J. Physiol. (1983), **343**, *pp.* 455–493
With 21 *text-figures*
Printed in Great Britain

THE EFFECT OF INTRACELLULAR CALCIUM ON THE SODIUM PUMP OF HUMAN RED CELLS

BY A. M. BROWN AND V. L. LEW*

*From the Department of Physiology and Pharmacology, University of Nottingham Medical School, Clifton Boulevard, Nottingham and the *Physiological Laboratory, Downing Street, Cambridge*

(*Received 15 February 1983*)

SUMMARY

1. The inhibitory effect of cytoplasmic Ca on Na-pump-mediated Na–K exchange was investigated in intact red cells under conditions of constant cell volume, membrane potential and inorganic ion composition. The ionized cytoplasmic Ca concentration ($[Ca^{2+}]_i$) was controlled using the ionophore A23187.

2. In normal cells, ouabain-sensitive ^{24}Na efflux was inhibited with an apparent affinity for $[Ca^{2+}]_i$ which depended on the concentration of A23187; 50% inhibition required 20–40 μM and 160–300 μM-cytoplasmic Ca^{2+} with 10 μM and 0·63 μM-A23187 respectively. Cytoplasmic Ca also affected cell ATP content which fell rapidly on addition of A23187 and subsequently increased, steadied or continued to fall more slowly depending on the Ca and A23187 concentrations. Half-maximal fall required 5–15 μM and 110–170 μM-cytoplasmic Ca^{2+} at 10 μM and 0·63 μM-A23187 respectively. Removal of Ca from the cells failed to reverse either the Na pump inhibition or the fall in cell ATP.

3. In ATP-enriched cells cytoplasmic Ca caused inhibition of ouabain-sensitive ^{24}Na efflux in an A23187-dependent manner with apparent affinities for $[Ca^{2+}]_i$ similar to those observed in the normal cells. Inhibition was complete at high $[Ca^{2+}]_i$. As in the normal cells, the ATP content of the cells fell in the presence of cytoplasmic Ca, but always remained above 1·2 m-mole/l. cells. This was higher than the ATP content of Ca-free normal intact cells.

4. A23187 had no effect on the inhibition by Ca of ouabain-sensitive ATPase activity in isolated red cell membrane preparations. Both under conditions near optimal for Na–K-ATPase activity and under conditions resembling those in the cytoplasm, inhibition was half-maximal at about 25 μM-Ca^{2+} and in the latter case complete at below 400 μM-Ca^{2+}.

5. The apparent ATP-dependence of ouabain-sensitive Na efflux in the presence of cytoplasmic Ca was distinctly different in the normal and ATP-enriched cells but in both groups of cells it was similar for data obtained with high and low concentrations of A23187.

6. The data for Na pump inhibition by cytoplasmic Ca in the intact cells were well fitted by several kinetic models involving either $[Ca^{2+}]_i$ or CaATP as the inhibitory species and a low affinity dependence of pump activity on MgATP or total ATP.

However, for any model, the apparent affinities for CaATP or for Ca^{2+} required to fit the ATPase data were 2·5–10 times higher than those required to fit the data for Na efflux.

7. From the kinetic models fitted to the Na efflux data, it is predicted that physiological (i.e. 1–10 μM) fluctuations in cell $[Ca^{2+}]_i$ will inhibit the Na pump by less than 35%.

INTRODUCTION

The inhibitory effect of cytoplasmic Ca on the Na-pump-mediated Na–K exchange (Hoffman, 1962; Dunn, 1974) and on the Na–K-ATPase (Skou, 1957, 1960; Dunham & Glynn, 1961; Albers & Koval, 1962; Epstein & Whittam, 1966; Davis & Vincenzi, 1971; Tobin, Akera, Baskin & Brody, 1973; Robinson, 1974; Lindenmayer & Schwartz, 1975) has been known for some time. The mechanism of inhibition, however, is not well understood (Epstein & Whittam, 1966; Tobin *et al.* 1973; Robinson, 1974; Fukushima & Post, 1978) and the observed inhibition by Ca varies so much from one study to another that its likely magnitude under physiological conditions is impossible to predict. We wanted to assess whether data from ATPase studies are consistent with, and admit a similar explanation as, the effects of cytoplasmic Ca on the Na pump in an intact cell under physiological conditions and also to establish whether pump inhibition by cytoplasmic Ca is likely to be physiologically relevant. Such an investigation requires knowledge of the precise dependence of Na pump inhibition on the ionized cytoplasmic Ca concentration ($[Ca^{2+}]_i$) in the intact cell as well as in the isolated membrane so that hypotheses on the inhibitory mechanism can be quantitatively tested and predictions made of the likely extent and time course of changes in pump activity following physiological fluctuations in $[Ca^{2+}]_i$. This $[Ca^{2+}]_i$-dependence has not been explored before in the intact cell since only recently has it become experimentally feasible to maintain controlled $[Ca^{2+}]_i$ levels in intact cells for periods of time long enough to allow Na-pump-mediated fluxes to be measured (Ferreira & Lew, 1976; Lew & Brown, 1979).

Using the ionophore A23187 and these recently developed techniques, we have measured the effect of varying the internal Ca^{2+} levels on the ouabain-sensitive Na efflux from intact fed human red cells in the presence of saturating external K concentrations ($[K]_o$) (Garrahan & Glynn, 1967*b*). The experiments were carried out under conditions which ensured constancy of volume, membrane potential and inorganic ion content of the cells during the flux measurements.

The results indicate that Ca inhibits Na-pump-mediated Na–K exchange in intact normal cells but with a lower apparent affinity than it does the Na–K-ATPase activity of a red cell membrane preparation under comparable conditions. Furthermore, at least part of the inhibitory effects of internal Ca on the Na pump in the cells are secondary to Ca-induced changes in cell ATP levels which arise from Ca-pump-mediated ATP hydrolysis exceeding, at least transiently, the rate of glycolytic ATP production. However, using ATP-enriched cells, it was possible to separate such effects from direct actions of Ca on the Na pump. From considerations of possible mechanisms which could explain the observed effects of Ca on the pump, it is

predicted that in excitable cells, Na-pump-mediated Na–K exchange could be transiently inhibited by up to 35 % within the physiological range of $[Ca^{2+}]_i$ variations (1–10 μM).

Preliminary reports of parts of this work have been published (Brown & Lew, 1981, 1982).

METHODS

Experiments with intact cells

Principle of the method

Na-pump activity was measured as ouabain-sensitive Na efflux into a medium containing a saturating K^+ concentration (40–75 mM), a condition in which this efflux measures only Na–K exchange through the pump (Garrahan & Glynn, 1967*b*). The Ca content of the cells was altered by addition to the medium of between 0·63 and 10 μM-A23187 and different concentrations of Ca. Under these conditions, Ca redistributes across the cell membrane and, within about 10 min in most cases (see e.g. Fig. 1), reaches a steady state which represents a balance between Ca extrusion by the Ca pump and passive A23187-mediated Ca entry. The cell Ca level obtained with a given steady external Ca concentration, $[Ca^{2+}]_o$, therefore depends on the relative sizes of the Ca pump flux and ionophore-mediated Ca 'permeability' and for A23187 concentrations up to about 10 μM, the same level of cell Ca can be obtained with a different A23187 concentration simply by varying $[Ca^{2+}]_o$ (Ferreira & Lew, 1976; Lew & Brown, 1979; Simonsen & Lew, 1980).

When the A23187 concentration is 10 μM or greater, the Ca 'permeability' is so high that the Ca pump is swamped at cell Ca levels greater than about 5–10 μmole/l. cells and cell Ca is then close to equilibrium with external Ca. In this condition, the following relationship holds (Ferreira & Lew, 1976; Lew & Brown, 1979):

$$[Ca^{2+}]_i^s = [Ca^{2+}]_o^s . r^2, \quad (1)$$

where $[Ca^{2+}]_i^s$ and $[Ca^{2+}]_o^s$ are the steady-state concentrations of ionized Ca in the cells and medium respectively and r is $[H^+]_i/[H^+]_o$ where $[H^+]_i$ and $[H^+]_o$ are the intracellular and extracellular H ion concentrations respectively. For red cells, since $[H^+]_i/[H^+]_o = [Cl^-]_o/[Cl^-]_i$ (extracellular and intracellular Cl concentrations respectively, e.g. Hladky & Rink, 1977), r is about 1·5 (see e.g. Flatman & Lew, 1980).

Ferreira & Lew (1976) showed that under these conditions, at least for cell Ca up to about 3 m-mole/l. cells, the relationship between total cell Ca at the steady state, $(Ca^T)_i^s$, and $[Ca^{2+}]_o^s$ is linear and passes close to the origin. Thus, writing the slope of this line as m:

$$(Ca^T)_i^s = m . [Ca^{2+}]_o^s. \quad (2)$$

This indicates that the red cells act as low affinity, high capacity Ca buffers and that $[Ca^{2+}]_i$ is a constant fraction of total cell Ca ($(Ca^T)_i$). Writing this fraction as α and eliminating $[Ca^{2+}]_o^s$ from eqns. (1) and (2) gives:

$$[Ca^{2+}]_i^s = (Ca^T)_i^s . r^2/m = \alpha . (Ca^T)_i^s. \quad (3)$$

Thus, in any experiment, α can be determined from the slope of the relationship between $(Ca^T)_i$ and $[Ca^{2+}]_o$ at the steady-state in the presence of 10 μM-A23187. Data from experiments with ATP-depleted cells indicate that the value of α is not affected by the concentration of A23187 between 0·1 μM and 6 μM (Lew & Ferreira, 1978; Simonsen, Gomme & Lew, 1982). The value of α obtained with 10 μM-A23187 can therefore reasonably be used to calculate $[Ca^{2+}]_i$ corresponding to any measured cell Ca level.

Raised $[Ca^{2+}]_i$ activates a K channel in red cells (see e.g. Lew & Ferreira, 1978) and in cells suspended in a plasma-like medium this leads to hyperpolarization and loss of cell K and water. To prevent this, high K media were used throughout, $[K^+]_o$ being chosen to be close to electrochemical equilibrium with $[K^+]_i$ at the normal red cell membrane potential. For normal-ATP red cells, $[K^+]_o$ was set at 75 mM which has been found to produce constant haematocrit and cell K concentration over a 1000-fold change in K permeability (Lew & Ferreira, 1976). In experiments with ATP-enriched cells, a lower $[K^+]_o$ was used because of the increase in r during the pre-incubation to increase cell ATP. Because of the variation in extent of the ATP increase in different experiments,

r was measured separately, as detailed later, for each experiment and the incubation medium with the appropriate $[K^+]_o$ then prepared.

A23187 transports Mg as well as Ca into red cells and produces a redistribution of intracellular and extracellular Mg bringing them into equilibrium (Flatman & Lew, 1977, 1980). To prevent net Mg movements in the presence of A23187, Mg in the incubation medium ($[Mg^{2+}]_o$) must therefore be at electrochemical equilibrium with cell Mg and for experiments with normal-ATP cells this requires a Mg concentration in the medium of about 0·15 mM (Flatman & Lew, 1977; Till, Petermann, Wenz & Frunder, 1977). In some early experiments, $[Mg^{2+}]_o$ was somewhat higher (0·25–0·4 mM) and in these experiments the cells gained Mg when A23187 was added. The concentration of Mg in the medium, together with an estimate of the ionized cell Mg at equilibrium, $[Mg^{2+}]_i^{eq}$, in each experiment, is given in the corresponding Figure legend. In experiments with ATP-enriched cells, which have higher concentrations of organic phosphates (Duhm, 1973) that may bind Mg (Flatman & Lew, 1980), 0·4 mM-extracellular Mg^{2+} was used and $[Mg^{2+}]_i^{eq}$ estimated from measurements of total cell Mg as described by Flatman & Lew (1980).

The metabolic substrate used in all experiments with normal-ATP cells was inosine as described by Flatman & Lew (1980). In experiments with ATP-enriched cells, adenosine + pyruvate was used instead of inosine. This enabled adequate ATP levels to be maintained in these cells when they were exposed to A23187 + Ca.

Composition of media and materials

All solutions were prepared in double-glass-distilled water and all reagents used were of A.R. grade or equivalent. EGTA, ouabain and Tris buffer were obtained from Sigma (London) Ltd. ATP (disodium salt) was from Boehringer. A23187 was a gift from the Lilly Research Centre.

The compositions of media referred to in subsequent sections were as follows: medium A (wash medium): 75 mM-NaCl, 75 mM-KCl, 10 mM-Tris Cl, pH 7·8 at 37 °C; medium B (incubation medium, normal-ATP cells): as medium A with addition of 10 mM-inosine and 0·15–0·5 mM-$MgCl_2$; medium C (incubation medium, high-ATP cells): 110 mM-NaCl, 40 mM-KCl, 10 mM-adenosine, 10 mM-pyruvate, 0·4 mM-$MgCl_2$, 10 mM-Tris Cl, pH 7·8 at 37 °C.

The KCl concentration in medium C was calculated as $[K^+]_i/r$ where $[K^+]_i$ was assumed to be 140 mM and r was determined from pH measurements in a suspension of the ATP-enriched cells after the pre-incubation to increase cell ATP (see below).

$CaCl_2$ containing tracer amounts of ^{45}Ca was added to the basic incubation media as concentrated stock solutions so that dilution of the media was less than 2%. Ca-free conditions contained 100 μM-Tris–neutralized EGTA and no added Ca. A23187 was added as concentrated stock solutions in ethanol, 10 μl. being added to 2·25 ml. of a 10% haematocrit cell suspension. Ouabain and metabolic substrates were added to the media on the day of the experiment. The final concentration of ouabain was in the 0·1–1 mM range. In the experiments on the reversibility of the effect of Ca on Na fluxes, sufficient Tris–EGTA (stock solution, 250 mM) was added to reduce the Ca concentration in the cell suspension to less than 10^{-6} M. The pH fall following EGTA addition to the cell suspensions in medium B was measured in one experiment to be less than 0·05.

Preparation of cells

Red cells were obtained either from blood freshly drawn into heparinized syringes or from blood from the Blood Bank which was in acid-citrate-dextrose preservative and was less than 5 days old when used. The whole blood was centrifuged at 2500 g for 5 min and the plasma and buffy coat aspirated. The red cells were then washed 5 times at 10–20 °C in at least five volumes of medium A, 100 μM-EGTA being included in the medium during the first two washes to remove externally bound Ca from the cells (Harrison & Long, 1968; Bookchin & Lew, 1980). At each wash, care was taken to remove all traces of white cells. After the final wash, the cells were packed to about 80% haematocrit and placed on ice ready for use.

Loading of cells with ^{24}Na

For experiments with normal-ATP cells, washed cells were incubated with gentle agitation for 4 hr at 37 °C at about 30% haematocrit in medium B to which had been added 0·05–0·15 mCi of ^{24}Na per millilitre of suspension (Amersham, $^{24}NaCl$ injection). The cells were then washed 4–6 times in ice-cold medium A, packed to about 80% haematocrit and kept on ice ready for use.

In experiments with ATP-enriched cells, ^{24}Na loading was achieved by addition of $^{24}NaCl$ to the pre-incubation medium used to increase cell ATP.

Preparation of ATP-enriched cells

The ATP content of intact fresh cells was increased by incubation of the cells with adenosine + pyruvate + phosphate which has been shown previously to increase the ATP levels of stored red cells (Duhm, 1973). Washed cells were incubated with gentle agitation for 4 hr at 37 °C at a haematocrit of 15% in a medium containing 60 mM-NaCl, 40 mM-KCl, 50 mM-Na phosphate, 10 mM-pyruvate, 10 mM-adenosine, 5 mM-Tris Cl, pH 7·3 at 37 °C. The cells were then washed twice in ice-cold medium A and incubated with magnetic stirring for 15 min at 10% haematocrit, 37 °C, in medium A containing 10 mM-adenosine + 10 mM-pyruvate to remove traces of inorganic phosphate which might have caused precipitation of Ca or Mg phosphates in subsequent incubations. During the last 5 min of this incubation, measurements were made of extracellular pH (pH_o) and intracellular pH (pH_i), the latter being made on a sample of cells packed by centrifugation at 8000 *g* for 30 sec and then rapidly frozen in liquid nitrogen and thawed. pH measurements were made at 37 °C using a Radiometer micro-electrode unit (type ES021a). r was estimated as $10^{(pH_o - pH_i)}$ and was found to be 4·2 and 7·5 in two experiments. A measurement on fresh cells suspended in the same medium gave a value of 1·8.

Finally, the cells were washed twice in ice-cold medium A, packed to about 80% haematocrit and kept on ice ready for use.

Experimental procedure

^{24}Na-efflux measurements. ^{24}Na-loaded cells were added to the incubation media to give a haematocrit of about 10%. The suspensions were incubated at 37 °C in small plastic vials with magnetic stirring. After 10 min to allow temperature equilibration, A23187 was added to give final concentrations between 0·63 and 10 μmole/l. cell suspension. At 10 min intervals thereafter, 100 μl. samples were taken to measure ^{24}Na activity in the medium and ^{45}Ca, Mg and ATP in the cell pellet. Each sample was quickly ejected into an Eppendorf microtube containing 0·9 ml. of ice-cold inactivation medium (medium A containing 2 mM-EGTA) and 0·4 ml. of di-*n*-butyl phthalate oil (BDH). The tube was rapidly capped, inverted to mix the contents and centrifuged at 8000 *g* for 10 sec. This procedure quickly reduces the A23187- and Ca-pump-mediated Ca transport and separates the cells from the medium (for more details of the method, see Lew & Brown, 1979). The tubes were then left either at room temperature or on ice (if cell ATP was being measured) until the end of the experiment for further processing. 100 μl. samples of the cell suspension were also taken for measurement of total ^{24}Na and ^{45}Ca activities and haematocrit and, where necessary, total Mg.

