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THE EFFECTS OF MEMBRANE POTENTIAL ON ACTIVE AND PASSIVE SODIUM TRANSPORT IN *XENOPUS* OOCYTES

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SUMMARY

1. The effects of membrane potential on the Na^+–K^+ pump were studied by measuring membrane current and $^{22}Na^+$ efflux in voltage-clamped *Xenopus* oocytes. The effects of inhibiting the Na^+–K^+ pump with strophanthidin were examined.

2. Strophanthidin produced an inward shift of membrane current which reversed on removal of the drug. In control oocytes the magnitude of this current was not significantly affected by changing membrane potential over the range −20 to −160 mV.

3. In another series of experiments the intracellular Na^+ concentration ($[Na^+]_i$) was elevated either by overnight Na^+–K^+ pump inhibition (strophanthidin or exposure to K^+-free solutions) or by loading with nystatin. This Na^+-loading increased the magnitude of the strophanthidin-sensitive current. The ratio of strophanthidin-sensitive $^{22}Na^+$ efflux:strophanthidin-sensitive current was consistent with that expected from a $3Na^+$–$2K^+$ exchange.

4. When $[Na^+]_i$ was elevated the strophanthidin-sensitive current was sensitive to changes of membrane potential. Hyperpolarization from −20 to −80 mV decreased the current to 60% of control. It is suggested that the current is not sensitive to membrane potential at normal $[Na^+]_i$ because the over-all reaction is rate limited by the availability of intracellular Na^+.

5. The application of strophanthidin decreased the rate of $^{22}Na^+$ efflux. Both the strophanthidin-insensitive and the strophanthidin-sensitive components of efflux were sensitive to changes of membrane potential. The strophanthidin-insensitive component was not greatly affected by hyperpolarization from −40 to −160 mV but was increased by depolarization to +40 mV.

6. In Na^+-loaded oocytes, the strophanthidin-sensitive component of $^{22}Na^+$ efflux was inhibited by hyperpolarization negative from −40 mV. Hyperpolarization from −40 to −160 mV decreased the efflux by 54±5%. Over the limited range of potentials for which a comparison could be made, the effects on $^{22}Na^+$ efflux were somewhat less than on the electrogenic Na^+–K^+ pump current. On average there was no significant effect of depolarizing from 0 to +40 mV. However, in some experiments a clear inhibition of the efflux was observed. If the oocytes were not Na^+ loaded there was no significant effect of membrane potential on the strophanthidin-sensitive Na^+ efflux.

7. These results show that the effects of membrane potential on the net reaction of the Na^+–K^+ pump (as measured by the electrogenic current) result partly from an inhibition of the forward mode of operation. However, there is also evidence to suggest a contribution from stimulation of the reverse reaction.

INTRODUCTION

In every tissue in which it has been examined, the Na^+–K^+ pump has been shown to be electrogenic. This arises because it transports more Na^+ ions out of the cell than it brings K^+ ions in. One consequence of this is that under physiological conditions it generates an outward membrane current. This means that the pump performs electrical work and its rate should therefore be sensitive to changes of membrane potential. Specifically sufficient hyperpolarization should first inhibit the exchange and then, in theory, reverse it so that rather than the pump producing net ATP hydrolysis accompanied by Na^+ efflux and K^+ influx it will result in net ATP synthesis and Na^+ influx and K^+ efflux. The effects of membrane potential on the fluxes through the Na^+–K^+ pump have been looked for in several studies in a wide range of preparations. Nevertheless, apart from recent reports on the squid axon (De Weer, Gadsby & Rakowski, 1986) and reconstituted vesicular preparations (Karlish, Rephaeli & Stein, 1985) no effect of membrane potential has been found (Cotterell & Whittam, 1971; Brinley & Mullins, 1974; Beauge & Sjodin, 1976; Zade-Oppen, Schooler, Cook & Tosteson, 1979). There have recently, however, been reports that the electrogenic current produced by the Na^+–K^+ pump which is a measure of the net rate of the Na^+–K^+ pump is sensitive to changes of membrane potential (de Weer & Rakowski, 1984; Turin, 1984; Gadsby, Kimura & Noma, 1985; Lafaire & Schwarz, 1985, 1986). These are contrary to previous work on the current which found no effect of membrane potential (Isenberg & Trautwein, 1974; Eisner & Lederer, 1980; Glitsch, Pusch, Schumaher & Verdonk, 1982).

In the light of the above controversy the aim of the work reported in the present paper was to investigate the effects of membrane potential on the Na^+–K^+ pump and to answer the following questions. (i) Why has some work found that the current produced by the Na^+–K^+ pump is affected by changes of membrane potential whereas other work has not found this effect? (ii) Is the reported effect of membrane potential on the electrogenic current accompanied by a corresponding change of Na^+ transport or, alternatively, does it result from changes of the stoicheiometry of the reaction? (iii) Finally, is the reported inhibitory effect of hyperpolarization on the over-all rate a consequence of decreasing the forward reaction of the pump or of stimulating the reverse reaction? In order to examine these questions we have measured isotopic Na^+ efflux and membrane current in *Xenopus* oocytes. The results show that under some conditions the Na^+ efflux is indeed sensitive to membrane potential.

A preliminary account of these results has been presented to The Physiological Society (Eisner, Valdeolmillos & Wray, 1986).

METHODS

Oocytes. Mature female *Xenopus laevis* frogs were anaesthetized with Tricaine (ethyl *m*-aminobenzoate, 0·1 % (w/v) solution in tap water). A small portion of the ovary was removed

through a lateral incision. The body wall and skin incisions were sutured and the animals allowed to recover. The frogs were kept in sinks with non-circulated tap water, kept on a 12 h day–night cycle all the year round and fed on fresh meat once a week. Individual oocytes were gently teased from the ovarian tissue and placed in Barth's solution (see below) containing streptomycin, 10 μg/ml. Only large (> 1 mm diameter) prophase-arrested oocytes with clearly distinct animal and vegetable poles were used.

Electrophysiological recording. Oocytes were transferred to a 100 μl Perspex bath and superfused with solution at 1·5 ml/min. Solution entered the bath by gravity and was removed by a peristaltic pump for γ-counting when required (see below). The membrane potential was controlled using a conventional two micro-electrode voltage-clamp technique. The micro-electrodes were filled with 3 M-KCl and had resistances in the range 1–3 MΩ. Membrane current and voltage were recorded on an FM tape recorder for subsequent analysis.

