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THE EFFECTS OF MAGNESIUM ON POTASSIUM TRANSPORT IN FERRET RED CELLS

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SUMMARY

1. The magnesium dependence of net and isotopic (using ^{86}Rb as tracer) potassium transport was measured in fed ferret red cells. Bumetanide (0·1 mM) was used to dissect total flux into two components: bumetanide sensitive and bumetanide resistant.

2. Increasing the external magnesium concentration from zero (added) to 2 mM stimulated bumetanide-sensitive uptake by 16% but inhibited the bumetanide-resistant component by about 20%.

3. Ionophore A23187 was used to control internal magnesium concentration. A23187 was usually present in the cells during measurement of isotopic fluxes but was washed away before measurement of net fluxes. The magnesium-buffering characteristics of fed ferret red cells were assessed during these experiments. The cytoplasm acts as a high-capacity, low-affinity magnesium buffer over most of the range. Some high-affinity binding was seen in the presence of A23187 and 2 mM-EDTA.

4. A23187 itself slightly inhibits bumetanide-sensitive potassium transport.

5. Bumetanide-sensitive potassium transport is strongly dependent on the concentration of internal ionized magnesium. Transport is 35% maximal at 10^{-7} M and increases up to the maximal rate at 1·3 mM. Further increase in ionized magnesium concentration to 3·5 mM has no additional effect. The curve relating activity to magnesium concentration is steepest at the physiological magnesium concentration. The effects of changing magnesium concentration are fully reversible.

6. Reduction of internal ionized magnesium concentration to 10^{-7} M with A23187 and EDTA approximately doubles bumetanide-resistant potassium transport.

7. Bumetanide-sensitive fluxes occur via the sodium–potassium–chloride co-transport system under the conditions used. Results described in this paper thus suggest that internal magnesium may be an important physiological controller of sodium–potassium–chloride co-transport activity.

INTRODUCTION

Ferret red cells provide an excellent experimental system in which to examine the effects of both intracellular and extracellular factors on the operation of the sodium–potassium–chloride co-transport system. This is because these cells have a

high co-transport capacity: the majority of basal sodium and about 98% of potassium transport occur through the system (Flatman, 1983; Hall & Ellory, 1985; Mercer & Hoffman, 1985). The activity of the system can be assessed by measuring bumetanide-sensitive potassium uptake. Ionophore A23187 can be used to control calcium and magnesium concentrations in these cells without the complication from ionophore-induced changes in sodium content seen in human red cells at low magnesium concentration (Flatman & Lew, 1977). Sodium is already close to electrochemical equilibrium across the ferret red cell membrane (Flatman & Andrews, 1983) so the introduction of an extra sodium transport system to a cell with an initially high sodium permeability (Flatman, 1983; Hall & Ellory, 1985) causes little perturbation of sodium content. In a recent paper (Flatman, 1987*a*), I have described the use of A23187 to control intracellular calcium concentration in ferret red cells. I showed that changes of calcium concentration over the physiological range did not affect co-transport activity in these cells, whereas high concentrations led to irreversible inhibition. This paper will examine the effects of another physiologically important divalent cation, magnesium. I shall show that changes of magnesium concentration within the physiological range alter co-transport activity and that magnesium may be an important regulator of the system. A brief account of this work was presented to the American Physiological Society (Flatman, 1987*b*).

METHODS

Blood samples were taken into EDTA from adult ferrets anaesthetized with intraperitoneal urethane, 1·5 g kg^{-1} body weight. Red cells were washed three times by centrifugation and resuspension in at least ten volumes of ice-cold FBM (ferret basic medium) (145 mM-NaCl, 5 mM-KCl, 10 mM-Na-HEPES buffer, pH 7·7 at 37 °C) containing 0·05 mM-Tris-EGTA. The cells were washed once more in FBM alone. The cells were either used immediately or were stored packed in FBM at 5 °C for up to 2 days. All experiments were carried out at 38 °C in suspensions with haematocrits of between 3 and 10%.

Haematocrit, haemolysis, cell magnesium content and chloride-distribution ratio were measured as described previously (Flatman, 1987*a*). Cell magnesium content was controlled with ionophore A23187. Ionized magnesium concentration inside cells at equilibrium ($[Mg^{2+}]_i$) in the presence of 10 μM-A23187 is given by the following equation:

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

where $[Mg^{2+}]_o$ is the external magnesium concentration at equilibrium and r is the ratio of external to internal chloride concentration (see Flatman & Lew, 1980). r^2 was 2·2 in these experiments. Final external equilibrium magnesium concentration was calculated from initial and final cell magnesium contents and initial external magnesium concentration. All external magnesium was assumed to be ionized. Internal bound magnesium concentrations were calculated by subtracting ionized from total concentrations.

Isotopic potassium fluxes were measured with ^{86}Rb which is an excellent tracer for potassium on all transport systems described here (Flatman, 1987*a*). Net potassium movements were measured in cells incubated in potassium-free media (150 mM-NaCl, 10 mM-Na-HEPES, pH 7·7 at 37 °C) containing 11 mM-glucose and 0·05 mM-EGTA. Cell potassium content was measured by atomic absorption spectroscopy (AAS) after separating cells from medium. 0·1 ml cell suspension was transferred to a 1·5 ml centrifuge tube containing 0·4 ml di-*n*-butylphthalate and 0·9 ml ice-cold inactivation medium (150 mM-choline chloride, 10 mM-Na-HEPES, 0·1 mM-bumetanide, 2 mM-EDTA). The tube was centrifuged at 12000 *g* for 25 s. Oil and supernatant were removed by suction and the walls and cap were cleaned with cotton swabs. The cell pellet was lysed in 1·2 ml double-glass-distilled water. Potassium concentration was measured in the lysate by AAS (Perkin Elmer 2280 spectrometer fitted with a red filter to absorb radiation shorter than 650 nm). Fluxes

were determined from the change in cell potassium content over the first 8 min by linear regression analysis.

