J. Physiol. (1981), **317**, *pp.* 163–187
With 10 *text-figures*
Printed in Great Britain

THE DEPENDENCE OF SODIUM PUMPING AND TENSION ON INTRACELLULAR SODIUM ACTIVITY IN VOLTAGE-CLAMPED SHEEP PURKINJE FIBRES

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(*Received 28 November 1980*)

SUMMARY

1. Intracellular Na activity (a^i_{Na}) was measured in sheep cardiac Purkinje fibres using a recessed-tip Na^+-sensitive micro-electrode. The membrane potential was controlled with a two-micro-electrode voltage clamp. Tension was measured simultaneously.

2. Removing external K produced a rise of a^i_{Na} and both twitch and tonic tension. On adding 4–10 mM-$[Rb]_o$ to reactivate the Na–K pump a^i_{Na} and tension declined. An electrogenic Na pump current transient accompanied the fall of a^i_{Na}.

3. The half-time of decay of the electrogenic Na pump current transient was similar to that of a^i_{Na}, (mean $t^{Na}_{0.5}/t^I_{0.5} = 0.97 \pm 0.03$ (S.E.M.; $n = 28$)). Following re-activation of the Na–K pump, the electrogenic Na pump current transient was linearly related to a^i_{Na}.

4. The duration of exposure to K-free, Rb-free solutions was varied to change the level of a^i_{Na}. On subsequently re-activating the Na–K pump with 10 mM-$[Rb]_o$, the ratio of charge extruded to the total change of a^i_{Na} was constant. It is concluded that the fraction of Na extruded electrogenically is unaffected by changes of a^i_{Na}. About 26% of the total Na extrusion appeared as charge transfer.

5. The relationship between tonic tension and a^i_{Na} was usually different during Na–K pump inhibition in a K-free, Rb-free solution compared with the relationship during Na–K pump re-activation. In general, a given a^i_{Na} was associated with a greater level of tonic tension during Na–K pump inhibition compared with that during pump re-activation. A similar hysteresis was often seen between twitch tension and a^i_{Na}.

INTRODUCTION

It is widely believed that the Na–K pump in a variety of cardiac tissues is electrogenic (e.g. Deléze, 1960; Page & Storm, 1965). Recently there have been reports suggesting that it is possible to measure directly the electrogenic Na pump

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current in voltage-clamped cardiac Purkinje fibres (Eisner & Lederer, 1979*b*, 1980*a*; Gadsby & Cranefield, 1979; Gadsby, 1980). This is achieved by loading the fibres with Na in a K-free medium and then re-activating the Na–K pump by introducing external K or another appropriate 'activator cation' such as Rb or Cs. It is known that the raised level of internal Na then stimulates a net Na extrusion (see e.g. Deitmer & Ellis, 1978*b*) and it is at this time that an outward current appears during voltage-clamp experiments. It quickly reaches a peak and then declines exponentially within a few minutes. The current transient is abolished by cardioactive steroids, becomes larger if the pre-loading with Na is prolonged, and is sensitive to the levels of external activator cation. It therefore closely resembles the outward Na–K pump current measured by Thomas (1969, 1972) in snail neurones following an intracellular ionophoretic injection of Na. He confirmed that it reflected enhanced electrogenic Na extrusion since the internal Na activity, a^{i}_{Na}, also declined exponentially and in parallel with the electrogenic outward current. There has so far been no similar demonstration that the outward current transient measured in Purkinje fibres reflects a decline of a^{i}_{Na}. However, quantitative analyses of this current have been based upon the assumption that it does (Eisner & Lederer, 1980*a*; Gadsby, 1980). This assumption was also made by Eisner & Lederer (1980*b*) when examining the relation between the outward current transient and the force of contraction in Purkinje fibres.

In the present work we have measured the membrane current in voltage-clamped sheep-heart Purkinje fibres whilst simultaneously measuring a^{i}_{Na} using a recessed-tip Na^{+}-sensitive micro-electrode, and also the twitch and tonic tension of the preparation. We have therefore been able to examine directly their interrelation whilst removing and re-adding external activator cations to change the Na–K pump rate. We find that, as in snail neurones, the decline of the electrogenic outward current during recovery from K-free solution is exponential and parallels the recovery of a^{i}_{Na}. Furthermore, the fraction of Na extruded electrogenically during this period appears to remain constant for quite large variations of a^{i}_{Na}. Finally, an increase and decrease of a^{i}_{Na} produced by altering Na–K pump activity is associated with positive and negative inotropic effects, although the exact relation between a^{i}_{Na} and tension under various experimental conditions is not as yet fully understood.

Preliminary accounts of some of the results of this paper have been published (Eisner, Lederer & Vaughan-Jones, 1980; Vaughan-Jones, Lederer & Eisner, 1981).

METHODS

General

With the exception of the addition of Na^{+}-sensitive micro-electrodes, the general methods have been described previously (Eisner & Lederer, 1979*a*). Sheep-heart Purkinje fibres (100–300 μm in total diameter) were shortened to 1–2 mm and mounted in a superfusion chamber. Voltage control was imposed with a two-micro-electrode clamp (Deck, Kern & Trautwein, 1964). Both the current-passing and the voltage-recording electrodes were bevelled (Lederer, Spindler & Eisner, 1979) from *ca.* 15 MΩ to *ca.* 3 MΩ. The preparation was connected to a tension transducer (Akers 803E piezoresistive element). The results were recorded on magnetic tape for subsequent analysis using a Hewlett-Packard FM tape-recorder. The final data were displayed on a Watanabe potentiometric pen-recorder, or on a Tektronix bistable storage oscilloscope.

Na^+-sensitive micro-electrodes

Recessed-tip Na^+-sensitive micro-electrodes were produced according to the method of Thomas (1970, 1978). Na^+-sensitive glass, 1 mm o.d., code NAS 11–18, was obtained from Microelectrodes Inc., U.S.A. Aluminosilicate glass (8049 'Supremax'; Schott Glass Ltd, West Germany) was used for the outer insulating glass pipette. This was re-drawn before making electrodes into capillary tubing 2 mm o.d. and *ca.* 1 mm i.d. The tip diameters of the completed Na^+-sensitive micro-electrodes were 0·5–1·0 μm and the exposed length of Na^+ glass in the 'recess' was 15–70 μm (see Thomas, 1978, for more details). The electrodes were calibrated in the perfusion chamber before and after each experiment. An electrode was acceptable if it produced 53–61 mV response for a tenfold change in Na activity (36 °C). The initial calibrating solution was 145 mM-NaCl. [Na] was then decreased by substituting K for Na, thus maintaining a constant ionic strength. K was chosen so that its presence in the calibrating solutions would compensate for any small interference of the high intracellular K on the electrode response. The relation between $\log_{10}$ [Na] and the electrode response (mV) was virtually linear from 145 mM-[Na] to *ca.* 4 mM-[Na], as reported previously by Ellis (1977). The activity coefficient of Na in the solutions is assumed to be 0·75 (36 °C) (Parsons, 1959), so that the Na activity:

$$a_{Na} = 0{\cdot}75\ [Na]. \quad (1)$$

All intracellular measurements have been expressed in terms of activity since this is what the Na^+-electrode measures. However, in order to express a change of internal Na activity, a^i_{Na}, in terms of an underlying net transmembrane Na flux it is first necessary to convert activities into ionic concentrations using a value for the intracellular activity coefficient for Na. Whilst there is evidence that a substantial portion of the total intracellular Na in muscle cells may be inaccessible to measurement by an intracellular ion-sensitive electrode it is generally assumed that the ionization of the Na in the cytoplasm is similar to that in the external medium (e.g. Vaughan-Jones, 1977; Ellis, 1977). Consequently, an intracellular activity coefficient of 0·75 has been assumed when estimating net Na fluxes, although this remains to be validated.

The present experiments required the measurement of comparatively rapid changes of a^i_{Na} with half-times ($t_{0·5}$) as low as 30–40 sec. It was therefore important to select electrodes with fast response times. This is particularly relevant when using the recessed-tip type of Na^+-electrode. The response time of an electrode was estimated by changing the solution flowing through the perfusion chamber from 145 mM-$[Na]_o$ to 14·5 mM-$[Na]_o$. If the half-time of the measured change of a_{Na} was greater than 6 sec, the electrode was not used. This procedure undoubtedly over-estimated $t_{0·5}$ by a few seconds or more since the solution in the bath itself took a finite time to change ($t_{0·5}$ *ca.* 3–4 sec as measured with a fast-responding, non-recessed Na^+-electrode: Hinke, 1959). Therefore, all electrodes used probably had a $t_{0·5}$ appreciably less than 6 sec. An attempt has been made to assess the errors that can occur when such electrodes are used to measure quite rapid changes of a^i_{Na}. This is presented in the Appendix.

Measurement of a^i_{Na} during voltage-clamp

In order to measure a^i_{Na} the potential recorded by the voltage electrode (the membrane potential, E_m) is subtracted from the Na^+-electrode signal. Ideally, then, E_m recorded by both electrodes should be the same. In the present experiments the preparation is polarized by the current-passing electrode. The resulting potential non-uniformity is minimized by using a short-fibre preparation (Deck *et al.* 1964) but some non-uniformity will necessarily persist. The ion-sensitive and voltage electrodes should therefore be inserted into the fibre as close together as possible. In practice, however, this can prove difficult because of movement during penetration. Fig. 1 shows the compromise spacing usually employed. The voltage and Na^+-electrodes were normally separated by 0·1–0·2 mm. With the arrangement shown, injection of current will change E_m at the proximal voltage electrode slightly more than at the distal Na^+-sensitive electrode. Therefore, because a^i_{Na} is measured as the difference between these two signals, passage of depolarizing current will result in an underestimate of a^i_{Na} and hyperpolarizing current will produce an over-estimate. Fig. 2*A* shows an experimental estimate of the resulting artifact and demonstrates that it is only a small source of error in the present work. E_m was depolarized to various levels requiring the observed change in voltage-clamp current, ΔI. The a^i_{Na} trace shows in each case a rapid transient deflexion associated with the initial potential change. This relaxes to a fairly steady value after a few seconds, probably reflecting the large RC time constant of the high-resistance Na^+-electrode. However, the

steady-state difference, ΔV (expressed in mV), produced by depolarization most likely represents the voltage non-uniformity. It is shown in the next paper (Eisner, Lederer & Vaughan-Jones, 1981) that a long-lasting depolarization of several minutes can produce a genuine fall of a^i_{Na}, but this effect will only slightly affect the present experiment since the depolarizations here are relatively brief (*ca.* 30 sec).

