Effect of intracellular magnesium on calcium extrusion by the plasma membrane calcium pump of intact human red cells

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1. The effect of varying the concentration of intracellular magnesium on the Ca^{2+}-saturated Ca^{2+}-extrusion rate through the Ca^{2+} pump (ϕ_{max}) was investigated in human red blood cells with the aid of the divalent cation ionophore A23187. The aim was to characterize the $[Mg^{2+}]_i$ dependence of the Ca^{2+} pump in the intact cell.

2. The initial experimental protocol consisted of applying a high ionophore concentration to obtain rapid sequential Mg^{2+} and $[^{45}Ca]CaCl_2$ equilibration, prior to measuring ϕ_{max} at constant internal $[Mg_T]_i$ by either the Co^{2+} block method or by ionophore removal. With this protocol, competition between Ca^{2+} and Mg^{2+} through the ionophore prevented Ca^{2+} equilibration at high $[Mg^{2+}]_o$. To provide rapid and comparable Ca^{2+} loads and maintain intracellular ATP within normal levels it was necessary to separate the Mg^{2+} and the Ca^{2+} loading–extrusion stages by an intermediate ionophore and external Mg^{2+} removal step, and to use different metabolic substrates during Mg^{2+} loading (glucose) and Ca^{2+} loading–extrusion (inosine) periods.

3. Intracellular Co^{2+} was found to sustain Ca^{2+} extrusion by the pump at subphysiological $[Mg^{2+}]_i$. Ionophore removal was therefore used to estimate the $[Mg^{2+}]_i$ dependence of the pump at levels below $[Mg_T]_i$ (~2 mmol (340 g Hb)$^{-1}$), whereas both ionophore removal and Co^{2+} block were used for higher $[Mg_T]_i$ levels.

4. $[Mg^{2+}]_i$ was computed from measured $[Mg_T]_i$ using known cytoplasmic Mg^{2+}-buffering data. The ϕ_{max} of the Ca^{2+} pump increased hyperbolically with $[Mg^{2+}]_i$. The Michaelis parameter ($K_{½}$) of activation was $0{\cdot}12 \pm 0{\cdot}04$ mmol (l cell water)$^{-1}$ (mean ± S.E.M.). Increasing $[Mg_T]_i$ and $[Mg^{2+}]_i$ to 9 mmol (340 g Hb)$^{-1}$ and 2·6 mmol (l cell water)$^{-1}$, respectively, failed to cause significant inhibition of the ϕ_{max} of the Ca^{2+} pump.

5. The results suggest that within the physiological and pathophysiological range of $[Mg^{2+}]_i$, from 0·3 mmol (l cell water)$^{-1}$ in the oxygenated state to 1·2 mmol (l cell water)$^{-1}$ in the deoxygenated state, the Ca^{2+}-saturated Ca^{2+} pump remains unaffected by $[Mg^{2+}]_i$ at normal ATP levels.

Magnesium ions are essential cofactors of the plasma membrane Ca^{2+} pump at the inner membrane surface (Schatzmann & Vincenzi, 1969). Phosphorylation of the pump enzyme from ATP requires Ca^{2+} and micromolar ATP concentrations, but not Mg^{2+} (Caride, Rega & Garrahan, 1986). At physiological ATP concentrations, in the millimolar range, Mg^{2+} ions control the rate of the configurational change which allows the spontaneous release of P_i from the phosphorylated enzyme, the last step in the overall ATPase cycle (Garrahan & Rega, 1978). By this mechanism, Mg^{2+} ions regulate the turnover rate of the Ca^{2+} pump. The concentration dependence of Ca^{2+}-pump function on Mg^{2+} has been investigated in purified enzyme preparations (Dunham & Glynn, 1961; Graf & Penniston, 1981) and isolated membranes (Caride *et al.* 1986) and found to be biphasic, with inhibition by high Mg^{2+}.

In circulating red cells, $[Mg^{2+}]_i$ oscillates with the state of oxygenation of haemoglobin. The $[Mg^{2+}]_i$ varies between about 0·4 mM in fully oxygenated normal human red cells and 0·62 mM in fully deoxygenated cells (Flatman, 1980). The oscillations result from the reversible binding of the main cytoplasmic Mg^{2+} buffers, ATP and 2,3-DPG, to deoxygenated haemoglobin (Bunn, Ransil & Chao, 1971; Flatman, 1980). The variation is more marked in dehydrated sickle cell anaemia red cells (SS cells), which have reduced 2,3-DPG/Hb and ATP/Hb molar ratios (Ortiz, Lew & Bookchin, 1990). Increased $[Mg^{2+}]_i$ or $[Mg^{2+}]_i$/ATP ratios in the deoxygenated state, when SS cell Ca^{2+} permeability is increased, may affect the delicate pump–leak balance which holds $[Ca^{2+}]_i$ just below the levels required for activation of the Ca^{2+}-sensitive K^+ channels, a main dehydration-triggering mechanism in SS cells. In

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addition, deoxygenation of normal and SS red cells causes partial inhibition of the Ca^{2+} pump and this was found to contribute to polymer-induced elevated $[Ca^{2+}]_i$ in SS cells (Tiffert, Etzion, Bookchin & Lew, 1993; Etzion, Tiffert, Bookchin & Lew, 1993). The mechanism of this inhibition is unknown. It was suggested that Ca^{2+}-pump inhibition on deoxygenation may be produced by excess $[Mg^{2+}]_i$ or by an increased $[Mg^{2+}]_i$/ATP ratio (Tiffert *et al.* 1993), an effect demonstrated for the Na^+ pump in dense SS cells (Ortiz *et al.* 1990).

Clearly then, defining the precise $[Mg^{2+}]_i$ dependence of Ca^{2+} pump-mediated Ca^{2+} extrusion in the intact cell is not only of academic interest but particularly relevant for the understanding of Ca^{2+} transport disfunction in sickle cell anaemia. Assumptions based on data concerning the effect of Mg^{2+} on the Ca^{2+} pump obtained from isolated preparations cannot be directly applied to whole erythrocytes as cell disruption compromises the complex system of interacting factors that controls Ca^{2+} pump function (Wang, Villalobo & Roufogalis, 1992). Xu & Roufogalis (1988) and Wu, Hinds & Vincenzi (1992) recognised the need to investigate the intact cell condition, and their pioneering work provided a preliminary characterization of the variation of Ca^{2+}-ATPase activity (Wu *et al.* 1992) and active Ca^{2+} efflux (Xu & Roufogalis, 1988) with $[Mg^{2+}]_o$ when red cells were exposed to the ionophore A23187. The specific questions that remained open, and which the present experiments were designed to answer, concerned the discrimination between effects of internal Co^{2+} and Mg^{2+}, and the precise dependence of the ϕ_{max} of the Ca^{2+} pump on $[Mg^{2+}]_i$ when Ca^{2+} efflux is measured at normal ATP levels and after comparable Ca^{2+} loads.

