Fig. 4). This is reminiscent of the well-known observation that the electrophysiological changes induced under these conditions are prevented by a modest increase in $[K]_o$ (Hoffman, 1972). We believe that the basis of these findings is a net stimulation of the Na^+–K^+ pump.

When the conditions are changed in a direction that favours toxic effects, i.e. an increased dose of ouabain or a reduction of $[K]_o$ in the bathing Tyrode, exactly the opposite effects were observed (Figs. 9, 11).

The current–voltage relation and E_{rev} changed in a way indicative of K^+ accumulation in the RES. This effect could be mimicked by increasing external $[K^+]$. We interpret these results as indicating blockage of the Na–K pump which is known to occur under these conditions. Changes attributable to pump blockage were only rarely reversible in our experiments, they usually persisted for hours after washout of the drug and were accompanied by depolarization, membrane potential oscillations and spontaneous activity. This agrees well with the changes in action potential configuration found by other groups in Purkinje fibres, ventricle, atrium, SA- and AV nodes, namely depolarization accompanied by a decrease of action potential duration which are also not reversible for several hours after washout of the drug (for references see Hoffman, 1972). We believe that blockage of the Na–K pump and the resulting accumulation of K^+ in the RES are the basis of these effects.

As can be seen from the calculations in the Appendix, the fractional change in pumping rate necessary to produce the observed effects would be very small indeed if the RES was totally separate from the bulk solution. So even if there is a considerable flux of ions between the external solution and the RES, our estimate is that a very small change in the pumping rate would be sufficient to change $[K]_e$. From this it follows that *in the steady state* only small changes in the electrogenic pump current will be observed (see below).

This result may appear to contradict that of Isenberg & Trautwein (1974) who measured quite substantial current changes that they attributed to i_p. However, it is important to remember that they applied a large dose of a fast-acting cardiac glycoside in order to measure the total pump current before the redistribution of ions becomes significant. With longer exposures to ouabain they also obtained indirect evidence

of extracellular K^+ accumulation. Moreover, some of our own results with inhibiting doses of ouabain resemble those of Isenberg & Trautwein (e.g. Fig. 6).

Stimulation of the pump by ouabain

The observation that ouabain may stimulate the Na–K exchange pump at low concentrations is perhaps unexpected but is not entirely without precedent. Some early work on glycoside-sensitive ATPases suggested that stimulation may occur in some circumstances (for references see Lee & Klaus, 1971). Moreover, in squid nerve, Baker & Willis (1972) found that concentrations of ouabain (10^{-6} M or greater) that produce inhibition of Na efflux in the steady state may first produce a transient stimulation of efflux. They did not use lower concentrations to determine whether steady-state stimulation could occur. Baker & Willis also noted they had no information concerning the question whether the transient stimulation of sodium efflux reflected an increase in Na–K exchange or in some other component of Na efflux. In our case, we can be certain that K movements are involved since we have measured K reversal potentials. Recently Peters *et al.* (1974) found that Na–K-sensitive ATPase activity from heart muscle was stimulated by therapeutic doses of ouabain.

Transient effects of ouabain

The results presented on the early effects of ouabain action suggest that when the ouabain dose level is high it is quite possible to see the effects of pump stimulation at early times after drug administration, where at later times pump blockage will predominate.

This could reflect the slow increase of ouabain concentration at its site of action in the Purkinje fibre, producing a stimulatory effect as long as the concentration is low and a net inhibitory effect as the ouabain concentration rises. On the other hand it could also mean that the binding of the drug to one site of the enzyme, which results in stimulation of the pump, is faster than the binding to another site, which results in inhibition of the pump (see Peters *et al.* 1974). Thus the transient stimulation could also reflect different rates of binding of cardiac glycoside to two different sites.

The sequence of events demonstrated in Figs. 15 and 16 could also account for the well-known diphasic effects of cardiac glycosides on the action potential and the membrane resistance of Purkinje fibres (Dudel & Trautwein, 1958). The transient stimulation of the Na–K pump could lead to a reduction of the K^+ concentration $[K]_e$ in the restricted extracellular space, which prolongs the action potential and increases the membrane resistance. The subsequent inhibition of the pump would

produce the opposite effects, shortening of the action potential and decrease of membrane resistance, which are observed at later times.

