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IRREVERSIBLE ATP DEPLETION CAUSED BY LOW CONCENTRATIONS OF FORMALDEHYDE AND OF CALCIUM-CHELATOR ESTERS IN INTACT HUMAN RED CELLS

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Calcium chelators which can be incorporated inside small cells without disruption have become useful tools to investigate the role of intracellular ionized calcium in the processes of cell activation and signal-effect mediation. In experiments designed to investigate further Ca^{2+} pump function in chelator-loaded human red cells we found that the chelator-loading procedure itself caused delayed Ca^{2+}-pump inhibition when pump function was explored by increasing the intracellular Ca^{2+} levels with the aid of the divalent cation ionophore A23187. Ca^{2+}-pump inhibition was found to be secondary to ATP-depletion, and ATP-depletion, in turn, could be attributed to formaldehyde, which was released during the hydrolytic incorporation of free chelator, from the cleavage of the four ester groups which anchor it to cell membranes on addition to cell suspensions. The evidence suggests that the formaldehyde released stays largely within the cells. Formaldehyde, in concentrations of up to 20 mmol/l cells had no direct effects on Ca^{2+} transport in red cells, other than through ATP depletion. Procedures to circumvent the difficulties arising from the formaldehyde effects are outlined and discussed.

Introduction

The unexpected observation of Ca^{2+}-pump inhibition in chelator-loaded intact red cells whose Ca^{2+} content was raised with the use of the calcium ionophore A23187, led us to the discovery that low concentrations of formaldehyde, such as those which evolve during the hydrolytic incorporation of the free chelator [1–6], cause a sharp and irreversible fall in red cell ATP levels. Since these chelators are becoming widely used as tools to study Ca^{2+} transport and metabolism in intact cells, it is important to know the side effects of formaldehyde, both alone and as produced during chelator ester hydrolysis, so as to be able to characterize safe conditions for chelator ester use.

Here we describe the effects of formaldehyde and of the esters of two calcium chelators, Benz2 and Quin2 [4,7], on the ATP levels, on the passive transport of calcium and cobalt and on the Ca^{2+}-pump mediated extrusion of calcium in intact human red cells. The results show (a) that at concentrations above 100 μmol/l cells Benz2 and Quin2 can cause substantial and irreversible ATP depletion, (b) that similar effects are obtained with formaldehyde alone, (c) that Benz2 and formaldehyde, per se, have no effects on calcium or cobalt transport other than Ca^{2+}-pump inhibition secondary to ATP depletion, but that in Quin2-loaded cells, in addition, the passive Ca^{2+} influx is

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Abbreviations: DMSO, dimethylsulphoxide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

increased 10-fold, and (d) that, as with indoacetamide [8,9], formaldehyde- and chelator ester-induced ATP depletion is accelerated by metabolic substrates.

Methods

Red cells were obtained from fresh blood drawn into heparin or citrate. The cells were washed three times with a solution containing 75 mM NaCl, 75 mM KCl, 10 mM Tris-HCl (pH 7.7 at 37 °C), and 0.1 mM Na-EGTA, and twice more with a solution containing 60 mM NaCl, 80 mM KCl, 0.2 mM $MgCl_2$, and 20 mM Hepes-Na (pH 7.55) (solution A). After the last wash the cells were packed to 80% haematocrit and then resuspended at 10–30% haematocrit in solution A, with or without 10 mM of glucose or inosine, as indicated. Additions of A23187, $CaCl_2$ or $^{45}CaCl_2$, $CoCl_2$ or $^{60}CoCl_2$, chelator esters, DMSO or formaldehyde to cell suspensions were done while stirring over vortex mixers or using teflon-coated magnetic bars over magnetic stirreres. The suspension was incubated at 37 °C and samples were drawn for measurements of haemoglobin, ATP, ^{45}Ca and ^{60}Co content. Specific details are provided with each experiment. When ATP-depleted cells were used, depletion was attained by preincubating the cells in media with 10 mM inosine and 6 mM iodoacetamide.

The composition of the stock solutions used for additions was as follows: A23187, 0.2 or 2 mM in absolute ethanol; Benz2 ester, 2 to 250 mM in DMSO; Quin2, 100 mM in DMSO; formaldehyde, original concentrated Analar solution, 38% w/v, diluted to 0.002–1 M with distilled water; ^{45}Ca and ^{60}Co (obtained from Amersham, U.K.), 20–200 mM solutions with specific activities in the 10^6–10^7 cpm per μmol range (approx. 1–10 μCi/μmol). Ethanol or DMSO were less than 1% in the final cell suspensions. Chelator loading of the cells was performed by adding the concentrated chelator to well stirred cell suspensions, followed by incubation at 37 °C, as indicated in each case.

The samples for ATP measurement were processed as follows. A volume of cell suspension, expected to render, after processing, a final ATP concentration in the 1–60 μM range, was mixed with ice-cold perchloric acid to a final concentrationof 0.6–1 M. After precipitating the protein, the perchloric acid was neutralized with K_2CO_3; the precipitate was then centrifuged and the neutralized supernatant compared with ATP standards by a luciferin-luciferase bioluminescence test, as described by Brown [10]. Neutralized supernatants from formaldehyde-poisoned cells containing little ATP (experiment of Table II) were added to ATP standards to explore possible effects of the poisoned cell extracts on the ATP assay. The readings of the standards did not change, indicating no interference by cell products with the bioluminescence test.

To measure the ^{45}Ca or ^{60}Co content of the cells, two different procedures were used. When the cell Ca content was much lower than that in the medium we used method 1. Samples of the cell suspensions (usually 50–200 μl) were diluted with, at least, 100 volumes of ice-cold wash medium containing Na-EGTA, 0.1 mM and albumin, about 1 g% (w/v), and then washed twice more in the same medium but without albumin. Trichloroacetic acid, 6%, was used to precipitate proteins and aliquots of the trichloroacetic acid supernatant were used for counting. In some experiments, when the cell associated radioactivity was comparable or higher than the concentration of radioactivity in the medium, the cells were separated by centrifugation through dibutyl-phthalate oil ($\delta = 1.042$) as described before [11,12] (method 2). The particular technique used is reported for each experiment. Endogenous cell Ca and Co, before experimental additions, were assumed to be negligible. The content, expressed in μmole per litre original cells (μmol/l cells), was calculated from the specific activity of Ca or Co, determined on samples of cell suspension, so that the quenching was similar to that of the cell samples.

In the experimental conditions used here, except in the experiment of Table II, red cell volume, as well as Na^+, K^+ and Mg^{2+} contents were expected to remain close to their original values despite large permeability changes induced by the ionophore A23187 and internal Ca^{2+} [11–13].

The original volume of cells was estimated from haemoglobin measurements. Haemoglobin was determined after lysis of samples from the cell suspensions in, at least, 100 volumes of distilled water. The absorbance at 540 nm was read and referred to a standard of 284 for packed cells.

Results

In the first part of the the Results we report the experiments that led to the discovery of the ATP-depleting effects of chelator esters and of formaldehyde. In the second part we characterize these in some detail. Many of the observations in the first part are marginal to the main point of this paper, namely the ATP-depleting effect of formaldehyde. They contain, however, important information on the use and applications of incorporated chelators in red cells.