We report here the development and application of experimental protocols for studying the Mg^{2+} dependence of the Ca^{2+} pump in intact human red cells at physiological ATP concentrations. The results defined the relation between Ca^{2+}-saturated Ca^{2+} extrusion by the Ca^{2+} pump, total intracellular magnesium concentration ($[Mg_T]_i$) and $[Mg^{2+}]_i$ over a wide concentration range.

METHODS

Preparation of the cells

Blood was drawn from healthy unpaid volunteers, after informed consent, using heparin as anticoagulant. The cells were washed four times with a solution containing (mM): KCl, 80; NaCl, 70; Hepes-Na (pH 7·6), 10; and Na-EGTA, 0·02. After each centrifugation (2500 *g* for 5 min) any remaining buffy coat was removed by aspiration. After the last wash, the packed cells were resuspended at about 10% haematocrit in either of two incubation solutions (A or B) containing (mM): KCl, 76; NaCl, 67; Hepes-Na (pH 7·6), 20; and Na-EDTA, 0·10. Solution B was the same as A except that it had no EDTA. The use of high K^+ equilibrium solutions was intended to prevent substantial changes in cell volume, pH, membrane potential and overall ion content (other than Mg^{2+} and Ca^{2+}) in cells with Ca^{2+}-activated K^+ permeability (Lew & García-Sancho, 1989).

Measurement of Ca^{2+}-saturated Ca^{2+}-pump rate in red cells with different $[Mg_T]_i$

The initial experimental protocol (one-stage protocol) was as follows. Cells suspended in solution A with appropriate metabolic substrates and different $[Mg_T]_o$ were incubated at 37 °C, and exposed to a high concentration of the ionophore A23187 (Boehringer). After Mg^{2+} equilibration [^{45}Ca]$CaCl_2$ (Amersham International plc), was added, and 2 min later, the ionophore-mediated Mg^{2+} and Ca^{2+} permeabilities were blocked either by addition of Co^{2+} in excess of Ca^{2+} (Dagher & Lew, 1988) or by ionophore washout with albumin-containing solutions (Lew & García-Sancho, 1989; Pereira, Samellas, Tiffert & Lew, 1993). The $[Ca^{2+}]_i$-saturated Ca^{2+}-extrusion rate through the Ca^{2+} pump, ϕ_{max}, was then estimated from the rate of decrease in cell [^{45}Ca]$CaCl_2$ content with time and related to the measured $[Mg_T]_i$ content of the cells. Failures of the one-stage protocol, analysed in Results, led to the development of a two-stage protocol with Mg^{2+} and Ca^{2+}-loading stages separated by an intermediate ionophore-washout step. Each procedure is described in detail below. The rationale for some of the steps will become apparent in Results.

Loading and depletion of cell magnesium

The ionophore A23187 was dissolved to 5 mM in DMSO/ethanol (20/80 v/v) and added, with rapid stirring, to a 10% suspension of cells in solution A. The resultant ionophore concentration in the suspension was 15 μM. To produce a range of $[Mg_T]_i$, the cells were then diluted to 1% haematocrit by dividing the suspension between several tubes each containing solution A plus 10 mM glucose but each with a different concentration of $MgCl_2$ in the 0–5 mM range. As the ionophore partitions between the cells and aqueous solution at a ratio of about 50/1 (Simonsen & Lew, 1980) the cell concentration of the ionophore was approximately 150 μmol (l cells)$^{-1}$, which was sufficient to allow complete equilibration of Mg^{2+} during a 15 min incubation at 37 °C (Flatman & Lew, 1980) even at the highest Mg^{2+} concentrations.

The one-stage protocol

Suspensions were centrifuged immediately after Mg^{2+} loading and concentrated again to 10% haematocrit. The suspensions were warmed to 37 °C, and [^{45}Ca]$CaCl_2$ was added to 200 μM to initiate ionophore-induced Ca^{2+} influx. The specific activity of [^{45}Ca]$CaCl_2$ used throughout was about 400 kBq (μmol)$^{-1}$.

The two-stage protocol

Following Mg^{2+} loading, suspensions were centrifuged immediately. Extracellular Mg^{2+} and the ionophore were removed by washing the cells twice in ice-cold wash solution with 1·5% albumin and twice in solution B. The cells were resuspended to a 10% haematocrit in solution B with 10 mM inosine. After warming the suspension to 37 °C, first [^{45}Ca]$CaCl_2$ and then A23187 were added to give concentrations of 120 μM and between 5 and 15 μM, respectively, in the cell suspension. During Ca^{2+} loading, some $[Mg_T]_i$ was lost through the ionophore into the Mg^{2+}-free external solution. Therefore, to produce Ca^{2+}-loaded cells with particular $[Mg_T]_i$ levels using this protocol, it was necessary to overload the cells with Mg^{2+} during the first stage of the experiment, as detailed in Results.

Measurement of active Ca^{2+} efflux

When excess Co^{2+} was used to block Ca^{2+} and Mg^{2+} transport through the ionophore (Tiffert, García-Sancho & Lew, 1984; Dagher & Lew, 1988; Pereira *et al.* 1993), $CoCl_2$ was added from a 100 mM aqueous solution to give 200–400 μM in the cell suspension. Following Co^{2+} addition, frequent, timed samples were taken to assess the rate of decline of total intracellular calcium concentration, $[Ca_T]_i$. For ionophore washout 2–4 ml of the cell suspension were added to 40 ml of ice-cold wash solution containing 1·5% albumin, rapidly mixed and centrifuged in the cold. This wash procedure was repeated once and then the albumin solution replaced with solution B for two more washes before the cells were packed. It was essential to keep the temperature below 4 °C throughout to prevent Ca^{2+} efflux through the pump (Lew & García-Sancho, 1989). To initiate the ϕ_{max} measurement, 200 μl of ice-cold packed cells were added to 1·60 ml of solution B at 42 °C to ensure rapid warming of the cells to a suspension temperature of 37 °C; samples were taken at 15–30 s intervals thereafter.

For $[Ca_T]_i$ measurement, 50 μl samples were delivered to 1·5 ml nominal capacity microfuge tubes containing 1·25 ml of ice-cold wash solution with 400 μM $CoCl_2$; the low temperature inhibited the Ca^{2+} pump while the excess Co^{2+} prevented loss of Ca^{2+} through the ionophore. After a 40 s centrifugation at 4 °C, the supernatants were aspirated and the cells deproteinized by addition of 500 μl of 5% trichloroacetic acid (TCA). The clear extract was processed for scintillation counting of ^{45}Ca radioactivity as previously described (Dagher & Lew, 1988).