^{24}Na activity was measured in the aqueous supernatants above the oil and in the samples of the total suspension and the Na efflux rate constant calculated from the data (see Garrahan & Glynn, 1967*a*).

Ca content of cells. ^{45}Ca activity was measured by scintillation counting of trichloroacetic acid (TCA) or perchloric acid (PCA) extracts of distilled-water-lysed cells. In experiments where ^{24}Na was used, ^{45}Ca samples were left for about a week before counting to allow the ^{24}Na to decay. Cell Ca and external Ca following A23187-induced Ca redistribution were calculated from the ^{45}Ca activity in the cell pellets and whole suspension and the specific activity of ^{45}Ca. The small amount of Ca associated with the EGTA-washed cells (about 6 μmole/l. cells (Bookchin & Lew, 1980)) and contaminant Ca in the medium (about 5 μM) were neglected, the error thus introduced being small except at very low Ca levels (Lew & Brown, 1979).

ATP content of cells. Cell ATP was measured in neutralized TCA or PCA extracts of 5–10 μl. of cells lysed in distilled water. ATP was determined by Firefly luminescence (luciferin–luciferase preparation: Sigma, FLE50) using the method of Glynn & Hoffman (1971) either as originally described or modified for use with a scintillation counter (Brown, 1982). Ca, at concentrations up to and above those likely to have occurred in the cell samples, had no effect on the assay. At cell ATP levels below about 50 μmole/l. cells, some loss of ATP occurs during the cell lysis and PCA extraction process (up to about 50% (Brown, 1982)) and, where appropriate, a correction for this was applied.

Mg content of cells. In some experiments, cell Mg was measured by atomic absorption spectrophotometry and $[Mg^{2+}]_i$ estimated as described by Flatman & Lew (1980). In experiments with normal-ATP cells where cell Mg was not measured, $[Mg^{2+}]_i^{eq}$ was calculated using data in the literature. Thus, for 0·1 mM < $[Mg^{2+}]_i$ < 1 mM, the relationship between total cell Mg (in m-mole/l. cells), $(Mg^T)_i$ and $[Mg^{2+}]_i$ can be approximated from the data of Flatman & Lew (1977, 1980) as:

$$(Mg^T)_i = (7 \cdot [Mg^{2+}]_i + 1) \cdot V_w / V_c \quad (4)$$

where V_w/V_c is the fractional cell water content. From this equation, the relationship between cell and external ionized Mg at equilibrium (Flatman & Lew, 1980), and assuming a Mg content of fresh cells of 2·35 m-mole/l. cells (Flatman & Lew, 1980), $[Mg^{2+}]_i$ at equilibrium in the presence of A23187 was estimated from the initial Mg concentration in the incubation medium and the haematocrit, assuming values of 2·25 and 0·67 for r^2 and V_w/V_c respectively.

Measurement of haematocrit. Haematocrit was measured from determinations of haemoglobin, either directly as oxyhaemoglobin or after conversion to cyan-methaemoglobin (Dacie & Lewis, 1975), by measurement of optical density at 540 nm. Values for packed cell absorbance, determined from measurements of absorbance and microhaematocrit, were between 285 and 350 for oxyhaemoglobin and between 230 and 280 for cyan-methaemoglobin.

Experiments with red cell membranes

Preparation of membrane fragments

Membranes were prepared by hypotonic lysis of red cells from fresh or Blood Bank blood in an EGTA-containing medium. This haemolysis condition was chosen to improve resolution of the inhibitory effects of Ca on the Na–K-ATPase. The rationale of this choice is that the Ca-stimulated ATPase activity of red cell membrane preparations is normally much greater than even the uninhibited Na–K-ATPase activity (see e.g. Dunham & Glynn, 1961) but may be reduced by as much as 50% when $[Ca^{2+}]$, during haemolysis, is below 10^{-10} M (Scharff & Foder, 1978). This is probably due to removal of calmodulin.

Packed, washed cells were lysed by squirting into ten volumes of an ice-cold medium containing 1 mM-HEPES, 2 mM-$MgCl_2$, 10 mM-Tris–EGTA, (pH 7·2 at room temperature) and stirred vigorously with a magnetic stirrer for 5 min. The lysate was then centrifuged at 12,000 *g* for 20 min at 0–4 °C, the supernatant removed and the membranes washed three times by resuspension in at least ten volumes of an ice-cold wash medium which contained 1 mM-HEPES, 1 mM-$MgCl_2$, 0·1 mM-Tris–EGTA, pH 7·2 and centrifugation at 18,000 *g*, 0–4 °C for 20 min. After the third wash, the packed membranes were transferred to 10 ml. Pyrex tubes and frozen and thawed twice in a solid-CO_2–acetone mixture to improve accessibility of ATP and ions to the intracellular surface of the membranes. The membranes were then washed three times in ice-cold wash medium and finally resuspended in this medium to about 80% of the original packed cell volume, thus giving a membrane suspension with an 'equivalent haematocrit' of about 100%. This suspension was divided into aliquots and stored at −20 °C.

Measurement of ATPase activity

ATPase activity was measured as the production of inorganic phosphate, P_i, determined as $^{32}P_i$ production from [γ-^{32}P] ATP. Na–K-ATPase activity was measured as the difference between the activities in the presence and absence of ouabain.

Incubations were at 37 °C in 1·5 ml. Eppendorf microtubes using a membrane concentration of about 5% equivalent haematocrit and an incubation volume of 200 μl. The incubation components were added at 0 °C with the tubes on ice and mixed by vortexing. The membranes and all co-factors including A23187, ethanol and ATP but excluding Ca and ouabain were pre-mixed and added in 100 μl. and Ca, Tris–EGTA ('Ca-free' conditions) and ouabain (final concentration, 1 mM) were added in water in a total volume of 100 μl. [γ-^{32}P] ATP was used as supplied as the triethylammonium salt in aqueous ethanol (0·5–3 Ci/m-mole, Amersham PB108) to give a final activity in each incubation tube of about 200 nCi. The incubation conditions are given in the Figure legends.

The incubation was started by transferring the tubes to a water bath at 37 °C and terminated by returning them to the ice-bath and allowing them to cool for 15 min before processing for P_i determination. In all experiments, the maximum ATP consumption was less than 10% of the total ATP present initially. A preliminary experiment showed that P_i production was approximately linear for at least 60 min both in the presence and absence of ouabain and in all subsequent experiments, an incubation time of 60–80 min was used. Measurements of P_i production were corrected for ATP hydrolysis in the absence of membranes. Preliminary experiments indicated that the $^{32}P_i$ activity in tubes incubated for 60 min at 37 °C without enzyme was similar to that in tubes with or without membranes or Ca kept on ice for 60 min and was about 1% of the total ^{32}P present. All measurements were the mean of three or more determinations.

$^{32}P_i$ in the tubes was determined after separation from [γ-^{32}P] ATP using a modification (Brown, 1982) of the procedure of Weil-Malherbe & Green (1951) in which P_i is converted to a

phosphomolybdate complex which is then extracted into isobutanol. To each tube was added 100 μl. of ice-cold 'molybdate mixture' which consisted of one volume of 55% TCA + five volumes of 5% ammonium molybdate in 4 N-sulphuric acid + 12 μl. of 250 mM-Na phosphate per millilitre. This mixture was prepared within an hour of use since a yellow precipitate forms on storage even for a few hours. 800 μl. of ice-cold isobutanol was then added to each tube and the tubes recapped. The tubes, which were held firmly in racks, were then shaken vigorously for 10 sec and returned to the ice-bath for 5 min. This process was then repeated twice more. This procedure ensured good mixing of the tubes which is essential since extraction of the phosphomolybdate complex into isobutanol is slow at 0–4 °C. The tubes were mixed by shaking rather than by vortexing since even with prolonged vortexing, extraction was found to be incomplete.

After the third shake, the tubes were centrifuged at 8000 *g* for 30 sec and returned to the ice bath. This centrifugation produced complete separation of clear isobutanol and aqueous phases with the membrane debris forming a layer at the interface between them. $^{32}P_i$ activity in 500 μl. of the now yellow isobutanol phase was determined by scintillation counting in 5 ml. of Bray solution (Bray, 1960) diluted 10% with water to prevent formation of a yellow precipitate in the scintillation vial overnight. Total $^{32}P_i$ in the incubation medium was also determined and differences in quenching between this sample and the isobutanol samples corrected for by internal standardization (Dyer, 1974). In calculating the P_i production, correction was made for an 8% shrinkage of the isobutanol phase during the extraction procedure.

Preliminary experiments indicated that extraction of $^{32}P_i$ from the incubation mixture by this procedure is approximately 100% provided that the total P_i in the 300 μl. volume of incubation mixture + 'molybdate mixture' is between 0·7 and 2 mM (i.e. P_i in the incubation less than 1·5 mM) and that the equivalent haematocrit is less than 10%.

Calculations

The concentrations of free and complexed Ca, Mg and ATP were calculated using dissociation constants of 217 μM for K_{CaATP} (Collier & Lam, 1970) and 83 μM for K_{MgATP} (Berger, Janig, Gerber, Ruckpaul & Rapoport, 1973). These values were both determined at 37 °C and with near physiological ionic strength and pH. For ATPase experiments the equations relating free, complexed and total concentrations of Ca, Mg and ATP were solved using the trigonometric solution for the roots of a cubic equation (eg. Selby, 1970) where necessary. For the analysis of the mechanism of inhibition of ouabain-sensitive Na efflux in intact cells, intracellular concentrations of MgATP, CaATP and free ATP were calculated from the experimentally determined values of $[Ca^{2+}]_i$, $[Mg^{2+}]_i$ and total cell ATP content. Binding of ATP to cell constituents other than Ca and Mg was neglected and the dissociation constants for MgATP and CaATP were assumed to be the same as in free solution.

RESULTS

Effects of $[Ca^{2+}]_i$ and A23187 on Na efflux from intact, fed 'normal-ATP' red cells

In order to examine the effect of intracellular Ca on Na–K exchange via the Na pump, we measured the ouabain-sensitive efflux of ^{24}Na from ^{24}Na-loaded, inosine-fed intact normal red cells incubated in high-K, Ca-containing media and made permeable to Ca by addition of A23187. The reversibility of the effect of Ca was also examined, Ca being removed from the cells after the first part of the experiment by addition of EGTA to the cell suspensions. It has been found previously that with the method for controlling cell Ca used in the present work, the apparent $[Ca^{2+}]_i$ affinity of the Ca-activated K channel of intact, fed red cells increases with the concentration of A23187 used to increase Ca permeability (Lew & Ferreira 1976). Since the present work was also concerned with the apparent $[Ca^{2+}]_i$ affinity of a membrane transport process, both high and low concentrations of A23187 were used to control cell Ca in the present experiments.

Fig. 1 shows, for one experiment, the time dependence of the Ca content of fed intact

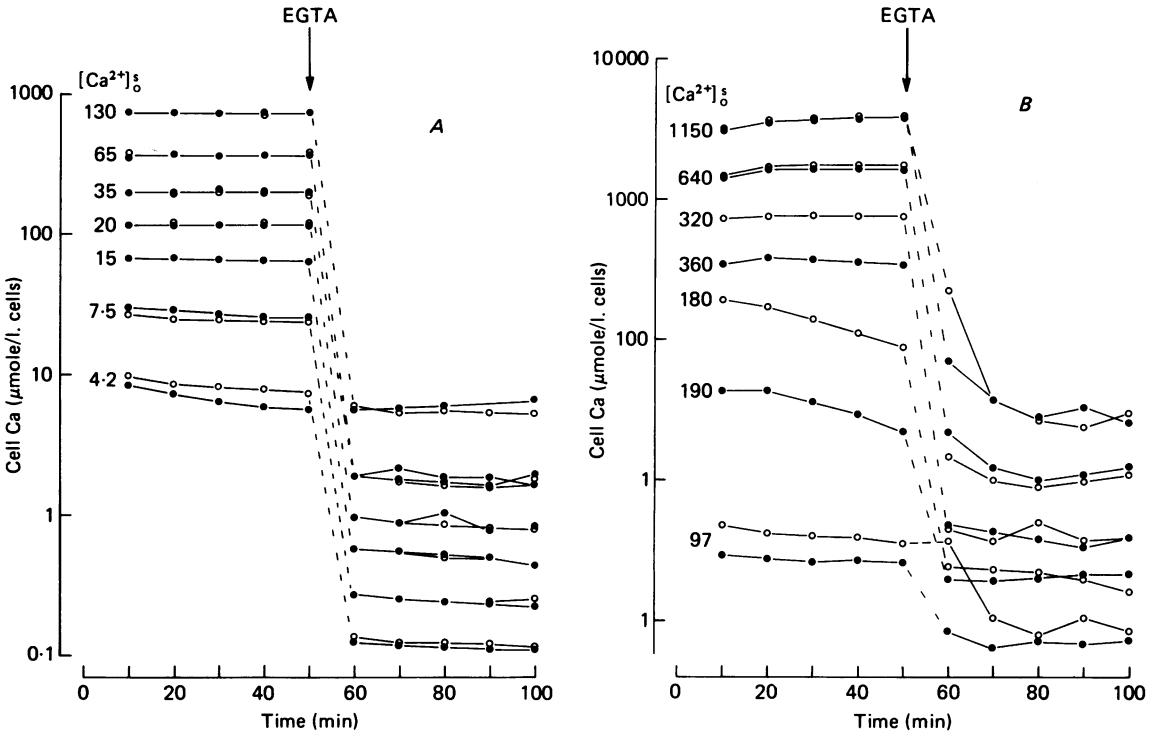

Fig. 1. Ca content as a function of time in inosine-fed, intact normal red cells in the presence of A23187 at 10 μM (*A*) or 0·63 μM (*B*), before and after chelation of external Ca. The cells were from 4-day old Bank blood and were incubated in medium B containing 0·5 mM-$MgCl_2$. A23187 was added at $t = 0$ min. At $t = 50$ min, the Ca concentration in each cell suspension was reduced to below 1 μM by addition of Tris–EGTA to a final concentration of 50–300 μM greater than the total Ca concentration. Estimated ionized cell Mg concentration at equilibrium $[Mg^{2+}]_i^{eq}$: 0·76 mM. The figures indicated as $[Ca^{2+}]_o^s$ are the calculated mean external Ca^{2+} concentrations between 10 and 50 min (*A*) or 20 and 50 min (*B*). In the Figure, the values for cell Ca have not been corrected for a trapped extracellular space of about 1 %. ○, no ouabain; ●, 1 mM-ouabain. One of more than five experiments giving similar results.

red cells incubated with two concentrations of A23187, 0·63 and 10 μM, with different initial extracellular Ca levels and with and without ouabain. In most cases, and particularly at higher Ca levels, steady levels of cell Ca were attained within about 10 min of A23187 addition and maintained until 50 min. Addition of EGTA to each cell suspension at 50 min rapidly restored cell Ca to levels below 10^{-7} M when corrected for extracellular trapped ^{45}Ca. Ouabain had little or no effect on cell Ca at the high Ca levels but, in this experiment, appeared to reduce somewhat cell Ca at the lower Ca levels. Though marked here, this effect of ouabain was not a consistent finding and in other experiments ouabain had little and sometimes opposite effects.

