Journal of Physiology (1988), **407**, *pp.* 569–586
With 10 *text-figures*
Printed in Great Britain

MAXIMAL CALCIUM EXTRUSION CAPACITY AND STOICHIOMETRY OF THE HUMAN RED CELL CALCIUM PUMP

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(*Received 12 March 1987*)

SUMMARY

1. The uphill calcium efflux through calcium-saturated pumps in intact red cells was investigated with the aid of a new method, in initial conditions of uniform ionophore A23187-induced calcium distribution among the cells. The method is based on findings by Tiffert, García-Sancho & Lew (1984) which show that cobalt can suddenly arrest passive calcium transport by the ionophore and expose, without noticeable interference, uphill calcium extrusion by the pump. The results comprise methodological aspects and questions concerning interactions between inner pump sites, ATP and Ca^{2+}, and the calcium:ATP stoichiometry of the calcium-saturated pump.

2. Ionophore-induced calcium influx was set to be far in excess of the maximal calcium pump capacity. This secured a uniform calcium distribution among the cells, and Ca^{2+} equilibration by 2 min or less of calcium permeabilization. Cobalt was added between 15 s and 5 min after ionophore addition. The calcium and ATP content of the cells was followed during ionophore-induced influx and cobalt-exposed efflux.

3. The external cobalt concentrations required to block completely ionophore-mediated calcium transport were similar or only marginally higher than those of calcium.

4. The reproducibility of independent cobalt-exposed calcium efflux measurements from single blood samples was within an 8% range.

5. During cobalt-exposed calcium efflux, the calcium content of subpopulations of cells, with and without active Ca^{2+}-sensitive K^+ channels, investigated by post-incubation of samples in low-K^+, thiocyanate (SCN^-) media (modified from García-Sancho & Lew, 1988*a*), was similar. This is consistent with the maintenance of a uniform calcium distribution among the cells during uphill calcium extrusion.

6. Cobalt-exposed calcium efflux was similar in the interval from 15 s to 5 min after calcium permeabilization although cell ATP levels had fallen by over 50% in that period. Therefore, cell ATP concentrations within the physiological range do not seem to be regulatory for calcium-saturated pumps in the intact red cell.

7. All cobalt-exposed calcium efflux curves were linear in time, at least until total cell calcium contents reached levels below 100 μmol/l cells. This suggests that

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internal calcium is not inhibitory for calcium-saturated efflux in intact cells in the 0·1–1 mmol/l cells range.

8. The cobalt-exposed calcium fluxes were in the range from 4 to 24 mmol/(l cells.h) for fresh cells and from 10 to 18 mmol/(l cells.h) for samples from the Blood Bank. This variability was documented for blood samples from different donors and also for the same donor in samples obtained on different days. This variability can be attributed to unknown factors that control the fraction of active pumps, or the turnover of calcium-saturated pumps, in intact red cells.

9. Simultaneous measurements of cobalt-exposed calcium efflux and of the calcium-dependent ATP fall following calcium permeabilization of fresh red cells incubated in the absence of substrates, were used to estimate the calcium:ATP stoichiometry of the calcium-saturated pump. The scatter of the experimental points was large but all fell within the region predicted for a 1:1 stoichiometry once allowance was made for the ATP-buffering effects of adenylate kinase.

INTRODUCTION

The experiments reported in this paper introduce a new method to estimate the maximal calcium extrusion rate and calcium:ATP stoichiometry of the calcium pump in intact human red cells, in conditions which secure a uniform calcium distribution among the cells (Lew & García-Sancho, 1985; García-Sancho & Lew, 1988*a*, *b*). Earlier attempts at measuring those parameters in intact red cells had ignored the heterogeneity of ionophore-induced calcium distribution (for reviews see Sarkadi, 1980; Schatzmann, 1982; Al-Jobore, Minocherhomjee, Villalobo & Roufogalis, 1984). The method is based on findings by Tiffert, García-Sancho & Lew (1984). It combines the use of the divalent cation ionophore A23187 and of cobalt in a way which affords precise determination of uphill calcium extrusion rate even at very short time intervals after the introduction of a pump-saturating calcium load. The results show no modulatory effects of physiological ATP concentrations, no inhibitory effects of saturating internal calcium concentrations, and support a calcium:ATP stoichiometry of 1:1 for a calcium-saturated pump in intact red cells. They also document substantial individual variability of maximal calcium extrusion rate for blood samples taken from single donors in different days, with high reproducibility within each day.

METHODS

Experimental design. Tiffert *et al.* (1984) showed that addition of cobalt in excess of calcium to a suspension of red cells containing the ionophore A23187 immediately blocks all calcium movements through the ionophore without disturbing the calcium pump-mediated fluxes. Cobalt is rapidly transported by the ionophore and buffered by cytoplasmic components (Tiffert *et al.* 1984; Brown & Simonsen, 1985; Scharff & Foder, 1986). It is not transported by the calcium pump to any measurable extent and, on short exposures at least, it does not seem to interfere with calcium transport by the pump. The potential of cobalt to suddenly arrest ionophore-mediated calcium fluxes can be used to expose uphill calcium extrusion by the calcium pump after an ionophore-induced calcium load. Following preliminary trials, the following general protocol was designed. Red cells from fresh or 2–3 week old bank blood were washed twice with a medium containing (in mM): KCl, 75; NaCl, 75; $MgCl_2$, 0·2; Tris-Cl (pH 7·5 at 37 °C), 10; and Na-EGTA, 0·1. After two more washes in the same medium but without EGTA (solution A), the cells were suspended at 10% haematocrit with the addition of inosine or ouabain as specified for each

experiment. In these conditions, cells treated with ionophore and calcium retain their initial volume, pH and overall ionic composition (Ferreira & Lew, 1976; Lew & Brown, 1979; Lew & García-Sancho, 1985). The specific activity of ^{45}Ca was in the 10^7–10^8 c.m.p./μmol range. The cell suspension was equilibrated at 37 °C with magnetic stirring for about 10–15 min. At zero time, ionophore A23187 was added from 2 mM-stock solutions in ethanol or dimethyl sulphoxide (DMSO) to give a final concentration of 10 μM in the suspension. The batch of ionophore used in these experiments gave values of P, the ionophore-induced calcium permeability, of about $[I]^{1.45}$ in units of h^{-1} (see also García-Sancho & Lew, 1988*b*). Within the range of external calcium concentrations used in the present experiments, this ionophore concentration was enough to rapidly secure a uniform calcium distribution in all cells (García-Sancho & Lew, 1988*a*, *b*). Such uniformity is essential if a measured mean flux is to be reliably related to the whole population of cells under study (García-Sancho & Lew, 1988*b*). Cobalt was added from 20 or 100 mM-stock solutions of $CoCl_2$, at the indicated times. Sampling was as frequent as possible for optimal statistics. The calcium and ATP content of the cells was estimated as described below. The values of α, the fraction of ionized calcium within the cell, were computed from the ionophore-induced equilibrium distribution of calcium assuming $r^2 = 2$ (Lew & Brown, 1979). Whenever EGTA was added in excess of calcium in the medium care was taken to neutralize the protons released using NaOH.

