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CELL ELECTRICAL POTENTIALS DURING ENHANCED SODIUM EXTRUSION IN GUINEA-PIG KIDNEY CORTEX SLICES

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SUMMARY

1. Experiments were performed on outermost slices of the guinea-pig kidney which are mainly made up of proximal tubular cells.

2. Kidney cells loaded with Na^+ by chilling at 0·6° C for 2·5 hr, when subsequently rewarmed to 25° C in a medium containing 16 mM-K^+ extrude Na^+ at enhanced speed for about 10 min. This Na^+ movement is accompanied by efflux of Cl and influx of K^+.

3. Measurements of cell potential during enhanced Na^+ extrusion show that cells hyperpolarize to values about 30 mV more negative than the K^+ equilibrium potential.

4. This hyperpolarization is only partly inhibited by 1 mM ouabain or by 2 mM ethacrynic acid but both agents added together suppress it completely.

5. With 16 mM-Rb instead of 16 mM-K the hyperpolarization is smaller.

6. A diminished extracellular K^+ concentration outside of the cells, within the slice, can account for only a small part of the hyperpolarization.

7. The hyperpolarization is proportional to the rate of Na^+ pumping.

8. Cl^- seems to shunt the hyperpolarization to a greater extent than K^+.

9. It is concluded that Na^+ extrusion is capable of transferring electric charge across the membrane.

INTRODUCTION

Although it is widely accepted that in the proximal kidney tubule active transport of Na at the peritubular cell border brings about Na extrusion from cell to peritubular space, it is still unclear whether Na extrusion is neutral or electrogenic (Orloff & Burg, 1971; Giebisch & Windhager, 1973). This is relevant to understanding the way in which passive ionic movements evolve, i.e. how K^+ enters the cell from the peritubular space and how Cl^- follows Na^+ extrusion out of the cell. If Na extrusion were neutral (and the membrane potential, V_m, mainly a K^+ diffusion potential) K could

be taken into the cell by the same mechanism that extrudes Na and the ensuing increased cell K concentration and K^+ diffusion would increase the cell negativity which could then drive Cl^- passively out of the cell. On the other hand, if Na extrusion were electrogenic (because the Na to K pump ratio is greater than one) the electrical potential directly generated by Na^+ extrusion (superimposed on the K^+ diffusion potential) could induce either Cl^- efflux to the peritubular space or K^+ influx from it, by a pathway different from that of the pump, in order to maintain the electroneutrality.

In kidney cells studied during the transition between steady states, it has been found that one Na^+ is expelled from the cell in exchange for one K^+ which is taken up into the cells (Whittam & Willis, 1963; Whittembury, 1965). Furthermore, V_m has been found to be nearly equal to the K equilibrium potential (E_K) over a wide range of high bathing fluid K concentrations (Giebisch, 1961; Whittembury, Sugino & Solomon, 1961; Whittembury, 1965; Boulpaep, 1967; 1971; Frömter, Müller & Wick, 1971). Thus, under these conditions the neutral mode of Na extrusion would be sufficient to account for the experimental observations. However, at low K concentrations, K seems to enter the cell against its electrochemical potential gradient (Whittembury, 1965). Also, under conditions in which Na extrusion was enhanced, net Na efflux to net K influx ratio has been found to vary between 2·1 and 0·9 and V_m has been found to be transitorily more negative than E_K (Whittembury, 1965). To account for these observations it was proposed that Na extrusion could be electrogenic (Whittembury, 1965).

To gain further insight into the possibility that Na extrusion is electrogenic, we decided to study the relationship between ion movements and the electrical potential under conditions in which Na extrusion would be maximally stimulated in kidney cells. This situation is realized at the outset of rewarming in a medium containing a high K concentration after cells of guinea-pig cortex slices have been loaded with Na and Na extrusion has been initially curtailed by lowering the temperature (Whittembury & Proverbio, 1970). When the temperature is raised (this procedure is denoted as 'rewarming') Na extrusion is triggered under conditions in which (due to increased intracellular Na and extracellular K concentrations and due to a diminished V_m) the electrochemical opposing Na extrusion is lowered. Ion movements and their relationship to V_m were analysed during the first 10 min of rewarming, the time during which net Na extrusion and net K and Cl movements proceeded at maximal speed. Ouabain and ethacrynic acid, which are known to inhibit Na extrusion differently (Whittembury & Proverbio, 1970), were also used to assess the relationship between Na extrusion and generation of the cell electrical potential. The

results provide additional evidence indicating that Na extrusion is electrogenic in kidney cells. Part of these investigations have been presented in a preliminary form (Whittembury & Proverbio, 1969; Whittembury, 1971; Giebisch, Boulpaep & Whittembury, 1971).

METHODS

Outermost slices (0·2–0·3 mm in thickness) were obtained from guinea-pig kidney, as described before (Whittembury, 1965). They were immersed in a 0-K medium (Table 1) at 0·6° C and shaken for 2·5 hr to induce gain of Na and Cl by, and loss of K from, the cells. The slices were subsequently re-immersed, still in the cold, in one of the bathing fluids shown in Table 1, i.e. either in the 16-K medium (control experiments) or in media containing 0-K, 16-Rb, 16-Cs or 16-K plus 1 mM ouabain (Sigma Chemical Company, St Louis, Mo.), 16-K plus 2 mM ethacrynic acid (Merck, Sharp and Dohme) or 16-K plus both inhibitors added simultaneously at the stated concentrations. This procedure carried out for 30 min in the cold allows extracellular

TABLE 1. Ionic composition of bathing fluids

(All bathing media contained mM: Na-acetate, 9; $NaHCO_3$, 15; NaH_2PO_4, 0·6; Na_2HPO_4, 2·4; $MgSO_4$, 1·2; Na_2SO_4, 0·6; Ca-gluconate, 1·0; glucose 5. To this basic medium, the required amounts of NaCl, KCl, RbCl or CsCl were added to obtain media with the Na, K, Rb, Cs and Cl concentrations that are described below. The osmolalities were kept between 290 and 300 m-osmole/kg. The pH was maintained between 7·2 and 7·6.)