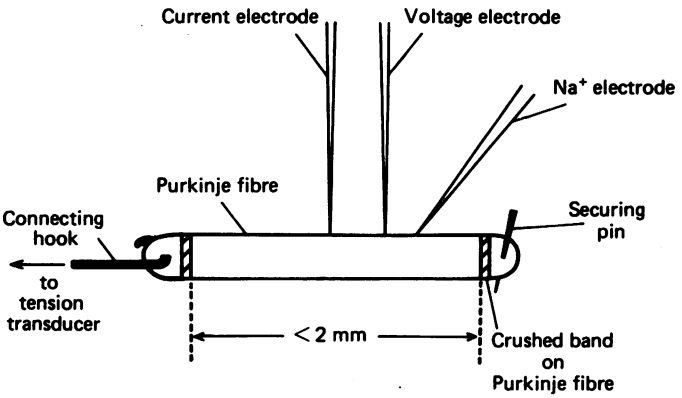

Fig. 1. Schematic diagram of Purkinje fibre preparation and electrode placement.

Fig. 2*B* shows ΔV as a function of ΔI. The relation is roughly linear, meaning that the larger the current being passed, then the larger is the error in the derived signal of a^i_{Na}. Nevertheless, with maximal currents of 30–40 nA this error is only about 2 mV. A full analysis of this type of voltage non-uniformity in short cable Purkinje fibres has been presented by Kass, Siegelbaum & Tsien (1979). They showed that the difference in potential between any two points along the fibre was monotonically related to the injected current. Therefore in our experiments it is possible in principle to correct for the current-passing artifact by reference to Fig. 2*B*. We have not done this, however, simply because the error is always small in comparison with the total changes of a^i_{Na} commonly detected. The experiment shown in Fig. 3 illustrates this point. It is part of the same experiment shown in Fig. 2: a continuous impalement with three electrodes. Upon re-adding external Rb (Fig. 3, described more fully in the Results) the peak of the electrogenic current transient is 16 nA and a^i_{Na} declines from 14·5 mM to 8·1 mM. Therefore, from Fig. 2*B*, the maximum error in the a^i_{Na} trace during this recovery will be just over 1 mV whereas the potential change in the a^i_{Na} trace as it declines is about 15 mV. Further demonstrations of adequate voltage uniformity are shown in Figs. 1, 6 and 7 of Eisner, Lederer & Vaughan-Jones (1981).

Previous measurements of a^i_{Na} in sheep cardiac Purkinje fibres have been performed in long-fibre preparations. In order to voltage clamp, however, the fibres must be shortened to less than 2 mm. It is possible that this could damage the fibre and raise the resting a^i_{Na}. We have found that at a mean holding potential of −60 mV the mean a^i_{Na} in 10 mM-$[Rb]_o$ is 6·7 ± 0·7 mM (S.E.M.; $n = 9$). Similar values are found in 4 mM-$[K]_o$ or 4 mM-$[Rb]_o$ and in previous work on long fibres (Ellis, 1977).

When it was necessary to elicit a twitch, brief (0·5 sec) depolarizing voltage pulses were applied. As shown in Fig. 2*A* these produce fast capacitive spikes on the a^i_{Na} trace. These spikes have been attenuated in all the other Figures, which were recorded at a slower chart speed with a low-pass filter (time constant 0·5–2 sec). The current records are similarly displayed and do not show the spikes produced by brief depolarizations.

Solutions

The modified Tyrode solution consisted of: 145 mM-NaCl, 4 mM-KCl, 2 mM-$CaCl_2$, 1 mM-$MgCl_2$, 10 mM-Tris HCl, 10 mM glucose. It was oxygenated with 100% O_2. Modifications to this standard solution are indicated in the text. A solution described as 4 mM-$[Rb]_o$ is the above solution with KCl removed and 4 mM-RbCl added. When more than 10 mM-RbCl was added, NaCl was reduced to maintain tonicity. The pH of all solutions was 7·4 ± 0·1. All experiments were performed between 35 and 37 °C ± 0·5 °C.

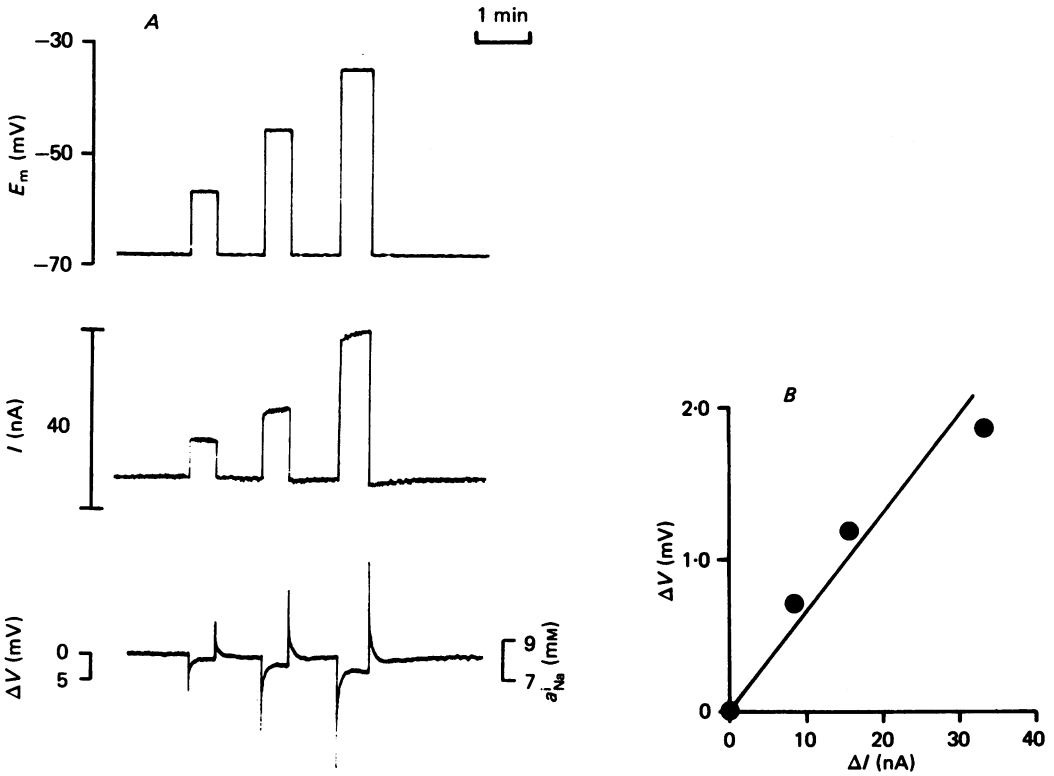

Fig. 2. Estimate of the artifactual alteration of measured a^i_{Na} due to potential non-uniformity produced by current injection. *A*, a series of three voltage-clamp pulses was applied from a holding potential of −68 mV to −56 mV, −45 mV and −34 mV respectively (top record). The middle record shows the current passed to produce the voltage changes while the bottom record shows measured a^i_{Na} (right-hand ordinate). Changes in the a^i_{Na} signal can alternatively be expressed as changes of potential (left-hand ordinate shows a 5 mV calibration). The spikes seen on the a^i_{Na} trace reflect the different RC time constants for the voltage and Na^+-sensitive electrodes. *B*, graph of ΔV *versus* ΔI (applied current change). ΔV is the artifactual change in the a^i_{Na} signal (expressed in mV) produced by an applied current change. Data from *A*. The values of ΔV were measured 20 sec after applying the depolarizing current. (Subsequently, there can also be a real fall of a^i_{Na} with depolarization (see Eisner *et al.* 1981). The beginning of such an effect is seen in the last depolarization in *A*.)

RESULTS

Simultaneous measurements of a^i_{Na}, membrane current and tension

Fig. 3 shows the effects on the internal Na activity, a^i_{Na}, membrane current, twitch tension and tonic tension of reducing and then increasing the activity of the Na–K pump. This was achieved by removing and then re-adding Rb to the external, K-free bathing solution. Rb is virtually equivalent to K in its ability to re-activate the Na–K pump (see next section, p. 171). Removing external Rb reduces the activity of the pump resulting in a rise of a^i_{Na} (cf. Ellis, 1977). The membrane potential was held constant at −68 mV by the voltage-clamp circuit and a 500 msec depolarizing pulse (to −34 mV) was applied once every 10 sec. This was sufficient to elicit a twitch. Both

the peak twitch tension and the tonic tension increased as a^i_{Na} increased. Six minutes later $[Rb]_o$ was raised to 4 mM. This re-activated the Na–K pump producing a rapid decrease of a^i_{Na} and also of twitch and tonic tension. The relationship between a^i_{Na} and tension is dealt with later (see p. 176). Of greater interest here is the current record, which shows a transient increase of outward current subsequently decaying with the fall of a^i_{Na}. This current is abolished by cardioactive steroids (Gadsby & Cranefield, 1979; Eisner & Lederer, 1979*b*, 1980*a*) and we find (not shown) that 10^{-5} M-strophanthidin equally inhibits the simultaneous fall of a^i_{Na}. The outward current transient has been attributed to stimulation of the Na–K pump in the presence of an elevated a^i_{Na}. It is thought to be produced by the electrogenic extrusion

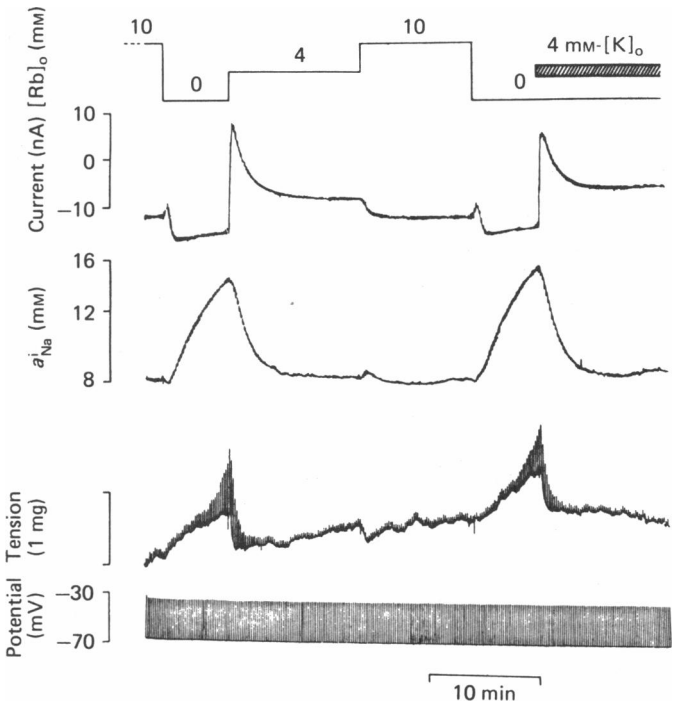

Fig. 3. Simultaneous measurement of membrane current, a^i_{Na}, tension and voltage-clamped membrane potential. Using a holding potential of −68 mV, a 500 msec depolarizing pulse to −34 mV was applied at 0·1 Hz (bottom). The other traces show (from top to bottom): membrane current, a^i_{Na} and tension. Alterations of the superfusing solution are shown above the Figure. From a control solution of 10 mM-$[Rb]_o$, $[Rb]_o$ was lowered to zero for 6 min and was then increased to 4 mM. After 12 min the preparation was returned to the control (10 mM-$[Rb]_o$) solution. After a period of 10 min, $[Rb]_o$ was again reduced to zero. Six minutes later 4 mM-$[K]_o$ was added to the superfusing solution.

of Na. In this case, the decay of current should reflect the fall of a^i_{Na} that occurs when the Na–K pump is re-activated.