Processing of samples. Frequent sampling, whether for determination of the mean calcium content of the cells or of density-separated cell fractions, left no time for spinning individual samples. The observation that no calcium is lost from high-calcium-loaded cells as long as the temperature is kept very near 0 °C (García-Sancho & Lew, 1988*a*) was here confirmed for high-ionophore conditions as well (see Fig. 3). This allowed delayed spinning of the samples without detectable differences from controls in which the cells had been immediately separated through the oil. For mean total cell calcium, 50 μl samples of cell suspension were delivered into 1·5 ml nominal capacity microcentrifuge tubes containing 0·9 ml of ice-cold wash-medium with 0·4 mM-$CoCl_2$ and 0·4 ml diethyl-phthalate oil, and kept in the ice-bath for up to 20 min before separating the cells through the oil. Processing of samples for density separation was modified from García-Sancho & Lew (1988*a*) as follows. Samples of 100 μl were added to 1 ml of ice-cold medium containing (mM): NaSCN, 70; NaCl, 70; KCl, 2; $CoCl_2$, 0·4; Na-HEPES (pH 7·5), 10 in a 1·5 ml microcentrifuge tube. After rapid mixing the tubes were kept for 20 min in the ice-bath. After this incubation, two 0·5 ml aliquots of the cell suspension were deposited, one over 0·4 ml dibuthyl-phthalate oil and the other over 0·4 ml diethyl-phthalate oil (which had been kept in tubes at room temperature to prevent hardening of the diethyl-phthalate) and immediately spun in a microfuge. In some experiments, arabinogalactan cushions of relative densities 1·042 and 1·090 were used, as well as phthalate oils (Fig. 3). After spinning, the supernatant, with or without light cells and oil, was sucked off, the tube cleaned with cotton swabs, and the cell pellets processed as described before (Lew & Brown, 1979). The ^{45}Ca activity was measured in an aliquot of the trichloroacetic acid supernatants. This method differs from that of García-Sancho & Lew (1988*a*) in that the ionophore removal steps are omitted, and in that cobalt is also used to arrest ionophore-mediated calcium transport during the dehydrating incubation in the low-K^+, SCN^- medium. The sustained presence of cobalt during dehydration is equivalent to the functional removal of the ionophore. When ionophore + calcium-induced dehydration was followed by light scattering at 650 nm, in the conditions described by García-Sancho & Lew (1988*a*), cobalt addition had no effect on Ca^{2+}-sensitive K^+ channel-mediated dehydration, as long as the cells retained sufficient calcium (not shown).

Determination of the stoichiometry of the calcium pump required simultaneous determination of the calcium and ATP content of the cells. This was done in separate samples from the same suspensions. ATP consumption by the pump was estimated from the initial calcium-dependent break-down of ATP in the absence of metabolic substrates, and in the presence of 0·1 mM-ouabain, to reduce background ATP break-down through the Na^+ pump. Cell ATP was measured by the method of Brown (1982), modified as follows. Samples (50 μl) of the 10% haematocrit cell suspension were mixed with 200 μl of ice-cold 0·1 M-HCl and kept on ice for up to 1·5 h before processing. Preliminary trials showed that such a treatment did not reduce ATP relative to that measured in neutralized perchloric acid supernatants from samples processed immediately. The acid-treated samples were spun in a microfuge and 25 μl of the supernatant were added to 2·5 ml of the combination buffer (Brown, 1982). This changed the pH of the buffer by less than 0·05 units and did not affect the bioluminescence measurements.

RESULTS

The experiments in Fig. 1 show the effect of increasing cobalt concentrations on the calcium efflux from cells loaded with calcium at two different external calcium levels. It can be seen that at cobalt concentrations in the suspension similar to those of calcium, or above, calcium efflux becomes maximal and is not affected by the presence of a large excess of cobalt. Excess cobalt in the medium and in the cells does not, therefore, seem to affect calcium fluxes through calcium-saturated calcium pumps. The experiments also illustrate the general design of the flux experiments. The initial samples after ionophore addition provide a minimal estimate of the ionophore-generated calcium influx. This was about 50 mmol/(l cells.h) at 56 μM-initial external calcium (Fig. 1*A*), and 75 mmol/(l cells.h) at 110 μM-initial external calcium (Fig. 1*B*). These net fluxes were in excess of the maximal calcium extrusion capacity of the calcium pump (up to about 30 mmol/l cells in the cells with the highest pumping rates; García-Sancho & Lew, 1988*b*) and ought to have secured rapid calcium equilibration in all the cells. From the distribution of calcium at equilibrium, the value of α, the fraction of ionized cell calcium (Ferreira & Lew, 1976; Lew & Brown, 1979), was calculated for each experiment and is reported in the corresponding figure legends. The cobalt-exposed calcium fluxes are uphill since they proceed from calcium-equilibrated cells against the calcium gradient. They are therefore calcium pump-mediated in line with them being strictly ATP dependent (Tiffert *et al.* 1984; Scharff & Foder, 1986). As shown in Fig. 1*C*, at maximal cobalt concentrations, calcium efflux was similar at the low and high calcium concentrations. This, together with the fact that the fall in cell calcium was linear in time to levels below 100 μmol/l cells of total calcium, suggests that for the range between 100 and 400 μmol/l cells of total cell calcium the calcium efflux was through a saturated pump uninhibited by excess $[Ca^{2+}]_i$.

The experiment of Fig. 2 was designed to test the reproducibility of the method in six identical experiments run in parallel. Figure 1 shows that the duplicate calcium efflux determinations agree within a range of 8%.

In the experiments of Figs 1 and 2 the tailing-off fluxes were followed for 15 min by which time total cell calcium had fallen to levels comparable to those measured before ionophore addition. These correspond to total cell calcium levels of less than 2 μmol/l cells, and comprise contaminant extracellular ^{45}Ca trapped within the cell pellets as well as residual calcium pools within the cells (García-Sancho & Lew, 1988*c*). This is in agreement with earlier observations that for brief calcium loads, such pools are negligible (Bookchin & Lew, 1980; García-Sancho & Lew, 1988*c*). It also shows that calcium extrusion in the presence of cobalt was to the lowest measurable cell calcium levels.

The presence or absence of inosine, adenosine or glucose made no difference to the measured calcium fluxes from initially normal-ATP cells within the short time intervals after calcium permeabilization in which those fluxes were investigated here (not shown). Metabolic substrates were therefore omitted in the following experiments reported here.

The experiments in Fig. 3, representative of seven others with similar results, report the fraction of dense cells and the calcium content of the various cell fractions

