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THE EFFECTS OF METABOLISM ON Na^+–K^+–Cl^- CO-TRANSPORT IN FERRET RED CELLS

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SUMMARY

1. The effects of altering metabolism on Na^+–K^+–Cl^- co-transport were studied in ferret red cells. Na^+–K^+–Cl^- co-transport was measured as the bumetanide-sensitive uptake of ^{86}Rb.

2. Glucose, but not inosine or adenosine, sustained metabolism and maintained cell ATP content ($[ATP]_i$) at the physiological level. $[ATP]_i$ could be reduced by prolonged incubation of cells in a substrate-free medium or more quickly by incubating cells with 2-deoxyglucose or with a mixture of iodoacetamide and glucose.

3. Na^+–K^+–Cl^- co-transport activity was inhibited when $[ATP]_i$ was reduced to below 100 μmol (l cell)$^{-1}$ by starvation or by treatment with 2-deoxyglucose. However, a unique relationship between $[ATP]_i$ and activity could not be found. $[ATP]_i$ and the method and time course of ATP depletion all influenced activity. The inhibition of Na^+–K^+–Cl^- co-transport caused by reducing $[ATP]_i$ could be partially reversed by restoring $[ATP]_i$ to normal.

4. Increasing the concentration of intracellular ionized magnesium ($[Mg^{2+}]_i$) did not stimulate co-transport activity in ATP-depleted cells. This contrasts with the substantial stimulation seen in cells with normal $[ATP]_i$.

5. Vanadate stimulated Na^+–K^+–Cl^- co-transport activity in ATP-depleted cells but not in cells with normal $[ATP]_i$. Fluoride did not affect activity at any $[ATP]_i$.

6. The effects of some sulphydryl reagents on Na^+–K^+–Cl^- co-transport were also examined. *n*-Ethylmaleimide (1 mM) inhibited Na^+–K^+–Cl^- co-transport while it stimulated bumetanide-resistant potassium transport. Dithiothreitol (1 mM) did not affect activity. Iodoacetamide (6 mM) appeared to reduce the inhibition of co-transport activity seen at low $[ATP]_i$ but also greatly increased cell fragility.

7. The data suggest that activity of the Na^+–K^+–Cl^- co-transport system is controlled by a cycle of phosphorylation and dephosphorylation with the phosphorylated form being active. Phosphorylation and transport appear to be almost maximal in ferret red cells with normal $[ATP]_i$. Reduction of $[ATP]_i$ may allow changes in phosphatase activity to manifest as changes in transport rate. Differences in the balance between phosphorylation and dephosphorylation may explain tissue-dependent variations in the response of the system to various stimuli.

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INTRODUCTION

Evidence to date suggests that all the energy required for the movement of sodium, potassium and chloride ions via the Na^+–K^+–Cl^- co-transport system is obtained from the combined chemical gradients of these ions (Geck, Pietrzyk, Burckhardt, Pfeiffer & Heinz, 1980; Haas, Schmidt & McManus, 1982; Duhm & Göbel, 1984; Saier & Boyden, 1984). For instance, the active movement of potassium up its gradient can be driven by the movement of sodium or chloride down their gradients. There is no evidence that additional energy input is needed, for example from the hydrolysis of ATP (Geck *et al.* 1980). On the other hand Na^+–K^+–Cl^- co-transport is inhibited when cells are starved for prolonged periods (Hall & Ellory, 1985) or treated with metabolic inhibitors (Rindler, McRoberts & Saier, 1982; Palfrey, 1983; Adragna, Perkins & Lauf, 1985; Dagher, Brugnara & Canessa, 1985; Mercer & Hoffman, 1985; Ueberschär & Bakker-Grunwald, 1985; Kim, Tsai, Franklin & Turner, 1988). Both procedures drastically reduce cell ATP content ($[ATP]_i$), suggesting some role for ATP in controlling transport activity (see Chipperfield, 1986; Haas, 1989). One possibility is that phosphorylation of the transporter itself or an auxiliary controls activity of the system (Palfrey & Rao, 1983; Saier & Boyden, 1984). Support for this view comes from the observation that phosphorylation of a 230 kDa intrinsic membrane protein and stimulation of Na^+–K^+–Cl^- co-transport occur with the same time course when turkey red cells are treated with isoprenaline or cyclic AMP (Alper, Beam & Greengard, 1980).

However, metabolic manipulation produces a wide range of cellular changes and it is difficult to isolate the true effector (if there is just one). The most compelling evidence for direct involvement of ATP is that co-transport activity in dialysed squid axons depends on the concentration of ATP in the dialysis medium (Russell, 1980; Altamirano, Breitwieser & Russell, 1988; Russell, 1990). The requirement for ATP appears specific, as hydrolysable ATP analogues such as α, β-methylene ATP, β, γ-methylene ATP and ATP-γ-S are incapable of supporting Na^+–K^+–Cl^- co-transport (Russell, 1980; DiPolo & Beaugé, 1987). Treatment of squid axons with fluoride or vanadate has little effect on activity when $[ATP]_i$ is normal. However, the presence of these ions markedly slows co-transport inactivation when axons are dialysed with ATP-free media. Taken together these data have been interpreted as indicating that activation of co-transport by phosphorylation is regulated by separate kinases and phosphatases and that the co-transporter is not an ATPase (Altamirano *et al.* 1988; Russell, 1990). The finding that Na^+–K^+–Cl^- co-transport can operate at about half-maximal activity for over an hour when the concentration of ionized intracellular magnesium ($[Mg^{2+}]_i$) is reduced to about 10^{-7} M in ferret red cells also argues against the co-transporter being an ATPase (Flatman, 1988).

The work described below explores how metabolic manipulations affect Na^+–K^+–Cl^- co-transport in ferret red cells. These cells have a very high co-transport activity and a high sodium and low potassium content (Flatman, 1983) which makes them particularly suitable for the purpose. Small changes in co-transport activity can easily be detected, and the absence of the Na^+ pump means that depletion of ATP does not produce large changes in the cellular concentrations of sodium and potassium which could complicate analysis of results. Comparisons are often made

between the properties of the Na^+–K^+–Cl^- and the possibly related K^+–Cl^- co-transport system (see Haas, 1989). Recent work on K^+–Cl^- co-transport indicates that the redox state of certain sulphydryl groups on this transporter may be influenced by the metabolic state of the cell and may in turn affect activity (Lauf, 1985; Dunham, 1990). It is also known that some procedures which are used to deplete cells of ATP may modify sulphydryl groups. Therefore, the effects of some sulphydryl reagents are also examined.