Standardization of measurements

As red cell volumes change slightly with different Mg^{2+} loads (Flatman & Lew, 1980), total cell solute concentrations were expressed per 340 g Hb, which represents 1 l of red cells at their initial volume, prior to Mg^{2+} loading. In this way total cell contents are always referred to the same number of cells. Haemoglobin was measured by the cyanomethaemoglobin method.

Measurement of $[Mg_T]_i$

When $[Mg_T]_i$ was estimated to be less than 200 μmol (340 g Hb)$^{-1}$, 500 μl samples of the 10% haematocrit cell suspensions were used, otherwise sample size was only 100 μl. Samples were mixed with 1·25 ml wash solution plus 400 μM $CoCl_2$ in 2 ml nominal capacity microfuge tubes and the cells washed twice to remove extracellular Mg^{2+}. The remainder of the procedure was that used previously by Flatman & Lew (1980). The cells were haemolysed in 700 μl water and a sample removed for haemoglobin estimation before the remaining 500 μl lysate was deproteinized by addition of 50 μl TCA (55%). After standing for 10 min, the tubes were centrifuged and the supernatant diluted with 640 μl water. The Mg concentration was then measured using atomic absorption spectroscopy. Sarstedt tubes (Sarstedt, No. 72.608), but not Eppendorff (Eppendorff, No. 3810) tubes (P. W. Flatman, personal communication), proved suitable for this assay as they contained no detectable Mg that could be leached out by acid.

The $[Mg^{2+}]_i$ levels (in mmol (l cell water)$^{-1}$), corresponding to each $[Mg_T]_i$ value (in mmol (340 g Hb)$^{-1}$), were calculated using the Flatman–Lew description of cytoplasmic Mg^{2+} buffering (Flatman & Lew, 1980). Correction for the minor changes in cell pH, ionic content and volume at high Mg^{2+} loads was done with the aid of the Lew–Bookchin red cell model (Lew & Bookchin, 1986), extended to incorporate divalent cation transport and cytoplasmic buffering (not shown). A compiled version of the programme of the extended model for use with IBM compatible computers is offered free on request, together with brief instructions and a full description of the extensions made to the original model.

Measurement of ATP

Cell ATP was measured by the method of Brown (1982) as modified by Dagher & Lew (1988). Samples (15 μl) of the 10% cell suspension were vortexed with 1·0 ml of ice-cold 100 mM HCl. It was possible to collect ATP samples over a 60 min period and assay them together as ATP was shown to be stable in cold HCl for 90 min (Dagher & Lew, 1988). After centrifugation, 15 μl of each extract was added to 2·5 ml of the combination buffer (Brown, 1982). Luciferase–luciferin (20 μl; FLE 50, Sigma) was added to the buffer with vigorous mixing and the resulting luminescence measured on a scintillation counter.

RESULTS

Search for optimal experimental protocol to measure Ca^{2+}-saturated Ca^{2+} extrusion through the pump at different $[Mg^{2+}]_i$ levels in the intact cell

Ideally, the protocol for investigating the $[Mg^{2+}]_i$ dependence of the Ca^{2+} pump through measurements of $[Ca^{2+}]_i$-saturated Ca^{2+} extrusion in intact cells should seek to attain: (i) sufficiently high Ca^{2+} loads to allow precise estimates of Ca^{2+}-extrusion rates before Ca^{2+} desaturation of the pump; (ii) uniform Ca^{2+} loading to prevent artifacts due to differences in Ca^{2+} content among the cells (García-Sancho & Lew, 1988*a*,*b*); (iii) moderately low, and comparable Ca^{2+} loads at all $[Mg^{2+}]_i$ levels, to minimize both inhibition by high $[Ca^{2+}]_i$ and differences in the extent of inhibition among cells with different $[Mg^{2+}]_i$; (iv) steady $[Mg^{2+}]_i$ levels during Ca^{2+}-extrusion measurements; (v) ATP concentrations within the non-limiting range for the Ca^{2+} pump at all $[Mg^{2+}]_i$ levels; and (vi) no interfering effects other than those of $[Mg^{2+}]_i$ on the Ca^{2+} pump. The experiments below report the development of the experimental protocol considered the best compromise.

The simplest experimental protocol to test the effects of $[Mg^{2+}]_i$ on transport in the intact red cell is that successfully applied in earlier studies on the $[Mg^{2+}]_i$ dependence of Na^+ pump-mediated transport (Flatman & Lew, 1981). The general design consists of incubating the cells in the presence of different $[Mg^{2+}]_o$, adding a high concentration of ionophore A23187 to induce rapid Mg^{2+} equilibration, and then adding the required tracer (substrate) to measure fluxes through the transporter under investigation, $[^{45}Ca]CaCl_2$ in this study.

The results of the experiments of Figs 1 and 2 illustrate some of the difficulties when this design is applied to study Ca^{2+} pump-mediated transport. In the experiment of Fig. 1, four aliquots of inosine-fed cells had each been loaded to different $[Mg_T]_i$ by addition of a high concentration of ionophore A23187 in the presence of different $[Mg^{2+}]_o$.

With the cells still suspended in the Mg^{2+}-loading solutions, [^{45}Ca]$CaCl_2$ was added, Ca^{2+} influx was followed for 10 min and, after addition of $CoCl_2$ in excess of $[Ca^{2+}]_o$, uphill Ca^{2+} extrusion was measured for a further 3 min (Fig. 1*A*). Throughout the periods of Ca^{2+} loading and extrusion, $[Mg_T]_i$ levels remained constant (Fig. 1*B*). It can be seen that cells depleted of Mg^{2+} ($[Mg_T]_i \approx 0{\cdot}03$ mmol (340 g Hb)$^{-1}$) accumulated Ca^{2+} rapidly, with $[Ca_T]_i$ reaching 600 μmol (340 g Hb)$^{-1}$ within 30 s of addition of $[Ca^{2+}]_o$, whereas at higher Mg^{2+} concentrations, both the rate of Ca^{2+} uptake and steady state $[Ca_T]_i$ levels decreased substantially. It took, for instance, cells with 7·0 mmol (340 g Hb)$^{-1}$ of $[Mg_T]_i$ about 5 min to reach a steady state $[Ca_T]_i$ level of only 360 μmol (340 g Hb)$^{-1}$. In the experiment of Fig. 2, a similar protocol to that used in the experiment of Fig. 1 was applied to ATP-predepleted cells (see legend to Fig. 2). It can be seen that here again increasing $[Mg^{2+}]_o$ levels slow down the rate of Ca^{2+} entry, but, in contrast to fed cells, the final $[Ca_T]_i$ levels attained prior to $CoCl_2$ addition were all similar. As expected from fuel-starved pumps, no uphill Ca^{2+} extrusion after Co^{2+} addition was observed.

