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EVIDENCE FOR A MAGNESIUM- AND ATP-DEPENDENT CALCIUM EXTRUSION PUMP IN DOG ERYTHROCYTES

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Summary

Intact dog erythrocytes, whose Ca^{2+} permeability had been increased with A23187 still maintained intracellular Ca^{2+} below electrochemical equilibrium indicating that they could extrude Ca^{2+}. This extrusion required no Na^+ gradient but apparently depended on intracellular ATP and Mg^{2+} suggesting that it was mediated by an ATP-fuelled Ca^{2+} pump.

Introduction

The dog erythrocyte is unusual in that it has a high intracellular Na^+ concentration [1,2] and no measurable ouabain-sensitive Na^+ pumping system [2—4]. It is, nevertheless, able to regulate its volume both in vivo [2] and in vitro [5] and it has been proposed that cell Na^+ and volume regulation in this cell occur via a Na^+/Ca^{2+} exchange system in which inward movement of Ca^{2+} down its electrochemical gradient fuels uphill Na^+ extrusion [5,6]. Since the lifetime of the dog erythrocyte in the circulation is about 100 days [7] and since dog erythrocytes are virtually Ca^{2+} free [6,8], a necessary requirement for the continuous operation of a mechanism dissipating a Ca^{2+} gradient is the presence of a Ca^{2+} pump to maintain that gradient. The presence of such a pump in this cell has not, to date, been explicitly demonstrated though Parker et al. [6] concluded from their data that there must be a Na^+-independent Ca^{2+} extrusion system in the cell. I present here evidence that the intact dog erythrocyte does have a plasma membrane Ca^{2+} pump and that, like the Ca^{2+} pump in human erythrocytes [9], it seems to require intracellular ATP and Mg^{2+} for its operation. It does not depend on the presence of an inwardly directed Na^+ gradient.

Methods

The approach used was essentially that described by Ferreira and Lew [10] and Lew and Brown [11] in which the divalent cation ionophore A23187 is used to increase the effective Ca^{2+} permeability of erythrocytes [12]. This allows the intracellular Ca^{2+} of intact cells to be increased and maintained at steady-state levels whose magnitude depend on the relative sizes of the Ca^{2+} pump and the ionophore-mediated fluxes. Measurement of these steady-state levels in cells incubated with different concentrations of Ca^{2+} and A23187 enable the presence or absence of a Ca^{2+} extrusion system in the cells to be determined and the Ca^{2+}-binding characteristics of the cells to be studied.

(a) Preparation of cells. Fresh blood from healthy, unanaesthetised mongrel dogs was centrifuged at $2500 \times g$ for 5 min and the plasma and buffy coat aspirated. The erythrocytes were then washed four times in about 10 vols of 'dog basic medium' which contained 145 mM NaCl, 5 mM KCl, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes), 0.25 mM $MgCl_2$ and 10 mM glucose, pH 7.7, at 20°C. 0.25 mM $MgCl_2$ was included so that the cell magnesium content would not change when the cells were incubated with A23187 (see Fig. 4). During the first two washes of the cells, 100 μM EGTA was included in the medium to remove externally bound cell calcium.

ATP-depleted cells, when required, were obtained by preincubation of washed fresh cells for 6 h at 37°C, 10% haematocrit in 'dog basic medium' with 6 mM Iodoacetamide added [13]. After preincubation, the cells were washed three times with glucose-free 'dog basic medium'.

Mg^{2+}-depleted cells were obtained using the method of Lew and Ferreira [14]. Washed fresh cells were suspended at 10% haematocrit, 37°C, in Mg^{2+}-free 'dog basic medium' containing 2 mM EDTA. A23187 was then added, as a concentrated solution in ethanol, to give a final concentration in the suspension of about 10 μM. After 5 min, to allow maximal removal of Mg^{2+} from the cells, the suspension was centrifuged for 5 min at $2500 \times g$, the supernatant aspirated and the cells washed five times with about 50 vols. of Mg^{2+}-free 'dog basic medium'. 100 μM EDTA was included in the first two washes to prevent uptake of contaminant Mg^{2+} by the cells while an active concentration of A23187 was still present.

After preparation, packed washed cells were resuspended at about 10% haematocrit either in 'dog basic medium' or in 'dog basic medium' with appropriate alterations (see figure legends) ready for distribution to the incubation vials.

