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VOLUME- AND TEMPERATURE-DEPENDENT PERMEABILITIES IN ISOLATED RAT LIVER CELLS

BY BRIGITTE BERTHON, M. CLARET, J. L. MAZET AND JOSIANE POGGIOLI

From the Laboratoire de Physiologie Comparée et de Physiologie Cellulaire Associé au CNRS, Université Paris-Sud, F-91405 – Orsay Cedex, France

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SUMMARY

1. Water, K, Na and Cl contents and fluxes of K and Na were determined in isolated rat hepatocytes incubated at 1 °C (90 min) then at 38 °C (60 min). At 1 °C cells progressively gained Na and Cl, lost K and increased their volume by 17 %.

2. Rewarming triggered a net *loss* of K and *gain* of Na. They were transitory (about 60 sec) being overcome rapidly by movements in the opposite direction until cells recovered their initial K and Na gradients.

3. Determination of time courses of the K rate constant (k'_K) and net Na influx (ϕ'_{Na}) in cells incubated in ouabain K-free media indicated that these paradoxical movements were due to a temporary shunting of the Na pump by sudden increases in K and Na permeabilities.

4. Increases in k'_K and ϕ'_{Na} were not sensitive to inhibitors of Ca-activated K channels such as quinine (10^{-3} M) or apamin (10^{-8} M), suggesting they were not dependent on internal ionized Ca.

5. In control media containing 1·8 mM-Ca divalent ionophore A23187, though stimulating the Ca pump (Ca efflux), presumably by increasing internal ionized Ca concentration, did not cause substantial and rapid changes in K permeability. This supports the hypothesis that Ca-sensitive K channels are lacking in rat hepatocytes.

6. A 10 % increase in cell volume provoked by a hypo-osmotic shock triggered increases in both k'_K and ϕ'_{Na} with time courses very similar to those brought about by rewarming.

7. It is proposed that transient changes in K and Na permeabilities are the consequence of the cell swelling, induced by cooling. These volume-dependent permeabilities are blocked at 1 °C and revealed by rewarming.

INTRODUCTION

A large part of our knowledge about the transport of ions and water in liver cells comes from studies carried out in tissue slices initially chilled to 1 °C then incubated at 38 °C, a convenient procedure which causes substantial and reversible movements of ions and water (for references see MacKnight & Leaf, 1977 and Russo, Galeotti & Van Rossum, 1977). At 1 °C, slices lose almost all their initial K, quadruple their Na content and increase their water volume by 60 %. On subsequent incubation at 38 °C, extrusion of Na from the cells begins immediately while K uptake starts only

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about 10 min later. There is no clear-cut interpretation of this apparent dissociation of Na and K transport. Because Na extrusion proceeds without delay, it was evident that the initial failure to take up K did not result from a transitory inhibition of the Na pump, and it has been attributed instead to an increased permeability to K selectively induced by an elevated cytosolic Ca concentration (Van Rossum, 1970). More recently, Russo *et al.* (1977) observed that a fraction of the Na extrusion was maintained on rewarming slices incubated in K-free media containing ouabain. They proposed that part of the Na transport was the result of a direct secretion of Na and Cl into vesicles which release their content after fusion with bile canaliculi plasma membranes.

In the present study these transport systems have been re-examined in isolated rat hepatocytes. Cells have been incubated at 1 °C then 38 °C. Their volume has been measured and the time course of passive fluxes under non-steady-state conditions has been determined using a fast centrifugation method (Ferreira & Lew, 1976). The results obtained differ from those previously observed in slices: at 1 °C isolated cells exhibit a relatively small passive loss of K and gain of Na, Cl and water. On rewarming, transient *loss* of K and *gain* of Na occurred due to temporary shunting of the Na pump by sudden increases in K and Na permeabilities. These permeability changes were not sensitive to quinine or apamin, suggesting that they are not mediated through an increase in internal Ca concentration. They seem more likely to be consequences of the cell's swelling at 1 °C, i.e. to reflect volume-dependent permeabilities. Moreover in no case was a net NaCl extrusion detectable as reported for liver slices in ouabain K-free media (Russo *et al.* 1977).