This hypothesis can now be tested directly by examining the decline of a^i_{Na} and clamp current in the same experiment. Fig. 4*A* shows the data of Fig. 3 re-plotted semi-logarithmically. The levels of current and a^i_{Na} above their steady-state levels in 4 mM-$[Rb]_o$ are plotted as ΔI (squares) and Δa^i_{Na} (filled circles) respectively. After

a short initial period of about 30 sec (during which time the current transient was rising to a peak value) a^i_{Na} declined exponentially ($t^{Na}_{0.5}$ = 50 sec). This provides important confirmation of the results of Deitmer & Ellis (1978*b*). They measured the decline of a^i_{Na} in non-voltage-clamped preparations and so the passive Na influx during their recoveries was not necessarily constant since both E_m and the voltage-sensitive Na permeability would have changed. This might seriously distort the time course of the recovery of a^i_{Na} (see following paper: Eisner *et al.* 1981). In our experiments, however, E_m was maintained constant and so the passive Na influx was

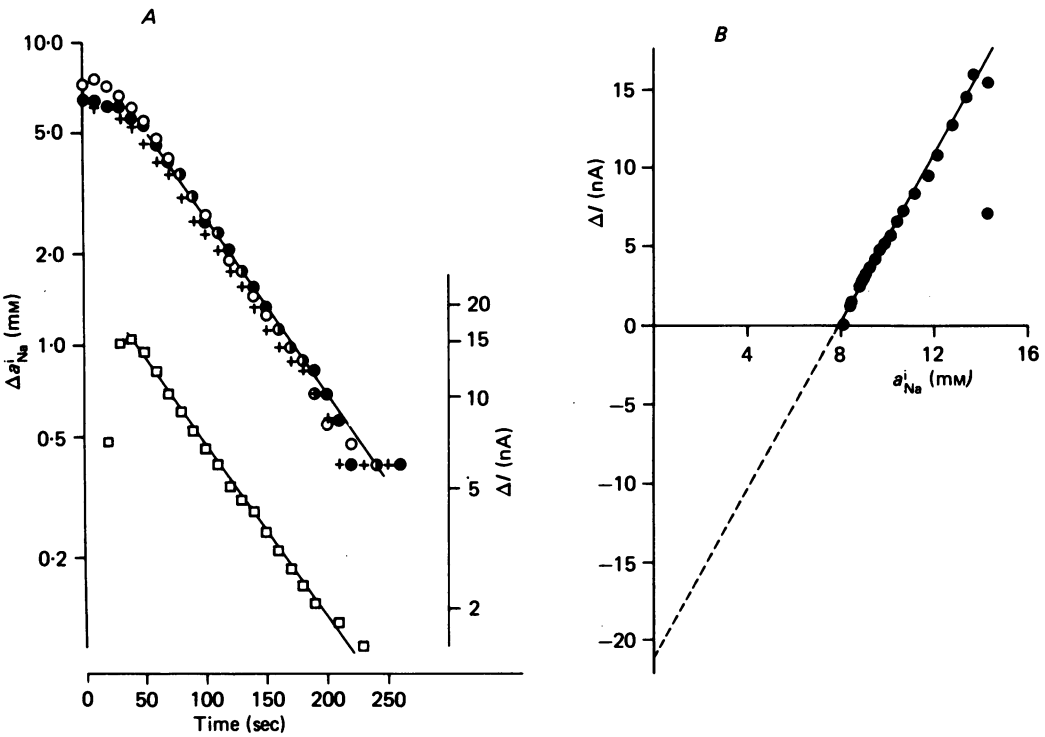

Fig. 4. The relationship between a^i_{Na} and current following Na–K pump re-activation. *A*, semi-logarithmic plot of the time course of decay of a^i_{Na} and current following Na–K pump reactivation. Following a 6 min exposure to a K-free, Rb-free superfusing solution the preparation was superfused with either 4 mM-$[K]_o$ or 4 mM-$[Rb]_o$. Current and a^i_{Na} are plotted as the magnitude above the steady-state value (respectively ΔI and Δa^i_{Na}) as a function of time after adding either 4 mM-$[Rb]_o$ (□, current; ●, a^i_{Na}) or 4 mM-$[K]_o$ (○, a^i_{Na}). The crosses show the a^i_{Na} record in 4 mM-$[Rb]_o$ corrected for the Na^+-electrode delay ($t_{0.5}$ = 6 sec). Regression lines are fitted to the Δa^i_{Na} and corresponding ΔI data obtained in 4 mM-$[Rb]_o$ (the first 30 sec following re-addition of Rb were ignored since they coincided with the rising phase of the electrogenic current transient). Data from Fig. 3. *B*, ΔI as a function of a^i_{Na}. The data are taken from the re-activation with 4 mM-$[Rb]_o$.

far less likely to vary. The decay of clamp current was also exponential (cf. Gadsby & Cranefield, 1979; Eisner & Lederer, 1980*a*), with $t^I_{0.5}$ = 51 sec. Thus both current and a^i_{Na} declined exponentially and at the same rate. In twenty-eight determinations from a total of eight preparations the average value of $t^I_{0.5}/t^{Na}_{0.5}$ was 0·97 ± 0·03 (S.E.M.). The excellent agreement between the two half-times confirms that the decline of the

outward current transient does indeed provide a good measure of the decline of a^i_{Na}. This in turn is strong evidence that the outward current transient reflects the electrogenic component of Na extrusion during recovery from Na-loading.

The correlation between current and a^i_{Na} can be expressed in another way, as in Fig. 4*B*. Here the magnitude of the current transient (ΔI) is plotted against a^i_{Na} for different time intervals following re-activation of the Na–K pump with external Rb (data from Fig. 3). After the first two points, which correspond to the rising phase of the current record, there is a linear relationship between ΔI and a^i_{Na}. The linear relationship confirms that a^i_{Na} and current decay in parallel. A similar linear correlation was found in other experiments (see e.g. Fig. 2*B* of Eisner *et al.* 1981). It was occasionally noted that the values of a^i_{Na} and current determined immediately after re-adding external Rb did not lie close to the line. In these particular cases a linear relationship was established 10–20 sec after the current transient had reached its peak.

Assuming that the recovery of a^i_{Na} represents a net efflux of Na from the fibre, Fig. 4 implies that a constant fraction of the Na extrusion is electrogenic. Our results are therefore in good agreement with those of Thomas (1969, 1972), who similarly found a parallel, exponential decline of both a^i_{Na} and electrogenic Na pump current in voltage-clamped snail neurones following an intracellular injection of Na. The recovery of a^i_{Na} in the present experiments was produced by adding back external Rb. It therefore seems likely that the coupling of Na extrusion to the pumped influx of Rb is remaining constant (although simultaneous movements of other ions cannot as yet be excluded).

It seemed possible that the slow response time of the Na^+-electrode ($t_{0.5}$ *ca.* 6 sec) might seriously distort the measured changes of a^i_{Na} that occurred upon re-activating the Na–K pump. This effect will be most serious when the recovery of a^i_{Na} is rapid ($t^{Na}_{0.5}$ *ca.* 30 sec in the fastest case). We have therefore attempted to correct for this effect by estimating the amount by which the activity of Na in the recess of the Na^+-electrode might lag behind the real activity of Na in the cell during fast changes of a^i_{Na}. The mathematical approach is outlined in the Appendix. By using eqn. (A 2) the measured change of a^i_{Na} in Fig. 4 upon re-activating the Na–K pump with Rb (●) can be re-plotted, allowing for the response time of the Na^+-electrode (crosses). Although these corrections must be regarded as a first approximation they will at least indicate whether the effect should be regarded as a serious problem. The corrected results re-plotted in Fig. 4*A* (crosses) indicate that the problem is minimal. The time constant of the corrected decline of a^i_{Na} is identical to the actual measured change. However, the measured values of a^i_{Na} lag behind the corrected values by a few seconds so that at any instant during the recovery the measured value of Δa^i_{Na} is about 12% higher than the corrected value. Such corrections affect the present results very little and certainly do not alter our conclusions. The corrections would slightly alter the slope of the line in Fig. 4*B* (from 2·6 nA mM^{-1} to 3·0 nA mM^{-1}), which would change the intercept on the ordinate from 21 nA to 24 nA. Since the corrections are small they have not been applied in subsequent experiments.

The use of Rb as an activator cation

In this and the following paper (Eisner *et al.* 1981) we have used variations of $[Rb]_o$ rather than $[K]_o$ to change the activity of the Na–K pump. For sheep cardiac Purkinje fibres the use of external K can introduce problems. Briefly, when the Na–K pump is re-activated following a period of Na loading, the level of activator cation (e.g. K or Rb) in the extracellular spaces may be reduced transiently (e.g. Kunze, 1977). Since changes in extracellular K markedly alter the shape of the Purkinje fibre current–voltage relationship, the magnitude and time course of the electrogenic Na pump current transient may be significantly distorted if K is used as the activator cation. Eisner & Lederer (1980*a*) showed that if external Rb is used instead of K, although depletion in the restricted extracellular spaces may still occur, there is little distortion of the current–voltage relationship of the preparation.

