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Sodium Transport through the Amiloride-sensitive Na-Mg Pathway of Hamster Red Cells

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Abstract. Previous work showed that in hamster red cells the amiloride-sensitive (AS) Na^+ influx of 0.8 mmol/liter cells/hr is not mediated by Na-H exchange as in other red cells, but depends upon intracellular Mg^{2+} and can be increased by 40-fold by loading cells with Mg^{2+} to 10 mM. The purpose of this study was to verify the connection of AS Na^+ influx with Na-dependent, amiloride-sensitive Mg^{2+} efflux and to utilize AS Na^+ influx to explore that pathway.

Determination of unidirectional influx of Na^+ and net loss of Mg^{2+} in parallel sets of cells showed that activation by extracellular [Na^+] follows a simple Michaelis-Menten relationship for both processes with a K_m of 105–107 mM and that activation of both processes is sigmoidally dependent upon cytoplasmic [Mg^{2+}] with a $[Mg^{2+}]_{0.5}$ of 2.1–2.3 mM and a Hill coefficient of 1.8. Comparison of V_{max} for both sets of experiments indicated a stoichiometry of 2 Na:1 Mg. Amiloride inhibits Na^+ influx and Mg^{2+} extrusion in parallel (K_i = 0.3 mM). Like Mg^{2+} extrusion, amiloride-sensitive Na^+ influx shows an absolute requirement for cytoplasmic ATP and is increased by cell swelling. Hence, amiloride-sensitive Na^+ influx in hamster red cells appears to be through the Na-Mg exchange pathway.

There was no amiloride-sensitive Na^+ efflux in hamster red cells loaded with Na^+ and incubated with high [Mg^{2+}] in the medium with or without external Na^+, nor with ATP depletion. Hence, this is not a simple Na-Mg exchange carrier.

Key words: Erythrocytes (red blood cells) — Amiloride — Magnesium-sodium exchange — Magnesium transport — Sodium transport — Hamster

Introduction

Although Mg^{2+} is the third or fourth most abundant cation in the cytoplasm, the concentration of ionized free Mg^{2+} is lower than that expected for equilibrium (Flatman, 1984). Accounting for the apparent energy-dependent extrusion of Mg^{2+} has made slow progress compared with that for Na^+ and Ca^{2+}. Squid giant axon apparently has a clear-cut Na-Mg exchanger, similar to that for Na-Ca exchange (Baker & Crawford, 1972; De Weer, 1976; Dipolo & Beaugé, 1988; Gonzalez-Serratos & Rasgado-Flores, 1990). Vertebrate red cells also show Na-dependent net Mg^{2+} extrusion that depends on metabolism and is inhibited by amiloride, but the specific properties vary among red cells from different species (Günther, Vormann & Förster, 1984; Féray & Garay, 1986, 1987; Lüdi & Schatzmann, 1987; Günther, Vormann & Höllriegl, 1990). In human red cells, evidence seems to suggest a direct dependence of the transport upon hydrolysis of ATP (Lüdi & Schatzmann, 1987; Frenkel et al., 1989), whereas in ferret red cells Mg^{2+} efflux is not dependent upon ATP at all (Flatman & Smith, 1990). Red cells of rat (Féray & Garay, 1987, Günther & Vormann 1989), hamster (Xu & Willis, 1991; Willis, Xu & Zhao, 1992) and guinea pig (Willis et al., 1992) have a much larger Na-dependent, amiloride-sensitive Mg^{2+} efflux than that so far described in other mammalian or avian red cells. In human red cells, hypotonic incubation has been reported to increase the Na-dependent Mg^{2+} transport (Féray & Garay, 1986).

The usual method for investigating the Na-dependent, amiloride-sensitive Mg^{2+} transport in red cells

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has been determination of Mg^{2+} loss into Mg-free medium from cells loaded with Mg^{2+} by equilibration with high concentrations in the medium in the presence of an ionophore. There has been almost no effort to characterize the pathway by unidirectional Na^+ influx. This is partly because there usually is at least one other pathway for Na^+ entry that is amiloride sensitive, namely, the Na-H exchanger, complicating the interpretation of Na^+ influx measurement. We have previously reported that amiloride-sensitive Na^+ influx in hamster red cells exhibits none of the characteristics of Na-H exchange (Xu & Willis, 1991; Willis et al., 1992). It is decreased, not increased, by cytoplasmic acidification and by cell shrinkage and it is not activated by phorbol esters. With their high rate of Na-dependent Mg^{2+} transport (50 times greater than that in human red cells) and their absence of a parallel amiloride-sensitive Na-H pathway, hamster red cells would seem to be a highly suitable model to investigate the kinetics of Na^+ entry via the Na-Mg exchange pathway.

We have used this cell model to compare the kinetics of activation of Na^+ entry by extracellular Na^+ and by cytoplasmic Mg^{2+} with the corresponding kinetics for Mg^{2+} extrusion, to explore the ATP dependence and the volume activation, and, finally, by measurement of Na^+ efflux, to determine whether the exchange of Na^+ for Mg^{2+} is reversible, as might be expected of a simple exchange carrier.

Materials and Methods

Animals

Syrian Hamsters (*Mesocricetus auratus*) were bred and raised by the animal center in the Department of Physiology, University of Illinois, and also were shipped to and housed in the animal care center at the University of Georgia. The protocol for this study was approved by the Animal Care Committee of the University of Illinois and the University of Georgia.

Preparation of Red Blood Cells

Hamsters were deeply anesthetized with Pentobarbital, and blood was then taken by heart puncture. The heparinized blood was washed three times with medium (mM: 150 NaCl, 7.5 KCl, 10 glucose, 5 adenosine, 10 MOPS, pH 7.4). The red cells were then suspended in the same medium at a hematocrit of about 10%. Before each experiment, cells were resuspended in media appropriate for the experiment.

Determination of Cell Magnesium

Total Mg^{2+} content of red blood cells was determined by atomic absorption spectrophotometry after separating them from incubation medium by centrifugation at 12,000 rpm for 10 sec through a layer of dibutylphthalate in a 1.5 ml microcentrifuge tube. Cell concentration of free Mg^{2+} was determined by the method of Flatman and Lew (1980), taking into account total Mg content, cell volume and chloride distribution ratio. In a medium with 1 mM Mg^{2+} (equivalent to the concentration in hamster blood plasma), the total Mg content was 2 mM, corresponding to a concentration of free Mg^{2+} in the cell of 0.3 mM. (Note: In this paper the term, Mg^{2+} is used to refer only to ionized or free Mg^{2+} and "$[Mg^{2+}]_i$" to cytoplasmic concentration of free Mg^{2+}.)

Modification of Intracellular Mg^{2+}

Intracellular concentrations of Mg^{2+} were altered by incubating cells in a loading medium with various concentrations of Mg^{2+} (0–10 mM) in the presence of the divalent cation ionophore, A23187. The loading medium contained 150 mM KCl, 5 mM NaCl, 0.3 mM EDTA, 10 mM HEPES, 0–10 mM $MgCl_2$, pH 7.7. The cell suspension was incubated at 37°C for 15 min with 3 μM A23187 with constant magnetic stirring. After Mg^{2+} loading, A23187 was removed by three quick washes in 40 volumes of the same loading medium but without A23187 and containing 0.1–0.5% bovine serum albumin. Cells were then resuspended at 10% hematocrit in the standard incubation medium. The free Mg^{2+} of cytoplasm corresponded closely with that of the equilibrating medium below 5 mM, but deviated slightly at higher concentrations (Fig. 1).

