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CALCIUM-INDUCED CONVERSION OF ADENINE NUCLEOTIDES TO INOSINE MONOPHOSPHATE IN HUMAN RED CELLS

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SUMMARY

1. When inosine-fed human red cells are permeabilized to calcium by exposure to the ionophore A23187, progressively larger proportions of the cell population become irreversibly depleted of ATP as calcium influx is increased (Brown & Lew, 1983; García-Sancho & Lew, 1988*b*). When calcium influx is over 30 mmol/(l cells.h), all cells become ATP depleted and calcium equilibrated (E cells) (García-Sancho & Lew, 1988*b*). When calcium influx is lower, E cells co-exist with cells able to maintain normal ATP and low calcium contents in vigorous pump–leak balance (B cells). The experiments reported here investigate why calcium-induced ATP depletion of E cells is irreversible.

2. The inosine monophosphate (IMP) content of cells after 30 min of calcium permeabilization increased with the magnitude of the calcium load, roughly in inverse proportion to the fall in ATP. The calcium-induced increase in IMP was confined to the fraction of cells which became osmotically resistant after SCN^- treatment (H cells), and which contained the E cells.

3. Cell nucleotides were measured after calcium permeabilization ($[A23187]_c$ = 100 μmol/l cells) in substrate-free media with different $[Ca^{2+}]_o$ (0–0·5 mM). Calcium entry caused rapid ATP fall, AMP and IMP accumulation, and delayed ADP fall at all $[Ca^{2+}]_o$ concentrations. Initial IMP formation increased with $[Ca^{2+}]_o$ along a sigmoid saturation-like curve whereas AMP accumulation and ATP fall were maximal at $[Ca^{2+}]_o = 20$ μM and declined at the higher $[Ca^{2+}]_o$. The rate of IMP formation correlated positively with cell ATP and negatively with cell AMP at all $[Ca^{2+}]_o$ values.

4. The AMP deaminase activity of red cell lysates was reversibly increased over tenfold by calcium. Half-maximal stimulation was observed at a Ca^{2+} concentration of about 50 μM.

5. These results suggest that the irreversibility of calcium-induced ATP depletion results from irreversible trapping of the adenine nucleotide as IMP, and help explain the mechanism of E cell formation.

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INTRODUCTION

When inosine-fed human red cells are loaded with calcium with the aid of the ionophore A23187 in conditions which prevent changes in cell volume, pH and ion contents other than $CaCl_2$, the cells become depleted of ATP (Ferreira & Lew, 1976; Brown & Lew, 1981; Scharff, Foder & Skibsted, 1982). Brown & Lew (1983) showed that ATP depletion persisted after calcium removal. Similar irreversibility of the calcium-induced ATP depletion was observed after ionophore removal and calcium extrusion (García-Sancho & Lew, 1988*b*). Calcium-induced ATP depletion is attributable to the imbalance between the limited metabolic capacity of the red cells (Bishop, 1964; Whittam, 1964; McMannus, 1967) and the powerful hydrolytic activity of a calcium-saturated pump (Lew & Beauge, 1979; Schatzmann, 1982). This view, though probably correct as to the main factors involved, fails to explain the irreversible nature of the depletion and ignores the complexities which arise from cell heterogeneity (García-Sancho & Lew, 1988*a*, *b*). The co-existence in the steady-state of low-calcium, normal-ATP cells in pump–leak calcium balance (B cells) and of high-calcium, low-ATP cells in equilibrium with external calcium (E cells), when a red cell population is exposed to uniform ionophore-induced calcium influx, suggests that the hydrolysis–synthesis imbalance must have critical values which differ from cell to cell.