In the case of cells incubated with lower Ca levels, and particularly those incubated with 0·63 μM-A23187 (Fig. 1 *B*), cell Ca showed a tendency to decrease with time. This effect also varied in extent in different experiments and the reason for it is unclear though one trivial possibility, haemolysis, can be ruled out since this was less

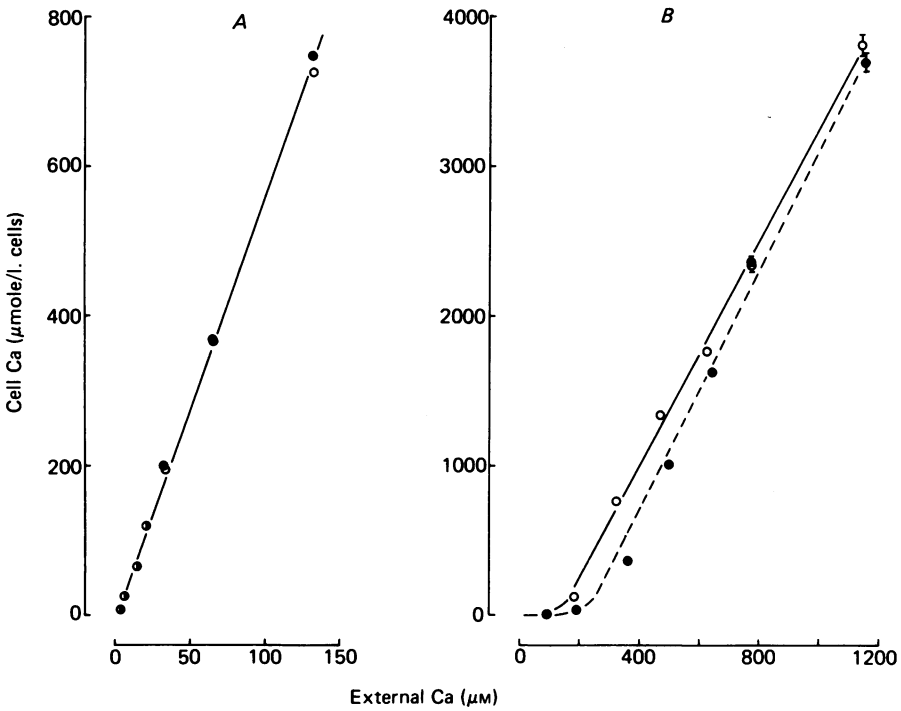

Fig. 2. Cell Ca ($(Ca^T)_i^s$) as a function of external Ca ($[Ca^{2+}]_o^s$) at steady state for inosine-fed intact normal red cells. The data are the mean values ±S.E. of the mean for cell and medium Ca between 10 and 50 min (*A*) or between 20 and 50 min (*B*), calculated from the data in Fig. 1. *A*: 10 μM-A23187; ○, no ouabain; ●, 1 mM-ouabain. Straight line fit to data obtained with and without ouabain: $(Ca^T)_i^s = 5{\cdot}67\ (\pm 0{\cdot}07).[Ca^{2+}]_o^s - 9{\cdot}08$, giving $\alpha = 0{\cdot}39$ assuming $r = 1{\cdot}5$. *B*: 0·63 μM-A23187; ○, no ouabain, ——, straight line fit: $(Ca^T)_i^s = 3{\cdot}77\ (\pm 0{\cdot}11).[Ca^{2+}]_o^s - 516\ (\pm 71)$. ●, 1 mM-ouabain, – – – –, straight line fit: $(Ca^T)_i^s = 4{\cdot}02\ (\pm 0{\cdot}21).[Ca^{2+}]_o^s - 906\ (\pm 137)$. Lines were fitted by linear regression.

than 5% in this experiment. It is interesting to note that a lack of steadiness in cell Ca levels under conditions similar to those used here has also been observed in dog red cells (Brown, 1979).

Fig. 2 shows the relationship between total cell Ca and external Ca at steady state for the cells incubated with the two concentrations of A23187, calculated from the data of Fig. 1. (These curves will be referred to in subsequent text as Ca distribution curves.) The data obtained with 10 μM-A23187 (Fig. 2 *A*) are well fitted by a straight line passing close to the origin, as found by Ferreira & Lew (1976). The fraction of cell Ca that is ionized, α, calculated from the slope of this line (see Methods) is 0·39 which is similar to that obtained by Ferreira & Lew (1976). Ouabain had no effect on either the slope or intercept of the line.

Fig. 2*B* shows the Ca distribution curve for cells incubated with 0·63 μM-A23187. The data at high Ca are well fitted by a straight line which is displaced to the right of the origin because of the action of the Ca pump maintaining cell Ca below equilibrium with external Ca (see e.g. Brown, 1979). Theoretical considerations indicate that the slope of this line should be the same at all ionophore concentrations

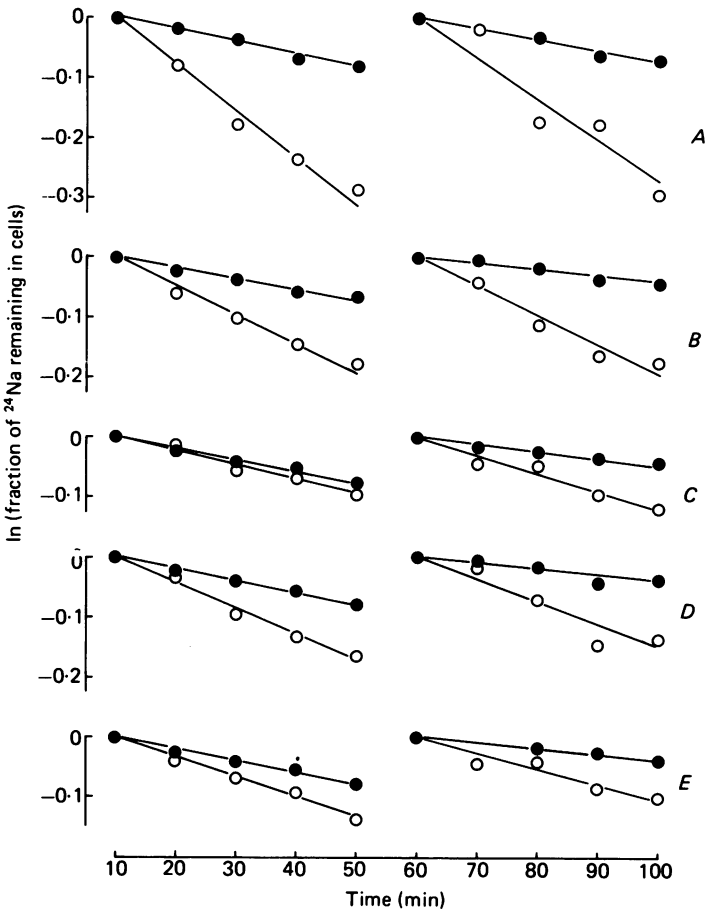

Fig. 3. Typical Na efflux curves from inosine-fed, intact normal red cells before and after Ca removal. Same experiment as in Figs. 1 and 2. A23187 concentration: *A*,*B*,*C*, 10 μM; *D*, *E*, 0·63 μM. Intracellular Ca^{2+} concentration in the steady state, before EGTA addition: *A*, 0 μM; *B*, 47 μM; *C*, 146 μM; *D*, 301 μM; *E*, 700 μM. ○, no ouabain; ●, 1 mM-ouabain.

if the Ca pump flux is a simple saturating function of $[Ca^{2+}]_i$ and the ionophore mediated Ca 'permeability' is independent of the Ca concentration (Ferreira & Lew, 1976; Lew & Brown, 1979). The finding here and in many other experiments that this slope is lower with the lower A23187 concentration indicates that at least one of these assumptions is invalid and there is evidence that this may be due to a slight extraction of the ionophore out of the membrane by increased external Ca, leading to a reduced Ca permeability (V. L. Lew & H. G. Ferreira, unpublished observations). Since neither of these possible complications will affect the Ca distribution when it is close to equilibrium, the best estimate for α is that calculated from the data obtained with 10 μM-A23187. In the present work, the value of α obtained in this way has been used to calculate $[Ca^{2+}]_i$ in cells incubated with both high and low concentrations of A23187.

Fig. 2*B* also shows the effect of ouabain on the Ca distribution curve obtained with

the low concentration of A23187. Covariance analysis indicates that the intercepts, but not the slopes, of the straight lines in the Figure were significantly different ($P < 0.01$). Since the intercept depends on the ratio of saturated pump flux to ionophore-induced Ca permeability (Ferreira & Lew, 1976; Lew & Brown, 1979), these results suggest that ouabain may have had some marginal effect on these parameters. The variability in the effect of ouabain, noted earlier, however, makes further analysis of these possibilities difficult.

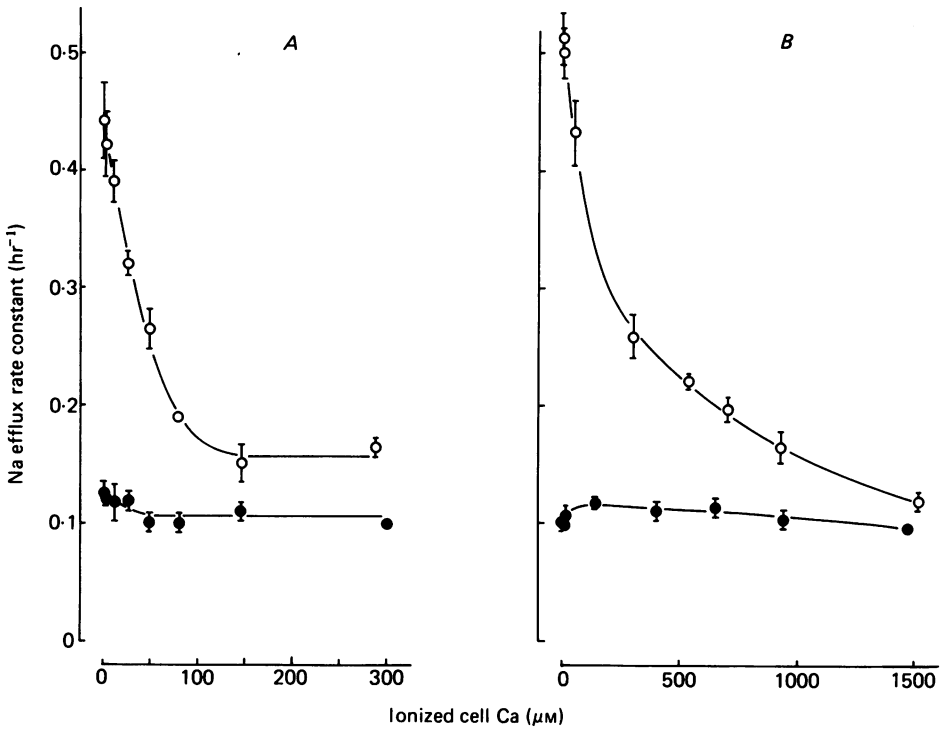

Fig. 4. Na efflux rate constant as a function of ionized cell Ca at steady state ($[Ca^{2+}]_i^s$) in inosine-fed intact red cells. *A*, 10 μM-A23187; *B*, 0·63 μM-A23187. Same experiment as in Figs. 1–3. $[Ca^{2+}]_i^s$ was calculated as α. $(Ca^T)_i^s$ (see eqn. (3) in Methods) with $\alpha = 0.39$ (see Fig. 2). ○, ouabain absent; ●, 1 mM-ouabain. Data are mean ±S.E. of the mean of slopes of regression lines from data as in Fig. 3.

Fig. 3 shows typical ^{24}Na efflux curves for cells incubated in the absence and presence of ouabain and at several concentrations of Ca. In each case, the data on the left show the efflux during the period when cell Ca was raised and those on the right the efflux following EGTA addition when the reversibility of the effect of Ca was studied. The efflux curves were in general well fitted by single exponentials though during the 'reversibility' period, there was generally more scatter. The reason for this is uncertain. Note, however, the similarity in the efflux rates before and after EGTA addition.

The rate constants for ^{24}Na efflux were calculated from the slopes of the lines obtained as in Fig. 3. Fig. 4 shows the rate constants for the first efflux period as

a function of $[Ca^{2+}]_i^s$, which was calculated from cell Ca at steady state using eqn. (3). With both ionophore concentrations there was a substantial decrease in Na efflux as $[Ca^{2+}]_i^s$ was increased in cells incubated without ouabain. In the presence of ouabain, however, Ca had little effect on the efflux rate constant.

The ouabain-sensitive ^{24}Na efflux rate constant at each $[Ca^{2+}]_i^s$ was calculated as the difference between the ^{24}Na effluxes measured in the absence and presence of

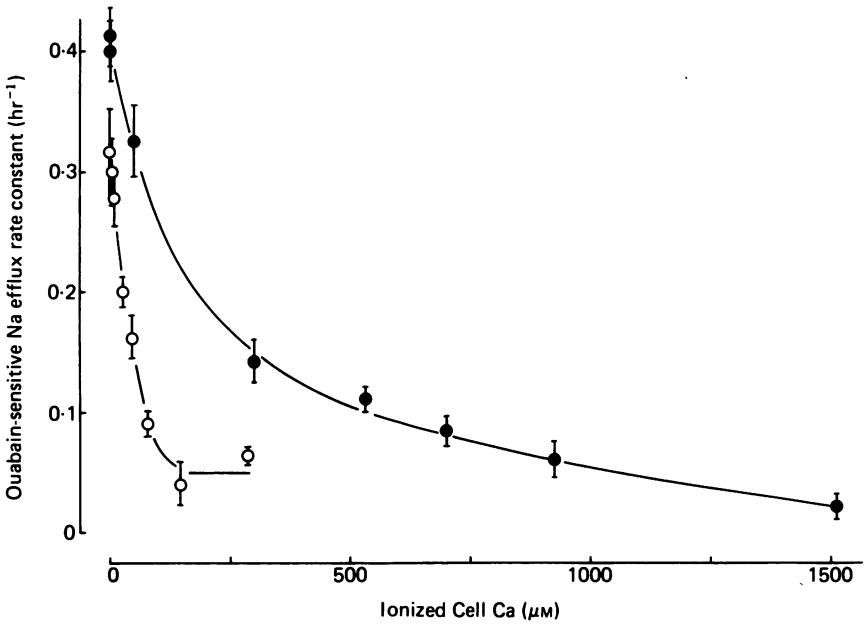

Fig. 5. Ouabain-sensitive Na efflux rate constant as a function of ionized cell Ca at steady state in inosine-fed, intact normal red cells. Rate constants calculated from the data in Fig. 4. ●, 0·63 μM-A23187; ○, 10 μM-A23187. One of four experiments giving similar results.

ouabain at similar $[Ca^{2+}]_i^s$ levels and Fig. 5 shows this rate constant as a function of $[Ca^{2+}]_i^s$ for cells incubated with the two concentrations of A23187. It is seen that in general agreement with previous work on the effect of Ca on the Na pump, there was a marked inhibition of Na efflux as $[Ca^{2+}]_i^s$ increased. The more striking finding, however, is that the apparent $[Ca^{2+}]_i$ sensitivity of the efflux depended on the concentration of A23187. With 10 μM-A23187, $[Ca^{2+}]_i^s$ at 50% inhibition (K_i^{app}) was about 40 μM while with 0·63 μM-A23187 it was about 165 μM. In other similar experiments with cells from different donors, values for K_i^{app} of 160–300 μM and 20–40 μM were obtained with 0·63 μM and 10 μM-A23187 respectively.

Preliminary measurements to test whether different ionophore concentrations had any effect on the ouabain-sensitive Na efflux in nominally Ca-free media showed a small but significant inhibitory effect of the high ionophore concentration in only two instances, both with cells from the blood bank (see Ca-free conditions in Fig. 5). In fresh cells, no such effects were seen (see Ca-free conditions in Fig. 17*B*), in agreement with the observations of Flatman & Lew (1981).

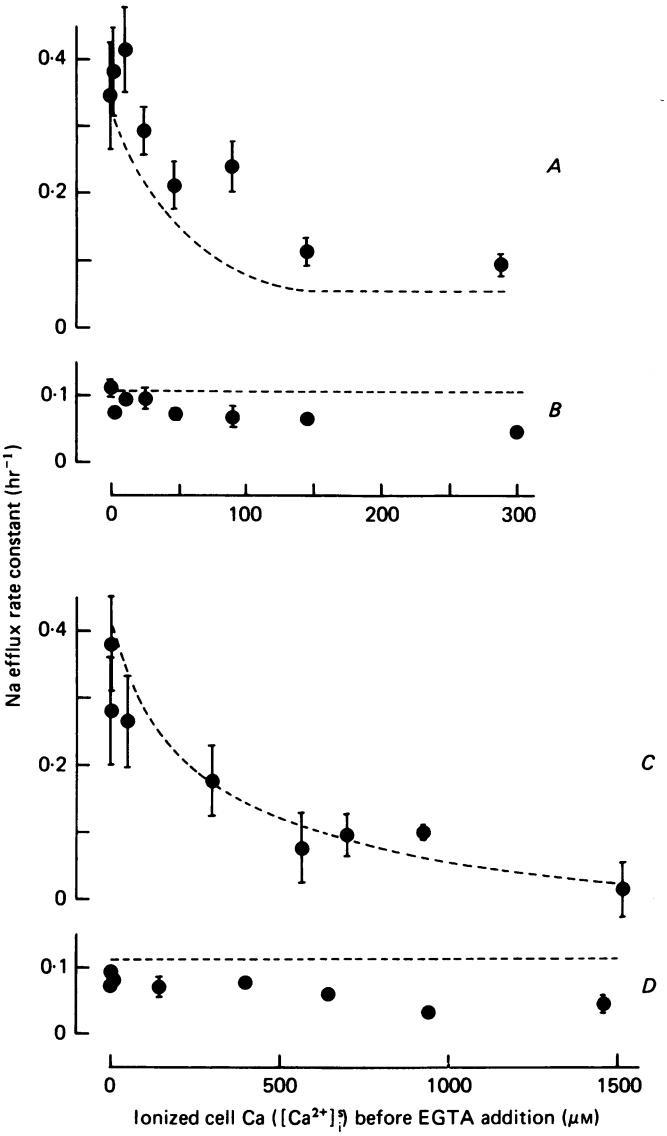

Fig. 6. Na efflux rate constants after removal of cell Ca in inosine-fed, intact normal red cells. Same experiment as in Figs. 1–5. Ouabain-sensitive (*A* and *C*) and ouabain-insensitive (*B* and *D*) rate constants obtained with 10 μM-A23187 (*A* and *B*) or with 0·63 μM-A23187 (*C* and *D*) are plotted as a function of $[Ca^{2+}]_i^s$ before addition of EGTA to each cell suspension. In each panel, the dashed lines represent the corresponding fluxes before EGTA addition, from Figs. 4 and 5. One of two experiments giving similar results.