The initial experiments of this series were designed to study Ca^{2+}-pump kinetics above physiological Ca^{2+} levels inside intact red cells, using incorporated calcium chelators as described by Lew et al. [4]. This required that the internal Ca^{2+} level in the pump-leak steady-state be increased. The simplest way to attempt increasing the internal Ca^{2+} level from its physiological value of 10–30 nM using normal Ca^{2+} permeation pathways is to increase external Ca^{2+}. As Fig. 1 shows,

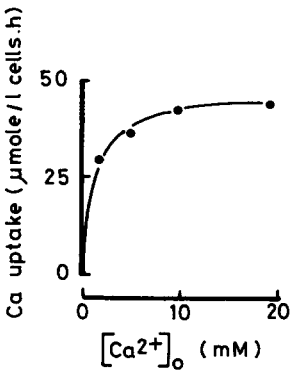

Fig. 1. Ca^{2+} influx as a function of the external Ca^{2+} concentration in Benz2-loaded intact red cells. Washed red cells were resuspended at about 30% haematocrit in solution A containing, in addition, 10 mM inosine and 0.1 mM EGTA. After addition of 1 mmol/l cells of Benz2 ester, the suspension was incubated for 90 min at 37 °C. After two washes with solution A, the cells were resuspended at 20% haematocrit in the same medium containing 10 mM inosine, the indicated external $^{45}Ca^{2+}$ concentrations and incubated at 37 °C. The Ca^{2+} content of cells was measured by method 1. Ca^{2+} uptake was linear during the first hour (samples at 1, 5, 10, 20, 40 and 60 min). The increase in tonicity and ionic strength caused by $CaCl_2$ addition was not compensated. The intracellular concentration of Benz2 was estimated by the method of Lew et al. [4] at 233 μmol/l cells.

however, Ca^{2+} uptake by chelator-loaded, inosine fed cells, saturated as external Ca^{2+} was increased. The $K_{1/2}$ was about 0.8 mM, similar to that described for ATP-depleted, chelator-free red cells [14]. Saturation of Ca^{2+} influx, which was also seen in neurons of *Helix aspersa* [15], does not allow experimental control of internal Ca^{2+} levels above normal simply by manipulating external Ca^{2+}. An alternative way of increasing $[Ca^{2+}]_i$ is to raise artificially the effective Ca^{2+} permeability of the cell membrane with the use of the divalent cation ionophore A23187 [12,16,17]. Varying the ionophore concentration allows precise control of the Ca^{2+} permeability over a wide range. This is well characterized for human red cells [17,18]. Rapid removal of the ionophore from the membrane can be achieved using albumin [19,20] making the permeability change to Ca^{2+} easily reversible.

In the experiment of Fig. 2, we used a low concentration of the ionophore A23187, which was unable, per se, to increase significantly the Ca^{2+} content of chelator-free, inosine-fed cells. The surprising result here was that the Ca^{2+} content of chelator-loaded cells increased to a level which did not change appreciably on further addition of a high concentration of the ionophore. This meant that Ca^{2+} distribution across the red cell membrane and virtually reached equilibrium at the low ionophore concentration in chelator loaded cells, in contrast with the behaviour of the chelator-free controls, suggesting that the Ca^{2+} pump had been inhibited.

Addition of excess EGTA caused further Ca^{2+} redistribution. The Ca^{2+} content of the control cells fell to below measurable levels whereas chelator-containing cells retained a relatively large amount of Ca^{2+}. Using the apparent dissociation constants for Ca^{2+} of EGTA and of the internal chelator under these experimental conditions we could estimate the incorporated chelator at about 930 μmol/l cells (see legend of Fig. 2).

We can see that the difference between the levels of Ca^{2+} in chelator-containing and chelator-free cells was lower than the chelator content of the cells measured after EGTA addition, as indicated in the legend of Fig. 2. Whenever such comparison was made, the same discrepancy was observed. One reason for this is that the usual

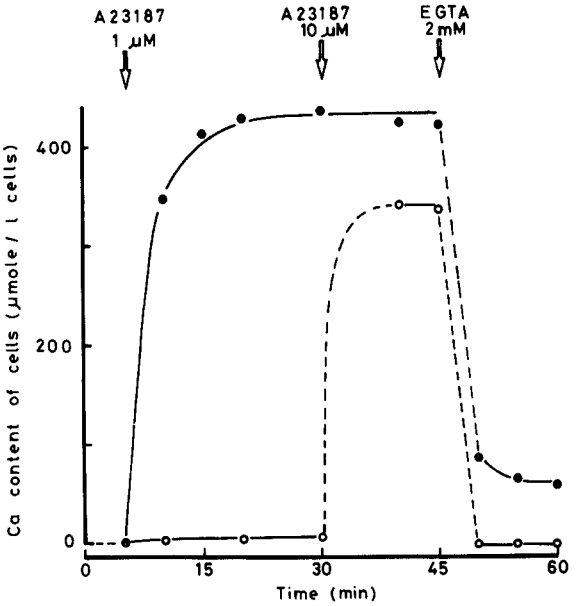

Fig. 2. Effect of low and high concentrations of ionophore A23187 on the Ca^{2+} uptake by Benz2-loaded intact red cells. Cells were preloaded with Benz2 as described for Fig. 1. (●), Benz2-loaded cells. Benz2-free controls (○) were incubated with an equal volume of DMSO alone. Final suspension at 20% haematocrit in solution A with 130 μM $^{45}CaCl_2$, and 10 mM inosine. Ionophore A23187 and EGTA were added as shown in the figure. Ca^{2+} content was measured by Method 2. The Benz2 content of the cells was estimated from the residual Ca^{2+} retained within the Benz2-loaded cells after EGTA addition using the equation:

$$B = Ca_i^T\left(K_B\left(\left(E - Ca_o^T\right)/r^2 K_E Ca_o^T\right)+1\right)$$

where B is the intracellular concentration of Benz2 (in μmol/l cells); E is the total extracellular concentration of EGTA (in mM); K_B and K_E are the apparent dissociation constants of Benz2 ($K_B = 50$ nM; [4]) and of EGTA ($K_E = 40$ nM) in the present experimental conditions; Ca_o^T is the total external Ca^{2+} concentration (in mM); $r^2 = ([H^+]_i/[H^+]_o)^2$ (see Ref. 12), and Ca_i^T is the total Ca^{2+} content (in μmol/l cells). This equation is easily derived by solving for B the three equations describing the equilibrium distribution of Ca^{2+} after EGTA addition to the medium. In red cells, internal Ca^{2+} binding other than to Benz2, when the intracellular Ca^{2+} concentration is below 200 nM, is negligible [4]. The three equilibrium equations are:

$$[Ca^{2+}]_i/[Ca^{2+}]_o = ([H^+]_i/[H^+]_o)^2; \quad (1)$$

$$Ca_i^T = B[Ca^{2+}]_i/\left(K_B + [Ca^{2+}]_i\right); \quad (2)$$

$$Ca_o^T = E[Ca^{2+}]_o/\left(K_E + [Ca^{2+}]_o\right). \quad (3)$$

Eqns. 2 and 3 apply when $B \gg Ca_i^T$ and $E \gg Ca_o^T$, as in this experiment after EGTA addition. r^2 was calculated from the pH values measured in the suspension, $[H^+]_o$, and after disrupting densely packed cell pellets by freezing and thawing, $[H^+]_i$.