$^{22}Na^+$-flux measurements. In order to monitor Na^+ efflux, in some experiments eight oocytes were incubated overnight in 60 ml of Barth's solution (see below) containing 10 μCi of $^{22}Na^+$. When an oocyte had been placed in the experimental chamber superfusion was begun and the solution leaving the bath was collected. The duration of the collection period varied from 30 s to 4 min as appropriate. In a control experiment we added 5 μl of $^{22}Na^+$to the bath in the absence of the egg in order to estimate the delay before the isotope was collected. We found that 99% of the isotope was collected within 30 s. In all experiments at least 1 min was allowed after changing the membrane potential before beginning to sample. At the end of the experiment the oocyte was removed from the bath and the total counts were measured. The apparent rate constant of Na^+ efflux corresponding to a given number of counts in the effluent could then be calculated by dividing the counts in the sample period by the total number of counts remaining in the egg. This latter quantity was estimated by adding together the counts lost subsequently in the experiment and the total number remaining at the end of the experiment.

Elevation of intracellular Na^+ concentration ($[Na^+]_i$). Two methods were used to elevate internal Na^+. (i) The oocytes were left overnight with the Na^+–K^+ pump inhibited either by exposure to K^+-free solutions or to strophanthidin (1 μM). (ii) The oocytes were exposed to the ionophore nystatin (Sigma, U.K.). In order to do this nystatin was made up at a concentration of 30 mg l^{-1} in Barth's solution in which Na^+ was elevated to the desired value at the expense of K^+. The solution also contained 60 mM-sucrose. The oocytes were left in this solution, in the dark at room temperature, for 1 h. The oocytes were then washed several times with the same solution without nystatin. Finally they were resuspended in Barth's solution. However, the 60 mM-sucrose was retained as experience showed that it increased the recovery of the oocytes. In order to check the condition of the oocytes in all the experiments we measured the membrane resistance and capacitance of the oocyte by passing square current pulses and recording the changes of membrane potential. The oocytes treated with nystatin had normal values.

Na^+-sensitive micro-electrodes. After pulling, normal glass micro-electrodes were left in an oven at 150 °C for at least 1 h to dry them. They were then exposed to silane vapour (tri-*n*-butylchlorosilane) at room temperature for 2 min and then left for a further 4 h in the oven. The electrode tips were filled with Na^+-sensitive cocktail (Fluka, Bucks) and the shanks with 10 mM-NaCl. The electrodes were calibrated with solutions containing various Na^+ concentrations between 5 and 80 mM. In these solutions Na^+ was replaced by K^+ such that $[Na^+]+[K^+] = 90$ mM. These electrodes gave Nernstian responses in Na^+ calibrating solutions with a slope of 50–60 mV per decade. All experiments were carried out at room temperature.

Solutions. The Barth solutions used had the following composition (mM): NaCl, 88; KCl, 3; $NaHCO_3$, 2·4; $Ca(NO_3)_2$, 0·3; $CaCl_2$, 0·41; $MgSO_4$, 0·82; HEPES, 14. The pH was adjusted to 7·5. All chemicals were of Analar quality from either BDH (Poole) or Sigma (Poole).

RESULTS

Fig. 1 shows the effects of strophanthidin on the membrane current recorded from a voltage-clamped oocyte. Strophanthidin produced an inward shift of current which reversed on washing off the drug. In another series of experiments we have examined the effects of membrane potential on this current. Because the strophanthidin-sensitive current is small in comparison to the changes of current required to change

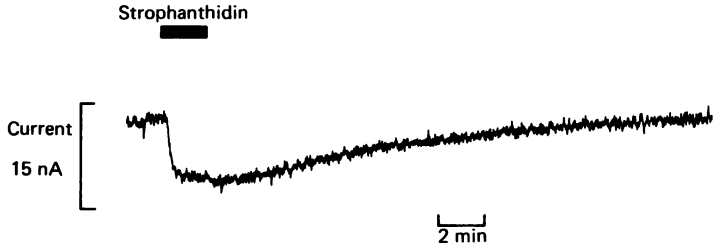

Fig. 1. The effects of strophanthidin on membrane current. The membrane potential was held at −20 mV and the trace shows the current. Strophanthidin (10 μM) was applied for the period shown. In this and all other Figures outward current is shown as an upward deflexion.

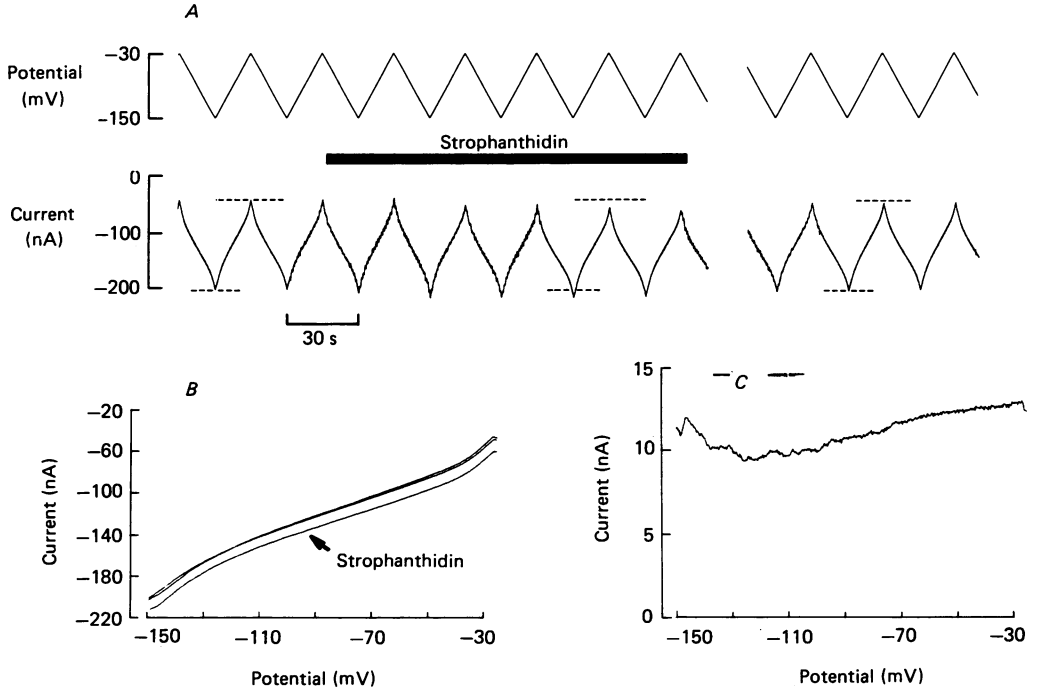

Fig. 2. The effects of membrane potential on the strophanthidin-sensitive current. *A*, original records. Traces show: top, membrane potential; bottom, current. The membrane potential was changed using the ramp protocol shown. The dotted lines have been drawn at the level of the maximum inward and outward currents in the control. Strophanthidin (10 μM) was applied for the period shown. Note that there is a break of 7 min in the record during the wash-off of strophanthidin. *B*, current–voltage relationships. These were obtained from the ramps during the control, application of strophanthidin and after washing off strophanthidin. *C*, the dependence of the strophanthidin-sensitive current on membrane potential. This shows the result of subtracting the current–voltage relationship in the presence of strophanthidin from that in the control.

the membrane potential these experiments were carried out by applying ramp changes of membrane potential. The original records shown in Fig. 2*A* show that strophanthidin produces a reversible inward shift of both the peak inward and outward current. In Fig. 2*B* current is plotted as a function of membrane potential. It is clear that strophanthidin produces an inward shift of current at all potentials.