	Composition (mM)						
Fluid designation	Na	K	Rb	Cs	Cl	Ouabain	Ethacrynic acid
16-K	134	16	—	—	120	—	—
16-K-Ouabain	134	16	—	—	120	1	—
16-K-Ethacrynic acid	134	16	—	—	120	—	2
16-K-Ouabain ethacrynic acid	134	16	—	—	120	1	2
16-Rb	134	—	16	—	120	—	—
16-Cs	134	—	—	16	120	—	—
0-K	150	—	—	—	120	—	—

equilibration without significantly changing the cell ion concentrations (Whittembury & Proverbio, 1970). Measurements of cell electrical potential were begun after 15 min re-immersion in these new media. Fifteen minutes later (i.e. 3 hr after the beginning of immersion at 0·6° C, rewarming to 25° C was suddenly induced by changing the bathing fluid for one of the same composition but at 25° C. Measurements of cell electrical potential were continued for about 50 min. Throughout incubation the solutions were gassed with a mixture of O_2 and CO_2 (95:5, v/v). Ion and water contents were evaluated in slices run in parallel to those used to measure cell electrical potentials. For this purpose several slices were incubated in the corresponding media and groups of two to three slices were taken out for analysis at the end of incubation in the cold, and at 1 min intervals for the first 10 min of

rewarming, and at 11–15 and 30–50 min of rewarming. The slices were blotted, weighed, desiccated overnight at 100° C, weighed again (to obtain their water content), and shaken for 48 hr in 2 ml. 1 N-HNO_3 to extract the ions. Na, K and Cl were determined as described previously (Whittembury, 1965; Whittembury & Proverbio, 1970). Cellular Na, K and Cl contents were calculated from the tissue analysis, by subtracting the extracellular content of these ions using a figure of 0·26 g/g tissue for the extracellular space. It has been previously found that under our experimental conditions the extracellular space of the slices in the 0-K medium or in the 16-K medium in the cold and at 25° C averages 0·26 (Whittembury & Proverbio, 1970). Na, K and Cl fluxes (in μmole/g.solids.min) were calculated from the changes in cell content of these ions measured at given time intervals after rewarming.

Electrical potential difference measurements

The technique has been described in detail previously (Whittembury, 1965). Briefly, the exploring and indifferent electrodes were symmetrical saturated calomel electrodes. The recording instrument was a 10^{14} Ω input impedance voltmeter (model 200-B Keithley Instruments, Inc., Cleveland, Ohio). The exploring micropipettes were drawn in a puller from Pyrex glass (1·0 mm o.d., 0·8 mm i.d.) to a tip angle of 4–6°, as observed under a water immersion objective at 600 ×; they were filled with 3 M-KCl and mounted in a holder on to a micromanipulator. Only those whose resistance and tip potential fell within the range 5–30 MΩ and 0–5 mV respectively were used. Membrane potential values were accepted only when the pipette characteristics had not changed with the impalement. The indifferent electrode was connected to the bathing solution by an agar bridge.

A kidney slice was mounted firmly between fibre glass nets in a lucite chamber with the outer surface upwards. The bathing medium, maintained at the desired temperature, was pumped from a reservoir (where it was gassed with the O_2 and CO_2 mixture) to circulate freely around the slice. A jet of the same gaseous mixture was blown on the fluid surface above the slice. During penetration with the microelectrode only about 0·3 mm fluid was allowed above the slice. A system of thermostated reservoirs in parallel allowed complete changes in solution or temperature to be achieved within 30 sec.

It has been calculated that a substance which diffuses into a 0·3 mm thick slice (with a diffusion coefficient of $8·6 \times 10^{-5}$ cm²/min) will be in 30 sec 99 % equilibrated with the medium at a depth of 0·1 mm and 90 % equilibrated at its centre (Whittembury, 1965). It may also be calculated that if the bath temperature changes, temperature will be 99 % equilibrated at the centre of such a slice in less than 0·1 sec after the bath has changed temperature, if a figure of $1·25 \times 10^{-3}$ cm²/sec for the thermal diffusivity is used (this figure is given by Hill, 1965, for the thermal diffusivity of muscle). In consequence, it seems unlikely that the present experimental results would be influenced by thermal diffusion delay since temperature equilibrium should be attained within the first second of rewarming at 25° C.

To facilitate penetration the kidney slice was mounted with its non-sectioned (external surface) uppermost. Thus, non-damaged, lumen-collapsed tubules could be observed and their cells impaled. The micropipette was advanced under a dissecting microscope at a magnification 120 ×. An oblique beam of point light illuminated the tissue. The potentials were selected when they had been established by a sudden jump to a stable level (Nastuk & Hodgkin, 1950). The micro-electrode measurements were begun in a solution at 0·6° C. Then the external solution was suddenly changed to one at 25° C and several cells were again sampled as a function of time. V_m has the conventional sign, the cell interior being negative.

RESULTS

Time course of cell potential after rewarming from 0·6 to 25° C in the 16-K medium

The average cell potential (V_m) measured in the cold in the 16-K medium was $-36{\cdot}8 \pm 0{\cdot}8$ mV. This value is not statistically different from a figure of -36 mV estimated for E_K, the K equilibrium potential. E_K was calculated using the Nernst equation from the ratio of $[K]_i/[K_o]$. The intracellular K concentration $[K]_i$ was obtained from the tissue analysis, an

TABLE 2. Membrane potentials (V_m, obtained in thirty-five slices) and K equilibrium potentials (E_K and E'_K) evaluated in slices run in parallel and analysed at different times of rewarming in the 16-K medium without inhibitors

(Values for E'_K were estimated using the Nernst equation from the calculated $[K]_i$ and bath $[K]_o$. The values for E'_K were calculated as described in the appendix. Several slices were incubated in experiments run parallel to those used for the membrane potential measurements. For each analysis 2–3 slices were taken out of the bath before rewarming and 1 min intervals after rewarming. Ion fluxes were calculated from the changes in cell ionic content measured at the times of rewarming given below.)