(b) Experimental procedure. Incubations were carried out at 37°C, 10% haematocrit in magnetically stirred plastic vials. Small volumes of concentrated $CaCl_2$ solutions containing tracer amounts of $^{45}CaCl_2$ were added to each vial to give the required initial extracellular Ca^{2+} concentrations and the cells were allowed 5 min to equilibrate. A23187 was then added from concentrated stock solutions in ethanol (10 μl added/2.25 ml cell suspension) to give final ionophore concentratios in the suspensions of 10 μM or 0.63 μM. At various times thereafter, samples (0.1 ml) of the suspensions were taken and processed as described previously for measurement of cell $^{45}Ca^{2+}$ [10,11] or cell magnesium [15], the cells being separated from the medium by rapid centrifugation through a layer of oil.

$^{45}Ca^{2+}$ and Mg^{2+} were measured in trichloroacetic acid extracts by liquid scintillation counting and atomic absorption spectrophotometry [15], respectively. Cell ATP was measured in neutralised perchloric acid extracts of cell lysates using a Firefly technique based on that described by Glynn and Hoffman [16]. The haematocrit in the cell suspensions was determined from 540 nm absorbance of a cell lysate after conversion of haemoglobin to cyanmethaemoglobin [17]; a measured value of 253 was used for the packed cell absorbance at 540 nm, 1 cm path length.

(c) Calculations. Cell calcium content was calculated from the $^{45}Ca^{2+}$ activity in the cell pellet and the specific activity of the Ca^{2+} added to the cell suspension. Correction was applied for an effective extracellular space of 0.9%. The external calcium concentration following A23187 addition was obtained by subtraction of the cell calcium from the total calcium added to the cell suspension. This method is both more convenient and more accurate than direct measurement of $^{45}Ca^{2+}$ in the supernatants which are diluted about ten times in the cell separation procedure [11].

In the calculations of cell and medium calcium, it was assumed that cell Ca^{2+} before addition of A23187 and contaminant Ca^{2+} in the incubation media were negligibly small compared with the amount of added Ca^{2+}. This assumption is reasonable in the high Ca^{2+} conditions but it may have caused the absolute values of cell and medium calcium to be underestimated in the low Ca^{2+} conditions. However, this would not affect the ratios of cell to medium calcium [11] and the qualitative conclusions from the experiments would not be altered.

Three points in the analysis of the results are worthy of note. First, the extracellular calcium is assumed to be completely ionised (and is therefore written as $[Ca^{2+}]_o$). This assumption may not be valid when haemolysis is high and this point is dealt with in detail in the legend to Fig. 3. Secondly, it is assumed that the A23187-mediated Ca^{2+} equilibria are potential dependent [12] because of the high anion permeability of erythrocytes (Ref. 18 and see below). Thirdly, it is assumed that the cell membrane potential is not affected by the experimental conditions. A23187 cannot affect the membrane potential directly through its action as an ionophore since it operates primarily as a neutral charge carrier and not as a conductance pathway [19]. However, indirect effects due to ionophore-induced increases in cell Ca^{2+} must also be considered since intracellular Ca^{2+} is known to activate a potassium-specific transport pathway in erythrocytes from some species [12,20]. Though calcium-induced changes in the conductance of the dog erythrocyte membrane cannot be ruled out, the membrane potential is not likely to have changed much under the experimental conditions used here because the external Na^+ and K^+ concentrations were set so that, even if their permeability increased, the membrane potential would stay near the Cl^- equilibrium potential which is probably equal to the normal membrane potential of dog erythrocytes. Accurate measurements of the Cl^- conductance of the dog erythrocyte membrane have not been reported but Parker et al. [21] were able to obtain a rather rough estimate for the permeability coefficient for Cl^- of $2.2 \cdot 10^{-8}$ cm/s. This is similar to the value of $2.1 \cdot 10^{-8}$ cm/s measured in human erythrocytes [18] and is between one and two orders of magnitude greater than the Na^+ permeability of the cells estimated from unidirectional Na^+ efflux ($1.3 \cdot 10^{-9}$ cm/s [8]) or from uni-

directional Na^+ influx ($3.3 \cdot 10^{-10}$ cm/s, calculated from data in Ref. 22). The permeability coefficients for Na^+ and K^+ are similar ($P_k/P_{Na} = 1.4$ [22]).