METHODS

Red cells were separated from blood obtained by cardiac puncture of adult ferrets anaesthetized with urethane, 1·5 g (kg body weight)$^{-1}$ as described by Flatman (1988). EDTA was used as anticoagulant. Cells were stored at 5 °C, packed at about 80% haematocrit in FBM (ferret basic medium: 145 mM-NaCl, 5 mM-KCl, 10 mM-Na-HEPES, pH 7·5 at 38 °C) until used. All media used during manipulations of metabolism or $[Mg^{2+}]_i$ and during subsequent washes and flux measurements contained 50 μM-EGTA to chelate contaminant calcium. Haematocrit, haemolysis and ^{86}Rb uptake were measured by the method of Flatman (1983). Cell ATP was measured by the firefly method (Flatman, 1988).

$[Mg^{2+}]_i$ was controlled with A23187 using the method of Flatman & Lew (1980). In the presence of 10 μM-A23187 $[Mg^{2+}]_i$ comes into equilibrium with external ionized magnesium ($[Mg^{2+}]_o$) and its value is given by:

$$[Mg^{2+}]_i = r^2[Mg^{2+}]_o,$$

where r is the chloride distribution ratio ($= [Cl]_o/[Cl]_i$, $[Cl]_o$ and $[Cl]_i$ are the external and internal chloride concentrations respectively). $[Mg^{2+}]_o$ was either measured directly or determined from the change in cell magnesium content ($[Mg]_i$). $[Mg]_i$ was measured by atomic absorption spectroscopy and the chloride distribution ratio was determined by adding ^{36}Cl to an aliquot of the suspension (Flatman & Lew, 1980).

Na^+–K^+–Cl^- co-transport activity was measured as the component of ^{86}Rb influx which was inhibited by 0·1 mM-bumetanide. ^{86}Rb was used because it is an excellent tracer for potassium on the co-transporter and because it has a more convenient half-life than ^{42}K (see Flatman, 1987). Uptake was measured over a 5 (total) or 15 min (bumetanide-resistant) period following the addition of tracer to the suspension. The rate constant (k) is given by the expression:

$$kt = -\ln(1-A_t/A_e),$$

where t is the time when the sample was taken and A_t and A_e are the activities measured in the cells at time t and at equilibrium. The value for k was obtained from the slope of the line relating $\ln(1-A_t/A_e)$ to t. The slopes (±standard error of the mean) were usually obtained by linear regression analysis of uptake measured at 5 time points. Unless otherwise stated these values are quoted in text and figures. The bumetanide-sensitive components were obtained by subtraction.

Cell ATP content was reduced by pre-incubating the cells at 38 °C at about 10–15% haematocrit in FBM containing 0·05 mM-Tris-EGTA with or without the addition of 11 mM-2-deoxyglucose or 6 mM-iodoacetamide and 11 mM-glucose. The suspensions were stirred intermittently. After the pre-incubations cells were washed twice in FBM before being used for flux determination. In some experiments the concentration of HEPES in the depletion medium was increased to 20 mM to minimize pH changes.

Concentrations of cell components, and the values of water contents and r are given as the mean (±S.E.M.) of three determinations unless otherwise stated.

Solutions were prepared in double-glass-distilled water with Analar grade reagents where possible (BDH Ltd). EGTA EDTA, HEPES, iodoacetamide, dithiothreitol, *n*-ethylmaleimide, sodium orthovanadate (Na_3VO_4) and 2-deoxyglucose were obtained from Sigma Chemical Co. Ltd. Bumetanide was a gift from Leo Laboratories Ltd, the purified firefly enzyme was obtained from LKB Ltd and A23187 from Calbiochem. ^{86}Rb and ^{36}Cl were obtained from Amersham International.

RESULTS

After an initial small rise, the ATP content of ferret red cells falls steadily when cells are incubated in a substrate-free medium (Fig. 1). The initial rise in $[ATP]_i$ is reproducible and is largest for cells in which $[ATP]_i$ has fallen most during storage at 5 °C. Glucose sustains metabolism and maintains $[ATP]_i$ in ferret red cells (Fig. 1) whereas adenosine (10 mM) or inosine (10 mM), which are effective in human red cells (Whittam & Wiley, 1968), do not. Thus incubation of cells in media containing inosine or adenosine causes $[ATP]_i$ to fall slowly as in the substrate-free condition (data not shown). Incubation of ferret red cells in a medium containing 11 mM-2-deoxyglucose causes a rapid fall of $[ATP]_i$ (Fig. 1) which reaches a steady level of about 10–30 μmol (l cell)$^{-1}$ after 2 h. Prolonging the incubation does not result in any further detectable fall in $[ATP]_i$. 2-Deoxyglucose enters cells and is phosphorylated by hexokinase in a reaction that consumes ATP and produces 2-deoxyglucose 6-phosphate. This product cannot be metabolized further and therefore accumulates in the cell but does not inhibit hexokinase (Sols & Crane, 1954). The finding that $[ATP]_i$ does not fall below 10 μmol (l cell)$^{-1}$ may indicate that ATP can still be produced perhaps from the metabolism of 2,3-bisphosphoglycerate. Submicromolar $[ATP]_i$ has been achieved in human red cells by incubating them with iodoacetamide and inosine or glucose (Lew, 1971). Incubation of ferret red cells with 6 mM-iodoacetamide and 11 mM-glucose also causes a rapid fall of $[ATP]_i$, though not to submicromolar levels (Fig. 1). Iodoacetamide prevents glycolytic production of ATP by inhibiting glyceraldehyde 3-phosphate dehydrogenase while glucose stimulates the consumption of ATP by the early steps in glycolysis (see Lew, 1971). Omission of glucose leads to a much slower fall in $[ATP]_i$ (data not shown). On several occasions incubation of cells in the presence of iodoacetamide (with or without glucose) caused complete lysis of the cells. Extrapolation of the time course of ATP depletion under these conditions suggests that the cells lysed when they still contained a substantial amount of ATP (> 200 μmol (l cell)$^{-1}$). As cells can survive for several hours with one-tenth this ATP content it seems that the lytic effect of iodoacetamide is not due simply to its effects on $[ATP]_i$.