lysis observed in stirred red cell suspensions at 37 °C in vitro, together with that additionally caused by the ionophore A23187 (see below), releases to the medium the chelator originally contained within the lysed cells. The fraction of ionized Ca^{2+} in the medium is therefore lower in the suspension containing chelator-loaded cells than in that of chelator-free cells and this effect may be substantial in low-Ca^{2+} media at high haematocrits, as here. The fraction of the internal Ca^{2+} not bound to chelator inside Benz2-loaded cells is then at equilibrium with a lower external Ca^{2+} concentration than that which exists in the suspension containing chelator-free cells. This invalidates the comparison of Ca^{2+} levels as an estimate of the free chelator content of the cells. The possible size of this effect was measured in the experiment of Fig. 5, of similar design to that of Fig. 2. At least 6% of the cells had haemolysed by the time Ca^{2+} equilibrium was reached (see Fig. 5C). The equilibrium ratio Ca_i^T/Ca_o^T in the controls was about 5.6. If all the Benz2 added as ester had been incorporated into the cells giving a uniform internal concentration of 1 mmol/l cells, its external concentration could have been as high as 25 μM, reducing the external ionized Ca^{2+} from 120 to about 95 μM (Fig. 5a). The total internal Ca at equilibrium with this level would have been about $95 \times 5.6 \cong 530$ μmol/l cells in this experiment. This gives a 'real' difference of about 500 μmol/l cells attributable to Benz2, a value still below the lower estimates of about 630 μmol/l cells obtained after EGTA addition by the method given in the legend of Fig. 2. The reason for this discrepancy is not clear. Tsien [1,7] observed a delayed activation of the Ca^{2+}-sensitive K^+ channel in Benz2-loaded red cells exposed to Ca^{2+} and the ionophore A23187. The delay was interpreted as reflecting the presence of Benz2 in all the cells. This is a valid argument but it does not rule out some heterogeneity in the distribution of the chelator. If the propensity to lyse affected preferentially the cells with a higher chelator content, this could account for the apparent discrepancies observed here. This interpretation, however, is not supported strongly by the measurements of the Benz2 content of cells after EGTA addition. If the cels with higher Benz2 had lysed already at the time of EGTA addition, the mean Benz2 content of the

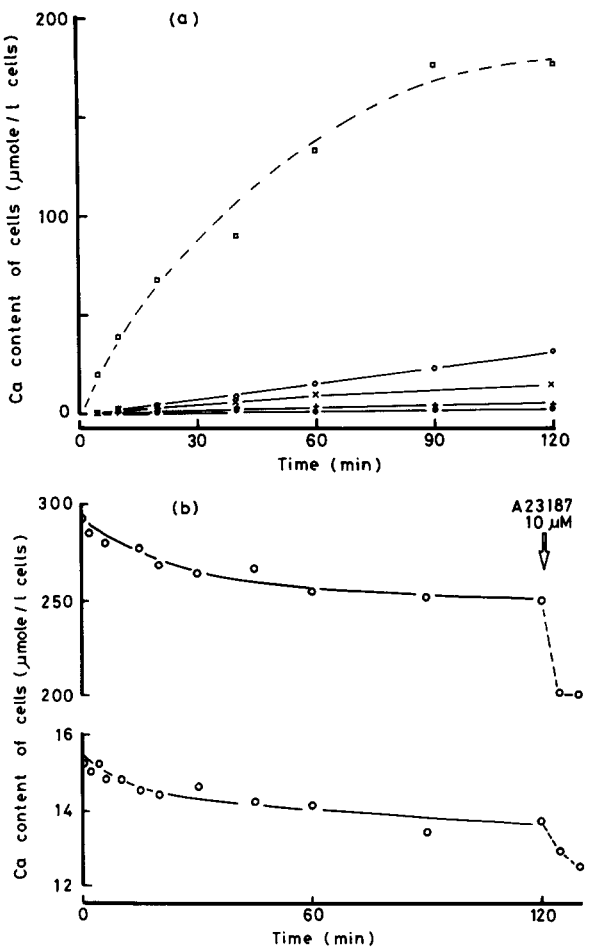

Fig. 3. (a) Ca^{2+} uptake by intact red cells during Benz2-loading. Red cells were washed and resuspended at 20% haematocrit in medium A with 10 mM inosine and 0.1 mM EGTA. To an aliquot of this suspension, 6 mM iodoacetamide was added to deplete the cells from ATP and both suspensions were incubated for 2 h at 37 °C. After this the cells were washed twice and resuspended at 20% haematocrit in Medium A, with 0.5 mM $^{45}CaCl_2$ and 10 mM inosine. The suspensions were prewarmed at 37 °C for about 10 min before addition of Benz2 (1 mmol/l cells) or DMSO, at $t = 0$. When present, the concentration of A23187 in the suspension was 0.2 μM. Conditions in suspension represented by the following symbols: (●) fed cells, controls; (●) fed cells + A23187; (+) ATP-depleted cells, controls; (×) ATP-depleted cells + Benz2; (○) fed cells + Benz2; (□) fed cells + A23187 + Benz2. Ca^{2+} content was measured using method 1. The shared symbol (●) represents experimental overlap.

(b) Ca^{2+} efflux from Benz2 and $^{45}Ca^{2+}$-loaded cells. The cells were treated in the same way as the fed cells + Benz2 (symbol (○) of Fig. 3a) except that in the high Ca^{2+} cells, the ionophore A23187 was added for 10 min only before the end of the incubation, at 120 min. After this time, external Ca^{2+}, and ionophore, were washed away and the cells resuspended in the same medium but with 0.1 mM EGTA instead of $CaCl_2$. Ca^{2+} release from the cells was followed for a further 2 h after which 10 μM A23187 was added. Cell Ca^{2+} was measured with method 1.

remaining cells should have been systematically lower. Despite the uncertainties of the method, which result from the variability in K_B [4], there is no clear indication that Benz2 content, measured after EGTA, was usually lower than what could have been incorporated maximally.

Ca^{2+}-pump inhibition was documented more precisely in the experiments of Figs. 3 and 4. Fig. 3a reports the way Ca^{2+} is taken up during Benz2-loading of inosine-fed or ATP-depleted cells, in the presence of 0.5 mM $CaCl_2$.

It can be seen (Figs. 3a and 4a) that Benz2-loading increases the Ca^{2+} uptake by inosine-fed and ATP-depleted cells, relative to controls. In the presence of Benz2 ester, inosine-fed cells take up Ca^{2+} about twice as fast as depleted cells. This represents either an inhibitory effect of iodoacetamide on the passive Ca^{2+} influx or a genuine metabolism-dependent component of passive Ca^{+} entry, as suggested before [4]. Addition of a very low ionophore (A23187) concentration, unable to cause a measurable rise in the Ca^{2+} content of control cells, induced a 20-fold increase of the Ca^{2+} entry rate in the presence of Benz2 ester. The surprising effect here was that Ca^{2+} uptake was maximal (240, and 360–550 μmol/l cells in the experiments of Figs. 3a and 4a, respectively) at the moment of chelator-ester addition, without detectable delays. If the internal ionized Ca^{2+} level remained not far from normal, as presumably happened in the ionophore-treated controls, the net Ca^{2+} gain must represent Ca^{2+} binding to rapidly incorporated free chelator. Alternatively, chelator addition may cause rapid pump inhibition. If this were the case, Ca^{2+} distribution would proceed towards equilibrium at a rate determined by the ionophore concentration and will not be revealing the kinetics of incorporation of free chelator. The Ca^{2+}-efflux measurement of Fig. 3b, obtained after washing the ionophore away, strongly suggest that the Ca^{2+} pump became inhibited during the chelator-loading period. Addition of ionophore at the end of the efflux period

