Journal of Physiology (1988), **407**, 523–539
With 6 text figures
Printed in Great Britain

HETEROGENEOUS CALCIUM AND ADENOSINE TRIPHOSPHATE DISTRIBUTION IN CALCIUM-PERMEABILIZED HUMAN RED CELLS

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(*Received 12 March 1987*)

SUMMARY

1. Calcium permeabilization of inosine-fed human red cells using the divalent cation ionophore A23187 induces pump–leak steady states in which the mean total calcium content of the cells may be held below electrochemical equilibrium for hours. A new method developed to detect and separate cells with different calcium contents revealed a striking heterogeneity of calcium contents in subpopulations of cells in pump–leak steady state (García-Sancho & Lew, 1988*a*). Most of the mean total cell calcium was found within a fraction of cells rendered dense by the separation procedure (H cells), with relatively little within the remaining light cells (L cells). The experiments in this paper were designed to study the nature and origin of the observed heterogeneity.

2. The fraction of steady-state H cells increased, and the mean ATP content of the cells fell, both linearly, as calcium influx was increased. The H/L divide is therefore the result of a continuous variation in cell properties. When calcium influx was above about 30 mmol/(l cells.h), all cells became dense, calcium distribution was at or near equilibrium, and cell ATP was 0·1–0·2 mmol/l cells.

3. Inosine-fed cells, subjected to ionophore-mediated net calcium influx of 13–15 mmol/(l cells. h), attained a steady state with mean calcium contents far below equilibrium. After ionophore removal and reincubation in calcium-free media, the initial calcium efflux was only a fraction of that required to sustain the previous steady state ($< 25\%$ for H cells, and $< 2\%$ for L cells). The ATP content of L cells was normal whereas that of H cells was irreversibly reduced. These results revealed a paradoxical discrepancy between leak influx and calcium pump efflux in H and L cells which were supposed to have been in steady-state pump–leak balance.

4. The changes in cell calcium and ATP were followed in time after calcium permeabilization to characterize the development of steady-state heterogeneity. Calcium influx triggered a sharp peak in the H cell fraction within 15 s of permeabilization. The mean calcium content of H cells increased towards steady-state values as their fraction decreased; most other cells transferred from H to L density fractions (HL cells) within the first 5 min of permeabilization.

5. In substrate-starved cells calcium influx triggered an immediate fall in cell

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ATP, steeper in H cells than in L cells. The initial calcium and density transients were unaffected. After about 5 min, when cell ATP was below 0·6 mmol/l cells, cell calcium and H cell fraction began to rise again. Addition of inosine reversed net calcium influx and ATP fall to give net calcium efflux and ATP rise. The extra ATP depletion caused by substrate deprivation was therefore reversible, unlike that caused by calcium influx alone.

6. Analysis of the flux paradox revealed that, behind the methodological H/L divide, the true calcium and ATP heterogeneity induced by calcium permeabilization was extreme. H cells contained a subpopulation of Ca^{2+}-equilibrated cells (E cells), irreversibly depleted of ATP. All other cells within the H and L cell fractions had normal ATP levels, and were able to sustain pump–leak balance (B cells) with low $[Ca^{2+}]_i$. The possible mechanism and implications of the E/B cell response to calcium permeabilization are discussed.

INTRODUCTION

In the previous paper (García-Sancho & Lew, 1987*a*) we described a density-separation method which revealed that the calcium contents of inosine-fed intact red cells, uniformly permeabilized to calcium with the divalent cation ionophore A23187, was markedly uneven. Large differences of calcium contents were revealed during ionophore-induced pump–leak steady states, when the mean total calcium content of the cells ($[Ca_T]_i$) was far below electrochemical equilibrium. It is well documented that $[Ca_T]_i$ can remain steady for hours under such conditions (Brown & Lew, 1983). Therefore the low-calcium cells which remained light (L cells) after the density-separation procedure, as well as the high-calcium cells which became dense (H cells), must both have maintained steady states with different but constant calcium contents for long periods of time.

Since ionophore contents and ionophore-induced divalent cation permeability were shown to be uniform, heterogeneity of calcium content had to arise from differences in active calcium extrusion rate among the cells. These, in turn, could result from variations in the state or the number of calcium pumps, in the supply of ATP to the calcium pump, or from a combination of these. To understand the origin of the heterogeneity it was therefore necessary to characterize the differences in calcium transport and ATP content in H and L cells in steady state, and to trace their development from the onset of calcium permeabilization.

We found a surprising pattern for the steady state. Most of the H cell calcium appeared to be in a subfraction of cells which had become irreversibly depleted of ATP, and were at or very near equilibrium with external calcium (E cells, for Ca^{2+}-equilibrated cells). The rest of the cells within the H and L subpopulations maintained high ATP levels and a pump–leak balance with an undetectable calcium transport pool (B cells, for the leak influx was almost completely balanced by pump-mediated efflux). L cells had a measurable calcium pool inaccessible to functional calcium pumps and K^+ channels. The properties of this pool are analysed in detail in the next paper (García-Sancho & Lew, 1988*b*). The mechanism of irreversible ATP depletion and extreme calcium-induced heterogeneity are considered in the paper by Almaraz, García-Sancho & Lew, (1988).

METHODS

Preparation of red cells, measurement of cell calcium contents and composition of solutions A, B and C were as detailed in the previous paper (García-Sancho & Lew, 1988*a*). Since there were substantial differences of aims and design among different experiments, the specific protocol followed for each experiment is given in the legends of figures.

The procedure for dense cell separation was standardized as follows. In the experiments of Figs 1 and 2 all the cell suspensions contained the ionophore A23187. At the end of the 30 min incubation period each suspension was mixed with 10–30 volumes of ice-cold solution B containing 1% albumin and 0·1 mM-EGTA; the cells were washed twice with this solution to remove the ionophore and external calcium. The cells were then washed once and resuspended at about 3% haematocrit in ice-cold solution C for 30 min. During this incubation at 0–4 °C in the thiocyanate (SCN^-) buffer, dense H cells were formed. Density separation was performed by placing 1 ml aliquots of this ice-cold cell suspension in solution C over 0·4 ml of diethyl-phthalate in 1·5 ml Eppendorf tubes and centrifuging for 1 min (12000 *g*) at room temperature. The cell pellets were lysed with distilled water and the lysate analysed for haemoglobin and ^{45}Ca as described before (García-Sancho & Lew, 1988*a*). Total cell calcium was similarly measured after centrifuging the cells through dibuthyl-phthalate. In samples taken soon after calcium permeabilization (experiments of Figs 4–7), special low-temperature conditions were used to prevent calcium loss through residual pumping (García-Sancho & Lew, 1988*a*).

When density separation was performed on samples of cell suspensions from which the ionophore had already been washed away (experiments of Fig. 3) 0·3 ml samples of the cell suspension were mixed directly with 10 ml of ice-cold solution C, washed and resuspended at about 3% haematocrit in solution C. After a 30 min incubation at 0–4 °C, density separation was performed by centrifugation through the phthalate oils.

The ATP content of the cells was measured in aliquots of the same lysates used for haemoglobin and ^{45}Ca determinations using a bioluminescence assay (Brown, 1982).

RESULTS

Figure 1 reports the changes in dense-cell fraction as a function of ionophore-mediated calcium influx. Calcium influx was increased by raising $[Ca^{2+}]_o$ at a constant ionophore concentration. The fraction of dense cells increased almost linearly with calcium influx (Fig. 1*A*), and cell ATP fell in inverse proportion to the fraction of dense H cells (Fig. 1*B*). When the ionophore-induced calcium influx exceeded 24 mmol/(l cells.h) more than 90% of the cells became dense (Fig. 1*A*). The continuous linear rise in the fraction of H cells seen as calcium influx was increased suggests that any cell may become an H cell, but there is a marked heterogeneity among the cells in the calcium influx required to transform into H cells. Figure 1*B* also suggests that the H cells have a lower mean ATP level. Similar results to those in Fig. 1 were obtained when calcium influx was varied by using different ionophore concentrations at constant $[Ca^{2+}]_o$.