Figures 2 and 3 show correlations between $[ATP]_i$ and Na^+–K^+–Cl^- co-transport activity. It can be seen that in most cases reduction of $[ATP]_i$ below about 0·1 mmol (l cell)$^{-1}$ inhibits co-transport activity. The data from cells pre-treated with 2-deoxyglucose can be described by Michaelis–Menten kinetics with the K_m for ATP ranging from 18 to 49 μmol (l cell)$^{-1}$ in different experiments. However, examination of the data from each experiment shows that the time for which cells are depleted has a clear effect on co-transport activity. Thus in the experiment shown in Fig. 4 $[ATP]_i$ only fell from 13 to 10 μmol (l cell)$^{-1}$ over a 4 h period while co-transport activity fell from 1·7 to 0·7 h^{-1}. In several experiments (e.g. Figs 2 and 3) there is evidence that activity is slightly inhibited at high $[ATP]_i$. This is supported by the observation that co-transport activity is reduced slightly when $[ATP]_i$ is increased above the initial level by prolonged feeding of the cells with glucose.

Reduction of $[ATP]_i$ content by starvation produces a minor initial stimulation of co-transport activity followed by an inhibition (Fig. 2). However, co-transport activity at each $[ATP]_i$ below 200 μmol (l cell)$^{-1}$ is significantly lower than when

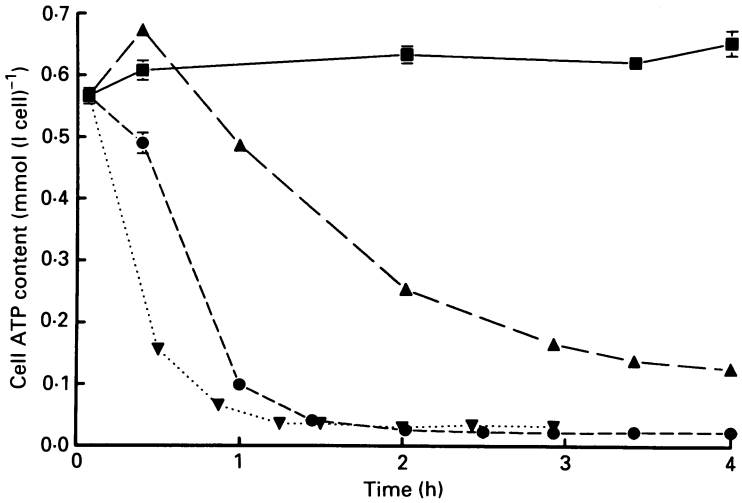

Fig. 1. ATP content of ferret red cells. Cells were incubated at about 13% haematocrit in FBM containing 0·05 mM-EGTA and the following additions: 11 mM-glucose, ■; none, ▲; 11 mM-2-deoxyglucose, ●; and 6 mM-iodoacetamide with 11 mM-glucose, ▼. Symbols show the means of three determinations with standard errors if these are larger than symbol size.

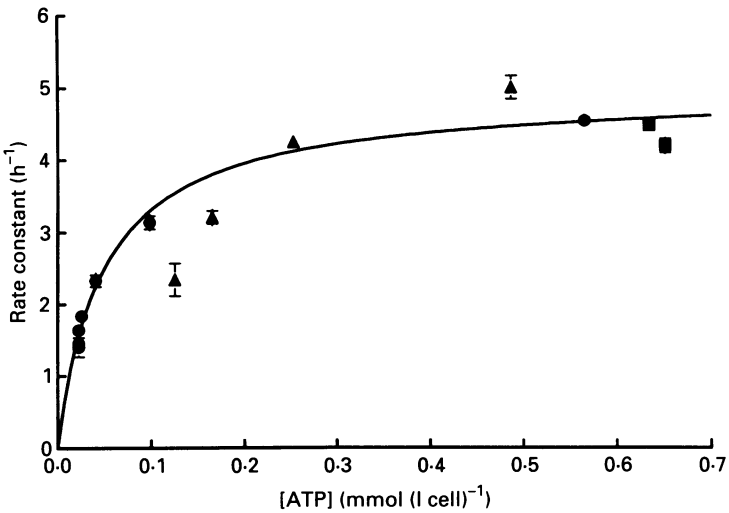

Fig. 2. Comparison of the effects of glucose, 2-deoxyglucose and starvation on the relationship between $[ATP]_i$ and co-transport activity. Cells were pre-incubated in FBM containing 0·05 mM-EGTA and the following additions: 11 mM-glucose, ■; 11 mM-2-deoxyglucose, ●; none, ▲. Samples of the cells were taken to measure bumetanide-sensitive ^{86}Rb uptake rate constant and $[ATP]_i$. The line is drawn through the data for cells incubated in 2-deoxyglucose. It assumes Michaelis–Menten kinetics and is fitted using non-linear regression analysis (Marquardt's method). The maximum rate is $4{\cdot}91 \pm 0{\cdot}20$ h^{-1} and the K_m is 49 ± 8 μmol (l cell)$^{-1}$.

$[ATP]_i$ is reduced by incubation with 2-deoxyglucose. As it takes much longer to achieve low $[ATP]_i$ by starvation than by treatment with deoxyglucose this supports the idea that the time spent with low $[ATP]_i$ is an important determinant of activity. Unfortunately it was not possible to examine the effects of starving cells to very low $[ATP]_i$ because of the very protracted incubations necessary.