Fig. 6 shows the results of the Na efflux measurements in the second part of the experiment of Fig. 5 where the reversibility of the effect of intracellular Ca on the fluxes was studied. During this part of the experiment, $[Ca^{2+}]_i^s$ was in all cases less than 1 μM (see text concerning Fig. 1) but the data have been plotted as a function of $[Ca^{2+}]_i^s$ during the first part of the experiment so that the fluxes before and after

Ca removal can be compared. It is seen that with both concentrations of A23187, there was substantial residual inhibition of the ouabain-sensitive Na efflux after removal of most levels of $[Ca^{2+}]_i$ though both in this and another similar experiment, there was some reversal of inhibition in cells incubated with 10 μM-A23187. The ouabain-insensitive flux was little affected by Ca removal but showed a slight tendency to fall, more in the conditions where the initial $[Ca^{2+}]_i^s$ was higher.

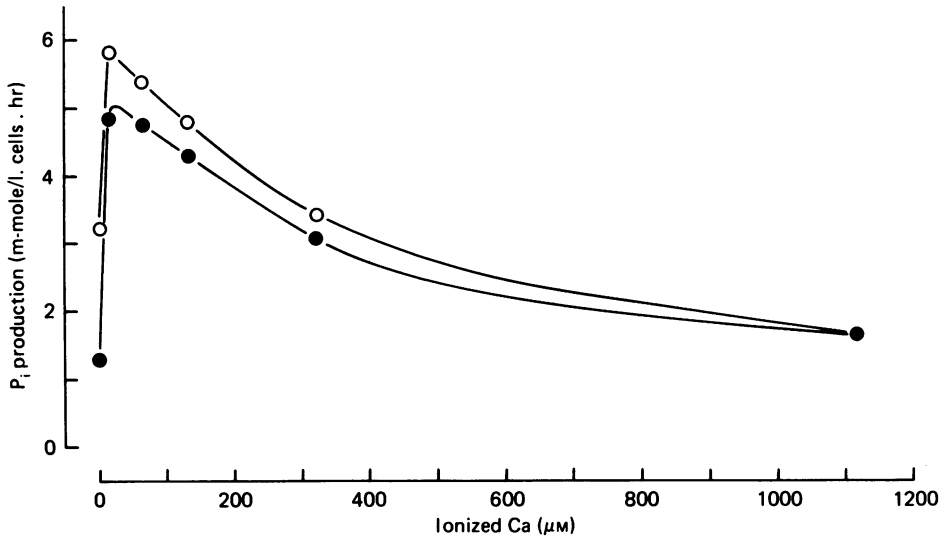

Fig. 7. ATPase activity of red cell membrane fragments as a function of ionized Ca under high Na, low K conditions. 2-day-stored membranes prepared from Bank blood were incubated for 60 min at 37 °C, 5 % equivalent haematocrit, with or without 1 mM-ouabain in a medium containing 100 mM-NaCl, 10 mM-KCl, 10 mM-Tris Cl (pH 7·8 at 37 °C), 1·5 mM-$MgCl_2$, 1·5 mM-Na_2ATP (with tracer [γ-^{32}P]ATP and various concentrations of $CaCl_2$ or EGTA (100 μM, 0 Ca^{2+} condition) and ethanol (4·25 μl./ml. of membrane suspension). Ca^{2+} was calculated as described in Methods. ○, ouabain absent; ●, 1 mM-ouabain. Each point is the mean of five measurements; the S.E. of the mean for each point is within the symbol.

Effects of Ca and A23187 on the Na–K-ATPase of red cell membranes

One possible explanation for the effect of A23187 on the apparent $[Ca^{2+}]_i$ sensitivity of the ouabain-sensitive Na efflux from intact cells is a direct effect of the ionophore on the Na pump. This is difficult to assess in intact cells because of the possibility of effects of A23187 and Ca on other co-factors for Na pump turnover. It seemed best to explore this possibility in the experiments aimed at investigating the inhibitory effects of Ca on the ATPase of human red cell membranes. The Na–K-ATPase activity, which is generally considered to be the enzymatic counterpart of Na–K exchange through the Na pump (see e.g. Dunham & Glynn, 1961), was measured as the ouabain-sensitive portion of the total ATPase activity in the presence of all necessary co-factors.

Fig. 7 shows the ATPase activity as a function of Ca^{2+} under control conditions, i.e. in the absence of A23187. The incubation conditions were chosen to be close to

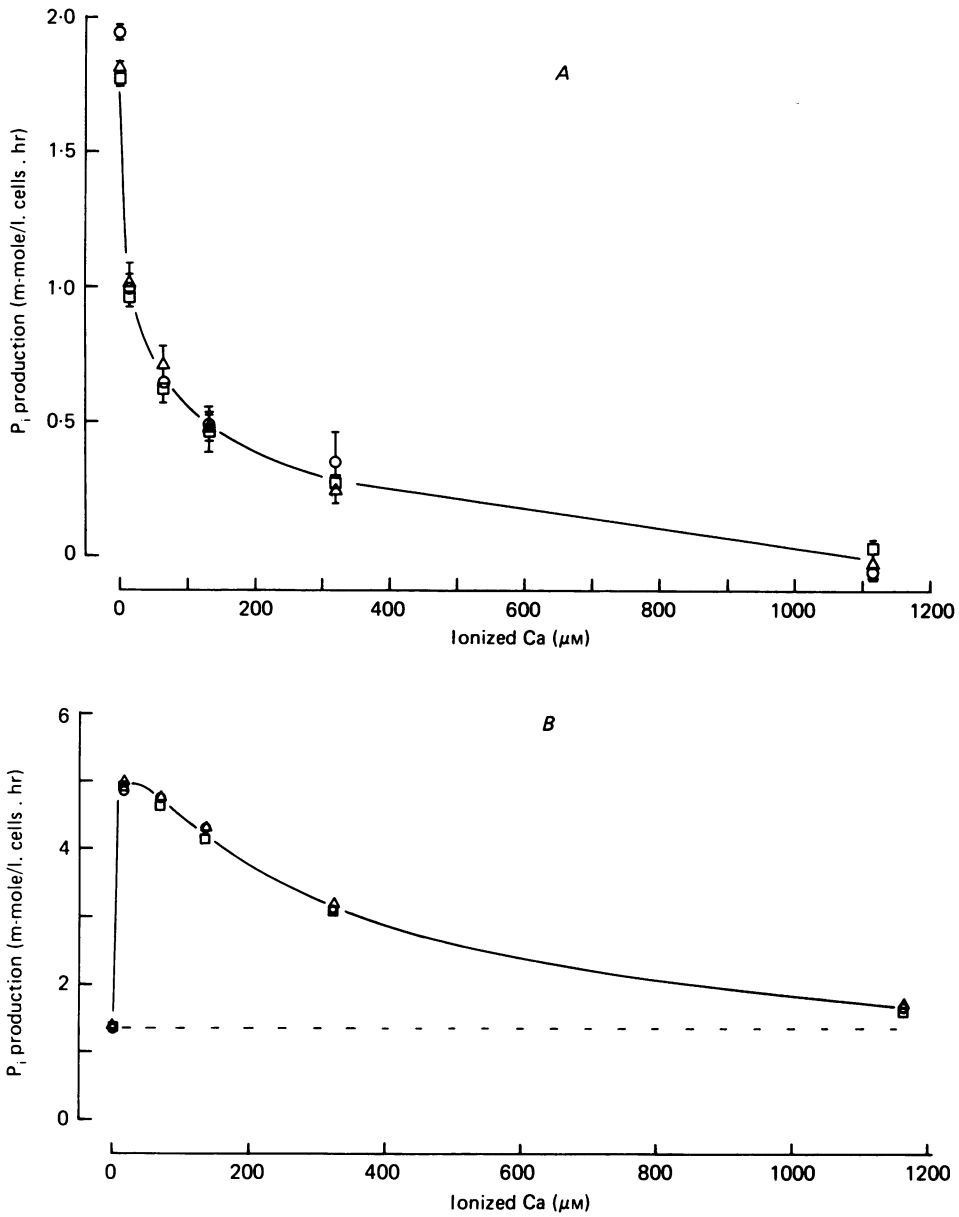

Fig. 8. Effect of A23187 on the Ca^{2+} dependence of ATPase activity of red cell membrane fragments under high-Na, low-K conditions. Same experiment as in Fig. 7. A23187 was added in ethanol (4·25 μl./ml. of membrane suspension) to give final concentrations (μmole/l. suspension) as follows: ○, 0; △, 0·55; □, 9. *A*, ouabain-sensitive activity; each point is the difference ±S.E. of the mean between the means of five measurements in the absence and presence of 1 mM-ouabain. *B* ouabain-insensitive activity; each point is the mean ±S.E. of the mean of five measurements.

optimal for the Na–K-ATPase (Dunham & Glynn, 1961). In the presence and absence of ouabain, low levels of Ca^{2+} produced a marked stimulation of ATPase activity which was maximal between 10 and 60 μM-Ca^{2+} and then decreased as Ca^{2+} was increased further. In the absence of Ca^{2+}, about 60% of the ATPase activity was ouabain-sensitive. This activity was about 1·9 m-mole P_i/l. cells . hr which is similar to that measured by Dunham & Glynn (1961).

Fig. 8*A* shows the ouabain-sensitive ATPase activity as a function of Ca^{2+} in the absence of A23187 and in the presence of 0·55 and 9 μmole A23187/l. of membrane suspension. These A23187 levels are similar to those used per litre of cell suspension in the experiments with intact cells (e.g. Figs. 1–6) but represent about twice the amount of A23187 present per unit amount of cell membrane. It can be seen that, in contrast to its effect on the apparent $[Ca^{2+}]_i$ sensitivity of the ouabain-sensitive Na efflux from intact red cells, A23187 had no effect on the inhibition of ouabain-sensitive ATPase by Ca. In all cases, inhibition was 50% at about 25 μM-Ca^{2+} and complete at about 1200 μM-Ca^{2+}. Fig. 8*B* shows that A23187 also had no effect on the ouabain-insensitive fraction of ATPase activity in this experiment.

Fig. 9 shows that there was still no effect of the ionophore on the ouabain-sensitive ATPase activity when the assay conditions were changed to be closer to those normally experienced by the intracellular surface of the Na pump (i.e. high K, low Na, $[Mg^{2+}]_i$ = 300–400 μM, [MgATP] = 800–1000 μM, total ATP ($[ATP]_T$) = 1·2 mM). In the absence of A23187 (Fig. 9*A*), the ouabain-sensitive ATPase activity was lower under low Na, high K conditions than in the presence of high Na and low K, even in the absence of Ca, presumably due to an inhibitory effect of high K competing with Na at the internal cation activating site of the pump (Garay & Garrahan, 1973). The ATPase activity was inhibited by Ca under both conditions, inhibition in each case being 50% at about 25 μM-Ca^{2+}, a value similar to that obtained from Fig. 8*A* for cells from a different donor. However, inhibition was biphasic at high Na, requiring much higher Ca^{2+} concentrations for full inhibition (see also Fig. 8*A*). Fig. 9*B* shows that 9 μM-A23187 had no effect on ATPase inhibition under high Na, low K conditions, confirming the result of Fig. 8*A*, and Fig. 9 *C* and *D* show a similar lack of effect of A23187 in the presence of low Na, high K. A23187 also had no significant effect of the Ca–Mg-ATPase and Mg-ATPase activities in the experiment of Fig. 9*A–C* but increased Ca–Mg-ATPase activity and decreased Mg-ATPase activity by 10–15% in the experiment of Fig. 9*D* (data not shown).

Effect of raised intracellular Ca and A23187 on the ATP content of intact, inosine-fed normal red cells

The experiments with red cell membrane fragments indicated that A23187 has no direct effect on ouabain-sensitive ATPase activity and suggested therefore that the effect of the ionophore on the apparent $[Ca^{2+}]_i$ sensitivity of ouabain-sensitive Na efflux from intact cells might involve A23187 + Ca-induced changes in some pump co-factor. Cell ATP is known to fall under these conditions (e.g. Reed, 1976; Allan, Watts & Michell, 1976; Ferreira & Lew, 1976; Taylor, Baker & Hochstein, 1977; Till *et al.* 1977, Till, Petermann, Wenz & Arese, 1981; Arese, Bosia, Pescarmona & Till, 1981) and might be able to mediate apparent $[Ca^{2+}]_i$-sensitivity changes because the apparent K_m for ATP (or MgATP) of the NA–K exchange or Na–K-ATPase activity

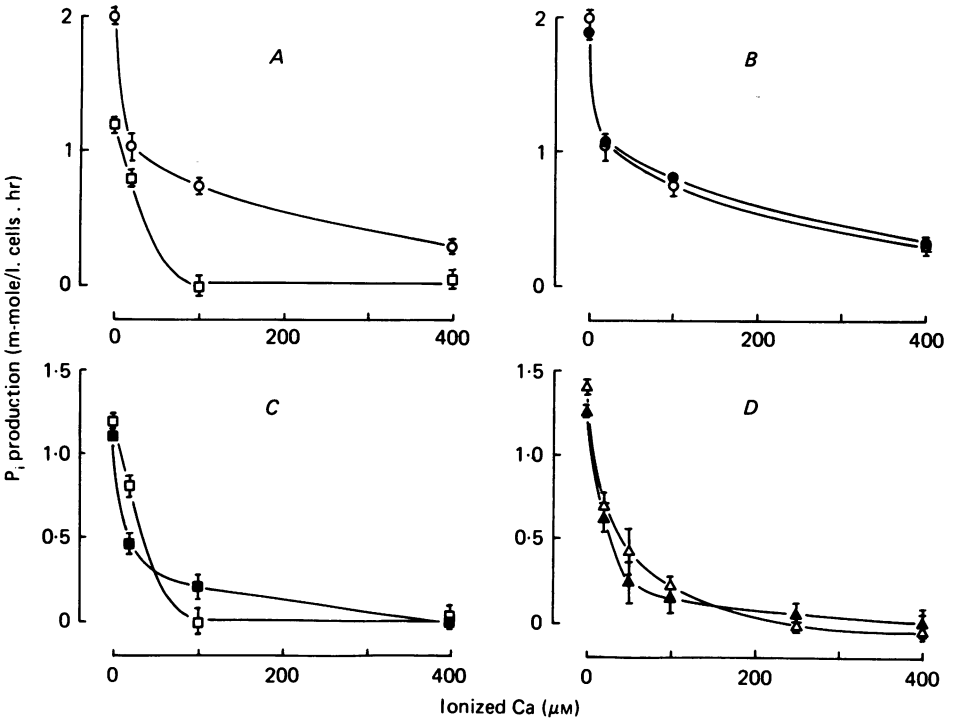

Fig. 9. Effects of A23187 Na and K concentration on the Ca^{2+} dependence of ouabain-sensitive ATPase activity of red cell membrane fragments. Experiments with membranes prepared from fresh cells (donor, V.L.L.) and stored for 2 days (*A*, *B*, *C*) or 3 days (*D*). The membranes were incubated at 37 °C for 80 min in a medium containing 10 mM-Tris Cl (pH 7·8 at 37 °C), 1·375 mM-$MgCl_2$, 1·2 mM-Na_2ATP (with tracer [γ-^{32}P]ATP) and either 100 mM-NaCl, 10 mM-KCl (high-Na conditions) or 10 mM-NaCl, 100 mM-KCl (low-Na conditions), various concentrations of $CaCl_2$ and ethanol (4·25 μl./ml. of membrane suspension) containing A23187 to give final concentrations of 0 (open symbols) or 9 (filled symbols) μmole/l. suspension. Each point is the difference ±S.E. of the mean between the means of four or more measurements in the absence and presence of 1 mM-ouabain. Ca^{2+} was calculated as described in Methods. *A*: no A23187; ○, high Na; □, low Na. *B*: high Na conditions. *C* and *D*: low Na conditions.

at saturating levels of K is between 100 and 800 μM (e.g. Dunham & Glynn, 1961; Robinson, 1967, 1974, 1976; Whittam & Wiley, 1967; Garrahan & Glynn, 1967*b*; Karlish & Glynn, 1974). Under normal conditions, therefore, cell ATP may be a rate limiting factor in Na pump turnover (Post, Hegyvary & Kume, 1972; Karlish, Yates & Glynn, 1978). If there were an effect of A23187 on the $[Ca^{2+}]_i$ dependence of the ATP changes, A23187-dependent differences in ouabain-sensitive Na efflux would be expected. The effects of Ca and A23187 concentrations on cell ATP were therefore examined in detail.