METHODS

Isolated cells

Liver cells were isolated from Wistar rats as described by Seglen (1972, 1973) and equilibrated with an Eagle's (Welcome TC 46) medium containing (mM): 143 Na; 5·4 K; 1·8 Ca; 0·8 Mg; 126 Cl; 25 HCO_3; 0·96 H_2PO_4; 11·1 glucose, various amino acids and vitamins, and oxygenated with 95% O_2–5% CO_2 at 38 °C. Cells were loaded with ^{22}Na or ^{36}Cl until isotopic equilibrium (30–45 min), centrifuged, then suspended (hepatocrit 0·6%) at 1 °C either in Eagle's medium ('control cells') or in K-free media containing 10^{-3} M-ouabain ('blocked cells') of the same specific activity; internal ^{22}Na and ^{36}Cl were thus at isotopic equilibrium throughout the experiments. After a 90 min period at 1 °C, the temperature was increased to 38 °C and was completed within 20 sec. The temperature was checked throughout by means of a thermocouple. The viability of the cells was tested by their ability to exclude Trypan Blue (5‰).

Materials

^{42}K, ^{22}Na, ^{36}Cl and [^{3}H]inulin were obtained from C.E.A. (Saclay, France) or the Radiochemical Centre (Amersham, England). Ionophore A23187 was a gift from Lillie. Quinine hydrochloride was a gift of V. L. Lew (Cambridge). Before use A23187 and quinine were dissolved in ethanol and the resulting solutions were added to the cell suspensions to give a 1:100 dilution. Apamin (a purified extract from the venom of the bee, *Apis mellifica*) was a gift of D. H. Jenkinson who received it from B.E.C. Banks (University College London). Before use it was kept at −20 °C in saline solution (100 μM).

Ion concentrations and volume of cells successively incubated at 38 °C, 1 °C and then 38 °C

Total K and ^{22}Na or total K and ^{36}Cl were measured in the same cell suspensions as a function of incubation time. At the times indicated, two samples of 300 μl. cell suspension were placed in 1·5 ml. Eppendorf Centrifugation tubes containing 400 μl. of either silicone oil (density 1·03)

or *n*-dibutylphtalate (density 1·043) and 800 μl. of isotonic choline chloride solution containing a small quantity of [^{3}H]inulin. All tubes were pre-equilibrated at 1 °C. Each sample was centrifuged immediately at 12,000 *g* for 20 sec. The supernatant and the oil were rapidly sucked off and the pellet of cells homogenized in 300 μl. deionised water. Aliquots of homogenate were used (i) for ^{22}Na/^{3}H or ^{36}Cl/^{3}H counting from which the ^{3}H activity provided a measure of extracellular water trapped in the cell pellet, (ii) for estimating total K content.

Cell weight was estimated from the difference between the weight and the [^{3}H]inulin space of the pellets of cell samples centrifuged at 5000 *g* for 1 min.

^{22}Na, ^{36}Cl and [^{3}H]inulin were determined by liquid scintillation counting, ^{42}K by gamma scintillation counting and the total K content by flame photometry after digestion of the cell pellet with 0·15 M-HNO_3.

K rate constant and net Na influx of cells incubated at 38 °*C*, 1 °*C and then* 38 °*C*

In control experiments the net flux of ions ($\vec{\phi}$) at each time is given by:

$$\vec{\phi} = \phi_i + \phi_o + \phi_p, \tag{1}$$

where ϕ_i and ϕ_o are the diffusional fluxes and ϕ_p the pump flux respectively. In K-free solutions containing ouabain, the Na–K pump is blocked and the K influx is suppressed. As a consequence internal K and Na cell contents progressively change and the membrane potential may differ from that in control cells. Assuming the diffusional K efflux to be proportional to internal K concentration, eqn. (1) reduces to, for K ions:

$$\vec{\phi}'_K = k'_K \times [K]'_i \tag{2}$$

where $\vec{\phi}'_K$, k'_K and $|K|'_i$ are the net efflux, the rate constant of diffusional efflux and the internal concentration of K ions respectively. k'_K was determined from the net flux and the internal K content of cells. Similarly, if we assume the diffusional Na efflux to be proportional to the internal Na concentration, eqn. (1) becomes, for Na ions:

$$\vec{\phi}'_{Na} = \phi'_{i,Na} + \phi'_{o,Na}[Na]'_i/[Na]_i \tag{3}$$

where $\vec{\phi}'_{Na}$ and $\phi'_{i,Na}$ are the net efflux, and the diffusional influx in 'blocked' cells. $[Na]'_i$ and $[Na]_i$ are the Na concentrations in 'blocked' and 'control' cells respectively. The product $(\phi'_{o,Na}[Na]'_i/[Na]_i)$ represents the diffusional efflux in 'blocked' cells. As long as $[Na]'_i$ and $[Na]_i$ are not largely different, the diffusional efflux in rat hepatocytes is small as compared with the diffusional influx $\phi'_{i,Na}$ (Claret, Claret & Mazet, 1973) so that during the first minutes of rewarming rapid changes in $\vec{\phi}'_{Na}$ reflect roughly the changes in $\phi'_{i,Na}$.

In a few experiments, cells were loaded with ^{42}K during the pre-equilibrium period at 38 °C, washed, then suspended for 90 min at 1 °C and for 60 min at 38 °C in control Eagle's medium containing no radioactivity. The rate constant was calculated from the ^{42}K efflux and ^{42}K cell content.

Passive ^{42}K and ^{22}Na unidirectional fluxes

In these experiments, the K rate constant and the Na influx were determined in cell suspensions incubated either at 38 °C or at 1 °C. After ^{42}K loading, the cells were washed then the ^{42}K efflux was followed over 90 min. The rate constant of K efflux was calculated from the ^{42}K content of cell samples centrifuged once at 5000 *g* for 1 min. The Na influx was determined from the ^{22}Na uptake by cells incubated for 1 min with labelled Na. Samples of the cell suspension were washed three times at 5000 *g* for 1 min.

Cell volume of hepatocytes after an osmotic shock

The mean cell volume was measured with a Coulter counter (model C1000, calibrated with 4712 fl. latex beads) in hepatocytes incubated in control media (ideal osmotic concentration = 348 m-osmole.l.$^{-1}$) and immediately after osmotic shocks (220 and 671 m-osmole.l.$^{-1}$). The control mean cell volume was 6040 ± 270 fl. (n = 8). It was increased to 7970 ± 340 fl. (n = 8) in hypo-osmotic medium and decreased to 4650 ± 190 fl. (n = 8) in hyperosmotic medium showing that hepatocytes behave as osmometers shortly after the shock.

Expression of results

All the experimental values are referred to the protein weight, assuming that 10^6 isolated rat liver cells contain 1·8 mg protein. Dispersions about the means in Figures and Tables represent ± S.E. of the mean.

RESULTS

Ion and water contents at the end of each equilibration period

Table 1 shows ion and water contents of cells successively incubated at 38 °C, 1 °C and 38 °C. For clarity only the values measured at the end of each equilibration period are presented. After 90 min at 1 °C, hepatocytes lost 89 n-mole K, and gained

TABLE 1. Total K, ^{22}Na, ^{36}Cl and water contents of cells equilibrated at 38 °C, then either in control or ouabain K-free media for 90 min at 1 °C and finally for 60 minutes at 38 °C. After 90 min at 1 °C, ion contents and volumes of 'control' and 'blocked' cells were not significantly different and the values were pooled. Membrane potentials were calculated from the Nernst equation, assuming that ^{36}Cl was at electrochemical equilibrium (Claret & Mazet, 1972). Mean ± S.E. of three to twenty-two experiments

			38 °C	
Temperature	38 °C	1 °C	'Control' cells	'Blocked' cells
K (n-mole mg^{-1} P)	319 ± 19	230 ± 17	319 ± 14	105 ± 5·6
Na (n-mole mg^{-1} P)	55·6 ± 4·5	105 ± 11	77·8 ± 4·5	226 ± 20
Cl (n-mole mg^{-1} P)	73·3 ± 3·3	110 ± 10	72·2 ± 7·8	117 ± 11
Weight (mg.mg^{-1} P)	3·27 ± 0·14	3·83 ± 0·12	3·06 ± 0·13	3·21 ± 0·11
E_m (mV)	−33·9	−26·0	−31·4	−21·8
E_m–E_K (mV)	54·6	38·9	60·0	