Measurement of Net Mg^{2+} Loss

The rate of Mg^{2+} outflow was determined by the measurement of net Mg^{2+} loss into (initially) Mg-free medium. Mg-loaded cells (0–10 mM) were washed two times with Na-free, choline chloride medium, resuspended in Mg^{2+} efflux medium at a hematocrit of 10–20%, and incubated at 37°C. The Mg^{2+} efflux medium contained various concentrations of NaCl, 0–150 mM (with choline chloride replacing NaCl osmotically), glucose 10 mM, adenosine 5 mM, MOPS 10 mM, pH 7.4, ouabain, 2 mM; with or without 2 mM of amiloride. Samples of the efflux suspension were taken at various times, cells were removed by centrifugation, and the supernatant was taken to measure the amount of magnesium in the solution by atomic absorption spectrophotometry. Net Mg^{2+} loss was calculated from the difference of Mg^{2+} loss at 5 min and 20 min at 37°C. The units of Mg^{2+} loss (Mg^{2+} "efflux") are mmol/liter cells/hr.

Modification of Intracellular Na^+

In certain experiments, intracellular Na^+ concentration was changed by incubating cells in the Na^+ loading medium (NaCl, 10–150 mM, KCl 0–140 mM, HEPES 10 mM, pH 7.4) at about 10% hematocrit with the presence of the ionophore, nystatin. The cell suspension was incubated at 0°C for 30 min with nystatin (30 μg/ml), centrifuged 10 sec at 12,000 rpm, resuspended with fresh Na^+ loading medium, and incubated at 0°C for another 30 min. After incubation, the remaining nystatin was quickly removed by washing cells three times with the same Na^+ loading medium containing 0.1–0.5% albumin. Cells were then resuspended in the appropriate medium.

Measurement of Cell Na^+

Cell Na^+ content was measured by emission flame photometry. A 0.2 ml sample of the cell suspension was centrifuged at 12,000 rpm for 15 sec. The supernatant was removed and the cells washed three times in washing medium (107 mM $MgCl_2$, 10 mM Tris, pH 7.8). The cells were then lysed in 1 ml 0.1% triton solution, and sodium content was measured by emission flame photometry.

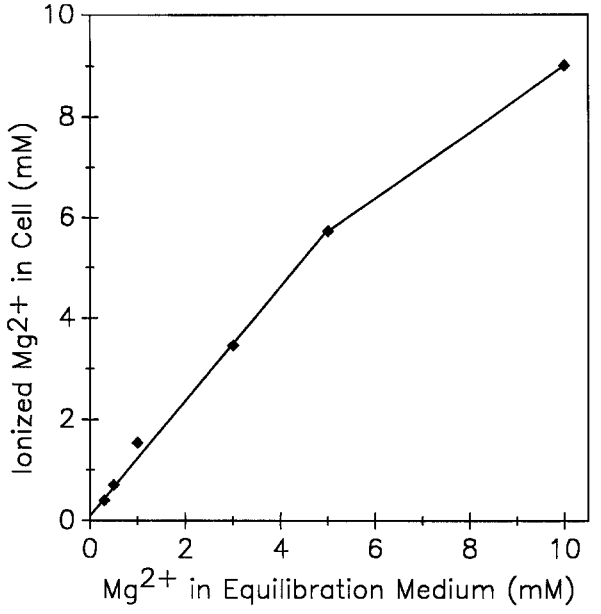

Fig. 1. Relationship between free Mg^{2+} in cytoplasm and Mg^{2+} in medium. Free cytoplasmic Mg^{2+} was estimated by the method of Flatman and Lew (1980). This required determination of total Mg content by atomic absorption spectrophotometry, determination of chloride ratio and determination of water content for cells incubated at each of the stated extracellular concentrations of Mg^{2+} in the presence of 3 μM A23187. These determinations were made in separate sets of cells from the same animal incubated in parallel under identical conditions. The points represent means of three to six such determinations. The values obtained were then used to compute cytoplasmic free Mg^{2+} ("$[Mg^{2+}]_i$") in Fig. 5.

ATP Depletion and ATP Measurement

The methods for depletion of red cell ATP were the same as those described by Marjanovic and Willis (1992). Briefly, fresh blood was washed four times with ATP depletion medium (*see below*), then incubated at 37°C for various times, up to 6 hr, to deplete the cell ATP. ATP-depleted red cells were washed and resuspended at 10% hematocrit in K-free and Mg-free glucose-free, 2-deoxyglucose containing, ATP depletion medium (mM: 150 NaCl, 7.5 KCl, 1.0 $MgCl_2$, 10 2-deoxy-D-glucose, 10 MOPS, pH 7.4) for the various other tests.

Cell ATP content was measured by the luciferin-luciferase method (Brown, 1982). Briefly, red cell suspension (0.1 ml) was first extracted with 1 ml ice-cold 3.35% perchloric acid (PCA). The extract was held in ice-water bath for 5 min, then centrifuged for 1 min at 12,000 rpm. A 0.5 ml aliquot of PCA supernatant was transferred to another microcentrifuge tube which contained 0.5 ml of ice-cold KOH buffer (mM: 200 HEPES, 100 KCl, 600 KOH). This sample was then later combined with the firefly tail extracts and substrates ("FLE") and the light emission measured in a liquid scintillation counter.

Radioisotopes

^{24}Na: Na_2CO_3 was radiated at the Nuclear Reactor Lab at the University of Illinois, and dissolved in 1 M HCl. Water, excess HCl and CO_2 were evaporated by heating under a 250 W lamp. The residual crystals of $^{24}NaCl$ were made up to a concentration of 150 mM by adding distilled water.

^{22}Na: $^{22}NaCl$, specific activity 100–1,000 mCi/mg Na, was purchased from Amersham, and diluted appropriately.

Measurement of Na^+ Influx

Unidirectional Na^+ influx was determined by the measurement of initial rate of uptake of ^{22}Na or ^{24}Na into the cells. Red cell suspension (10% hematocrit) was prepared in influx medium lacking isotope and inhibitors, and 0.3 ml suspension was placed in 1.5 ml Eppendorf plastic microcentrifuge tubes containing 0.7 ml K-free, Mg-free influx medium with ^{24}Na (1 μCi) or ^{22}Na (0.2 μCi) and 2 μmol ouabain and with or without 2 μmol amiloride. The resulting 1 ml samples of 3% suspension of red cells were then incubated at 37°C for 5 or 20 min. The samples were then put in an ice-water bath for 5 min, washed three times with ice-cold washing medium (*see above*). The pellets were treated with 5% TCA to precipitate protein and were spun for 1 min, then the radioactivity of the supernatant was counted in water (as Cerenkov radiation, ^{24}Na) or in scintillation cocktail (^{22}Na) in a liquid scintillation counter. The rate of Na^+ influx (mmol/liter cells/hr) was calculated from the difference of Na^+ uptake at 5 min and 20 min at 37°C, by using the specific activity of ^{24}Na or ^{22}Na and the cell volume.