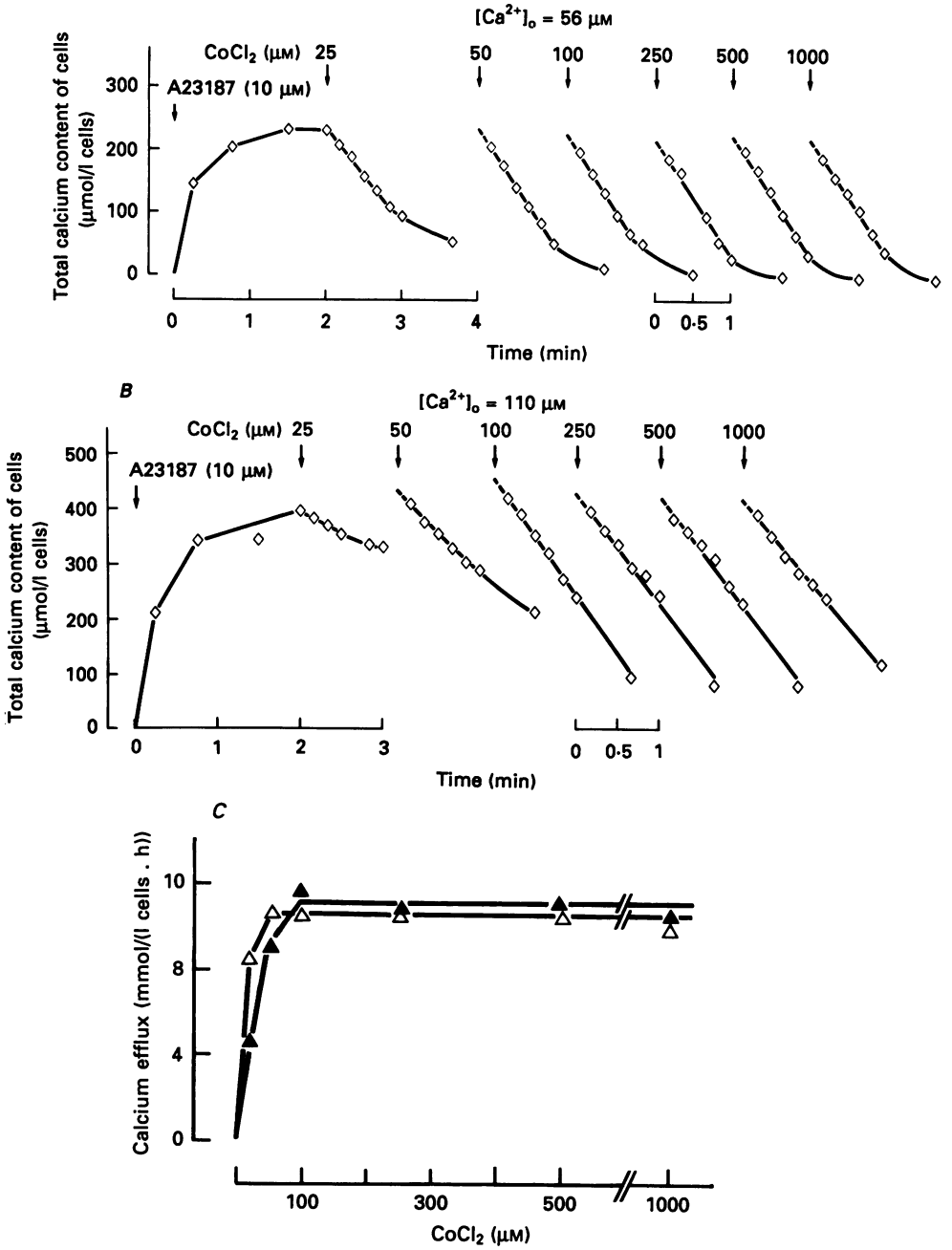

Fig. 1. Ionophore-induced and cobalt-exposed calcium fluxes in human red cells. The cells were from the Blood Bank, and were incubated in the presence of inosine (10 mM). ^{45}Ca was added to give initial $[Ca^{2+}]_o$ of 56 μM in *A* and of 110 μM in *B*. The estimated ionophore-induced net calcium influx was (mmol/(l cells.h)) 33 in *A* and 55 in *B*. Cobalt was added 2 min after ionophore in parallel conditions, to give the final concentrations in the suspension indicated in the figure. The calcium extrusion rate was calculated by linear regression analysis of the linear part of the efflux curve after cobalt addition, and is reported in units of mmol/(l cells.h). Correlation coefficients of the efflux curves were always $\geqslant 0{\cdot}98$. The calcium efflux as a function of the added cobalt concentration is given in *C*. △, values from *A*; ▲, values from *B*. α was in the range 0·28–0·33.

after SCN^- treatments, as well as the mean cell calcium content of all the cells, following ionophore-induced calcium permeabilization and cobalt-induced uphill calcium efflux. The aim of these experiments was to investigate heterogeneity of pumping rates from cells which had initially been equilibrated to the same $[Ca^{2+}]_i$ (Simonsen, Gomme & Lew, 1982). Calcium content of all the cells and of the dense cells is expressed per 340 g haemoglobin (equivalent to 1 l of normal packed cells). It can be seen that all the cells became dense after SCN^- treatment following ionophore

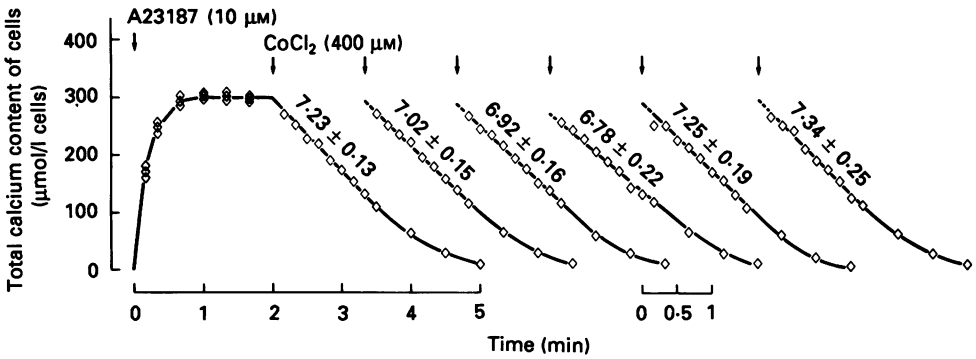

Fig. 2. Reproducibility of calcium efflux measurements with the cobalt-exposure method. The cells were from freshly drawn blood and were incubated in the presence of inosine (10 mM). The initial $[Ca^{2+}]_o$ was 56 μM. The six experiments were run consecutively using the same cell suspension. Calcium influx after A23187 was 65 mmol/(l cells.h). Calcium efflux is reported as the slope ± standard error of the slope of the curves reporting total cell calcium content as a function of time after cobalt addition, and is given in units of mmol/(l cells.h). α was 0·15.

addition and stayed so after cobalt addition until the mean calcium content of the cells had declined to less than 40% of its equilibrium level. After about 2 min of cobalt addition, calcium had been extruded to below activatory levels for calcium-sensitive K^+ channels in all the cells. The results show that the mean calcium content of the dense cells is similar to that in the whole cell population even during the tailing-off of the pump-mediated calcium efflux. This suggests that there is no detectable heterogeneity of pumping rates from Ca^{2+}-pre-equilibrated cells and that the factors responsible for the density distribution during the tailing-off of the calcium fluxes are related to differences in the response to calcium of K^+ channels belonging to different cells and not to differences in total calcium content of the cells.

Since calcium-dependent ATP break-down occurs as soon as calcium is admitted into the cells (García-Sancho & Lew, 1988*b*), and uniformly so at the high-ionophore-induced calcium fluxes applied here (García-Sancho & Lew, 1986*b*), it was necessary to see whether calcium efflux declined in time due to partial ATP depletion. This was investigated by varying the time interval at which cobalt was added after ionophore. The results in Fig. 4 show that there was no meaningful difference in calcium efflux whether cobalt was added 15 s or 5 min after ionophore to fresh (Fig. 4*A*) or to bank (Fig. 4*B*) cells; neither did it matter whether the cells had already reached calcium equilibrium or were only about half-way there. These results indicate either that

there are no changes in the state of the pumps or in cell metabolism that could affect saturated calcium fluxes through the pump in the interval from 15 s to 5 min after calcium permeabilization, or else that the changes have opposite and compensatory effects on the calcium fluxes. There is no evidence that ATP becomes limiting for calcium extrusion through the pump in intact cells. Desaturation of the pump by the falling $[Ca^{2+}]_i$ levels must be the predominant factor in the tail-off fluxes since these do not start at higher cell calcium levels after late cobalt addition, when ATP levels must have been lowest (see below).