The lack of ATP recovery after calcium removal suggested that both transport and metabolism became simultaneously deactivated by calcium extraction (Brown & Lew, 1983). Brown & Johnston (1983) confirmed this prediction and demonstrated that calcium entry into inosine-fed red cells causes a large stimulation of lactic acid production. This explains the mechanism by which B cells can sustain normal ATP levels (García-Sancho & Lew, 1988*b*). For, in order to match the calcium influx set by the ionophore without becoming depleted of ATP, these cells ought to be able to synthesize ATP at rates much higher than those observed in calcium-free states (McManus, 1967). The problem that remains unresolved is why E cells do not reverse from net breakdown to net synthesis whenever the calcium pump becomes deactivated. The use of inosine should secure that recovery since this substrate enters the glycolytic pathway as ribose-phosphate, bypassing the hexokinase reaction. It is therefore, unlike glucose, not limited in its metabolism by the low initial ATP levels within the E cells (Dunham, 1957; Whittam, 1964; Lew, 1971; Lew & Ferreira, 1978; Scharff *et al.* 1982; García-Sancho & Lew, 1988*b*). The irreversible nature of E cell depletion therefore remains puzzling and unexplained. The main aim of the experiments in this paper was to find the reason why calcium-induced ATP depletion of E cells was irreversible.

A clue to a possible mechanism was found in early work on nucleotide conversions in metabolically poisoned red cells (Lew, 1971). When rapid ATP depletion of human red cells was induced by iodoacetamide in conjunction with inosine or glucose, almost all the adenine nucleotide was converted to inosine monophosphate (IMP) (Lew, 1971; Lew & Ferreira, 1978; Plagemann, Wohlhueter & Kraupp, 1985). Human red cells are known to possess high levels of AMP deaminase activity (Conway & Cooke, 1939; Askari & Franklin, 1965) and of adenylate kinase activity. When ATP is consumed inside the cell in the process of phosphorylating an intermediate in a

metabolic or transport reaction, one ADP molecule is formed per molecule of ATP consumed. If the break-down of ATP is fast enough, the adenylate kinase-catalysed equilibrium will secure the conversion of most of the ATP to AMP, thus building up the concentration of the substrate for the deaminase reaction. Activation of this enzyme by AMP is highly sigmoidal (Askari, 1966; Askari & Rao, 1968) and this may trigger rapid IMP formation as soon as the AMP concentration is increased. The equilibrium of the deaminase reaction is strongly shifted towards IMP (Lee, 1957). The mature red cell has no alternative pathways to reconvert IMP to adenine nucleotide (Bishop, 1964). IMP can be hydrolysed to inosine which is phosphorylized to hypoxanthine and rapidly lost from the cells (Plagemann *et al.* 1985). Hence, IMP production is essentially irreversible. IMP trapping of the adenine nucleotide then has all the necessary ingredients of a plausible working hypothesis on the mechanism of irreversible ATP depletion in E cells. Additional effects of calcium on the AMP deaminase also seemed worth exploring, although the brain enzyme was unaffected by calcium (Weil-Malherbe & Green, 1955).

We investigated IMP formation and distribution in calcium-permeabilized human red cells, and the effects of calcium on the AMP deaminase activity of red cell lysates. The results demonstrate calcium-induced IMP formation; in the steady state IMP was found only in cells which become dense (H cells) after SCN^- treatment and which include, as a subgroup, the calcium-equilibrated E cells (García-Sancho & Lew, 1988*b*). Calcium was found to cause over tenfold stimulation of the AMP deaminase activity of red cell lysates, with half-maximal effect at an ionized calcium concentration of about 50 μM.

METHODS

Preparation of red cells, calcium loading, and nomenclature of solutions was as detailed in the first paper in this series (García-Sancho & Lew, 1988*a*). Fresh cells were used throughout.