Cell ATP content was measured by the fire-fly assay. ATP was extracted from cells by a modified version of Brown's method (1982). 0·05 ml cell suspension was added to 0·35 ml ice-cold 10 mM-EDTA containing 0·2% Triton X-100. 0·1 ml 2·8 M-ice-cold perchloric acid was added to the lysate which was then vortexed and allowed to stand at 0 °C for 5 min. Precipitated protein was removed by centrifugation at 12000 *g* for 30 s. 0·3 ml supernatant was then transferred to a separate tube containing 0·3 ml 700 mM-KOH, 200 mM-HEPES and 100 mM-KCl. Potassium perchlorate was removed by brief centrifugation. 0·01 ml supernatant was added to a mixture of 0·2 ml purified fire-fly enzyme and 0·79 ml 100 mM-Tris-Cl or Tris-acetate (pH 7·7 at room temperature) containing 2 mM-Tris-EDTA. Bioluminescence was determined in a luminometer (model 1250, LKB Ltd). Sample luminescence was measured and then 0·01 ml standard ATP (normally 2 μM) was added and the luminescence was measured again. All measurements were well within the linear range of the assay so sample ATP content was calculated from the luminescence of the sample and the increase in luminescence after addition of standard. Luminescence did not decay significantly during the assay.

All solutions were prepared with double-glass-distilled water and where possible Analar grade reagents (BDH Ltd). EGTA, EDTA, HEPES and Tris base were obtained from Sigma Chemical Co. Ltd. A23187 was obtained from Calbiochem and was dissolved in ethanol to form a 2·1 mM stock. Bumetanide was a gift from Leo Laboratories Ltd. All isotopes were obtained from Amersham International plc. Vials of the purified fire-fly enzyme were obtained from LKB Ltd (product No. 1243–200). The contents of each vial were dissolved in 10 ml distilled water just before use.

Abbreviations

EGTA: ethyleneglycol-bis-(β-aminoethylether)-*N,N,N',N'*-tetraacetic acid; EDTA: ethylenediaminetetraacetic acid; Tris: tris(hyroxymethyl)aminomethane; HEPES: *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid.

RESULTS

Effect of external magnesium on ^{86}Rb uptake

Figure 1 shows that increasing external magnesium concentration from zero (none added to medium, maximum contamination in medium about 10 μM) to 2 mM stimulated total ^{86}Rb uptake by about 16% whereas bumetanide-resistant uptake was slightly reduced (see later). Consequently the bumetanide-sensitive flux must have been stimulated by 16%. This was an unexpected result since increasing external magnesium inhibits all three components of potassium transport in human red cells (Ellory, Flatman & Stewart, 1983). The same results were obtained in two repeats of this experiment. In all cases the experiments were designed to ensure that internal magnesium could not have changed during the course of the experiment. Cells were added to the medium 5 min before the isotope and total fluxes were measured within 5 min of this time. In similar experiments changes in cell magnesium content could not be detected over these time and concentration ranges. The chelation of contaminant magnesium in the medium with 2 mM-EDTA caused a small increase in total ^{86}Rb uptake in the experiment shown in Fig. 1. However, in another experiment a small reduction was seen. In this case the addition of EDTA caused a drop of 0·07 units in the pH of the medium. Since ^{86}Rb uptake is highly pH sensitive in ferret red cells, the effects reported above were probably due to small differences in pH caused by the addition of EDTA. In separate experiments Tris, which was used to neutralize the EDTA, was shown not to affect ^{86}Rb uptake into ferret red cells up to a concentration of 9 mM.

Control of internal magnesium with A23187

Cell magnesium content was controlled with ionophore A23187. Figure 2 shows how cell magnesium content changed when 10 μM-A23187 was added to cells suspended in media with different magnesium concentrations. Magnesium entered cells when external magnesium concentration was above 0·3 mM and left when the

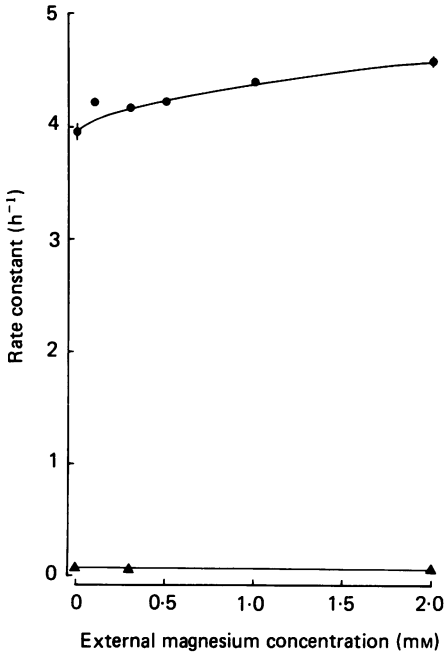

Fig. 1. Effect of external magnesium on ^{86}Rb uptake by ferret red cells. Red cells were incubated in FBM containing 11 mM-glucose, 50 μM-EGTA and the magnesium concentrations indicated. ^{86}Rb was added 5 min after the cells were added to the medium. Total fluxes (●) were measured over a 5 min period after the addition of isotope. Bumetanide-resistant fluxes (▲) were measured over a 15 min period to improve resolution because of their small size. Rate constants were determined from five time points and are plotted here with their S.E.M. if these are larger than the points.

concentration was less than 0·3 mM. Equilibrium was reached in less than 10 min for the entire range of magnesium concentrations used and magnesium contents were stable for the next hour. Cell lysis in the presence of A23187 became significant when external magnesium concentrations exceeded 2 mM. Hence experiments were limited to external concentrations below this level. EGTA (50 μM) was always present in the media to chelate contaminant calcium and thus prevent an increase in internal calcium in the presence of ionophore.