Reduced Ca^{2+} influx at high $[Mg^{2+}]_o$ is the result of decreased effective Ca^{2+} permeability through the ionophore due to competition between Ca^{2+} and Mg^{2+} (Ferreira & Lew, 1976; Xu & Roufogalis, 1988) and is observed in both fed and ATP-depleted cells (Figs 1 and 2). Sub-equilibrium steady-state $[Ca_T]_i$ levels are only observed in fed cells (compare Fig. 1 with Fig. 2) and are thus the expression of pump–leak balance between Mg^{2+}-modified pump and leak fluxes. Sub-equilibrium $[Ca_T]_i$ levels and slow Ca^{2+} entry both introduce artifacts which will be considered in turn.

Earlier results (García-Sancho & Lew, 1988*a*, *b*) demonstrated that sub-equilibrium pump–leak $[Ca_T]_i$ steady states are not attained with a uniform Ca^{2+} distribution among cells but with extreme heterogeneity. This heterogeneity is well established by about 30 min after ionophore addition, but is less pronounced at shorter times (García-Sancho & Lew, 1988*b*). Therefore, Ca^{2+} extrusion measured from $[Ca_T]_i$ levels far below equilibrium (as at the higher $[Mg_T]_i$ conditions of Fig. 1) does not represent pump-mediated Ca^{2+} efflux from the bulk of the cell population. This limits comparability among conditions in

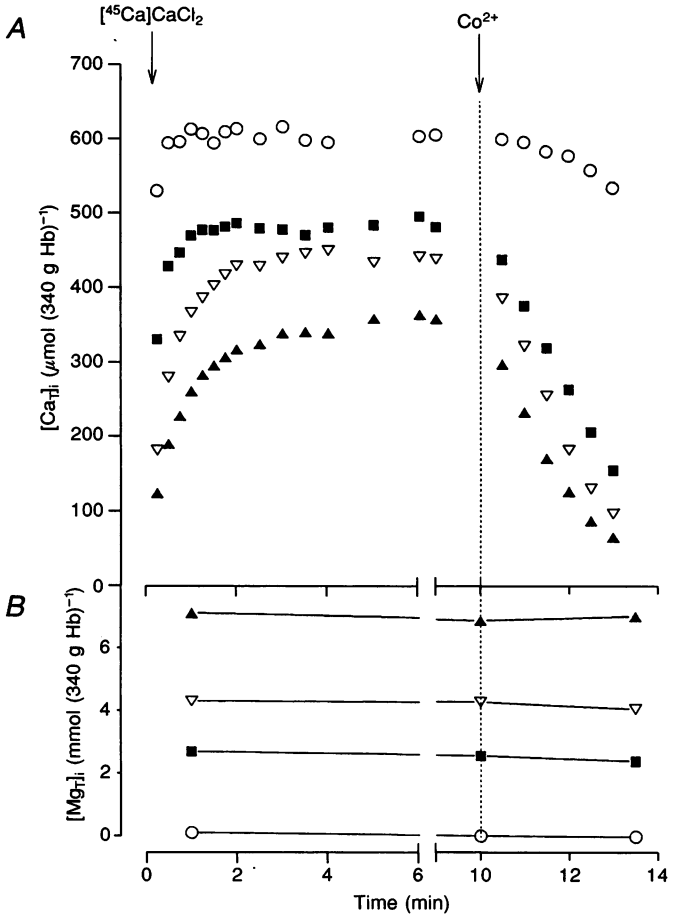

Figure 1. Ionophore-induced Ca^{2+} uptake, steady-state $[Ca_T]_i$ and pump-mediated Ca^{2+} extrusion in inosine-fed red cells loaded to various $[Mg_T]_i$

The cells were prepared by the one-stage protocol (see Methods). Inosine at 10 mM was present throughout the experiment. The ionophore A23187 was added to a final concentration of ~150 μmol (l cells)$^{-1}$. $[Mg_T]_o$ for Mg^{2+} loading or depletion was 0 (○), 0·5 (■), 1·0 (▽) or 2·0 (▲) mM. Calcium uptake (*A*) was initiated by addition of [^{45}Ca]$CaCl_2$ to 200 μM. After 10 min, ionophore-mediated Ca^{2+} transport was blocked with 400 μM $CoCl_2$ exposing the active Ca^{2+} efflux. *B* shows $[Mg_T]_i$ values measured during Ca^{2+} loading and efflux.

which differences in $[Mg_T]_i$ are combined with marked differences in sub-equilibrium $[Ca_T]_i$ levels.

Even if it were possible to load all the cells with Ca^{2+} to near equilibrium in the presence of different $[Mg^{2+}]_o$, slow Ca^{2+} entry at the higher $[Mg^{2+}]_o$ would generate variable degrees of ATP depletion among cells loaded with different $[Mg_T]_i$ and assayed for Ca^{2+}-pump activity long after the onset of the Ca^{2+} load. This is because ATP hydrolysis by the pump begins as soon as Ca^{2+} enters the cells, and no substrate can sustain glycolytic ATP synthesis at a rate to match ATP breakdown by a non-inhibited Ca^{2+} pump (Lew & García-Sancho, 1989). Rapid Ca^{2+} equilibration is therefore a necessary condition to prevent ATP depletion to limiting levels for pump activity. Previous results (Dagher & Lew, 1988) indicated that loading periods of up to 2 min cause minimal ATP fall, and this was aimed for in the subsequent experimental design.

With these considerations in mind, all that may be concluded from experiments such as that in Fig. 1, and others with similar protocols in the literature (Xu & Roufogalis, 1988), is that when $[Mg_T]_i$ is reduced below 0·05 mmol (340 g Hb)$^{-1}$ (○, Fig. 1), the pump is inhibited.

Preliminary experiments aimed at bypassing the limitations discussed above by increasing the ionophore concentration within the non-lytic range failed to reduce sufficiently the differences in sub-equilibrium $[Ca_T]_i$ levels. The alternative strategy was to separate the Mg^{2+}-loading from the Ca^{2+}-loading stage. By excluding $[Mg^{2+}]_o$ during Ca^{2+}-loading, it was expected that Ca^{2+} would rapidly distribute to near-equilibrium levels at all $[Mg_T]_i$.