Rb might be expected to behave in a manner similar to K in its effects on the Na–K pump in cardiac Purkinje fibres (Eisner & Lederer, 1980*b*). Fig. 3 compares the relative abilities of K and Rb to decrease a_{Na}^{i}. Following the re-activation with 4 mM-$[Rb]_o$, the preparation was again exposed to a Rb-free, K-free solution. After 6 min in this solution the preparation was superfused with 4 mM-$[K]_o$. a_{Na}^{i} recovered at a similar rate in 4 mM-$[Rb]_o$ or 4 mM-$[K]_o$. In Fig. 4*A* these re-activations are plotted on semi-logarithmic co-ordinates. It is clear that the rate of decay of a_{Na}^{i} in 4 mM-$[K]_o$ (open circles; $t_{0.5}^{Na} = 48$ sec) is similar to that in 4 mM-$[Rb]_o$ (●; $t_{0.5}^{Na} = 50$ sec). In three fibres the mean ratio of the two half-times (Rb/K) was 0.98 ± 0.07 (S.E.M.) (two fibres with 4 mM-$[K]_o$ or 4 mM-$[Rb]_o$; one fibre with 10 mM-$[Rb]_o$ or 10 mM-$[K]_o$), indicating that the abilities of each of these two cations to activate the Na–K pump are very similar. This confirms the finding of Eisner & Lederer (1980*b*) and furthermore supports the use of Rb as a substitute for K in this and the following paper (Eisner *et al.* 1981). Glitsch & Pusch (1980) have compared the effects of $[K]_o$ and $[Rb]_o$ on the rate of fall of a_{Na}^{i} in sheep Purkinje fibres following exposure to K-free, Rb-free solutions. In contrast to our results they found that the rate constant of decay in $[Rb]_o$ was slightly less than in $[K]_o$.

Effect of changes of a_{Na}^{i} *on the rate of Na extrusion*

In a further series of experiments a_{Na}^{i} was elevated to different levels in a Rb-free, K-free solution before reactivating the Na–K pump with 10 mM-$[Rb]_o$. In this way we could examine the dependence of electrogenic Na extrusion on a_{Na}^{i}. Fig. 5 illustrates one of three such experiments. Depolarizing voltage-clamp pulses were not applied in this experiment and so only tonic tension at the holding potential was recorded. Different levels of a_{Na}^{i} were achieved by varying the exposure time to Rb-free solution. During the longer exposures a_{Na}^{i} tended to level off around 15–20 mM as already described by Deitmer & Ellis (1978*b*). The beginning of this effect can be observed in the last exposure of Fig. 5. Upon re-adding 10 mM-$[Rb]_o$ there was, in all cases, a rapid recovery of a_{Na}^{i} associated with the rise and fall of the electrogenic Na pump current transient. As expected this current transient became larger as the amount of Na extruded was increased. Fig. 6*A* shows that the outward current transient and a_{Na}^{i} each fell exponentially at virtually the same rate. Fig. 6*B* shows that varying the initial level of a_{Na}^{i} has no great effect on the half-time of the

subsequent decline of current and a^i_{Na}; $t_{0.5}$ varies by less than 20 % over the measured range of a^i_{Na} (2·7–11·3 mM). It is not clear whether this small change is significant. In seven re-activations from two other preparations, the $t_{0.5}$ values remained similar for large variations of a^i_{Na} (5–16·5 mM) although again there was some decrease in $t_{0.5}$ as the degree of Na loading was increased. However, the maximum decreases of $t_{0.5}$ were only 5·6 and 10 %. Eisner & Lederer (1980*a*) report no significant effect of Na loading on the half-time of recovery of the electrogenic Na pump current transient in sheep cardiac Purkinje fibres under similar conditions. No change of $t_{0.5}$ has also been reported for canine Purkinje fibres following short (1–5 min) periods of Na loading (Gadsby & Cranefield, 1979).

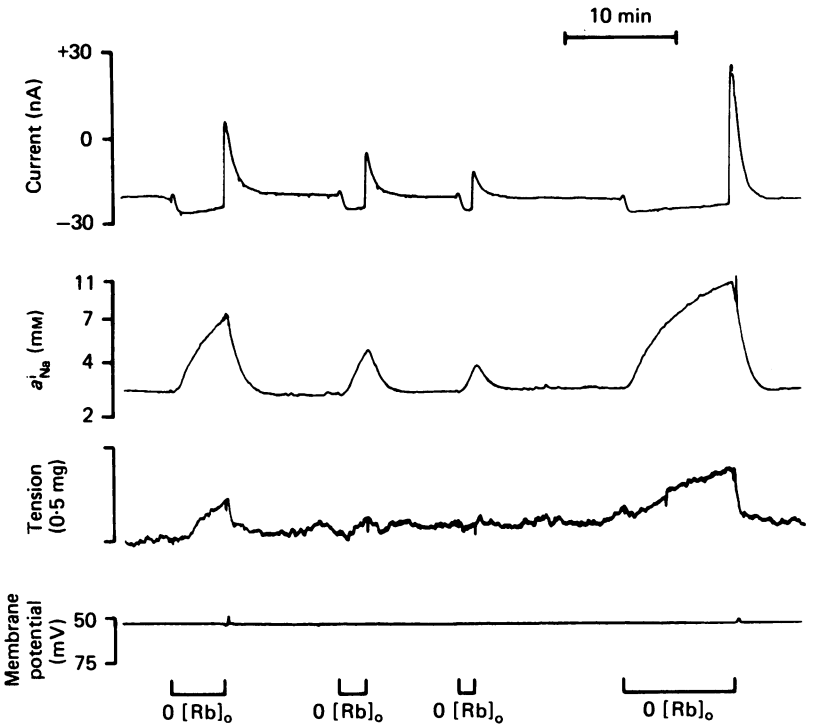

Fig. 5. The effect of varying the duration of exposure to 0 $[Rb]_o$ on current, a^i_{Na} and tonic tension. From top to bottom the records show: current, a^i_{Na}, tension, membrane potential. The membrane potential was held at −54 mV. No pulses were applied. $[K]_o$ was zero throughout the experiment. The control solution was 10 mM-$[Rb]_o$. As shown by the bars, $[Rb]_o$ was lowered to zero for periods of 5, 2·5, 1·5 and 10 min. Following these exposures $[Rb]_o$ was increased back to 10 mM-$[Rb]_o$.

In general, then, our results suggest that during re-activation of the Na–K pump both the *total* net Na extrusion rate and the *electrogenic* Na extrusion rate are linearly related to a^i_{Na}. This result complements that of Fig. 4*B* which showed a linear relationship between the electrogenic extrusion of Na and a^i_{Na}. The data indicate no sign of saturation as a^i_{Na} is increased. This is also consistent with the observation of Deitmer & Ellis (1978*b*) who examined the recovery of a^i_{Na} in sheep Purkinje fibres under non-voltage-clamped conditions and found that the maximum rate of recovery of a^i_{Na} increased linearly with a^i_{Na} up to levels as high as 31 mM. Of course, our results

do not exclude a more complex, non-linear relationship either at a very high or very low a^i_{Na}. Glitsch, Pusch & Venetz (1976) using chemical analysis of internal Na suggest that the active Na efflux from guinea-pig auricles saturates as internal Na levels are raised ($K_{0.5}$ = 22 mM-$[Na]_i$; equivalent to 17 mM-a^i_{Na}). In other tissues the relationship between Na efflux and internal Na appears to be either linear (e.g. squid axon: Baker, Blaustein, Keynes, Manil. Shaw & Steinhardt, 1969) or saturating (e.g. erythrocyte: Garay & Garrahan, 1973).

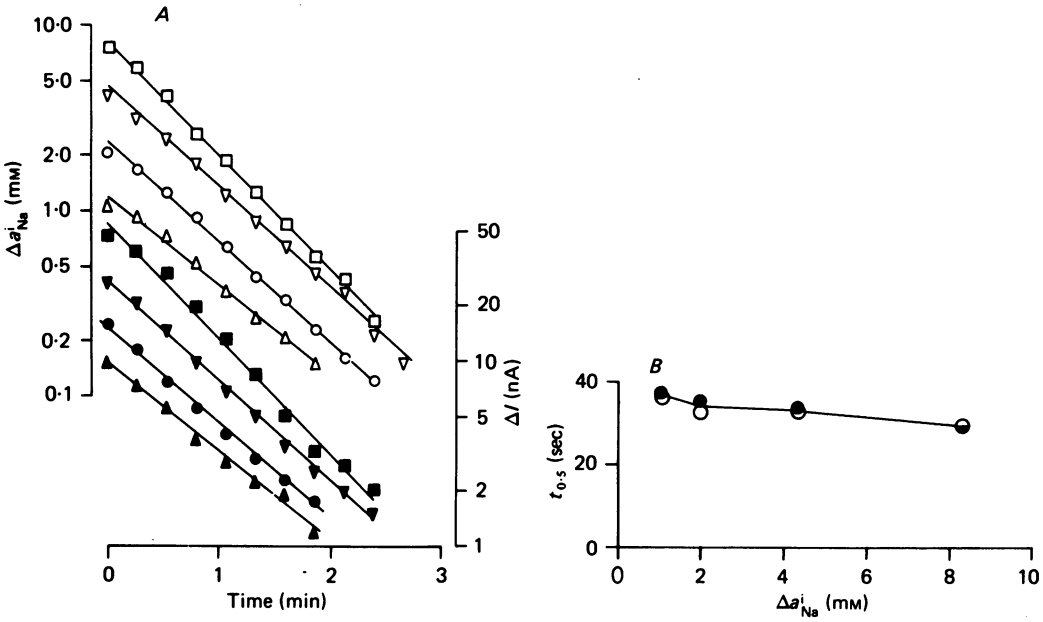

Fig. 6. The relationship between a^i_{Na} and current on reactivating the Na–K pump with 10 mM-$[Rb]_o$ following different durations of exposure to 0 $[Rb]_o$. *A*, semi-logarithmic plot of decays of a^i_{Na} and current. Measurements begin at the peak of the electrogenic current transient. Open symbols show Δa^i_{Na} and filled symbols current, ΔI, following exposures to 0 $[Rb]_o$: (□■), 10 min; (▽▼), 5 min; (○●), 2·5 min; (△▲), 1·5 min. Lines fitted by least-squares. Data from Fig. 5. *B*, half-time of decay of a^i_{Na} and current as a function of initial a^i_{Na}. The ordinate shows $t_{0.5}$ for current (●) and a^i_{Na} (○), the abscissa plots the increase of a^i_{Na} (Δa^i_{Na}) produced by the previous exposure to 0 $[Rb]_o$. Data from *A*.