Measurement of Na^+ Efflux

Na^+ efflux was determined by measuring the initial rate of loss of intracellular ^{22}Na into the efflux medium. Red cells were suspended at about 20% hematocrit in the Na^+ loading medium (NaCl 10–150 mM, KCl 0–140 mM, HEPES 10 mM, pH 7.4), plus ^{22}Na (0.25–1.00 μCi/ml), then incubated at 37°C for 30 min. Cells were quickly washed three times with flux medium (mM: NaCl 0–150, MOPS 10, glucose 10, adenosine 5, pH 7.4) to remove the ^{22}Na in the medium. After the final wash, cells were resuspended to about 5% hematocrit in the flux medium, with 2 mM ouabain, with or without 2 mM amiloride, and with or without 20 mM $MgCl_2$; incubated at 37°C for various times (typically 5, 20 and 35 min). The sample of 1 ml was centrifuged at 12,000 rpm for 15 sec, then 0.8 ml of supernatant was directly transferred into a scintillation vial for the determination of the radioactivity in a liquid scintillation counter. The efflux rate was calculated as described by Hall and Willis (1984).

Analysis of Kinetics of Na^+ Influx and Mg^{2+} Loss

Analysis of the kinetics of Na^+ influx and net Mg^{2+} loss with $[Na^+]_o$ was based on the Eadie-Hofstee equation:

$$v = V_{max} - (K_m \cdot v/s) \quad (1)$$

Analysis of the kinetics of Na^+ influx and net Mg^{2+} loss activated by $[Mg^{2+}]_i$ was based on the Hill equation:

$$v/V_{max} = [Mg^{2+}]^h/(([Mg^{2+}]_{0.5})^h + [Mg^{2+}]^h) \quad (2)$$

where $[Mg^{2+}]_{0.5}$ is the magnesium concentration for half-maximal stimulation, and h represents the Hill coefficient. (V_{max} was obtained from curve fitting.)

Measurement of Cell Volume

Cell volume was calculated from the optical density reading at 540 nm of diluted lysate of the red cell suspension. Absorption was then

converted to original cell volume by the relationship between absorption at 540 nm in diluted lysate of whole blood and the hematocrit of whole blood (*see* Hall & Willis, 1984).

Results

In fresh hamster red cells, the total Na^+ influx was 3.04 ± 0.17 mmol/liter cells/hr (mean ± SE, $n = 19$), and it was reduced by 2 mM amiloride to 2.24 ± 0.12 ($P < 0.001$, paired t-test for 19 cases), yielding an amiloride-sensitive Na^+ influx of 0.8. When red cells were incubated in a Mg-depleting medium (mM: 145 KCl, 5 NaCl, 10 HEPES, 1 EDTA) at 37°C with the ionophore, A23187, for 15 min, which decreased their total cellular Mg content to less than 0.01 mmol/liter cells, the influx rose linearly with extracellular Na^+, and was not different from the influx in 2 mM amiloride (Fig. 2).

Mg^{2+} loss into Mg-free medium from red cells loaded in medium with 10 mM Mg^{2+} was reduced 81% in Na-free medium. The Mg^{2+} loss in medium with 150 mM Na^+ and 2 mM amiloride was not significantly different from that in Na-free medium (Fig. 3).

Coupling of Amiloride-sensitive Na^+ Influx to Na-dependent Mg^{2+} Loss

Inhibition of Na^+ Influx and Mg^{2+} Loss by Amiloride

The results above confirmed that amiloride-sensitive Na^+ influx in hamster red cells is totally dependent upon the presence of free Mg^{2+} in the cell and that Mg^{2+} extrusion is dependent upon the presence of Na^+ in the medium. To establish a coupling between Na^+ influx and Mg^{2+} extrusion, it was necessary to make comparisons between the two in parallel under various conditions causing stimulation or inhibition.

Mg^{2+} extrusion or "efflux" was determined by observing the net Mg^{2+} loss into Mg-free medium from cells loaded in 10 mM Mg^{2+} with A23187.

Incubation of Mg-loaded cells with amiloride caused the same relative inhibition of unidirectional Na^+ influx and net Mg^{2+} loss (Fig. 4) with a maximum inhibition at 5 mM and an inhibition constant (K_i) of 0.3 mM.

Activation of Na^+ Influx and Mg^{2+} Loss by Intracellular Mg^{2+}

If the Mg^{2+}-dependent Na^+ influx in hamster red cells represents only Na-Mg transport, increasing $[Mg^{2+}]_i$ should activate both amiloride-sensitive Na^+ influx and amiloride-sensitive Mg^{2+} loss with the same kinetic constants. The dependence of the amiloride-sensitive Na^+ influx on intracellular Mg^{2+} is shown in Fig. 5.

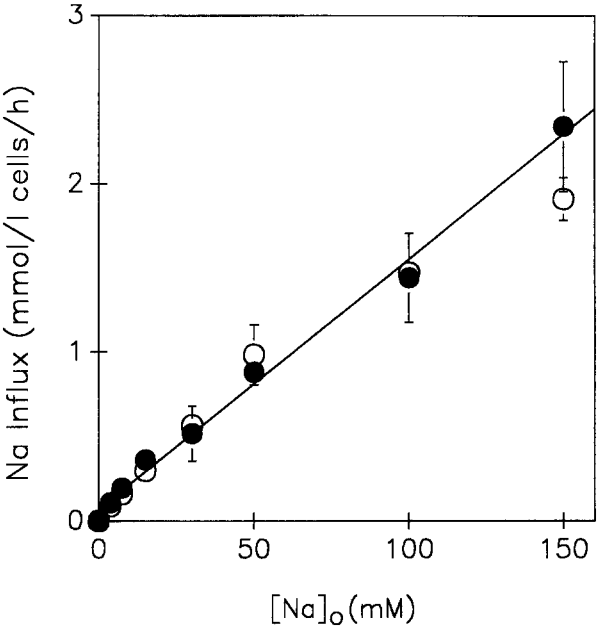

Fig. 2. Na^+ influx in Mg^{2+}-depleted and in amiloride-blocked hamster red cells as a function of $[Na^+]_o$. Filled circles: Na^+ influx in Mg^{2+}-depleted cells. Open circles: Na^+ influx in fresh cells incubated in the presence of 2 mM amiloride. Points represent the mean ± SE of four experiments. The linear regression for Na^+ influx shown is for Mg^{2+}-depleted cells only and has the equation:

$$Na^+ \text{ influx} = 0.07 + 0.015[Na^+]_o. \quad (3)$$

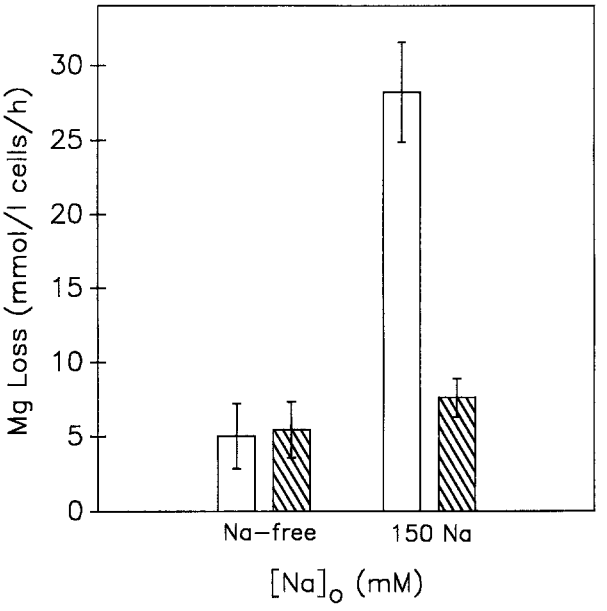

Fig. 3. Mg^{2+} loss from hamster red cells. Cells were equilibrated in medium with 10 mM Mg^{2+} and ionophore, A23187. After A23187 was removed as described in Materials and Methods, cells were placed in Mg-free medium either containing 150 mM NaCl or with NaCl replaced osmotically with choline Cl. Open bars: Mg^{2+} loss in medium without amiloride; hatched bars: Mg^{2+} loss in medium with 2 mM amiloride. Means ± SE of three experiments are represented.

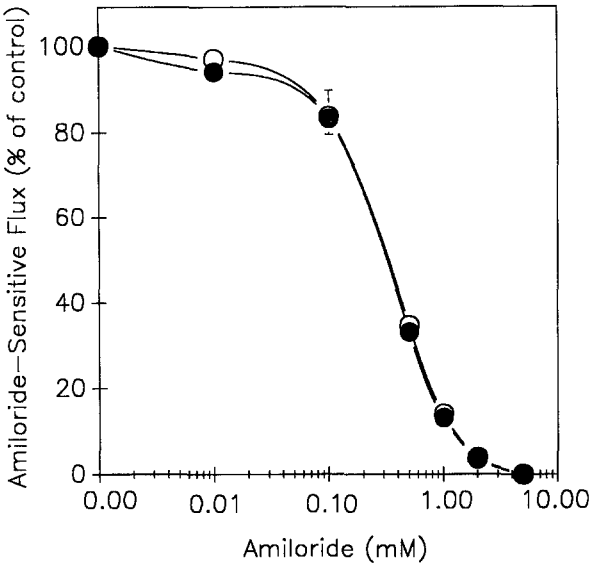

Fig. 4. Effect of amiloride on Na^+ influx and Mg^{2+} extrusion in hamster red cells. Cells were loaded with Mg^{2+} by incubation in medium with 10 mM $[Mg^{2+}]_i$ and ionophore, as described in Materials and Methods. Na^+ influx and net Mg^{2+} loss were measured in separate sets of cells from the same animal treated in parallel as described in Materials and Methods. The incubation medium contained (mM) 150 NaCl, 10 glucose, 5 adenosine, 10 MOPS buffer and 2 ouabain. The ordinate represents percent reduction of flux between control cells (Na^+ influx, 37.52 ± 2.81 mmol/liter cells/*h*); Mg^{2+} efflux, 19.19 ± 1.44) and cells incubated in 5 mM amiloride (Na^+ influx, 1.94 ± 0.31; Mg^{2+} efflux, 2.92 ± 0.51). Open circles: Na^+ influx; filled circles: net Mg^{2+} loss. Points represent means of three to four experiments. SE bars are shown where larger than the symbols.

When the intracellular Mg^{2+} concentration was increased from 0.3 to 5 mM, the rate of amiloride-sensitive Na^+ influx increased by about 40-fold. The amiloride-sensitive Na^+ influx in hamster red cells exhibited a saturation at a high concentration of $[Mg^{2+}]_i$ (above 5 mM), and the data could be fitted to the Hill equation (Fig. 5*A*). Kinetic analysis of $[Mg^{2+}]_i$-activated amiloride-sensitive Na^+ influx based on the data of seven experiments shows a sigmoidal dependence on $Mg^{2+}{}_i$ with a $[Mg^{2+}]_{0.5}$ of 2.1 mM and a Hill coefficient of 1.8 (Fig. 5). The maximum transport rate (V_{max}) was 40.8 ± 1.1 mmol/liter cells/hr (mean ± SE, $n = 4$).

The corresponding experiment for $[Mg^{2+}]_i$-activated Mg^{2+} loss into the Mg-free medium containing 150 mM NaCl is also shown in Fig. 5. The amiloride-sensitive net Mg^{2+} loss also saturated at high $[Mg^{2+}]_i$ (above 5 mM), and all data could be fitted to the Hill equation (Fig. 5*A*). The kinetics of $[Mg^{2+}]_i$-activated amiloride-sensitive Mg^{2+} loss based on graphic analysis of data of seven experiments yields essentially the same values for net Mg^{2+} loss as for the amiloride-sensitive Na^+ influx: $[Mg^{2+}]_{0.5}$ of 2.3 and h of 1.8. V_{max} of net Mg^{2+} loss was 19.8 ± 0.1 mmol/liter cells/hr (mean ± SE, $n = 4$), yielding a Na:Mg stoichiometry of 2.1. (V_{max} for Na^+, 40.8, above).

Dependence of Na^+ Influx and Mg^{2+} Loss on Extracellular Na^+

If the transport of Na^+ and of Mg^{2+} are coupled to each other in hamster red cells, both amiloride-sensitive Na^+ influx and amiloride-sensitive Mg^{2+} loss should also depend on extracellular Na^+ in the same way. Accordingly, parallel determination of Na^+ influx and Mg^{2+} loss was done in cells loaded with 10 mM Mg^{2+}. The amiloride-sensitive Na^+ influx increased as the extracellular Na^+ concentration increased (Fig. 6), and the data could be fitted to a simple Michaelis-Menten equation (Fig. 6*A*). Kinetic constants were obtained by the analysis of an Eadie-Hofstee plot of all the data of eight experiments, and yielded a V_{max} of 62.9 ± 4.3 mmol/liter cells/hr (mean ± SE).

The measurements of amiloride-sensitive Mg^{2+} loss made on cells from the same individual hamsters at the same Na^+ concentrations are shown in Fig. 6. The data could also be fitted to a simple Michaelis-Menten equation and the kinetic constants were obtained in the same way as for Na^+ influx, and yielded virtually the same K_m from both Na^+ influx and Mg^{2+} loss (107 ± 16 and 105 ± 13 mM, respectively). V_{max} of Mg^{2+} loss was about half of that of Na^+ influx (ratio of transported Na:Mg = 1.9:1).

Characteristics of Na-Mg Exchange in Hamster Red Cells

The results above indicated that in hamster red cells amiloride-sensitive Na^+ influx is a faithful indicator of the activity of the Na-Mg exchange. On this basis, therefore, we attempted to characterize this mechanism and its regulation.