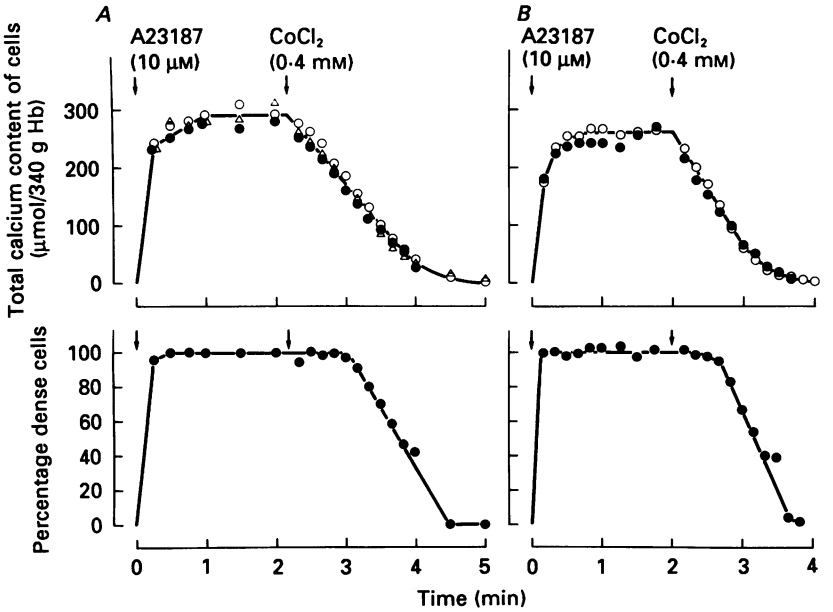

Fig. 3. Calcium content of SCN^--treated, density-separated cell fractions, and fraction of dense SCN^--treated cells during ionophore- and cobalt-induced calcium fluxes (calcium content expressed per 340 g haemoglobin (Hb) which is equivalent to 1 l normal packed cells). Cells were from freshly drawn blood from two donors (*A* and *B*). The initial $[Ca^{2+}]_o$ was 56 μM. Samples were processed as described in Methods. Influx rates were 63 and 61 μmol/(l cells.h) for panels *A* and *B*, respectively, and calcium efflux was $8{\cdot}97 \pm 0{\cdot}25$ (*A*) and $12{\cdot}10 \pm 0{\cdot}31$ mmol/(l cell.h) (*B*). α was 0·15 for *A* and *B*. ○, untreated cells spun through dibuthyl-phthalate oil ($\delta = 1{\cdot}042$). ●, SCN^--treated cells spun through diethyl-phthalate oil ($\delta = 1{\cdot}118$). ▲, SCN^--treated cells spun through isotonic stractan ($\delta =$ 1·090–1·098).

Figure 5 compiles all the independent calcium efflux measurements performed. The fluxes are plotted as a function of the mean total calcium content of the cells at equilibrium. The diamonds (◇) represent isolated determinations from different donors. The open triangles (△) represent cells from the Blood Bank. The other three symbols report repeated flux determinations in fresh cells from three donors. The lower three abscissa scales are meant to represent the full range of possible cytoplasmic ionized calcium levels within the cells during the efflux measurements (see legend of Fig. 5). It can be seen that within a general pattern of decline in flux as cell calcium is increased, there is a bewildering variability in the absolute value of

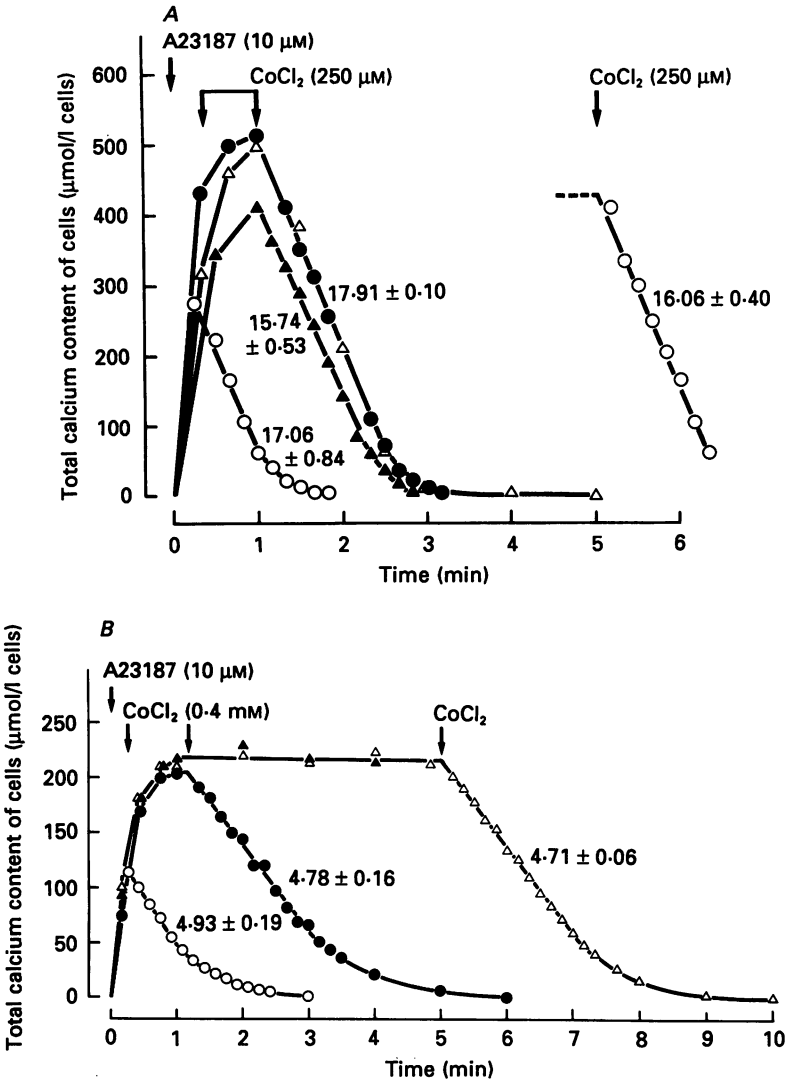

Fig. 4. Cobalt-exposed calcium efflux as a function of time after A23187 addition. Cells from fresh blood suspended with 56 μM $[Ca^{2+}]_o$ for *A* and Bank cells with 110 μM $[Ca^{2+}]_o$ for *B*. Ionophore-induced net influx was (mmol/(l cells.h)) 41 for *A* and 112 for *B*. Cobalt was added at the indicated times, from 15 s to 5 min after A23187. α was 0·28 for *A* and 0·22 for *B*. In *A*, the open triangles are from samples kept 20 min on ice after cobalt addition. In *B*, the closed triangles are from an aliquot of the cell suspension pre-incubated at 37 °C prior to A23187 addition. Open triangles and closed circles are from duplicate conditions.

the saturated calcium efflux at comparable calcium contents even in cells from the same donor obtained on different days. This contrasts with the good reproducibility observed in cells from the same blood sample (see Fig. 2). The range of variation goes from 4 to 25 mmol/(l cells.h) in fresh cells and from 10 to 18 mmol/(l cells.h) in samples from the Blood Bank.