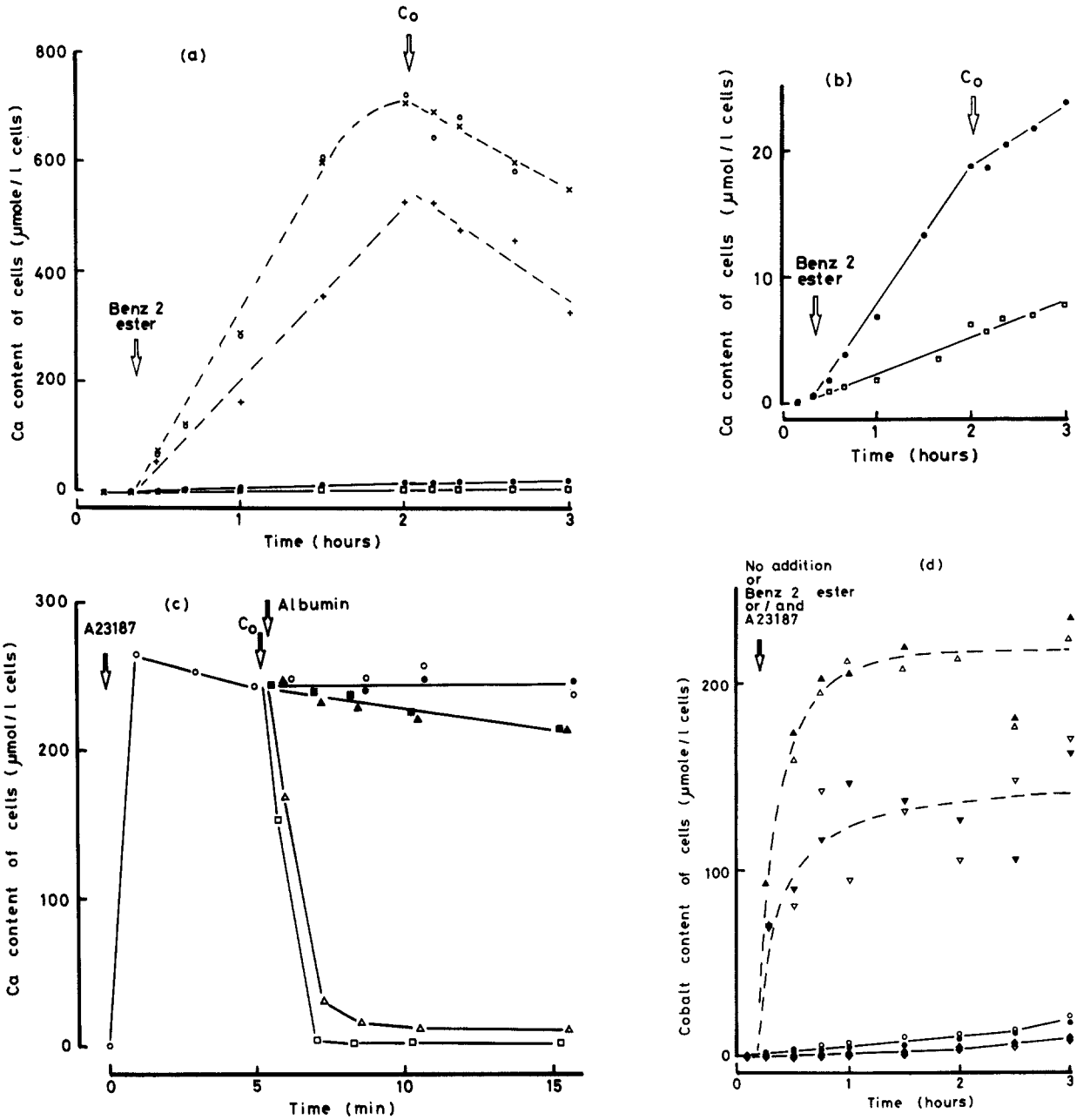

Fig. 4. (a and b). Effect of Benz2, A23187 and cobalt on the Ca^{2+} uptake by inosine-fed intact human red cells. To cells pretreated as the fed cells in Fig. 3a, A23187 and $CaCl_2$ were added at $t = 0$ as follows: (●) controls without A23187; (+) 0.2 μM A23187; (×) 0.3 μM A23187; (○) 0.4 μM A23187; (□) 0.4 μM A23187 + 200 μM $CoCl_2$. The suspensions were incubated at 37 °C with the indicated further additions as follows: 1 mM Benz2, 200 μM $CoCl_2$. Cell Ca^{2+} was measured using method 1.

Fig. 4b shows the conditions represented by symbols (●) and (□) of Fig. 4a drawn on an expanded ordinate scale.

(c) The effect of cobalt on the Ca^{2+}-pump-mediated Ca^{2+} extrusion in intact cells. The experiment was designed to explore the effect of cobalt on the ATP-dependent release of Ca^{2+} from cells preequilibrated with Ca^{2+} for 5 min in the presence of a high ionophore (A23187) concentration. Fresh red cells were resuspended at 10% haematocrit in solution A containing, in addition, 50 μM $^{45}CaCl_2$ and 10 mM inosine. The suspension was divided in two lots: one was preincubated for 2 h at 37 °C with 250 μM formaldehyde to deplete the cells from ATP (the ATP-depleting effect of formaldehyde is similar to that of iodoacetamide, as discussed in the text; the

revealed that the internal ionized Ca^{2+} level was high in the ionophore-pulsed cells, and yet Ca^{2+} efflux was extremely low.

The extent of pump-inhibition is better seen in the experiment of Fig. 4. The experimental protocol here was to preincubate cells with increasing ionophore concentrations and then add the chelator ester. If the ester caused immediate pump-inhibition, Ca^{2+} influx should have increased with ionophore concentration. If, on the other hand, the initial Ca^{2+} gain reflected the rate of free-chelator incorporation, and the pump is not immediately inhibited, Ca^{2+} uptake should have remained similar at ionophore concentrations above a limiting value. The results (Fig. 4a) indicate that this is indeed the case and that there is no initial pump inhibition.

In order to explore late Ca^{2+}-pump inhibition, we used cobalt ions. Cobalt ions effectively block all movement of calcium through the ionophore but have no significant effect on Ca^{2+}-pump-mediated fluxes, as evidenced by the rapid, ATP-dependent uphill Ca^{2+} extrusion which followed Co^{2+} addition to fed cells preequilibrated with Ca^{2+} at a high ionophore concentration (Fig. 4c). In the experiment of Fig. 4b the presence of Co^{2+} not only blocked completely Ca^{2+} entry at the highest ionophore concentration added, it also inhibited passive Ca^{2+} entry by about 50%. Besides blocking Ca^{2+} transport other than through the Ca^{2+} pump, Co^{2+} is itself rapidly transported by A23187, and this transport is unaffected by Ca^{2+} (Fig. 4d). Co^{2+} also binds to internal buffers (Fig. 5b) and may be able to compete and displace Mg^{2+} and Ca^{2+}. Comparison of ionophore-induced steady-state gradients of Co^{2+} in fed (Fig. 4d) and ATP-depleted cells (Fig. 5b) suggests also that Co^{2+} may be transported weakly by the Ca^{2+} pump. A more detailed study of binding and transport of Co^{2+} is outside the scope of this paper. What is relevant to role out is Ca^{2+}/Co^{2+} counterransport through A23187 or other pathways. The fact that addition of Co^{2+} to ATP-depleted cells in the experiment of Fig. 4c failed to induce Ca^{2-} release, together with the lack of effect on Ca^{2+} influx of doubling the external Co^{2+} concentration in the experiment of Fig. 4b make Ca^{2+}-Co^{2+} exchange implausible. Co^{2+} is therefore a useful tool to expose Ca^{2+}-pump fluxes in ionophore treated cells, and this was used in the experiment of Fig. 4a to investigate whether the Ca^{2+} pump had been inhibited after 2h with Benz2 ester. Addition of Co^{2+} revealed Ca^{2+} extrusion rates of only about 0.2 mmol/l cells. The Ca^{2+} pump, therefore, had become substantially inhibited after Benz2 incorporation.