Requirement for ATP

ATP is required for the Na-dependent Mg^{2+} loss (*reviewed by* Flatman, 1991). The ATP content in fresh hamster red cells is 1.06 ± 0.05 mM (mean ± SE, $n = 8$). When $[ATP]_i$ was decreased from 1.06 mM to 0.07 mM, amiloride-sensitive Na^+ influx fell from 0.58 ± 0.02 (mean ± SE, $n = 4$) to 0.10 ± 0.01 (mean ± SE, $n = 4$) mmol/liter cells/hr ($P \leq 0.05$, Fig. 7). Similar results were observed in red cells loaded with 1 mM Mg^{2+}, and the maximum of amiloride-sensitive Na^+ influx still occurred down to about 0.15 mM $[ATP]_i$. Thus, an apparently high affinity for cell ATP was observed in Mg-loaded cells, with half saturation at about 90 μM, but the kinetics was not simple.

This requirement for ATP might represent either the need for an energy source or the role of ATP as a regulatory activator of the Na-Mg transporter. As a test for the latter possibility, fresh or Mg-loaded cells were in-

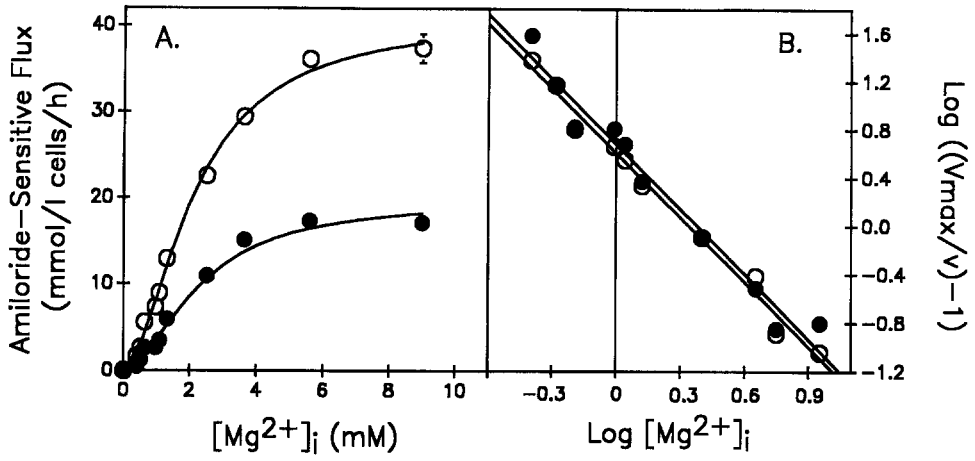

Fig. 5. Activation of amiloride-sensitive Na^+ influx and Mg^{2+} loss by intracellular Mg^{2+}. Cells were preloaded to various concentrations of $[Mg^{2+}]_i$ by A23187, then Na^+ influx (open circles) and net Mg^{2+} loss (filled circles) were measured in separate sets of cells from the same individuals, incubated under the same conditions as described in Fig. 4. $[Mg^{2+}]_i$ was determined from the equilibration curve in Fig. 1. (*A*) The curves drawn were based on values of kinetic variables obtained from Hill plots (*see B*). Points represent means ± SE, for three to five cases. (*B*) Hill plots of effect of $[Mg^{2+}]_i$ on Na^+ influx and Mg^{2+} extrusion. To compute the kinetic variables, "*h*" and "$[Mg^{2+}]_{0.5}$", values for V_{max} were first obtained by fitting data above 1 mM to a Michaelis-Menten curve. V_{max} (mmol/liter cells/hr) was 40.8 ± 1.1 (mean ± SE, $n = 4$) for Na^+ influx and 19.8 ± 0.1 (mean ± SE, $n = 4$) for net Mg^{2+} loss, and the ratio of transported Na:Mg^{2+} was 2.1. Points are based on the same data as shown in *A*. For Na^+ influx, *h* (Hill coefficient) = 1.8 ± 0.1, $[Mg^{2+}]_{0.5}$ = 2.1. For Mg^{2+} efflux, $h = 1.8 \pm 0.1$, $[Mg^{2+}]_{0.5}$ = 2.3 mM).

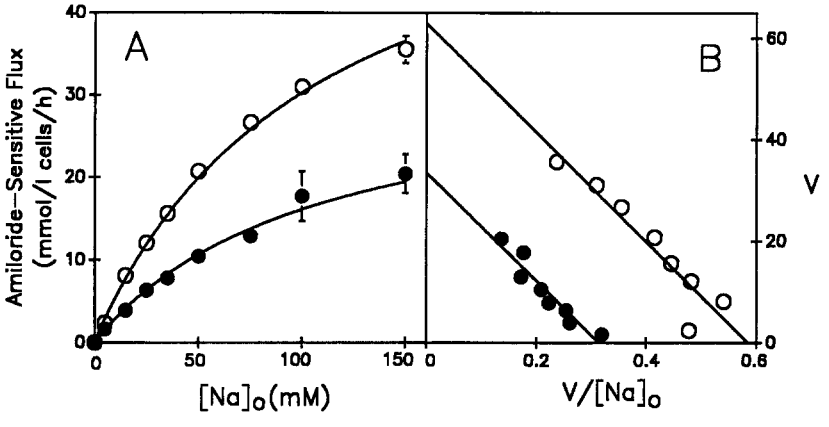

Fig. 6. Activation of amiloride-sensitive Na^+ influx and Mg^{2+} loss by extracellular Na^+. The cytoplasm of cells was equilibrated with 10 mM $MgCl_2$ as described in Materials and Methods. Na^+ influx (open circles) and net Mg^{2+} loss (filled circles) were measured in separate sets of cells from the same animals incubated in parallel under the same conditions and with samples for the fluxes collected over the same intervals. Choline chloride was used to replace NaCl osmotically. (*A*) Michaelis-Menten curves whose values of K_m and V_{max} were obtained from Eadie-Hofstee plots (*see B*). Points represent means ± SE of three to five experiments. (*B*) Eadie-Hofstee plots based on the same data as shown in *A*. The intercept represents V_{max} and the slope is $-K_m$. For Na^+ influx, V_{max} = 62.9 ± 4.3 mmol/liter cells/hr and K_m = 107 ± 16 mM; for Mg^{2+} efflux, V_{max} = 33.2 ± 2.1 and K_m = 105 ± 13.

cubated with dibutyryl-cAMP, with kinase promoter phorbol ester, with phosphatase inhibitors, vanadate and okadaic acid. None of these agents caused any significant effect on the rate of amiloride-sensitive Na^+ influx (*data not shown*).

Effect of Osmolarity of Influx Medium

Na-dependent Mg^{2+} loss in human red cells is stimulated by incubating cells in hypotonic medium (Féray & Garay, 1986). The following experiments were designed to see if stimulation of amiloride-sensitive Na^+ influx by hypotonic incubation could be observed in fresh and Mg-loaded hamster red cells. Table 1 shows that incubation in hypotonic medium (190–210 mOsM) significantly stimulated amiloride-sensitive Na^+ influx in fresh and Mg-loaded (1–10 mM) hamster red cells ($P \leq 0.05$, paired *t*-test).