50 n-mole Na and 37 n-mole Cl per mg of protein. They swelled by 0·56 μl. mg^{-1} which represents a 17 % increase over their initial weight. These ionic and water movements are small as compared with those observed under the same conditions in slices from rat liver: when suspended in cooled media, these lose about 200 n-mole K and gain 400 n-mole Na mg^{-1} dry wt. and increase their water volume by 60 % (for references see Macknight & Leaf, 1977; see also Judah & McLean, 1962 and Russo *et al.* 1977). The calculated membrane potential was only slightly lower than at 38 °C, which is in keeping with the maintenance of a significant driving force for K ions.

Sixty min after returning to 38 °C, 'control' cells recovered their initial ion and water contents and their calculated membrane potential, indicating that the effect of a 90 min period at 1 °C on liver cells is totally reversible. Cells suspended in ouabain K-free media exhibited high Na and Cl and low K contents but had a normal volume. This observation confirms that the volume regulation in hepatocytes can occur with the Na–K pump blocked (Macknight, Pilgrim & Robinson, 1974; Claret, Mazet & Poggioli, 1976; Russo *et al.* 1977). The membrane potential was significantly lower than in control cells probably as a result of the decrease in K gradient.

K and Na unidirectional fluxes as measured from the ^{42}K loss and ^{22}Na uptake were significantly altered at 1 °C indicating that K and Na permeabilities were inhibited by the low temperature. At 38 °C, the K rate constant k_K was 0·56 ± 0·03 hr^{-1} ($n = 5$)

and the Na influx was 8·85 ± 1·16 n-mole . mg^{-1} min^{-1} (n = 14). At 1 °C, k_K was decreased to 0·21 hr^{-1} which, on the basis of 319 n-mole K mg^{-1} (Table 1) corresponds to an efflux of 1·11 n-mole mg^{-1} min^{-1}. The Na influx was reduced to 1·16 n-mole mg^{-1} min^{-1}. If allowance is made for the slight over-estimation of the diffusional Na influx due to the presence of a Na for Na exchange (Claret & Mazet, 1972) these values are similar to the passive net fluxes estimated from the change in ion content at 1 °C (0·99 n-mole K and 0·56 n-mole Na mg^{-1} min^{-1} respectively).

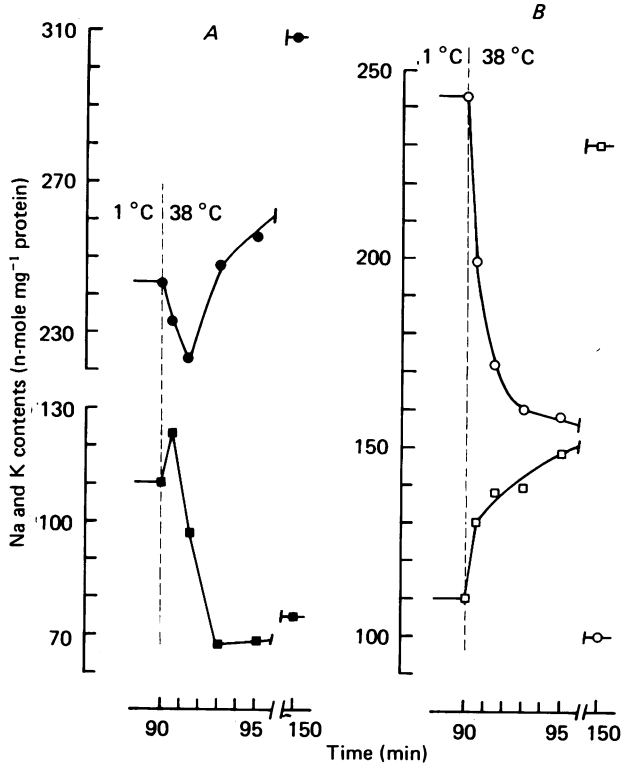

Fig. 1. Time course of K (● ○) and ^{22}Na (■ □) contents measured in the same cell suspensions equilibrated for 90 min at 1 °C then for 60 min at 38 °C in control media (● ■) or in ouabain K-free media (○ □). Mean of two experiments.