The fact that the control and wash-off relationships are almost superimposed shows the reversibility of the action of strophanthidin. The change of current produced by strophanthidin is plotted as a function of membrane potential in Fig. 2*C*. It can be seen that this difference current is not significantly affected by membrane potential. Similar experiments showed that over the range −160 to −20 mV there was no significant effect of membrane potential.

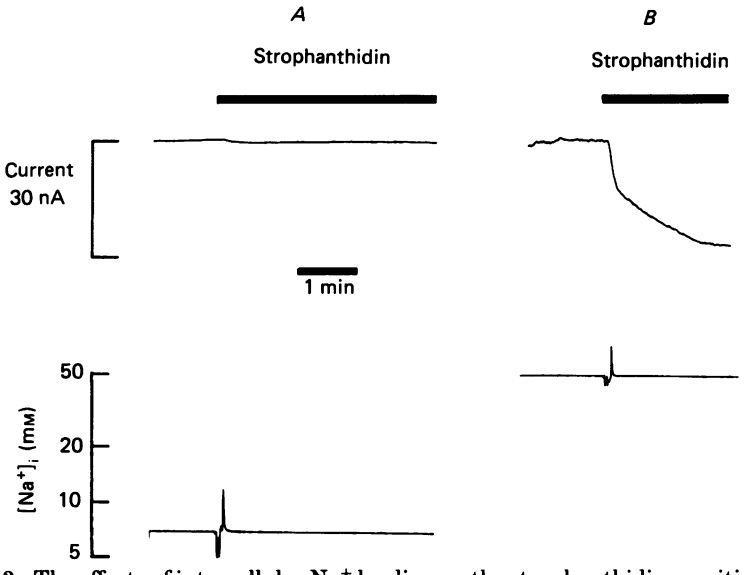

Fig. 3. The effects of intracellular Na^+ loading on the strophanthidin-sensitive membrane current. In both panels traces show: top, membrane current; bottom, $[Na^+]_i$. Strophanthidin was applied for the period shown above. *A*, control oocyte. The oocyte had been kept in Barth's solution containing 3 mM-K^+. *B*, nystatin-treated oocyte. The oocyte had been exposed to nystatin in a solution containing 60 mM-Na^+.

The previous experiments have shown that, under control conditions, the electrogenic Na^+–K^+ current is comparatively small. The magnitude of this current can, however, be increased by elevating $[Na^+]_i$. We have used two methods to elevate $[Na^+]_i$. In most of the experiments $[Na^+]_i$ was elevated by leaving the oocytes overnight with the Na^+–K^+ pump inhibited either by exposure to strophanthidin or by incubating in a K^+-free solution. In ten experiments either protocol elevated $[Na^+]_i$ from 8·0±0·2 to 30·0±2·3 mM (mean±S.E. of mean). A different approach was to treat the oocytes with the ionophore nystatin in a solution containing 60 mM-Na^+ and 30 mM-K^+ (see Methods). The result shown in Fig. 3*A* was obtained from a control oocyte. $[Na^+]_i$ was 7 mM and the application of strophanthidin produced a very small inward shift of current. The oocyte illustrated in Fig. 3*B* was taken from the same batch but had been treated with nystatin. The Na^+-electrode record shows that $[Na^+]_i$ is elevated and the strophanthidin-sensitive current is greatly augmented.

The experiment illustrated in Fig. 4 was designed to investigate the effects of membrane potential on the strophanthidin-sensitive current. The oocyte had been

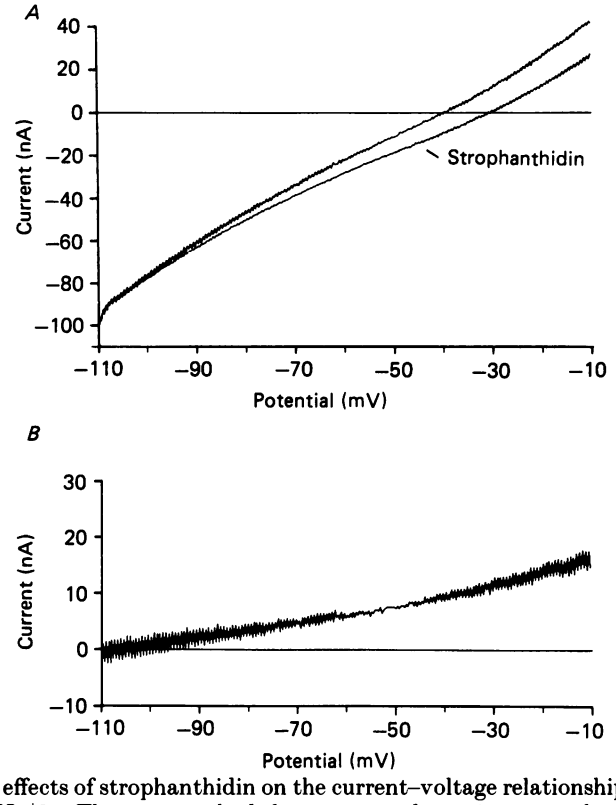

Fig. 4 The effects of strophanthidin on the current–voltage relationship of an oocyte with elevated $[Na^+]_i$. The oocyte had been exposed to 1 μM-strophanthidin overnight. Strophanthidin was then washed off. *A*, current–voltage relationships obtained before and after subsequently applying strophanthidin (10 μM). The current–voltage relationships were obtained using ramp changes of membrane potential (see Fig. 2). *B*, difference current–voltage relationship. The result of subtracting the strophanthidin from the control current–voltage relationship.

exposed to strophanthidin (1 μM) overnight in order to elevate $[Na^+]_i$. After the oocyte had been penetrated with the micro-electrodes it was left for 30 min for the strophanthidin to wash off. Fig. 4*A* shows that the effect of strophanthidin on the current–voltage relationship is decreased at more negative voltages. This voltage dependence of the Na^+–K^+ pump current is emphasized in Fig. 4*B*. It can be seen that the difference current becomes very small at negative potentials.