Figs. 10 and 11 show the results of such an experiment using fresh inosine-fed red cells pre-incubated for 4 hr under the same conditions as in the experiment of Figs. 1–6. The A23187 concentrations used were the same as in that experiment and the time course and distributions of cell Ca were qualitatively similar to those of Figs. 1 and 2. Fig. 10 shows the time course of cell ATP in the cells incubated with 0·63 μM

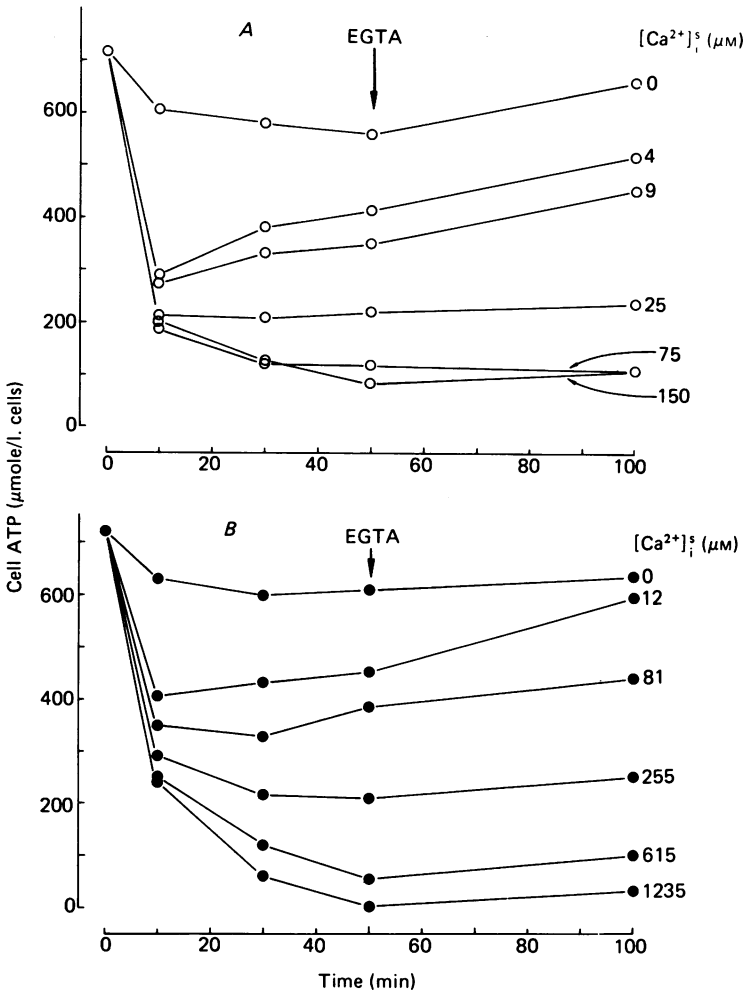

Fig. 10. ATP content as a function of time in inosine-fed, intact normal red cells. Washed fresh cells (donor, V.L.L.) were pre-incubated as if for ^{24}Na loading (see Methods) and then incubated in medium B containing 0·25 mM-$MgCl_2$. A23187 was added at $t = 0$ and steady-state ionized cell Ca concentration ($[Ca^{2+}]_i^s$) in each condition (calculated as α. $(Ca^T)_i^s$ (eqn. (3) in Methods) with $\alpha = 0{\cdot}23$) is indicated at the right of each curve. Immediately after sampling for cell ATP at 50 min, Tris–EGTA was added to each cell suspension to reduce cell Ca to below 1 μM (see legend to Fig. 1). The ATP content indicated for $t = 0$ min was measured in the 0 Ca condition just before addition of A23187. *A*, 10 μM-A23187; *B*, 0·63 μM-A23187. Estimated ionized cell Mg concentration at equilibrium, $[Mg^{2+}]_i^{eq}$: 0·44 mM. One of four experiments giving similar results.

and 10 μM-A23187 at several $[Ca^{2+}]_i^s$ levels. In the first 10 min following A23187 addition there was a rapid fall in cell ATP in all conditions where Ca was present, the extent of this fall increasing with increasing $[Ca^{2+}]_i^s$. In the subsequent 40 min, the direction of the ATP changes depended on the $[Ca^{2+}]_i^s$ level. In the absence of Ca, cell ATP fell slightly at first in all experiments and then changed little. At 50 min, EGTA was added to each condition reducing $[Ca^{2+}]_i^s$ to levels not measurably

different from zero and it can be seen from Fig. 10 that this had little effect on cell ATP which was either similar or only slightly increased 50 min later. In another similar experiment where only a high and a low Ca level were used with each A23187 concentration, the ATP changes were almost identical to those here with measurements 10, 30 and 50 min after EGTA addition showing steady ATP levels throughout this time.

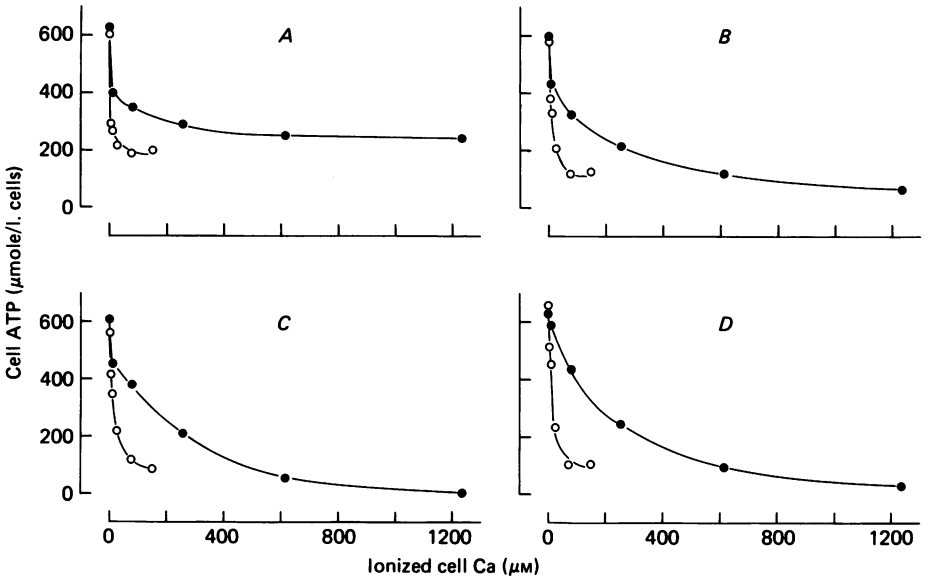

Fig. 11. ATP content as a function of ionized cell Ca ($[Ca^{2+}]_i^s$) in inosine-fed intact normal red cells. Data from the experiment of Fig. 10, plotted as a function of $[Ca^{2+}]_i^s$ measured before EGTA addition. Cell ATP is shown at a different time after A23187 addition: *A*, 10 min; *B*, 30 min; *C*, 50 min; *D*, 100 min, i.e. 50 min after EGTA addition. ●, 0·63 μM-A23187; ○, 10 μM-A23187.

Fig. 11 shows the data of Fig. 10 re-plotted to show cell ATP as a function of $[Ca^{2+}]_i^s$ at each time point. At all four times, the effect of A23187 was similar. In each case, the ATP levels in the Ca-free cells and the general form of the relationship between cell ATP and $[Ca^{2+}]_i^s$ were similar with both ionophore concentrations. However, the ATP level was much more sensitive to $[Ca^{2+}]_i$ in cells incubated with 10 μM-A23187 than in those incubated with 0·63 μM-A23187. 50% reduction in cell ATP required 110–170 μM and 5–15 μM-steady-state intracellular Ca^{2+} concentration at 0·63 μM and 10 μM-A23187 respectively. Note that in Fig. 11*D*, cell ATP is plotted as a function of the $[Ca^{2+}]_i^s$ which was in the cells before EGTA addition. This Figure shows that the changes in cell ATP were largely irreversible.

An additional point to note is that at 10 min after A23187 addition in cells incubated with both A23187 concentrations, there appears to be a minimum level to which cell ATP falls (about 200 μmole/l. cells) suggesting either that the rapid initial fall in cell ATP is caused by a process which saturates at about 600 μM and 25 μM-steady-state intracellular Ca^{2+} concentration with 0·63 μM and 10 μM-A23187 respectively or that a mechanism is present which prevents cell ATP falling below this level.

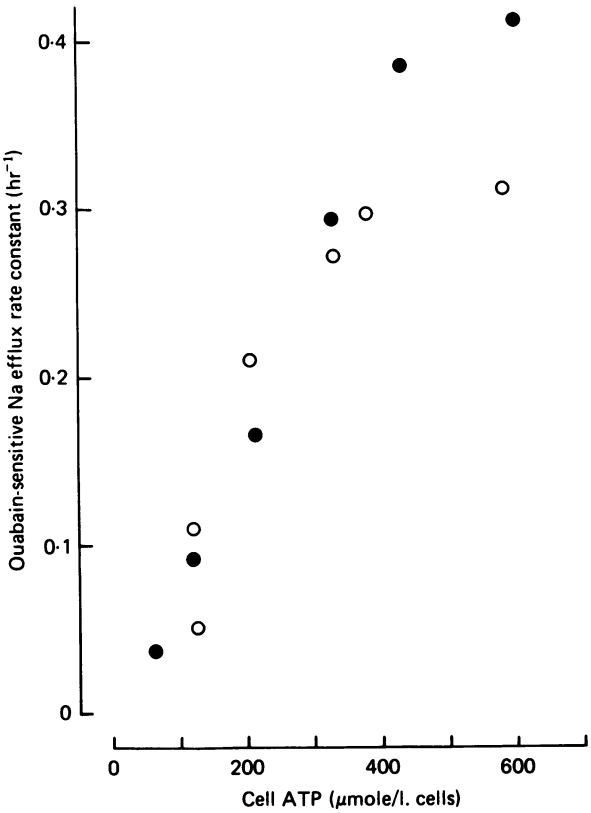

Fig. 12. Ouabain-sensitive Na efflux rate constant as a function of cell ATP in inosine-fed, intact normal red cells. Each point shows data for a different combination of A23187 and ionized cell Ca concentrations ($[Ca^{2+}]_i^s$). The data for cell ATP are from Fig. 11*B* and those for Na efflux are interpolated with respect to $[Ca^{2+}]_i^s$ from Fig. 5. ●, 0·63 μM-A23187; ○, 10 μM-A23187.

Relationship between ouabain-sensitive Na efflux and cell ATP

Three observations suggest that changes in cell ATP might play a central role in the Ca + A23187-induced inhibition of ouabain-sensitive Na efflux in the intact cell. Firstly, the ATP changes caused by incubation of the cells with Ca + A23187 are large enough to produce changes in Na pump turnover via the low affinity ATP regulatory site. Secondly, removal of cell Ca with EGTA fails to reverse either the inhibition of ouabain-sensitive Na efflux (Fig. 6) or the reductions in cell ATP (Fig. 10). Finally, there is a strong quantitative correlation between the effects of Ca and A23187 on the two variables as shown in Fig. 12. Here, ouabain-sensitive Na efflux (interpolated from Fig. 5) is plotted against cell ATP at 30 min (from Fig. 11) for various $[Ca^{2+}]_i^s$ and A23187 concentrations. Although the flux and ATP data are from experiments on cells from different donors, there is a strong suggestion that the data for both ionophore concentrations fall close to a single line which passes close to the origin and whose shape is that expected for a simple low affinity dependence of Na efflux on intracellular ATP concentration. Indeed, one possible interpretation of this Figure

is that Ca, *per se*, may have no direct inhibitory effect at all on the Na pump in intact cells. However, this would seem to be inconsistent with the ATPase results.

To explore this possibility and to examine in more detail the roles of ATP and $[Ca^{2+}]_i$ in pump inhibition in the intact cell, cell Ca, ATP and Na efflux were all measured in the same cells with normal and artificially increased ATP contents. A

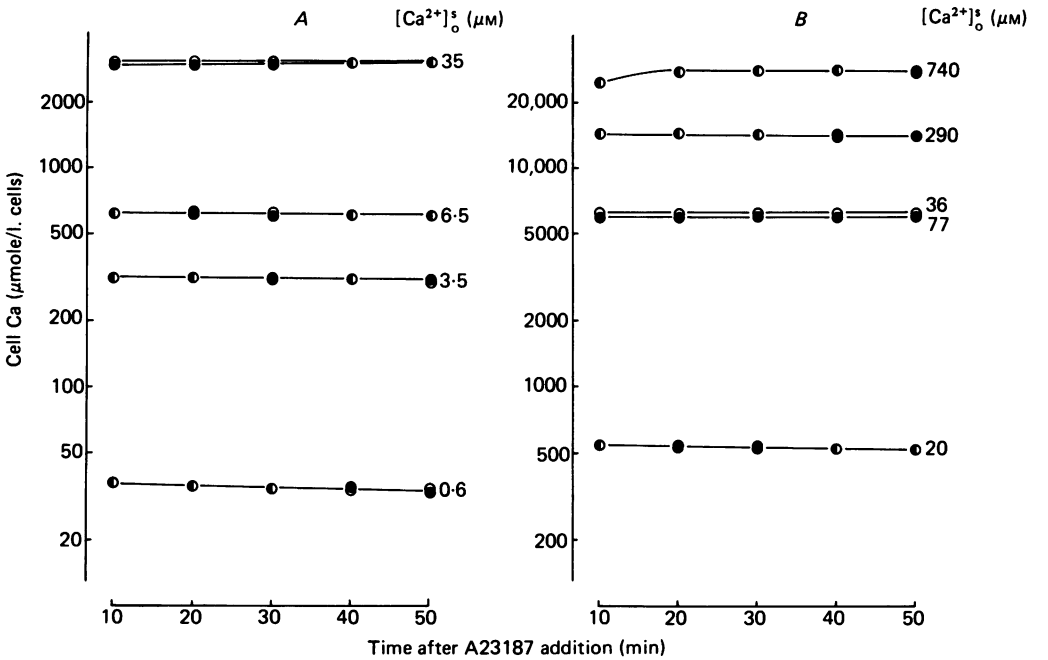

Fig. 13. Ca content as a function of time in adenosine + pyruvate-fed, ATP-enriched intact red cells, in the presence of A23187 at 10 μM (*A*) or 1 μM (*B*). Washed fresh red cells (donor, V.L.L.) were loaded with ^{24}Na and ATP and then incubated in medium C with (●) or without (○) 1 mM-ouabain. A23187 was added at $t = 0$. The figures indicated as $[Ca^{2+}]^s_o$ show the calculated mean external Ca^{2+} concentrations between 10 and 50 min. Estimated ionized cell Mg concentration at equilibrium, $[Mg^{2+}]_i^{eq} = 0{\cdot}81$ mM.

preliminary experiment showed that, as demonstrated by Duhm (1973), cells with an appropriately increased level of cell ATP could be obtained by incubation of fresh red cells with 10 mM-adenosine + 10 mM-pyruvate + 50 mM-phosphate (APP) for several hours. Even after a subsequent incubation of these cells with a high level of intracellular Ca, cell ATP remained higher than in freshly drawn red cells.

Figs. 13–17 show the results of an experiment similar in design to that of Figs. 1–5 to examine the effect of Ca and A23187 on Na efflux from cells pre-incubated with APP for 4 hr. The concentrations of A23187 used, 1 μM and 10 μM, were similar to those used in the experiments with normal cells. Fig. 13 shows that the method for controlling cell Ca using A23187 works well with ATP-enriched cells, steady levels of cell Ca being maintained throughout the 10–50 min Na-efflux-measurement period in all but one condition. Ouabain did not affect these levels.

Fig. 14 shows the steady-state Ca distribution curves for the cells incubated with the two concentrations of A23187 and it is clear that these are somewhat different

from those obtained with normal ATP cells (cf. Fig. 2). The data obtained with 10 μM-A23187 (Fig. 14*A*) are still well fitted by straight lines, but the slopes of these lines are 5–10 times larger indicating that a much higher proportion of the Ca in each cell suspension was associated with the cells. This is the cause of the larger than normal errors in the values for steady-state external Ca which are calculated from the

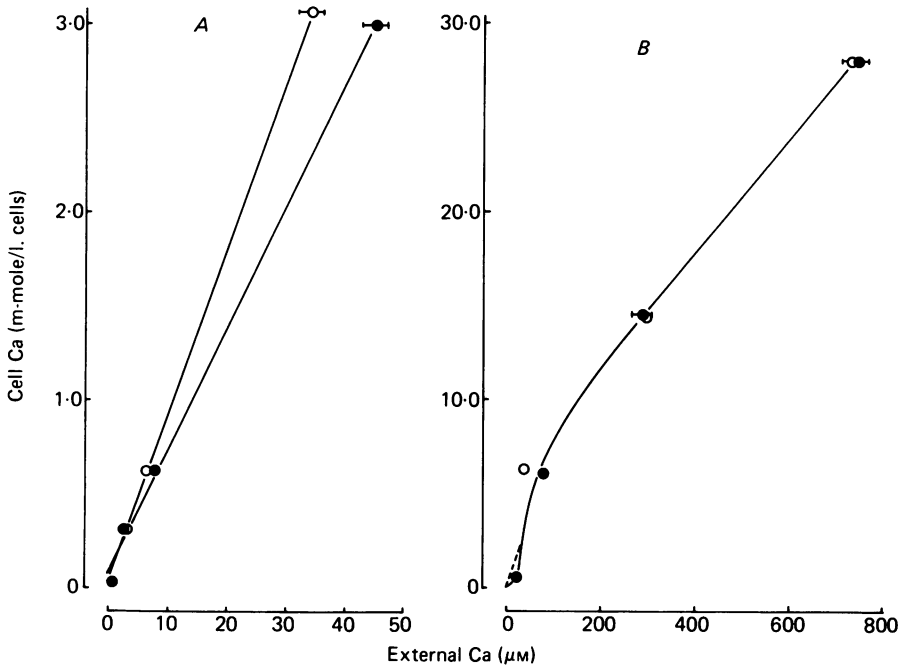

Fig. 14. Cell Ca ($(Ca^T)_i^s$) as a function of external Ca ($[Ca^{2+}]_o^s$) at steady state for adenosine + pyruvate-fed, ATP-enriched intact red cells. The data are the means ± S.E. of the mean for cell and external Ca between 10 and 50 min, calculated from the data of Fig. 13. *A*, 10 μM-A23187; O—O, no ouabain; straight line fit: $(Ca^T)_i^s = 89 (\pm 2).[Ca^{2+}]_o^s + 23$. ●—●, 1 mM-ouabain; straight line fit: $(Ca^T)_i^s = 64 (\pm 2).[Ca^{2+}]_o^s + 85$. r determined by pH measurements (see Methods) was 4·2 and the mean slope for data obtained with and without ouabain (76) gives $\alpha = 0{\cdot}23$. *B*, 1 μM-A23187. O, no ouabain; ●, 1 mM-ouabain. The dotted line represents the data for 10 μM-A23187, from *A*.

differences between cell and total Ca in each cell suspension. The size of these errors renders meaningless the observed differences in Ca distribution with and without ouabain. The data obtained both with and without ouabain have therefore been fitted by a single line whose slope is 76 ± 6. Assuming a value of r^2 of 17·4, based on the estimate from pH measurements after pre-incubation with APP, a value of 0·23 is obtained for α which is similar to the values of 0·20 and 0·21 obtained in experiments with fresh cells from the same donor. This suggests that the large increase in organic phosphate content of the cells during pre-incubation with APP (from 3 to 49 μmole P/g red cells in 3 hr (Duhm, 1973)) has little effect on the Ca buffering properties of the cells and that the increase in cell Ca content of ATP-enriched cells at a particular $[Ca^{2+}]_o^s$ is due mainly to cell hyperpolarization increasing $[Ca^{2+}]_i^s$ (and hence also bound Ca).