Transient ion movements on rewarming

Fig. 1 shows the transient K and Na movements which occurred when the temperature was increased from 1 °C to 38 °C. In control cells, after the slow decline in K and increases in Na and Cl contents at 1 °C, rewarming induced sudden loss of K and gain of Na. These paradoxical fluxes were observed in thirteen out of eighteen K experiments and five out of nine Na experiments. They were transitory, being rapidly overcome by movements in the opposite direction until the cells completely recovered their initial K and Na gradients. In ouabain K-free media the net rapid loss of K and gain of Na slowed though without reaching completion 60 min after the return to 38 °C. In no case was a net loss of NaCl detectable as reported for liver slices incubated in ouabain K-free media.

An extrusion of chloride occurred during the first minutes of rewarming both in 'control' and 'blocked' cells (not shown), suggesting that the cell membrane hyperpolarized during this period. The cell volume returned to its normal value during the same period: 5 min after rewarming it was not significantly different from that before cooling (Table 1) for 'control' as well as for 'blocked' cells.

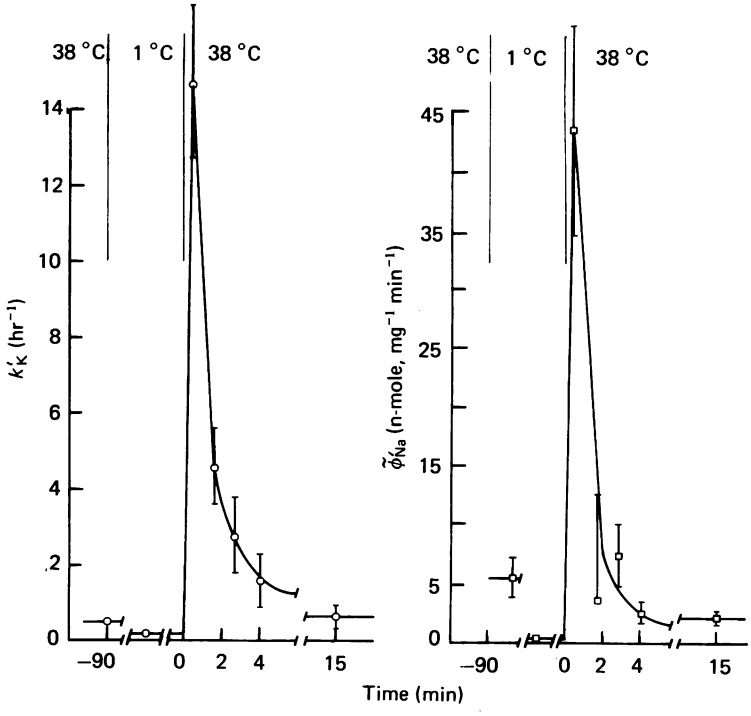

Fig. 2. Time courses of changes in K rate constant and net Na influx. Rewarming triggers a transient activation of K and Na permeabilities. Mean ± S.E. of five to fourteen experiments.

These results suggest that rewarming triggers the opening of K and Na channels and that as long as their amplitudes remain elevated (about 60 sec), the Na pump is unable to balance the resulting net K efflux and Na influx. An estimate of the time course of these transports may be obtained from experiments carried out in the presence of ouabain and in the absence of external K (see Methods). Fig. 2 shows values of the K rate constant (k'_K) and the Na influx ($\tilde{\phi}'_{Na}$) calculated from eqns. (2) and (3): k'_K and $\tilde{\phi}'_{Na}$, inhibited at 1 °C, drastically increased on rewarming. Twenty to thirty sec later, the rate constant was 26 times that measured in normal conditions; it then fell progressively down to reach to its original value within 15 min. The relative increase in $\tilde{\phi}'_{Na}$ was 6·6 times, suggesting that (in so far as variations in k'_K and $\tilde{\phi}'_{Na}$ reflected variations in K and Na permeabilities) the temperature effect was more marked on P_K than on P_{Na}. This observation reinforces the supposition that at the moment of rewarming there was an increase in membrane potential both in 'control' and in 'blocked' hepatocytes.