Competition between ouabain and K^+

Our finding that the level of extracellular K determines the magnitude and direction of action of a given dose of ouabain is clearly consistent with the antagonism between cardiac glycoside and K^+ observed clinically. Digitalis toxicity is observed more frequently during hypokalaemia and may be prevented by increasing plasma K before glycoside administration (Scherf & Schott, 1973). This has been explained by competition between ouabain and K^+ by analogy with the results obtained in red cells (Glynn, 1957) and in nerve (Baker *et al.* 1969).

Nevertheless, it is premature to conclude that all the effects of K^+ may be attributed to simple competition. Thus, the stimulatory effect of ouabain on Na^+–K^+ ATPase has not so far been found to be K^+ dependent. Moreover, the situation in the case of the inhibitory action is more complex than a single-site competition (see Schwartz, Matsui & Laughton, 1968; Akera, Baskin, Tobin & Brody, 1973; Hansen & Skou, 1973).

Mechanism of inotropic action

The observation that pump stimulation occurs at ouabain concentrations that are generally considered to be therapeutic creates obvious difficulties for the hypothesis that the positive inotropic action of cardiac glycosides is a secondary consequence of inhibition of the Na–K pump (Langer & Serena, 1970). Such models would predict a *negative* inotropic action during pump stimulation. It should, of course, be emphasized that our results were obtained on Purkinje fibres and we do not know whether our conclusions also apply to the myocardium itself. However, Blood (1975) has shown that positive inotropic effects may be recorded in Purkinje fibres exposed to ouabain concentrations that produce pump stimulation in our experiments. Whether or not our results are applicable to the myocardium, it is clearly very difficult to explain positive inotropic action in the Purkinje fibre only by pump inhibition.

Moreover, there are indications that in other parts of the heart also the therapeutic action of cardiac glycosides is not well correlated with inhibition of the pump. If depolarization and shortening of the action potential are used as indicators of net K^+ loss during pump inhibition, there is a striking discrepancy between the extremely slow reversibility of such changes recorded electrophysiologically and the much faster reversal of the therapeutic action in atrium (Peters *et al.* 1974), ventricle (Okita, 1972; Okita *et al.* 1973; Ten Eich, Bassett & Okita, 1973) and AV node (Toda & West, 1969). The diphasic time course of action of ouabain on

the duration of the action potential has not only been recorded in Purkinje fibres, but also in ventricular muscle (cf. Isenberg & Trautwein, 1974) and atrium (Sleator, Furchgott, de Gubaneff & Krespi, 1964). Taking these observations together with our own, we are inclined to reject the hypothesis that the positive inotropic action is causally related to net pump inhibition.

There are several other possibilities that remain to be considered. First, the inotropic action may be quite unrelated to actions on the Na–K ATPase and on ion pumping. Ouabain may have other actions on the cell. Secondly, it is possible that the positive inotropic effect may be related to ouabain actions on membrane ATPase sites other than those influencing ion transport. Finally, it is conceivable that the inotropic effects may be correlated with the direction of action on ion pumping but that the correlation is in the opposite direction to that suggested by Langer & Serena (1970), i.e. that the positive inotropic action is associated with the stimulatory effect on the pump (though not necessarily with *net* stimulation).

At present, it is not possible to eliminate any of these possibilities. Moreover, they each pose their own difficulties. These arise partly from the fact that net stimulation or net inhibition of the pump may conceal an underlying heterogeneity of action. For example, pump stimulation might occur at some sites even when net inhibition is observed. If these sites were those most involved in the inotropic action then it would be possible to obtain positive inotropic effects causally dependent on pump stimulation even when net inhibition occurs. Similarly, we might suppose that the relevant sites are inhibited even when net pump stimulation occurs, the inhibition being marked by a greater degree of pump stimulation at sites that are not related to inotropic mechanisms. This possibility would allow a more sophisticated version of the hypothesis suggested by Baker *et al.* (1969) and Langer & Serena (1970). Unfortunately, it is difficult at the present time to see how such heterogeneity may be investigated experimentally. All we can say with certainty is that positive inotropic effects occur during *both* net stimulation *and* net inhibition of the pump and that this fact, at least, eliminates some of the simpler hypotheses concerning the mechanism of the inotropic effect.