The calcium : ATP stoichiometry of the calcium-saturated pump was studied next. The effect of ionophore and cobalt on the basal, calcium-independent, rate of ATP break-down in substrate-free, ouabain-poisoned fresh red cells was investigated in the experiment of Fig. 6. It can be seen that, in the absence of calcium, the presence or absence of ionophore and cobalt within the range of concentrations used for the stoichiometry experiments had no effect on the basal rate of ATP break-down, which

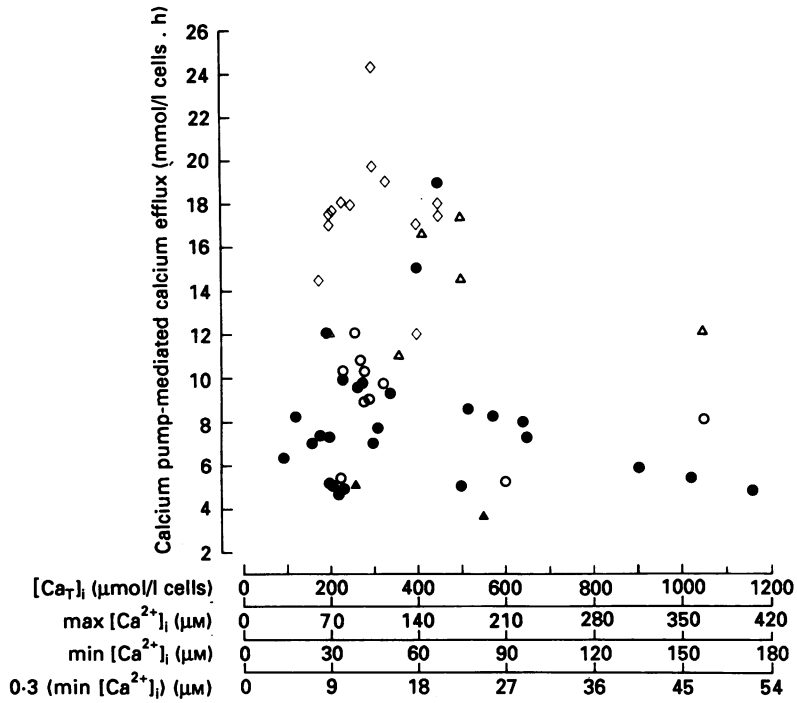

Fig. 5. Cobalt-exposed calcium efflux as a function of cell calcium content at equilibrium. For explanation of symbols see text. $[Ca_T]_i$ is total cell calcium content. α values of 0·35 and 0·15 were used to calculate the maximal (max $[Ca^{2+}]_i$) and minimal (min $[Ca^{2+}]_i$) $[Ca^{2+}]_i$ levels at equilibrium (in μM), respectively. The 0·3 (min $[Ca^{2+}]_i$) scale represents approximate cell ionized calcium levels after 70% calcium extrusion, when the calcium fluxes were still at their calcium-saturated level in all experiments.

in this experiment was about 0·5 mmol/(l cells.h). In the experiments of Fig. 7, the basal rate of ATP break-down was investigated after ionophore-induced calcium loads and after rapid calcium removal by addition of excess EGTA(OH) to the medium. EGTA was added 3 (panel *A*) or 6 (panel *B*) min after ionophore to measure the break-down rates when the residual ATP was still high and when it was decreased. The results showed that pre-exposure of the cells to different calcium levels had no effect on the rate of ATP hydrolysis after calcium removal, and that basal ATP break-down falls with the ATP level within the cells. Because of this and also because of the variation in rates observed in different experiments, the calcium dependent ATP break-down was estimated from the difference between the measured

rates of ATP fall during the calcium load and after calcium depletion or removal in each experiment.

The protocol followed is illustrated in the experiments of Fig. 8. The ionophore was added to cell suspensions containing different calcium levels. Cobalt or excess EGTA were added about 2 min after ionophore and the calcium and ATP content of the cells was determined at the indicated times. It can be seen that after cobalt addition, the basal rate of ATP break-down was only measurable for a sufficient length of time at the lowest calcium levels, for it was only reached after all cell calcium had

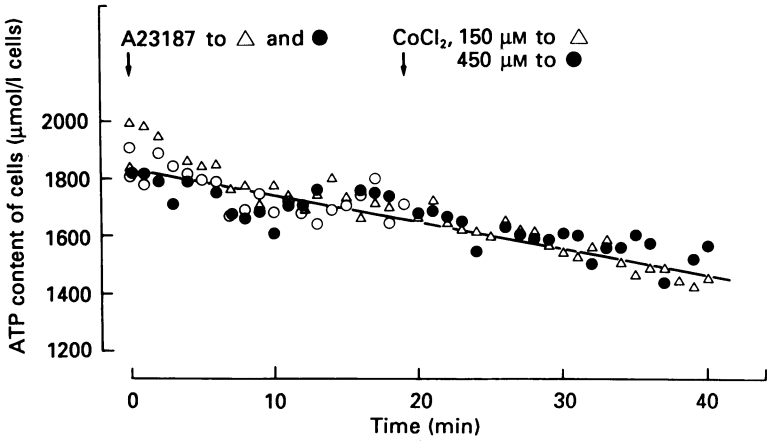

Fig. 6. Effect of A23187 and cobalt on the calcium-independent rate of ATP break-down in substrate-starved red cells. Fresh cells were incubated in the presence of 0·1 mM-ouabain. ○, controls, no additions. At $t = 0$, 10 μM-A23187 was added to the conditions represented by △ and ●. At $t = 19$ min, 150 μM-cobalt was added to the suspension represented by △, and 450 μM to that represented by ●. The ATP break-down rate was calculated by linear regression analysis and was $0{\cdot}52 \pm 0{\cdot}03$ mmol/(l cells.h).

been extruded. This made it necessary to include in the high-calcium experiments a condition where rapid calcium removal was induced by EGTA addition. The calcium-dependent ATP break-down rate was estimated by difference between that observed after ionophore addition and that recorded after calcium depletion. This procedure seemed justified in that there was no detectable difference in the basal rate of ATP break-down revealed by cobalt or EGTA addition other than that resulting from calcium depletion, as measured in parallel samples of the same suspension. The results of two typical experiments are shown in Fig. 8, and those of all the stoichiometry experiments performed are displayed in Fig. 9. In this figure, the simultaneously determined calcium extrusion and calcium-dependent ATP break-down rates are plotted as a function of the total calcium content of the cells, with parallel abscissa scales for the possible range of ionized cytoplasmic calcium levels as explained in the legend of Fig. 5. Despite substantial scatter, calcium-dependent ATP break-down was generally lower than the corresponding calcium efflux through the pump.

The points in Fig. 9 were plotted again in Fig. 10 (open circles) in order to

estimate the calcium : ATP stoichiometry of the calcium pump. The lines represent calcium : ATP stoichiometries of 1 : 1 and 2 : 1, on the assumption that the calcium-induced ATP fall had been determined exclusively by the activity of the calcium