The magnesium binding curve for ferret red cells is shown in Fig. 3. Magnesium binding is well described by a single binding constant for ionized concentrations above 0·1 mM. When the data in Fig. 3 were replotted in the form of a Scatchard plot a straight line was obtained (correlation coefficient = −0·997) giving a binding

constant of 1·68 mM and a maximal binding capacity of 12·55 mM (Fig. 4). In other experiments binding constants of 2·12 and 1·91 mM and capacities of 15·44 and 16·7 mM were obtained. Ferret red cells were found to contain between 30 and 50 μmol magnesium/l cells when incubated in media containing 2 mM-EDTA and 10 μM-A23187. Ionized magnesium concentration under these conditions is about

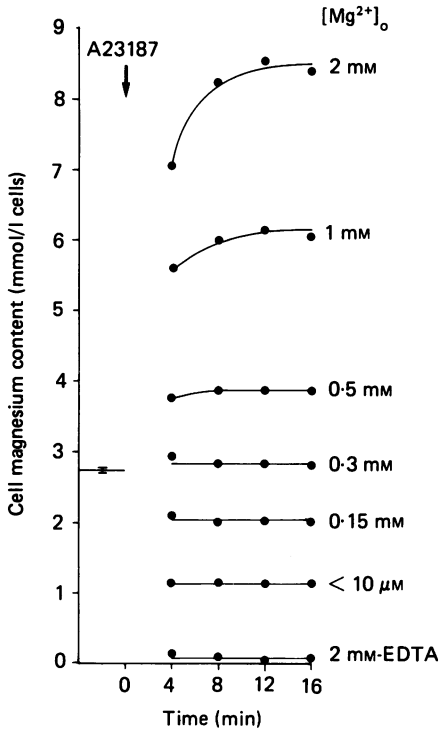

Fig. 2. Magnesium content of ferret red cells incubated in the presence of A23187. Red cells were incubated in FBM containing 11 mM-glucose, 50 μM-EGTA and the initial magnesium concentrations ($[Mg^{2+}]_o$) shown to the right of the Figure. Samples were taken to assess the initial cell magnesium content and then 10 μM-A23187 was added at time zero. New cell magnesium content was measured over the next 16 min.

10^{-7} M and thus the concentration of bound magnesium is much greater than predicted from the data above. There are three possible explanations. First, there may be high-affinity binding sites (with low capacity) in ferret red cells as described for human red cells (Flatman & Lew, 1980). Second, the ionophore may not be evenly distributed amongst the cells. The result could be explained if about 2% of cells received no ionophore and thus retained their normal magnesium content. Third, the ionophore itself may act as the high-affinity site. The observed level of binding is consistent with two ionophore molecules binding each magnesium ion.

Effect of internal magnesium on bumetanide-sensitive ^{86}Rb uptake

The magnesium concentration inside ferret red cells was controlled with A23187 while ^{86}Rb fluxes were measured. Figure 5 shows bumetanide-sensitive uptake of ^{86}Rb as a function of external ionized magnesium concentration in the presence of

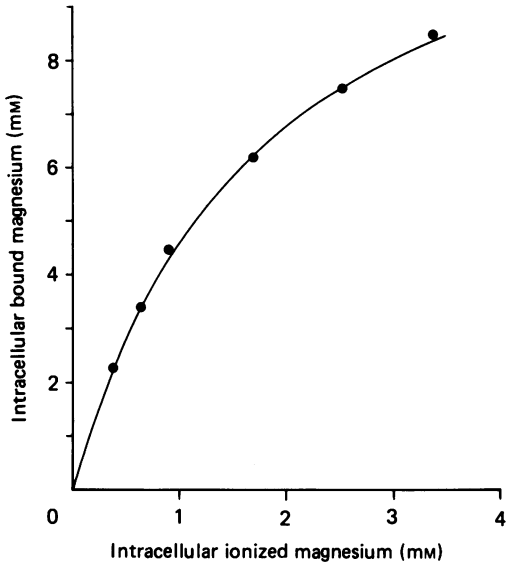

Fig. 3. Magnesium binding curve for fed ferret red cells. Concentration of bound magnesium is plotted as a function of ionized intracellular magnesium concentration. Circles represent mean values; the S.E.M. are smaller than circle size. The line through the points was drawn according to the equation:

$$[Mg]_{bound} = \frac{12{\cdot}55[Mg^{2+}]_i}{(1{\cdot}68+[Mg^{2+}]_i)} \text{ mM}.$$

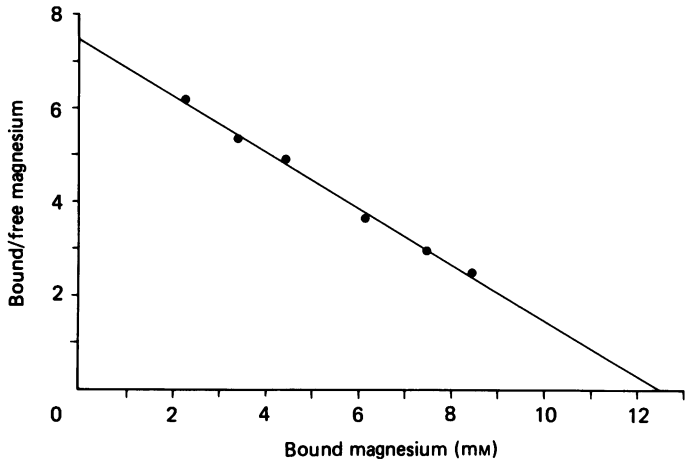

Fig. 4. Scatchard plot of magnesium binding data for ferret red cells. Data from Fig. 3 have been plotted and the line drawn by linear-regression analysis (correlation coefficient = −0·997). Maximal binding, 12·55 mM, was obtained from the intercept on the abscissa and the dissociation constant of the binding site, 1·68 mM, from the reciprocal of the slope.