Delayed Ca^{2+}-pump inhibition suggested that perhaps the mechanism of inhibition was indirect. We first explored the possibility that chelator incorporation somehow caused metabolic inhibition and ATP depletion, thus reducing the fuel to the Ca^{2+} pump.

During chelator incorporation, four molecules of formaldehyde evolve from each molecule of fully hydrolysed chelator ester [1,7]. It was therefore important to test the effect of formaldehyde as well as of Benz2 ester. Since formaldehyde is also released by other chelator esters, like Quin2 ester [1,7], if formaldehyde was involved in the process causing late Ca^{2+}-pump inhibition, such inhibition shoul be observed with all chelator esters prepared with the same acetoxymethyl ester.

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other was incubated for 15 min at 37 °C. At $t = 0$, 10 μM A23187 was added to each suspension and, after 5 min, to allow for Ca^{2+} to equilibrate, aliquots were transferred to vials containing concentrated $CoCl_2$ and albumin to give final concentrations of 200 μM and 1.6% (w/v), respectively. 100-μl samples were taken at the indicated times and processed by method 2. (○) controls, fed, (●) controls, depleted; (□) cobalt, fed; (■) cobalt, depleted; (△) albumin, fed; (▲) albumin, depleted. It can be seen that only fed cells extrude Ca^{2+} against the gradient, after albumin or cobalt addition. Albumin acts by removing ionophore [23], and reveals a pumping rate of 9.6 mmol/l cells. Co^{2+} blocks Ca^{2+} movement through the ionophore, probably displaces Ca^{2+} from common binding sites and is itself transported by the ionophore (see also Figs. 4a, 4b, 4d and 5b). What this experiment shows is that Co^{2+} does not interfere to a measurable extent with Ca^{2+} transport by the Ca^{2+} pump. The pumping rate in the presence of Co^{2+} was 10 mmol/l cells.

(d) Effect of Benz2, A23187 and $CaCl_2$ on the cobalt uptake by inosine-fed intact red cells. Washed red cells were resuspended in medium A with 10 mM inosine and $^{60}CoCl_2$, 100 μM and divided in two groups: Controls (open symbols) and +200 μM $CaCl_2$ (dark symbols). After 10 min incubation at 37 °C, Benz2 (0.75 mM) and A23187 (0.5 μM) were added as follows: (◇,◆) controls, no additions; (○,●) Benz2; (▽,▼) A23187, (△,▲) Benz2 + A23187. Co^{2+} in cells was measured by method 1. We have no technical explanation for the scatter observed in the presence of 0.5 μM A23187. Genuine oscillatory behaviour cannot be ruled out.