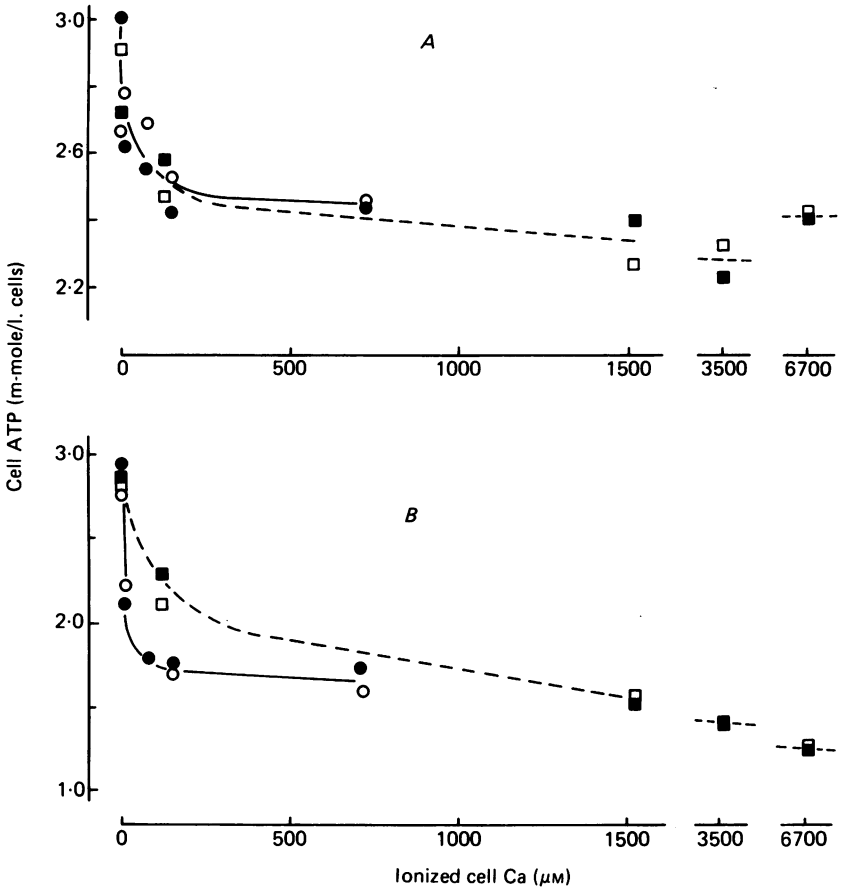

Fig. 15. ATP content of adenosine + pyruvate-fed, ATP-enriched intact red cells as a function of ionized cell Ca at steady state ($[Ca^{2+}]_i^s$). Same experiment as Figs. 13 and 14. Cell ATP measured 10 min (*A*) and 50 min (*B*) after A23187 addition. $[Ca^{2+}]_i^s$ was calculated as $\alpha . (Ca^T)_i^s$ (eqn. (3) in Methods) with $\alpha = 0{\cdot}23$ (see Fig. 14*A*). Open symbols, no ouabain; filled symbols, 1 mM-ouabain. □, ■, ---, 1 μM-A23187; ○, ●, ——, 10 μM-A23187. Curves are fitted by eye.

Fig. 14*B* shows the steady-state Ca distribution curve for cells incubated with 1 μM-A23187. The lowest $(Ca^T)_i^s$ point was well below $[Ca^{2+}]_o^s . r^2/\alpha$ indicating the influence of the Ca pump on the Ca distribution at this ionophore concentration. At higher Ca levels the slope of the distribution curve decreases suggesting that at least some of the intracellular buffers are saturating. Although the data are insufficient to warrant detailed discussion of the distribution curves, it should be noted that at Ca levels where Ca buffers are saturating, the fraction of cell Ca that is ionized is no longer constant but increases with increasing $(Ca^T)_i$ so that $\alpha . (Ca^T)_i$ is an underestimate of the true $[Ca^{2+}]_i$. Without information about the Ca buffers in the cell, it is not possible to allow for this in the estimation of $[Ca^{2+}]_i^s$ so that values for $[Ca^{2+}]_i^s$ greater than about 1500 μM represented in subsequent Figures are only approximate.

Fig. 15 shows the effects of intracellular Ca on the ATP content of the ATP-enriched

cells 10 and 50 min after addition of the two different concentrations of ionophore in the experiment of Figs. 13 and 14. It had been found in a preliminary experiment with ATP-enriched cells that, as with normal-ATP cells (Fig. 10), there were two phases of ATP change following ionophore addition in the presence of Ca, an initial rapid Ca-dependent fall followed by a slower change. Unlike in normal cells, however, cell ATP in all cases decreased during this slower phase at nearly constant, Ca-dependent rates. The data of Fig. 15 confirm these preliminary findings. Fig. 15*A* shows that there was a rapid Ca-dependent fall in cell ATP in the first 10 min after A23187 addition though it is interesting to note that, in contrast to the findings in normal ATP cells, this fall was little affected by the ionophore concentration, half maximal fall requiring 30–60 μM-steady-state intracellular Ca^{2+} concentration with both 1 and 10 μM-A23187. Based on data for 10 min after ionophore addition, the maximum initial rate of ATP fall in the high ATP cells, both in this and the preliminary experiments, corresponds to 4–5 m-mole/l. cells . hr which is quite similar to the rate in the equivalent period in normal-ATP cells (3–3·5 m-mole/l. cells . hr). However, it is clear from other experiments with normal-ATP and ATP-enriched cells (A. M. Brown & V. L. Lew, unpublished results) that this is a substantial underestimate of the rate of ATP fall in the first 2–3 min after ionophore addition. The strikingly high level of ATP remaining in the cells after 50 min, even at the highest Ca levels, will be considered later (see Results on Fig. 17*A*).

Fig. 15*B* shows that 50 min after A23187 addition, an effect of the ionophore on the cell ATP levels was evident, indicating, therefore, also an effect on the rate of ATP fall between 10 and 50 min. This fall was slower than in the period immediately following A23187 addition, the maximal rate of Ca-dependent net ATP consumption at Ca steady state being only about 1·2–1·7 m-mole/l. cells . hr. 50% fall occurred with about 10 μM and 100–300 μM-steady-state intracellular Ca^{2+} concentration at 10 and 1 μM-A23187 respectively, levels similar to those causing half maximal changes in cell ATP in the normal cells at similar A23187 concentrations (Fig. 11). Ouabain had no effect on cell ATP levels either in the ATP-enriched cells (Fig. 15) or in normal-ATP cells (data not shown) suggesting that in the presence of raised cell Ca, ATP consumption by the Na pump is a small fraction of the total ATP turnover rate in these cells.

^{24}Na efflux from the ATP-enriched cells was linear in all conditions, as in Fig. 3, and Fig. 16 shows the $[Ca^{2+}]_i$ dependence of Na efflux in the presence and absence of ouabain in cells incubated with the two A23187 concentrations in the experiment of Figs. 13–15. The data are similar to those for the normal cells (Fig. 4), the only notable difference being that in the cells incubated with 1 μM-A23187, higher $[Ca^{2+}]_i^s$ levels were used than in the experiments with normal cells and at $[Ca^{2+}]_i^s$ greater than about 1500 μM the Na efflux both in the presence and absence of ouabain increased markedly. This effect was too large to be accounted for by the observed minimal increase in rate of cell lysis at high cell Ca levels, and so appears to indicate an increased Na permeability of the cells. (Incidentally, high ATP seems to have some protective effect against Ca-induced lysis since it is smaller than in normal ATP cells at equivalent Ca contents (Lew & Ferreira, 1978).) A similar Ca-induced increase in Na permeability has been reported in metabolically poor, stored red cells containing 1–3 m-mole/l. cells (Romero & Whittam, 1971).

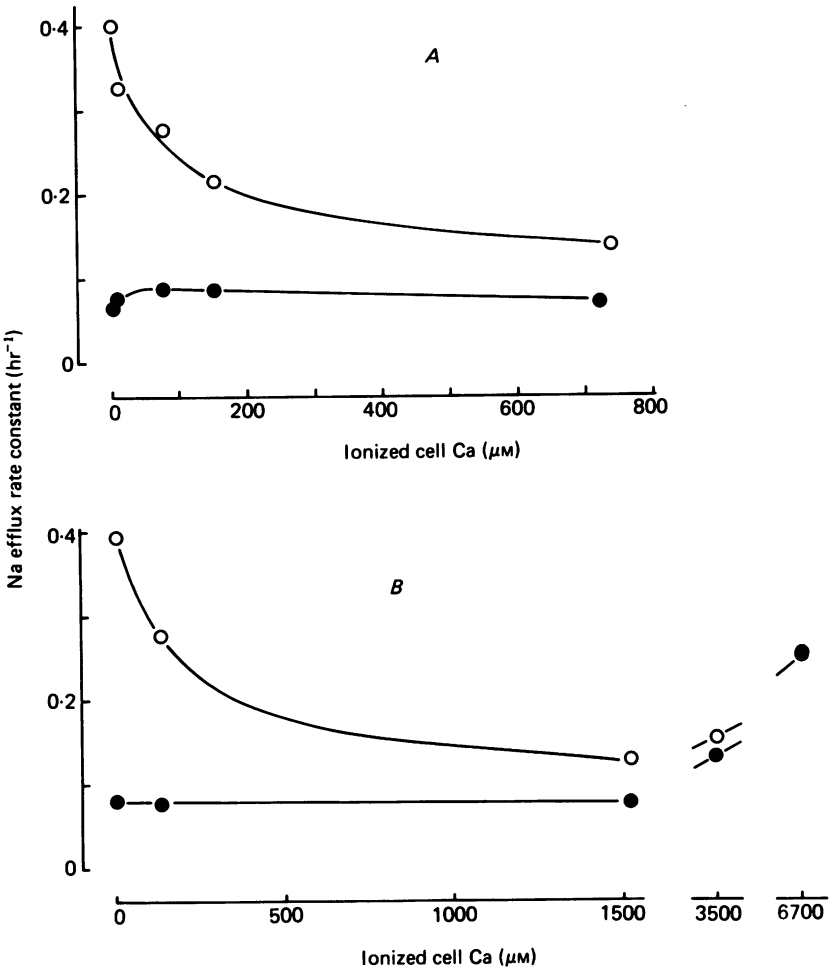

Fig. 16. Na efflux rate constant as a function of ionized cell Ca at steady state ($[Ca^{2+}]_i^s$) in adenosine+pyruvate-fed, ATP-enriched intact red cells. Same experiment as Figs. 13–15, the data being the mean slopes of regression lines similar to those in Fig. 3. *A*, 10 μM-A23187; *B*, 1 μM-A23187. ○, no ouabain; ●, 1 mM-ouabain. Curves fitted by eye. Values for $[Ca^{2+}]_i^s$ above about 1500 μM are only approximate (see text).

Fig. 17*A* shows the $[Ca^{2+}]_i$ dependence of ouabain-sensitive Na efflux at the two ionophore concentrations in the ATP-enriched cells, calculated from the data of Fig. 16, and Fig. 17*B* shows a similar plot for normal-ATP cells from the same donor but measured in a different experiment. The curves correspond to predictions of a competitive model for pump inhibition discussed below. It is seen that $[Ca^{2+}]_i$ had qualitatively similar effects in both lots of cells although the effect of A23187 on the apparent $[Ca^{2+}]_i$ sensitivity of inhibition was less marked in the ATP-enriched than in the normal cells. This may have been due in part to the slightly higher 'low' ionophore concentration used although in the same experiment, there was still a very marked effect of the ionophore concentration on the apparent $[Ca^{2+}]_i$ sensitivities of the Ca-activated K channel (A. M. Brown & V. L. Lew, in preparation). Furthermore,

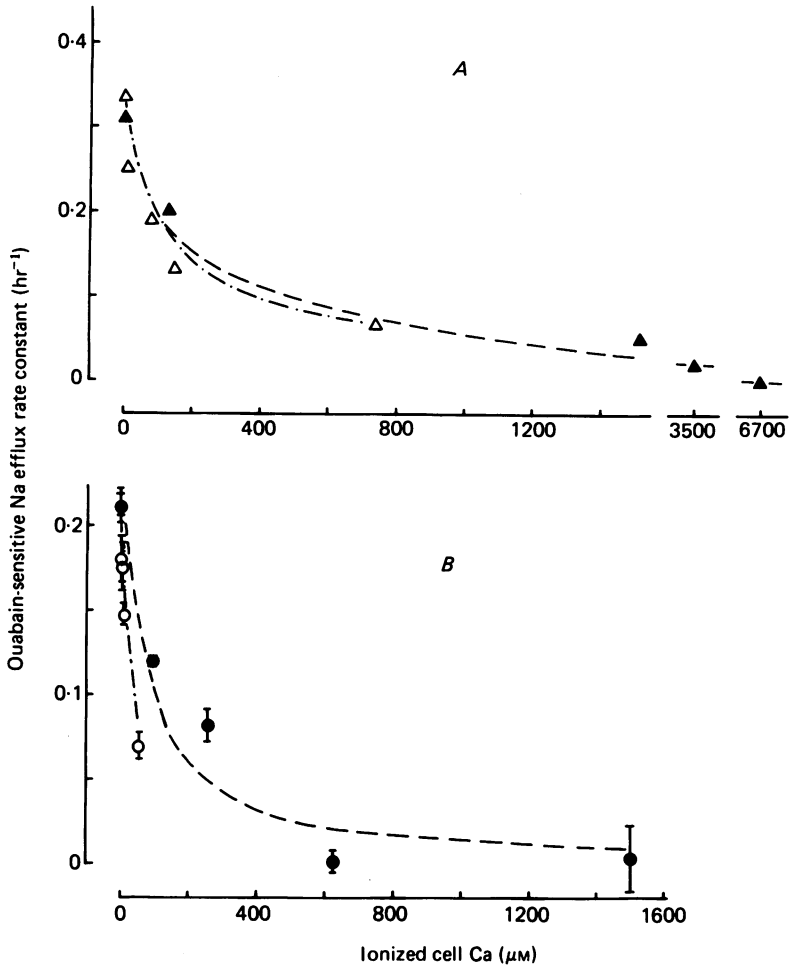

Fig. 17. Ouabain-sensitive Na efflux rate constant (v_{Na}) as a function of ionized cell Ca at steady state. *A*, adenosine + pyruvate-fed, ATP-enriched intact red cells, calculated from the data of Fig. 16. *B*, inosine-fed normal red cells from the same donor (V.L.L.) as in *A* but measured in a different experiment. △, ○, 10 μM-A23187; ▲, 1 μM-A23187; ●, 0·63 μM-A23187. The dashed lines show the fluxes predicted by the simple competitive model:

$$v_{Na} = 0{\cdot}39 \,.\, [MgATP]/([MgATP] + 800\ (1 + [CaATP]/50))$$

for data obtained with 10 μM-A23187 (– . – . –) or with 1 or 0·63 μM-A23187 (– – –) (see text for details). The S.E. of the mean for each point lies within the symbol in *A*.

the $[Ca^{2+}]_i^s$ required for half maximal inhibition with the high ionophore concentration was also somewhat higher for the ATP-enriched cells (80–100 μM) than for the normal-ATP cells (20–30 μM). As well as differences between the effects of Ca on the two groups of cells, there was also a difference between the fluxes measured in the absence of Ca which is probably due, at least in part, to the low affinity modulation of Na pump-mediated Na–K exchange by cell ATP (Karlish & Glynn, 1974).

An important finding in Fig. 17*A* is that at very high $[Ca^{2+}]_i^s$ levels, the ouabain-sensitive Na efflux was completely inhibited. Fig. 15 showed that both at

the beginning and end of the flux measurement period, cell ATP was well above the levels seen in normal cells, the minimum measured level of cell ATP being about 1·25 m-mole/l. cells, which corresponds to about 2 mM in cell water. This is 4–5 times higher than the estimated K_m for ATP of ouabain-sensitive Na–K exchange (Karlish & Glynn, 1974) so that the maximum fall in ouabain-sensitive Na efflux likely to occur solely due to ATP depletion during the incubation (i.e. from about 3 to 1·25 m-mole/l.