Preliminary experiments were carried out to investigate whether ionophore-induced calcium influx triggered IMP formation in red cells, and, if so, whether IMP accumulation occurred in all cells or preferentially in cells which become dense (H) or stay light (L) after density separation, or are separated into identical H and L subgroups by differential osmotic lysis (García-Sancho & Lew, 1988*a*). In these experiments relatively crude nucleotide assays were applied. For the experiment of Table 1, cells were incubated in solution B containing 10 mM-inosine, 0·2 mM-$CaCl_2$ and the different ionophore concentrations indicated. The nucleotide content was determined in the supernatant (lysed L cells) and in the residual cell pellet (H cells) after haemolysis in 75 mM-NaCl (García-Sancho & Lew, 1988*a*). Lysates or cell pellets were deproteinized with trichloroacetic acid (TCA); TCA was extracted with ether, and the ether was blown off with air. Total nucleotides were estimated from the absorbance at 258 nm (with a correction for the absorbance of IMP, when present in high concentrations). ATP was estimated by the luciferin–luciferase method (Brown, 1982). AMP and (ADP+IMP) were separated by ion-exchange chromatography on Dowex-1 × 4-400 resin (Garrahan & Glynn, 1967; Glynn & Lew, 1970; Lew, Glynn & Ellory, 1970; Lew, 1971). AMP was estimated directly from the absorbance of the eluent at 258 nm. IMP was determined by the xanthine-oxidase method of Plesner & Kalckar (1956) in the (ADP+IMP) eluent which was free of inosine. Briefly, 0·5 ml of the supernatant were mixed with 0·5 ml of a solution containing 0·2 mM-Na-EDTA and 50 mM-glycyl-glycine buffer, pH 7·5. The hypoxanthine content was estimated from the increase in absorbance at 290 nm after addition of about 0·04 U/ml of xanthine-oxidase (Sigma, grade I). Stable readings were obtained after 30 min at room temperature. Absorbance varied linearly with hypoxanthine concentration between 5 and 30 μM. The absorbance due to adenine, under these conditions, was less than 2% that of hypoxanthine. ADP was estimated from the absorbance of the (ADP+IMP) fraction by subtracting the value

corresponding to IMP. There are large errors associated with these measurements: high non-specific background absorbance from unknown contaminants, as well as low and barely measurable AMP, ADP and IMP concentrations in the diluted eluents from controls and low calcium influx samples. This renders artificially high calculated values for those conditions. Therefore only large differences may be considered significant. Preliminary experiments such as that reported in Table 1 were nevertheless of value in revealing the occurrence and distribution of calcium-induced IMP formation in inosine-fed red cells.

Detailed and more precise investigation of the effects of calcium permeabilization on cell nucleotides was performed using high-pressure liquid chromatography (HPLC), with which the detection limits for cell nucleotides were better than 10 μmol/l original cells, and the reproducibility of duplicates was better than 5%. The cells were suspended at 10% haematocrit in solution B and permeabilized to calcium by adding the ionophore A23187 (2 mM in ethanol) to give a final concentration of 10 μM in the suspension. Samples of the cell suspension (0·5 ml), taken just before ionophore addition and during the incubation, were delivered to ice-cold Eppendorf tubes containing, layered from top to bottom, 0·5 ml of solution A, 0·4 ml of dibutyl-phthalate oil ($\delta \approx$ 1·042) to minimize the time from sampling to enzyme inactivation and so prevent possible changes in cell nucleotides during sample processing. After a 15 s centrifugation at 12000 *g* the top aqueous layer and most of the oil were aspirated, the acid extract was diluted with 0·5 ml of ice-cold distilled water and centrifuged again. An aliquot of the diluted extract was neutralized with concentrated $KHCO_3$ and $KClO_4$ was sedimented by centrifugation in the cold. Analysis of nucleotides was performed by reverse-phase HPLC on μBondapak C_{18} column (300 × 4 mm, Waters). After a 5 min isocratic run with water: Pic A: 2% acetonitrile, a linear gradient to 15% acetonitrile was developed in 5 min. The same mixture was then run for eight additional min. The order of elution was IMP, AMP, ADP and ATP. 2,3-Diphosphoglycerate was measured using a commercial kit (Boehringer 148334). In two of the pilot experiments, cell IMP before ionophore addition was found to be relatively high (50–200 μmol/l cells), and AMP barely detectable, as if the AMP deaminase had been activated before calcium permeabilization. The heparinized blood samples from which those cells were obtained had been stored at 4 °C for over 3 h. All the experiments described below utilized blood stored for shorter periods.