Is the electrogenic fraction of net Na extrusion independent of a^i_{Na}*?*

During any individual re-activation, the fact that a^i_{Na} and the electrogenic Na pump current decline in parallel implies that the fraction of Na extruded electrogenically remains constant and is thus independent of a^i_{Na}. This idea can be tested by comparing the charge extruded by the electrogenic Na–K pump with the total amount of Na extruded. During recovery from Rb-free solution, the area under the outward current transient (i.e. the time-integral of the current) is a measure of the amount of Na extruded electrogenically by the Na–K pump (Rang & Ritchie, 1968; Thomas, 1969, 1972; Eisner & Lederer, 1980*a*) whereas the overall change of a^i_{Na}, Δa^i_{Na}, will be a function of the *total* amount of Na extruded. The ratio between 'area' and Δa^i_{Na} should therefore be constant when comparing recoveries from different

levels of a^i_{Na} as long as the electrogenic fraction of Na extrusion remains constant. The theoretical relationship is expressed by the equation

$$Q^e = \Delta a^i_{Na}\, r \frac{FV}{\gamma}, \tag{2}$$

where Q^e is the integral of the electrogenic Na pump current transient, F is the Faraday, V is the intracellular volume of the Purkinje fibre, r is the fraction of Na extrusion which is electrogenic (i.e. $r = 0{\cdot}33$ for an Na–K pump which transports 3 Na for 2 K ions), Δa^i_{Na} is the change of a^i_{Na} on re-activating the Na–K pump and γ is the intracellular activity coefficient for the Na ion (assumed to be 0·75: see Methods).

Fig. 7*A* shows that the relationship between the area under the electrogenic Na pump current transient (coulombs) and Δa^i_{Na} appears to be reasonably linear over a range of a^i_{Na} from 2·7 to 11·2 mM ($\Delta a^i_{Na} = 0$–8·3 mM). The results of eleven

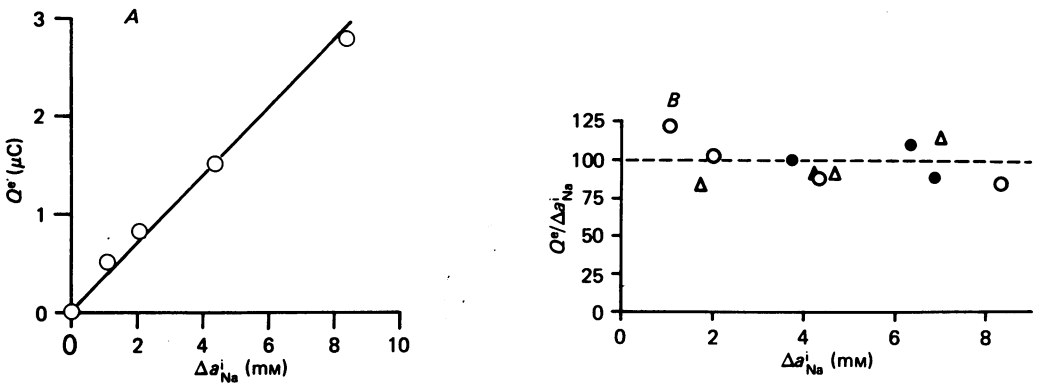

Fig. 7. The relationship between the charge extruded during the electrogenic Na pump current transient and the decrease of a^i_{Na}. *A*, data from Fig. 5. The ordinate is the area under the electrogenic Na pump current (μC). The abscissa shows the decrease of a^i_{Na} (Δa^i_{Na}) produced by Na pump re-activation. *B*, normalized ratio of charge extruded (Q^e) to Δa^i_{Na}. The ordinate shows $Q^e/\Delta a^i_{Na}$ as a percentage of the mean value in a particular experiment. The abscissa is the decrease of a^i_{Na} produced by Na pump re-activation with 10 mM-$[Rb]_o$. The different symbols show data from three experiments.

re-activations with 10 mM-$[Rb]_o$ obtained from three experiments (see e.g. Fig. 5) are summarized in Fig. 7*B*. In order to compare different experiments, the ratio $Q^e/\Delta a^i_{Na}$, has been calculated for each individual re-activation and expressed as a percentage of the mean ratio found in the same experiment. Although there is some scatter, it is evident that when different amounts of Na are pumped out of the fibre (Δa^i_{Na}) then the fraction which is extruded electrogenically (proportional to $Q^e/\Delta a^i_{Na}$) appears to remain constant, varying within ±20% of the mean.

From eqn. (2) it is clear that a comparison of the fraction of Na extruded electrogenically requires that the intracellular volume, V, be constant. However, the fibres may swell as Na–K pump activity is reduced in Rb-free solution and a^i_{Na} rises and then shrink again upon re-activation of the pump. Such volume changes are unlikely to be large enough to distort the present results. For example, consider the largest rise of a^i_{Na} in Fig. 5. Upon re-adding external Rb, a^i_{Na} decreased by 8·3 mM. If this represents a net efflux unaccompanied by any net influx of external cations, then in the absence of other regulating processes the cell would be expected to shrink by nearly 6% (Boyle

& Conway, 1941). This is likely to be an over-estimate since about 70% of the efflux will be accompanied by an influx of external Rb (see next section) and so involve no net exchange of water. The residual shrinkage might then be about 2–3%. Such a volume change would distort the measured 8·3 mM fall of a^i_{Na} by less than 0·5 mM and this effect is unlikely to alter the type of analysis shown in Fig. 7.

If the fraction of Na extruded electrogenically represents the amount of Na extruded by the pump in excess of the pumped influx of Rb, then our results suggest that the coupling coefficient of the Na–K(Rb) transport system is independent of substantial alterations of a^i_{Na}. Such a conclusion was also reached by Thomas (1969, 1972) for snail neurones. Furthermore, the results support similar conclusions drawn from less direct experiments examining the Na pump current in cardiac Purkinje fibres (Eisner & Lederer, 1980*a*; Gadsby & Cranefield, 1979).

The coupling ratio of the Na–K pump

If the intracellular volume is known then, from eqn. (2), it is possible to calculate a value for the electrogenic fraction of Na extrusion. However, reliable estimates of volume are difficult to obtain, mainly because the Purkinje cells are surrounded by a connective tissue sheath. Our standard method of determining volume from measurements (length and diameter) using a binocular microscope may therefore be inaccurate and this will produce errors in our estimate of the absolute value of the electrogenic fraction of Na extrusion. It should, however, be noted that determinations of relative changes in any individual fibre will not be affected by this problem (e.g. Fig. 7).

The mean value of the electrogenic fraction obtained from eight preparations (twenty-eight re-activations with usually three to five determinations per fibre) was $0{\cdot}26 \pm 0{\cdot}06$ (S.E.M; $n = 8$). Assuming that the recovery of a^i_{Na} represents a net efflux from the fibre, then, on average, 26% of this departs electrogenically as net charge. If it is assumed that the remainder leaves in exchange for a pumped influx of Rb, then this value will represent the mean coupling coefficient of the Na–K(Rb) pump. It is equivalent to a stoichiometry in the range of 4 Na:3 Rb. Clearly this is only a rough estimate because of the uncertainties of volume measurement. It is also possible that part of the total Na extrusion is mediated by systems other than the Na–K pump (Deitmer & Ellis, 1978*a*). In this case the Na–K pump may be more electrogenic than is suggested by the above value. The mean value is, nevertheless, remarkably close to the mean value of 0·28 obtained by Thomas (1969) for snail neurones recovering from an intracellular injection of Na and similar to the value of 0·33 estimated for the Na–K pump in erythrocytes (Garrahan & Glynn, 1967; Post & Jolly, 1957).

Another possible way of estimating the electrogenic fraction of Na extrusion is illustrated with reference to Fig. 4*B* (data from Fig. 3). This showed that the electrogenic current was linearly related to a^i_{Na}. If this remains true for all levels of a^i_{Na} then the difference between the currents at the steady-state level of a^i_{Na} (8·1 mM) and at zero a^i_{Na} will equal the steady-state electrogenic Na pump current. The extrapolated line in Fig. 4*B* indicates that this is 21 nA. This is equivalent to a resting electrogenic efflux of 2·4 p-mole cm^{-2} sec^{-1} (the measured volume was 0·023 μl. and the volume to surface area ratio was assumed to equal $2{\cdot}56 \times 10^{-4}$ cm (Mobley & Page, 1972)). This value can now be compared with the total resting Na pump efflux. When the Na–K pump is inhibited the maximum rate of rise of a^i_{Na} should reflect the net resting Na–K pump efflux (Deitmer & Ellis, 1978*b*). Removing external K or Rb largely inhibits the pump and the maximum rate of rise of a^i_{Na} in Fig. 3 was 1·55 mM min^{-1}, equivalent to a net Na influx and hence a resting Na–K pump

efflux of 8·82 p-mole cm^{-2} sec^{-1}. This may be an underestimate since pump inhibition is more complete with maximal doses of strophanthidin (Deitmer & Ellis, 1978*b*) and in two experiments we found that the maximum rate of rise of a^i_{Na} was 1·15 and 1·5 times faster with 10^{-5} M-strophanthidin than with Rb-free, K-free solution. Nevertheless, for Fig. 3, the fraction of the resting Na efflux that appears to be electrogenic is 2·4/8·82 = 0·27. Twenty re-activations from five fibres were analysed in this way giving an overall mean of 0·54 ± 0·07 (S.E.M.; $n = 5$). This value would be closer to 0·4 if the rise of a^i_{Na} in Rb-free solution is assumed to underestimate the resting Na efflux. In general, the electrogenic fraction of Na extrusion was higher than that determined by the previous method (e.g. the same twenty re-activations yielded a mean of 0·22 when using the previous method). That the two estimates are not greatly different may seem encouraging but our determination of the *resting* Na pump current remains to be validated. It is puzzling that removal of external Rb (Figs. 3 and 5) shifts the holding current in an inward direction by only a few nA and not by about 21 nA as might be expected if electrogenic Na extrusion were inhibited. Even if the inhibition were incomplete one would expect a large shift unless some other membrane current changed simultaneously in the opposite direction. At present this seems unlikely since re-adding 10 mM-$[Rb]_o$ in the presence of cardioactive steroids (sufficient to inhibit the Na–K pump) produces an outward step of steady base line current but no large, transient overshoot (see Gadsby & Cranefield, 1979, Fig. 2; Eisner & Lederer, 1980*a*, Fig. 2). This suggests that the *resting* electrogenic pump current in Fig. 3 may be much less than 21 nA. The subsequent determination of the electrogenic fraction of Na efflux would then be erroneously high and it is notable that such determinations are on average higher than those made using the previous method. We therefore suggest that our extrapolated value of *resting* Na pump current (Fig. 4*B*) is an upper limit. It would be lower, for example, if the relation between a^i_{Na} and Na pump current did not remain linear at very low levels of a^i_{Na}.