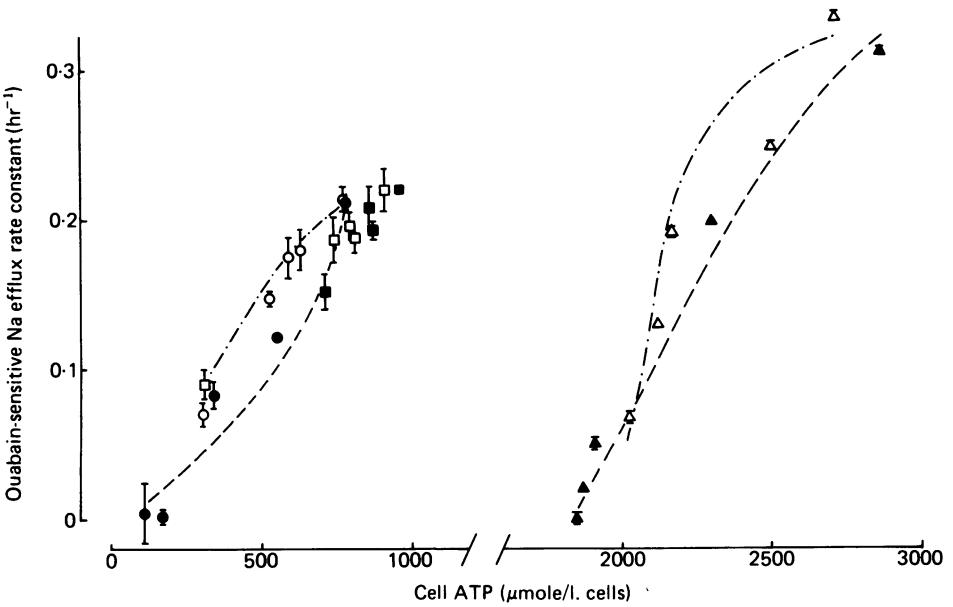

Fig. 18. Ouabain-sensitive Na efflux rate constant (v_{Na}) as a function of mean cell ATP. Results from three experiments with cells from the same donor (V.L.L.) are shown. Cell ATP is the mean of two (△, ▲), three (□, ■) or five (○, ●) measurements between 10 and 50 min after A23187 addition. Triangles: adenosine+pyruvate-fed, ATP-enriched intact red cells, same experiment as in Figs. 13–17*A*; ▲, 1 μM-A23187; △, 10 μM-A23187. Circles and squares: inosine-fed normal red cells. ●, 0·63 μM-A23187; ○, 10 μM-A23187: same experiment as Fig. 17*B*. □, ■, A23187 concentration uncertain but 10 times higher in □ than in ■. The dashed lines show the fluxes predicted by the simple competitive model:

$$v_{Na} = 0{\cdot}39 \,.\, [MgATP]/([MgATP]+800\,(1+[CaATP]/50))$$

for data obtained with 10 μM-A23187 (–·–·) or with 1 or 0·63 μM-A23187 (–––) (see text for details).

cells) is only about 10%. The results in Fig. 17*A* therefore indicate that Na pump inhibition is not caused solely by the Ca+A23187-induced changes in cell ATP and consistent with this view is the maintenance of linear ^{24}Na effluxes in the absence of ouabain in all conditions (not shown), even at Ca levels where cell ATP fell substantially during the flux measurement. However, the finding that the apparent $[Ca^{2+}]_i$ sensitivities of both ouabain-sensitive Na efflux and cell ATP fall were reduced in the ATP-enriched cells suggests once again that ATP changes might be involved in the pump inhibition. This view is further supported by the data of Fig. 18 which shows the dependence of ouabain-sensitive Na efflux on mean cell ATP during the

efflux. (Again, the curves correspond to predictions by the competitive inhibition model discussed below.) Both in normal-ATP and in ATP-enriched cells, the relationship between flux and cell ATP is similar at different ionophore concentrations. However, the intercepts and slopes of these relationships are different for the cells in the different initial metabolic states.

Analysis of the effects of Ca, ATP and A23187 on the ouabain-sensitive Na efflux from intact cells

The relationships between ouabain-sensitive Na efflux, cell ATP, $[Ca^{2+}]_i$ and A23187 suggest that inhibition of the Na pump by intracellular Ca in intact cells depends on both intracellular Ca and ATP concentrations. One way this might occur is through an interference by the Ca complex of ATP, CaATP, with the pump. Although the identity of the true nucleotide substrate for the pump is uncertain, i.e. whether it is free ATP, MgATP or both (see e.g. Glynn & Karlish, 1975), some data from ATPase studies are consistent with inhibition of the Na–K-ATPase by Ca being mediated by an interaction between CaATP and MgATP. We therefore investigated whether the inhibition of ouabain-sensitive Na efflux in the intact cells could be explained in this way.

Before the results of such an analysis are evaluated, however, it is worth questioning the value and limitations of such an exercise. We show below that the fine detail of the data can be reasonably explained by various inhibition models, the intrinsic limitations of which are discussed at the end. However, our data may not even be acceptable for such an analysis since the possibility that all inhibitory effects caused by $[Ca^{2+}]_i$ in the intact cells are mediated by ATP changes, though perhaps unlikely, cannot be entirely ruled out. The reason for this is that, as is apparent from Fig. 18, if some of the ATP contained in the ATP-enriched cells (about 1·8 m-mole/l. cells) were compartmentalized away from the Na pump, the behaviour of normal and ATP-enriched cells would become practically identical (except for differences in the absolute rates of ouabain-sensitive Na efflux which might reflect a change in the levels of other pump modulators). This would mean that Ca-induced inhibition of the Na pump of intact red cells could be explained simply by Ca-induced reductions in the cell ATP levels under all conditions. Since such an explanation would not apply to the Ca-induced inhibition of the ouabain-sensitive ATPase activity (where the Ca-induced changes in ATP concentration were less than 10%), this would imply a genuine difference between the Ca-reactivity of the Na pump *in situ* and in the isolated membrane. This possibility must remain open at present.

Analytical approach used

Mean values for cell [CaATP] and [MgATP], $[CaATP]_m$ and $[MgATP]_m$, during the efflux were calculated from the experimentally determined mean values for $[Ca^{2+}]_i$, $[Mg^{2+}]_i$ and cell ATP, as described in Methods. For convenience in these calculations, $[Mg^{2+}]_i$ for a particular experiment was assumed to be the same in all Ca and A23187 conditions, the value used being that determined in the absence of Ca. Experimental support for the validity of this approximation in normal cells is provided by Fig. 19 which shows that in the presence of both low and high concentrations of A23187, there was at most only a 25% increase in $[Mg^{2+}]_i^{eq}$ as $[Ca^{2+}]_i^s$

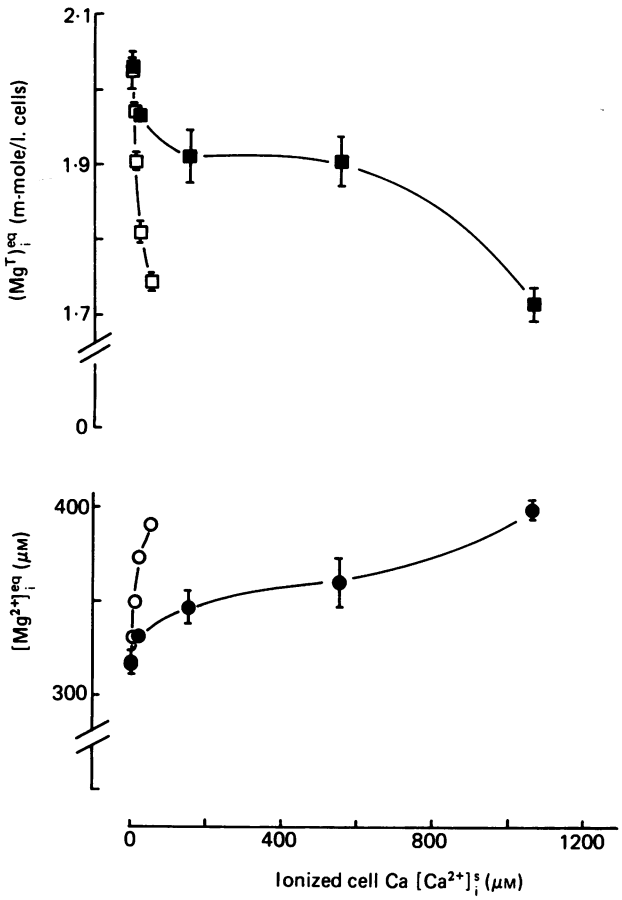

Fig. 19. Mg content, $(Mg^T)_i^{eq}$, and ionized cell Mg, $[Mg^{2+}]_i^{eq}$, at equilibrium as a function of ionized cell Ca at steady state, $[Ca^{2+}]_i^s$, in inosine-fed normal red cells. Washed fresh cells (donor, A.M.B.) were pre-incubated as if for ^{24}Na loading and then incubated with A23187 and Ca or EGTA in medium B containing 0·15 mM-$MgCl_2$. Cell Ca and Mg contents were determined at 10 min intervals between 10 and 40 min after A23187 addition when the distributions of both cations were steady. $[Ca^{2+}]_i^s$ was calculated as $\alpha . (Ca^T)_i^s$ (eqn. (3) in Methods) with $\alpha = 0{\cdot}19$. Squares: $(Mg^T)_i^{eq}$; circles: $[Mg^{2+}]_i^{eq}$. ○, □, 10 μM-A23187; ●, ■, 0·63 μM-A23187. Note the expanded ordinate scale; the change in $[Mg^{2+}]_i^{eq}$ is less than 25% under all conditions.

was increased within the ranges studied in the present work. This increase in $[Mg^{2+}]_i^{eq}$ reflects a 0–15% decrease in total cell Mg as cell Ca was increased (Fig. 19) and is the result of the consequent increase in $[Mg^{2+}]_o^{eq}$ (which occurred because the total Mg in the cell suspension was the same for all Ca concentrations) in the presence of a Ca-independent membrane potential clamped by the use of the high-K medium (see Methods). The effect of neglecting this $[Ca^{2+}]_i$-dependent change in $[Mg^{2+}]_i^{eq}$ in the consideration of the mechanism of pump inhibition is to decrease the predicted normalized efflux rate constant (see below) by at most 0·05 and in most conditions by less than 0·03.

It was clear from the values of $[CaATP]_m$ and $[MgATP]_m$ obtained from these

calculations that the standard methods such as the Lineweaver–Burk plot for analysis of inhibitory mechanisms could not be applied directly since neither quantity remained constant in different conditions. An attempt was made to derive relationships between ouabain-sensitive Na efflux and $[MgATP]_m$ at different constant $[CaATP]_m$ levels by interpolation from plots of efflux *vs.* $[CaATP]_m$ and $[MgATP]_m$ *vs.* $[CaATP]_m$ at different A23187 concentrations and in different experiments. However, this approach proved inconclusive because of insufficient data to provide reliable interpolations. A trial-and-error method of analysis was therefore adopted in which an inhibitory mechanism was assumed, Na fluxes predicted from the pairs of values of $[CaATP]_m$ and $[MgATP]_m$, and the result compared with the experimentally measured efflux. This procedure was then repeated using different values for the parameters of the model so that the quality of agreement between the model and the data could be examined.

Analysis of a competitive model

(*i*) *Application to normalized fluxes measured in the presence of intracellular Ca.* The first inhibitory mechanism considered was a simple competition between CaATP and MgATP. Some data in the literature are consistent with such a mechanism for inhibition of the Na–K-ATPase (Epstein & Whittam, 1966; Robinson, 1974). The analysis was based on the following descriptive equation:

$$v_{Na} = \frac{V_{Na}^{max} \cdot [MgATP]}{[MgATP] + K_m (1 + [CaATP]/K_i)},$$

where v_{Na} is the ouabain-sensitive Na efflux rate constant, V_{Na}^{max} the maximum rate constant, and K_m and K_i are the apparent affinities for MgATP and CaATP respectively at the pump. Fig. 17 showed that the efflux rate constant in the absence of raised intracellular Ca was somewhat higher in the ATP-enriched cells than in the normal-ATP cells from the same donor. Since this may be due in part to factors other than the difference in cell ATP levels, no attempt was made to take this difference into account in the initial analysis. Instead, the data for normal and ATP-enriched cells were analysed separately and normalized so that the predicted and measured fluxes for the Ca-free condition had values of 1·0.

Fig. 20 shows the results of this analysis for the data from normal-ATP cells. Each symbol shows the efflux predicted for a particular pair of values for $[CaATP]_m$ and $[MgATP]_m$ and the dotted line shows the approximate position of the cluster of experimental data in Fig. 18. It is seen that as K_i is increased, the fluxes predicted from data obtained with the two ionophore concentrations move closer to a common line and that the position of this line is closest to that seen experimentally with K_m between 400 and 4000 μM. Fig. 21 shows the results of this procedure applied to the data for the ATP-enriched cells. In this case, the efflux predicted from data at the two ionophore concentrations lie close to a common line with all combinations of K_m and K_i tested but this line is closest to that observed experimentally when the K_m/K_i ratio is about 20.

The values of K_m and K_i required to give a good agreement between the competitive inhibition model and the experimental data can be examined further by comparing the predicted and observed normalized efflux rate constants as functions

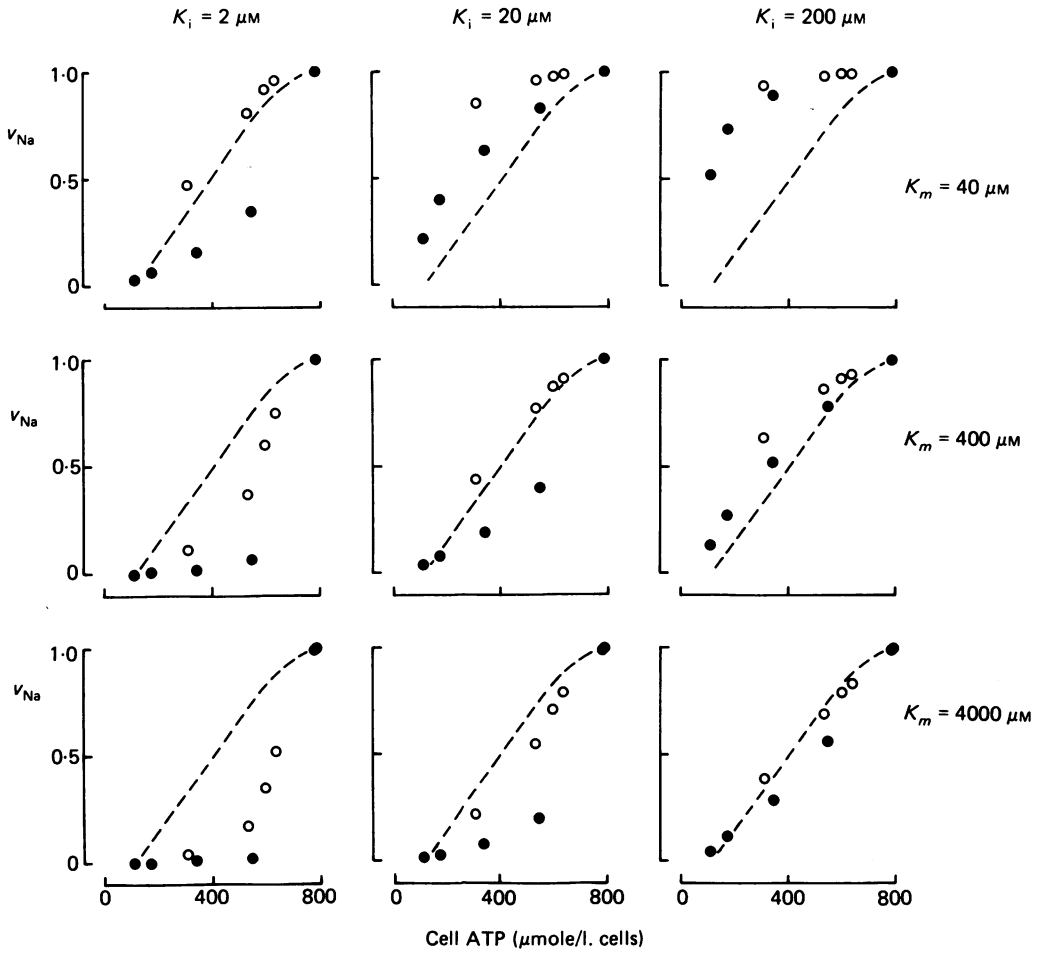

Fig. 20. Normalized ouabain-sensitive Na efflux rate constant (v_{Na}) as a function of mean cell ATP for inosine-fed normal red cells; comparison between experimental data and values predicted by the simple competitive inhibition model:

$$v_{Na} = V_{Na}^{max} . [MgATP]/([MgATP] + K_m (1 + [CaATP]/K_i)).$$

Cell ATP is the mean of five measurements between 10 and 50 min after A23187 addition in the experiment of Fig. 17*B* and Fig. 18 (circles). Each row of panels represents the comparison with a single value of K_m (indicated at right) and each column the comparison with a single value of K_i (indicated at top). For each value of K_m, V_{Na}^{max} is chosen so that v_{Na} is 1·0 for the Ca-free condition. The symbols show the values predicted by the model (see text for details); filled symbols: 0·63 μM-A23187; open symbols, 10 μM-A23187. The dotted line in each panel shows the best fit by eye to the experimental data (○, ● in Fig. 18) after normalization to give v_{Na} = 1·0 in the absence of Ca.