For the same reason, the potential is also unlikely to be affected by the changes in external Na^+ concentration in the range used in the experiment of Figs. 1 and 2. This conclusion is supported by the finding of Parker [8] that, in the absence of agents which increase the Na^+ permeability, dog erythrocytes do not show measurable hyperpolarisation when suspended in hypotonic choline solutions.

Results and Discussion

Fig. 1 shows typical Ca^{2+} uptake curves for fresh, glucose-fed dog erythrocytes incubated with a high (10 μM in the suspension) or a low (0.63 μM) concentration of A23187. With the higher ionophore concentration (panel a) substantial Ca^{2+} entry occurred at all levels of external Ca^{2+} and steady levels of intracellular Ca^{2+} were maintained throughout the 50 min incubation in each case. From the external Ca^{2+} at steady state, $[Ca^{2+}]_0^s$, indicated for each condition, the ionised cell calcium required for electrochemical equilibrium with the external Ca^{2+} can be calculated as $[Ca^{2+}]_i^{eq} = [Ca^{2+}]_0^s \cdot \exp(-2FE/RT) \cdot V_w/V_c$

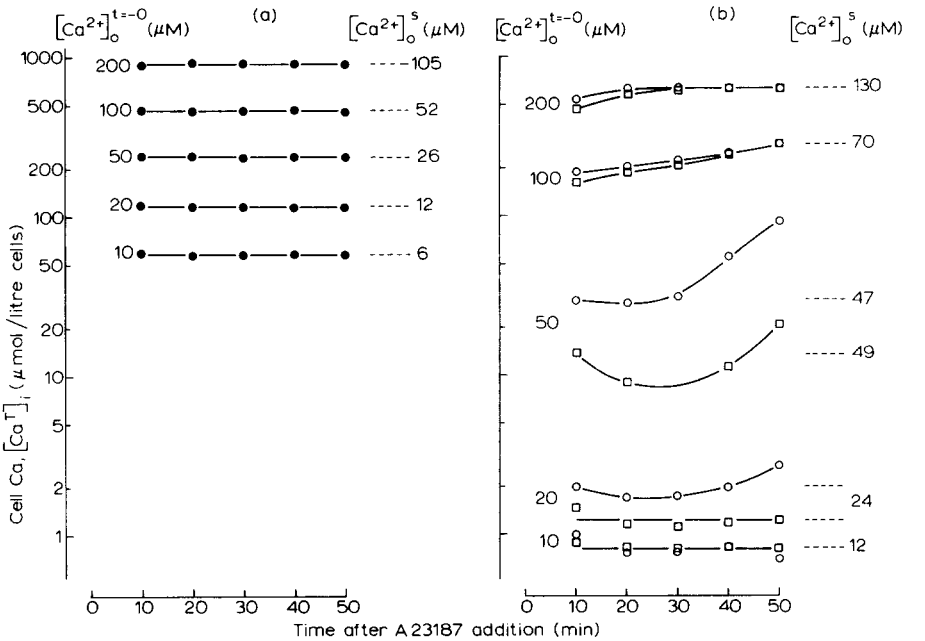

Fig. 1. Ca^{2+} content of dog erythrocytes as a function of time at different external Ca^{2+} and Na^+ concentrations and in the presence of two concentrations of A23187. Washed fresh cells were suspended either in 'dog basic medium' (final external Na^+ concentration, $[Na^+]_0$, = 150 mM (●,○)) or in 'dog basic medium' in which 52 mM NaCl had been replaced by 52 mM choline chloride ($[Na^+]_0$ = 100 mM, allowing for mixing with the washing medium trapped with the cells (□)). A23187 was added at $t = 0$ to give final concentrations in the suspensions of 10 μM (panel a) or 0.63 μM (panel b). The external Ca^{2+} concentration prior to A23187 addition ('$t = -0$') is shown at the left of each curve ($[Ca^{2+}]_o^{t=-0}$) and (· · · · · ·) to the right of the curves show the average steady-state cell calcium levels, $[Ca^T]_i^s$. The figures adjacent to the dotted lines are the corresponding external Ca^{2+} concentrations at steady state, $[Ca^{2+}]_0^s$.