Figure 2 shows the change in the proportion of total cell calcium contained in the dense cells ($[Ca_T]_H/[Ca_T]$, panel *A*) and the extent to which the H cells are equilibrated with $[Ca^{2+}]_o$ ($[Ca_T]_H/[Ca_T]_{Eq}$, panel *B*), as a function of the percentage of dense cells formed. Despite some variability, the trends are clear; the higher the fraction of dense cells, the higher the proportion of the mean total cell calcium which is in the H cells and the nearer the mean calcium content of the H cells to equilibrium. In general, with 1 μM-A23187 in the suspension, and $[Ca^{2+}]_o$ at 200–250 μM, corresponding to calcium influx values around 11–15 mmol/(l cells.h), 40–70% of the cells became dense. These conditions were used in subsequent

experiments because they gave good working fractions of L and H cells for the studies required to characterize the L and H states.

The main aim of the experiments in Fig. 3 was to estimate the calcium pumping rate from H and L cells during the steady state by measuring extrusion of calcium after removal of ionophore and external ^{45}Ca. In addition, the ATP contents of H and

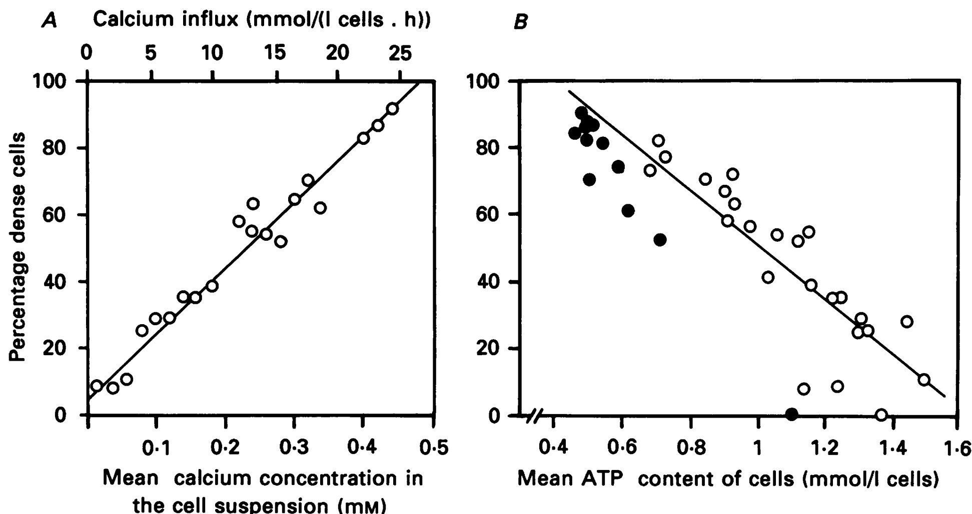

Fig. 1. Fraction of steady-state dense cells formed as a function of ionophore-induced calcium influx (panel *A*), and of mean cell ATP levels (panel B). The solid symbols in panel *B* correspond to a separate experiment in which the proportion of dense cells was compared only with the ATP levels. The cells were incubated for 30 min at 37 °C and 10% haematocrit in solution B containing 10 mM-inosine, 1 μM-A23187 and different concentrations of $CaCl_2$ as indicated in the abscissa of panel *A*. The corresponding unidirectional calcium influx values, computed from the equations described in Results, are given in the top axis. At the end of the incubation, samples were processed for determination of the dense-cell fraction (haemoglobin in diethyl-phthalate pellet/ haemoglobin in dibutyl-phthalate pellet), and of ATP, as described in Methods. The straight lines correspond to least-square fits. The values of r were 0·98 and 0·88 for panels *A* and *B*, respectively.

L cells were measured to establish whether the lower levels predicted for the H cells, from the results in Fig. 1, were indeed confined to this cell fraction only.

Calcium release from high-ionophore-treated cells

The initial sample of the cells treated with high ionophore (circles in Fig. 3) reports the calcium content of calcium-equilibrated cells at the end of the preceding incubation period to be 800–900 μmol/l cells (panel *A*, inserts). Over 85% of the initial calcium content of the cells came out at rates of 3–4 mmol/(l cells.h) (panel *A*), similar to those observed at the high calcium loads of the experiment in Fig. 1 of the previous paper (García-Sancho & Lew, 1988*a*). The ATP concentration in these cells remained low and steady (circles, panel *B*), at about 0·2 mmol/l cells, despite the continued presence of inosine, suggesting that ATP depletion was irreversible. About 90% of the high-ionophore-treated cells in the first sample became dense after SCN^-

treatment. The proportion decreased sharply by 12 min incubation, when the mean total calcium level had fallen below 200 μmol/l cells.

Calcium release from low-ionophore-treated cells

The initial calcium content of low-ionophore-treated cells (triangles in Fig. 3, panel *A*) reports the calcium content of the cells during the preceding steady state, since,

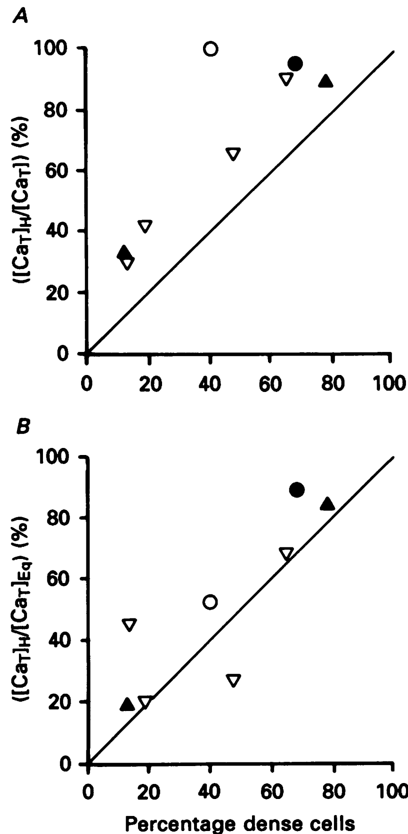

Fig. 2. Fraction of the total cell calcium which is contained within dense cells (panel *A*), and fractional calcium equilibration within dense cells (panel *B*), as a function of the percentage of dense cells formed. The points report results from seven different experiments, two with fresh blood (filled symbols) and five with bank blood (open symbols). Triangles and circles identify different donors. All conditions were as for the experiment of Fig. 1. In addition, $[Ca_T]_{Eq}$, the level of calcium in the cells at equilibrium with $[Ca^{2+}]_o$, was measured using a high ionophore concentration (10·μM). The identity lines through the origin are for reference.

as demonstrated before (García-Sancho & Lew, 1988*a*), there was no significant calcium loss during ionophore wash-out or density separation procedures. Cell calcium was about 200 μmol/l cells for H cells, and about 45 μmol/l cells for L cells in each of the duplicate experiments (*a* and *b*). Since at equilibrium total cell calcium was 800–900 μmol/l cells (inserts of panel *A*), the calcium levels of low-ionophore-treated cells during the steady state were far below equilibrium in both H and L cell