Figure 3 shows the results of a typical two-stage experiment. In the first stage (not shown) the cells were loaded to different $[Mg_T]_i$ by incubation in the presence of A23187 and different $[Mg^{2+}]_o$. After Mg^{2+} equilibration, the cells were washed in ice-cold albumin solution to remove ionophore and $[Mg^{2+}]_o$. The ionophore was removed to minimize loss of $[Mg_T]_i$ during washes and subsequent procedures. After the washes, the cells were resuspended in Mg^{2+}-free solution B containing 120 μM $[^{45}Ca]CaCl_2$. On addition of a high concentration of A23187 to these suspensions Ca^{2+} rapidly entered the cells to similar and near-equilibrium levels within 2 min despite the wide range of $[Mg_T]_i$ remaining in the cells (0·02 to 11 mmol (340 g Hb)$^{-1}$; Fig. 3*A*). It can also be seen that Ca^{2+} entry on addition of ionophore was coincidental with a sharp fall in

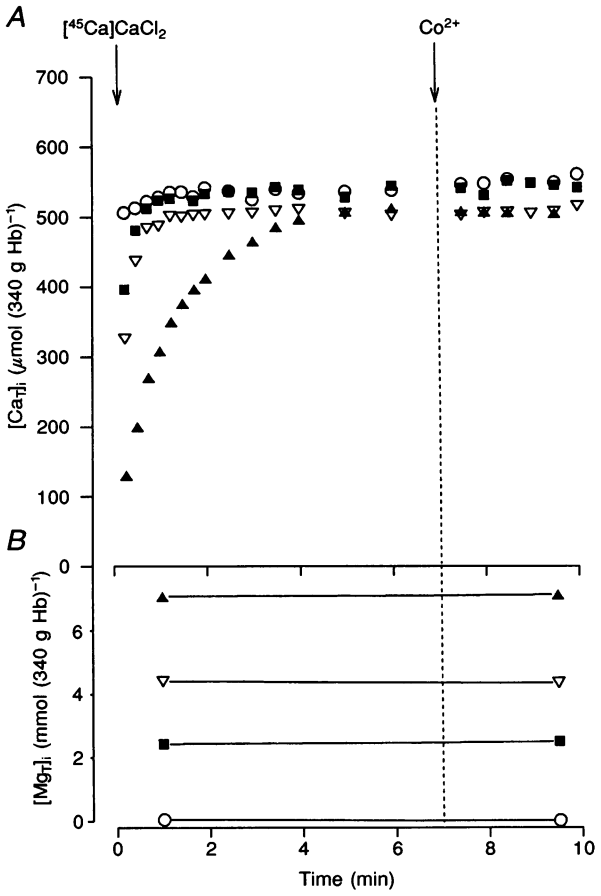

Figure 2. Ionophore-induced Ca^{2+} uptake and steady-state $[Ca_T]_i$ in ATP-depleted red cells loaded to various $[Mg_T]_i$

The cells were first incubated for 60 min at 37 °C with 10 mM inosine and 6 mM iodoacetamide. $[Mg_T]_i$ control and Ca^{2+} loading (*A*) were carried out as for Fig. 1. Seven minutes after the addition of $[^{45}Ca]CaCl_2$, $CoCl_2$ was added to 400 μM in the suspension. $[Mg_T]_i$ values are shown in *B*.

Table 1. Effect of $[Mg_T]_i$ on the maximum $[Ca_T]_i$ levels obtained during Ca^{2+} loading using the two-stage protocol

$[Mg_T]_i$ mmol (340 g Hb)$^{-1}$	A23187 μmol (340 g Hb)$^{-1}$	$[Ca_T]_i$ μmol (340 g Hb)$^{-1}$
0·04	50	639 ± 3
0·31	50	626 ± 7
1·0	50	588 ± 2
1·8	50	572 ± 7
2·4	150	625 ± 3
3·3	150	620 ± 9
4·1	150	585 ± 3
6·7	150	551 ± 5

The cells were albumin washed after Mg^{2+} loading and resuspended in solution B (Mg^{2+} free) with 120 μM $[^{45}Ca]CaCl_2$ before ionophore was added to initiate Ca^{2+} uptake. The reported $[Ca_T]_i$ was that measured 2 min after this addition of A23187 (mean ± S.E.M. of three measurements). For all cells except those with the highest $[Mg_T]_i$, $[Ca_T]_i$ had reached its maximum value and levelled off by 1 min; when $[Mg_T]_i$ was 6·7 mmol (340 g Hb)$^{-1}$, the $[Ca_T]_i$ was still increasing 2 min after ionophore addition.

$[Mg_T]_i$, and that further Mg^{2+} loss was limited by accumulation of Mg^{2+} in the external solution and then fully prevented by the addition of Co^{2+} (Fig. 3*B*). Thus, during the measurement of the Co^{2+}-exposed uphill Ca^{2+} extrusion through the pump, $[Mg_T]_i$ remained steady at all $[Mg_T]_i$ levels. Table 1 reports the range of Ca^{2+} loads and final steady $[Mg_T]_i$ levels obtained in another experiment similar to that illustrated in Fig. 3. The two-stage design, therefore, approaches the first four requirements of the 'ideal' protocol.

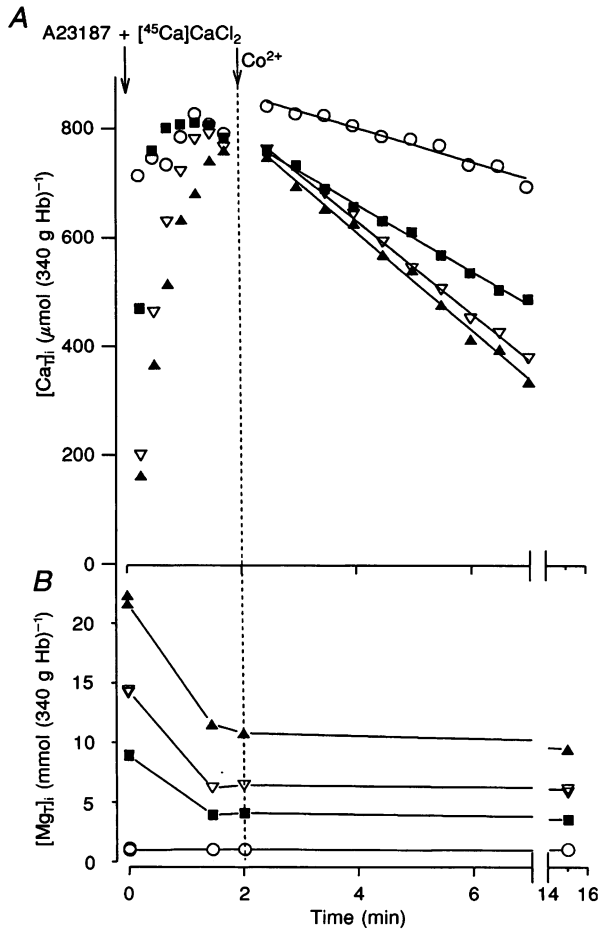

Figure 3. $[Mg_T]_i$ and $[Ca_T]_i$ during Ca^{2+} loading and Ca^{2+} efflux using the two-stage protocol

Cells were prepared using the two-stage protocol (see Methods). Cells loaded with various $[Mg_T]_i$ were suspended in medium B containing 120 μM $[^{45}Ca]CaCl_2$; A23187 was added to 150 μmol (l cells)$^{-1}$ to initiate the Ca^{2+} loading seen in *A*. Two minutes later, active Ca^{2+} efflux was exposed by the addition of 300 μM $CoCl_2$. $[Mg_T]_i$ was measured during Ca^{2+} loading and efflux and the results are shown in *B*.