Effects of Divalent Cations

When either Mg^{2+} or Mn^{2+} (1 mM) was included in the incubation medium, about 90% of the amiloride-sensitive Na^+ influx was inhibited, but higher concentra-

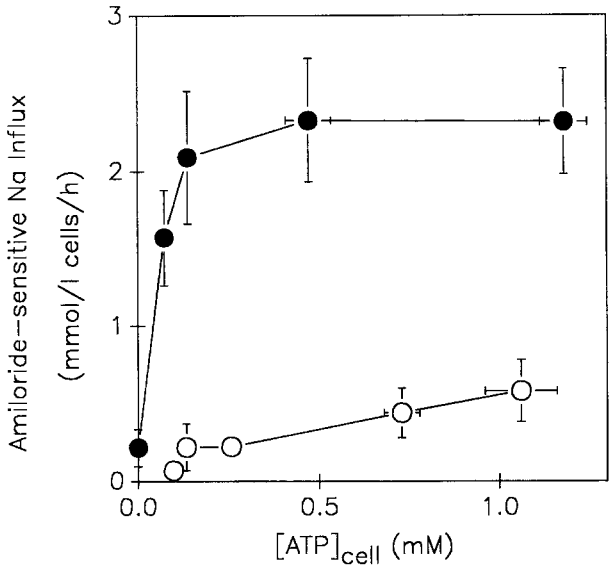

Fig. 7. ATP dependence of amiloride-sensitive Na^+ influx in hamster fresh and Mg-loaded red cells. To deplete their ATP, cells were incubated for varying periods in glucose-free, 10 mM 2-deoxy-glucose medium. For Mg-loaded cells, after depleting cell ATP, intracellular $[Mg^{2+}]_i$ was set to 1 mM by using A23187. Na^+ influx was determined in influx medium without glucose and with 2 mM ouabain. Open circles, fresh cells; filled circles, Mg-loaded cells. Points are means ± SE of four to six experiments.

tions produced no further inhibition (Fig. 8). Extracellular Ca^{2+} (1.25 mM) had no effect on amiloride-sensitive Na^+ influx (*data not shown*).

Attempts to Measure Amiloride-sensitive Na Efflux

Assuming that Na-dependent Mg^{2+} extrusion in vertebrate red cells is mediated by a common mechanism, the reaction mechanism of that transporter has not been resolved. By some accounts, it would appear to be a relatively simple exchanger (Günther & Vormann, 1990) and by others it appears to have the properties of an ATP-dependent pump (Lüdi & Schatzmann, 1987; Frenkel et al., 1989). A simple exchanger ought to show the property of reversibility dependent only on gradients of the transported ligands, as for example do the Na-Ca exchanger, the Na-H exchanger and the Cl-HCO_3 exchanger. A pump on the other hand might be expected to show a change in specificity from one side of the membrane to the other as the Na-K pump does. To investigate the reversibility of the exchanger in hamster cells from the standpoint of Na^+ movement, we measured Na^+ efflux under a variety of conditions.

Na^+ Efflux in Mg-rich, Na-free Medium (Reversal)

The first experiment on Na^+ efflux was designed to see if there was any amiloride-sensitive Na^+ efflux in hamster red cells activated by extracellular Mg^{2+}, and, if so, whether it could be stimulated by loading cells with higher concentrations of Na^+. Accordingly, unidirectional Na^+ efflux was measured with isotopic ^{22}Na in hamster red cells. As described in Materials and Methods, the efflux medium contained 20 mM $MgCl_2$ but no NaCl. Choline Cl was used to substitute osmotically for NaCl. The results, shown in Fig. 9, indicate that there was no amiloride-sensitive Na^+ efflux in hamster red cells loaded with 10 mM Na^+, ($P > 0.05$, compared with zero by paired *t*-test). Increasing $[Na^+]_i$ also did not stimulate amiloride-sensitive Na^+ efflux in hamster red cells (Fig. 9).

Na^+ Efflux in Medium with Na (Na-Na Exchange)

Although there was no amiloride-sensitive Na^+ efflux stimulated by extracellular Mg^{2+} in hamster red cells, we could not exclude the possibility of Na^+ efflux via Na-Na exchange through the Na-Mg pathway. Therefore, we incubated cells loaded with 50 mM Na^+ in a medium with or without Mg^{2+} and with and without extracellular Na (Table 2). There was no amiloride-sensitive Na^+ efflux when the efflux medium contained no Na^+ and no Mg^{2+} ($P > 0.05$, compared with zero by paired *t*-test), nor when the medium contained 150 mM Na^+. Nor was there any increase in Na^+ efflux in the presence of Na^+ compared with the absence of Na^+ (Table 3). The presence of Mg^{2+} (20 mM) in the medium did not stimulate amiloride-sensitive or Na-dependent Na^+ efflux in hamster red cells loaded with 50 mM Na^+.

Depletion of ATP by incubation in glucose-free medium with 2-deoxyglucose did not promote Na^+ efflux into Na-free medium in cells loaded with 50 mM Na^+ (Table 2). In two experiments, depletion of Mg^{2+} combined with depletion of ATP also did not promote Na-dependent, amiloride-sensitive Na^+ efflux (*data not shown*).

Finally, hyposmotic incubation, which activates amiloride-sensitive Na^+ influx, did not induce an amiloride-sensitive Na^+ efflux in cells loaded with 50 mM Na^+ and incubated in 150 mM Na^+ and 20 mM Mg^{2+} (*data not shown*).

Na^+ Efflux in Rat Red Cells

Günther et al. (1990) reported a large amiloride-sensitive Na^+ efflux in Na-loaded rat red cells by measuring $^{22}Na^+$ efflux. They found that amiloride-sensitive Na^+ efflux increased from 0.98 to 12.75 mmol/liter cells/15 min when $[Na^+]_i$ was raised from 10 to 150 mM. To see if the discrepancy between their results and ours was due to the difference in species, we repeated the determination of Na^+ efflux in rat red cells (Table 3). The amiloride-sensitive Na^+ efflux from rat red cells loaded

Table 1. Effect of osmolarity on amiloride-sensitive Na^+ influx in hamster red cells

Experimental conditions	N	Amiloride-sensitive Na^+ influx (mmol/liter/hr)	
		200 (mOsM)	300 (mOsM)
Fresh cells	5	1.46 ± 0.47*	0.50 ± 0.13
$[Mg^{2+}]$ in loading medium (mM):			
1	4	6.23 ± 0.31*	4.67 ± 0.38
3	3–4	23.89 ± 1.29**	19.07 ± 0.49
10	5	39.19 ± 5.66*	31.24 ± 3.19

Cell Mg^{2+} concentration was changed by preloading cell with 0–10 mM $MgCl_2$ using A23187 at 37°C for 15 min. All values are means ± SE. Osmotic concentration of hyposmotic Na^+ influx media was adjusted by adding water. The designations, "200" and "300" mean that the osmolarity of the influx medium was 190–210 and 290–310 mOsM, respectively. *$P \leq 0.05$ by paired t-test, compared with "300 mOsM" group. **$P < 0.01$ by t-test, compared with "300 mOsM" group.