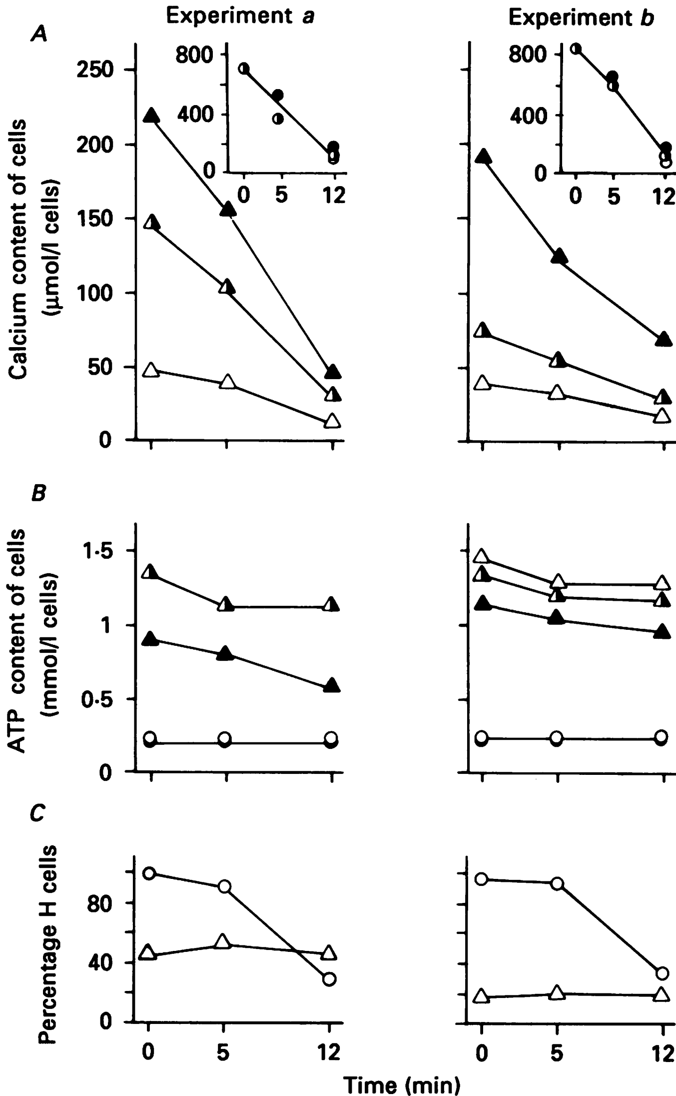

Fig. 3. Calcium (A) and ATP (B) contents of different cell fractions, and percentage dense cells (C), as a function of time during net calcium efflux from inosine-fed, calcium pre-loaded cells. The results of two similar experiments with cells from two donors are shown (a and b). Fresh cells were suspended at 10% haematocrit in solution B containing 10 mM-inosine. $^{45}CaCl_2$ was added to a final concentration of 250 μM in the cell suspension. After about 10 min at 37 °C the suspension was divided into two lots and the ionophore A23187 was added to give final concentrations of 1 and 10 μM, respectively. Thirty minutes after ionophore addition the cells were washed twice with ice-cold solution B containing 0·1 mM-EGTA and 1% albumin to remove the ionophore and the extracellular calcium. After the washes the cells were resuspended again at 10% haematocrit in cold solution B with 0·1 mM-EGTA and 10 mM-inosine. At $t = 0$, after initial samples were taken, the suspensions were placed in the bath at 37 °C and further samples were taken at 5 and 12 min of incubation. These samples were carried through the density separation procedure described in Methods. Each value represents the mean of four individual measurements with S.E.M. smaller than 10%. Triangles refer to cells pre-loaded with

fractions. The initial ATP levels (panel *B*) were about 1 mmol/l cells in H cells and 1·5 mmol/l cells in L cells. Calcium efflux was 0·6 to 0·9 mmol/(l cells. h) for H cells, and 0·1–0·2 mmol/(l cells. h) for L cells.

These results are puzzling for a number of reasons. To sustain pump–leak steady states with calcium contents far below equilibrium, the unidirectional calcium efflux through the pump must match the net calcium influx through the ionophore. The inward leak through the ionophore was over 10 mmol/(l cells.h) see below for more precise estimates. The main puzzle is that the observed calcium efflux rates of 0·1 to 0·9 mmol/(l cells.h) are clearly no match for such influx. In addition, both the H and L cells from the low-ionophore group, with near normal ATP levels, had much lower apparent pumping rates than the calcium-equilibrated cells with very low ATP levels. Furthermore, within the low-ionophore group, the L cells, with more ATP and less calcium than the H cells, also seem to pump less than the H cells.

To estimate the magnitude of the discrepancy between net inward calcium leak and outward pump fluxes in the low-ionophore-treated cells, it is necessary to calculate first the size of the ionophore-mediated calcium fluxes in the experiments of Fig. 3. If calcium were uniformly distributed in the H cells, and we represented its value by $[Ca_T]_H$, the ionophore-mediated net calcium influx (ϕ_{net}) could be calculated from the equation $\phi_{net} = P(r^2[Ca^{2+}]_o - \alpha[Ca_T]_H)$, where P represents the ionophore-induced calcium permeability, $r^2 = ([H^+]_i/[H^+]_o)^2 \approx 2$ in the present experimental conditions (Brown & Lew, 1983), and α represents the fraction of the total cell calcium which is in ionized form. The value of α is usually in the 0·14–0·45 range for human red cells (Ferreira & Lew, 1976; Lew & Brown, 1979; Simonsen, Gomme & Lew, 1982; Simons, 1982). Its approximate value in any particular experiment can be calculated from the distribution of calcium at equilibrium, as in the high-ionophore conditions in the experiments of Fig. 3, using the equation above. At equilibrium, $\phi_{net} = 0$ and $\alpha = r^2 ([Ca^{2+}]_{o,\,Eq}/[Ca_T]_{Eq})$, where $[Ca^{2+}]_{o,\,Eq}$ and $[Ca_T]_{Eq}$ are the concentrations of Ca^{2+} in the medium and of total calcium in the cells at equilibrium, respectively. $[Ca^{2+}]_{o,\,Eq}$ is obtained from the redistribution equation: $[Ca^{2+}]_{o,\,Eq} = ([Ca_T]_s - (Ht[Ca_T]_{Eq})/(1-Ht))$, where $[Ca_T]_s$ is the total calcium concentration in the suspension, which remains constant throughout, and Ht is the haematocrit. In experiment *a* of Fig. 3, $[Ca^{2+}]_{o,\,Eq} = (250-(0{\cdot}1\times720))/0{\cdot}9 = 198$ μM, and in experiment *b* $[Ca^{2+}]_{o,\,Eq} = (250-(0{\cdot}1\times850))/0{\cdot}9 = 183$ μM; the approximate values of α were: $\alpha_a = 2\times198/720 = 0{\cdot}5$ and $\alpha_b = 2\times183/850 = 0{\cdot}4$. In order to estimate the net flux we still need to know $[Ca^{2+}]_o$ in the low-ionophore steady state. This we calculate from the redistribution equation using $[Ca_T]$ instead of $[Ca_T]_{Eq}$. For experiment *a* $[Ca^{2+}]_o = (250-(0{\cdot}1\times146)/0{\cdot}9 = 262$ μM and for experiment *b*, $[Ca^{2+}]_o = (250-(0{\cdot}1\times74)/0{\cdot}9 = 270$ μM. The value of P, the ionophore-induced calcium permeability, was estimated from direct measurements of calcium influx in ATP-depleted cells or by ^{45}Ca tracer exchange in steady-state or equilibrium conditions (seven experiments, not shown). For the batch of ionophore used in the experiments reported here, $P(h^{-1}) \approx ([I]_c)^{1{\cdot}45}$, where $[I]_c$ is the ionophore concentration in the cells (in μmol/l cells) assuming full cell partition (Lew & Simonsen, 1980; Simonsen & Lew, 1980). In the experiments of Fig. 3 then, ϕ_{net}, $a = 10^{1{\cdot}45}$ $[(2\times0{\cdot}26)-(0{\cdot}5\times0{\cdot}22)] = 12$ mmol/(l cells.h) and ϕ_{net}, $b = 10^{1{\cdot}45}$ $[(2\times0{\cdot}27)-(0{\cdot}4\times0{\cdot}199)] = 13$ mmol/(l cells.h). These values represent the estimated ionophore-mediated net influx of calcium which must have existed in the H cells during the steady state. Because the steady-state calcium content (Fig. 3, $t = 0$) of L cells ($[Ca_T]_L$) was smaller than that of the H cells ($[Ca_T]_H$), the inward Ca^{2+} concentration gradient for L cells in steady state was higher. Net calcium influx into L cells in steady state must therefore have been higher than that into H cells; calculations similar to those above for H cells render values of about 15 mmol/(l cells.h). In steady