The maintenance of near-normal ATP levels is considered next. Inosine had been shown to maintain red cell ATP levels more efficiently than glucose when the Ca^{2+} pump is fully active (Lew & García-Sancho, 1989). In agreement with these observations, the ATP level of inosine-fed cells with $[Mg_T]_i$ at either 0·02 or 6·2 mmol (340 g Hb)$^{-1}$ (and for intermediate levels, not shown) remained within 75% of the initial values throughout Ca^{2+} loading and the period of Ca^{2+}-pump rate measurement (Fig. 4*B*). Therefore, inosine can sustain non-limiting ATP levels during the final Ca^{2+}-loading and efflux stage at all $[Mg_T]_i$ investigated. During the initial Mg^{2+}-loading stage, however, inosine caused a substantial and unexpected decline in ATP levels, slightly steeper the higher the Mg^{2+} load (Fig. 4*A*). With glucose, on the other hand, ATP levels were maintained to within 85% of initial values throughout the 15 min, Mg^{2+}-loading step (Fig. 4*A*). Glucose and inosine were thus used sequentially during first and second stages, respectively, in all experiments reported below.

A final methodological consideration concerns the use of Co^{2+} to investigate the Mg^{2+} dependence of the Ca^{2+} pump. At normal cell $[Mg^{2+}]_i$ and ATP, the Ca^{2+}-pump activity of intact red cells, measured by Co^{2+} exposure (with high cell Co^{2+} (Tiffert *et al.* 1984)) or after ionophore washout (in the absence of Co^{2+}) gives identical results (Pereira *et al.* 1993). However, cobalt ions are known to be good substitutes for Mg^{2+} on the Na^+–K^+-ATPase (Richards, 1988) and to enter red cells via A23187 (Tiffert *et al.* 1984). It was also claimed that Co^{2+} could partially support Ca^{2+} pump-mediated Ca^{2+} accumulation within inside-out vesicles from red cell membranes in the absence of Mg^{2+} (Sarkadi, Enyedi & Gardos, 1981) but as these experiments were performed at 1 μM ATP it is doubtful whether Co^{2+} was catalysing the same configurational change for which Mg^{2+} requires high ATP. Taken together though, these results suggest the possibility that Co^{2+} may replace Mg^{2+} on the Ca^{2+} pump. It was therefore necessary to determine whether using Co^{2+} to study the Mg^{2+} dependence of the pump was appropriate. To this end, active Ca^{2+} extrusion from cells preloaded to different $[Mg_T]_i$ levels was measured in parallel using both Co^{2+} exposure and ionophore washout methods (Fig. 5).

Figure 5*A* reports Ca^{2+} efflux measured in the presence of Co^{2+} and in its absence. When $[Mg_T]_i$ was 0·027 mmol (340 g Hb)$^{-1}$, Ca^{2+} efflux after Co^{2+} addition was 3·1 ± 0·1 mmol (340 g Hb h)$^{-1}$ whereas after ionophore washout it was only 0·75 ± 0·13 mmol (340 g Hb h)$^{-1}$. This suggests that at low $[Mg_T]_i$, Co^{2+} can partially substitute for Mg^{2+} to sustain Ca^{2+} extrusion by the pump. The Co^{2+} effect is apparent at $[Mg_T]_i < 2$ mmol (340 g Hb)$^{-1}$ but at near-normal and higher $[Mg_T]_i$ levels the regression lines reporting pump-mediated Ca^{2+} efflux measured by Co^{2+} exposure or after ionophore washout become indistinguishable. Similar results obtained in another of three additional experiments are shown in Fig. 5*B*. All these results indicate that at the $[Co^{2+}]_i$ level reached in the present experiments, Co^{2+} can substitute for Mg^{2+} and stimulate the intact cell pump when it is inhibited by sub-physiological $[Mg_T]_i$, but that it does not interfere with Mg^{2+} at higher $[Mg_T]_i$ levels. Therefore, the results obtained

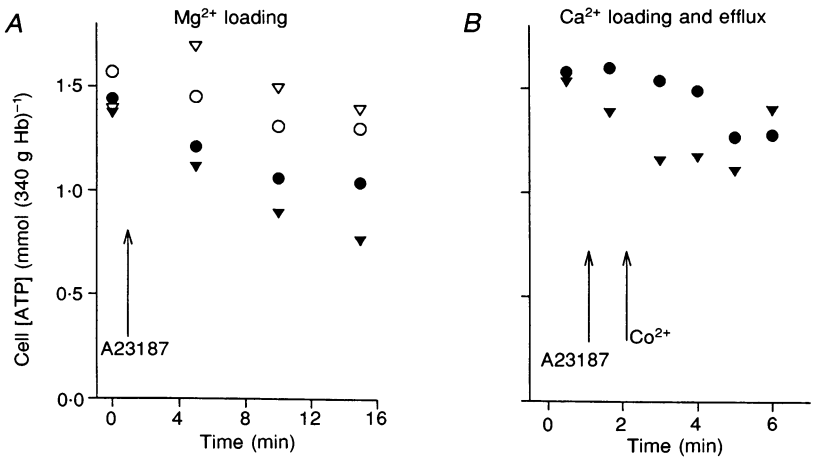

Figure 4. The effect of different glycolytic substrates on cell ATP content during the two-stage protocol

ATP concentrations seen in *A* were measured during $[Mg_T]_i$ loading or depletion (see Methods) in suspensions with 10 mM inosine with (▼) or without (●) 10 mM $[Mg_T]_o$ or in 10 mM glucose with (▽) or without (○) 10 mM $[Mg_T]_o$. A23187 was added at the time indicated (arrow). ATP concentrations were measured during Ca^{2+} loading and efflux in the presence of 10 mM inosine and the results are reported in *B*. The cells had been prepared according to the two-stage protocol with 10 mM glucose present during $[Mg^{2+}]_i$ loading. A23187 (50 μmol (l cells)$^{-1}$) and $CoCl_2$ (400 μM) were added at the times indicated (arrow). The $[Mg_T]_i$ values after $CoCl_2$ addition were 0·02 (●) or 6·7 (▼) mmol (340 g Hb)$^{-1}$.

with the Co^{2+} exposure method reliably report Ca^{2+}-pump activity when $[Mg_T]_i$ is in the 2–7 mmol (340 g Hb)$^{-1}$ range. At $[Mg_T]_i < 2$ mmol (340 g Hb)$^{-1}$, only the ionophore washout method returns reliable results on the Mg^{2+} dependence of the pump.