where $[Ca^{2+}]_i^{eq}$ is the ionised cell calcium at equilibrium expressed in μmol/l cells, $[Ca^{2+}]_0^s$ is in μM, V_w/V_c is the cell water volume fraction, E is the membrane potential measured relative to the external medium and F, R, T have their usual meanings. When the total cell calcium at steady state, $[Ca^T]_i^s$, is less than $[Ca^{2+}]_i^{eq}$ in the presence of an ionophore-induced Ca^{2+} leak, Ca^{2+} extrusion must be taking place; when $[Ca^T]_i^s$ exceeds $[Ca^{2+}]_i^{eq}$ there is intracellular calcium binding and conclusions concerning Ca^{2+} extrusion can only be drawn if the properties of this calcium binding are known. Assuming a membrane potential of −9 mV [21] and a water fraction of 0.66 [2], it is readily seen from Fig. 1 (panel a) that with the higher ionophore concentration, $[Ca^T]_i^s$ exceeded $[Ca^{2+}]_i^{eq}$ at all calcium levels and that substantial calcium binding occurred.

With the higher concentration of A23187, any Ca^{2+}-extruding ability of the cells would have been swamped by the inward Ca^{2+} flux through the large ionophore-induced Ca^{2+} leak. This was not the case, however, when the lower concentration of ionophore was used (panel b). Comparison of $[Ca^T]_i$ with $[Ca^{2+}]_i^{eq}$ calculated from the indicated values for $[Ca^{2+}]_0^s$ shows that at the lower Ca^{2+} concentrations the cells were able to maintain their Ca^{2+} at steady-state levels far below $[Ca^{2+}]_i^{eq}$ in the presence of an ionophore-induced Ca^{2+} leak. This demonstrates clearly that the cells have a Ca^{2+} extrusion system. At higher Ca^{2+} concentrations, the cells gained large amounts of Ca^{2+}, indicating that the cells' ability to maintain their calcium below equilibrium was not due to failure of this concentration of A23187 to raise the Ca^{2+} permeability of the cells. At these higher calcium levels, there was always a trend for $[Ca^T]_i$ to rise with time. It would seem that the Ca^{2+} extrusion capacity begins to decline after a few minutes when more than about 30 μmol/l cells Ca^{2+} is forced into the cells.

If the average 'steady-state' levels of cell calcium (see legend to Fig. 1) are plotted as a function of $[Ca^{2+}]_0^s$, the curves in Fig. 2 are obtained. Also shown in Fig. 2 is the line relating cell $[Ca^{2+}]_i^{eq}$ and $[Ca^{2+}]_0^s$. The presence of experimental data in the region between this line and the abscissa is the best evidence that the cells have a Ca^{2+} extrusion system. Data lying between this line and the ordinate, on the other hand, indicates the presence of calcium-binding sites in the cells; the good fit of a straight line to the data obtained with the high ionophore concentration indicates that the cytoplasm of dog erythrocytes, like that of human erythrocytes, acts as a low affinity, high capacity Ca^{2+} buffer in the Ca^{2+} range studied [23]. From the slope of this line, 8.5, the concentration of ionised calcium in cell water can be calculated to be about $0.2 \times [Ca^T]_i$ (see Ref. 10 for methods).

An additional point shown by the data of Figs. 1 and 2 is that the Ca^{2+} extrusion from these cells is not dependent on the presence of a Na^+ gradient across the cell membrane. Since the cells have a high Na^+ content ($[Na^+]_i \approx$ 160 mM in cell water [2]), cell Na^+ is approximately in electrochemical equilibrium with an extracellular Na^+ concentration of 115 mM so that at the normal plasma Na^+ concentration of 165 mM [2], a small inward Na^+ gradient is present. However, when the external Na^+ concentration was experimentally set either above, close to, or below electrochemical equilibrium with Na_i^+ the time dependence of cell Ca^{2+} uptake and the curves relating $[Ca^T]_i^s$ and $[Ca^{2+}]_0^s$