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calcium using low ionophore (1 μM), and circles to those pre-loaded with high ionophore (10 μM). Measurements in L cell fractions were carried out after spinning the cells recovered over diethyl-phthalate oil through dibutyl-phthalate oil. Calcium and ATP were determined independently in all cells (half-filled symbols), H cells (filled symbols) or L cell (open symbols).

state, therefore, H and L cells must have been pumping at rates of between 12 and 15 mmol/(l cells.h) in order to maintain their calcium contents at the observed levels. The measured rates of 0·6–0·9 mmol/(l cells.h) and 0·1–0·2 mmol/(l cells.h) for H and L cells, respectively, cannot therefore represent the pumping rates of these cells in the steady state. Despite approximations and indirect estimates, it is difficult to find errors that could invalidate the conclusions derived from these calculations. The largest error is in α because it was estimated from a single calcium distribution value instead of the usual curve (Ferreira & Lew, 1976; Lew & Brown, 1979). This error propagates little in the ϕ_{net} equation and, if anything, because α appears overestimated, ϕ_{net} may, in fact, be higher. The functional relation between P and cell ionophore concentration, as well as the reliability of the $[Ca_T]_H$ measurements in steady state have been sufficiently documented (Simonsen *et al.* 1982; García-Sancho & Lew 1988*a*).

In the steady state, 30 min after exposure to low-ionophore and calcium, L cells contain variable and sometimes large amounts of calcium (see also Table 2 in García-Sancho & Lew, 1988*a*). In the experiments shown in Fig. 3 the calcium content of L cells at $t = 0$ was about 35–45 μmol/l cells. This cannot represent the pool of calcium from which pumping proceeded during the steady state (i) because the measured extrusion rate was only 0·1–0·2 mmol/(l cells.h) rather than the saturated pump rate expected with ionized calcium levels of about 40% of the $[Ca_T]_i$, and (ii) because the measured efflux obviously cannot balance the calculated net influx of about 15 mmol/(l cells.h). The fact that L cells do not dehydrate during SCN^- treatment also indicates that this calcium pool may be unavailable to Ca^{2+}-sensitive K^+ channels. These considerations suggest that the measured L cell calcium pool represents some sort of 'residual calcium' unavailable to calcium pumps or K^+ channels, and that the real calcium transport pool is too small to be detected by our methods. The demonstration of such residual calcium pools and their properties will be analysed separately in the paper by García-Sancho & Lew (1988*b*).

In the experiments of Fig. 3, L cells were exposed to more processing steps than those in the sample for totals or for H cells, since they had to be collected from the top layer, after centrifugation of the SCN^- suspended samples through diethyl-phthalate oil at room temperature, and respun through dibutyl-phthalate oil. In experiment *a* calculation of the initial calcium content of the L cells ($[Ca_T]_L$ by difference between $[Ca_T]$ and $[Ca_T]_H$, $(146-(0{\cdot}42\times220)/0{\cdot}58 = 92$ μmol/l cells, gives about twice the measured amount of 46 μmol/l cells, and in experiment *b*, the calculated $[Ca_T]_L$, $(74-(0{\cdot}18\times192)/0{\cdot}82 = 48$ μmol/l cells, is only 10–12 μmol/l cells higher than measured. It is possible then that a small calcium transport pool was lost during the processing of the L cells. It is also possible that L cells may have had active Ca^{2+}-sensitive K^+ channels in the steady state which became deactivated during the density separation procedure by rapid loss of the small calcium transport pool.

In summary, L cells seem to contain two different calcium pools, one in rapid pump–leak turnover comprising a small fraction of total cell calcium, and another much larger pool of residual calcium unable to activate pumps or channels. The L cells also had the highest ATP levels, essentially normal. Since the hydrolysis of ATP by the calcium pump alone must be in the 7–15 mmol/(l cells.h) range, depending on the stoichiometry of the pump, ATP as well as calcium must have been in rapid turnover in the steady state.

In the experiments in Fig. 3, the persistent fall in ATP of the H cells in the low-ionophore group, as well as the sustained low ATP of all the high-ionophore-treated cells, suggests that ATP depletion is irreversible (see also the experiment of Fig. 4 in the paper by García-Sancho & Lew (1988*b*), which shows that the ATP level of the

H cells remains steady for at least 2 h, long after calcium pumping has ceased). This conclusion is consistent with earlier observations in unfractionated cells (Brown & Lew, 1983) in which calcium pump deactivation was induced by external calcium chelation in the presence of ionophore.

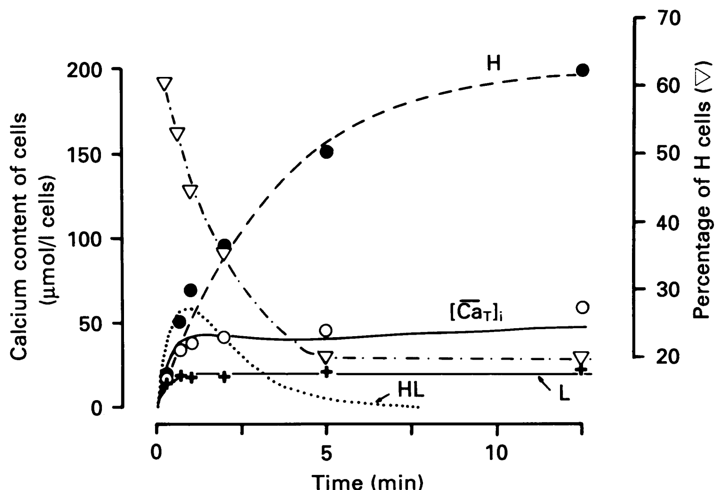

Fig. 4. Time course of changes in dense cell fraction (H cells) and in cell calcium content following ionophore-induced calcium influx. Fresh washed red cells were suspended at 10% haematocrit in solution B containing ^{45}Ca (100 μM) and inosine (10 mM) and incubated at 37 °C. The ionophore A23187 was added at $t = 0$ to give concentration of 1 μM in the suspension. The mean total calcium in unfractionated cells (○) and in H cells (●) was measured, and that in the L cells (+) was estimated by the difference. All the curves, except that reporting the percentage dense cells (▽), were computed with the model given in the text. The curves report the calcium content as a function of time in H cells (dashed line), HL cells (dotted line), L cells (continuous line through crosses), and in unfractionated cells (continuous line through open circles).