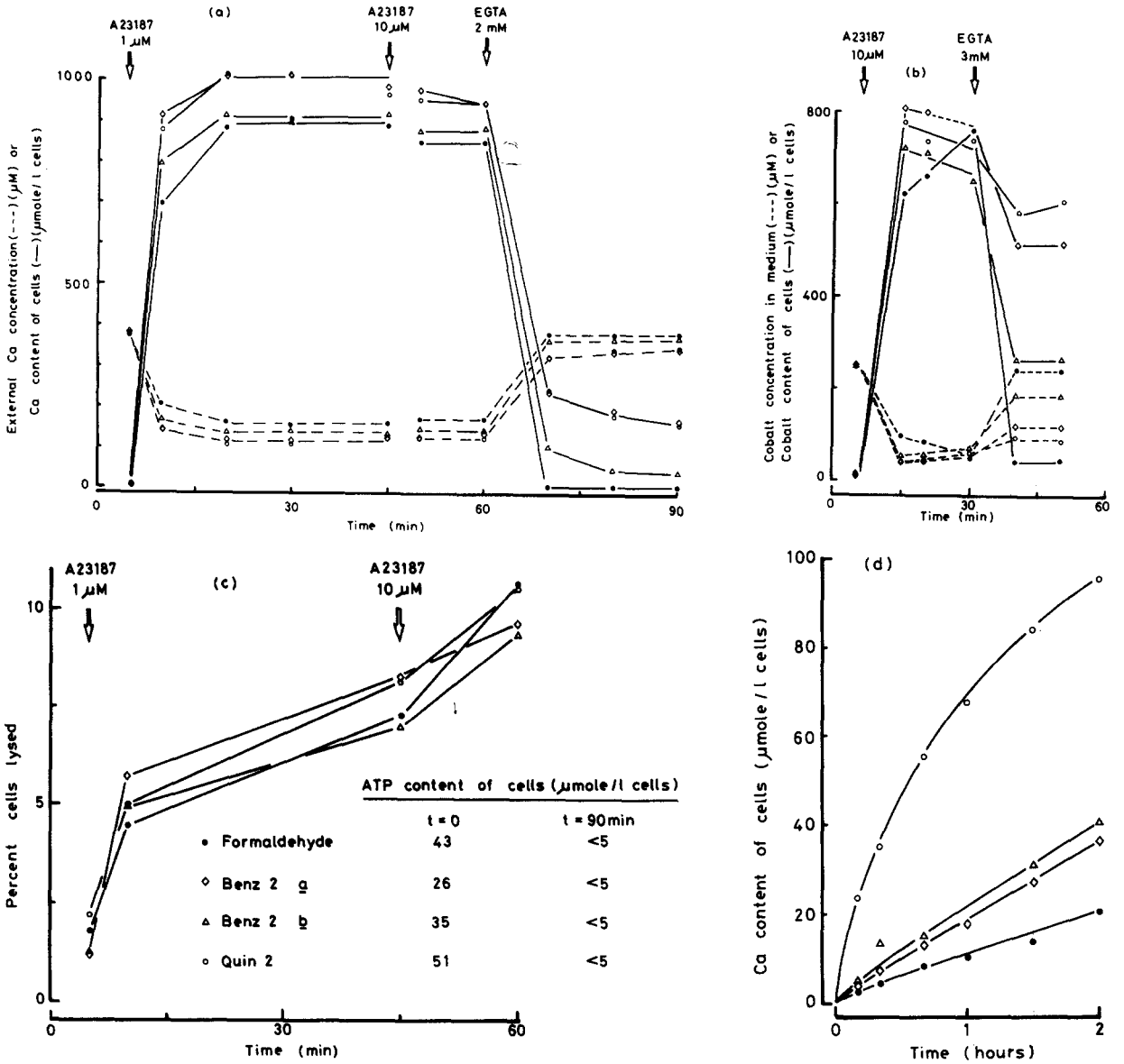

Fig. 5. (a) Effect of low and high ionophore A23187 concentrations on the Ca^{2+} uptake by inosine-fed cells pretreated with formaldehyde, Benz2 ester of two different sources (*a*, prepared by R.Y. Tsien; *b*, prepared by Calbiochem, CA, U.S.A.), and Quin2 ester. Cells were loaded with the esters of Benz2 *a* (◇), Benz2 *b* (△) and Quin2 (○), all at 1 mmol/l cells, as described in Fig. 1. Controls with 4 mM formaldehyde (●). After formaldehyde treatment or chelator loading, the cells were washed twice and resuspended at 20% haematocrit in solution A with 10 mM inosine. At the beginning of the final incubation at 37 °C 0.3 mM $^{45}CaCl_2$ was added to all suspensions. Dashed lines: Ca^{2+} concentration in medium. Additions of A23187 and EGTA, as indicated on figure. Cell Ca^{2+} was measured with method 2. The fraction of ionized Ca^{2+} (α_{Ca}) in chelator-free cells was calculated from the equilibrium distribution of Ca^{2+} as described before [11,12]. α_{Ca} was about 0.2. The chelator content of the cells was calculated from the Ca^{2+} distribution after EGTA addition, as detailed in the legend of Fig. 2. Benz2 *a* content was 1.0 mmol/l cells; Benz 2 *b* was 0.46 mmol/l cells, and Quin2 was 0.94 mmol/l cells. The apparent Ca^{2+} dissociation constant of Quin2 at an estimated intracellular Mg^{2+} level of 0.4 mM, was calculated at about 100 nM.

(b) Ionophore-induced cobalt distribution in formaldehyde-treated and chelator-loaded intact red cells. The protocol and symbols correspond to the conditions described in Fig. 5a, except that 200 μM of $^{60}CoCl_2$ instead of $CaCl_2$ were added at $t = 0$. The fraction of ionized Co^{2+} (α_{Co}) in chelator-free cells was calculated as for Ca^{2+}. This is at best a crude estimate, since the complete

We therefore measured the effects of formaldehyde, of two different sources of Benz2 ester and of Quin2 ester on active and passivle Ca^{2+} and Co^{2+} transport, on the equilibrium distributions of these ions and on the ATP content of the cells. The results are reported in Fig. 5. Previous work [4] had shown that neither DMSO nor formaldehyde had any effect on the Ca^{2+} fluxes in glucose-fed intact red cells loaded with about 100 μmol/l cells of Benz2. Cell ATP was not measured in these experiments, but Ca^{2+} efflux from chelator-loaded cells was found to be fully inhibited only in the cells that had been preincubated with iodoacetamide and inosine, a treatment known to reduce ATP levels to about 1 μM [8]. In the presence experiments, the concentrations of chelator ester used were higher, and inosine, instead of glucose, was used as substrate. It was therefore important to test the effects of formaldehyde and chelator esters under the present experimental conditions, where Ca^{2+}-pump inhibition was observed in supposedly fed cells.

It can be seen (Fig. 5c) that fresh, inosine-fed red cells, preincubated for 90 min with 4 mM formaldehyde or with 1 mM of chelator esters of Benz2 or Quin2, have, at the start of the final incubation, very reduced ATP levels, sufficiently low to explain the failure of the Ca^{2+} pump to balance the leak fluxes induced by the ionophore, even at the very low initial concentration used (Fig. 5a). ATP depletion appears to be irreversible, as it progressed even further during the second incubation after having washed the cells and without further additions of formaldehyde or chelator esters. These results strongly suggest that formaldehyde causes ATP depletion in the presence of glycolytic substrate (inosine) without the need of Ca^{2+}, and that the ATP-depleting and Ca^{2+}-pump inhibitory effects of the chelator esters are mediated by the hydrolytic release of formaldehyde. The experimental results also show (a) that passive Ca^{2+} influx (Fig. 5d) is within values found before [4] for cells pretreated with formaldehyde and Benz2, but 10-fold higher in Quin2-loaded cells; (b) that chelator-free cells bind Co^{2+} stronger than Ca^{2+}; an amount equivalent to about 40 μmol/l cells appears to be particularly tightly bound (Fig. 5b), and (c) that the incorporated chelators bind Co^{2+} more tightly than Ca^{2+} and, at least, ten times more tightly than EGTA (see legend of Fig. 5b). Fig. 5c shows the haemolysis pattern during the final incubation. Addition of a low concentration of A23187 caused sudden lysis of about 3% of the cells which then progressed under the present conditions, at a rate of about 5% per hour. The lytic release of free chelator from preloaded cells precludes assessment of internal chelator levels from differences in equilibrium levels of internal Ca^{2+}, as discussed before.

The effect of formaldehyde on the ATP and Ca^{2+} content of inosine-fed red cells in the presence and absence of ionophore, is shown in the experiment of Fig. 6. It can be seen (a) that formaldehyde causes immediate ATP depletion with a half time of about 20 min in the controls without ionophore (Fig. 6a); (b) that the effect is saturated at a concentration of 0.5 mM in a 10% haematocrit cell suspension; (c) that only in the presence of formaldehyde does an increased ionophore-induced Ca^{2+} influx accelerate the rate of ATP depletion ($T_{1/2} = 10$–12 min); (d) that ionophore-induced Ca^{2+} influx (Fig. 6b) causes no significant increase in cell Ca^{2+} during the first 8 min, while ATP, though falling fast, is still above 0.6 mmol/l cells and (e) that as ATP falls further to less than 5% of its original value, Ca^{2+} reaches

Co^{2+}-buffering curve has not yet been characterized. The equilibrium level of Co^{2+} in the cells was about 760 μmol/l cells, from which 43 μmol/l cells of tightly bound Co^{2+} were subtracted. α_{Co} was calculated at about 0.1. From the Co distribution after EGTA addition, the tabulated values [24] of the Co^{2+} binding constants of EGTA and the estimated chelator contents of Fig. 5a, approximate values of the apparent Co^{2+} dissociation constants for the incorporated chelators could be estimated by solving the equation given in the legend of Fig. 2, for K_B instead of for B. The apparent dissociation constants of Co^{2+} for Benz2 *a*, Benz2 *b* and Quin2 under the present experimental conditions were (in nM), 0.03, 0.02 and 0.008, respectively. These values indicate that Benz2 and Quin2 bind Co at least three orders of magnitude tighter than they bind Ca^{2+}. These calculations do not consider Co^{3+} binding.

(c) Record of haemolysis and cell ATP contents during the Ca^{2+} uptake measurements reported in Fig. 5a. Haemoglobin in the supernatants was measured as described in Methods.

(d) Ca^{2+} uptake by formaldehyde-treated and chelator-loaded red cells. Aliquots of cells treated as described in Fig. 5a were incubated in medium A with 10 mM inosine. 1.5 mM $^{45}CaCl_2$ was added at $t = 0$. Ca^{2+} content was measured by method 1. No ionophore (A23187) additions. Symbols as in Fig. 5c.