In experiments such as that reported in Fig. 4, the AMP deaminase activity was determined in membrane-free lysates of fresh red cells following Askari & Franklin (1965) and Askari & Rao (1968). Packed cells were lysed in five volumes of ice-cold distilled water. After centrifugation at about 40000 *g* for 20 min, the supernatant was mixed in equal volumes with an ice-cold solution containing (in mM): KCl, 150; K-HEPES, 50, pH 7·5; and AMP, 1–4. The reaction was started by incubation at 37 °C. Samples of 0·2 ml were withdrawn at different times and mixed with 0·1 ml of 2 M-perchloric acid; 0·1 ml of the clear supernatant was mixed with 3 ml of distilled water and the absorbance at 265 nm determined. The activity of the enzyme was estimated from the decrease in absorbance.

RESULTS

Table 1 reports the distribution of nucleotides in H/L cell fractions from calcium-permeabilized cells. Calcium influx was varied by increasing the ionophore concentration at a constant external calcium concentration. The results, and those of four other preliminary experiments, showed that calcium influx induces IMP accumulation in inosine- or glucose-fed intact red cells, as well as in cells incubated in the absence of substrates. Within the error margins of the measurements reported in Table 1 (see Methods), IMP accumulation became significant only when calcium influx was high enough to generate over 50% of H cells. Maximal IMP accumulation was obtained at the highest ionophore concentrations, when all the cells became calcium equilibrated. As with the ATP fall (García-Sancho & Lew, 1988*b*), any detectable IMP increase was confined to H cells. Since the total nucleotide was conserved and, if anything, ADP + AMP in the H cell fraction declined at the higher calcium fluxes, IMP accumulation appears to be stoichiometrically linked to the fall in ATP.

The earlier experiments of García-Sancho & Lew (1988*b*) suggested that the ATP fall after calcium permeabilization was confined to the calcium-equilibrated E cells within the H cell fraction. The link noted above between IMP accumulation and ATP fall suggests that both events occur in the same cells. Therefore, to study the detailed effects of calcium permeabilization on cell nucleotides it seemed best to make all cells behave like E cells, by using high ionophore concentrations in the experiments of Figs 1–3.

TABLE 1. Effect of varying calcium influx on the nucleotide content of H and L cell fractions after 30 min incubation at 37 °C (experimental details in Methods)

[A23187] (μM)	Total ATP content of cells (μmol/l cells)	Cell density fraction	Cells in density fraction (%)	Nucleotide content of cells (μmol/l cells)				
				ATP	ADP	AMP	IMP	Total
0	820	L	98	817	500	257	258	1946
		H	2	—	—	—	—	—
0·5	855	L	89	897	560	228	289	2002
		H	11	—	—	—	—	—
0·6	893	L	90	925	492	235	239	1913
		H	10	618	—	—	—	—
0·7	714	L	70	849	377	316	274	1942
		H	30	399	640	452	284	2213
0·8	667	L	67	845	436	273	319	1959
		H	33	299	486	34	378	1838
1·0	423	L	44	817	421	395	364	2061
		H	56	117	147	131	829	1495
1·9	97	L	10	—	—	—	—	—
		H	90	81	419	198	1015	1994
10	78	L	14	—	—	—	—	—
		H	86	67	415	68	1069	1897