Early changes in cell calcium and ATP contents following calcium permeabilization

These experiments were designed to characterize the development of the differences in calcium distribution in H and L cells in the steady state. The experiments of Figs 4 and 5 report the ionophore-induced changes in the fraction of dense cells and in the calcium and ATP contents of unfractionated and density-separated cells. Inosine was omitted in the experiment of Fig. 5, which was otherwise identical to that of Fig. 4. Ionophore addition triggered large and transient changes in the H cell fraction, and different changes in the calcium contents of the various cell subpopulations. Comparison of Figs 4 and 5A suggests that these changes were affected little by the presence or absence of inosine during the first 5 min. The falling ATP levels (Fig. 5*B*) therefore did not become limiting to the calcium pump during this period, when cell ATP were was above ≈ 600 μmol/l cells. The mean ATP content of the starved cells fell at a steady rate of 5·6 mmol/(l cells.h) for the first 5–7 min after calcium permeabilization (Fig. 5*B*). The fall was slightly sharper in H than in L cells (about 7·5 and 6·0 mmol/(l cells.h) respectively, after the first minutes); if, as analysed in the Discussion, the ATP fall in the H cell group is

confined to a small subpopulation, the difference in rate of decline of ATP between L cells and that subpopulation of H cells may be quite large. Since H cells were gaining more calcium than L cells exposed to the same calcium influx, H cells must have been pumping less calcium than L cells. The fact that ATP fell faster in H cells,

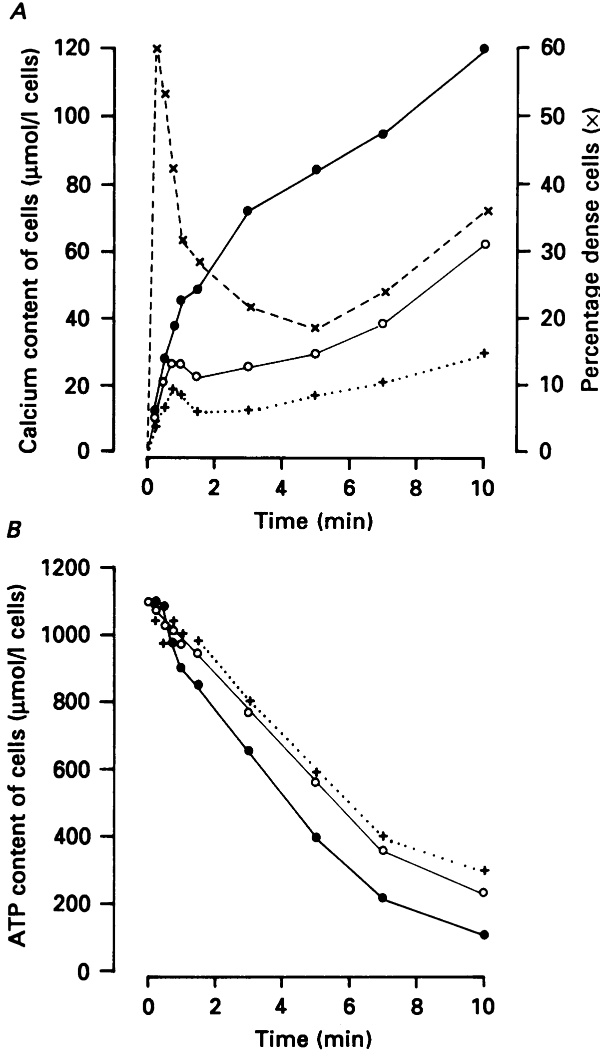

Fig. 5. Time course of changes in dense (H) cell fraction, cell calcium and ATP in substrate-starved cells during ionophore-induced calcium influx. Fresh washed red cells were suspended at 10% haematocrit and 37 °C in solution B with ^{45}Ca (40 μM). Ionophore (1·1 μM) was added at $t = 0$. Panel *A* shows changes in H cell fraction (×), and in calcium content of unfractionated cells (○), of H cells (●), and of L cells (+). Panel *B* shows ATP contents of unfractionated cells (○), of H cells (●), and of L cells (+).

then, indicates that factors other than calcium pump-mediated ATP hydrolysis contribute to the different depletion rates in H and L cells. These factors seem to be active within a minute after calcium permeabilization and may play a role in the irreversible depletion of H cell ATP.

The fraction of dense cells was highest in the first sample, at 15 s. The subsequent steep decline suggests that, if it had been possible to sample earlier, the peak might have been even higher. The H cell fraction declined from 60 to 20% within 5 min in both experiments (Figs 4 and 5). Therefore, at least 40% of cells transferred from H to L cell fractions (HL cells). After 5 min the fraction of H cells showed a secondary slow rise in the absence of substrate (Fig. 5), but remained steady in the presence of inosine (Fig. 4). A large fraction of steady-state L cells must therefore be HL cells. The mean calcium content of H cells increased as their fraction was reduced whereas that of the L cells remained fairly constant and relatively low (Figs 4 and 5*A*).

We will consider next the cells that transferred from H to L (HL cells). Since H cells differ from L cells in that their Ca^{2+}-sensitive K^+ channels stay open during the density separation procedure, a cell that transfers from H to L must have its channels deactivated, either by a fall in $[Ca^{2+}]_i$, or by a decrease in the $[Ca^{2+}]_i$ sensitivity of the channels. There is ample documentation of peak transients in the mean calcium content of human red cells following ionophore-induced calcium permeabilization (Ferreira & Lew, 1976; Scharff, Foder & Skibsted, 1983; Scharff & Foder, 1986), but no evidence of time-dependent changes in the $[Ca^{2+}]_i$ sensitivity of the K^+ channels. If we assume the latter to be constant in time, then analysis of the results in Fig. 4 indicates that the HL cells must have undergone peak calcium transients (even when, as in that experiment, there was no apparent peak in mean total cell calcium).

The measured change in H cell calcium content with time can be described by an exponential with a rate constant of about 0·3 min^{-1}, to a final level of 200 μmol/l cells: $[Ca_T]_H = 200(1-\exp(-0{\cdot}3t))$. Values for the calcium content of L cells, obtained by difference between mean total cell calcium and H cell calcium weighted by the fraction of H cells, fitted an exponential with rate 3·5 min^{-1} and final value of 20 μmol/l cells: $[Ca_T]_L = 20(1-\exp(-3{\cdot}5t))$. These equations for $[Ca_T]_H$ and $[Ca_T]_L$ describe the time course of the changes in calcium content within the 20% of cells which start and stay dense (H), and within the 40% of cells which start and stay light (L). The time course of change in the calcium content of the remaining 40% of HL cells ($[Ca_T]_{HL}$), estimated as the difference between the measured $[Ca_T]_i$ and $(0{\cdot}2[Ca_T]_H + 0{\cdot}4[Ca_T]_L)$ at each time, is shown as the dotted line in Fig. 4. The equation describing this curve, $[Ca_T]_{HL} = 200\{1-[\exp(-0{\cdot}9t)\exp(0{\cdot}7t)]\}$, can be interpreted using the following model. HL cell calcium initially rises towards a similar asymptotic value as that of H cells (200 μmol/l cells) with a rate constant of about 0·9 min^{-1}. Subsequently, HL cells extrude the calcium gained at the rate found for H to L cell transfer (about 0·7 min^{-1}). The only weak assumption in the computation of $[Ca_T]_{HL}$ concerns the existence of cells that start and stay L. If it had been possible to process samples taken 5 s after calcium permeabilization, for example, all cells might have initially been H. Eighty per cent of cells would then be HL cells, but $[Ca_T]_{HL}$ would still rise and fall, with an even sharper 'peak'. This only reinforces the main conclusion from the present analysis, that all or part of the cells which become steady-state L cells do undergo peak calcium transients during the first few minutes following calcium permeabilization, whether or not this is evident in the mean total calcium curves.