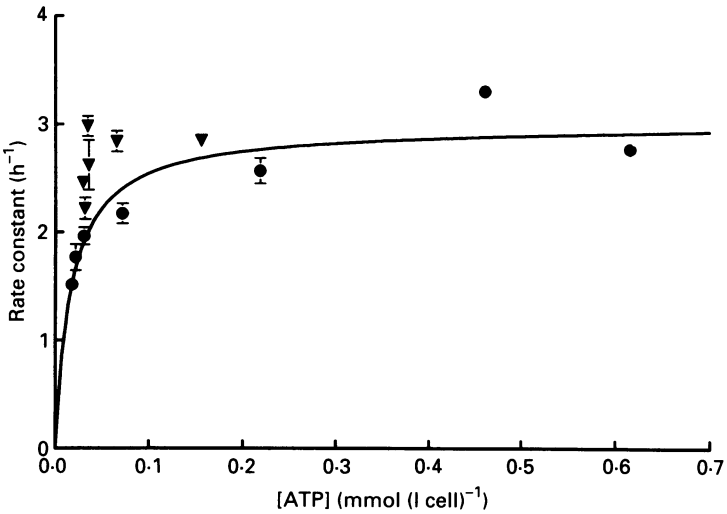

Fig. 3. Comparison of the effects of 2-deoxyglucose and iodoacetamide on the relationship between $[ATP]_i$ and co-transport activity. Cells were pre-incubated in FBM containing 0·05 mM-EGTA and 11 mM-2-deoxyglucose (●) or 6 mM-iodoacetamide and 11 mM-glucose (▼). Samples were taken to measure bumetanide-sensitive ^{86}Rb uptake rate constant and $[ATP]_i$. The line is drawn through the data for cells incubated in 2-deoxyglucose. It assumes Michaelis–Menten kinetics and is fitted by non-linear regression analysis. The maximum rate is $2{\cdot}99 \pm 0{\cdot}16$ h^{-1} and the K_m is 18 ± 4 μmol (l cell)$^{-1}$. Co-transport activity in control, fed cells from this batch was $2{\cdot}86 \pm 0{\cdot}03$ h^{-1}.

Reduction of $[ATP]_i$ by treating cells with iodoacetamide and glucose initially spares co-transport activity compared to cells treated with 2-deoxyglucose (Fig. 3). Activity does not fall significantly until cells, with $[ATP]_i$ of only about 30 μmol (l cell)$^{-1}$, have been incubated for more than 2 h. After this time activity begins to fall and the cells become very fragile. Iodoacetamide appears to react with a cell constituent to slow the inhibition caused by reduced $[ATP]_i$. Iodoacetamide inhibits glycolysis by interacting with a critical sulphydryl group on glyceraldehyde 3-phosphate dehydrogenase. It also interacts with sulphydryl groups on other proteins. The observations here perhaps suggest that a sulphydryl group, which can interact with iodoacetamide, is involved in the metabolic control of Na^+–K^+–Cl^- co-transport. In view of this possibility the effects of another sulphydryl reagent were investigated. Cells were incubated for 20 min in 1 mM-*n*-ethylmaleimide (NEM), a reagent which alkylates sulphydryl groups. In this case co-transport was inhibited by about 83% (from $5{\cdot}4 \pm 0{\cdot}1$ to $0{\cdot}9 \pm 0{\cdot}1$ h^{-1}) while the bumetanide-resistant flux almost trebled (from $0{\cdot}15 \pm 0{\cdot}02$ to $0{\cdot}41 \pm 0{\cdot}02$ h^{-1}). These results suggest that iodoacetamide and NEM may interact with different sulphydryl groups. A similar

explanation has been made to account for the effects of these agents on the K^+–Cl^- co-transporter (Lauf, 1987).

A fall in $[ATP]_i$ may explain the reduction in co-transport activity (measured at 38 °C) seen in ferret red cells which have been stored for long periods in FBM

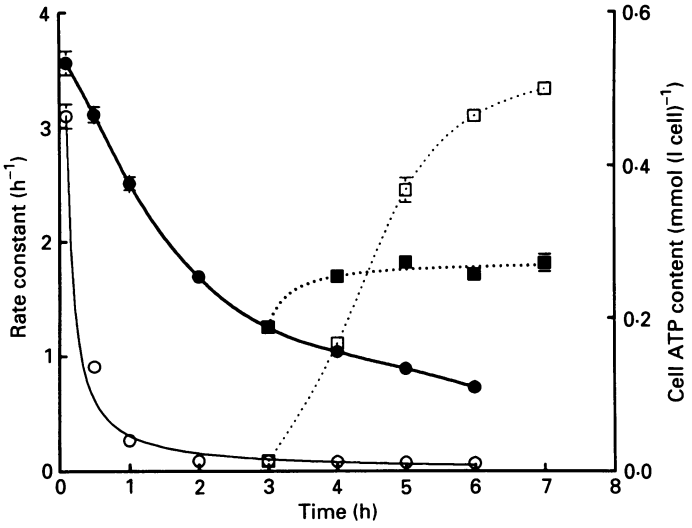

Fig. 4. Reversibility of the effects of ATP depletion on co-transport activity. Cells were incubated in FBM containing 20 mM-Na-HEPES, 0·05 mM-EGTA and 11 mM-2-deoxyglucose. After 3 h an aliquot of these cells was washed twice to remove 2-deoxyglucose and then resuspended in FBM containing 1 mM-sodium phosphate (pH 7·5) and 11 mM-glucose. At the times indicated, samples were taken from these suspensions to measure the bumetanide-sensitive ^{86}Rb uptake rate constant (left axis) and $[ATP]_i$ (right axis). ○, $[ATP]_i$ in 11 mM-2-deoxyglucose; □, $[ATP]_i$ in depleted cells reincubated in 11 mM-glucose; ●, rate constant in 2-deoxyglucose; ■, rate constant of depleted cells incubated in glucose. Lines through data are drawn by eye. Non-linear regression analysis of co-transport activity as a function of $[ATP]_i$ in 2-deoxyglucose gives a maximum rate of $3·79 \pm 0·25$ h^{-1} and a K_m of 26 ± 5 μmol (l cell)$^{-1}$.

(+0·05 mM-EGTA) at 5 °C. For instance co-transport activity only fell from 3·7 (±0·1) to 3·6 (±0·1) h^{-1} during storage of cells for 11 days while $[ATP]_i$ fell from 0·6 to 0·19 mmol (l cell)$^{-1}$. Storage for a further 5 days resulted in a fall of $[ATP]_i$ to 0·09 mmol (l cell)$^{-1}$ and a fall in co-transport activity to 2·5 (±0·1) h^{-1}. The possibility that some of this inhibition may have been due to the oxidation of critical sulphydryl groups was investigated by incubating cells, previously stored for 16 days, for 20 min in FBM containing 1 mM-dithiothreitol (DTT). Activity in the presence of this sulphydryl reducing agent was 102 ± 5 % of the activity seen in stored cells incubated in the absence of DTT. This indicates that DTT does not affect Na^+–K^+–Cl^- co-transport in ferret red cells and is consistent with observations made in human red cells (see discussion in Bergh, Kelley & Dunham, 1990 of results of Wiater & Dunham, 1983).