Cause of the transient increase in K and Na permeabilities

Rat liver slices on rewarming after cold incubation exhibit an immediate ejection of Na and a delayed accumulation of K. This led Van Rossum (1970) to propose that the lag in K recovery was the consequence of a transient rise in K permeability caused by a high level of ionized cytoplasmic Ca previously accumulated at 1 °C (Gardos effect). This observation prompted us to test (i) the effects of agents that

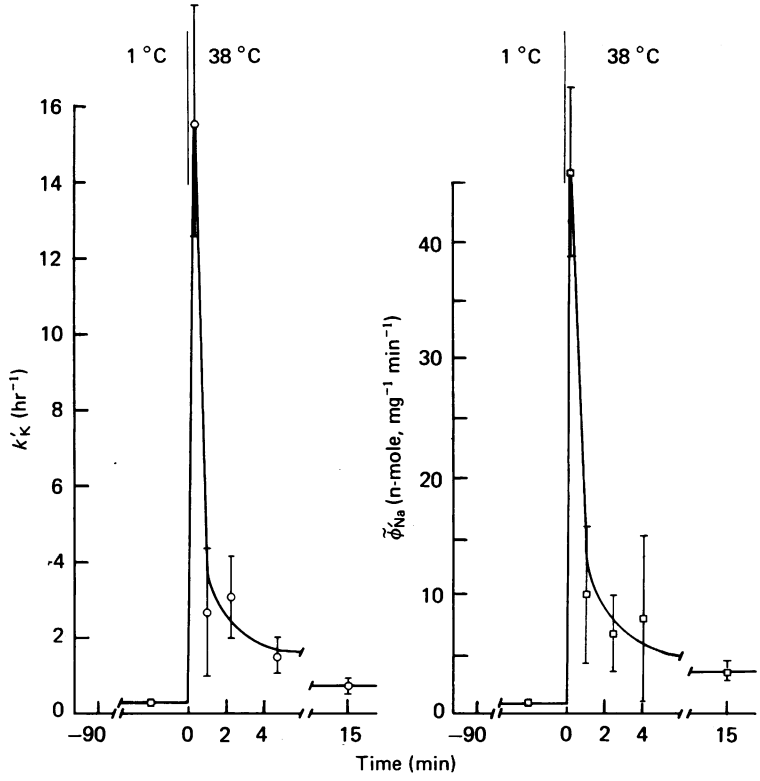

Fig. 3. Quinine (10^{-3} M), added 10 min before rewarming, has no apparent effect on the K and Na channels activated by rewarming. Mean ± S.E. of five experiments.

blocked specifically Ca-dependent K channels on the permeabilities triggered by rewarming, (ii) the effect of an increase in ionized Ca concentration induced by ionophore A23187 on the K permeability. In such a scheme the inhibitors of Ca-dependent K channels would be expected to block increases in k'_K and $\tilde{\phi}'_{Na}$ and the divalent ionophore to initiate an increase in K efflux.

Effect of quinine (10^{-3} M) *and apamin* (10^{-8} M)

Quinine has been shown to completely inhibit the Ca-induced increase in K permeability in red blood cells (Armando-Hardy, Ellory, Ferreira, Fleminger & Lew, 1975) in guinea-pig hepatocytes (Burgess, Claret & Jenkinson, 1979) and in other tissues (Hanani & Shaw, 1977) at a concentration of 10^{-5} to 10^{-3} M. Apamin is a neurotoxin purified from bee venom which is believed to block, at 10^{-10} to 10^{-8} M, the same

Ca-dependent channel in guinea-pig hepatocytes and rabbit liver slices (Banks, Brown, Burgess, Burnstock, Claret, Cocks & Jenkinson, 1979). The K rate constant and net Na influx were determined in cells incubated for 90 min at 1 °C and for 60 min at 38 °C in K-free, ouabain media. Fig. 3 shows that quinine (10^{-3} M) added 10 min before returning to 38 °C had no detectable effects on k'_K and ϕ'_{Na}. Similarly, apamin (10^{-8} M) was also without effect on the K permeability increase.