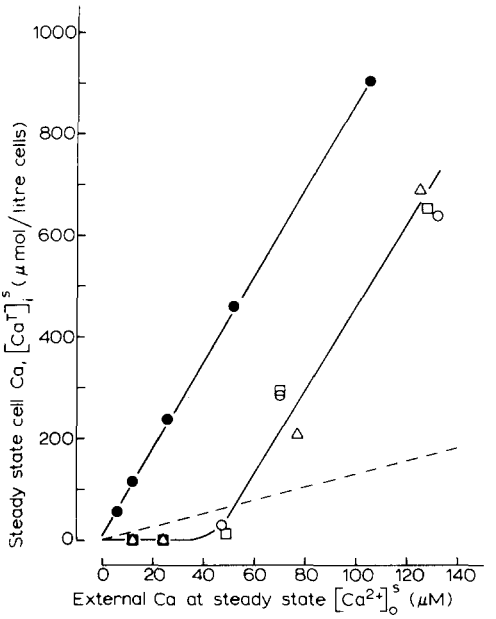

Fig. 2. Steady-state calcium content of dog erythrocytes as a function of external Ca^{2+} at steady state in the presence of different concentrations of A23187 and at different external Na^+ concentrations. A23187 concentration in the medium was either 10 μM (●) or 0.63 μM (○,□,△) and the extracellular Na^+ concentration was 150 mM (●,○), 100 mM (□) or 50 mM (△). The data for cell calcium, $[Ca^T]_i^s$, and external calcium, $[Ca^{2+}]_0^s$, at 150 mM and 100 mM external Na^+ are the average steady-state levels indicated by the dotted lines and adjacent figures in Fig. 1. The data for 50 mM external Na^+ are from Ca^{2+} uptake data similar to those in Fig. 1 and measured in the same experiment. These data were not shown in Fig. 1 for the sake of clarity. The dashed line shows the cell calcium content expected at equilibrium if there were no intracellular Ca^{2+} binding, calculated from $[Ca^{2+}]_i^{eq} = [Ca^{2+}]_0^s \cdot \exp(-2FE/RT) \cdot V_w/V_c$ (see text for details).

(Fig. 2) were similar. It is therefore concluded that calcium extrusion via a Na^+/Ca^{2+} counter transport system is negligible compared with the total calcium extrusion seen in these cells under the present conditions. This seems to be in contrast to some of the findings of Parker et al. [6] that in dog erythrocytes, the rate of Ca^{2+} extrusion against an electrochemical gradient was increased by measurable amounts when the external Na^+ concentration was increased. The reason for this disagreement is unclear but in view of the Na^+ independence of the Ca^{2+} extrusion in the present experiments all further experiments were carried out using 150 mM $[Na^+]_0$ because that is closer to the physiological condition and also avoids complications which might arise when other cations are substituted for external Na^+.

Fig. 3 shows that Ca^{2+} extrusion is reduced in ATP-depleted cells suggesting a dependence of the transport mechanism on intracellular ATP. Curves (a) and (b) show the relationships between $[Ca^T]_i^s$ and $[Ca^{2+}]_0^s$ in fresh glucose-fed cells, determined using the same concentrations of A23187 as in the experiment of Figs. 1 and 2. Curves (c) and (d) show the $[Ca^T]_i^s$ vs. $[Ca^{2+}]_0^s$ relationships determined in cells from the same blood sample but depleted of ATP by 6 h preincubation with 6 mM iodoacetamide plus 10 mM glucose [13]. It can be seen that at the higher concentration of A23187, there was only a small difference between the Ca^{2+} uptake by fresh cells and by depleted cells. This

difference may be an artifact due to cell lysis but even if genuine it is so small that ATP depletion could have caused only small changes in intracellular calcium binding or membrane potential. This in turn means that in Fig. 3, the line relating $[Ca^{2+}]_i^{eq}$ and $[Ca^{2+}]_0^s$, which was calculated for fresh cells, can also be used in analysing the data for depleted cells. The data obtained with the lower ionophore concentration then show that while fresh cells were able to maintain their intracellular Ca^{2+} below $[Ca^{2+}]_i^{eq}$ with $[Ca^{2+}]_0^s$ as high as 70 μM, the ATP-depleted cells gained Ca^{2+} to levels far above $[Ca^{2+}]_i^{eq}$ even at the