Relationship between a^i_{Na} and tension

The experiments have so far concentrated on the relation between membrane current and a^i_{Na}. We were also able to measure the twitch tension and tonic tension of the Purkinje fibre under the same voltage-clamped conditions. Their relation to a^i_{Na} during inhibition and re-activation of the Na–K pump will now be considered.

Twitch tension. Fig. 8*A* shows an experiment where Rb-free solution was superfused for 10 min, during which time a^i_{Na} rose from 5·3 to 11·8 mM. This was accompanied by a pronounced positive inotropy shown by an increase of twitch tension. Fig. 8*B* shows oscilloscope tension records in 10 mM-$[Rb]_o$ and during exposure to 0 $[Rb]_o$. On changing to Rb-free solution there was an artifactual tonic transient (Fig. 8*A*, arrow) caused by a surge in the solution flow. This was not seen upon re-adding 10 mM-$[Rb]_o$ and was rarely seen in other experiments (see e.g. Figs. 3, 5 and 9). Re-adding external Rb produced a rapid decline of twitch tension back to control levels and a decline of a^i_{Na}. In a total of five fibres, removal of external Rb always produced a development of positive inotropy for twitch tension at a rate comparable to the rate of rise of a^i_{Na} (cf. Figs. 3 and 8). This argues against the conclusion of Ehara (1974) that the onset of low-K inotropy (in frog ventricle) is too rapid to be associated with an elevation of a^i_{Na}. In sheep Purkinje fibres at least, our results are consistent with the proposal (Baker *et al.* 1969; Langer, 1970; Glitsch, Reuter & Scholz, 1970) that during Na–K pump inhibition a rise of a^i_{Na} is an intermediate step in the development of positive inotropy. This then produces an elevation of internal Ca ions, possibly by means of a Na–Ca exchange system (Reuter & Seitz, 1968).

Eisner & Lederer (1980*b*) have recently proposed that during Na–K pump inhibition the step which limits the rate of development of positive inotropy in cardiac muscle is the rate of rise of a^i_{Na} rather than the secondary rise of a^i_{Ca}. Although our

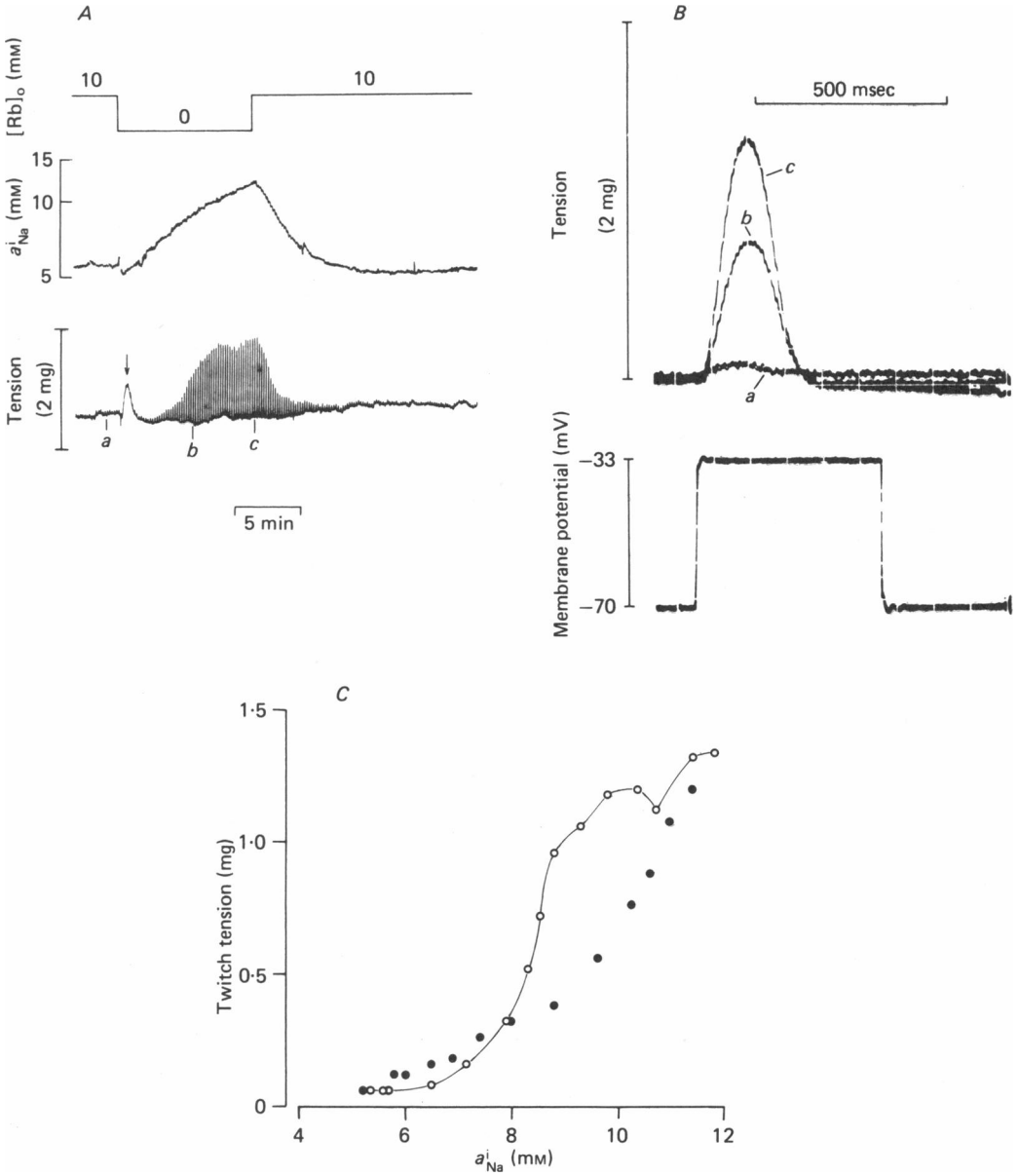

Fig. 8. The relationship between a^{i}_{Na} and twitch tension. *A*, time course of change of a^{i}_{Na} and tension produced by Na pump inhibition and activation. Records show a^{i}_{Na} (above) and tension (below). The membrane potential was held at −70 mV and a 0·5 sec depolarizing voltage-clamp pulse was applied at 0·1 Hz to −33 mV in order to elicit a twitch. After a period of exposure to control solution (10 mM-$[Rb]_o$) the superfusing solution was changed to 0 $[Rb]_o$ for 10 min. $[Rb]_o$ was then increased to 10 mM. *B*, typical tension records. Membrane potential and tension records at a faster time base. The letters *a*, *b*, *c* refer to the corresponding points on *A*. *C*, hysteresis plot of a^{i}_{Na} and twitch tension. The ordinate shows twitch tension as a function of a^{i}_{Na} during the exposure to 0 $[Rb]_o$ (○) and following the addition of 10 mM-$[Rb]_o$ (●).

experiments are consistent with this idea, a further observation has emerged. It is that a given level of a_{Na}^i in a fibre can correspond to more than one level of twitch tension dependng on whether a_{Na}^i is rising during reduced Na–K pumping (positive inotropy) or falling during enhanced Na–K pumping (negative inotropy). This is illustrated in Fig. 8*C* (data from Fig. 8*A*). The open circles compare a_{Na}^i and twitch tension during superfusion with Rb-free solution. The filled circles make the same comparison after the Na–K pump has been re-activated with 10 mM-$[Rb]_o$. In this experiment there is considerable hysteresis in the relationship. A given value of a_{Na}^i that corresponds to a low twitch tension during pump reactivation will be associated with a higher twitch tension during Na–K pump inhibition. Thus, following Na pump re-activation, the fall of a_{Na}^i seems to lag behind the decline of twitch tension. It is unlikely that this discrepancy simply results from the slow response time of the Na^+-electrode. We have allowed for this using eqn. (A2) (Appendix) and find that the hysteresis remains. Three of the five fibres analysed for twitch tension and a_{Na}^i showed hysteresis of the form described in Fig. 8*C*. One fibre showed hysteresis in the *opposite* direction and one fibre (Fig. 9) displayed little sign of hysteresis. In this last preparation, manipulating a_{Na}^i by removing and then re-adding external Rb produced an essentially unique relationship between twitch tension and a_{Na}^i.

Tonic tension. In many experiments changes of a_{Na}^i produced by removing and re-adding external Rb or K were accompanied by changes of tonic tension (e.g. Fig. 3). Tonic tension may also hysterese with respect to a_{Na}^i and the effect is far more obvious than for twitch tension. This is evident in Fig. 3 where tonic tension and a_{Na}^i rise at comparable rates. However, on re-activating the Na–K pump with either 4 mM-$[Rb]_o$ or 4 mM-$[K]_o$ the tonic tension clearly falls much more rapidly than a_{Na}^i. A similar tonic tension hysteresis is also evident in Figs. 5 and 9*A*. The tonic tension hysteresis in Fig. 9*A* is replotted in Fig. 9*B* (open and filled circles: see legend). Tonic tension was sufficiently stable to be analysed in six fibres. Five showed pronounced hysteresis with tonic tension falling faster than a_{Na}^i. The other fibre showed no hysteresis.

Even in the same fibre, the behaviour of twitch and tonic tension with respect to a_{Na}^i can be different. This is apparent in Fig. 9, where tonic tension falls quickly on re-activating the Na pump whereas twitch tension decays at approximately the same rate as a_{Na}^i. The fibre was initially in 10 mM-$[Rb]_o$ (Fig. 9*A*). External Rb was then removed for 5 min and 8 mM-$[Rb]_o$ finally added to re-activate the Na–K pump. Fig. 9*B* shows both twitch and tonic tension plotted as a function of a_{Na}^i. The tonic tension relationship displays considerable hysteresis: the open circles denote the period of exposure to Rb-free solution and the filled circles the subsequent period in 8 mM-$[Rb]_o$. In contrast, the relationship between a_{Na}^i and twitch tension is essentially the same during exposure to both solutions (∇: period in Rb-free solution; $\blacktriangledown$: subsequent period in 8 mM-$[Rb]_o$).