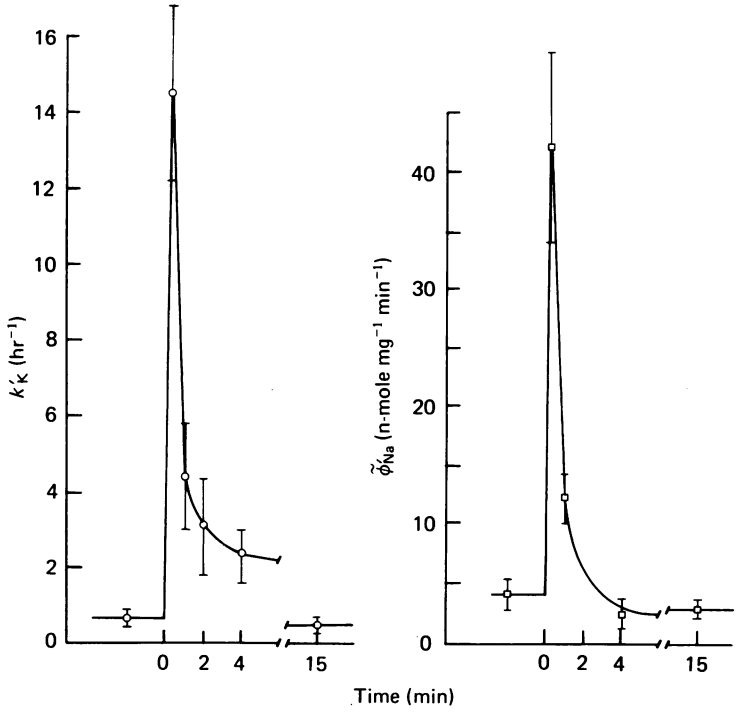

Fig. 4. Effect of a moderate osmotic shock (10%) on the K rate constant and the net Na influx. Mean ± S.E. of three to six experiments.

Effect of A23187 on ^{42}K efflux

After ^{42}K loading, the cells were suspended in Eagle's medium containing 1·8 mM-Ca. The addition of 4 μM-A23187 (corresponding to a final cell concentration of 230 μmole l.$^{-1}$ cells) did not initiate substantial and rapid changes in ^{42}K efflux observed on rewarming, whereas at these doses it causes a strong acceleration of the Ca pump in rat and in guinea-pig hepatocytes as well as a net K loss in the latter tissue (Chen, Babcock & Lardy, 1978; Blackmore, Brumley, Marks & Exton, 1978; Burgess *et al.* 1979; B. Berthon, unpublished observation).

Volume-induced K and Na permeabilities

The above results strongly suggest that K and Na channels induced by rewarming are not activated by a mechanism dependent on intracellular Ca. An alternative hypothesis to explain these is that both the K and the Na permeability increases were caused by the small increase in cell volume, being blocked at 1 °C and revealed by

the rewarming. To test this hypothesis, the K rate constant and the net Na influx were measured in cells equilibrated at 38 °C then submitted to a sudden increase in cell volume. Results reported in the Methods indicate that hepatocytes behave as osmometers shortly after osmotic shocks. So, in the present experiments, an increase in cell volume was provoked by reducing osmotic pressure of control media by 10 %. Fig. 4 shows the time course of k'_K and ϕ'_{Na}. The osmotic shock induced sudden increases in both K and Na fluxes very similar to those brought about by rewarming.