Influence of ouabain on pump activity in the steady state

We drew attention to the need to distinguish between the transient and steady-state actions of ouabain and have already indicated that the steady-state change in pump activity, and hence in pump current, may be quite small. The argument is in fact even stronger than we have already suggested. This arises from our observation that the value of

$[K]_o$ has little effect on the level of inward background current. This fact is the basis of a quantitative demonstration that steady-state changes in i_p will be very small. It is also a necessary condition for this to be the case.

The equation for membrane current (eqn. (1)) simplifies at the resting potential, since i_i is then zero. We then have

$$i_{in_B} = -(i_K + i_p). \tag{3}$$

Following the action of ouabain, and supposing once again that we wait for steady-state conditions to prevail, we have

$$i^1_{in_B} = -(i^1_K + i^1_p). \tag{4}$$

In general the cell will now lie at a different resting potential and the value of $[K]_e$ will have changed. However, provided these changes occur over the range of values for which i_{in_B} is nearly constant, $i \quad = i \quad$ and

$$i_K + i_p = i^1_K + i^1_p. \tag{5}$$

Now if the coupling ratio of the pump is unaltered and if i_{in_B} is the passive resting Na influx, then

$$i_K/i_{in_B} = i^1_K/i_{in_B} \tag{6}$$

eqns. (5) and (6) are satisfied only when

$$i_K = i^1_K \tag{7}$$

and

$$i_p = i^1_p. \tag{8}$$

Hence the steady-state pump current remains unchanged. This implies that transient changes due to stimulation or inhibition by ouabain are countered by the effects on pump activity of the ion concentration changes produced. Thus, if the pump is stimulated, the value of $[K]_e$ will fall and the cell will hyperpolarize (cf. Fig. 3). These changes will continue until the activity of the pump is reduced again by the fall in $[K]_e$ until it is just adequate to balance the K^+ loss and the Na^+ gain. Eqns. (4)–(8) simply express the fact that if the Na^+ gain is constant (i_{in_B} constant), the final steady state must be one in which the pump activity is restored to its original value. Finally, it is clear that the relative constancy of i_{in_B} is a necessary condition for the relative constancy of i_p. If i_{in_B} were to vary substantially with $[K]_e$ or E_m, the final value of i_p would have to adjust, via its action on $[K]_e$, to vary in proportion to the change in i_{in_B}, always assuming of course that the coupling ratio remains unchanged.

It should be noted that the derivation of eqn. (8) does not depend in any way on the properties of the extracellular space since the manner in which the pump adjusts to allow a steady-state condition to be achieved is not specified. Nevertheless, the presence of a restricted extracellular space greatly influences the speed at which the steady state may be

approached. As shown in the Appendix, $[K]_e$ will alter rapidly in response to changes in pump activity. In this way the restricted extracellular space in cardiac muscle may act as a 'buffer' to allow the net steady-state pump activity to adjust rapidly following changes in the external conditions, including the action of cardiac glycosides. This adjustment may also be achieved without requiring large changes in intracellular ion concentrations.

APPENDIX

The rate of active Na^+–K^+ transport in Purkinje fibres

The aim of this calculation is to relate results obtained from current–voltage relations of Purkinje fibres to the rate of Na^+–K^+ exchange through active transport. The assumptions underlying the calculation are listed below:

(1) The current measured at E_K reflects the current necessary to counter the K^+ efflux at the resting potential. This requires that the inward background current remains relatively unaffected by small changes in potential (i.e. the difference between the resting potential and the K equilibrium potential). This is substantiated in the results section of this paper (see section on 'Influence of extracellular K^+ in absence of ouabain').

(2) The reversal potential for i_{K_2} represents the true potassium equilibrium potential (see Cohen *et al.* 1976).

(3) The relative magnitude of intracellular and extracellular volumes within a Purkinje fibre are as reported by Hellam & Studt (1974). The percentage of the fibre occupied by intracellular material is 99·7 %, while that occupied by extracellular space (clefts) is 0·3 % of the total fibre volume.