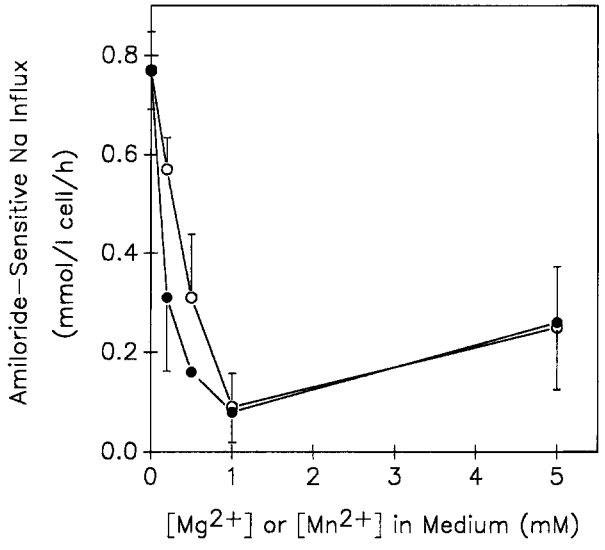

Fig. 8. The effects of extracellular Mg^{2+} and Mn^{2+} on amiloride-sensitive Na^+ influx in hamster red cells. Open circles, Mg^{2+} in the influx medium; filled circles, Mn^{2+} in the influx medium. Points represent means ± SE of three experiments.

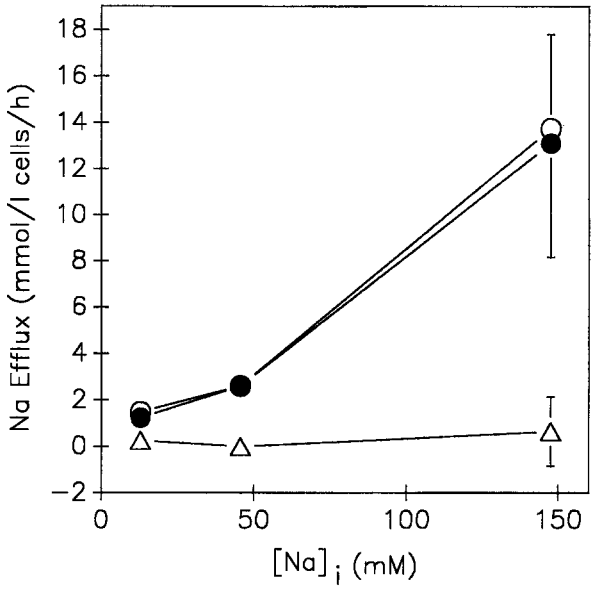

Fig. 9. Na^+ efflux into Na-free medium. Concentrations of $[Na^+]_i$ were set by equilibration of cells with various $[Na^+]_o$ in the presence of nystatin as described in Materials and Methods and were then equilibrated with ^{22}Na at 37°C for 30 min. Unidirectional Na^+ efflux was determined in Na-free medium containing 20 mM $MgCl_2$ and 2 mM ouabain. Amiloride-sensitive Na^+ efflux (open triangles) was obtained from the difference between total Na^+ efflux (open circles) and amiloride-insensitive Na^+ efflux (filled circles). Points represent means ± SE of three to four experiments.

with 150 mM Na (0.14 mmol/liter cells/hr, Table 2), though statistically significant, was negligible compared with that reported by Günther et al. (1990), and compared with the amiloride-sensitive Na^+ influx in both rat and hamster red cells (Willis et al., 1992).

The method used by Günther et al. (1990) differed slightly from ours in that their loading of the cells with Na^+ was carried out at 37°C, whereas ours was done at 0°C. Therefore, we repeated the determination, using the same method for loading as described in Günther et al. (1990). The results still showed an amiloride-sensitive Na^+ efflux of only 0.2 (Table 3).

Discussion

This study had two goals, to confirm that amiloride-sensitive Na^+ influx in hamster red cells was through the Na-Mg pathway and, assuming this to be the case, to use amiloride-sensitive Na^+ influx as a tool for investigating the mechanism of the Na-Mg pathway.

The first of these goals was met in several ways: the kinetics of activation of amiloride-sensitive Na^+ influx by cytoplasmic Mg^{2+} and by extracellular Na^+ matched those for activation of Mg^{2+} efflux; the kinetics of inhibition by amiloride of Na^+ influx matched that of Mg^{2+} efflux measured in the cells of the same animals at the same time; and, finally, amiloride-sensitive Na^+ influx displayed the same sort of dependence upon ATP and stimulation by cell swelling that had been observed

Table 2. Absence of Na-dependent, amiloride-sensitive Na^+ efflux in hamster red cells

Condition of Cells	N	$[Mg^{2+}]_o$ (mM)	Na Efflux (mmol/liter cells/hr)		
			150 $[Na]_o$	+Amil (Medium)	0 $[Na]_o$
Na-loaded	3	0	2.0 ± 0.1	1.9 ± 0.2	3.8 ± 0.3
Na-loaded	3	20	2.0 ± 0.3	2.9 ± 0.1	2.6 ± 0.4
Na-loaded, starved	3	0	2.2 ± 0.4	2.2 ± 0.5	3.4 ± 0.3

Cells were loaded with 50 mM Na^+ in the presence of nystatin as described in Materials and Methods. "Starved" cells were first incubated in glucose-free medium containing 2-deoxyglucose for 3–4 hr to decrease cytoplasmic ATP to less than 0.1 mM. Cells were then loaded with Na^+. Efflux medium contained 2 mM ouabain. Medium labeled "Amil" contained 150 mM Na and 2 mM amiloride. Medium labeled "0 [Na]" was Na free with choline replacing NaCl. Means ± SE are shown.

Table 3. Na^+ efflux in rat red cells

$[Na]_i$ (mM)	Condition	Na Efflux (mmol/liter cell/15 min)		
		This study		Günther et al. (1990)
		Note[a]	Note[b]	
10	No amiloride	0.36 ± 0.03	0.39	1.54
	+Amiloride (1 mM)	0.32 ± 0.02	0.32	0.56
	Amiloride-sensitive	0.04 ± 0.01*	0.07	0.98
150	No amiloride	1.27 ± 0.04	1.80	14.70
	+Amiloride (1 mM)	1.13 ± 0.08	1.60	1.95
	Amiloride-sensitive	0.14 ± 0.04*	0.20	12.75

[a] Na loading of cells and Na^+ efflux were done according to procedure described for hamster cells in Materials and Methods. Data are means ± SE of three to four experiments. *$P \leq 0.05$ for difference from 0. [b] According to Günther et al. (1990), cells were preloaded to 150 mM Na^+ by 30 μg/ml nystatin, then nystatin was removed by washing the cells four times with Na loading medium plus 1% bovine serum albumin. For measuring Na^+ efflux, the cells loaded with 10 or 150 mM Na^+ were loaded with ^{22}Na by incubating with $^{22}NaCl$ in the Na^+ loading medium at 37°C for 30 min. Extracellular ^{22}Na was removed by washing cells in Na^+ loading medium. Na^+ efflux was done in Mg-free and Na^+ (150 mM) flux medium. Data are means of two experiments.

by others for Na-dependent Mg^{2+} transport (Féray & Garay, 1986; Frenkel et al., 1989). Inhibition of the pathway by extracellular Mg^{2+} and Mn^{2+} was also in accord with expectation since these ions have been shown to exchange with intracellular Mg^{2+} via this pathway (Féray & Garay, 1987; Günther & Vormann, 1987).