The delayed effects of calcium permeabilization in substrate-starved red cells (Fig. 5) become apparent after about 5 min, when the mean ATP content of the cells has fallen to 50% or less of the initial values. The increase in H cell fraction (Fig. 5*A*) indicates net Ca^{2+} gain within cells transferring from L to H density fractions, presumably as a result of the progressive imbalance between a failing, ATP-deprived pump, and a constant calcium influx. As noted above, calcium-induced ATP depletion of inosine-fed cells was irreversible (Brown & Lew, 1981, 1983) and, in the steady state, was confined to H cells (Fig. 3). Even in the absence of inosine, H cells,

or cells within this fraction at any given time, depleted faster than L cells (Fig. 5*B*), signalling a specific and early difference between H/L subpopulations.

It is important to investigate whether ATP depletion within cells transferring from L to H cell fractions is irreversible. For if it were not, it would indicate that low ATP and a rising cell Ca^{2+} *per se* do not generate the intracellular conditions that make the depletion of steady-state H cells irreversible. It would also suggest that the

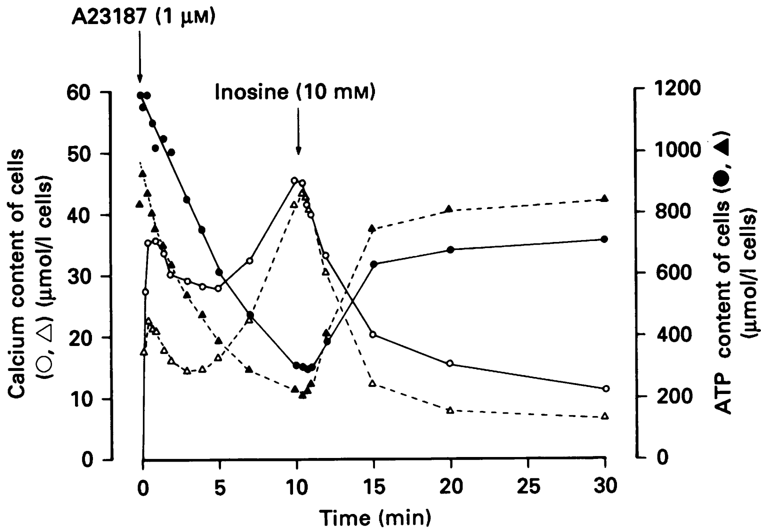

Fig. 6. Effect of delayed inosine addition on cell calcium and ATP levels of calcium permeabilized cells. The results of two experiments are shown in which the cells were pre-incubated without substrate for either 5 min (circles) or 50 min (triangles). At $t = 0$, the ionophore A23187 (1 μM) was added. Samples were taken at the times shown to measure cell-associated ^{45}Ca (open symbols) or ATP (closed symbols). At $t = 10$ min, inosine was added to give a concentration of 10 mM in the cell suspensions.

factors which determine the steady-state H/L divide observed with fed red cells (Figs 1 and 4) act immediately after calcium permeabilization to affect the fraction of cells destined to become H cells at each calcium influx level. The reversibility of L cell depletion was investigated in the experiment of Fig. 6 by initiating calcium influx in substrate-starved red cells, as in the experiment of Fig. 5, and observing the effects of adding inosine 10 min after ionophore addition. The initial rates of ATP depletion in this experiment were about 7 and 12 mmol(l cell.h), for cells that had been pre-incubated without substrate for 5 and 50 min respectively. Within 30–60 s after inosine addition, net calcium influx and ATP fall reverted to net efflux and ATP gain. This reversal represents a net change in calcium pumping rate of about 0·8–0·9 mmol/(l cell.h). For ATP, a net break-down of about 3–6 mmol/(l cells.h) just before inosine addition was reverted to net synthesis of about 9 mmol/(l cells.h). ATP recovery was partial, to about 60–85 % of the initial value. These results show that the extra depletion caused by substrate deprivation is reversible unlike the depletion caused by calcium influx alone (Fig. 3). Therefore, high calcium and low ATP levels *per se* cannot be responsible for the irreversibility of ATP depletion.

DISCUSSION

Analysis of the results centres on the flux paradox of Fig. 3, since its explanation reveals the basic heterogeneity generated by calcium permeabilization. H/L cell fractionation serves only as a tool, the single one available at present, with which this heterogeneity can be exposed.

In the low-ionophore-treated cells of the experiments reported in Fig. 3, the calcium pumping rate of 0·6–0·9 mmol/(l cell.h) measured in H cells did not match the net influx rate of 12–13 mmol/(l cells.h) required for the maintenance of a pump–leak steady state. The calculation implicitly assumed uniformity of calcium content and pumping rate within the H cells. This assumption may be invalid; the results in Figs 4 and 5 suggest, rather, large heterogeneities of calcium and ATP content within subpopulations of H cells, at least during the period preceding steady state. The crucial question, then, is whether a particular heterogeneous distribution of calcium and ATP could explain all results as well as the flux paradox. Let us consider first the simplest case, that of extreme heterogeneity within the H cell fraction, by assuming that all of the H cell calcium is contained within a subpopulation of cells designated 'E' cells. Then the product of total E cell calcium content, $[Ca_T]_E$, and its fraction in the total population, p_E, would equal the corresponding product for the H cells, $p_H \times [Ca_T]_H$. Since E cells are a subpopulation of H cells, $p_E < p_H$. If all of the measured calcium efflux from the H cells, ϕ_H, originates in the E cell fraction, with calcium extrusion rate ϕ_E, then $p_E \times \phi_E = p_H \times \phi_H$. If, in addition, E cells are to explain the flux paradox, they have to obey the pump–leak steady-state equation $\phi_E = P(r^2[Ca^{2+}]_o - \alpha[Ca_T]_E)$. This gives us a set of three equations from which the fraction (p_E), calcium content ($[Ca_T]_E$) and calcium pumping rate (ϕ_E) of the hypothetical E cells can be computed using only the measured quantities p_H, $[Ca_T]_H$ and ϕ_H. For experiment *a* (Fig. 3) the three equations give: $p_E \times [Ca_T]_E = 0{\cdot}42 \times 0{\cdot}22$; $p_E \times \phi_E = 0{\cdot}42 \times 0{\cdot}87$, and $\phi_E = 10^{1{\cdot}45}((2 \times 0{\cdot}26) - 0{\cdot}5\,[Ca_T]_E)$, and for experiment *b* $p_E \times [Ca_T]_E = 0{\cdot}2 \times 0{\cdot}19$; $p_E \times \phi_E = 0{\cdot}2 \times 0{\cdot}60$, and $\phi_E = 10^{1{\cdot}45}((2 \times 0{\cdot}27) - 0{\cdot}4\,[Ca_T]_E)$. The solutions are: $p_E = 12\%$, $[Ca_T]_E = 750\ \mu mol/l$ cells, and $\phi_E = 3{\cdot}0$ mmol/(l cells.h), for experiment *a*; and $p_E = 3{\cdot}6\%$, $[Ca_T]_E = 1{\cdot}05$ mmol/l cells, and $\phi_E = 3{\cdot}3$ mmol/(l cells.h) for experiment *b*.

This is a surprising outcome. Firstly, the extreme calcium heterogeneity explanation neatly fits the results without internal contradictions, and also explains the flux paradox. Secondly, the characteristics of the E cells correspond closely to those of the high-ionophore, calcium-equilibrated cells found in the experiments of Fig. 3.

Note that the calculated calcium content of the E cells is higher than that measured in the calcium-equilibrated high-ionophore cells of the same experiment, under apparently identical experimental conditions. This is expected since, at equal total calcium concentration in the suspension, the extracellular $[Ca^{2+}]_o$ in the steady state was higher in the low-ionophore than in the high-ionophore suspension because calcium remained excluded from many of the low-ionophore cells (L cells).