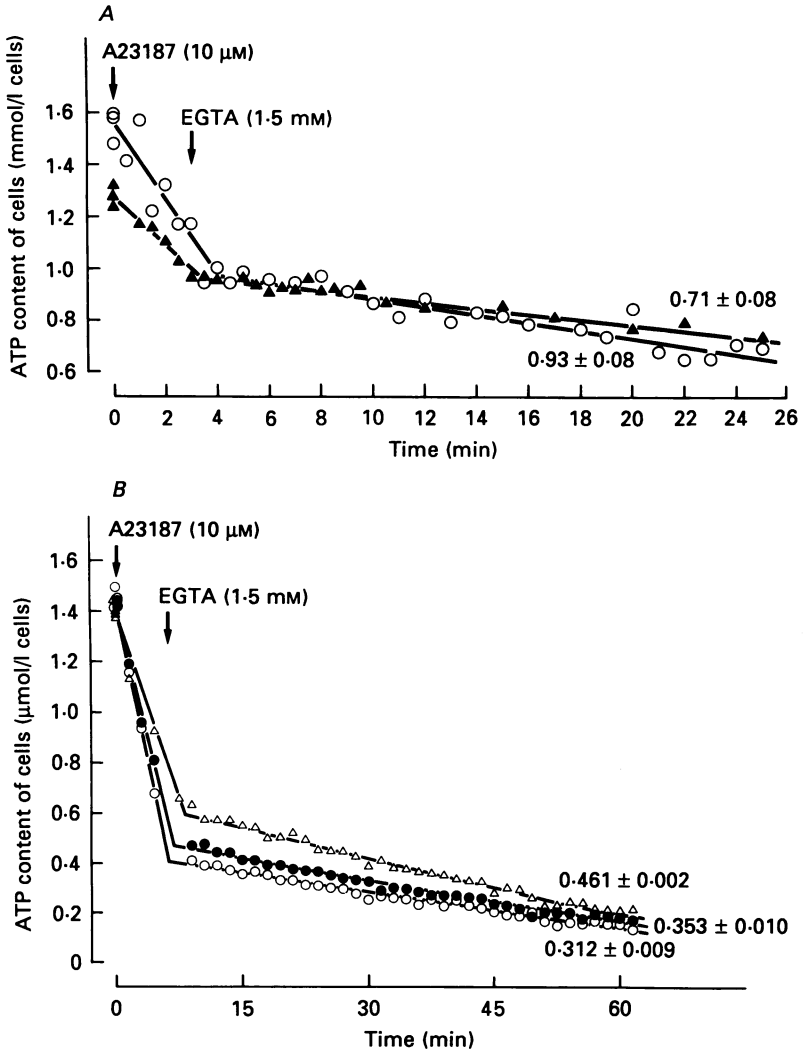

Fig. 7. Consecutive measurement of calcium-induced and calcium-independent fall in cell ATP. Fresh cells were suspended with 0·1 mM-ouabain and different initial $[Ca^{2+}]_o$. At $t = 0$, A23187 was added to initiate cell calcium loading, and EGTA(OH) was added in excess of $[Ca^{2+}]_o$ at 3 (*A*) and 6 (*B*) min after ionophore. The initial $[Ca^{2+}]_o$ were (μM): ▲ (*A*), 25; ○ (*A*), 150; ○ (*B*), 15; ● (*B*), 50; △ (*B*), 100. The numbers by the lines correspond to ATP break-down rates after EGTA addition (in mmol/(l cells.h)).

pump. The real ATP fall, however, is known to be influenced by at least two additional and powerful red cell enzymes: adenylate kinase and calcium-stimulated AMP deaminase (see Almaraz *et al.* 1988, and references therein). In estimating the stoichiometry of the calcium pump, therefore, the experimental results have to be

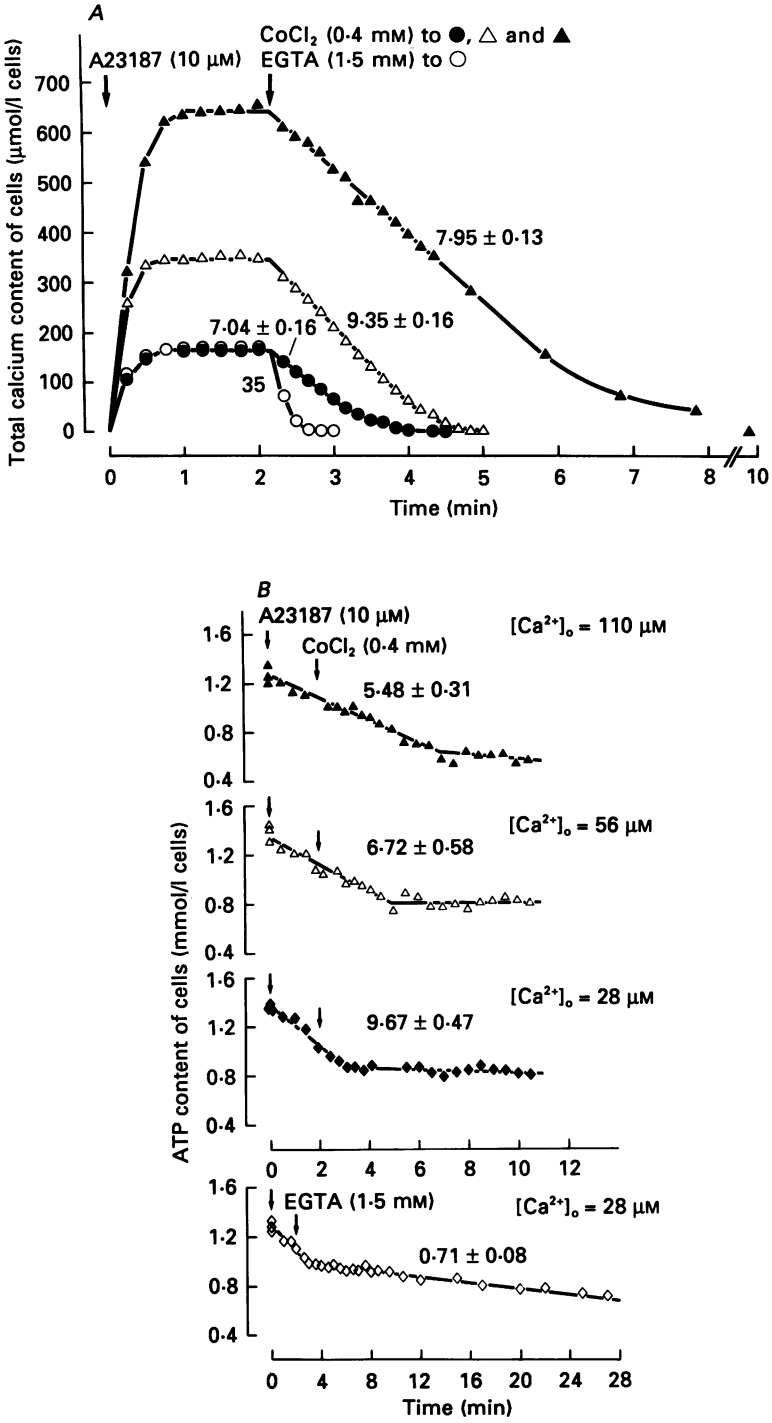

Fig. 8. For legend see facing page.