The ATP content of cells which has been reduced to a low level by incubation with

2-deoxyglucose can be returned to the physiological level by washing away the 2-deoxyglucose and then incubating the cells in media containing 11 mM-glucose and 1 mM-phosphate (Fig. 4). Omission of phosphate substantially slows the rate of recovery (data not shown). Repletion of $[ATP]_i$ produces some recovery of co-

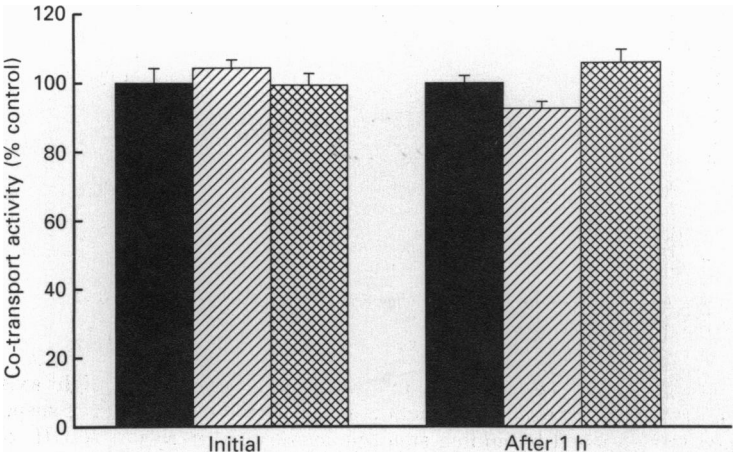

Fig. 5. Effect of phosphate on co-transport activity. Cells were incubated in FBM containing 0·05 mM-EGTA and either 0 (filled bars), 1 (hatched bars) or 4 mM (cross-hatched bars) sodium phosphate. Bumetanide-sensitive ^{86}Rb uptake was measured immediately after addition of cells to medium and 1 h later. Activity is expressed as a percentage of the activity in control incubations taken at the same time.

transport activity (Fig. 4) but even prolonged incubation in the presence of glucose and phosphate does not return it to the control value. The possibility that this reduction in activity is due to the presence of phosphate is examined in the experiment shown in Fig. 5. Activity is not affected by incubating cells in media containing 1 or 4 mM-phosphate for 5 min or 1 h. Thus neither external phosphate nor phosphate which has entered the cells over a 1 h period appears to affect co-transport activity.

Alteration of red cell metabolism might affect Na^+–K^+–Cl^- co-transport activity indirectly through changes in cell volume, cell sodium or potassium content, or pH. It was therefore important to monitor these variables during the experiments. $[ATP]_i$ was depleted to below 30 μmol (l cell)$^{-1}$ by incubating cells for 3 h with 2-deoxyglucose. This had no detectable effect on cell water content which remained constant at 0·65 ($\pm$0·002) g water (g wet weight)$^{-1}$ and had little effect on cell sodium and potassium contents which changed from initial values of 102·5 ($\pm$0·6) and 6·30 ($\pm$0·03) to final values of 101·9 ($\pm$0·5) and 6·42 ($\pm$0·05) mmol (l cell)$^{-1}$ respectively. It is thus unlikely that any of the effects reported are due to changes in cell volume or sodium and potassium content. pH is an important determinant of co-transport activity in ferret red cells. Figure 6 shows that activity rises steeply with pH across the physiological range whereas the activity of the bumetanide-resistant pathways remains constant. In view of this sensitivity particular care was taken during experiments to ensure that suspension pH was initially at the control value

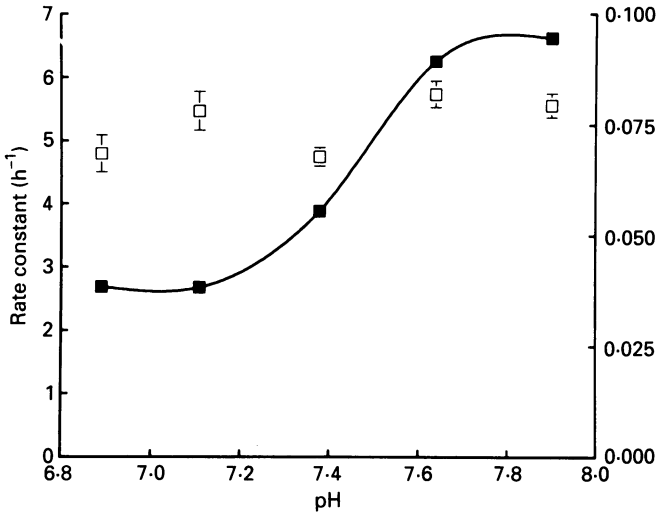

Fig. 6. Bumetanide-sensitive (■, left axis) and bumetanide-resistant (□, right axis) ^{86}Rb uptake rates constants are plotted as a function of suspension pH. The pH of suspensions of cells in FBM was adjusted by adding small quantities of 1 M-HCl or NaOH. pH was monitored at the start and end of incubations. The line is drawn by eye.

TABLE 1. Effect of fluoride on Na^+–K^+–Cl^- co-transport in ferret red cells

	Bumetanide-sensitive ^{86}Rb uptake rate constant (h^{-1})	
Condition	Fed	Depleted
Control	3·42±0·10	1·48±0·09
1 mM-NaF	3·62±0·09	1·32±0·09
1 mM-NaF+0·1 mM-$AlCl_3$	3·45±0·10	1·30±0·08

Fluxes in fed cells were measured in media containing 11 mM-glucose. Depleted cells had been incubated in 11 mM-2-deoxyglucose for 4 h and then washed in FBM before measuring fluxes in FBM. Depleted cells contained 10·3 (±0·1, $n = 6$) μmol (l cell)$^{-1}$ ATP. Cells were added to the flux media containing the additions shown about 6 min before the isotope.

of about 7·5 and did not change significantly throughout the experiment. However, even at constant external pH, internal pH can change if the metabolic manipulations affected membrane potential. Experiments showed that depletion of cell ATP by incubation with 2-deoxyglucose for 3 h had only a very minor effect on membrane potential (r was 1·39±0·01 for fed and 1·34±0·02 for depleted cells) and thus inadvertent changes in cell pH (or cell [Cl]) were not the cause of changes in co-transport activity.