A major aim of the present work was to compare the effects of strophanthidin on membrane current with those on Na^+ fluxes. Although the ramp protocol is convenient for current measurement it is unsuitable for flux measurements which require that the membrane potential be held at a fixed level for several minutes. Therefore in the experiment illustrated in Fig. 5 the effects of strophanthidin were examined at a fixed voltage. In Fig. 5*A* the membrane potential was −20 mV and the application of 10 μM-strophanthidin produced a large inward shift of current. Strophanthidin was then washed off. After the membrane current had recovered the membrane potential was changed to −105 mV and strophanthidin was reapplied (Fig. 5*B*). The change

of current is now significantly less than in *A*. Finally, after washing off strophanthidin, the membrane potential was returned to −20 mV and strophanthidin reapplied. As shown in Fig. 5*C*, this resulted in a shift of current of similar magnitude to that in the control. In three experiments the strophanthidin-sensitive current at −80 mV was 60% of that at −20 mV. This result is in reasonable agreement with that of Lafaire & Schwarz (1986) and should be compared with the insignificant effects of membrane potential on Na^+–K^+ pump current at normal $[Na^+]_i$ (Fig. 2).

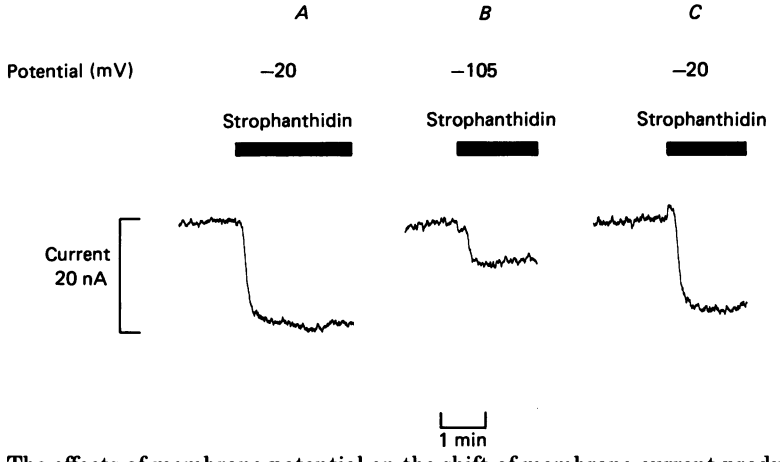

Fig. 5. The effects of membrane potential on the shift of membrane current produced by strophanthidin. $[Na^+]_i$ had been elevated by overnight exposure to strophanthidin (1 μM). In each panel the records show the effect on membrane current of adding strophanthidin (10 μM). The membrane potential was: *A*, −20 mV; *B*, −105 mV; *C*, −20 mV. Following each exposure to strophanthidin the drug was washed off for 30 min before changing the membrane potential and repeating the application of strophanthidin.

$^{22}Na^+$ efflux experiments

In the experiments described in the rest of this paper the efflux of $^{22}Na^+$ was measured. As described in Methods, the eggs were loaded with isotope by overnight exposure to $^{22}Na^+$. This was done either in the absence or the presence of strophanthidin depending on whether a high or low $[Na^+]_i$ was desired. A single oocyte was then put in the experimental bath and the electrodes inserted and the membrane potential clamped at −40 mV. The solution flow was then started and, in the experiment of Fig. 6, the effluent was collected in 4 min periods. It can be seen that the counts are high during the first period and then decay quickly over the next few minutes before then falling more slowly. Fig. 6*B* shows the calculated rate constant for $^{22}Na^+$ efflux. Apart from the first 4 min this is fairly constant. It should be noted that the apparent high rate constant of efflux seen in the first few minutes simply reflects the fact that this collection period includes all the isotope which left the egg during the period when the electrodes were being inserted.

The effects of strophanthidin on $^{22}Na^+$ efflux are shown in Fig. 7. As shown above strophanthidin produces an inward shift of membrane current. This can now be seen to be accompanied by a decrease of $^{22}Na^+$ efflux, in this experiment to 15% of control.

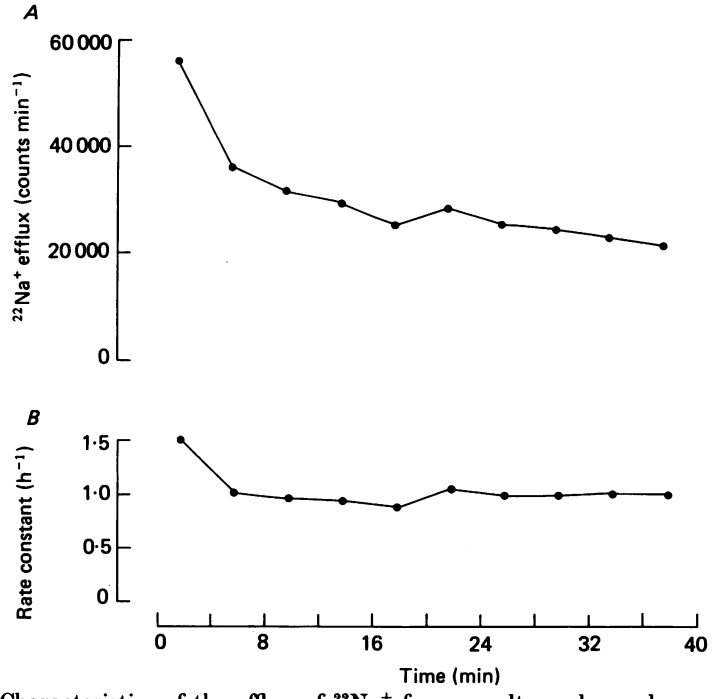

Fig. 6. Characteristics of the efflux of $^{22}Na^+$ from a voltage-clamped oocyte. *A*, radioactivity of effluent solution from the experimental bath. The samples were collected for periods of 4 min duration. The first collection period began immediately after starting the flow of superfusing solution (see text). *B*, calculated rate constant of efflux of $^{22}Na^+$ (see Methods).