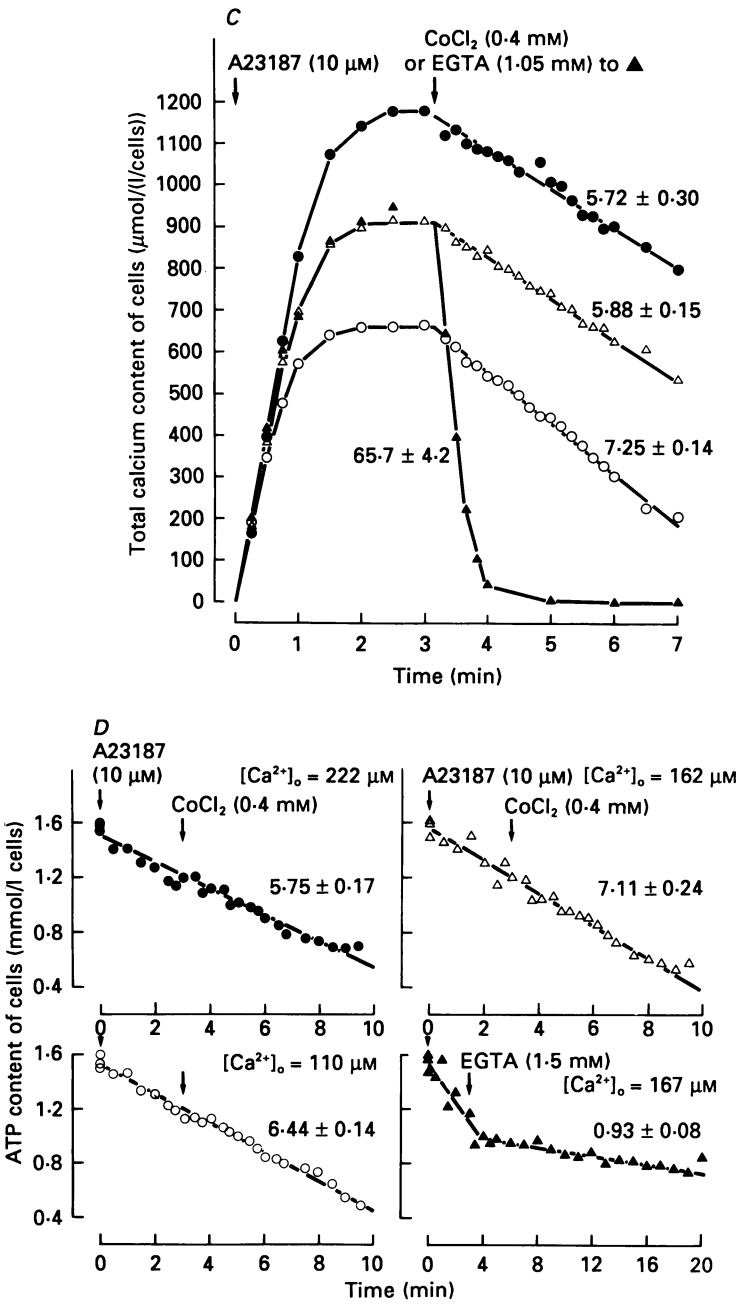

Fig. 8. Simultaneous measurements of cobalt-exposed calcium efflux (*A* and *C*) and rate of calcium-dependent ATP fall (*B* and *D*). Data from two experiments (*A* and *B*, *C* and *D*) with fresh cells suspended in the presence of ouabain (0·1 mM). A23187 was added at $t = 0$ and cobalt, or EGTA(OH), were added at $t = 2$ min (*A* and *B*) or at $t = 3$ min (*C* and *D*). The initial $[Ca^{2+}]_o$ were (μM), for *A* and *B*: ○, ●, ◇ and ◆, 28; △, 56; ▲, 110; for *C* and *D*: ○, 110; △, 162; ▲, 167; ●, 222. The numbers by the lines correspond to ATP break-down rates after ionophore or EGTA additions (in mmol/(l cells.h)).

compared with the global effects predicted for the interactions among calcium pump ATPase, adenylate kinase and AMP deaminase. In Fig. 10, all symbols other than open circles represent theoretical predictions. These were computed as indicated in the legend of that figure, to allow for the buffering effects of adenylate kinase on the ATP fall. This enzyme restores some of the ATP hydrolysed by the pump. by resynthesis from AMP and ADP. The fall in ATP is therefore less than anticipated

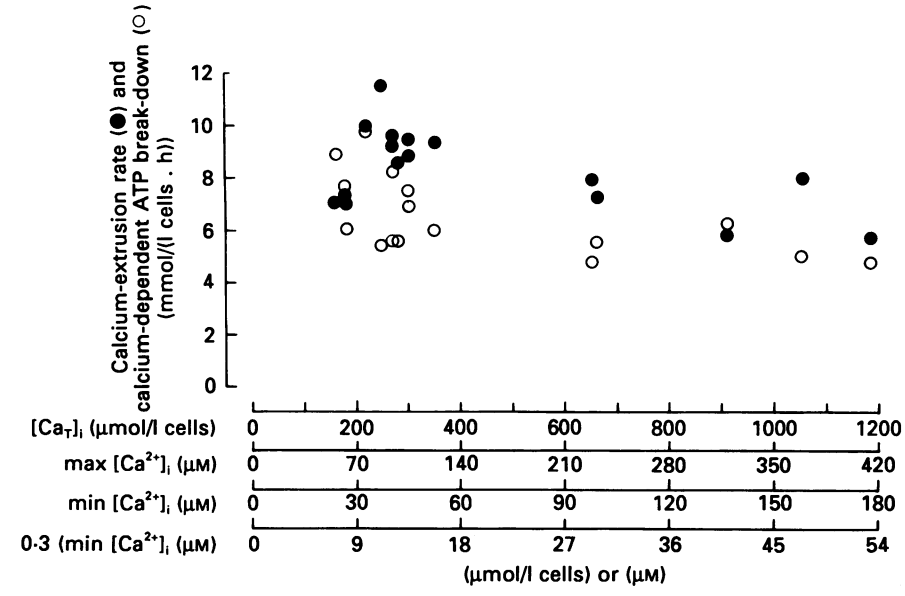

Fig. 9. Cobalt-exposed calcium efflux and calcium-dependent rate of ATP fall as a function of cell calcium content at equilibrium. Compiled data from sixteen simultaneous measurements of calcium efflux (●) and rate of ATP fall (○) in five different experiments with fresh cells. Meaning of the abscissa labels as in Fig. 5.

from pump-mediated hydrolysis alone. The computations assumed stoichiometries of 1:1 or 2:1 for the calcium pump, and that the concentrations of ATP, ADP and AMP were maintained at the equilibrium levels determined by adenylate kinase activity. The possible effects of IMP formation (Almaraz *et al.* 1988) on the initial rate of ATP fall were also considered in the calculations, but were found to be small relative to the scatter of the experimental points. It can be seen that most of the experimental points fell around the points predicted for a 1:1 stoichiometry. The predictions based on a 2:1 stoichiometry for the pump fell to the left of the 2:1 line, far from any experimental points (not shown). This suggests that the results are compatible with a 1:1 stoichiometry for calcium-saturated pumps in intact red cells.

DISCUSSION

The results in this paper describe a procedure which enables the precise measurement of uphill calcium extrusion by calcium-saturated pumps at short intervals after ionophore-induced calcium loads in intact red cells. The procedure is based on earlier

findings by Tiffert *et al.* (1984) and differs from that of Scharff & Foder (1986), based on the same findings, in several important aspects of methodology and use. The new methodological aspects which are established here are the excess cobalt over calcium required for full blockage of ionophore-mediated calcium transport, the reproducibility of independent calcium-efflux measurements, and the absence of detectable