Effects of fluoride were studied in order to investigate the possible involvement of phosphoprotein phosphatases and cyclic AMP in the regulation of Na^+–K^+–Cl^- co-transport in ferret red cells. Table 1 shows that Na^+–K^+–Cl^- co-transport is not affected by incubating control or ATP-depleted cells in media containing 1 mM-NaF

(with or without 0·1 mM-$AlCl_3$). Similarly, the presence of 2 mM-NaF does not affect the time course of ATP depletion or the relationship between $[ATP]_i$ and co-transport when cells are incubated with 2-deoxyglucose (data not shown).

Vanadate is a potent inhibitor of some phosphoprotein phosphatases as well as of transport ATPases (see Post, 1986). Effects of vanadate on co-transport are explored

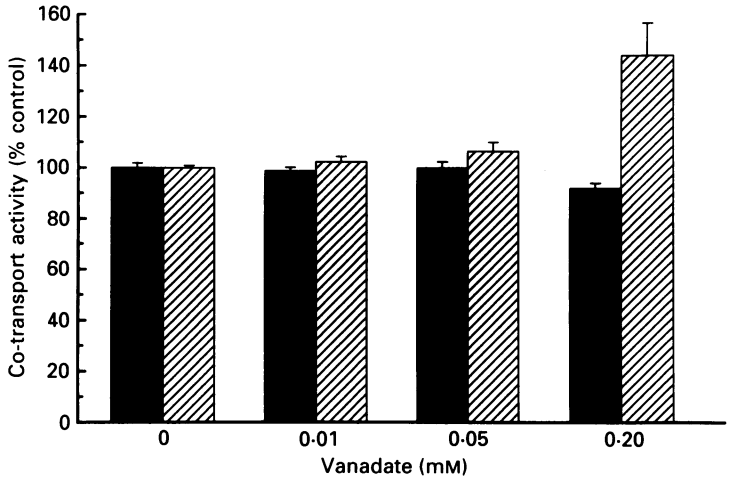

Fig. 7. The effect of vanadate on Na^+–K^+–Cl^- co-transport in ferret red cells. Bumetanide-sensitive ^{86}Rb uptake was measured in fed (filled bars) and depleted (hatched bars) cells which initially contained 589 (±6) or 19 (±0·1) μmol (l cell)$^{-1}$ ATP respectively. Cells were added to the medium containing the vanadate concentrations indicated about 10 min before the fluxes were measured. The bars show the means with S.E.M. of three determinations of rate expressed as a percentage of the rate observed in the absence of vanadate. The control fluxes (mean ± S.E.M., $n = 3$) were 2·26 (±0·04) and 0·90 (±0·01) h^{-1} in the fed and depleted cells respectively. Vanadate increased $[ATP]_i$ measured 10 min after addition of cells to medium. $[ATP]_i$ (μmol (l cell)$^{-1}$) increased to 664 (±10) and 883 (±19) in fed cells and 24·4 (±0·3) and 64·7 (±1·2) in depleted cells incubated in the presence of 50 or 200 μM-vanadate respectively.

in Fig. 7. Pre-incubation of cells for about 10 min with 10 or 50 μM-vanadate has no effect on co-transport activity when the cells have normal $[ATP]_i$. A slight inhibition is seen when the vanadate concentration is increased to 200 μM but this could be due to the marked increase in $[ATP]_i$ (to 883 μmol (l cell)$^{-1}$) which occurs under these conditions. Vanadate stimulates activity in ATP-depleted cells. This effect can be observed within 10 min of adding vanadate to the cell suspension and is substantial (44%) when 200 μM-vanadate is used. Vanadate also increases $[ATP]_i$ in these ATP-depleted cells. The inclusion of 50 μM-vanadate in the depletion medium containing 2-deoxyglucose very slightly slows the rate of ATP depletion and raises the steady-state $[ATP]_i$ achieved after 2 h incubation from 17 to 34 μmol (l cell)$^{-1}$. Co-transport activity is stimulated about 8–10% in these cells compared to vanadate-free controls over a range of $[ATP]_i$ from 35 to 125 μmol (l cell)$^{-1}$.

$[Mg^{2+}]_i$ is a powerful determinant of co-transport activity in ferret red cells with normal $[ATP]_i$ (Flatman, 1988). Figure 8 explores the effects of changing $[Mg^{2+}]_i$ in

cells with very low $[ATP]_i$. $[Mg^{2+}]_i$ was altered with A23187 after $[ATP]_i$ had been reduced to 38 μmol (l cell)$^{-1}$ by incubating cells for 3 h with 2-deoxyglucose. Changing $[Mg^{2+}]_o$ alone (no A23187) has no effect on co-transport activity in these depleted cells. After the addition of 10 μM-A23187, which brings $[Mg^{2+}]_i$ into