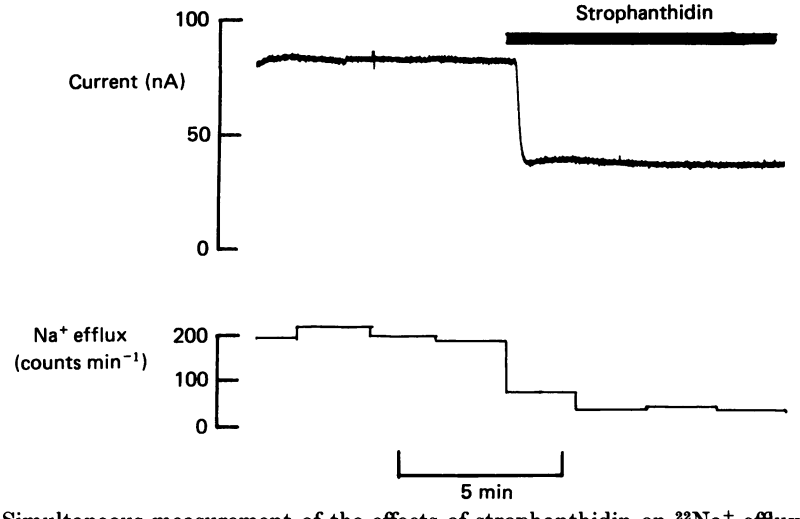

Fig. 7. Simultaneous measurement of the effects of strophanthidin on $^{22}Na^+$ efflux and membrane current. Traces show: top, membrane current; bottom, radioactivity of effluent. Strophanthidin (10 μM) was applied for the period shown above.

In eleven experiments at a potential of −40 mV 58±4% of the total Na^+ efflux was sensitive to strophanthidin and therefore through the Na^+–K^+ pump. Other experiments (not presented) showed that the strophanthidin-sensitive component of Na^+ efflux was almost completely abolished by removing extracellular K^+. This result shows that there is little Na^+–Na^+ exchange through the Na^+–K^+ pump even in the absence of external K^+. This result is in agreement with the findings of Dick & Fry (1975) on toad and frog oocytes. The significance of this result will be addressed in the Discussion.

TABLE 1. Comparison of strophanthidin-sensitive $^{22}Na^+$efflux and current

Na^+ efflux (pmol s^{-1} $oocyte^{-1}$)	Equivalent current (nA)	Measured current (nA)	Charges per Na^+
1·44±0·33	139±22	34·3±5·5	0·35±0·10

The Na^+ efflux (first column) was measured from the strophanthidin-sensitive $^{22}Na^+$ efflux and the specific activity of the loading solution. The second column shows the current equivalent to this Na^+ efflux and is the current expected if all the strophanthidin-sensitive Na^+ efflux carried current. The third column is the change of current produced by adding strophanthidin (10 μM) at a holding potential of −40 mV. The fourth column shows the result of dividing column three by column two. The data are the mean (±S.E. of mean) of four experiments.

Direct comparison of the magnitude of current and $^{22}Na^+$ efflux. We have attempted to obtain an estimate of the stoicheiometry of the Na^+–K^+ pump by comparing the strophanthidin-sensitive $^{22}Na^+$ efflux with the strophanthidin-sensitive current. In order to calculate the number of Na^+ ions which leave the cell one has to know the specific activity of intracellular Na^+ ions in the cytoplasm. Unfortunately this cannot be measured directly and we have assumed that after the prolonged (12–18 h) loading period the cytoplasm will have almost reached isotopic equilibrium with the external solution (Dick & Lea, 1964). Table 1 gives the mean values for both the rate constant and the actual value of the strophanthidin-sensitive efflux. When this is compared with the current one obtains the result that each Na^+ ion pumped by the Na^+–K^+ pump is accompanied by, on average, about 0·35 net charges. This compares with the value of 0·33 expected for a $3Na^+$–$2K^+$ pump.

The effects of membrane potential on Na^+ efflux

Na^+-loaded oocytes. The experiment illustrated in Fig. 8*A* shows the effects on $^{22}Na^+$ efflux of changing membrane potential in an oocyte which had been Na^+ loaded by overnight exposure to strophanthidin (1 μM). At the start of the record the oocyte was in a control solution. Depolarization increased Na^+ efflux and hyperpolarization decreased it. Again the application of strophanthidin decreased Na^+ efflux. The $^{22}Na^+$ efflux is, however, still sensitive to membrane potential. These results are presented graphically in Fig. 8*B*. Both the strophanthidin-sensitive and the insensitive components are affected by membrane potential. However, the shape of the voltage dependence is different in the two cases. The strophanthidin-insensitive flux is very small at potentials up to 0 mV and then increases on further depolarization. On the other hand the Na^+–K^+ pump flux gradually increases as the membrane potential is depolarized from −160 mV before reaching a maximum at 0 mV and then declining on further depolarization.