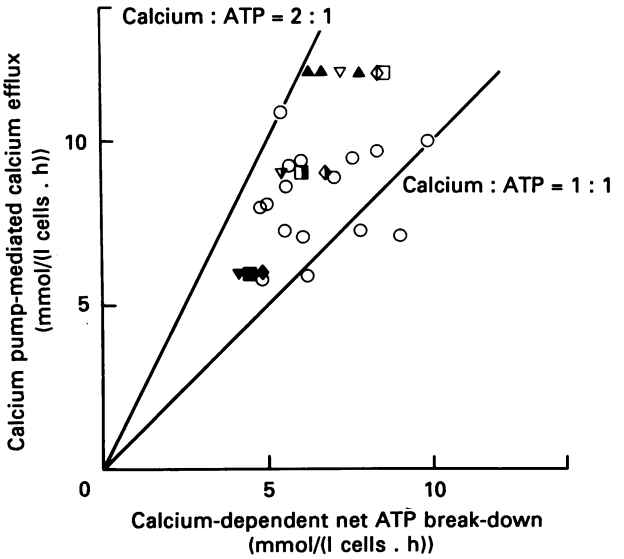

Fig. 10. Calcium efflux as a function of calcium-dependent ATP fall. ○, experimental results in Fig. 9. The lines correspond to calcium:ATP stoichiometries of 1:1 or 2:1, assuming that the measured calcium-dependent ATP fall was only due to calcium pump-mediated ATP break-down. The ATP-buffering effects of adenylate kinase, influenced or not by AMP deaminase activity, were simulated for a 1:1 stoichiometry with a highly simplified model. For each integration interval, dt, calcium pump hydrolysis was represented by $d[ATP] = -k_P\,dt$ and $d[ADP] = -dATP$, where k_P is the simulated rate of ATP break-down by the pump. The AMP deaminase, if active (see Almaraz *et al.* 1988), will catalyse $d[IMP]/dt = -k_D[AMP]$ and $d[IMP] = -d[AMP]$, where k_D is the rate constant for IMP formation from AMP. The adenylate kinase equilibrium will reset the adenine nucleotide transferred from ATP and AMP to ADP within each dt interval. Computer simulations were performed with k_P values of 6, 9 and 12 mmol/(l cells.h); with (squares and diamonds) and without (triangles) AMP deaminase activity at $k_D = 0{\cdot}1\ min^{-1}$, as required to give the measured rates of IMP accumulation observed before (Almaraz *et al.* 1988), and at different total nucleotides concentrations to fit the range observed in the present experiments. The scatter of the experimental points is so large that it cannot resolve any alternative other than that of providing general support for the 1:1 stoichiometry. As can be seen by doubling the height of the predicted points on the ordinate scale, points simulated with a 2:1 stoichiometry would fall to the left of the 2:1 line, away from all experimental data.

differences in the calcium content of subpopulations of cells with and without activated K^+ channels during uphill calcium efflux.

The differences in use relate to the conditions in which it is valid to apply this method. Here cobalt was applied only to uniformly loaded or calcium-equilibrated cells. These conditions were secured by inducing ionophore-mediated calcium fluxes,

much larger than the maximal pump flux observed, of about 30 mmol/(l cell.h) (García-Sancho & Lew, 1988*b*). By applying cobalt shortly after calcium permeabilization, it was possible to prevent the effects of marked ATP depletion on saturated calcium pump fluxes (García-Sancho & Lew, 1988*b*; Almaraz, García-Sancho & Lew, 1988; see below). The use of calcium-equilibrated cells, together with the apparent uniformity of calcium distribution during uphill calcium efflux (Fig. 3), justifies attribution of the measured calcium fluxes to the bulk of the cell population under study.

Addition of cobalt to suspensions containing cells with different calcium concentrations, as during initial calcium transients after calcium permeabilization (García-Sancho & Lew, 1988*b*), will produce a measurable release of calcium which may be impossible to translate into meaningful fluxes from identifiable subpopulations of cells. Therefore, application of the cobalt method in conditions where calcium distribution is not uniform may be useful, but only if additional information can be obtained on the fraction of cells from which the flux originates and on their calcium contents, or if the fluxes from the different subpopulations can be measured independently. Two subpopulations of cells, for instance, having identical calcium extrusion rates of 10 mmol/(l cells.h), and comprising, say, one 10% and the other 20% of the cells, will render apparent calcium effluxes of 1 and 2 mmol/(l cells.h), respectively, when attributed to the whole cell population and expressed per litre cells. Conversely, a truly larger efflux from fewer cells may appear smaller. In the experiments of Scharff & Foder (1986), for instance, the cobalt-exposed calcium pump efflux from the 'up' part of the peak may appear lower than that from the 'down' part simply because during the 'down', the measured mean cell calcium, even when smaller than during the 'up', may have been contained within more cells. If calcium had been within more cells during the 'up', the analysis performed by Scharff & Foder (1986) will underestimate the genuine difference.

The method applied here was expected to reveal whether some well-established regulatory interactions between the pump enzyme, ATP (Richards, Rega & Garrahan, 1978; Garrahan & Rega, 1978; Muallem & Karlish, 1979, 1983; Rossi, Rega & Garrahan, 1985) and internal Ca^{2+} (Lichner & Wolf, 1980; Kratje, Garrahan & Rega, 1985) had a clear correlate on the performance of the calcium pump in the intact cell, and what was the calcium:ATP stoichiometry of the saturated calcium pump.

The absence of significant differences in calcium efflux from cells whose ATP levels must have been reduced by half from the first measurement at 15 s to the start of the last measurement at 5 min (experiments of Fig. 4; ATP data from Figs 7 and 8), suggests that, at least for the calcium-saturated fluxes investigated here, the physiological ATP levels do not exert a regulatory function in the intact cells, and that the low-affinity ATP site on the pump, thought to be an allosteric regulatory site (Garrahan & Rega, 1978; Muallem & Karlish, 1983), is probably saturated.

If the intracellular Ca^{2+} concentrations used in the present experiments had been supramaximal and inhibitory to the calcium pump the curves reporting the cobalt-exposed calcium effluxes would have been expected to increase in slope as internal Ca^{2+} was reduced. This was never seen. The curves were always remarkably linear down to Ca^{2+} desaturation levels. It is conceivable that the linearity is only apparent and due to compensating factors changing within the cell in these conditions (rising inorganic phosphate levels, nucleotide conversions (Almaraz *et al.* 1988), $[Ca^{2+}]_i$-induced metabolic changes (Brown & Johnston, 1983)). What the present results indicate is either that within the range explored (Fig. 5) $[Ca^{2+}]_i$ is not inhibitory to

the intact cell pump, or, if it is, that the fall in ATP is not the compensatory factor. Lack of inhibition is consistent with the observations of Kratje *et al.* (1985) in resealed ghosts which show that the K_I for internal Ca^{2+} is around 1–1·5 mM. The highest $[Ca^{2+}]_i$ value in the experiments reported here did not exceed 0·42 mM.

The calcium : ATP stoichiometry of the calcium-saturated pump was investigated here by comparing simultaneously, and in the same cells, the fall in calcium and ATP contents. The ATP fall was triggered by ionophore addition whereas that of calcium was triggered by cobalt addition, 2 min later. As shown in the experiments of Fig. 8, cobalt addition had no measurable effect on the rate of ATP fall. The calcium-induced decrease in cell ATP in the absence of metabolic substrates, as in the present experiments, is the result of calcium pump-mediated hydrolysis and adenylate kinase-mediated resynthesis (Bishop, 1964). The net ATP fall is therefore less than expected from pump-mediated break-down alone. When allowing for the ATP-buffering effect of adenylate kinase, the experimental points fall within the range of values expected for a 1 : 1 stoichiometry.

The variability in calcium efflux values observed (Fig. 5) seems genuine in the sense that it is not the result of inconsistencies in the method but the outcome of reproducible measurements. It was also documented for blood samples from the same donor in different days. The widest oscillations were sometimes seen in consecutive samples, without any systematic pattern. This variability suggests the operation of factors which affect the saturated pump rate of intact red cells in ways that cannot be explained by any of the known pump interactions identified so far in isolated pump preparations or resealed ghosts. Since there is no protein synthetic activity in mature human red cells, the number of pumps must have been fairly constant at least in some of the consecutive samples from the same donor in which variability was seen. The highest flux recorded for each donor is thus likely to represent the nearest estimate of the true pump V_{max}. The observed variability must therefore be due to factors that control the fraction of active pumps, the turnover of the pumps, or both. The results in Fig. 3 suggest that, whatever the nature of these factors, they seem to affect the pumps of all red cells in any given blood sample to a more-or-less uniform extent.

We thank the Fondation pour la recherche Médicale (France) for personal support to G. D., the Wellcome Trust Foundation and the Medical Research Council of Great Britain for funds. We are grateful to R. M. Bookchin for useful comments on the manuscript, and to Mrs J. Gray for skilful technical assistance.

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