Condition					Net ion fluxes (μmole/g solids.min)		
Temperature (°C)	Time (min)	V_m (−mV)	E_K (−mV)	E'_K (−mV)	Na efflux	Cl efflux	K influx
0·6	—	$36{\cdot}8 \pm 0{\cdot}8$	36	36	0	0	0
25·0	0–1	$75{\cdot}7 \pm 1{\cdot}3$	40	43	41	25	18
	1–2	$75{\cdot}5 \pm 1{\cdot}7$	43	46	33	19	16
	2–3	$72{\cdot}6 \pm 1{\cdot}7$	46	48	27	12	14
	3–4	$71{\cdot}6 \pm 1{\cdot}8$	48	50	22	8	13
	4–5	$69{\cdot}5 \pm 1{\cdot}4$	50	51	17	6	11
	5–6	$65{\cdot}2 \pm 1{\cdot}4$	51	52	15	5	10
	6–8	$64{\cdot}1 \pm 1{\cdot}0$	53	51	10	2	9
	8–10	$61{\cdot}4 \pm 1{\cdot}5$	55	55	7	0	7
	11–15	$60{\cdot}1 \pm 1{\cdot}0$	54	54	0	0	0
	30–50	$52{\cdot}7 \pm 1{\cdot}1$	56	56	0	0	0

extracellular space of 0·26 and an extracellular K concentration, $[K]_o$, 16 mM. As may be seen in Fig. 1 and Table 2, within 1 min of rewarming V_m hyperpolarized about 40 mV to values near -75 mV. The hyperpolarization was marked for about 5 min, V_m values being significantly more negative than the values calculated for E_K. Later on V_m values became less negative and approached the E_K values in about 10 min. This form of the change in V_m is denoted here as a 'hyperpolarization well'. It may be seen in Fig. 1, that the time course of hyperpolarization runs parallel to the

magnitude of net Na efflux. This observed hyperpolarization may be accounted for by electrogenic Na extrusion if the extrusion of Na^+ out of the cell were to increase directly the negativity of the cell interior. However, before reaching this conclusion one must make sure that the values

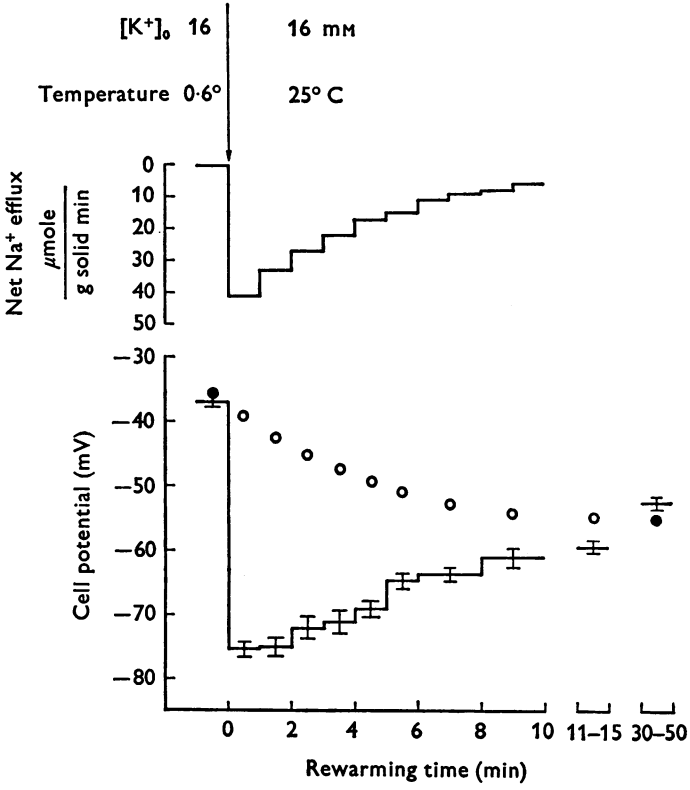

Fig. 1. Time course of cell potential (V_m) after rewarming in a medium with 16 mM-K. Control experiment. Measurements of V_m were performed at 0·6° C for 15 min. Then the bathing medium temperature was raised to 25° C (at time zero in the graph). Five to six satisfactory mesurements could be obtained in each run with a slice. The continuous line gives the mean + S.E. of measurements obtained in thirty-five slices. Circles are values of E_K calculated using the Nernst equation and the measured intra and extracellular K concentrations in a state of zero net K flux (filled circles) and when cells were gaining K (open circles). At the top of the figure the net Na efflux is depicted with the scale downwards to show its relation to V_m.

calculated for E_K are correct. In the calculations of the values of E_K represented in Fig. 1 and Table 2 a value of $[K]_o$ 16 mM was used. However, the K concentration in the immediate neighbourhood of the cell membrane could be lower than 16 mM. As Adrian & Slayman (1966) have pointed out,

if the rate of net influx of K^+ into the cell across the cell membrane were larger than its rate of diffusion across the extracellular space, the actual K concentration in immediate proximity of the tubule cell (which we denote as $[K]_o^x$ will be lower than $[K]_o$, the K concentration in the bath. Since $[K]_o^x < [K]_o$, then $[K]_i/[K]_o^x > [K]_i/[K]_o$, and the value of E_K calculated from $[K]_i/[K]_o$ would be less negative than the value calculated from $[K]_i/[K]_o^x$ (which we denote as E'_K). We have used several approaches to examine this possibility: measurements of V_m in the 0-K medium, recalculation of the values of E_K using an estimate of $[K]_o^x$ instead of $[K]_o$, comparison of the values of V_m measured using other cations that should diffuse in the extracellular space at the same rate as K, and curtailment of Na extrusion by means of inhibitors.