DISCUSSION

Incubating hepatocytes at 1 °C results in a blockade of the Na pump and a reduction in the passive ion fluxes. As a consequence, cells progressively gain Na, Cl and water and lose K with a resulting decrease in ion gradients and an increase in cell volume. Rewarming triggers sudden though transient increases in K and Na permeabilities. These are maximal after 30–60 sec and subside to close to the control values within a few min. The absence of an effect of quinine and apamin indicates that the channels concerned differ not only from the K channels activated by rises in internal Ca in rabbit and guinea-pig hepatocytes (Banks *et al.* 1979) red blood cells (Ferreira & Lew, 1976) or barnacle photoreceptors (Hanani & Shaw, 1977), but also from the potential-activated K and Na conductances in squid axons which are totally inhibited by quinidine (Yeh & Narahashi, 1975). In agreement with these observations is the finding that ionophore A23187 has no effect on ^{42}K efflux, though inducing a stimulation of the Ca pump presumably by elevating cytosolic Ca (Blackmore *et al.* 1978; Chen *et al.* 1978; B. Berthon, personal observations). Rat liver hepatocytes could thus be devoid of Ca-sensitive K channels or these channels could be very insensitive to Ca (see Burgess *et al.* 1979).

The similarity of the time courses of the changes in K rate constant (k'_K) and the net Na influx (ϕ'_{Na}) on rewarming and following an osmotic shock suggest that the K and Na permeability increases are triggered by the same mechanism, the increase in cell volume. It has already been shown in red cells (Kregenow, 1971) in fibroblasts (Roti-Roti & Rothstein, 1973; Buckhold-Shank, Rosenberg & Horowitz, 1973) and in renal tubules (Grantham, Lowe & Dellasega, 1976) that a hypoosmotic shock increases K and/or Na permeabilities, which remain elevated as long as the cells are swollen. Since the same seems to hold in liver cells, it could be supposed that the volume-dependent cation permeabilities are blocked at 1 °C. The sudden increase in P_K and P_{Na} would be triggered by rewarming and the time course of the subsequent closing of the channels would then be determined by the restoration of the cell volume. This is in keeping with the finding that after 5 min permeabilities as well as volume are close to their initial values.

The sudden increases in K and Na permeabilities result in a shunt of the cell membrane, so that the Na pump becomes transiently unable to restore ionic gradients. Thus in control cells, rewarming induces for a short period (30–60 sec) a loss of K and a gain of Na. The Na pump is nevertheless instantaneously activated. A rough estimate of its activity may be made when permeabilities have reached their maximal values: at that particular moment, the membrane is shunted and it may be assumed that the membrane potential in 'control' cells is little different from that in 'blocked'

cells. Combining eqns. (1) and (3), the Na pump flux $\phi_{p,Na}$ can be expressed by:

$$\phi_{p,Na} = \tilde{\phi}_{Na} - \tilde{\phi}'_{Na} - \phi_{0,Na}\{1 - [Na]'_i/[Na]_i\}. \quad (4)$$

At the beginning of rewarming $[Na]'_i$ and $[Na]_i$ are not yet very different and $\phi_{0,Na}$ is small as compared with net fluxes (membrane potential inside negative) so that the product $\phi_{0,Na}(1-[Na]'_i/[Na]_i)$ may be neglected. Thirty seconds after returning to 38 °C, $\tilde{\phi}_{Na} = 19{\cdot}3 \pm 14{\cdot}8$ n-mole mg^{-1} min^{-1} (not shown) and $\tilde{\phi}'_{Na} = 43{\cdot}4 \pm 9{\cdot}2$ n-mole mg^{-1} min^{-1} (Fig. 2). The Na-pump flux then amounts to 24 n-mole mg^{-1} min^{-1} which is about four times higher than the net Na influx (Fig. 2) measured before the temperature change, which is itself close to the steady-state Na pump flux.

The transient loss of K and gain of Na on rewarming were not observed in rat liver slices (see MacKnight & Leaf, 1977 and Russo *et al.* 1977). This is probably because the net ion movements at 1 °C are much more substantial in slices than in the present experiments. Such movements would tend after 90 min to reduce considerably the driving forces for K and Na ions. Increases in permeabilities may then fail to generate net K efflux and Na influx of the kind seen in isolated cells. Similarly, the NaCl extrusion via a mechanism insensitive to ouabain and external K (vesicle–membrane fusion) reported in slices regulating their volume in ouabain K-free media (Russo *et al.* 1977) and apparently absent in isolated cells, may result from the difference between the 'load' on the transport system of the two preparations: at the end of the 90 min period at 1 °C the internal Na concentration in slices is about four times that observed in isolated hepatocytes. This might activate other transport systems which would not otherwise be functional. This remains to be examined.

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