A23187. Internal ionized magnesium concentration is 2·2 times larger than the external concentration under these conditions. It is clear that changing internal free magnesium concentration has a profound effect on the bumetanide-sensitive flux. At the lowest ionized magnesium concentration (about 0·08 μM in medium, equivalent to about 0·16 μM internally) the rate was reduced to about 40% of the value seen at physiological magnesium concentrations (approximately 0·65 mM, Flatman, 1987*a*).

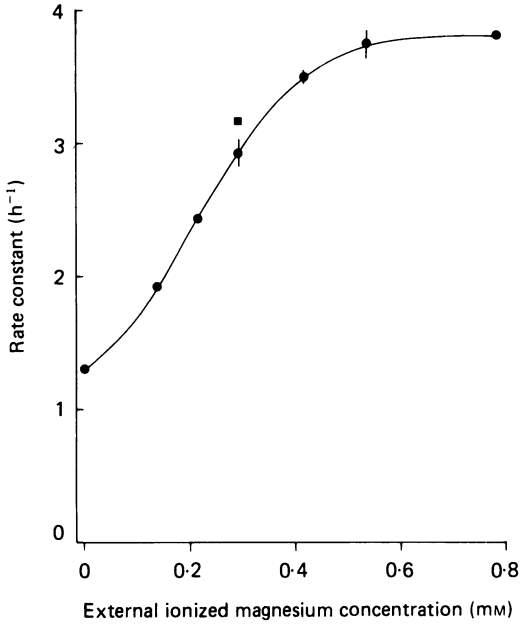

Fig. 5. Bumetanide-sensitive ^{86}Rb uptake as a function of external magnesium concentration in the presence of A23187. Cells were suspended in FBM containing 11 mM-glucose, 50 μM-EGTA and magnesium, with and without 0·1 mM-bumetanide. 10 μM-A23187 was added and 8 min were allowed for magnesium equilibration. Samples were then taken to measure the new cell magnesium content. ^{86}Rb was added 13 min after A23187 and five samples were taken at 1 or 3 min intervals to measure total or bumetanide-resistant ^{86}Rb flux respectively. Bumetanide-sensitive rate constants (●) were calculated for each magnesium concentration. ■, bumetanide-sensitive rate constant measured in cells incubated in a medium not containing ionophore. Standard errors are less than point size unless indicated.

Increasing the magnesium concentration stimulated transport to a maximum when external magnesium was about 0·6 mM (1·3 mM internally). Maximal activity was about 30% higher than the rate seen with physiological magnesium levels. The curve relating activity to concentration is sigmoid and cannot be described by simple Michaelis–Menten kinetics. It is steepest within the physiological range of ionized magnesium concentrations for ferret red cells (Flatman, 1987*a*). The square on Fig. 5 shows transport in cells not treated with A23187. Ionized magnesium concentration in these cells was found to be 0·61 mM by the null method (Flatman, 1987*a*). The square has been plotted at the external magnesium concentration which would have given this internal concentration had ionophore been present. It is clear that the

ionophore itself causes a slight inhibition of co-transport. This inhibitory effect of A23187 was seen in all experiments.

When analysing the data shown in Fig. 5 it must be remembered that magnesium can affect transport at both sides of the membrane when A23187 is present. Comparing the data in Fig. 5 with those in Fig. 1 shows that increasing the external

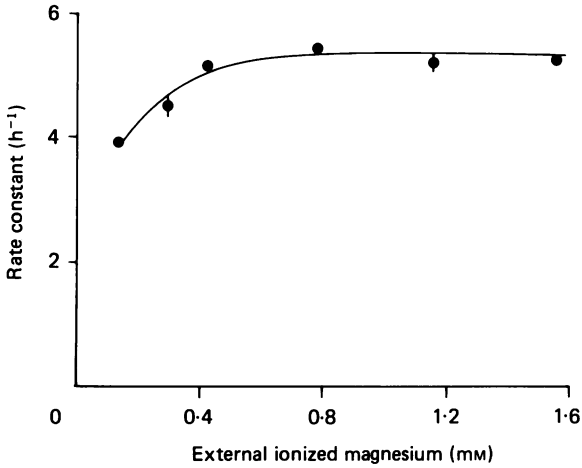

Fig. 6. Bumetanide-sensitive ^{86}Rb uptake as a function of high external magnesium concentrations in the presence of A23187. Experimental protocol was similar to that described in Fig. 5.

magnesium from 0 to 0·75 mM stimulates co-transport by 92% in the presence of ionophore and by only 9% in its absence. Clearly magnesium has a much greater effect inside the cell than outside. It might be asked whether the magnesium content of the cells could be changed with A23187 which is washed away before the fluxes are measured. In my experience it is only possible to wash the ionophore away completely when low magnesium concentrations are used. This is true even when bovine serum albumin is present in the wash media (see Fig. 9 and later; also P. W. Flatman & L. O. Simonsen, unpublished data). It is therefore not possible to examine the dependence of potassium transport on internal magnesium in the complete absence of A23187.