Time course of cell potential after rewarming in the 0-K medium

Na extrusion has been shown to occur upon rewarming in the 0-K medium with some net loss from the cells (without net entry of K) (Whittembury & Proverbio, 1970). If the hyperpolarization well shown in Fig. 1 were due to the establishment of a region with extracellular K concentrations, $[K]_o^x$, lower than 16 mM, the hyperpolarization should be markedly exaggerated by rewarming in the 0-K medium. Fig. 2 shows that within 1 min of rewarming in the 0-K medium V_m values were only slightly more negative than those obtaining in the 16-K medium. This experiment indicates that $[K]_o^x$ would have needed to drop from 16 mM to values near zero to account for the hyperpolarization observed in Fig. 1. This extreme reduction of the extracellular K concentration seems highly unlikely.

Recalculation of E_K values taking into account diffusion delay in the extracellular space

The real K concentration in the extracellular space, $[K]_o^x$ is a function of the size of the extracellular compartment, of the diffusion coefficient for K in the extracellular space and of the net flux of K into the cells. A figure for $[K]_o^x$ may be calculated using the treatment developed by Keynes (1954) and Adrian & Slayman (1966). The equations and the calculations given in the appendix yield an estimated value for $[K]_o^x$ not lower than 12 mM at a depth of 30 μm. At this depth, which was usually reached in our penetrations, it would be expected that a superficial kidney tubule should have been impaled by the micro-electrode. Using this figure of 12 mM, E'_K values may be recalculated. The values obtained are only 2–3 mV more negative than those obtained for E_K (Table 2). Even at a depth of 60 μm values calculated for E'_K are still 20–30 mV less negative than the observed

V_m values. Also, measurements of V_m in deeper regions of the slice were not more negative than those obtained in the superficial tubules usually punctured. Thus, although some hyperpolarization can be accounted for by a diminished extracellular K concentration close to the cells, its magnitude is much too small to account for the large hyperpolarization well observed during rewarming in the 16-K medium.

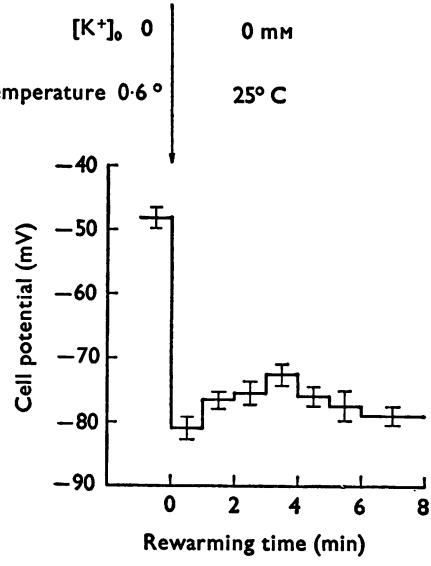

Fig. 2. Time course of cell potential after rewarming in the 0-K medium. Measurements begun after 2·5 hr immersion at 0·6° C. Thirty min thereafter rewarming was effected (time zero). In each run 5–6 satisfactory measurements could be obtained with a slice. The continuous line gives mean ± S.E. of measurements obtained in twenty-five slices. The value for V_m obtained in the cold in the 0-K medium is significantly more negative than that obtained in the 16-K medium, as would be expected from the virtual absence of K^+ in the bathing medium.

Time course of membrane potential upon rewarming in the 16-Rb and 16-Cs fluids

The time course of Na extrusion in media containing Cs or Rb is similar to that observed with K (Whittembury & Proverbio, 1968; and unpublished experiments). The diffusion of Rb^+, Cs^+ and K^+ in the extracellular space of the slices would be expected to be comparable since their diffusion coefficients in free solution are similar. Diffusion coefficient at infinite dilution, 2·055, 2·044, 1·994 cm^2/sec for Rb^{Cl}, Cs^{Cl} and K^{Cl}, respectively (Robinson & Stokes, 1959). On the other hand, net influx of Cs into the cells of kidney slices rewarmed in a 16-Cs solution is of about the same

magnitude as that of K, while Rb influx is twice that of K (Whittembury & Proverbio, 1968). If the hyperpolarization depicted in Fig. 1 were attributable to the possibility that diffusion of K^+ in the extracellular space was smaller than net influx of K^+ into the cell, one would expect that hyperpolarization in the presence of Rb would be larger than with K, because net Rb influx into the cells (being larger than net K influx) should lead to a larger decrement of the Rb concentration in the immediate

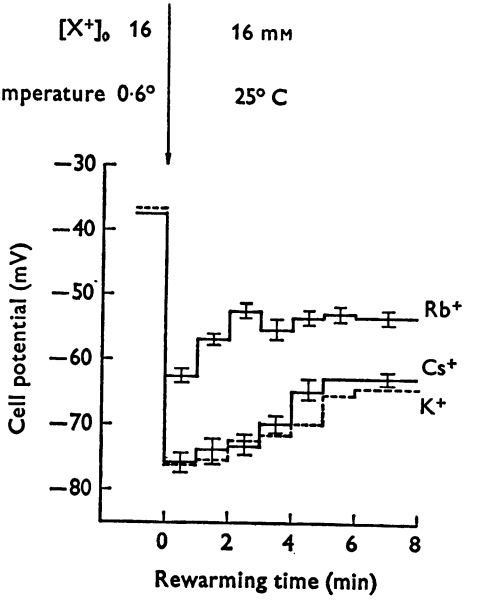

Fig. 3. Time course of cell potential after rewarming in the 16-Rb and in the 16-Cs media. Measurements were performed at 0·6° C for 15 min. Then the bathing medium temperature was raised to 25° C (time zero). In each run 5–6 satisfactory measurements could be obtained with a slice. The continuous lines represent mean ± S.E. of measurements obtained in eleven slices (Rb) and in sixteen slices (Cs). For comparison the dashed line represents the values obtained with 16-K taken from Fig. 1. The difference between V_m values obtained in the cold in the 16-K medium and in the 16-Rb and 16-Cs medium is not statistically significant.

neighbourhood of the cell membrane than that expected to occur with K. With this idea in mind V_m was measured during rewarming in the 16-Rb and in the 16-Cs fluid. The results depicted in Fig. 3 show that in the presence of Rb the hyperpolarization is significantly less than with Cs or K. This could be explained if Rb^+ as the cation that penetrates at a faster rate than K^+ were more effective in shunting the membrane potential. As expected from the similar net influx of Cs^+ as compared to K^+, there is

no difference in the degree of hyperpolarization observed with Cs^+ as compared to K^+. These experiments provide another indication that diffusion delay is not the main cause of the hyperpolarization well depicted in Fig. 1.