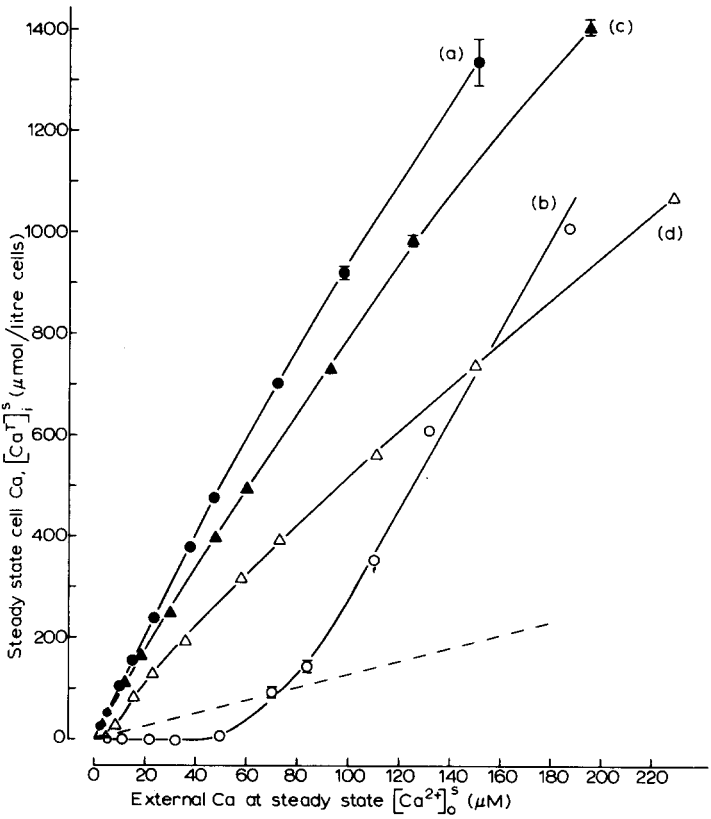

Fig. 3. Steady-state calcium content of fresh and ATP-depleted dog erythrocytes as a function of external Ca^{2+} at steady state in the presence of two concentrations of A23187. The incubation media were 'dog basic medium' for the fresh cells and glucose-free 'dog basic medium' for the ATP-depleted cells. The ATP content of the fresh cells was 0.32 mmol/l cells at the beginning of the incubation and between 0.11 and 0.38 mmol/l cells at the end (40 min). The ATP content of the ATP-depleted cells was about 40 μmol/l cells immediately following depletion and this fell during incubation with A23187 and Ca^{2+} to below 10 μmol/l cells. The incubation conditions are denoted by the following symbols: curve (a), ●, fresh cells, 10 μM A23187; curve (b), ○, fresh cells, 0.63 μM A23187; curve (c), ▲, depleted cells, 10 μM A23187; curve (d), △, depleted cells, 0.63 μM A23187. As in Fig. 2, (- - - - -) shows the cell calcium expected at equilibrium if there were no intracellular calcium binding. Cell lysis during incubation of the depleted cells was as much as 50% in the high Ca^{2+} conditions and this was allowed for in the calculations of cell calcium and ATP. However, no correction was made for Ca^{2+} binding to cell debris in the extracellular medium so that calculated values for extracellular Ca^{2+} may be overestimates of the extracellular free Ca^{2+}. The effect of this would be to reduce the free intracellular Ca^{2+}, and hence $[Ca^T]_i^s$, corresponding to a given value on the abscissa and so decrease the slopes of the lines fitting the data for the depleted cells. This may in part explain the difference in the slopes of the lines fitting the high ionophore data for the fresh and depleted cells.

lowest Ca^{2+} concentration used. ATP depletion had clearly reduced the cells' ability to extrude Ca^{2+}. The finding that the calcium levels in the depleted cells were still dependent on the ionophore concentration suggests that Ca^{2+} pumping by the cells had not stopped completely and this might be the cause of the fall in the residual cell ATP from about 40 to less than 10 μmol/l cells during incubation with A23187 and Ca^{2+}.

Fig. 4 shows the effect of Mg^{2+} depletion on the Ca^{2+} uptake by fed dog erythrocytes incubated with a low concentration of A23187. When Mg^{2+}-depleted cells were incubated in the presence of 0.6 mM $MgCl_2$ and A23187, their Mg^{2+} content increased to a level similar to that in control cells whose Mg^{2+} had not previously been altered. Since the Mg^{2+} content of the control cells did not change during incubation with A23187, the external Mg^{2+} (0.25 mM) must have been in electrochemical equilibrium with the intracellular free Mg^{2+} indicating that the concentration of free Mg^{2+} in fresh dog erythrocytes is about 0.5 mM in cell water. This is similar to the value reported for human erythrocytes [15].