Figure 1 shows the time course of cell nucleotide changes after permeabilization at two different external Ca^{2+} concentrations. It can be seen that calcium entry triggered large initial changes in ATP and AMP, variable changes in IMP, and slower changes in ADP, and that by 10–20 min all nucleotides had nearly levelled off to their new values. At 20 μM-Ca^{2+} (Fig. 1A) ATP depletion was relatively fast. ADP declined to low levels after a transient early increase. AMP accumulated, becoming the dominant nucleotide after 10 min of incubation. There was a slow increase in IMP which levelled off at about 100 μmol cells after 10 min, even though the concentration of AMP must have exceeded 1 mM in cell water. At 200 μM-Ca^{2+} (Fig. 1*B*) ATP depletion was slower than at 20 μM-Ca^{2+}, IMP increased faster than AMP and became the most abundant nucleotide after 10 min. The fact that much more IMP was formed at 200 μM $[Ca^{2+}]_o$ than at 20 μM $[Ca^{2+}]_o$, where the concentration of AMP was highest, indicates that availability of AMP, the substrate of the AMP deaminase, cannot be the main factor controlling IMP production. Within the critical initial 5–10 min, when nucleotide changes were largest, the main differences between cells in high and low Ca^{2+} were in their ATP, AMP, IMP and Ca^{2+} contents. Since the levels of AMP showed no direct correlation with the different levels of IMP production at low and high Ca^{2+}, we considered the possible effects of cell ATP, Ca^{2+}, and 2,3 diphosphoglycerate (2,3-DPG).

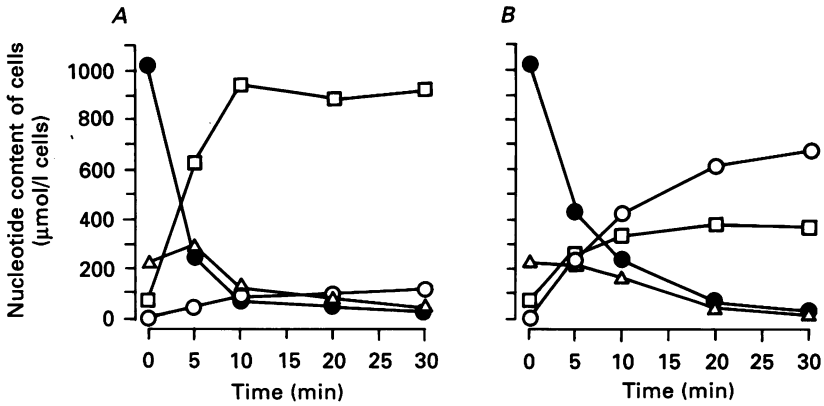

Fig. 1. Time course of changes in red blood cell nucleotides after calcium permeabilization with ionophore A23187 (10 μM). Haematocrit was 10%. $[Ca^{2+}]_o$ was either 20 μM (*A*) or 200 μM (*B*). ●, ATP; △, ADP; □, AMP; ○, IMP.

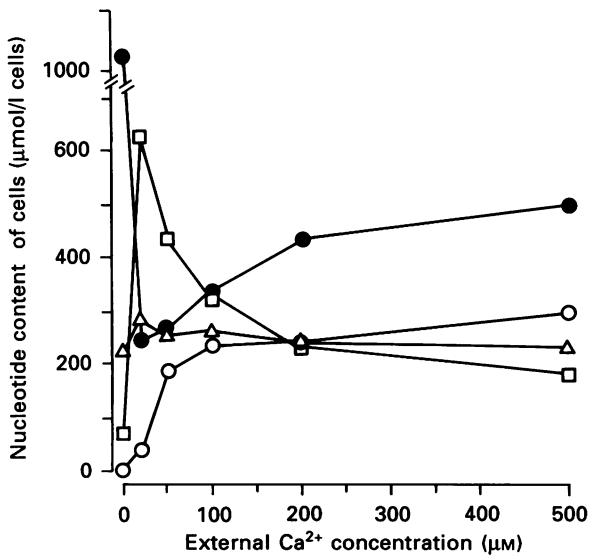

Fig. 2. Nucleotide content of cells after 5 min of calcium permeabilization ([A23187] = 100 μmol/l cells) as a function of $[Ca^{2+}]_o$. Conditions and symbols as in Fig. 1.