Effect of the use of transport inhibitors on the hyperpolarization produced by rewarming in the 16-K medium

It has been found that ouabain and ethacrynic acid inhibit differently Na extrusion and the accompanying fluxes of K^+ and Cl^-. Thus, during rewarming in the 16-K medium, in the presence of 1 mM ouabain, extrusion of Na^+ in exchange for K^+ is mainly curtailed, while kidney cells can still extrude Na^+ with Cl^- and water reducing their volume. On the other hand, in the presence of 2 mM ethacrynic acid (in the 16-K medium) kidney cells can still gain K^+ and extrude some Na^+. However, they are unable to extrude Na^+ with Cl^- and water. In consequence, they maintain a higher water, Na^+ and Cl^- content than those treated with ouabain. If 1 mM ouabain and 2 mM ethacrynic acid are used together during rewarming in the 16-K medium, cells neither extrude any Na^+ nor take up any K^+. They remain swollen with a high Na^+ and Cl^+ content and with a low K^+ content (Whittembury & Proverbio, 1970). Therefore, it was thought that a study of the time course of V_m upon rewarming in the 16-K medium in the presence of ouabain or of ethacrynic acid should further help to illuminate the relationship between Na^+ extrusion and the hyperpolarization of V_m shown in Fig. 1. For instance, if the hyperpolarization were primarily due to a lowered $[K]_o$ in the vicinity of the cell membrane, as discussed above, the hyperpolarization should be abolished by ouabain which drastically curtails net K influx into the cells. Hyperpolarization should not be affected by ethacrynic acid which does not affect K influx. Results of the ouabain experiments show (Fig. 4) that there is less hyperpolarization than in the control experiments without ouabain. However, a hyperpolarization well about 20 mV more negative than the values calculated for E_K is still present, particularly during the first 3 min of rewarming. In the presence of ethacrynic acid (Fig. 5), although the initial hyperpolarization well is absent, V_m values are generally still about 20 mV more negative than the E_K values. In the presence of both inhibitors (Fig. 6) no significant hyperpolarization is observed.

DISCUSSION

We have previously noticed interesting similarities and differences between kidney cells (Whittembury & Proverbio, 1970) and muscle fibres (Cross, Keynes & Rybova, 1965). Consequently in this work we have

followed lines of thought similar to those used by Adrian & Slayman (1966) to study muscle fibres. The observations reported in this work have been made in proximal tubular cells, which are the main constituents of the outermost slices of the guinea-pig kidney cortex (Whittembury, 1965). The main finding of the present experiments is the observation that during a period of enhanced Na extrusion, the cell membrane potential of proximal

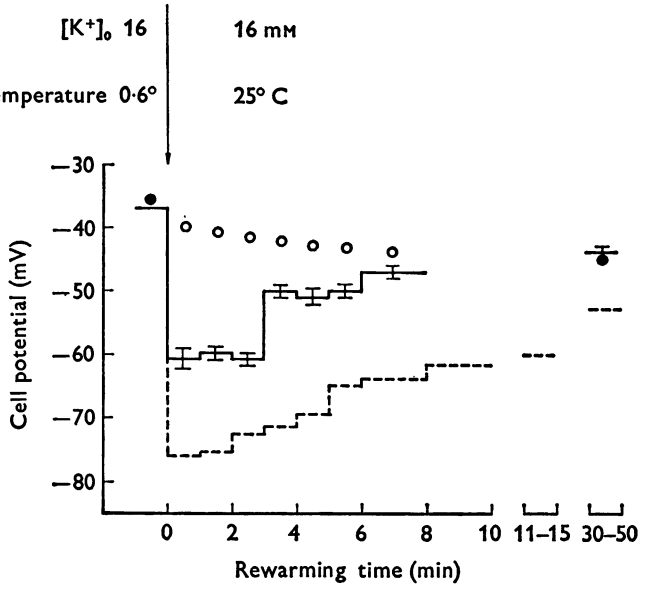

Fig. 4. Time course of cell potential after rewarming in the 16-K-ouabain medium. Experiment similar to that illustrated in Fig. 1 except for the presence of 1 mM ouabain. In each run 5–6 satisfactory measurements could be obtained with a slice. The continuous line shows mean ± S.E. of measurements performed in ten slices. The dashed line shows values obtained under control conditions taken from Fig. 1. There is no difference between V_m values obtained in the cold in the presence or absence of ouabain (controls). Circles represent E_K values calculated from the tissue analyses performed in slices taken out for analysis at the times indicated in the figure. These slices were run in parallel to those used to perform the cell potential measurements.

kidney tubular cells hyperpolarizes to values about 40 mV more negative than the values calculated from the Nernst equation. This hyperpolarization is transitory, being most marked during the first few minutes of rewarming at a time in which net Na extrusion, net K influx and net Cl efflux proceed at maximal speed. It is smaller if 16 mM-Rb, instead of 16 mM-K, is used in the bathing fluid. Although ouabain clearly diminished the magnitude of the hyperpolarization, there remained a hyperpolarization of some 20 mV more negative than E_K. Although ethacrynic acid

clearly blocks much of the hyperpolarization well, the tubule cells still hyperpolarized about 20 mV beyond the value calculated for E_K. On the other hand, no hyperpolarization beyond E_K was observed if ouabain and ethacrynic acid were given simultaneously. It is possible that extracellular depletion of K or Rb (or Cs in the experiments presented in Fig. 3) accounts for some part of the hyperpolarization observed during enhanced pumping of Na. But it is difficult to see how a neutral pump can account for the observed hyperpolarization. The conclusion may be reached that Na extrusion in proximal tubular cells is electrogenic. A somewhat similar