Dependence of Ca^{2+}-saturated Ca^{2+} extrusion through the Ca^{2+} pump on $[Mg_T]_i$

Figure 6 reports the combined results obtained in five experiments using the two-stage protocol. Stable $[Ca^{2+}]_i$ within the narrow range 550–650 μmol (340 g Hb)$^{-1}$ was achieved in these experiments prior to the Ca^{2+}-efflux measurement, despite a range of $[Mg_T]_i$ loads from 0·04 to 9·0 mmol (340 g Hb)$^{-1}$. For the suspensions with $[Mg_T]_i < 2{\cdot}0$ mmol (340 g Hb)$^{-1}$, only results obtained with the ionophore washout method are reported; for $[Mg_T]_i > 2{\cdot}0$ mmol (340 g Hb)$^{-1}$, results obtained by Co^{2+} exposure are also given. Figure 6*A* shows the flux data as a function of $[Mg_T]_i$. In Fig. 6*B*, pump activity is expressed as a function of $[Mg^{2+}]_i$. The results, fitted by Michaelis-type equations, show that activation of a Ca^{2+}-saturated pump by internal magnesium tends to saturate, perhaps with a mild inhibitory component at high Mg^{2+}. Least-squares fits of the data in Fig. 6*B* gave a half maximal activation of the Ca^{2+} pump ϕ_{max} by $[Mg^{2+}]_i$ of 0·12 ± 0·04 mmol (l cell water)$^{-1}$ (mean ± S.D.).

DISCUSSION

The results presented here define the Mg^{2+} dependence of Ca^{2+}-saturated Ca^{2+} efflux through the Ca^{2+} pump in the intact red cell. The estimated $K_{½}$ for $[Mg^{2+}]_i$ activation of ϕ_{max} was 120 ± 40 μmol (l cell water)$^{-1}$ (Fig. 6*B*) which is in reasonable agreement with the value of 88 μM obtained by Caride *et al.* (1986) using ATPase measurement in red cell membrane preparations at saturating $[Ca^{2+}]$ of 100 μM. Cobalt was also shown to activate the Ca^{2+} pump in whole red cells. For the single concentration investigated ($[Co^{2+}]_o = 400$ μM; $[Co^{2+}]_i < 250$ μmol (340 g Hb)$^{-1}$; Brown & Simonsen, 1985), activation by Co^{2+} was only observed when $[Mg^{2+}]_i$ was depleted ($[Mg^{2+}]_i < 300$ μmol (l cell water)$^{-1}$. At higher $[Mg^{2+}]_i$, with the Ca^{2+} pump almost fully saturated with Mg^{2+}, the effect of added Co^{2+} was negligible. These results indicate that, as with the Na^+ pump (Richards, 1988), both Co^{2+} and Mg^{2+} bind at a common activation site(s) on the Ca^{2+} pump. Our investigation of the Co^{2+} effect was motivated by the need to define conditions under which Co^{2+} could be used to block Ca^{2+} transport by A23187 without interfering with pump function. A more detailed investigation of the effect of Co^{2+} on the pump was thus beyond the scope of this study.

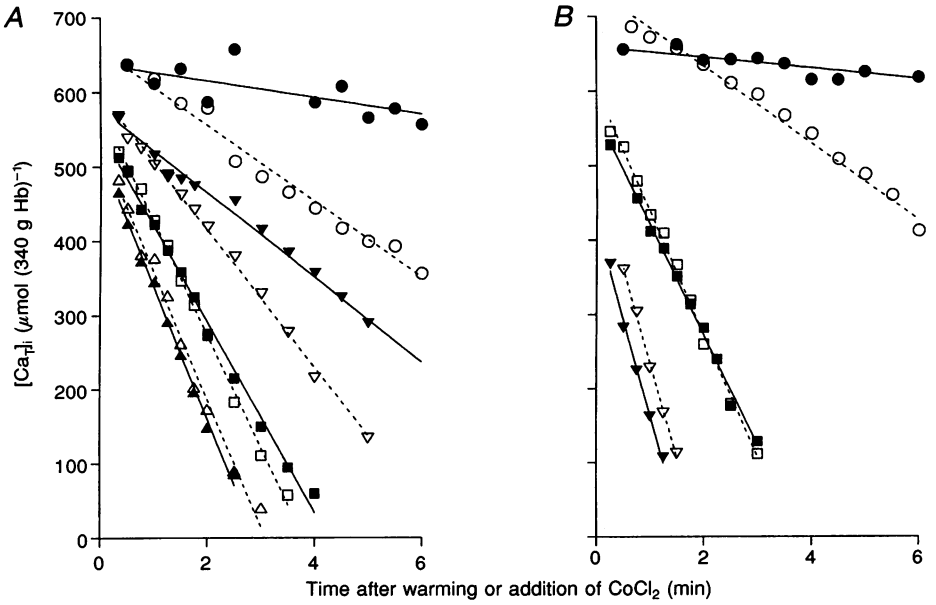

Figure 5. Ca^{2+} pump-mediated Ca^{2+} extrusion as a function of $[Mg_T]_i$ in the presence or absence of 400 μM $CoCl_2$

In two experiments (*A* and *B*), cells were prepared using the two-stage protocol (see Methods). Ca^{2+} efflux was exposed either by the addition of $CoCl_2$ (open symbols and dashed lines) or by washing the cells with ice-cold albumin solution followed by rapid warming (closed symbols and continuous lines). The Ca^{2+}-pump rate for each condition was evaluated as the slope of the straight line fitted onto the data. The $[Mg_T]_i$ values during Ca^{2+} efflux were, for *A*: 0·027 (○,●), 0·47 (▽,▼), 1·5 (□,■) and 2·2 mmol (340 g Hb)$^{-1}$ (△,▲); and for *B*: 0·02 (○,●), 1·8 (□,■), 7·2 (▽) and 6·4 mmol (340 g Hb)$^{-1}$ (▼). The symbols (□,■) correspond to control cells which were not exposed to A23187 until the Ca^{2+}-loading stage.

When the Mg^{2+} concentration exceeds that of the available ATP, the Ca^{2+}-ATPase activity of the purified enzyme (Graf & Penniston, 1981) and of disrupted red cell membranes (Caride *et al.* 1986), is inhibited by further increases in Mg^{2+}. However, significant inhibition requires very substantial elevation of Mg^{2+}; at 20 mM Mg^{2+} the Ca^{2+}-ATPase activity of disrupted red cell membranes is still 70% of the maximum value (Caride *et al.* 1986). Producing a comparable Mg^{2+} concentration within red cells would lead to extensive alteration of the physical and chemical state of the cells (Flatman & Lew, 1980).