The finding that Mg^{2+} depletion of hamster red cells resulted in Na^+ influx that was linearly dependent upon extracellular $[Na^+]$ and equivalent to Na^+ influx of nondepleted cells in the presence of amiloride (Fig. 2), supports our previous conclusion (Willis et al., 1992) that amiloride-sensitive Na^+ influx into hamster red cells is *only* through the Na-Mg pathway and that, in particular, the alternative Na-H pathway is not operative in these cells. [In guinea pig red cells, which possess a Na-H exchanger, Mg depletion does not eliminate amiloride-sensitive Na^+ influx (Zhao & Willis, 1993).]

The form of the kinetics observed here, both for amiloride-sensitive Na^+ influx and Na-dependent Mg^{2+} efflux, was generally similar to those seen by others for Mg^{2+} efflux alone in mammalian red cells, slight sigmoidicity for dependence on cytoplasmic Mg^{2+} and rectangularly hyperbolic for dependence upon extracellular $[Na^+]$ (Féray & Garay, 1986, 1987; Lüdi & Schatzmann, 1987). [Grouped data of Lüdi & Schatzmann (1987) for Na^+ activation of Mg^{2+} loss from human red cells showed a slight sigmoidicity, $h = 1.34$, but results from individual experiments did not.] The stoichiometry of $Na:Mg^{2+}$ exchange that we observed appears to be an electroneutral 2:1. This is lower than the value of 3:1 attributed to the process in rat red cells by Féray and Garay (1988), but they were unable to account for possible entry of Na^+ by other pathways. Given this stoichiometry, the sigmoidal dependency on cytoplasmic $[Mg^{2+}]$ would seem to imply a regulatory effect of Mg^{2+} on the cytoplasmic side of the membrane, analogous to that of H^+ in the Na-H exchange mechanism.

A stoichiometry of 2 Na:1 Mg ought, conversely, to predict a sigmoidicity of Na^+ dependence, yet this has not been observed here or elsewhere (*see above*). A formally analogous situation exists in human red cells, where, in the absence of extracellular Na^+, the kinetics of activation of the Na-K pump by extracellular K^+ appear hyperbolic, even though the stoichiometry involved

is 2 K^+ moved per pump cycle (Garrahan & Glynn, 1967). Analysis of this situation (Lew, Hardy & Ellory, 1973; Cavieres, 1977) indicated that one of the extracellular K sites (in the absence of extracellular Na^+) had an affinity of binding for K far higher than the other, so that only the binding of Na^+ to the low affinity site appeared to govern the transport event. Other possible explanations for the Na-Mg system are possible: the coupling ratio may be 2:1 only at high extracellular [Na^+], at lower Na^+ concentrations other ions may substitute for one of the Na^+ ions (e.g., H^+, as in the Na-K pump).

With regard to the mechanism of transport, the most important unresolved question is the source of energy for Mg^{2+} extrusion. On the face of it there appear to be two sources available: ATP and the Na^+ gradient. There have been suggestions that a Na- and Mg-dependent or amiloride-inhibitable ATPase may be present in human (Lüdi & Schatzmann, 1987) and rat (Günther et al., 1990) red cells that might correlate with this transport system. The strong ATP dependence of the system observed here for Na^+ influx and by others for Mg^{2+} extrusion could equally well reflect a regulatory role rather than an energetic one, as has been documented for Na-Ca exchangers in general and for a Na-Mg exchanger in squid giant axon (DiPolo & Beaugé, 1988) and suggested for a Na-Mg exchanger in ferret red cells (Flatman & Smith, 1991). However, our efforts to demonstrate such a role were inconclusive; neither kinase promoters, nor phosphatase inhibitors, nor dibutyryl-cAMP had any effect on amiloride-sensitive Na^+ influx. On the other hand, we had no independent means to verify the efficacy of these agents on their primary targets.

With a stoichiometry of 2 Na:1 Mg, there should be enough energy available in the Na^+ gradient to account for a Mg^{2+} gradient 30 to 60 times greater than that observed. However, Lüdi and Schatzmann (1987) found that when the Na^+ gradient was reversed in human red cells by loading them with Na^+, there was virtually no uptake of Mg^{2+} from the medium into cells with initially low cytoplasmic free Mg^{2+} concentration. From this they concluded that the system was not a readily reversible carrier in which the Na^+ gradient could be assumed to be the sole operative source of energy for extrusion of Mg^{2+}. The relatively large concentration of free cytoplasmic Mg^{2+} (0.3–0.6 mM in unmolested cells), compared with an equilibrium value for an exchange carrier (in the range of 0.01 mM), may, alternatively, be a reflection of the low affinity for Mg^{2+} of the Na-Mg transporter ($[Mg^{2+}]_{0.5} = 2.1$), combined with a significant inward leak.

Our observation that there is no amiloride-sensitive Na^+ efflux in hamster red cells loaded with Na^+ and incubated in Na-free medium with high Mg^{2+} concentration implies that the system is effectively irreversible at the Na^+ entry step. This accords with the observation of Lüdi and Schatzmann (1987) of irreversibility of the overall reaction, but it appears to conflict with their conclusion that the carrier is passively mobile when it binds Na^+. Indeed, the absence of even exchange diffusion (amiloride-sensitive Na-dependent Na^+ efflux) through the pathway, with or without high cytoplasmic ATP and with or without appreciable free cytoplasmic Mg^{2+}, indicates that Na^+ entry is even more irreversible than might be expected of an ATP-dependent pump!

[We are unable to account for the disparity between our results with hamster and rat red cells and those of Günther et al. (1990) on rat red cells. Günther et al. (1990) did not consider the possibility of a Na-H exchanger as a parallel amiloride-sensitive Na pathway. It should also be noted that the amiloride-sensitive Na^+ efflux reported by those authors, in cells loaded with 10 mM Na^+, was considerably in excess of the rate seen in guinea pig red cells, where, in addition to a Na-Mg transporter a robust Na-H exchanger is also present (Zhao & Willis, 1993).]

We conclude that the Na-Mg transport pathway in hamster red cells, like that in human red cells, is not a simple, reversible exchange carrier. Whether it is an ATP-utilizing pump remains problematic. The properties observed by us and by Schatzmann and his coworkers (Lüdi & Schatzmann, 1987; Frenkel et al., 1989) favor this hypothesis, yet the peculiar form of the ATP kinetics observed here and the absence of any inhibition by vanadate (Frenkel et al., 1989; Xu, 1993) suggest that this is no ordinary transport ATPase of the "E_1E_2" type. Whatever the outcomes of these uncertainties, red cells of hamster should provide a useful resource for resolving them.

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