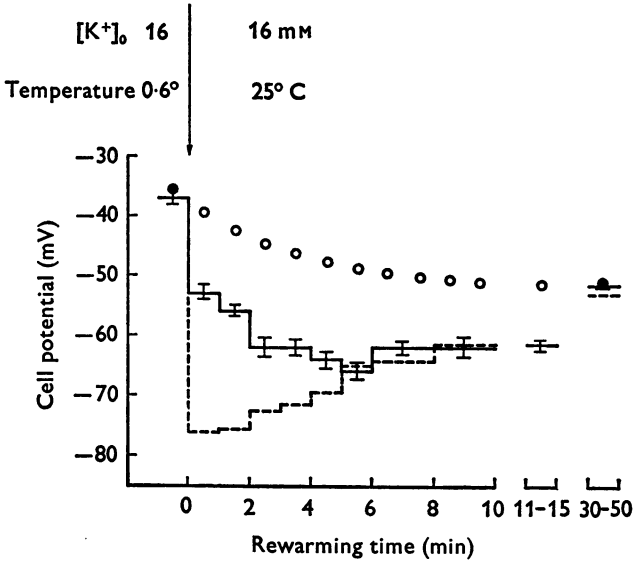

Fig. 5. Time course of cell potential after rewarming in the 16-K-ethacrynic acid medium. Experiment similar to that illustrated in Fig. 1 except for the presence of 2 mM ethacrynic acid. In each run 5–6 satisfactory measurements could be obtained with a slice. The continuous line shows mean ± S.E. of measurements performed in seventeen slices. The dashed line shows values obtained under control conditions taken from Fig. 1. There is no difference between V_m values obtained in the cold in the presence or absence of ethacrynic acid (control). Circles represent E_K values calculated from the tissue analyses performed in slices taken out for analysis at the times indicated in the figure.

behaviour has also been observed in muscle fibres (Kernan & Tangney, 1964; Cross *et al.* 1965; Adrian & Slayman, 1966). However, muscle fibres show a hyperpolarization that is larger with Rb than with K and which can be abolished by ouabain. It can be partly accounted for by a lowered K concentration in the external aspects of the cells due to diffusion delay (Adrian & Slayman 1966).

As illustrated in Fig. 7 there is a good correlation between the net ionic movements taking place during the first 10 min of rewarming (net Cl^- efflux, net K^+ influx and net Na^+ efflux) and the magnitude of the concomitant values of the membrane potential. The correlation coefficients are $r = -0{\cdot}926$ for Cl^-, $r = -0{\cdot}978$ for K^+ and $r = 0{\cdot}958$ for Na^+. As

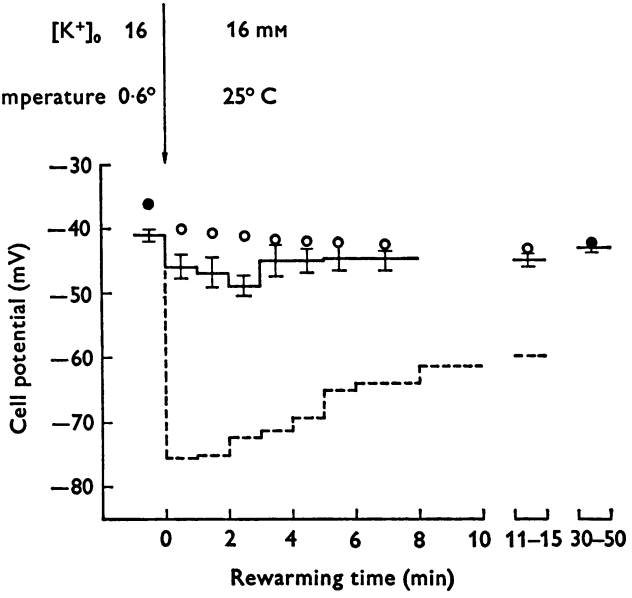

Fig. 6. Time course of cell potential after rewarming in the 16-K-ouabain-ethacrynic acid medium. Experiment similar to that illustrated in Fig. 1 except for the presence of 1 mM ouabain and 2 mM ethacrynic acid. In each run 5–6 satisfactory measurements could be obtained with a slice. The continuous line shows values obtained under control conditions taken from Fig. 1. In this series of experiments V_m value obtained in the cold in the presence of inhibitors was 3·7 mV more negative than the value obtained in the controls (no inhibitors, filled circle to the left) ($P = 0{\cdot}05$). We have no explanation for this difference. Circles represent E_K values calculated from the tissue analyses performed in slices taken out for analysis at the times indicated in the figure.

expected for ions moving passively, the magnitude of the net Cl^- efflux and the net K^+ influx is related to the membrane potential in such a way that the largest net flux is observed when the electrical driving force is the largest, and the smallest net flux is observed when the membrane potential is less negative. On the other hand, it may be seen (Fig. 7) that in the case of the net Na efflux, the extra potential is proportional to the rate of Na pumping. In other words, the largest amount of Na is extruded

when the magnitude of the electrical force opposing net Na efflux is the largest. This indicates that Na extrusion may transfer electric charge across the membrane and may generate a potential which is shunted to some extent by net Cl^- efflux and net K^+ influx. Thus it may be concluded that Na extrusion is electrogenic in kidney proximal tubule cells. Influx of K^+ shunts the potential to a lesser extent than efflux of Cl^- because the