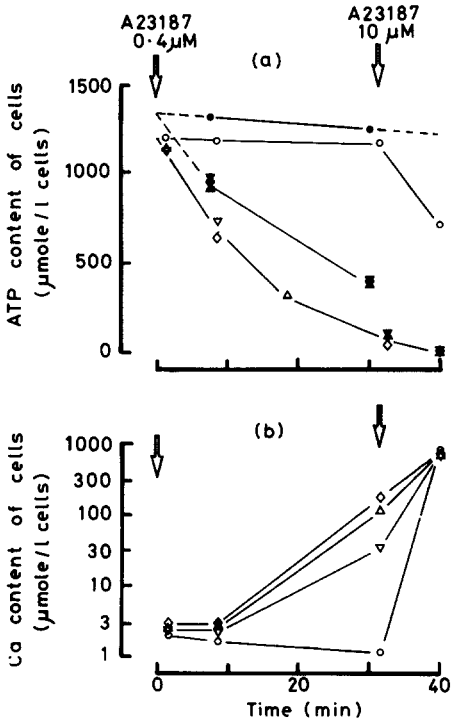

Fig. 6. Effect of formaldehyde on the ATP and Ca^{2+} content of inosine-fed red cells in the presence and absence of ionophore A23187. Washed red cells were resuspended at 10% haematocrit in solution A containing 180 μM $^{45}CaCl_2$ and 10 mM inosine. This suspension was divided it two sets of four equal aliquots; (○,●) controls, without formaldehyde; (△,▲)+ 0.5 mM formaldehyde; (▽,▼)+1 mM formaldehyde; (◇,◆)+ 1.5 mM formaldehyde. After 5 min preincubation at 37 °C, 0.4 μM of ionophore A23187 was added only to those conditions represented by open symbols. High ionophore (10 μM) was added to all conditions after the 30 min sample.
(a) ATP content of cells. (b) Ca^{2+} content of ionophore-treated cells. The Ca^{2+} content of cells in the absence of ionophore remained below measurable levels. Note logarithmic scale of ordinate.

its equilibrium distribution as measured in the high-ionophore controls. This means that (1) formaldehyde only causes appreciable Ca^{2+}-pump inhibition after the ATP levels are reduced, and that (2) hydrolysis of ATP by the pump, at low ionophore concentrations, contributes to deplete the cells from ATP only in the presence of formaldehyde. These results support the notion that Ca^{2+}-pump inhibition observed after chelator ester addition is due to formaldehyde released during the hydrolytic incorporation of the chelator and that it is secondary to ATP depletion caused by formaldehyde.

The experimental results in Table I show that Benz2 ester causes ATP depletion with glucose or inosine as substrates. With inosine, however, the ATP levels after one hour are about 5-times lower than with glucose, whatever the haematocrit and Benz2 ester-addition conditions. This is reminiscent of the accelerating effect of substrates on the iodoacetamide induced ATP-depletion [8,9] and suggests that perhaps formaldehyde acts via a similar mechanism as iodoacetamide. The experiment of Table II shows again the accelerating

TABLE I

EFFECT OF SUBSTRATE AND HAEMATOCRIT ON THE ATP CONTENT OF CELLS 1 h AFTER Benz2 ESTER ADDITION (1 MMOL/l CELLS)

Washed cells were suspended in ice-coled solution A containing the substrates shown and at the indicated haematocrits. The suspensions were divided in two equal lots. To one Benz2 ester was added at 0 °C, before the start of the incubation at 37 °C. To the other it was added after 10 min at 37 °C.

Substrate	Haematocrit	Cell ATP after 1 h at 37 °C (μmol/l cells)				
		Before Benz2 ester addition (control)	Benz2 ester added at 0 °C	% of controls	Benz2 ester added at 37 °C	% of controls
Glucose	33	1490	575	39	559	38
Inosine	41	1174	98	9	76	6
Glucose	13	1116	509	46	559	50
Inosine	13	880	108	12	94	11

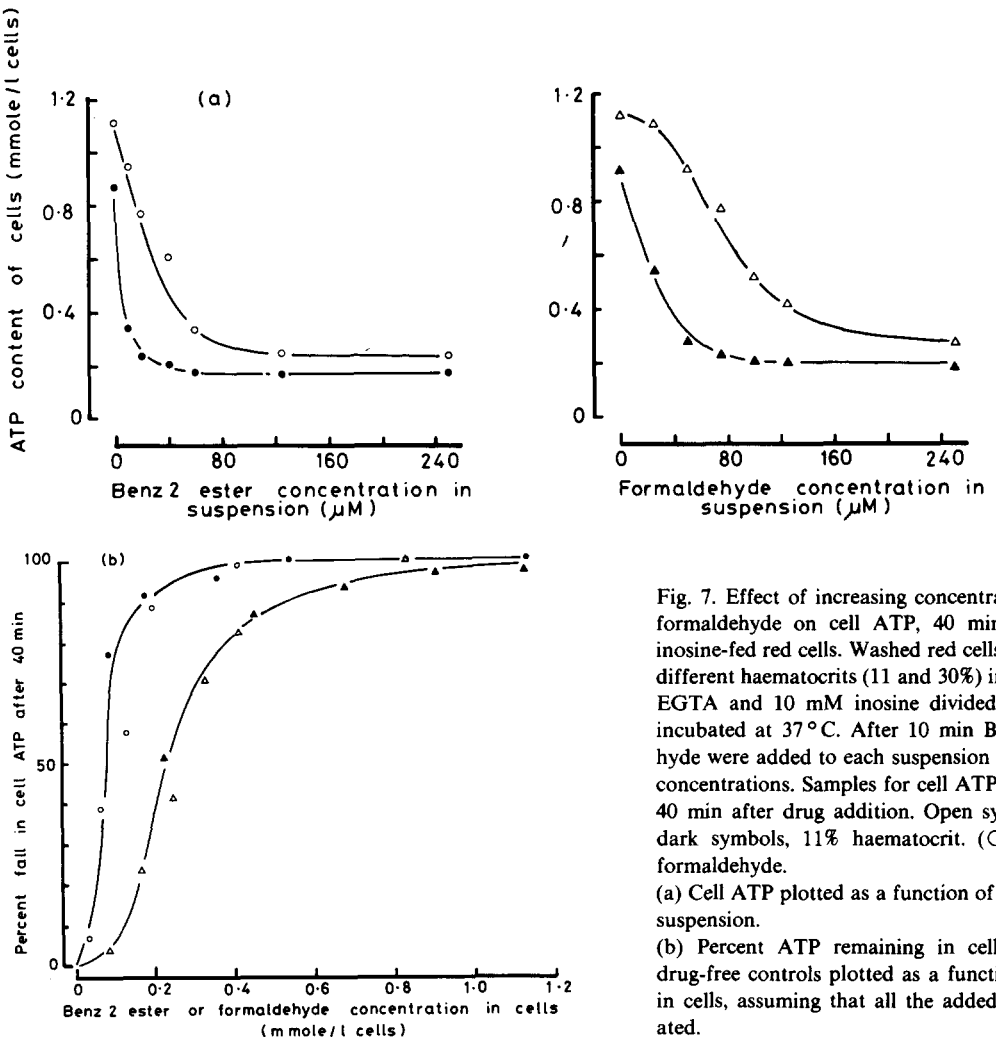

Fig. 7. Effect of increasing concentrations of Benz2 ester and formaldehyde on cell ATP, 40 min after drug addition to inosine-fed red cells. Washed red cells were resuspended at two different haematocrits (11 and 30%) in medium A with 0.1 mM EGTA and 10 mM inosine divided in seven equal lots and incubated at 37 °C. After 10 min Benz2 ester and formaldehyde were added to each suspension to give the indicated final concentrations. Samples for cell ATP measurement were taken 40 min after drug addition. Open symbols, 30% haematocrit; dark symbols, 11% haematocrit. (○,●), Benz2 ester; (△,▲), formaldehyde.

(a) Cell ATP plotted as a function of drug concentration in the suspension.

(b) Percent ATP remaining in cells at 40 min relative to drug-free controls plotted as a function of drug concentration in cells, assuming that all the added drug became cell-associated.

effect of inosine on the rate of ATP depletion induced by Benz2, but now relative to substrate-free controls. The experiment also shows that depletion is irreversible and that the concentrations of Na^+, K^+, Hepes or Tris in the isotonic media have no detectable effects.