Experiments with human red cells (Ellory *et al.* 1983) suggest that high internal magnesium concentrations inhibit co-transport. Preliminary experiments with ferret red cells suggest a similar finding (Flatman, 1987*b*). However, care must be taken when analysing the ferret data because high magnesium causes haemolysis. Figure 6 shows the result of an experiment where haemolysis was low and did not measureably increase throughout the experiment. In this case the rate of transport plateaued at the maximum rate as external magnesium concentration was increased from 0·8 to 1·6 mM (equivalent to about 1·7–3·5 mM internally). Above this level haemolysis increased dramatically and it was not possible to distinguish a genuine inhibitory effect of magnesium from haemolysis.

Figure 5 shows that transport is reduced to about half its normal level when 2 mM-

EDTA and 10 μM-A23187 are present in the medium. A much larger reduction is predicted by extrapolation of the other data in Fig. 5 suggesting a possible overestimate of transport in the presence of EDTA. For instance, magnesium may not have come into equilibrium within 13 min of ionophore addition when EDTA was present and thus ionized intracellular magnesium concentration could still have

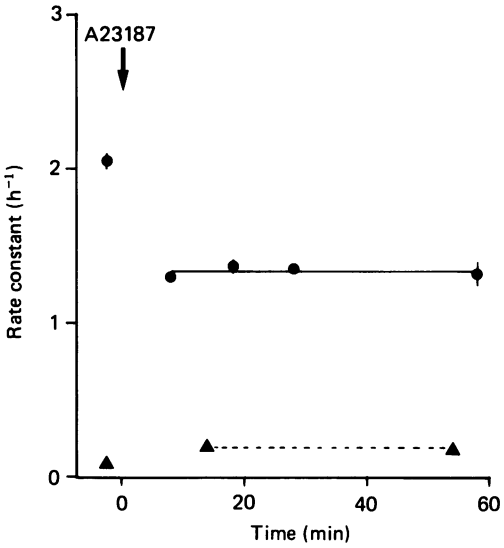

Fig. 7. Effect of EDTA on ^{86}Rb fluxes in the presence of A23187. Ferret red cells were incubated in FBM containing 50 μM-EGTA, 2 mM-EDTA and 11 mM-glucose, with and without 0·1 mM-bumetanide. 10 μM-A23187 was added as indicated. Aliquots of the suspensions were transferred to separate vials containing ^{86}Rb to measure fluxes at the times indicated. Rate constants were determined from five samples taken at 1 (total flux, ●) or 3 min intervals (bumetanide-resistant flux, ▲). Points are given with their S.E.M. if these are bigger than the symbol. Initial cell magnesium content was 3·26 ± 0·006 mmol/l cells (n = 5) and fell to 31 ± 4 μmol/l cells (n = 5) after A23187.

been falling when the ^{86}Rb fluxes were measured. A more detailed examination of the effects of EDTA is shown in Fig. 7. The rate constant for bumetanide-sensitive potassium uptake in the absence of A23187 was 1·96 h^{-1}. It fell to about 1·1 h^{-1} within 8 min of the addition of 10 μM-A23187 and remained at that level for the next 50 min. There was no sign of any decrease in activity with longer pre-incubations with EDTA and A23187 nor was a decrease detected in cell magnesium content (31 μmol/l cells). Therefore, magnesium had sufficient time to reach equilibrium and transport rate reach its minimum when EDTA was present in the experiments described in Fig. 5 and the sigmoid shape of the curve appears to be genuine.

Reversibility of the effect of magnesium on ^{86}Rb fluxes

Figure 8 shows that the effect of magnesium depletion on bumetanide-sensitive ^{86}Rb transport can be fully reversed by returning the magnesium content of the cells to normal. Cell magnesium content was reduced to a final level of 47 μmol/l cells by incubating them in 2 mM-EDTA and 10 μM-A23187 for 15 min. The ionophore was

then removed by washing the cells twice in FBM containing 2 mM-EDTA and 1 mg bovine serum albumin/ml and then twice more in FBM alone. The effectiveness of this washing procedure is shown by the fact that magnesium uptake by washed cells was 1 μmol/(l cells.min) when incubated in media containing 0·69 mM-magnesium compared with at least 700 μmol/(l cells.min) in the presence of 10 μM-A23187. The magnesium-depleted cells were then incubated in fresh media containing magnesium

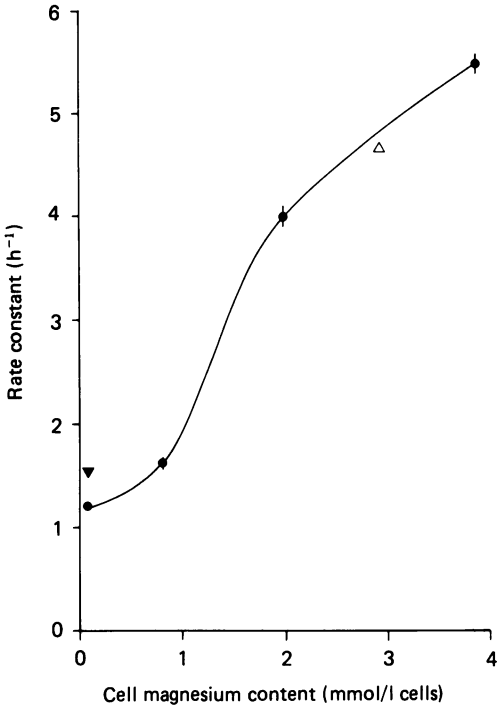

Fig. 8. Reversibility of the effect of magnesium removal. The magnesium content of ferret red cells was depleted to 47 μmol/l cells by treatment with 2 mM-EDTA and 10 μM-A23187 which was then washed away. The depleted cells were resuspended in FBM containing 50 μM-EGTA, 11 mM-glucose, magnesium and 10 μM-A23187, with and without 0·1 mM-bumetanide. After 6 min four samples were taken at 1 min intervals to measure the new magnesium content. ^{86}Rb was then added and five samples were taken at 1 or 3 min intervals to measure total or bumetanide-resistant fluxes respectively. Bumetanide-sensitive rate constant (●) is plotted as a function of the new magnesium content of the cells. △, rate measured in control cells incubated in media containing 0·3 mM-magnesium and 10 μM-A23187. ▼, rate measured in depleted cells incubated in media containing 0·69 mM-magnesium but no ionophore. Points are given with S.E.M. if these are bigger than symbols.