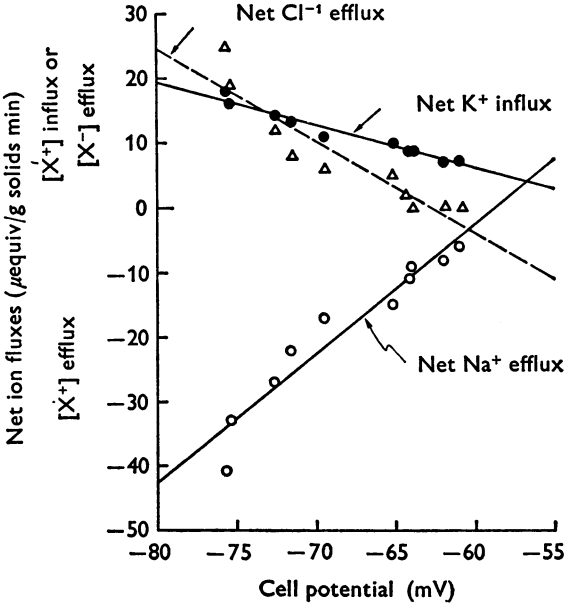

Fig. 7. Average ion fluxes during the first 10 min rewarming in the 16-K medium plotted as a function of the cell potential measured concomitantly (data taken from Table 2). In the ordinate influx and efflux of positive charges are plotted upwards and downwards respectively.

slope relating the K influx to V_m (0·681 equiv/V.g.min) is about one half of that relating the Cl flux to the membrane potential (1·435 equiv/V.g.min). This indicates that twice as much Cl^- moves in response to a given potential difference as compared with K^+. Although these slopes have the character of a conductance, one should not calculate conductances from these lines since the *real* contributions of these ions to current flow is unknown.

Ionic movements common to the steady-state condition at 25° C and those prevailing during rewarming are illustrated with the aid of Fig. 8. In the steady state, Na has to be extruded actively (Na^a in Fig. 8) only at a slow rate in order to balance the small passive entry of Na^+ (Na^p)

(Whittembury & Proverbio, 1970). Therefore, even with an electrogenic Na pump, any electrical activity due to Na extrusion would be masked by the potential due to K^+ diffusion. Since the cell membrane is permeable

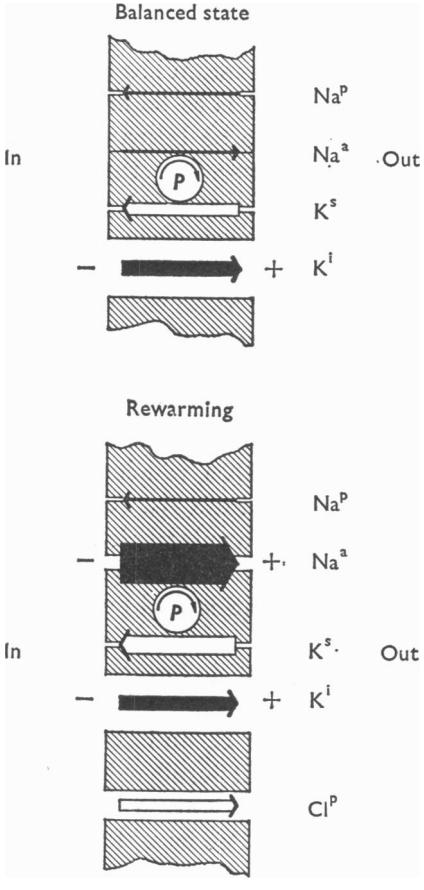

Fig. 8. Schematic representation of ionic fluxes prevailing in the balanced state (upper panel) and out of the balanced steady state, upon rewarming in the 16-K medium (lower panel). IN and OUT refer to the cell inside and outside respectively. Na^p, passive Na influx; Na^a, active Na extrusion; *P*, Na extruding mechanisms; K^s, K influx that may occur uphill at $[K]_o < 8$ mM; K^i, K diffusion that generates the potential. Upon rewarming, increased Na^+ extrusion enhanced Cl^- efflux and K^+ influx are underscored by the large arrows. (Cl^- fluxes have not been included in the upper panel.)

to K^+, diffusion of K constitutes the main origin of the potential (represented in Fig. 8 by K^i and by the largest arrow). At $[K]_o$ higher than 8 mM, V_m equals E_K. Under these conditions K^+ diffusion outwards should be just balanced by V_m. At $[K]_o$ lower than 8 mM the effect of passive entry of

Na^+ (Na^p) on the potential is apparent (Whittembury, 1965). Under these conditions V_m is less negative than E_K (for example at $[K]_o$ of 2 mM, E_K is estimated to be -95 mV and V_m is -70 mV (Whittembury, 1965; see also Giebisch, 1961; Whittembury *et al.* 1961; Boulpaep, 1967, 1971; Frömter *et al.* 1971). Therefore, under these circumstances V_m is insufficient to drive K^+ inwards. A similar situation has been described in muscle (Cross *et al.* 1965). If the steady state is to be maintained, the K being lost by diffusion out of the cell has to be taken back by an active mechanism (K-pump) which needs to overcome the adverse K^+ electrochemical gradient. It is conceivable that at least part of this active K influx is coupled to Na extrusion (and may be in part electrically silent). This is indicated in Fig. 8 by the closeness of the arrow representing K entry to the wheel representing the active Na extrusion mechanisms.

The large arrow (Na^a) in the lower panel of Fig. 8 underscores the conclusion that an electrical potential is generated by enhanced Na extrusion while passive Na influx stays small. As a consequence cell negativity is increased beyond the potential generated by K diffusion (K^i). Passive Cl efflux (Cl^p) and net K influx (K^s), which are augmented by the increased negativity, should tend to diminish V_m. Study of the cell potential under conditions in which Cl has been substituted by an impermeant anion like sulphate is desirable. This possibility was not tested because kidney cells change volume during substitution and are known to accumulate sulfate (Deyrup & Ussing, 1955).