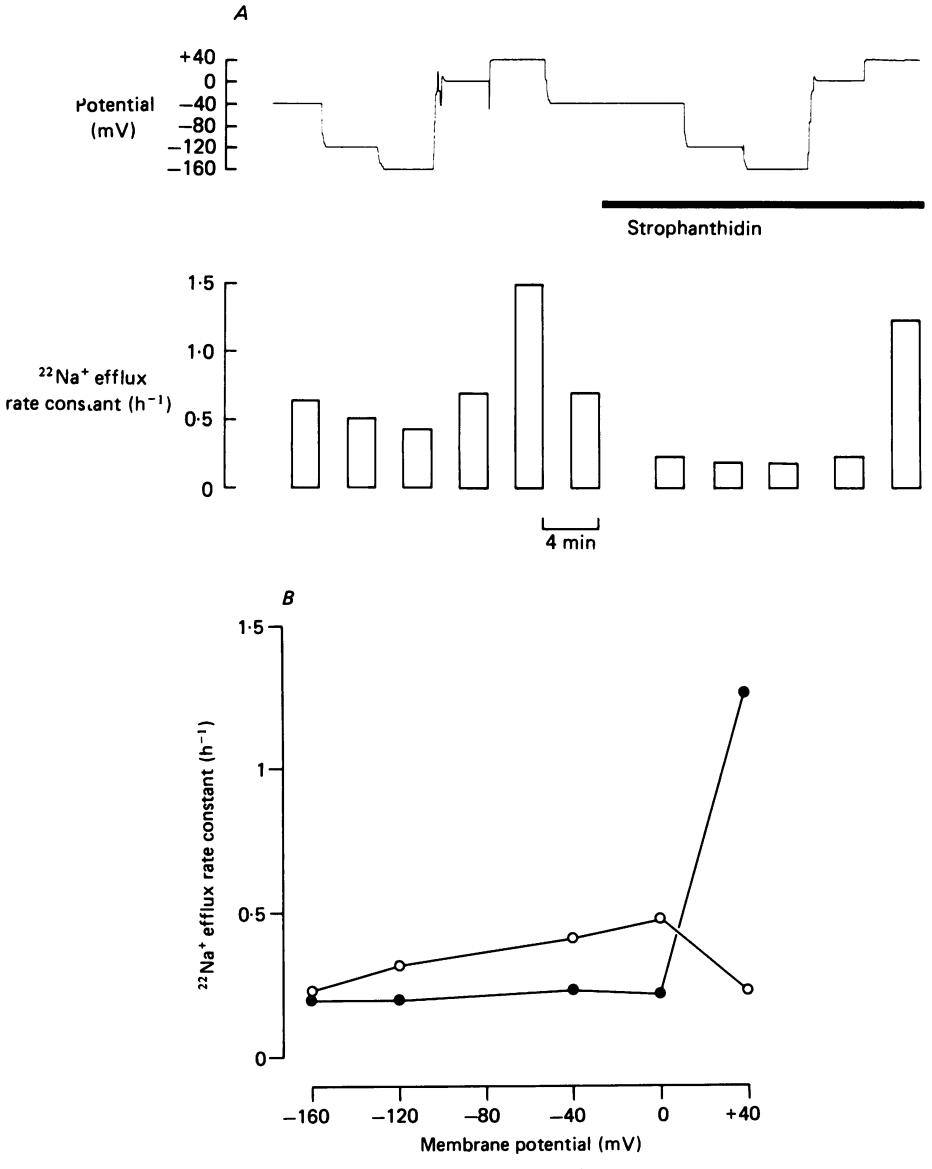

Fig. 8. The effects of membrane potential on $^{22}Na^+$ efflux. *A*, original data. The top trace is an original record of voltage-clamped membrane potential. The bottom trace shows the rate constant of $^{22}Na^+$ efflux measured over 1 min duration periods. Strophanthidin (10 μM) was applied for the period shown. $[Na^+]_i$ had been elevated by overnight exposure to strophanthidin (1 μM). *B*, graph of the relationship between $^{22}Na^+$ efflux and membrane potential. Symbols show: (○), strophanthidin-sensitive efflux; (●), strophanthidin-insensitive flux.

Averaged data from eight experiments are shown in Fig. 9. In this Figure the rate constants have been normalized to the level at −40 mV. Fig. 9*A* shows the strophanthidin-insensitive fluxes. As shown in the previous Figure, this component increases with depolarization beyond 0 mV. The dashed line shows the predictions

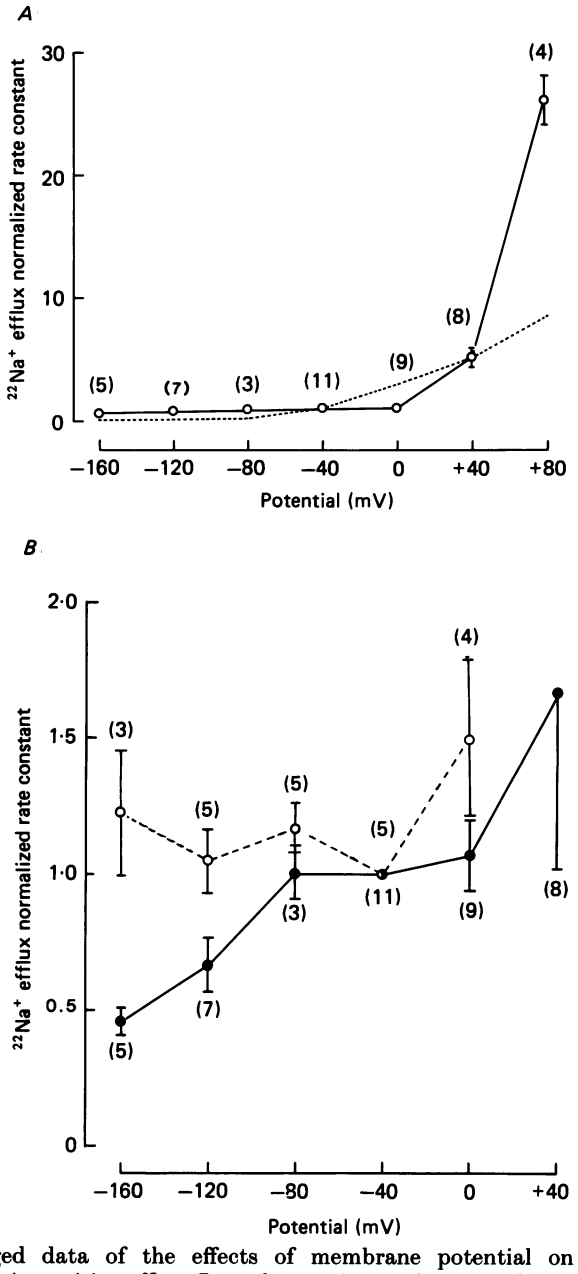

Fig. 9. Averaged data of the effects of membrane potential on $^{22}Na^+$ efflux. *A*, strophanthidin-insensitive efflux. In each experiment the points have been normalized to the strophanthidin-insensitive Na^+ efflux at −40 mV. The mean value of this rate constant at −40 mV in eleven oocytes was $0{\cdot}36 \pm 0{\cdot}05$ h^{-1} (mean ± S.E. of mean). The dashed line has been drawn according to the form of eqn. (1) scaled to go through unity at −40 mV. All the points were obtained from experiments in which $[Na^+]_i$ had been elevated. *B*, strophanthidin-sensitive efflux. The filled symbols are from Na^+-loaded oocytes and the open ones from controls. The points have been normalized to the strophanthidin-sensitive efflux at −40 mV. In Na^+-loaded oocytes this had a mean value in eleven oocytes of $0{\cdot}38 \pm 0{\cdot}05$ h^{-1} and in controls of $0{\cdot}31 \pm 0{\cdot}07$ h^{-1} ($n = 7$).

of a constant-field permeability model for the efflux. This has been drawn according to the equation:

$$\text{efflux} = \frac{k\,V}{1-\exp(-VF/RT)}, \tag{1}$$

where k is a constant and the other symbols have their usual meanings. It should be noted that the increase of efflux on depolarization to +80 mV is substantially greater than that predicted by this equation. The Na^+ efflux through the Na^+–K^+ pump is shown in Fig. 9*B*. At negative potentials the relationship is similar to that shown for the experiment of Fig. 8. However, at positive potentials the results were very variable as shown by the large standard error on the +40 mV point. We have no simple explanation for this variability although it is likely that it results from the large size of the strophanthidin-insensitive component and the errors produced by subtracting it off from the total flux.