and ionophore. The circles in Fig. 8 represent bumetanide-sensitive ^{86}Rb uptake as a function of the final total magnesium content of the cells. The activity in fresh cells treated with 10 μM-A23187 and 0·3 mM-magnesium (magnesium content = 2·92 mmol/l cells, which compares well with magnesium content of cells not treated with ionophore, i.e. 2·88 mmol/l cells) is shown by the open triangle. This activity is very similar to the value predicted by interpolation of the data for predepleted cells and indicates that the effects of magnesium removal are reversible.

Figure 8 also provides more evidence on the sidedness of magnesium's effect. The filled triangle shows the transport rate in depleted cells incubated in 0·69 mM-magnesium with no ionophore. The magnesium content of the cells remained low and was 67 μmol/l cells at the end of the experiment. ^{86}Rb uptake rate was only 26% higher than that seen in depleted cells incubated in the presence of 2 mM-EDTA and 10 μM-A23187. This stimulation was due to high external magnesium concentration and the absence of ionophore. The data show that the majority of magnesium's effect on bumetanide-sensitive transport is at the internal surface since transport was stimulated by more than 450% when A23187 was added to cells suspended in a medium initially containing 0·69 mM-magnesium.

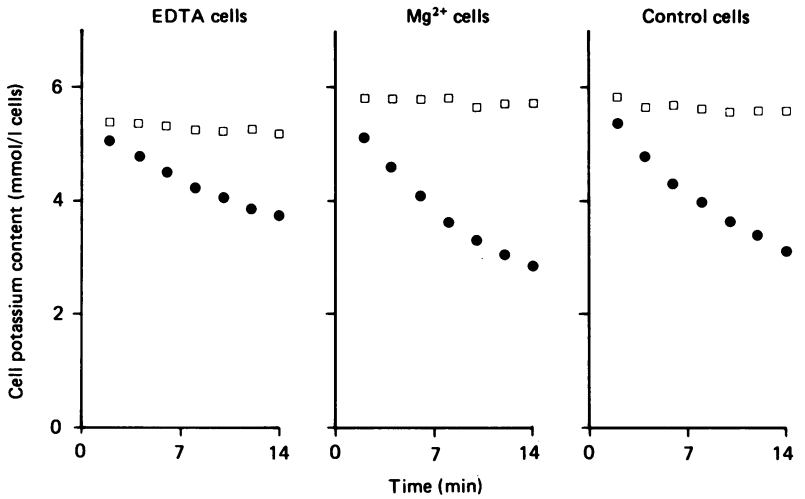

Fig. 9. Net potassium fluxes as a function of cell magnesium content. Cell potassium content was measured by AAS after addition of cells to a potassium- and magnesium-free medium with (□) or without (●) 0·1 mM-bumetanide (see Methods). Three types of cells were used. EDTA cells (left panel) had been treated with 2 mM-EDTA and 10 μM-A23187 and then washed free of A23187. Their magnesium content was 7 ± 1 μmol/l cells ($n = 3$). Mg^{2+} cells (middle panel) had been treated with 0·3 mM-magnesium and 10 μM-A23187. Cells were washed twice with albumin-containing media and twice with FBM alone but some ionophore must have remained since magnesium left the cells during the experiment (see text). Their magnesium content was $1{\cdot}58 \pm 0{\cdot}007$ mmol/l cells ($n = 3$) 20 min after cell addition to medium. Control cells (right panel) had not been treated with ionophore. Their magnesium content was $2{\cdot}77 \pm 0{\cdot}01$ mmol/l cells ($n = 3$).

Effect of magnesium on net potassium fluxes

A model has recently been proposed to describe sodium–potassium–chloride co-transport in duck red cells (McManus, 1987) which is probably applicable to transport in other red cells too. It integrates the many bumetanide-sensitive cation movements into a single scheme and sees these as partial reactions of the co-transport system. In this model isotopic movements of ^{42}K or ^{86}Rb may represent not only co-transport itself but also potassium–potassium exchange. Therefore in assessing the physiological importance of magnesium in controlling co-transport it is necessary to show that magnesium affects net movement of potassium, which is the physiologically important variable, as well as isotopic flux. Figure 9 shows the result

of an experiment in which net bumetanide-sensitive potassium fluxes were measured as a function of internal magnesium. Cell magnesium content was altered by treating cells with A23187 and either 2 mM-EDTA or 0·3 mM-magnesium. The cells were then washed with FBM containing albumin to try to remove A23187. Washed cells were transferred to a potassium-free medium and their potassium content was followed by AAS. The initial rate of bumetanide-sensitive potassium loss from cells not treated with A23187 was 12·7 mmol/(l cells.h) whereas that from cells pretreated with EDTA and A23187 (magnesium content = 7 μmol/l cells) was reduced by 45% to 6·93 mmol/(l cells.h). Clearly magnesium affects net fluxes in the same way as isotopic fluxes. The experiment using cells pretreated with 0·3 mM-magnesium and A23187 was not completely successful since it was not possible to remove all A23187 from the cells. The magnesium content of these cells fell at a rate of about 60 μmol/(l cells.min) throughout the experiment. The initial rate of bumetanide-sensitive potassium loss in these cells was about 14 mmol/(l cells.h), similar to the control value.