DISCUSSION

The relationship between the electrogenic Na pump current and a_{Na}^i

Our results confirm that, when using external Rb to re-activate the Na–K pump, the recovery of a_{Na}^i from Rb-free solution is well matched by the decline of an outward current transient. This provides powerful evidence that in cardiac Purkinje fibres the

outward current is carried by Na that is extruded electrogenically, as suggested by Eisner & Lederer (1979, 1980*a*) and Gadsby & Cranefield (1979). The electrogenic Na pump current transient is found to depend linearly on a^i_{Na} in the range investigated (2·7–16·5), although a more complex relationship at either a very high or very low a^i_{Na} cannot be excluded. The parallel exponential recovery of a^i_{Na} and current means that the fraction of the total Na extrusion which is electrogenic does not vary during the fall of a^i_{Na}, which implies that it is independent of a^i_{Na}. This is confirmed by the fact that the charge transfer during re-activation of the Na–K pump is proportional to the total change of a^i_{Na}. If the recovery of a^i_{Na} is mediated solely by the Na–K pump this means that the coupling coefficient of the pump (i.e. the ratio of Na to K or Rb transported) is constant.

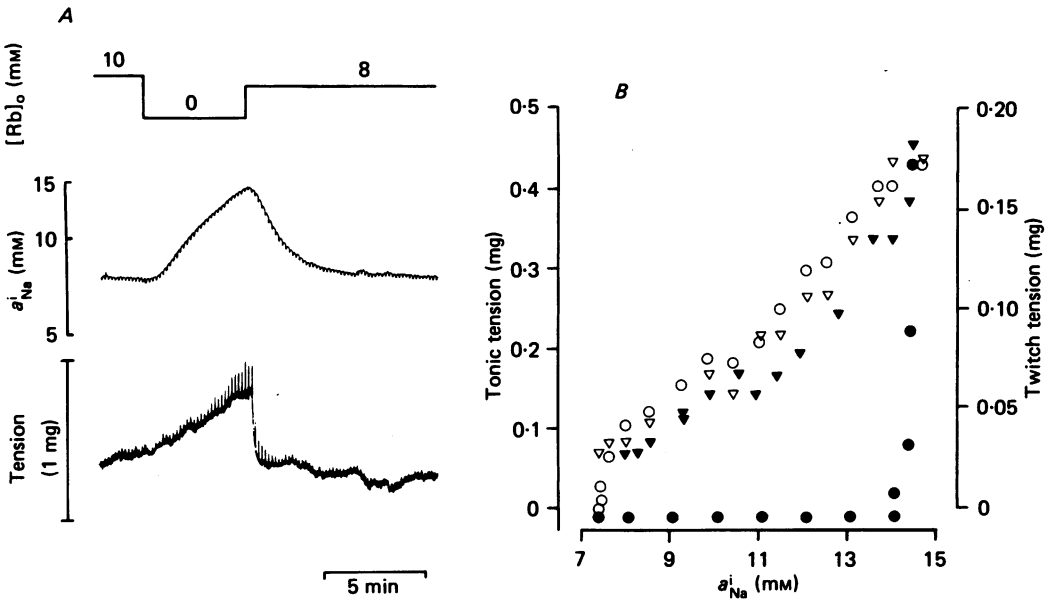

Fig. 9. The relationship between twitch and tonic tension and a^i_{Na}. *A*, tension and a^i_{Na} during Na–K pump inhibition and re-activation. Upper trace a^i_{Na}; lower trace tension. The holding potential was −70 mV. Voltage-clamp depolarizing pulses of 0·5 sec duration to −33 mV were applied at 0·1 Hz to elicit a twitch. The superfusing solution was initially 10 mM-$[Rb]_o$. $[Rb]_o$ was reduced to zero for 5 min and then increased to 8 mM. *B*, 'hysteresis' plot of twitch and tonic tension *versus* a^i_{Na}. Twitch and tonic tension are plotted as a function of a^i_{Na} during exposure to 0 $[Rb]_o$ (twitch, ▽; tonic, ○) or on Na pump re-activation with 8 mM-$[Rb]_o$ (twitch, ▼; tonic, ●). Data from *A*. The points for tonic tension only show the period in 0 $[Rb]_o$ plus the first 2 min of Na–K pump re-activation. They therefore do not include the subsequent slow decline of tonic tension. The inclusion of such later points would not affect the hysteresis.

Deitmer & Ellis (1978*a*, *b*, 1980) have, however, shown that other Na extrusion mechanisms, possibly Na–Ca and Na–H exchange, exist and may also be involved in the regulation of a^i_{Na}. These might then affect our examination of the Na–K pump. It should be noted that our experiments show that the electrogenic Na–K pump current and also the *total* Na efflux (produced by all mechanisms) are linearly related to a^i_{Na}. If other Na transport mechanisms are indeed operating then the linear

relationship between the Na–K pump current and a^i_{Na} (Fig. 4*B*) could arise in two ways. First, the coupling ratio of the Na–K pump could be constant and Na extrusion by the Na–K pump linearly related to a^i_{Na}. Alternatively the coupling ratio could vary with a^i_{Na}. In this case Na extrusion by the Na–K pump would have to depend on a^i_{Na} in a non-linear manner such that the electrogenic Na–K pump current was linearly related to a^i_{Na}. It would seem, however, extremely fortuitous that the dependence of the coupling ratio on a^i_{Na} should exactly cancel out a non-linearity of the dependence of Na extrusion on a^i_{Na}. Undoubtedly the simplest explanation of these results is that (i) Na–K pumping is linearly dependent on a^i_{Na} and (ii) the coupling ratio of the Na–K pump is fixed.

A fixed stoichiometry has been reported for frog skeletal muscle (Sjodin & Ortiz, 1975) although earlier reports had suggested that it varied with $[Na]_i$ (Keynes & Rybova, 1963; Adrian & Slayman, 1966), an idea that has also been proposed for the squid giant axon (e.g. Sjodin & Beaugé, 1968; Mullins & Brinley, 1969). Thomas (1969), however, concluded that, in snail neurones, it remained constant for small changes of a^i_{Na} (3–4 mM). We have examined a wider range of a^i_{Na} in Purkinje fibres (2·7–16·5 mM, equivalent to 3·6–22 mM-$[Na^+]_i$) and, like Thomas, conclude that the coupling ratio is not sensitive to a^i_{Na}.

The electrogenic fraction of Na pumping

It is difficult to obtain an exact value for the fraction of Na extrusion which is electrogenic. Our results suggest that, on average, 26 % of the net Na extrusion which occurs during a recovery from Rb-free solution is electrogenic. This is consistent with a stoichiometry of 4 Na:3 Rb. A second, more tentative estimate based upon assumptions concerning the resting level of electrogenic Na extrusion placed the electrogenic fraction nearer to 40 %. However, of the two estimates the second is probably less accurate since it requires extrapolating the graph of electrogenic Na pump current *versus* a^i_{Na} (Fig. 4*B*) to zero a^i_{Na}, a procedure which has yet to be validated.

The coupling ratio of the Na pump is classically assumed to be 3 Na:2 K, based upon the well-known experiments with erythrocytes (Post & Jolly, 1957; Garrahan & Glynn, 1967). The evidence for a similar coupling ratio in excitable cells is less convincing (see Thomas, 1972, for a review). Thomas (1969) concluded that, in snail neurones, the coupling was in the range 4 Na:3 K to 3 Na:2 K. Our value for cardiac muscle must be regarded as an approximation. This is because of uncertainty concerning the intracellular volume, and the intracellular Na activity coefficient, γ, as well as the possible participation of other Na transporting systems. For example, if γ is less than 0·75, then the electrogenic fraction of Na extrusion will be proportionally less. Conversely, if some Na is extruded by other systems during recovery from Rb-free solution then the Na–K pump will be more electrogenic than appears from our experiments. Nevertheless, the reasonable agreement of our mean value with that quoted for the erythrocyte and the snail neurone suggests that the stoichiometry of the Na–K pump is probably similar in these three tissues.

The magnitude of the steady-state Na extrusion

The steady-state level of Na extrusion can be estimated by inhibiting the Na pump and then measuring the rate of rise of a_{Na}^{i} (Deitmer & Ellis, 1978*b*). The mean rate of rise of a_{Na}^{i} produced by removing $[Rb]_o$ in the present experiments was $1{\cdot}1 \pm 0{\cdot}16$ mM min^{-1} (S.E.M.; $n = 9$ fibres). These determinations were made at a mean membrane potential of -60 mV. This value is considerably larger than that reported by Ellis (1977) of 0·3 mM min^{-1}. Interestingly, the rate constant of recovery of a_{Na}^{i} was considerably faster in our experiments than in previous work. The mean half-time of recovery of a_{Na}^{i} on re-activating with 10 mM-$[Rb]_o$ was 65 ± 7 sec (S.E.M.; $n = 8$ fibres). By contrast, one can estimate from the results of Deitmer & Ellis (1978*b*, Fig. 3) a value of 166 sec in 10 mM-$[K]_o$. Glitsch & Pusch (1980) find a mean half-time of decay of 133 sec in 5·4 mM-$[K]_o$, which is intermediate between the value of Deitmer & Ellis and ours. One explanation of the different kinetics is a variation of the surface area to volume ratio.

From the mean rate of rise of a_{Na}^{i} in K-free, Rb-free solution one obtains a resting Na pump flux of 6·3 p-mole cm^{-2} sec^{-1} using the value for the volume to surface ratio for sheep Purkinje fibres of $2{\cdot}56 \times 10^{-4}$ cm (Mobley & Page, 1972). Since complete Na–K pump inhibition does not occur in 0 K, 0 Rb (Ellis, 1977, and present results), this resting pump flux may be even larger. Therefore, if our present estimate of the electrogenic fraction of Na extrusion is reasonable (26 %) the rate of *electrogenic* Na extrusion at equilibrium will be in excess of 1·6 p-mole cm^{-2} sec^{-1}.

Since the Na pump in cardiac muscle is electrogenic does it contribute to the resting membrane potential? An electrogenic efflux of 1·6 p-mole cm^{-2} sec^{-1} is equivalent to 0·15 μA cm^{-2}. This gives a mean resting electrogenic current of about 13 nA for the fibres we have used (mean volume = 0·023 μl.). The effect of the electrogenic Na pump current on the membrane potential depends on the slope conductance. This is typically (in 4 mM-$[K]_o$) about 1–2 μS in the high conductance region close to the resting potential (negative to -70 mV) and it decreases to very low values and may even become negative at more depolarized potentials (plateau region). This suggests that the contribution to the resting potential will be 6–13 mV. This is similar to the estimate obtained from the effects of strophanthidin on the resting potential (Deitmer & Ellis, 1978*b*; Gadsby & Cranefield, 1979). A similar value has also recently been suggested for guinea-pig ventricular muscle (Daut & Rudel, 1980). It seems likely, then, that the direct contribution of the electrogenic Na–K pump to the resting potential is greater than the 1–2 mV found in snail neurones (Thomas, 1969) or the squid giant axon (de Weer & Geduldig, 1978). It should also be noted that in a fibre which is depolarized to plateau potentials and displays low-voltage oscillations (Hauswirth, Noble & Tsien, 1969), the effects of the electrogenic current on membrane potential will be increased by the decrease of membrane conductance. Therefore the electrogenic Na pump current may significantly influence these low-voltage oscillations.