Figure 2 shows the levels of AMP, IMP, ATP and ADP attained after 5 min of calcium permeabilization in different external Ca^{2+} concentrations. Analysis of the results centres on the two main types of Ca^{2+}-induced effects: symmetrical (inverse) changes in ATP and AMP, and IMP production. The initial rates of ATP fall and AMP formation were maximal at low Ca^{2+} concentrations. The mechanism of the decline in the initial rate of net ATP fall at the higher Ca^{2+} concentrations is unclear, from currently available data. With a different experimental design (Dagher & Lew, 1988), internal Ca^{2+} in the concentration range explored here showed no detectable inhibition of the calcium pump ATPase. On the other hand, the symmetries and cross-overs of the ATP and AMP curves can easily be explained if the powerful

adenylate kinase is assumed to determine the distribution of the adenine nucleotides ([ATP] [AMP]/[ADP]^2 = constant) at all times (Fig. 1) and at all $[Ca^{2+}]_i$ levels (Fig. 2).

The initial rate of IMP production increased with Ca^{2+} concentration along a sigmoid, saturation-like curve. The increase in the initial rate of IMP production was accompanied by decreased rates of AMP accumulation and ATP depletion. ADP levels changed little. The level of 2,3-DPG (not shown in the figures), initially 4·7 mmol/l cells, declined slowly in the calcium-free controls at a rate 0·6–0·7 mmol/

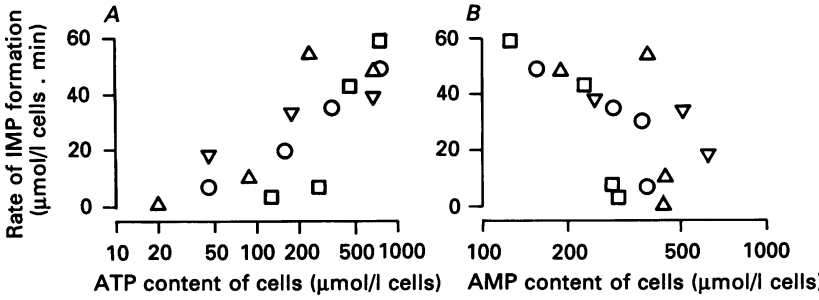

Fig. 3. Correlation between the rate of IMP formation and the cell levels of ATP (*A*) and AMP (*B*) in calcium-permeabilized red cells. Data from four experiments similar to those of Fig. 1. Rates of IMP formation were computed from the differences in IMP content between successive cell samples. Cell ATP and AMP levels are the mean values during the corresponding period. Symbols correspond to the following $[Ca^{2+}]_o$ (in μM): □, 500; ○, 200; △, 100; ▽, 50. At $[Ca^{2+}]_o$ = 20 μM, IMP formation rates were too low for significant estimates (see Fig. 1*A*). Note the logarithmic scale of the ATP abscissa.

(l cells.h); Ca^{2+} entry increased that rate to 1–1·2 mmol/(l cell.h), with less than 10% variation among the different Ca^{2+} concentrations tested. Therefore the changes in 2,3-DPG concentration during the first 5–10 min of calcium permeabilization were minimal, and could not have contributed significantly to the observed differences in IMP production. This leaves ATP and Ca^{2+} as the main candidates for such a role.

ATP was shown to be a powerful activator of AMP deaminase in red cell lysates, in the presence of 2,3-DPG (Askari & Franklin, 1965). To understand the possible effects of ATP on IMP production in intact cells, the rate of IMP formation, between successive samples in four experiments with different $[Ca^{2+}]_o$ (e.g. as in Fig. 1), was plotted against the mean ATP (Fig. 3*A*) and AMP (Fig. 3*B*) concentrations during corresponding periods. The rate of IMP formation correlated positively with ATP and negatively with AMP. From the known kinetics of AMP deaminase activation by AMP and ATP (in the presence of 2,3-DPG; Askari & Franklin, 1965; Askari, 1966; Askari & Rao, 1968), a positive correlation with both AMP and ATP was expected. The observed positive correlation with ATP and negative correlation with AMP in the calcium-permeabilized intact cell, suggests that activation of the enzyme by ATP was stronger than the reduction in activity due to lower substrate (AMP) levels.