of $[Ca^{2+}]_i^s$. Using the same range of values for K_m and K_i as for Figs. 20 and 21, this comparison indicates that (i) K_m must be greater than 400 μM in order that the fluxes predicted for the normal ATP cells incubated with low and high A23187 concentrations lie on separate curves, (ii) K_i must be greater than 25 μM to produce a good fit between the predicted and experimental data for the normal cells incubated with

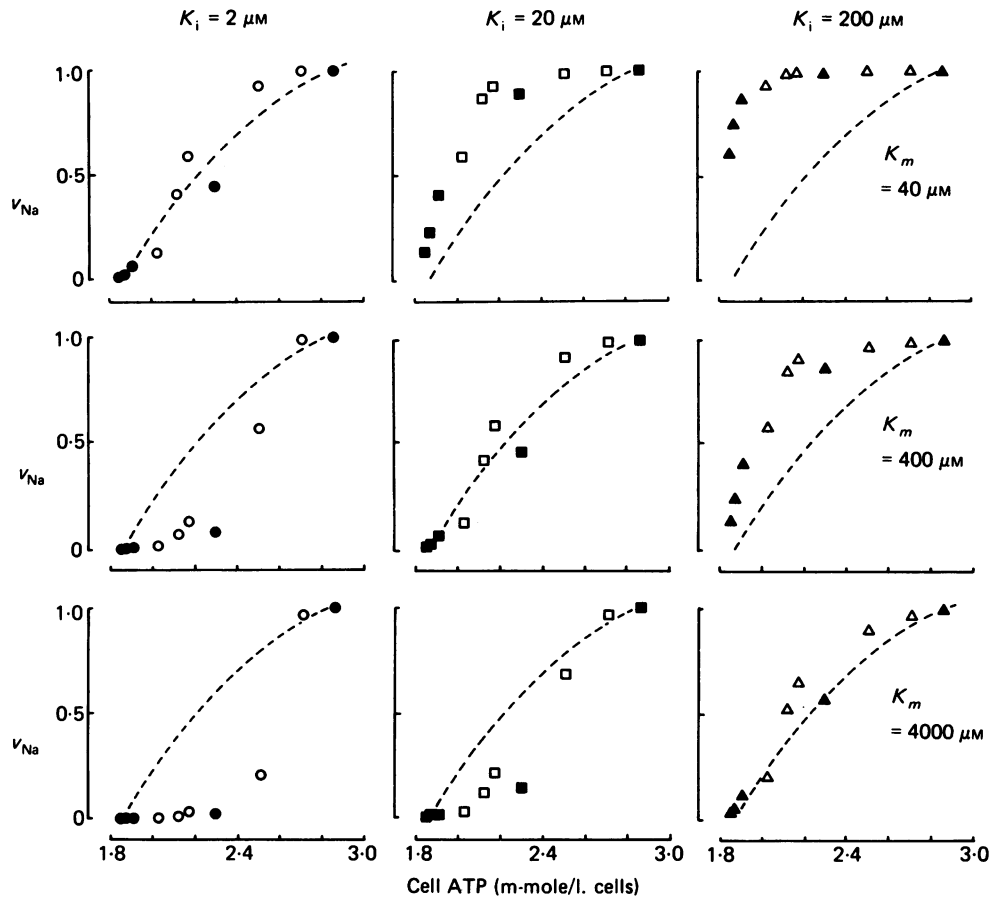

Fig. 21. Normalized ouabain-sensitive Na efflux rate constant as a function of mean cell ATP for adenosine + pyruvate-fed, ATP-enriched intact red cells; comparison between experimental data and values predicted by a simple competitive inhibition model (see legend to Fig. 20). Cell ATP is the mean of measurements 10 and 50 min after A23187 addition in the experiment of Figs. 13–17*A* and Fig. 18 (triangles). Each panel represents the comparison for a different combination of values for K_m and K_i (see legend to Fig. 20) with V_{Na}^{max} chosen so that v_{Na} is 1·0 for the Ca-free condition. The symbols show the values predicted by the model; filled symbols, 1 μM-A23187; open symbols, 10 μM-A23187. The dotted line in each panel shows the best fit by eye to the experimental data (△, ▲ in Fig. 18) after normalization.

0·63 μM-A23187 and (iii) for the ATP-enriched cells, there is little to choose between the three pairs of K_m and K_i values which gave the best fits in Fig. 21; however, in no case is a separation obtained between the data pertaining to the low and high ionophore conditions though this separation was in any case less marked for the ATP-enriched than for the normal cells (see Fig. 17). Thus, a reasonable fit between the competitive model and the normalized experimental data for both normal and ATP-enriched cells can be obtained with K_m between 400 and 4000 μM and K_i between 20 and 200 μM. A similar result is obtained from a consideration of the pooled data for normal cells used for Fig. 12.

(*ii*) *Extension of competitive model to un-normalized data.* So far the analysis of the competitive model has been based on normalized flux data to minimize constraints on the choice of values of K_m and K_i required to fit the experimental flux data. However, it seems likely that the difference in cell ATP levels is a major factor responsible for the efflux rate constant in the Ca-free conditions being higher in the ATP-enriched cells than in the normal cells. If so, the values of K_m and V_{Na}^{max} in the simple competitive model are defined by the fluxes in these conditions and it is then of interest to see whether this model can still provide a reasonable fit to the experimental fluxes measured in the presence of intracellular Ca.

The values obtained for K_m and V_{Na}^{max} in this way are 800 μM and 0·39 hr^{-1} respectively. The former falls within the range for K_m required to fit the normalized flux data and is of the same order as estimates for the apparent K_ms of ouabain-sensitive Na–K exchange for ATP in red cell ghosts (Karlish & Glynn, 1974) and of Na–K-ATPase activity for MgATP (Robinson, 1974). With these values defined, K_i was then chosen to optimize the agreement (by eye) between the competitive model and the experimental flux data. The value obtained for K_i is about 50 μM (i.e. $K_m/K_i = 16$) and the resulting fit, which is reasonably good, is shown by the dotted lines in Figs. 17 and 18. Note that in this analysis, differences between the $[Mg^{2+}]_i^{eq}$ levels in the normal and ATP-enriched cells have been neglected. This is reasonable for, although increasing $[Mg^{2+}]_i$ is known to have a stimulatory effect on Na–K exchange at concentrations up to about 1 mM, an increase in $[Mg^{2+}]_i$ from 0·35 to 0·8 mM increases the exchange by less than 10% (Flatman & Lew, 1981). This model, therefore, suggests that CaATP inhibits by displacing MgATP from a low affinity site for which it has a higher affinity. This would correspond, presumably, to the well characterized low affinity ATP site which controls the rate of K release from its occluded location on the Na–K-ATPase (Post *et al.* 1972; Karlish *et al.* 1978).

(*iii*) *Prediction of irreversibility of Na pump inhibition in intact cells by the competitive model.* A feature of the flux data in the intact cells not considered so far in the analysis is the irreversibility of the inhibition of the ouabain-sensitive fluxes following Ca removal (Fig. 6). A detailed analysis of this is not possible since cell ATP and the fluxes were not measured in the same cells. It is clear, however, that the lack of change in cell ATP following Ca removal (Fig. 10) together with a K_m of the pump for MgATP of between 400 and 4000 μM means that the model will predict substantial irreversibility of pump inhibition following Ca removal.

Limitations to the analysis of the inhibition mechanism

There are several limitations to the analysis just presented. Firstly, the good fit to the experimental data is not unique to the competitive model. An analysis similar to that just described was also carried out for two other types of model, simple non-competitive inhibition by CaATP (see e.g. Laidler & Bunting, 1973) and direct inhibition by $[Ca^{2+}]_i$ (of the general form $a/(b+[Ca^{2+}]_i)$, where a and b are constants containing or not concentrations of pump co-factors which remained constant in these experiments, like Mg^{2+}, K or Na) together with a low affinity dependence of the pump on ATP. Both of these models produced fits to the ATP and $[Ca^{2+}]_i$ dependences of the fluxes which, by eye, were at least as good as for the competitive model (data not shown). The parameters giving these best fits were as follows: non-competitive

model: K_m (MgATP) = 800 μM, K_i (CaATP) = 150–200 μM; direct inhibition models: K_m (ATP) = 1020 μM, b = 200 μM. Both mechanisms also predict substantial irreversibility of pump inhibition on removal of cell Ca. Thus, the present data do not permit firm conclusions about the precise mechanism of pump inhibition.

A second limitation in the analysis is an uncertainty about the identity of the normal pump substrate involved. Free ATP and MgATP are in constant proportion at constant $[Mg^{2+}]_i$ and in the present work, $[Mg^{2+}]_i$ was kept reasonably constant to prevent direct effects on the Na pump (Flatman & Lew, 1981). It is therefore not possible to determine from the flux data which of these two species is more likely to be the substrate whose interaction at the Na pump is interfered with by CaATP.

Thirdly, the parameters of the models which give a good fit to the flux data do not describe the ATPase data well. Using an analysis similar to that described above, reasonable fits could be obtained with all three models but with the K_m set as required to fit the flux data, the required value of K_i was either about 10–20 % (low Na, high K conditions, Fig. 9*C* and *D*) or about 15–40 % (high Na, low K conditions, Fig. 9*B* and also Fig. 8) of that needed to fit the flux data.

The final point which deserves mention is the effect of possible heterogeneity of cell Ca content on the analysis of the inhibition mechanism. Hoffman, Yingst, Goldinger, Blum & Knauf (1980) raised the possibility that, at low ionophore concentrations, most of the cell-associated Ca might be contained within only a fraction of the cells and proposed that such heterogeneity of cell Ca content was the explanation for the low apparent $[Ca^{2+}]_i$ sensitivity of the Ca-activated K channel observed by Lew & Ferreira (1976). They suggested that this heterogeneity arose through non-uniform distribution of the ionophore-induced increase in Ca permeability among the cells. Although it has been shown since that such variations in Ca permeability are absent in ATP-depleted cells (Simonsen *et al.* 1982), it is still unknown whether this is true in normal-ATP and ATP-enriched cells or whether heterogeneity of Ca content might even be present at uniform A23187 distribution due to differences in the Ca-extrusion activity of different cells.

To allow for the possible effects of heterogeneity in the analysis of the present results would have required precise information about the Ca, ATP and Na-flux distributions among the cells and these cannot be measured at present. Instead, therefore, a uniform Ca distribution was assumed not only in the high-ionophore conditions, where this is always the case since Ca is at equilibrium, but also in the low-ionophore conditions where the Ca distribution is still to be explored. Thus the present analysis of the low ionophore data represents one possible interpretation of these data. As it turns out, however, the conclusions from this analysis are similar to those that would be derived from an analysis based on the high-ionophore results alone. This can be seen clearly for the consideration of the competitive inhibition model. The ratio of K_m/K_i giving the best fit to the combined high and low ionophore data was also that giving the best fit to the high-ionophore data alone (Figs. 20 and 21) and the final choice of K_m was determined by the fluxes measured in the absence of Ca in the normal-ATP and ATP-enriched cells (Fig. 18). Together, these results yield a value for K_i of 40 μM which compares well with the value of 50 μM obtained by optimizing the fit of the model to the low and high-ionophore data (Figs. 17 and 18). A similar conclusion also applies to the analysis of the other models considered.

DISCUSSION

The present results describe, for the first time, the precise concentration dependence of the inhibitory effect of internal Ca on the ouabain-sensitive Na–K exchange in intact red cells. Unlike in previous work (Dunn, 1974), the internal Ca concentrations, cell inorganic ion content, volume and membrane potential were kept constant during the flux measurements and it was possible to estimate the bound and ionized Ca forms at each total Ca level. In intact cells, however, internal Ca also causes substantial changes in the concentrations of other pump co-factors, particularly ATP (Reed, 1976; Ferreira & Lew, 1976; Taylor *et al.* 1977; Till *et al.* 1977, Arese *et al.* 1981, Brown & Lew, 1981; Figs. 10–15 in the present work), MgATP (see Results, above), and $[Mg^{2+}]_i^{eq}$ (Fig. 19) and in the analysis and interpretation of the Ca effects in terms of inhibitory mechanisms, these changes have also been considered.

A fundamental problem with the present observations is that for any particular inhibition model, it seems impossible to explain, with a single set of parameters, the inhibitory effects of Ca on both pump-mediated fluxes and ATPase activity. Whether $[Ca^{2+}]_i$ or CaATP is considered to be the inhibitory substrate, the concentration required for half maximal inhibition of the ATPase is 2·5 to 10 times lower than for the ouabain-sensitive Na efflux with any model. Since the inhibitory effect of Ca on the Na–K-ATPase activity was determined in conditions which, at least for Na, K, ATP_T, Mg^{2+} and MgATP, mimic closely those prevailing at the intracellular surface of the pump in the experiments with intact cells, the difference in inhibitory constants must reflect differences in some other inner surface co-factor(s) or in Na pump configuration between intact cell and membrane preparation. The 'protective' agent is probably not P_i since P_i accumulation in cells above the levels allowed in the ATPase assay is more likely to increase flux inhibition slightly (K_i for substrate inhibition by P_i = 23 mM (Hexum, Sanson & Himes, 1970)) than to antagonize Ca inhibition at constant $[Ca^{2+}]_i$. ADP cannot be ruled out as a possible agent since it was present in the intact cells but not in the ATPase measurements. Another possibility is that part of the tightly bound Mg observed in intact red cells (Flatman & Lew, 1980, 1981) is associated with the Na pump in a Ca-excluding configuration and that this association is lost during hypotonic lysis. Whatever its origin, the effect is such that for any particular Ca^{2+} level, the Na pump flux is inhibited less in the intact cell than is the Na–K-ATPase activity measured under comparable conditions in a membrane preparation from the same cells. One consequence of this finding is that the likely extent of Na pump inhibition by intracellular Ca under physiological conditions cannot be predicted reliably from ATPase measurements made in a membrane preparation.

In intact red cells, it seems possible to account for the differences in inhibitory kinetics of the Na pump at different ionophore concentrations in terms of changes in ATP levels and, as analysed in the Results section, equally good explanations can be found regardless of whether total ATP, free ATP or MgATP is the true substrate and whether Ca^{2+} or CaATP is the true inhibitor. What remains difficult to explain at present is the mechanism or mechanisms which regulate the cell ATP levels at the various Ca and A23187 concentrations and prevent their recovery when Ca-pump mediated ATP consumption is halted by Ca removal from the cells. It is clear that Ca-pump mediated Ca extrusion is maintained throughout since $[Ca^{2+}]_i$ during the

ionophore-mediated steady states is kept far below equilibrium with external Ca^{2+} at high Ca permeability. ATP production must therefore be increased under these conditions to match consumption by the pump and it is remarkable that the ATP level should not change significantly on Ca removal. This means that extra production and Ca-pump mediated consumption must be switched off simultaneously. Elucidation of the mechanism of this effect will require a careful study of the effects of Ca on the glycolytic metabolism when inosine is used as the substrate but it would seem that in the steady state, either internal Ca is required for increased ATP production or ATP production is somehow linked to Ca pump activity. A close association between the Na pump and part of the red cell glycolytic pathway has been proposed previously (see e.g. Proverbio & Hoffman, 1977).

The maximum extent to which the Na pump might become inhibited when the internal Ca^{2+} concentration rises within physiological limits can be estimated from the different models discussed in the Results section. Models, rather than direct results must be used for this estimate because the ATP concentrations in the relevant mammalian tissues are above those present in normal red cells. If the normal intracellular concentrations of Mg^{2+} and ATP in mammalian tissues (e.g. brain, kidney, liver, red cells) are assumed to be, respectively, 0·3–1·9 mM (Veloso, Guynn, Oskarsson & Veech, 1973; Flatman & Lew, 1980; Fig. 19 in the present work) and 1–8 mM (e.g. Veloso *et al.* 1973; Newsholme & Start, 1976; Till *et al.* 1977, 1981; Flatman & Lew, 1981; Fig. 10 in the present work) and the maximum $[Ca^{2+}]_i$ during stimulation/excitation assumed to be of the order of 10 μM (Marban, Rink, Tsien & Tsien, 1980), then the model for CaATP/MgATP competition with K_m of 400–800 μM and K_i of 20–100 μM predicts that the pump might be as much as 35 % inhibited during cell excitation. During rest, however, when $[Ca^{2+}]_i$ is below 1 μM (Marban *et al.* 1980; Coray, Fry, Hess, McGuigan & Weingart, 1980; Lew, Tsien, Miner & Bookchin, 1982), inhibition would be less than 5 % compared with a Ca-free condition. The non-competitive model discussed in the Results predicts similar values while the model for direct inhibition by Ca^{2+} predicts only 5 % and 0·5 % inhibition during excitation and rest respectively. Thus, it appears that under physiological conditions, there might be transient partial inhibition of the Na pump in some excitable cells. Although this is unlikely to alter the contribution the Na pump fluxes make to the internal Na and K levels, a small and brief depolarization due to the reduction in electrogenic transport may be a part of the physiological response to raised intracellular Ca.

We wish to thank Mrs J. Gray for expert technical assistance and the Wellcome Trust and M.R.C. for funds.

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