Effect of magnesium on bumetanide-resistant ^{86}Rb influx

The rate constant for bumetanide-resistant ^{86}Rb equilibration in ferret red cells is very low, typically in the order of 0·05–0·07 h^{-1} (Flatman, 1983). Analysis of a large number of experiments suggests that increasing external magnesium concentration from zero to 2 mM causes a small fall in rate constant. For instance in the experiment shown in Fig. 1 the rate fell from $0·0728 \pm 0·0036$ to $0·0596 \pm 0·0033$ h^{-1} ($\pm$S.E.M., $n = 5$ in both cases). The data in Fig. 10 also show inhibition by external magnesium. Inhibition is also sometimes seen when external magnesium concentration is increased from 0·1 to 1·6 mM in the presence of 10 μM-A23187 but it is difficult to assess the contribution of internal and external magnesium in this process. However, when most magnesium is removed from cells with A23187 and 2 mM-EDTA a significant stimulation, often amounting to a doubling, of bumetanide-resistant ^{86}Rb uptake is seen (Fig. 10). This stimulation is due to removal of internal magnesium and not to low external concentration since it only occurs in the presence of A23187 (Fig. 10).

Effect of magnesium on ATP content of ferret red cells

Magnesium is an important cofactor for a wide range of enzymes (Walser, 1967; Ebel & Gunther, 1980; Wacker, 1980) so changing internal magnesium might seriously affect the cell ATP content which might in turn affect co-transport rate (Hall & Ellory, 1985; Mercer & Hoffman, 1985; Adragner, Perkins & Lauf, 1985; Dagher, Brugnara & Canessa, 1985; Lauf, 1986). Figure 11 shows the effect of A23187 and magnesium on cell ATP levels. Cell ATP content was approximately 0·45 mmol/l cells and fell in all cases by about 13% within 1 min of the addition of 10 μM-A23187. What happened next depended on the external magnesium concentration. In the presence of 2 mM-EDTA, ATP concentration continued to fall to about 74% of the initial value 30 min after ionophore addition. In the presence of 0·3 mM-magnesium, a condition which prevents change in magnesium content, ATP concentration increased to about 90% of the initial value. At higher magnesium concentrations, either 1 or 2 mM, ATP content increased above the initial level reaching 115 and 107% respectively 30 min after ionophore addition.

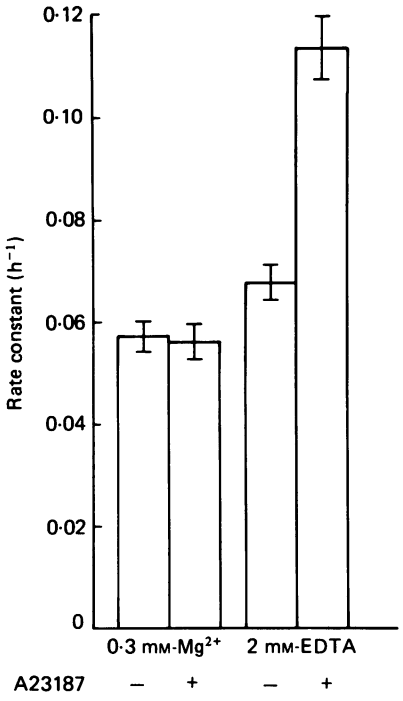

Fig. 10. Bumetanide-resistant ^{86}Rb uptake by ferret red cells. Red cells were incubated in FBM containing 11 mM-glucose, 50 μM-EGTA, 0·1 mM-bumetanide and the additions shown. ^{86}Rb fluxes were measured over a 15 min period after addition of isotope. When used, A23187 (10 μM) was added 4 min before ^{86}Rb. Total ^{86}Rb uptake rate constants in the presence of 10 μM-A23187 and 2 mM-EDTA or 0·3 mM-magnesium were $2{\cdot}52 \pm 0{\cdot}05$ and $3{\cdot}76 \pm 0{\cdot}05$ h^{-1} respectively. Mean rate constants are given with S.E.M. ($n = 5$).

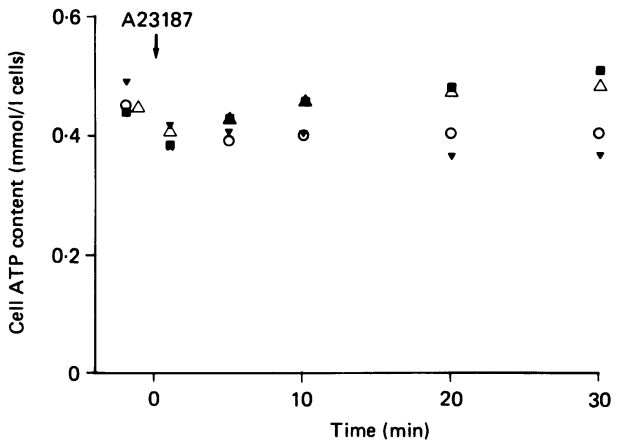

Fig. 11. Effect of A23187 and magnesium on the ATP content of ferret red cells. Ferret red cells were incubated in FBM containing 11 mM-glucose, 50 μM-EGTA and the following: 2 mM-EDTA (▼); 0·3 mM-Mg^{2+} (○); 1 mM-Mg^{2+} (■); 2 mM-Mg^{2+} (△). 10 μM-A23187 was added at time zero and cell ATP content was measured at the times indicated.