Wu *et al.* (1992) reported a significant inhibition of Ca^{2+}-pump ATPase in intact cells by excess Mg^{2+}. In their experiments, Ca^{2+}-ATPase activity was measured by the decline in ATP in iodoacetic acid-poisoned cells exposed to external Ca^{2+}, Mg^{2+} and A23187. This indirect estimate of Ca^{2+}-ATPase activity is unreliable when uncorrected for adenylate kinase and adenosine monophosphate deaminase activity (Dagher & Lew, 1988), or for the effects of $[Mg_T]_i$ on the overall ATP metabolism of the poisoned cells. By contrast, Xu & Roufogalis (1988) found no inhibition of Ca^{2+}-ATPase activity or Ca^{2+} efflux from intact red cells even at $[Mg^{2+}]_i$ levels tenfold higher than normal. At such high Mg^{2+} levels, Ca^{2+} uptake was markedly reduced so that their measurements of the Ca^{2+}-pump rate would have been compromised by variations in Ca^{2+} loading among the cells (for a detailed discussion of this point see Results). As a consequence, it is difficult to determine whether the results show a genuine lack of inhibition as claimed (Xu & Roufogalis, 1988) or whether inhibition was masked by heterogeneity.

In the experiments presented here, $[Mg^{2+}]_i$ was increased to a maximum of 2·6 mmol (l cell water)$^{-1}$ without a conclusive demonstration of inhibition of the Ca^{2+}-saturated Ca^{2+} pump. Inclusion of a non-competitive inhibition factor in the Michaelis equation (Fig. 6) did not significantly improve the fit (note the 70% coefficient of variation returned for the inhibition constant (K_i) value in Fig. 6*B*). Therefore, the Ca^{2+} pump appears to differ from the Na^+ pump (Ortiz *et al.* 1990), in that high $[Mg^{2+}]_i$, up to 2·6 mmol (l cell water)$^{-1}$, does not significantly inhibit the Ca^{2+} pump of intact cells with normal ATP levels. It remains to be established whether or not an inhibitory effect of excess $[Mg^{2+}]_i$ becomes apparent when ATP is reduced, or at lower (non-saturating) levels of $[Ca_T]_i$.

Deoxygenation of normal or sickle cell anaemia red cells inhibits both the Na^+ pump and the Ca^{2+}-saturated Ca^{2+}

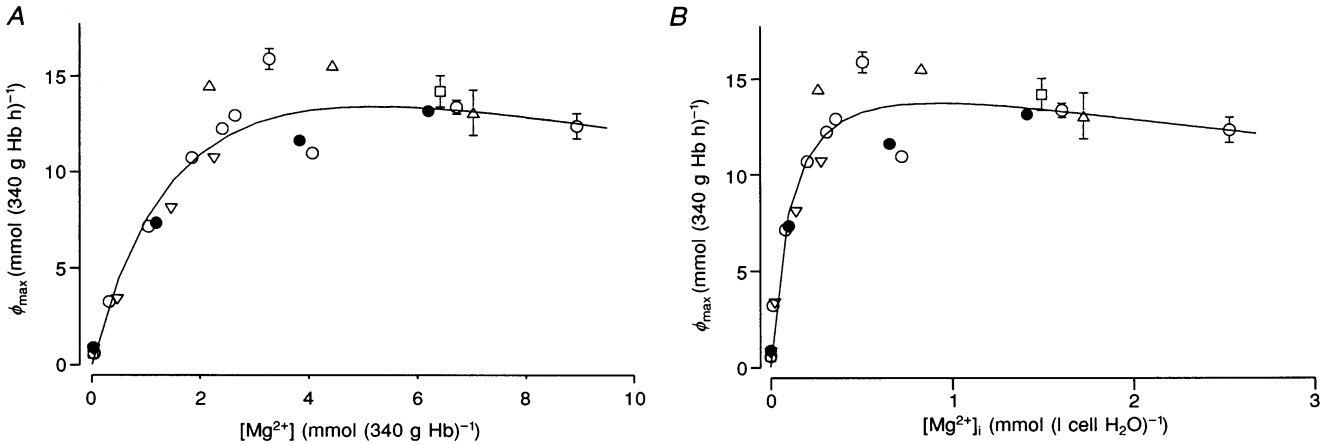

Figure 6. The dependence of Ca^{2+}-pump Ca^{2+} extrusion on $[Mg_T]_i$ and $[Mg^{2+}]_i$

The different symbols represent data from five different experiments in which cells were prepared using the two-stage protocol with 10 mM glucose as the metabolic substrate during Mg^{2+} loading. Calcium efflux was measured in the presence of 10 mM inosine after addition of 300 μM $CoCl_2$ when $[Mg_T]_i > 2$ mmol (340 g Hb)$^{-1}$ or by albumin wash. The curves represent the equation:

$$\phi_{max} = \phi'_{max}([Mg]/(K_{½} + [Mg]))(K_i/(K_i + [Mg])),$$

where ϕ_{max} is the Ca^{2+}-saturated Ca^{2+} efflux through the Ca^{2+} pump, ϕ'_{max} is the maximum Ca^{2+}-saturated Ca^{2+} efflux through the Ca^{2+} pump and [Mg] represents $[Mg_T]_i$ or $[Mg^{2+}]_i$ for panels *A* and *B*, respectively. Parameter values (± S.D.) obtained by least-squares fit of the equation to the data for *A* are $K_{½} = 2·9 \pm 2·4$ mmol (340 g Hb)$^{-1}$; $K_i = 9·5 \pm 16$ mmol (340 g Hb)$^{-1}$; $\phi'_{max} = 32 \pm 21$ mmol (340 g Hb h)$^{-1}$ and for *B*: $K_{½} = 0·12 \pm 0·04$ mmol (l cell water)$^{-1}$; $K_i = 7·3 \pm 5·0$ mmol (l cell water)$^{-1}$; $\phi'_{max} = 17·0 \pm 2·3$ mmol (340 g Hb h)$^{-1}$. The $[Mg^{2+}]_i$ values (*B*) were calculated from the measured $[Mg_T]_i$ data (*A*) using the Flatman–Lew description of cytoplasmic Mg^{2+} buffering (Flatman & Lew, 1980) and the Lew–Bookchin red cell model (Lew & Bookchin, 1986) to correct for cell volume changes at the high Mg^{2+} loads (see Methods).

pump (Tiffert *et al.* 1993; Etzion *et al.* 1993). Elevation of the $[Mg^{2+}]_i$/[ATP] ratio was suggested as the cause of Na^+-pump inhibition (Ortiz *et al.* 1990) and the same mechanism was proposed for Ca^{2+}-pump inhibition (Tiffert *et al.* 1993). The present results (Fig. 6*B*) have shown that throughout the range of physiological or pathophysiological $[Mg^{2+}]_i$ variations that can be induced by oxygenation–deoxygenation of red cells (i.e. from 0·3 to 1·2 mM; Bunn *et al.* 1971; Flatman, 1980; Ortiz *et al.* 1990), the Ca^{2+} pump was virtually saturated with Mg^{2+} but no inhibition occurred. It is therefore unlikely that an increased $[Mg^{2+}]_i$/[ATP] ratio *per se* is the cause of deoxygenation-induced Ca^{2+}-pump inhibition.

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