Non-Na^+-loaded oocytes. In agreement with the measurements of membrane current, we have found that the strophanthidin-sensitive Na^+ efflux from control (i.e. not Na^+-loaded) oocytes was not significantly affected by changes of membrane potential. Averaged data showing this lack of effect are shown as the open symbols (dashed line) in Fig. 9*B*.

DISCUSSION

In the present paper we have shown that it is possible to measure both the isotopic Na^+ efflux and also the electrogenic current produced by the Na^+–K^+ pump. The small surface to volume ratio of the large oocyte makes the measurement of Na^+ efflux much easier than in smaller cells. Furthermore the oocyte has the advantage over other large cells such as the squid axon (De Weer & Rakowski, 1984) and the barnacle muscle (Lederer & Nelson, 1984) inasmuch as the oocyte has a much higher electrical resistance and it is therefore much easier to (i) measure the electrogenic Na^+–K^+ pump current and (ii) clamp the membrane potential to extreme levels.

The strophanthidin-insensitive Na^+ efflux

The results have shown that depolarization increases the strophanthidin-insensitive Na^+ efflux. This result is consistent with the strophanthidin-insensitive efflux being largely a passive efflux of Na^+ from the cell. Depolarization will increase the driving force for Na^+ efflux. However, Fig. 9*A* shows that increase of Na^+ efflux. on depolarization is considerably greater than would be expected simply from the constant-field theory. In particular our experiments show that the Na^+ efflux at +80 mV was about 5 times that at +40 mV whereas a constant-field model (eqn. (1)) would predict a ratio of about 1·7. In other words this depolarization has increased the apparent permeability by about threefold. Previous work (Baud, Kado & Marcher, 1982) has shown that depolarization activates an inward Na^+ current which activates with a half-time of about 10–20 s (Baud & Kado, 1984). This current does not inactivate on maintained depolarization. The results of Fig. 3 of Baud *et al.* (1982) show that depolarization from +40 to +80 mV increases the Na^+ conductance by a factor of about 2·8. Under the conditions of their experiments ($[Na^+]_o$ = 84 mM,

$[Na^+]_i$ = 6 mM) the constant-field equation would predict that the Na^+ conductance at +40 mV should be 1·4 times that at +80 mV. Therefore the increase of Na^+ conductance by a factor of 2·8 requires an increase of Na^+ permeability of $2{\cdot}8 \times 1{\cdot}4 = 3{\cdot}9$. Given the large number of assumptions, this is in reasonable agreement with our value of 3.

The effects of membrane potential on the Na^+–K^+ pump

Our results have shown that the electrogenic current produced by the Na^+–K^+ pump in *Xenopus* oocytes is inhibited by hyperpolarization. This is in agreement with recent work in the same preparation (Lafaire & Schwarz, 1986) and also with studies on blastomeres (Turin, 1984) and on isolated cardiac myocytes (Gadsby *et al.* 1985). However, other studies have found no effect on this current (Isenberg & Trautwein, 1974; Eisner & Lederer, 1980; Glitsch *et al.* 1982). Previous studies which have measured the active fluxes of Na^+ and K^+ have found no effect of membrane potential. These negative results would only be consistent with the effect of membrane potential on the current if some change in stoicheiometry of the exchange were felicitously cancelling out the effect. However, the observation in the present work that the efflux of $^{22}Na^+$ is inhibited means that the inhibition of the electrogenic current by hyperpolarization can be accounted for. The fact that the efflux of $^{22}Na^+$ is inhibited has another implication for the mechanism of the Na^+–K^+ pump. The electrogenic Na^+–K^+ pump current can be thought of as a measure of the net rate of the pump and therefore represents the difference between the forward rate (Na^+ efflux) and the reverse reaction (Na^+ influx). *A priori* the inhibitory effect of hyperpolarization on the electrogenic Na^+–K^+ pump current could result from: (i) an inhibition of the forward reaction or (ii) a stimulation of the reverse reaction. The observation that the efflux is inhibited along with the current suggests that (i) is at least partly responsible for the inhibition. However, there are also indications that this may not be the only effect of changes of membrane potential. We were unable to look at the effects of membrane potential on the strophanthidin-sensitive current and flux simultaneously. However, a comparison of the results from the two sets of experiments suggests that membrane potential may have more effect on the electrogenic current than on the $^{22}Na^+$ efflux. For example although a hyperpolarization from −20 to −80 mV inhibited the current by 40% it had little effect on the flux (Fig. 9*B*). Our data on flux measurements can also be compared with the more complete study on the Na^+–K^+ pump current of Lafaire & Schwarz (1985). These authors found that hyperpolarization from 0 to −160 mV decreased the strophanthidin-sensitive current to 10%. This is a much larger inhibition than that found for the $^{22}Na^+$ efflux in the present work. One explanation of the greater effect of membrane potential on current than flux is that hyperpolarization may increase the reverse mode of operation of the pump or convert the Na^+ efflux from Na^+–K^+ to Na^+–Na^+ exchange. A direct way to test this possibility would be to investigate the effects of membrane potential on either the strophanthidin-sensitive Na^+ influx or K^+ efflux. However, these fluxes are both small in comparison to the passive fluxes and we have therefore not been able to perform this experiment.

Our experiments gave equivocal results for the effects of depolarization from −40 to +40 mV on the strophanthidin-sensitive fluxes. Although some experiments (e.g.

Fig. 8) showed that depolarization decreased the flux, the averaged result was insignificant (Fig. 9). A significant inhibition of the electrogenic Na^+–K^+ pump current by depolarization has been reported by Lafaire & Schwarz (1986) who attributed it to the Na^+–K^+ pump having at least two voltage-dependent steps of which one is inhibited by depolarization. In contrast Goldshlegger, Karlish, Rephaeli & Stein (1987), investigating purified Na^+–K^+-ATPase reconstituted into lipid vesicles found that depolarization positive to zero increased the flux.