The possible effects of calcium on the AMP deaminase activity of human red cells were investigated directly in cell lysates (Fig. 4). Calcium was found to have a

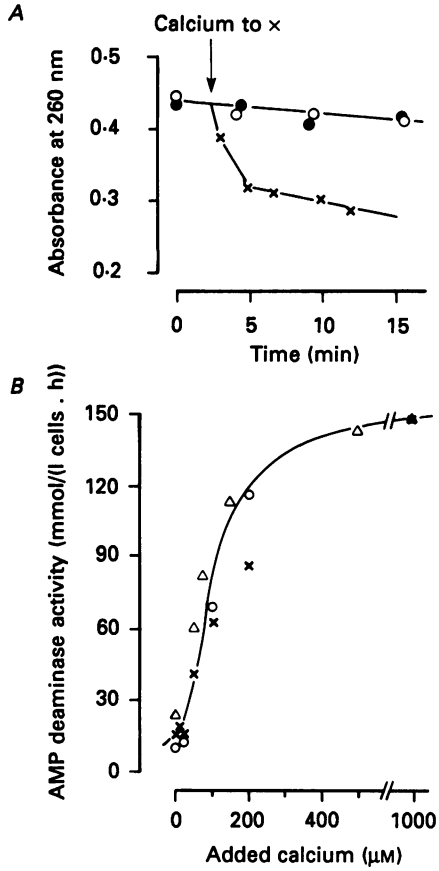

Fig. 4. Effects of calcium on the AMP deaminase activity of red cell lysates. Panel *A* shows the increase of activity, measured as the rate of decrease in absorbance at 260 nm, on addition of 200 μM-calcium (see Methods). ○, nominally calcium-free medium; ●, medium containing 0·1 mM-EGTA. The increase in activity during the first 45 s after addition of calcium was over fortyfold greater than the control value with no calcium. The subsequent fall in activity (indicated by a lower rate of decrease in absorbance) is due to consumption of AMP. Panel (*B*) shows the effects of the addition of different concentrations of calcium on the initial rate of AMP deamination. The concentration of Ca^{2+} was about half that shown on the abscissa due to the calcium-buffering capacity of the lysate. Different symbols represent different experiments. In that shown by ×, 0·15 mM Mg-ATP was added to the assay medium. In all the assays the concentration of AMP was 2 mM and the equivalent haematocrit in the test conditions was about 10%.

powerful stimulatory effect which was fully reversible upon addition of excess EGTA (not shown). Saturating calcium concentrations increased the enzyme activity at least ten times above basal levels. Half-maximal activation was obtained at 100 μM-total calcium in the medium, equivalent to about 50 μM-ionized calcium in the presence of lysate proteins (Schatzmann, 1973).

DISCUSSION

The experiments presented here show that calcium (i) induces IMP formation in intact human red cells permeabilized to calcium with the ionophore A23187, and (ii) strongly stimulates the AMP deaminase activity of cell lysates, in the absence of ionophore. The irreversible reduction in the cells' pool of adenine nucleotides resulting from conversion to IMP could account for the irreversibility of calcium-induced ATP depletion (Brown & Lew, 1981, 1983; García-Sancho & Lew, 1988*b*).

These results also provide important clues about the mechanism of E cell formation, which may help explain the observed calcium-induced heterogeneity. Calcium entry has two major effects on nucleotide metabolism. Firstly, it stimulates the AMP deaminase activity with a low apparent Ca^{2+} affinity ($K_{\frac{1}{2}} \approx 50\ \mu M$; Fig. 4). This should induce early IMP formation in the cells that gain more calcium on permeabilization. Secondly, it triggers ATP hydrolysis (via the calcium pump ATPase) which, in turn, has opposing effects on IMP production. On one hand, the higher the rate of ATP hydrolysis, the higher the rate of formation of AMP (Fig. 1). ATP hydrolysis is therefore essential to sustain a supply of substrate for the AMP deaminase. On the other hand, as shown in Fig. 3, a relatively high ATP level is more effective than a high substrate level in stimulating AMP deaminase activity and IMP production in calcium-loaded cells. Therefore the critical intracellular conditions for early IMP formation are the simultaneous presence of relatively high calcium and ATP levels within the first few minutes after calcium permeabilization.