DISCUSSION

The main conclusion of this paper is that changes in magnesium concentration significantly alter bumetanide-sensitive potassium transport in ferret red cells. Both isotopic (^{86}Rb uptake) and net potassium fluxes are similarly affected. Increase in internal or external magnesium concentration stimulates transport but the system is about five times more sensitive to internal magnesium. Maximal activity is seen when internal ionized magnesium concentration is about 1·7 mM and remains at this level up to a magnesium concentration of 3·5 mM. The effect of further increase in magnesium concentration could not be investigated because high internal magnesium concentrations caused haemolysis. Bumetanide-resistant potassium uptake is inhibited by an increase in external magnesium concentration and doubled by a reduction of internal free magnesium concentration to 10^{-7} M. Bumetanide-sensitive potassium uptake by ferret red cells is via the sodium–potassium–chloride co-transport system (Flatman, 1983; Hall & Ellory, 1985) so the results reported indicate that this co-transport system is magnesium dependent.

Ellory *et al.* (1983) have examined the effects of magnesium on potassium uptake by human red cells. Increasing external magnesium concentration was found not only to inhibit bumetanide-resistant transport as in ferret red cells but also bumetanide-sensitive flux. Indeed, external magnesium either inhibited or had no effect on all the transport systems examined. Thus the finding that external magnesium stimulates bumetanide-sensitive potassium uptake in ferret red cells is unique and may indicate a specific interaction between external magnesium and the transport system, possibly by means of a specific magnesium binding site. Changing internal magnesium concentration in human red cells had complex effects which can now be more fully understood in the light of the data reported here with ferret red cells and by recent work on human and low-potassium (LK) sheep red cells.

Ellory *et al.* (1983) showed that reduction of internal magnesium with EDTA and A23187 reduced bumetanide-sensitive potassium uptake in human red cells by about one-half while stimulating bumetanide-resistant uptake fivefold. Taken in conjunction with data on chloride-sensitive potassium flux this was taken to indicate that bumetanide was not effective at low magnesium concentrations. This suggestion may not be correct. In ferret red cells treated with A23187 and EDTA 0·1 mM-bumetanide inhibited more than 95% of potassium uptake (see Fig. 10) and thus appears just as effective at low as at normal magnesium concentrations. An alternative explanation of the human red cell data is that removal of magnesium may activate a chloride-dependent potassium transport system which is poorly inhibited by bumetanide and is thus distinct from sodium–potassium–chloride co-transport. Such a transport system is latent in human red cells (Lauf, Adragna & Garay, 1984) but can be stimulated by *n*-ethylmaleimide (see also Wiater & Dunham, 1983). Recently, Brugnara & Tosteson (1987) have found this potassium transport system in the least-dense fraction of human red cells and have shown that it is inhibited by high internal magnesium concentrations. This system is also found in LK sheep red cells where it is stimulated by removal of magnesium (Lauf, 1985*a*, *b*; see discussion by Flatman, 1987*b*). Thus Ellory *et al.* (1983), who went on to measure chloride-dependent fluxes, probably looked at the effects of magnesium on two

transport systems: the potassium–chloride system, which was stimulated by magnesium removal, and the sodium–potassium–chloride system, which was inhibited.

The effects of magnesium on bumetanide-sensitive potassium transport reported in this paper are not secondary to changes in ATP content caused by magnesium. Figure 11 shows that changes in ATP content were less than 30% over the entire range of conditions used. ATP content never fell below 0·35 mmol/l cells whereas it must fall below 0·1 mmol/l cells before transport is affected in ferret cells (P. W. Flatman, in preparation). A similar finding has been reported in human red cells (Dagher *et al.* 1985). Under carefully controlled conditions, which prevent changes in magnesium and increases in calcium content, A23187 itself inhibits bumetanide-sensitive potassium transport and also causes a fall in ferret red cell ATP content. Although it is attractive to assume that transport inhibition is due to ATP depletion, experiments reported above suggest this is not so. Dunham & Ellory (1981) have reported a similar inhibitory effect of A23187 on potassium–chloride co-transport in LK sheep red cells.

There is considerable controversy over the role of metabolism and in particular ATP in chloride-dependent potassium transport (Dagher *et al.* 1985; Lauf, 1986; Chipperfield, 1986). Sodium–potassium–chloride co-transport often disappears in cells whose ATP content has been depleted to very low levels ($< 10^{-7}$ M), suggesting that ATP is necessary for activity of this system. ATP could have any of three functions. It could be required as a cofactor, as an energy source or as a source of phosphate to maintain the transporter in a phosphorylated state (see Palfrey & Rao, 1983). The last two require phosphoryltransferase activity. Magnesium is an essential cofactor for phosphoryltransferases (Wacker, 1980) and reduction of magnesium to 10^{-7} M with EDTA would be expected to reduce their activity to a very low level. For instance sodium pump fluxes are reduced to less than 5% of maximal rate under these circumstances (Flatman & Lew, 1981). The finding that co-transport can be maintained at 50% of maximal rate for over an hour in cells containing about 10^{-7} M-ionized magnesium supports the idea that ATP is not a direct energy source for co-transport (Geck, Pietrzyk, Burckhardt, Pfeiffer & Heinz, 1980) and suggests that if a phosphorylated protein is involved in control of the system (Palfrey & Rao, 1983; Saier & Boyden, 1984) then this must have a very low turnover at low magnesium concentrations.

This paper shows that changes in the concentration of ionized intracellular magnesium within the physiological range have profound effects on the activity of the sodium–potassium–chloride co-transport system. Thus magnesium may well be an important physiological regulator of this transport system and defects in magnesium metabolism may be responsible for some defects in co-transport function. The relationship between magnesium and co-transport in disease now merits investigation.

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