Why has previous work not generally found voltage dependence of the Na^+–K^+ pump? As mentioned above, much previous work has failed to find a significant effect of membrane potential on the Na^+–K^+ pump. One explanation for this discrepancy is provided by our observation that the inhibitory effects of hyperpolarization are only seen if $[Na^+]_i$ is elevated. A similar result can be seen in Fig. 7 of Lafaire & Schwarz (1986). A similar $[Na^+]_i$ dependence of the effects of membrane potential may also occur in cardiac muscle. Work on sheep Purkinje fibres with $[Na^+]_i$ either at control levels of about 10 mM (Isenberg & Trautwein, 1974) or elevated to 10–20 mM (Eisner & Lederer, 1980) has found no effect of membrane potential on the Na^+–K^+ pump current. Subsequent work (Eisner, Lederer & Vaughan-Jones, 1981) has shown that, at these values of $[Na^+]_i$, the Na^+–K^+ pump would have been far from saturation. In contrast a recent study of single cardiac cells (Gadsby *et al.* 1985) found that the Na^+–K^+ pump current was markedly voltage dependent. In the study of Gadsby *et al.* (1985) $[Na^+]_i$ was 34 mM and the Na^+–K^+ pump would have been completely saturated ($K_{0.5}$ = 10 mM; Gadsby & Nakao, 1986). The high affinity for intracellular Na^+ is presumably due to the fact that Cs^+ replaced intracellular K^+. Therefore in both the *Xenopus* oocyte and in cardiac muscle the voltage dependence of the Na^+–K^+ pump seems to require that the intracellular Na^+ site of the Na^+–K^+ pump be more saturated than is the case under control conditions. This Na^+ dependence of the inhibitory effects can be explained if, at normal $[Na^+]_i$, the over-all rate is limited by the availability of $[Na^+]_i$ and therefore not very sensitive to the voltage-dependent step(s). However, if $[Na^+]_i$ is elevated then the voltage-dependent step may become rate limiting.

It has been suggested (Glynn, 1984) that the voltage-dependent step may be the conformational change from the E_1 to the E_2 forms of the enzyme. Fig. 10*A* shows such a scheme. The enzyme initially exists in a form with ATP bound (E_1ATP). It then binds intracellular Na^+ and the next reaction is the phosphorylation and release of ADP. This is then followed by the conformational reaction ($E_1P \cdot Na \rightarrow E_2P \cdot Na$). Finally Na^+ is released at the extracellular surface. The dashed line indicates the rest of the reactions (involving K^+ transport) which follow before the original form of the enzyme is regained. A compressed version of this scheme is shown in Fig. 10*B*. We have replaced all the steps from E_1ATP to $E_1P \cdot Na$ by one pair of rate constants (a and b). The conformational change is represented by the rate constants c and d and the rest of the reaction by one forward rate constant e.

A steady-state analysis gives the over-all rate of Na^+ transport as:

$$\text{rate} = E_T \frac{c}{c+\beta} \frac{e\,a}{(a+e)},$$

where $\beta = (d+e)\,(a+b)/(a+e)$ and E_T is the total concentration of Na^+–K^+ pump.

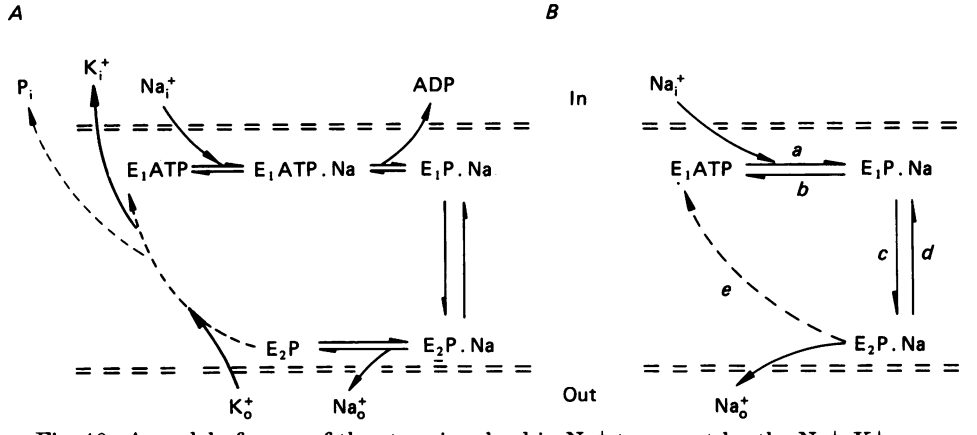

Fig. 10. A model of some of the steps involved in Na^+ transport by the Na^+–K^+ pump. *A*, detailed model. This shows some of the steps involved in Na^+ transport. Note that all the reactions following Na^+ release at the extracellular surface have been represented by the dashed line. Further details are given in the text. *B*, simplified model. Several of the intermediates have been eliminated. The rate constants are indicated (*a*–*e*). Note that, for simplicity, the reaction represented by the dashed line has been assumed to be irreversible. This assumption has no qualitative effect on the general conclusions. P_i, inorganic phosphate; K_i^+, intracellular K^+; Na_i^+, intracellular Na^+; K_o^+, extracellular K^+; Na_o^+, extracellular Na^+.

If we assume that c is decreased by hyperpolarization then this equation predicts that the effects of hyperpolarization on the over-all rate will be greatest when β is large. The predicted effects of changes of $[Na^+]_i$ on over-all rate therefore depend on its effects on β. Increasing $[Na^+]_i$ increases a and an increase of a will only increase β if $b < e$. Therefore the observation in the present paper that increasing $[Na^+]_i$ increases the voltage dependence is consistent with the model of Fig. 10 if $b < e$. This can be explained as follows. If e is large then, provided that a is increased sufficiently the over-all forward rate will be limited by reaction c and therefore the voltage dependence of the over-all rate will represent that of reaction c. In contrast, if b is large in relation to e, the over-all rate will be limited by reaction e and not c.

The conclusion that rate constant b is small is consistent with the observation that the magnitude of Na^+–Na^+ exchange is small even in the absence of external K^+. The fact that Na^+–Na^+ exchange is much smaller than Na^+–K^+ exchange means that either or both of b and d must be small in comparison to a and c.

In contrast to the work discussed above, Goldshlegger *et al.* (1987) found that, in reconstituted vesicles, the voltage dependence of the Na^+–K^+ pump was greatest at *low* $[Na^+]_i$. These results can be accounted for on the scheme of Fig. 10 if $b > e$. A further complication arises if membrane potential affects the reaction $E_2P.Na \rightarrow E_1P.Na$ (*d*). If hyperpolarization increases d it is easy to show that the effects of membrane potential will be greatest at elevated $[Na^+]_i$ if $b > e$. Conversely the effects of membrane potential will be greatest at low $[Na^+]_i$ if $b < e$. This analysis shows therefore that the model of Fig. 10 can account qualitatively for an increase of $[Na^+]_i$ either increasing or decreasing the voltage dependence of the Na^+–K^+ pump.

The present results have shown that the Na^+ efflux through the Na^+–K^+ pump is sensitive to changes of membrane potential. Further work is required to localize the exact reaction which is affected by membrane potential and to test the model of

Fig. 10. To this end it will be useful to examine the effects of membrane potential not only on Na^+–K^+ exchange but also on the other fluxes catalysed by the pump such as Na^+–Na^+ and K^+–K^+ exchange.

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