Although authors differ in their interpretation, there is general agreement that in kidney cells ouabain inhibits largely that fraction of Na extrusion which exchanges for K, and that kidney cells can extrude Na in the presence of ouabain (Kleinzeller & Knotkova, 1964; Kleinzeller, 1972; Macknight, 1968, 1969; Willis, 1968; Whittembury & Proverbio, 1970; Podevin & Boumendil-Podevin, 1972). The present observations show that even in the presence of 1 mM ouabain an electrogenic component of Na extrusion is still active (initial hyperpolarization well). If Na extrusion in exchange for net K influx were electrically neutral, its inhibition (by ouabain) should not affect the hyperpolarization. The observation that ouabain inhibits partly the hyperpolarization indicates that Na extrusion in exchange for K is electrogenic and suggests that only part of net K influx shunts the membrane potential. From electrophysiological measurements across the peritubular cell boundary, as a function of sudden changes in the ionic concentration, it has been concluded that the cell membrane conductance to K^+ is higher than that to Cl^- (Boulpaep, 1966, 1967, 1971; Giebisch, 1968; Frömter *et al.* 1971; Giebisch & Windhager, 1973). Therefore, it would be expected that K flux would short-circuit the membrane potential to a greater extent than Cl flux. The ratio

1:2 obtaining for the slopes of K and Cl in Fig. 7 suggests that part of the net influx of K observed at the outset of rewarming has a smaller short-circuiting effect than Cl^- efflux because it carries less electric charge into the cell interior than Cl ions moving outwards.

Since about two thirds of the K flux and about one half of the Rb flux are sensitive to ouabain at external K and Rb concentrations of 16 mM (Whittembury & Proverbio, 1968, and unpublished experiments), about one third of the K flux and about one half of the Rb flux may be related to a mode of entry, independent of ouabain-sensitive Na extrusion. These ouabain-insensitive moieties of the Rb and K fluxes are probably carrying electric charges across the membrane as evidenced by their shunting the hyperpolarization (Rb influx shunts the potential to a greater extent than K). In this respect kidney cells differ from muscle fibres in which only 10% of the Rb influx seems to carry electric charges across the membrane.

As illustrated in Fig. 5, ethacrynic acid diminishes the initial hyperpolarization in a manner different from that induced by ouabain (cf. Fig. 4). It has been previously reported that ouabain inhibits mainly a fraction of net Na^+ efflux that is extruded in exchange for K^+ and that ethacrynic acid inhibits mainly a fraction of net Na^+ efflux that is extruded accompanied by Cl^- and water. Since in the presence of ouabain, V_m is more negative than E_K and in the presence of ethacrynic acid V_m is also more negative than E_K, it seems fair to conclude that both modes of Na^+ extrusion are electrogenic.

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APPENDIX

Estimation of the K concentration at a given depth within a kidney slice

In this calculation we follow the approach used by Adrian & Slayman (1966). $[K]_o^x$, the K concentration within the tissue at a place located at a given distance x from the centre of a slice of half thickness b, is a function of x, of e, the size of the extracellular space, and of D' the diffusion coefficient of K in the tissue (which should take into account the hindrance of the extracellular space to the diffusion of K). Diffusion of K from the bath supplies K to the place located within the slice and the net influx of K

into the cells, ϕ (in μmole/cm^3.min) withdraws K from that place. $[K]_o^x$ is also a function of ϕ. Eqn. 8 of Keynes (1954) may be written as

$$\partial[K]_o^x/\partial t = D'.\partial^2[K]_o^x/\partial^2 - \alpha^2 D'\,[K]_o^x, \tag{1}$$

where $\alpha^2 = (1-e)\,\phi/eD'\,[K]_0$. We assume a steady extracellular distribution to be reached in a short time so that $\partial[K]_o^x/\partial t = 0$. This assumption seems justified since the slice is left to equilibrate in the cold with the 16-K solution for 30 min before rewarming. Since diffusion takes place from both surfaces $[K]_o^x$ should be a minimum at $x = 0$, the centre of the slice $\partial[K]_o^x/\partial x = 0$, and at $x = b$, $[K]_o^x = [K]_o^b = 16$ mM. Eqn. 1 may be solved to yield

$$[K]_o^x/[K]_o^b = \cosh \alpha x/\cosh \alpha b. \tag{2}$$

This useful equation allows to obtain values for $[K]_o^x$ at a distance x from the centre of the slice, provided α is known. Our V_m measurements were performed in slices 200–300 μm in thickness at about 30 μ from the surface. We shall use $b = 150$ μm, $x = 120$ μm. There are no values for D' for K kidney tissue. Conway & Fitzgerald (1942) measured a value of $8{\cdot}65 \times 10^{-5}$ cm^2/min for the diffusion of urea in kidney tissue. The free diffusion coefficient of urea ($1{\cdot}12 \times 10^{-5}$ cm^2/sec; Conway, 1952) is about 0·6 times the diffusion coefficient of K in free solution ($1{\cdot}993 \times 10^{-5}$ cm^2/sec, Robinson & Stokes, 1959). Dividing by 0·6, we obtain from the value of Conway & Fitzgerald a figure of 14×10^{-5} cm^2/min which we shall use in the calculation. The highest K influx value (Table 2) of 18 μmole/g solids.min is equivalent to one of $\sim$ 6 μmole/cm^3 cell min, since 1 g solids correspond to about 3 cm^3 cells (1 g tissues has $\sim$ 0·22 g solids, 0·74 g cells and a specific gravity of 1·05. Thus 1 g solids corresponds to $0{\cdot}74/(1{\cdot}05 \times 0{\cdot}22) \cong 3{\cdot}2$ cm^3 cells). It is important to realize that the measured influx may be an underestimate of the renal flux if there is delay in the diffusion of K, and should be corrected. The corrected flux may be obtained from eqn. 3 which is derived from eqn. 17 of Keynes (1954):

$$\phi'/\phi = (\tanh \alpha b)/\alpha b, \tag{3}$$

where ϕ' is the measured flux calculated above and ϕ is the real flux to be used in eqn. (2). With the help of eqn. (3) it may be calculated that the real flux would be 1·6 times the measured flux. Even if we use a value of 12 μmole/cm^3 min for ϕ, the calculated value for $[K]_o^x$ is 12 m-mole/l. at a depth of 30 μm from the surface. It should be realized that in these calculations we are using values which would tend to lead to extremely high correction factors. Despite this, as explained in the text, these calculations lead to corrected values for E'_K which can explain only a small part of the hyperpolarization (Table 2).

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