Thus it is possible to explain the paradoxical mismatch between pump and leak of the H cells if we assume that during the pump–leak steady state, most of the mean calcium content of the cells is accumulated near equilibrium in a small proportion of

cells, p_E, with calcium transport properties like those of the calcium-equilibrated cells. All the other cells, some within the H group (HB cells), and all of the L group, have a relatively low calcium concentration and must be able to sustain balancing pump fluxes of about 15 mmol/(l cells.h), matching the net ionophone-mediated influx (balanced cells, or B cells).

The ATP results in Fig. 3 provide an independent way of testing the E/B extreme heterogeneity hypothesis (where B = HB + L). If E cells are like the calcium equilibrated cells from the high-ionophore condition, their ATP should be at the 0·2 mM level. If HB cells are like L cells, their ATP should be similarly high. According to this, the lower mean ATP level of H cells should be entirely due to the presence of the E cells. To see whether this view is compatible with the observed ATP levels in the initial samples, we ought to solve for ATP_B the equation $ATP_T = p_E\ ATP_E + p_B\ ATP_B$, where $p_B = 1 - p_E$ and ATP_T, ATP_E and ATP_B are the mean total ATP content of unfractionated cells, E and B cells, respectively. For experiment *a* $ATP_B = (1{\cdot}34 - (0{\cdot}12 \times 0{\cdot}2))/0{\cdot}88 = 1{\cdot}5$ mmol/l cells and for expt *b* $ATP_B = (1{\cdot}33 - (0{\cdot}036 \times 0{\cdot}23))/0{\cdot}964 = 1{\cdot}4$ mmol/l cells. These values should be compared with an ATP_L of $(1{\cdot}33 - (0{\cdot}42 \times 0{\cdot}9))/0{\cdot}58 = 1{\cdot}6$ mmol/l cells obtained by difference for experiment *a* and with that of $1{\cdot}45 \pm 0{\cdot}11$ (mean ± S.D.) measured directly for experiment *b*. This agreement confers independent support for the E/B structure. Since the ATP fall measured in H cells is confined to E cells, the observed irreversibility implies that inosine-fed E cells cannot restore ATP even after calcium influx was terminated by ionophore removal. The reason for the irreversibility of calcium-induced ATP depletion is investigated in the paper by Almaraz *et al.* (1988).

This analysis then suggests that the concealed E/B heterogeneity, rather than the observed H/L heterogeneity, represents the true calcium distribution in the steady state, since it explains the flux paradox. The E/B structure can be outlined as follows. At any value of calcium influx only two subpopulations of cells evolve: high calcium cells (E cells) and low-calcium cells (B cells). The higher the calcium influx, the higher the proportion of E cells. The E cell state is defined by the behaviour observed in calcium-equilibrated cells obtained by high-ionophore treatment, i.e. (i) total calcium content near equilibrium, (ii) calcium extrusion by the pump in the 2·5–6 mmol/(l cells.h) range, rate-limited by low ATP levels, and perhaps additionally inhibited by excess calcium over ATP, (iii) irreversibly reduced constant ATP level in fast turnover (7–20 times the pool size per hour), (iv) all E cells are in the H cell fraction, and (v), as Fig. 2 suggests, the proportion of E cells within the H cell fraction increases with calcium influx.

The B cell state is defined by (i) small and difficult-to-detect calcium transport pool, in extremely rapid turnover, (ii) the ionophore-mediated unidirectional calcium influx balanced almost entirely by calcium pump-mediated calcium extrusion, (iii) ATP level at or very near normal and also in rapid turnover, (iv) B cells make up all the L cell fraction and a variable proportion of the H cell fraction (HB cells), (v) at least those B cells in the L cell fraction have a variable residual calcium pool which fails to activate calcium pumps or K^+ channels. The existence of a non-pumping residual calcium pool in B cells as well as in E cells will be analysed in detail in the paper by García-Sancho & Lew (1988*b*).

The results of the experiments reported in Figs 4–6, and the analysis above, allow

a description of the events leading from calcium permeabilization to steady-state E/B cell distribution. If the calcium influx induced in a particular cell is below its maximal calcium extrusion capacity, then, after an initial peak $[Ca_T]_i$ transient, the cell will establish a pump–leak steady state with very low $[Ca^{2+}]_i$, and with normal ATP levels in calcium stimulated turnover (B cell). If the induced calcium influx exceeds the maximal calcium extrusion capacity of the cell, it will gain calcium and become a Ca^{2+}-equilibrated, irreversibly ATP-depleted, E cell. The factors which determine the E or B fate of each cell act immediately after calcium influx is induced (Figs 4 and 5) and are related to the conditions which make the calcium-induced ATP depletion in E cells irreversible. These are analysed in detail in the paper by Almaraz *et al.* (1988).

The origin of the peak $[Ca_T]_i$ transients at the onset of ionophore-induced calcium influx was attributed to delayed pump activation by Scharff, Foder & Skibsted (1983). The idea was based on the observation of slow calcium-dependent binding of calmodulin to the pump, an association which was found to be required *in vitro* by the Ca^{2+}–Mg^{2+}-ATPase for conversion from a low-activity, low-Ca^{2+}-sensitivity state to a high-activity, high-affinity state (Scharff & Foder, 1982; Agre, Gardner & Bennet, 1983). Scharff, Foder & Skibsted (1982) tested delayed pump activation by measuring the time course of calcium-induced ATP fall after ionophore-induced calcium influx in substrate-starved cells, since delayed pump activation would be expected to be associated with delayed ATP hydrolysis. These authors found a lag of over a minute in the onset of the ATP fall, in agreement with the predicted delay. Our results in Figs 5 *B* and 6, on the other hand, do not show any measurable lag. Perhaps residual substrate may account for the lag observed by Scharff *et al.* (1982). Peak $[Ca_T]_i$ transients, however, do indicate delayed activation of reserve pump capacity, but their mechanism remains to be elucidated.

We will consider now the wider implications of the present results for red cells and other cell types. From an experimental point of view the E/B response to calcium permeabilization represents a serious obstacle to the study of Ca^{2+} transport and of the effects of Ca^{2+} on transport and metabolism in intact red cells because we cannot control the cytoplasmic Ca^{2+} levels within critical concentration ranges. Such control remains at present attainable only in metabolically depleted red cells (Ferreira & Lew, 1977; Lew & Ferreira, 1978). It is necessary then to re-evaluate results in the literature in which calcium influx was measured within the critical range and in which the cytoplasmic Ca^{2+} levels were computed on the erroneous assumption of a uniform calcium distribution among the cells (Lew & Ferreira, 1976; Burgin & Schatzmann, 1979; Brown & Lew, 1983; Scharff *et al.* 1983; Cala, 1983). In those experiments the observed effects were reported as a function of ionized cell calcium levels computed from mean total cell calcium values assumed to be uniform. The interpretation conveyed may therefore be wrong and misleading. Thus, for instance, the curves which show low apparent calcium sensitivities of the K^+ channels in inosine-fed red cells (Lew & Ferreira, 1976), or which describe the $[Ca^{2+}]_i$ dependence of calcium pump-mediated calcium extrusion (Sarkadi, Szász & Gárdos, 1976; Sarkadi, Szász, Gerloczy & Gárdos, 1977; Scharff, Foder & Skibsted, 1982, 1983) do not provide the originally intended information. In terms of the E/B hypothesis, the tracer fluxes reporting the channel and pump activities proceeded mainly from the fraction of E cells formed at each external calcium level. Since the fraction of E cells as well as their calcium content increased with the external calcium level, the tracer fluxes were actually reporting effects from cells whose calcium concentration was

varying over a much higher range than that assumed from uniform distribution, and whose ATP levels had been substantially depleted. If these effects are normalized to the fraction of E cells that might have formed at each calcium level, rather than attributed to the whole cell population, as originally done, what appeared as activation would tend to flatten, whereas inhibition would become steeper. Reinterpretation of the results in terms of E/B cell fractions, therefore, suggests that those experiments contain little information on the Ca^{2+} activation kinetics of pumps or channels.