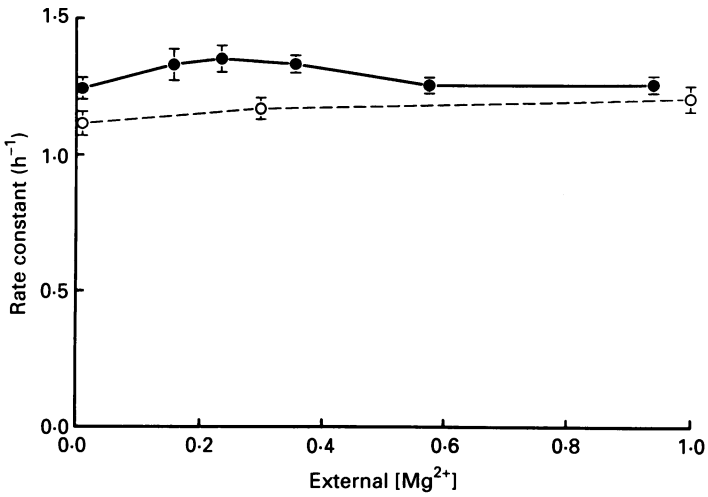

Fig. 8. The effect of changing internal or external $[Mg^{2+}]$ on bumetanide-sensitive ^{86}Rb uptake rate constant in ferret red cells. Red cell ATP content was reduced to 38 (±0·6, $n = 18$) μmol (l cell)$^{-1}$ by incubating cells in 2-deoxyglucose for 3 h. Cells were then washed twice and incubated in fresh FBM for the determination of fluxes. $[Mg^{2+}]_i$ was controlled by adding 10 μM-A23187 to the final suspensions about 10 min before addition of ^{86}Rb. $[Mg^{2+}]_i$ is 1·8 $[Mg^{2+}]_o$ in the presence of A23187. $[Mg^{2+}]_o$ is calculated from the change in cell magnesium content measured 5–8 min after ionophore addition (see Flatman & Lew, 1980). ●, Bumetanide-sensitive rate constant in the presence of 10 μM-A23187; ○, rate constant in the absence of A23187. Symbols show the means (±S.E.M.) of three measurements.

equilibrium with $[Mg^{2+}]_o$, a small stimulation is seen at all $[Mg^{2+}]$ but there is no significant change in activity as $[Mg^{2+}]_o$ is changed from 10^{-7} to 9×10^{-4} M. This corresponds to a change in $[Mg^{2+}]_i$ from about 2×10^{-7} to $1{\cdot}6 \times 10^{-3}$ M and indicates that $[Mg^{2+}]_i$ does not activate co-transport in ATP-depleted cells. This is in contrast to the large (approximately 3-fold) stimulation seen in cells with normal $[ATP]_i$ over a similar range of $[Mg^{2+}]_i$ (Flatman, 1988, Fig. 5).

It is unlikely that any of the changes in Na^+–K^+–Cl^- co-transport activity reported in this paper are due to an increase in intracellular ionized $[Ca^{2+}]$ concentration ($[Ca^{2+}]_i$). First, all solutions to which cells were exposed contained 50 μM-EGTA to chelate contaminant calcium. This would prevent the accumulation of calcium by cells depleted of ATP or treated with A23187, which can occur as a result of either Ca^{2+} pump inhibition or increased membrane calcium permeability. Second, $[Ca^{2+}]_i$ has to increase substantially before changes in co-transport activity are observed (Flatman, 1987). At these levels, calcium also activates bumetanide-resistant potassium transport systems. No such activation was observed (see below)

indicating that any increase in $[Ca^{2+}]_i$ due either to changes in calcium buffering or transport is insufficient to affect co-transport.

During the course of the experiments described above to measure co-transport activity it was necessary to measure the bumetanide-resistant ^{86}Rb fluxes. These fluxes are small (typically 2–3% total flux) and take about 20 min to measure accurately. Therefore it was not possible to obtain as much information about the time course of the effects of metabolism on bumetanide-resistant pathways. Reduction of $[ATP]_i$ to below 30 μmol (l cell)$^{-1}$ by treating cells with 11 mM-2-deoxyglucose or 6 mM-iodoacetamide inhibited bumetanide-resistant fluxes from control values of about 0·1–0·12 h^{-1} to about 0·05–0·07 h^{-1}. On the other hand starvation of cells for more than 3 h which reduced $[ATP]_i$ to 140 μmol (l cell)$^{-1}$ had no effect on these fluxes. Vanadate (0·2 mM) did not affect bumetanide-resistant fluxes in cells with normal $[ATP]_i$ but reduced them from 0·080 ($\pm$0·004) to 0·056 ($\pm$0·001) h^{-1} in cells with $[ATP]_i$ of 20–65 μmol (l cell)$^{-1}$. Taken together these data are consistent with the idea that there is a low level of K^+–Cl^- co-transport in ferret red cells which can be activated by NEM and which is inhibited by reducing $[ATP]_i$ (see Wiater & Dunham, 1983; Lauf, 1985, 1987).

DISCUSSION

The consensus of recent opinion is that the Na^+–K^+–Cl^- co-transport system does not need energy from the hydrolysis of ATP to drive ion transport and is probably not an ATPase. However, this important transport system, which plays prominent roles in volume control and secretion, is, perhaps not surprisingly, sensitive to the cell's metabolic state. The major problem is to identify the nature of the link between metabolism and co-transport activity.

Taken in isolation, the data presented here from ferret red cells suggest that co-transport activity is controlled by the concentration of a material, not necessarily ATP, which reflects metabolic state, However, because the properties of the co-transport system are very similar in a wide range of cells (Haas, 1989) these data taken with those from other cell types (particularly squid axon; Russell, 1990) suggest ATP is probably an important link between metabolism and co-transport activity in ferret red cells. Changes in $[Mg^{2+}]_i$ and [NADPH] may be other links. Unequivocal proof for the involvement of ATP in controlling co-transport activity in ferret red cells is difficult to obtain as it has not yet proved possible to make resealed ghosts with co-transport activity from these cells. However, the strong correlations between $[ATP]_i$ and activity (Figs 2 and 3) suggest involvement of ATP. Both the time course of ATP changes and the method used to cause change have profound effects on activity and there appears to be a substantial time lag between changes in $[ATP]_i$ and activity. It was not possible to find a unique relationship between $[ATP]_i$ and activity which held under all conditions in ferret red cells. This indicates that ATP is not simply a co-factor. Kinetic analysis of the data of Figs 2, 3 and 4 shows that models which assume the existence of an intermediate, the concentration of which depends on $[ATP]_i$ and which slowly declines when $[ATP]_i$ is reduced to low levels, describe the data better than simple Michaelis–Menten kinetics. Detailed analysis of these models is not yet justified given the amount of

data available. Analysis would be substantially improved if it were possible to clamp $[ATP]_i$ at chosen levels for longer periods of time.