(4) The cell surface membrane is divided into two groups. The greatest fraction, about 90 %, is facing the extracellular clefts within the fibre. About 10 % of the membrane faces the outside of the fibre (Mobley & Page, 1972).

(5) The fibre can be represented by a right circular cylinder.

(6) The Na^+–K^+ exchange pump operates by pumping three Na ions out for each two K ions that it pumps in. Therefore the pump current can be represented as equal to one half the K^+ efflux at the resting potential, and equal in magnitude but opposite in direction to one third the Na^+ influx at the resting potential.

(7) There is no substantial resting anion, or calcium current at the resting potential.

With these assumptions, a sample calculation for the fibre from Fig. 15 is given below.

Measurements from the fibre show that the radius was 0·09 mm, while

the length was 2·2 mm. The inward background current at E_K, $i(E_K)$ was 0·1 μA. The volume of the fibre is easily calculated, from assumption (5):

$$\pi r^2 h = 5{\cdot}6 \times 10^{-5} \text{ ml.}$$

The intracellular volume is assumed to be 99·7 % of the total by assumption (3). This is $5{\cdot}58 \times 10^{-5}$ ml. The extracellular or cleft volume is 0·3 % of the total fibre volume or $1{\cdot}68 \times 10^{-7}$ ml.

From assumption (6) the Na^+–K^+ exchange pump current is equal to one half the K^+ efflux at the resting potential, this is one half of $i(E_K)$. Thus the pump current at the resting potential is 0·05 μA. This is equal to $5{\cdot}18 \times 10^{-13}$ mole/sec.

The aim is to calculate the time necessary to reduce the internal K^+ concentration by the amount required to change E_K by 12 mV, the amount found experimentally in Fig. 15 of the results section. The total intracellular K^+ is equal to the concentration × volume, which is $8{\cdot}43 \times 10^{-9}$ M. Initially the external $[K]^+$ surrounding the fibre can be calculated by the Nernst equation:

$$E_K = -61 \log [K]_i/[K]_o,$$
$$92 = -61 \log 151/[K]_o,$$
$$[K]_o = 4{\cdot}69 \text{ mM}.$$

Where $[K]_i$ is assumed to be 151 mM (Robertson & Dunihue 1954) and −92 mV is the maximum value of E_K during the experiment. In order to reduce E_K by 12 mV the internal K^+ must be reduced by 34 % to 96·1 mM. This would require 44 min in the presence of total Na^+–K^+ exchange pump blockage. Thus in order for a change in internal K^+ to account for the experimental time of 18 min for the E_K change, a rate of K^+ efflux 2·5 times the normal rate would have to be assumed.

External accumulation of K^+ in a restricted extracellular space can account for the results. The large surface area facing the clefts of small volume provide an ideal location for restricted diffusion. The total K^+ in the clefts of the Purkinje fibre in the experiment of Fig. 16 is concentration × volume = $7{\cdot}90 \times 10^{-13}$ M. The K^+ efflux into these clefts is 90 % of the total K^+ efflux or $9{\cdot}32 \times 10^{-13}$ mole/sec. Thus in a totally restricted extracellular space (no diffusion into the bulk space) all the K^+ is exchanged every second. The required accumulation of K^+ in the clefts to reduce E_K by 12 mV could occur with 100 % pump blockage in less than 0·5 sec. Or if it was to occur in 18 min less than 0·01 % pump blockage is required.

A similar calculation on the results shown in Fig. 14 suggest that this cell would have to accumulate K^+ at 3× the normal pumping rate if internal K^+ concentration changes are to account for the shift in E_K

from −96 to −108 mV. However, if depletion in a restricted extracellular space were to account for the results, the necessary loss of extracellular K^+ could occur in less than 1 sec in the presence of total diffusion block, with 100% pump stimulation, or in 45 min with less than 0·01% pump stimulation.

We should like to acknowledge the support of the Muscular Dystrophy Association of America, the Rhodes Foundation and the Medical Research Council. We should also like to thank Mr A. J. Spindler for his expert technical assistance and the staff of Hedges (Abingdon) slaughterhouse for their help in providing fresh sheep hearts.

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