How general is the E/B phenomenon? Does the all-or-none E/B response, by similar or different mechanisms, occur in other cell types? Is this a purely experimental phenomenon, or could it be part of physiological responses? Calcium permeabilization of cell populations by electroporation, by physiological agonists and by ionophores is widely used to study the role of $[Ca^{2+}]_i$ as second messenger and modulator of intracellular signals. In all such experiments submaximal responses are used to assess the kinetics of the investigated processes, with the implicit assumption that the recorded signals reflect the mean of more-or-less uniform individual cell responses. There is no direct evidence for or against the assumption of uniformity in conditions of documented uniform action. Many of the responses show all-or-none appearance, with the submaximal effects levelling off in time at values below those of the maximal response (Rink, Smith & Tsien, 1982; Knight & Baker, 1982; Gomperts & Fernandez, 1985). This indirectly suggests that submaximal responses to uniform action may potentially originate from large-scale heterogeneities in the responses of individual cells. If this were true, it would have important physiological and methodological implications; physiological, because of the crucially different strategies involved in graded or all-or-none cell responses, and methodological, because information on the kinetics of all-or-none responses could only be derived from isolated cell studies.

We thank EMBO, the British Council, the Wellcome Trust Foundation and the Medical Research Council of Great Britain for funds. We are grateful to R. M. Bookchin for helpful comments on the manuscript, and to Mrs J. Gray for skilful technical assistance.

REFERENCES

AGRE, P., GARDNER, K. & BENNET, V. (1983). Association between human erythrocyte calmodulin and cytoplasmic surface of human erythrocyte membranes. *Journal of Biological Chemistry* **258**, 6528–6565.

ALMARAZ, L., GARCÍA-SANCHO, J. & LEW, V. L. (1988). Calcium-induced conversion of adenine nucleotides to inosine monophosphate in human red cells. *Journal of Physiology* **407**, 557–567.

BROWN, A. M. (1982). ATP and ATPase determinations in red blood cells. In *Red Cell Membranes. A Methodological Approach*, ed. ELLORY, J. C. & YOUNG, J. D. pp. 223–238. London: Academic Press.

BROWN, A. M. & LEW, V. L. (1981). Does intracellular calcium stimulate ATP production in red blood cells? *Journal of Physiology* **319**, 98*P*.

BROWN, A. M. & LEW, V. L. (1983). The effect of intracellular calcium on the sodium pump of human red cells. *Journal of Physiology* **343**, 455–493.

BURGIN, H. & SCHATZMANN, H. J. (1979). The relation between net calcium, alkali cation and chloride movements in red cells exposed to salicylate. *Journal of Physiology* **287**, 15–32.

CALA, P. M. (1983). Cell volume regulation by *Amphiuma* red blood cells. *Journal of General Physiology* **82**, 761–784.

FERREIRA, H. G. & LEW, V. L. (1976). Use of ionophore A23187 to measure cytoplasmic Ca buffering and activation of the Ca pump by internal Ca. *Nature* **259**, 47–49.

FERREIRA, H. G. & LEW, V. L. (1977). Passive Ca transport and cytoplasmic Ca buffering in intact red cells. In *Membrane Transport in Red Cells*, ed. ELLORY, J. C. & LEW, V. L., pp. 53–91. New York: Academic Press.

GARCÍA-SANCHO, J. & LEW, V. L. (1988*a*). Detection and separation of human red cells with different calcium contents following uniform calcium permeabilization. *Journal of Physiology* **407**, 505–522.

GARCÍA-SANCHO, J. & LEW, V. L. (1988*b*). Properties of the residual calcium pools in human red cells exposed to transient calcium loads. *Journal of Physiology* **407**, 541–556.

GOMPERTS, B. D. & FERNANDEZ, J. M. (1985). Techniques for membrane permeabilization. *Trends in Biochemical Sciences* **10**, 414–417.

KNIGHT, D. E. & BAKER, P. F. (1982). Calcium-dependence of catecholamine release from bovine adrenal medullary cells after exposure to intense electric fields. *Journal of Membrane Biology* **68**, 107–140.

LEW, V. L. & BROWN, A. M. (1979). Experimental control and assessment of free and bound calcium in the cytoplasm of intact mammalian red cells. In *Detection and Measurement of Free Ca^{2+} in Cell*, ed. ASHLEY, C. C. & CAMPBELL, A. K. pp. 423–432. Amsterdam: Elsevier North Holland.

LEW, V. L. & FERREIRA, H. G. (1976). Variable Ca sensitivity of a K-selective channel in intact red-cell membranes. *Nature* **263**, 336–338.

LEW, V. L. & FERREIRA, H. G. (1978). Calcium transport and the properties of a calcium-activated potassium channel in red cell membranes. In *Current Topics in Membranes and Transport*, vol. 10, ed. KLEINZELLER, A. & BRONNER, F., pp. 217–277. New York: Academic Press.

LEW, V. L. & SIMONSEN, L. O. (1980). Ionophore A23187-induced calcium permeability of intact human red blood cells. *Journal of Physiology* **308**, 60*P*.

RINK, T. J., SMITH, S. W. & TSIEN, R. Y. (1982). Cytoplasmic free Ca^{2+} in human platelets: Ca^{2+} threshold and Ca^{2+}-independent activation for shape change and secretion. *FEBS Letters* **148**, 21–26.

SARKADI, B., SZÁSZ, I. & GÁRDOS, G. (1976). The use of ionophores for rapid loading of human red cells with radioactive cations for cation pump studies. *Journal of Membrane Biology* **26**, 357–370.

SARKADI, B., SZÁSZ, I., GERLOCZY, A. & GÁRDOS, G. (1977). Transport parameters and stoichiometry of active calcium ion extrusion in intact human red cells. *Biochimica et biophysica acta* **464**, 93–107.

SCHARFF, O. & FODER, B. (1982). Rate constants for calmodulin binding to Ca^{2+}-ATPase in erythrocyte membranes. *Biochimica et biophysica acta* **691**, 133–143.

SCHARFF, O. & FODER, B. (1986). Delayed activation of calcium pump during transient increases in cellular Ca^{2+} concentration and K^+ conductance in hyperpolarizing human red cells. *Biochimica et biophysica acta* **861**, 471–479.

SCHARFF, O., FODER, B. & SKIBSTED, U. (1982). Two states of Ca^{2+}-ATPase and activation of Ca^{2+}-pump of human erythrocytes. *Acta physiologica latino americana* **32**, 93–95.

SCHARFF, O., FODER, B. & SKIBSTED, U. (1983). Hysteretic activation of the Ca^{2+} pump revealed by calcium transients in human red cells. *Biochimica et biophysica acta* **730**, 295–305.

SIMONS, T. J. B. (1982). A method for estimating free Ca within human red blood cells, with an application to the study of their Ca-dependent K permeability. *Journal of Membrane Biology* **66**, 235–247.

SIMONSEN, L. O., GOMME, J. & LEW, V. L. (1982). Uniform ionophore A23187 distribution and cytoplasmic calcium buffering in intact human red cells. *Biochimica et biophysica acta* **692**, 431–440.

SIMONSEN, L. O. & LEW, V. L. (1980). The correlation between ionophore A23187 content and calcium permeability of ATP-depleted human red blood cells. In *Membrane Transport in Erythrocytes, Alfred Benzon Symposium 14,* ed. LASSEN, U. V., USSING, H. H. & WIETH, J. O., pp. 208–212. Copenhagen: Munksgaard.