Data reported in this paper are consistent with the idea (Altamirano & Russell, 1988) that Na^+–K^+–Cl^- co-transport activity is controlled by a cycle of phosphorylation and dephosphorylation of some key component of the system, where ATP influences activity by donating phosphate. In this model phosphorylation is necessary for activity but there need not be a stoichiometric relationship between ATP consumed and ion transport. Activity of the transporter may be controlled through specific kinases and phosphatases just as has been suggested for the K^+–Cl^- co-transporter (Jennings & Al-Rohil, 1990). Differences in the mechanisms controlling phosphorylation and dephosphorylation may explain tissue-dependent variations in the response of the transporter to a variety of stimuli. In the case of ferret red cells the transporter is almost fully activated when cells are isolated from the circulation suggesting that the system is almost maximally phosphorylated. Reduction of $[ATP]_i$ to very low levels produces a slow fall in activity perhaps revealing a slow spontaneous rate of dephosphorylation. Activity in these depleted cells is increased when $[ATP]_i$ is returned to normal. However, activity does not return completely to the control value suggesting that the presence of ATP alone may not be sufficient to activate transport fully and that other factors, which may not return to control levels quickly on feeding with glucose, may be required. For instance the cell's ability to provide NADPH to keep critical sulphydryl groups in a reduced state may be impaired.

With this model in mind it is possible to re-examine in more detail some of the results presented here.

$[Mg^{2+}]_i$ is an important regulator of co-transport activity (Flatman, 1988). It is not clear, however, whether $[Mg^{2+}]_i$ acts as a co-factor, perhaps binding directly to the transporter to regulate activity, or whether it is necessary in some other capacity. The finding that $[Mg^{2+}]_i$ no longer stimulates activity when $[ATP]_i$ is low argues against the idea that magnesium affects activity simply by binding to a regulatory site on the transporter. It seems more likely that $[Mg^{2+}]_i$ is required for phosphorylation of the system just as it is required for other reactions involving transfer of phosphate groups. Activity remains substantial when $[Mg^{2+}]_i$ is reduced to about 10^{-7} M both at normal and low $[ATP]_i$. This argues against the idea that the transporter is an ATPase and also suggests that removal of $[Mg^{2+}]_i$ may slow the rate of dephosphorylation perhaps trapping the system in a partially activated state.

The actions of fluoride were examined for a number of reasons. Its presence slows the rate of co-transport inactivation seen when squid axons are dialysed with ATP-free media (Altamirano & Russell, 1988; Russell, 1990). It was suggested that inhibition of a phosphoprotein phosphatase by fluoride might therefore maintain the co-transport system in a phosphorylated, active state. Fluoride also activates adenylate cyclase to increase cell cyclic AMP which may affect co-transport through its effects on kinases (see Haas, 1989). Fluoride, however, had no detectable effect on co-transport in ferret red cells at both low and high [ATP] levels nor did its presence during procedures to deplete ATP influence activity measured subsequently. Fluoride's ineffectiveness might have been due to the routine presence of EGTA in the experiments. Some effects of fluoride can be explained by the formation of

complexes with aluminium which may mimic phosphate (see MacDonald & Martin, 1988). The presence of EGTA, which chelates contaminant aluminium normally present in the medium, may have prevented the formation of these complexes. However, experiments in which excess (0·1 mM) aluminium was added along with the fluoride also failed to show any significant effect on activity.

The effects of vanadate are interesting for a number of reasons. Not only does vanadate inhibit transport ATPases but it also inhibits some phosphatases (see Swarup, Cohen & Garbers, 1982; Post, 1986). The results reported here show that vanadate has little effect on co-transport activity in cells with normal $[ATP]_i$ but that it stimulates activity in cells with low $[ATP]_i$. These findings support the idea that the transporter is not an ATPase. They also suggest that vanadate either inhibits a phosphatase thus increasing the level of phosphorylation or that binding of vanadate, perhaps to a site which normally binds phosphate, activates the system. Both effects may be more noticeable at low ATP levels. With normal ATP the model presumes that the rate of phosphorylation via the kinase is much faster than dephosphorylation via the phosphatase and so most of the system is in the phosphorylated active state. Minor changes to the activity of the phosphatase may have little impact on the level of phosphorylation. On the other hand, in cells with low $[ATP]_i$ where the rate of phosphorylation via the kinase is greatly slowed, the level of phosphorylation may become much more sensitive to the activity of the phosphatase. If this is so the phosphatase in ferret red cell differs from that in squid axon in not being affected by fluoride.

In many respects the metabolic control of Na^+–K^+–Cl^- co-transport appears to be the opposite of that found for K^+–Cl^- co-transport. Thus the active form of the Na^+–K^+–Cl^- co-transport system appears to be phosphorylated whereas that of the K^+–Cl^- co-transport system appears to be dephosphorylated (Jennings & Al-Rohil, 1990). Metabolic manoeuvres which activate one often inhibit the other. A major exception is that ATP depletion inhibits K^+–Cl^- as well as Na^+–K^+–Cl^- co-transport. K^+–Cl^- co-transport's apparently paradoxical requirement for ATP has been explained in terms of the ATP requirement shown by many phosphatases and by the belief that high phosphatase activity is necessary for K^+–Cl^- co-transporter activity (Jennings & Al-Rohil, 1990). There has been much discussion about the possibility that Na^+–K^+–Cl^- and K^+–Cl^- co-transport may be different aspects of a common transporter revealed under different conditions but recent evidence suggests that the ion movements themselves may be via independent routes (Haas, 1989). It may be possible to combine these ideas in a model where the transporters themselves are separate molecular entities which share some regulatory machinery. Thus phosphorylation of a regulatory molecule (which may shuttle between the two systems) may activate Na^+–K^+–Cl^- co-transport while inhibiting K^+–Cl^- co-transport. The opposite occurs when the molecule is dephosphorylated. This kind of complementary control would help explain how stimuli which activate one system (perhaps to cause cell swelling) must inactivate the other (which would cause cell shrinkage if active). The model may also explain the often complementary effects of sulphydryl reagents on the two systems. In addition to possible direct effects on the transporters themselves these agents may affect the common regulatory machinery tending to activate one system while inhibiting the other. These effects may be more apparent

at either high or low $[ATP]_i$ depending on the balance of kinase and phosphatase activities in the particular tissue. The idea that the common regulatory machinery may be affected is supported by the finding that some phosphoprotein phosphatases have sulphydryl groups which are critical for activity and which are particularly susceptible to sulphydryl reagents (Li, 1982). In addition some are inhibited by oxidized glutathione (Li, 1982) which may accumulate in cells with blocked metabolism or in those treated with sulphydryl reagents. The role of glucose metabolism in providing reducing power for the co-transporter through the pentose phosphate pathway is a much neglected area. It deserves exploration.

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