The relation between a^i_{Na} and tension

The results presented in this paper show that the increase in twitch tension in K-free solutions is closely associated with an increase of a^i_{Na}. This is consistent with the hypothesis that a raised a^i_{Na} can increase a^i_{Ca} and hence tension by means of an exchange of Ca for Na across the sarcolemma (Baker *et al.* 1969; Langer, 1970; Glitsch *et al.* 1970). However, we do not exclude the possibility that a^i_{Na} affects tension by other mechanisms. For example, it is known that changes of a^i_{Na} during Na–K pump inhibition can affect pH_i (Deitmer & Ellis, 1980). A change of pH_i could then have either a direct effect on the contractile apparatus (Fabiato & Fabiato, 1978) or act indirectly by changing a^i_{Ca} (Hess & Weingart, 1980). Lee, Kang, Sokol & Lee (1980) report a linear relationship between a^i_{Na} and twitch tension during Na–K pump inhibition with dihydro-ouabain. The physical significance of this observation is not clear. In some experiments we find that the relationship is markedly non-linear (Fig. 8) whereas in others it is virtually linear (Fig. 9). It should be emphasized, however, that we have only investigated the relationship when both tension and a^i_{Na} are changing, whereas Lee *et al.* related tension to a^i_{Na} under various equilibrium conditions. Nevertheless, since tension depends non-linearly on a^i_{Ca} (Fabiato & Fabiato, 1978) there is no reason to assume that the level of a^i_{Ca} at the peak of the twitch bears any simple relationship to a^i_{Na}. The observation of Lee *et al.* (1980) may therefore be coincidental.

A puzzling and somewhat variable observation has been that the apparent relationship between a^i_{Na} and tension during Na–K pump re-activation often differs from that during the preceding Na–K pump inhibition. The relationship differs between preparations and the hysteresis is more consistent for tonic than twitch tension (see Fig. 9). This means that, depending on the conditions, a given level of a^i_{Na} cannot be uniquely related to a single level of twitch or tonic tension. We have no satisfactory explanation for this effect.

One explanation could be that a^i_{Na} is not homogeneous throughout the Purkinje fibre: resting levels may differ in the different cells of the bundle. Consequently, it would be difficult to relate precisely a^i_{Na} measured inside one cell to tension developed by the whole preparation. A further problem could arise if a^i_{Ca}, and hence tension, were related to a^i_{Na} immediately under the sarcolemma rather than to the bulk cytoplasmic a^i_{Na} which is measured by the Na^+-electrode. If during Na–K pump re-activation the Na efflux were sufficiently rapid to make the sub-sarcolemmal Na activity less than that in the cytoplasm then tension would change faster than cytoplasmic a^i_{Na}. Whilst considering these problems it is worth remembering that the electrogenic Na pump current is an efflux of Na which presumably reflects a^i_{Na} under the sarcolemma. This current has the same kinetics as a^i_{Na} measured in the cytoplasm, which is hard to reconcile with the idea that sub-sarcolemmal a^i_{Na} and cytoplasmic a^i_{Na} are substantially out of phase. The observation also demonstrates that an efflux of Na measured from the whole surface of the preparation (i.e. the electrogenic Na pump current) bears a constant relationship to a^i_{Na} measured at a single site, suggesting that inhomogeneities of a^i_{Na} throughout the Purkinje fibre are probably minimal.

We are left, then, with the observation that, when comparing inhibition and re-activation of the Na–K pump, there can be hysteresis in the relationship between

a^i_{Na} and both twitch and tonic tension. Furthermore, there is the added complication that even in the same fibre twitch and tonic tension may behave differently. The resolution of this problem will probably require additional knowledge of the sources of activator Ca for contraction.

APPENDIX

An estimate of possible errors due to the slow response of the Na^+-sensitive electrode

The Na activity measured with a recessed-tip Na^+-sensitive micro-electrode is the level present inside the recess rather than the activity in the test solution. These values will be equal at equilibrium but as a^i_{Na} in the test solution changes, a^i_{Na} in the recess will lag behind the real change by an amount determined by the time taken for Na to re-equilibrate inside the recess.

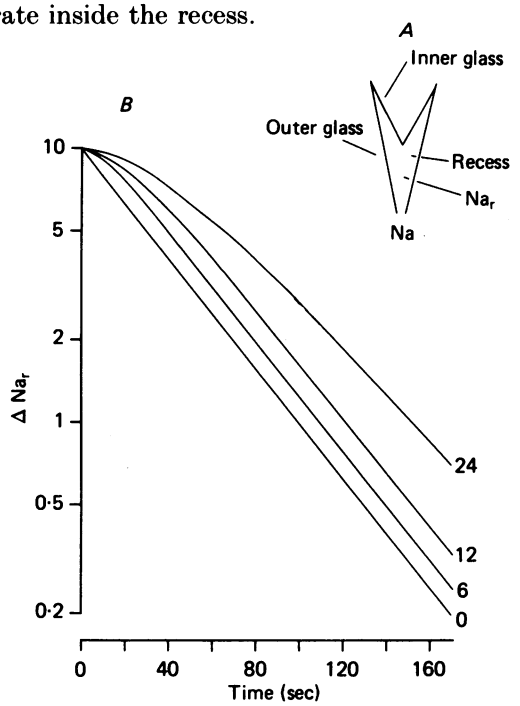

Fig. 10. Effect of diffusion into recess on response time of Na^+-sensitive electrode. *A*, schematic diagram of tip of electrode. The end of the Na^+-sensitive inner glass and the outer insulating glass is shown. Na_r is the Na activity in the recess. Na is the Na activity outside the tip of the electrode. *B*, the calculated time course of decay of Na_r when Na falls with a half-time of 30 sec. The traces show the calculated decay of Na_r for the indicated half-times of the Na electrode (in seconds).

Model. We assume that the electrode recess can be treated as a well-mixed compartment (Fig. 10*A*). This is plausible because of the small dimension of the recess (length 15–70 μm). Let the activity of Na in the test solution be Na and that in the recess Na_r. Let the recess have unit volume. The tip of the electrode limits the movement of Na into the recess giving a time constant τ_e such that

$$\frac{dNa_r}{dt} = \frac{1}{\tau_e}(Na - Na_r). \qquad (A\ 1)$$

Therefore if Na is changed instantaneously, Na_r will relax with a time constant, τ_e. This has been confirmed experimentally by measuring Na_r on rapidly changing Na in the bath. Rearranging eqn. (A 1) gives:

$$Na = Na_r + \tau_e \, dNa_r/dt. \tag{A 2}$$

Therefore Na_r differs from Na by the term $\tau_e \, dNa_r/dt$. By calculating dNa_r/dt and knowing τ_e one can correct the Na_r record for the electrode delay and obtain Na. This has been done in Fig. 4*A* (crosses).

Estimate of the magnitude of the error term. In a typical experiment the Na^+-electrode records an exponential recovery of Na activity on re-activating the Na–K pump. This represents the time course of change of Na activity in the recess (Na_r). How different is this from the real Na activity (Na) during the recovery? ΔNa_r is the value of Na activity in the recess *above* its steady-state level. This changes exponentially with time constant τ_r.

$$\Delta Na_r = a \, e^{-t/\tau_r}.$$

Therefore substituting in eqn. (A 2) gives:

$$\text{Error term} = Na - Na_r = \frac{\tau_e}{\tau_r} \Delta Na_r. \tag{A 3}$$

The error term will therefore be a constant fraction (τ_e/τ_r) of the level of Na_r above its steady-state level. For example, in the experiment of Fig. 4, the fractional error of Na_r is 12% ($\tau_e = 6$ sec, $\tau_r = 50$ sec). It should be noted that the steady-state measurements of Na are not subject to this error since the recess will be equilibrated with the solution.

The effect of the electrode delay on the measured time constant

We assume that the *real* Na activity (Na) decays exponentially with time constant, τ, to a steady level:

$$\Delta Na = b e^{-t/\tau}. \tag{A 4}$$

Substituting in eqn. (A 1):

$$\frac{d(\Delta Na_r)}{dt} = \frac{1}{\tau_e}(b e^{-t/\tau} - \Delta Na_r),$$

which has the solution

$$\Delta Na_r = \frac{b}{\tau - \tau_e}(\tau \exp^{-t/\tau} - \tau_e \exp^{-t/\tau_e}). \tag{A 5}$$

Therefore ΔNa_r is described by the difference of two exponentials, the faster having the same time constant as that of the electrode and the slower that of ΔNa. Fig. 10*B* shows a semi-logarithmic plot calculated from eqn. (A 5).

ΔNa_r is calculated assuming that Na falls with a half-time of 30 sec ($\tau = 43{\cdot}3$ sec), which is the fastest value recorded in the present experiments. Difference values for the half-time of response of the electrode have been used (0, 6, 12, 24 sec). When the half-time is zero the plot is equal to the real decline of Na. As the half-time of the electrode is increased, an initial delay appears (equal to about τ_e). In each case, however, after a time equal to about $2 \times \tau_e$ the rate constant of fall of Na_r is equal

to that of Na. The half-times of the Na electrodes used in the present experiments were certainly less than 6 sec (see p. 165 of Methods). Therefore, provided measurements are made after about 15 sec, the rate constant of fall of Na_r will be equal to that of Na.

We are grateful to D. Ellis and P. W. Flatman for comments on the manuscript. We thank D. Noble and A. J. Spindler for valuable discussion. The work was supported by a grant from the British Heart Foundation to D. Noble. This work was done during the tenure of a British–American research fellowship of the American Heart Association and the British Heart Foundation and part of this work has been supported by NIH grant HL-25675 (W. J. Lederer). R. D. Vaughan-Jones is an M.R.C. Senior Fellow. D. A. Eisner was an M.R.C. scholar. We thank R. A. Rixons Ltd, and especially Mr R. Shock, for supplying sheep hearts.

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