Following Ca^{2+} addition, steady-state levels of cell calcium were rapidly attained. In the control cells, cell calcium remained well below $[Ca^{2+}]_i^{eq}$ throughout the incubation while in the Mg^{2+}-depleted cells $[Ca^T]_i^s$ was much larger than $[Ca^{2+}]_i^{eq}$. Mg^{2+} depletion had substantially reduced the ability of the cells to extrude Ca^{2+}. In Mg^{2+}-depleted cells which had been repleted with Mg^{2+}, $[Ca^T]_i^s$ was once again much lower than in the Mg^{2+}-depleted cells though it was only slightly below $[Ca^{2+}]_i^{eq}$. However, unless the intracellular calcium

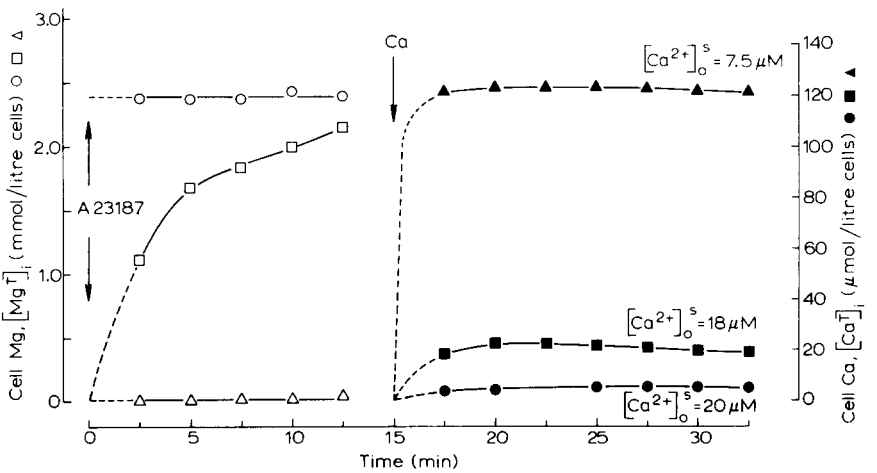

Fig. 4. Mg^{2+} and Ca^{2+} uptake in fresh and Mg^{2+}-depleted dog erythrocytes in the presence of A23187. Cells depleted of Mg^{2+} by preincubation with A23187 + EDTA were resuspended either in Mg^{2+}-free 'dog basic medium' (△,▲) or in 'dog basic medium' containing 0.6 mM $MgCl_2$ (□,■). Control cells not previously exposed to A23187 were suspended in normal 'dog basic medium' (○,●). A23187 was added to each suspension at $t = 0$ to give a final concentration of 0.63 μM in the suspension and cell Mg^{2+} was measured several times over the next 15 min (open symbols and scale on left hand ordinate). A small volume of a concentrated $CaCl_2$ solution containing tracer $^{45}CaCl_2$ was then added to each suspension to give an initial Ca^{2+} concentration in the medium of 20 μM and cell calcium was measured at various times thereafter (closed symbols and scale on right hand ordinate). The figures adjacent to each set of Ca^{2+} data are the external Ca^{2+} concentrations at steady state, $[Ca^{2+}]_o^s$. Cell ATP was measured in each condition at the beginning and end of the incubation and was between 0.18 and 0.28 mmol/l cells in each case.

buffers had been dramatically reduced, which is very unlikely, the ionised intracellular calcium would still have been substantially below electrochemical equilibrium with external Ca^{2+}. Thus, restoration of intracellular Mg^{2+} to near its original level had reversed much of the effect of Mg^{2+} removal on Ca^{2+} extrusion suggesting a direct role of Mg^{2+} in the Ca^{2+} extrusion. Certainly it is unlikely that the effects of changing intracellular Mg^{2+} were mediated by changes in the level of cell ATP since this was similar in all three conditions (0.18—0.28 mmol/l cells).

In conclusion, the data presented indicate that dog erythrocytes have a Ca^{2+} extrusion system which is not affected by the electrochemical gradient for Na^+ but which can be substantially inactivated by removal of intracellular Mg^{2+} or by depletion of cell ATP. Although these data do not yet show direct coupling between Mg^{2+}-dependent ATP consumption and Ca^{2+} extrusion, the Mg^{2+} and ATP requirements resemble those of the human erythrocyte Ca^{2+} pump [9]. Since Ca^{2+} extrusion by this system is not linked to the Na^+ gradient, the system could be used to maintain an inward Ca^{2+} gradient whose potential energy then fuels movement of other ions, such as Na^+, against their electrochemical gradients.

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