The present results may also explain the observation that ATP depletion was faster in H cells than in L cells within the first minute following calcium permeabilization (Fig. 5 of García-Sancho & Lew, 1988*b*). As noted before, the faster ATP fall could not have been due to increased hydrolysis by the calcium pump, since H cells must have been pumping less than L cells at equal calcium influx to sustain their larger calcium gain. The following mechanism accounts for apparent calcium-induced ATP hydrolysis, not mediated by the calcium pump. The increased Ca^{2+} levels within the H cells, higher if confined to the E cell fraction, would activate AMP deaminase activity and IMP production from AMP. The reduced AMP levels would drive the adenylate kinase reactions in the direction of AMP formation at the expense of ATP. Calcium-stimulated IMP formation may therefore act as a drag on ATP via the adenylate kinase reactions, and thus simulate the effects of increased ATPase activity.

If a sufficient fraction of the adenine pool becomes converted irreversibly into IMP, in the first few minutes after calcium permeabilization (Fig. 1), glycolytic substrates can no longer restore ATP to non-limiting levels for the calcium pump. Continued calcium gain would stimulate further IMP production, until a new steady state is reached with high IMP, increased AMP, low ATP, and $[Ca^{2+}]_i$ near equilibrium. In this state, IMP production is again low despite the high $[Ca^{2+}]_i$, because of the reduced ATP concentration (Fig. 3*A*). The distribution of adenine nucleotides is determined by the activity of adenylate kinase. In the absence of a glycolytic substrate, as in the experiment illustrated in Fig. 1*B*, the adenylate kinase equilibrium is with higher AMP than ATP. In the presence of a substrate, as in E cells, the observed steady-state balance between $[Ca^{2+}]_i$-stimulated glycolysis and

ATP hydrolysis by residual calcium pumping is with low ATP (Table 1; García-Sancho & Lew, 1988*b*), but the precise AMP/ATP ratio in these conditions has yet to be established.

The first critical step in the sequence of events leading to E cell formation is therefore a sustained initial calcium gain. Such a gain was found only in H cells, and shown to be little affected by the presence or absence of inosine (Figs 4 and 5 of García-Sancho & Lew, 1988*b*). The cell calcium concentration, if the gain is confined to the prospective E cells within the H cell fraction, must have been considerably higher in those E cells than the reported mean H cell. Since normal ATP levels are non-limiting for the calcium pump in intact cells (García-Sancho & Lew, 1988*b*; Dagher & Lew, 1988), the calcium pumps of the cells which experience that gain must have either a relatively low maximal extrusion capacity or a longer delay in the activation of spare pumping capacity. Such delays were described before (Scharff *et al.* 1983; Scharff & Foder, 1986) and attributed to slow $[Ca^{2+}]_i$-dependent calmodulin association to the pump. Whatever limits the calcium extrusion capacity of each cell, the limiting factor(s) must be responsible for the pump–leak imbalance producing the sustained initial calcium gain. Therefore, the distribution of pumps among the cells, or cell differences in the factors modulating spare pump activation, may determine the fractions of steady-state E and B cells at each level of calcium influx. Alternatively, these fractions may be determined by cell differences in factors controlling calcium stimulation of AMP deaminase activity, unrelated to variations in pump performance among cells. If so, the initial $[Ca^{2+}]_i$ surge during peak calcium transients would activate AMP deaminase activity in a fraction of cells (E) and, by triggering IMP formation and ATP drag, cause sustained calcium gain and irreversible ATP depletion.

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