The results in Fig. 7 report the concentration-dependence of the ATP-depleting effects of formaldehyde and Benz2 on inosine-fed fresh red cells at two different haematocrits (11 and 30%).

Substrate (inosine) and incubation time (40 min) were chosen so as to minimize errors and maximize depletion of ATP. It can be seen that the depleting effects of Benz2 and formaldehyde, plotted as a function of concentration in the suspension (Fig. 7a) appear dependent on the proportion of cells. Plotted as a function of the concentration per unit cell volume and normalized (Fig. 7b), the experimental points fall reasonably well within single curves for both Benz2 ester and formaldehyde. This suggests that most of the formaldehyde added partitions and stays bound to the cells. Half maximal depletion is obtained with about 90 μmol/l cells of Benz2 ester and with 240 μmol/l cells of formaldehyde. The higher inhibitory affinity for Benz2 ester is to be expected if formaldehyde is the inhibitor. Gradual hydrolysis of Benz2 ester as well as impurities in the starting

TABLE II

CELL ATP BEFORE AND AFTER Benz2 ESTER ADDITION UNDER VARIOUS EXPERIMENTAL CONDITIONS

Washed red cells were divided in two lots and resuspended at 10% haematocrit (a) in medium A with 10 mM inosine and 0.1 mM EGTA, or (b) in a low-K medium containing 145 mM NaCl, 5 mM KCl, 10 mM Hepes-Na (pH 7.5) and 0.1 mM EGTA. This last suspension was divided in two equal portions and 10 mM inosine added to one of them. All suspensions were incubated at 37 °C for 30 min before Benz2 ester (1 mM) addition. One hour after Benz2 ester addition, all cells were washed, resuspended (10% haematocrit) in the same solutions as before (but without Benz2 ester) and incubated for further 2 h at 37 °C.

Condition	Cell ATP (μmol/l cells)		
	Before Benz2 ester	1 h after Benz2 ester	2 h after end of Benz2 loading
High-K medium + inosine, 10 mM	1160	116	10
Low-K medium without substrate	487	344	30
Low-K medium + inosine, 10 mM	861	136	10

material may well account for an observed affinity ratio (240/90) of less than 4. Precise kinetic analysis of the inhibition curves is not justified because the ATP level at forty minutes is a complex parameter whose relation to the primary inhibitory event is unknown. It is important to note, however, that even if the concentration-dependence of the depleting effect were similar under all conditions, the extent of ATP depletion at any particular time may vary a great deal depending on the presence and nature of the substrate and on whether the Ca^{2+} pump is causing or not additional ATP hydrolysis.

Discussion

The results reported here show that formaldehyde causes rapid ATP depletion in intact human red cells. Most of the formaldehyde added to cell suspensions becomes cell-associated and half maximal depletion is obtained with a concentration of 0.24 mmol/l cells. Concentrations of up to 20 mmol/l cells cause no measurable effects on passive Ca^{2+} fluxes, as seen before [4]. Formaldehyde, Benz2 and Quin2 esters all cause ATP-depletion and, consequently, Ca^{2+}-pump inhibition. The higher depleting affinity of Benz2 ester relative to formaldehyde suggests that the chelator ester effects are mediated by formaldehyde released during hydrolytic incorporation of the free chelator. No additional hypotheses are necessary to explain the present observations. Direct effects of the chelator ester or of the free chelator, however, cannot be ruled out yet.

The mechanism by which formaldehyde causes irreversible ATP depletion is unknown, but similarities with the depletion patterns observed with iodoacetamide [8,9] suggest that the enzyme glyceraldehydephosphate dehydrogenase may be the specific target of formaldehyde. As with iodoacetamide, the rate of ATP depletion, is accelerated in the presence of glucose relative to substrate-free controls and, even more, in the presence of inosine. The increasing depleting efficiency parallels the relative feeding efficiency of these substrates in human red cells. This suggests that ATP consumption by the substrates in the early steps of their glycolytic metabolism proceeds unimpaired whereas ATP production is fully and irreversibly blocked. In a preliminary experiment designed to investigate whether exogenous glyceraldehydephosphate dehydrogenase (from yeast and from muscle; obtained from Sigma, London) could restore the ATP levels in lysates from formaldehyde poisoned cells supplied with inosine and adenine, added glyceraldehydephosphate dehydrogenase succeeded in halting and reversing a spontaneous ATP declining trend observed in controls but had no effect on the ATP levels from poisoned cells. This is not yet evidence that enzymes other than glyceraldehydephosphate dehydrogenase must be inhibited since (a) we may not have used the right conditions for recovery, or (b) the new added enzyme may have also become poisoned by formaldehyde displaced from weakly adsorbing molecules. In other preliminary experiments, we investigated whether dithiothreitol (5 mM), 2 mercaptoethanol (5 mM) or urea (50 mM) had any protective effect on formaldehyde-induced ATP depletion, but with negative results.

Inhibition of glycolysis could affect ATP production in cells with oxidative metabolism depending on the relative importance of glucose to other endogenous substrates. Tsien et al. [3] found that the ATP levels of mouse thymocytes and splenocytes were reduced to within 80% of normal with Quin2 loads of up to 2–3 mM. Further falls in ATP, to 40% of normal, were obtained with Quin2 loads of 4–5 mM. More recently, Hesketh et al. [5] reported a steeper fall in the ATP content of thymocytes measured one hour after loading with [^{3}H]Quin2 and washing of the cells. With 1 mM internal Quin2, The ATP level was about 70% of normal. It is not clear whether differences in the time at which the samples were taken could explain the discrepancy between the two sets of data. The effect of formaldehyde alone was not investigated by this group. Shoback et al. [6] found ATP levels within $91 \pm 5\%$ of controls after loading dispersed bovine parathyroid cells with 0.7–0.8 mM of Quin2. In all these cells, the ATP-depleting effects of Quin2 ester were much weaker than those observed with the esters of Quin2 and Benz2 and with formaldehyde in human red cells.

The cells in which the physiological pump-leak and Ca^{2+}-pump-mediated Ca^{2+} extrusion were studied [4] had been loaded with about 100 μmol/l cells of Benz2 using glucose as substrate, and the fluxes measured in conditions in which we would now predict ATP levels in the 0.4–0.6 mmol/l cells range. Ca^{2+}-pump inhibition would then have been minimal. If there had been some inhibition of the Ca^{2+} pump, the physiological internal Ca^{2+} levels might be even lower than those reported at the time (10–30 nM range).

From the present results it is possible to outline some general rules and conditions for the safe use of incorporated chelators to study Ca^{2+} transport in red cells. It is clear that Benz2 and formaldehyde do not primarily affect Ca^{2+} fluxes. Benz2 is therefore, in principle, a safe tool to study passive and active Ca^{2+} transport. Quin2, on the other hand, seems to increase passive Ca^{2+} influx in red cells and may not be used safely until the nature of this effect is understood or better characterized. R.Y. Tsien (personal communication) suggested that since Quin2 binds Mg better than Benz2 does, the increased Ca^{2+} influx in Quin2-loaded cells may result from a hitherto unknown dependence of this flux on the internal Mg^{2+} level. This has not yet been investigated. Since Benz2 ester, even at the lowest concentrations required for useful loading, inhibits ATP production without impairing substrate consumption of ATP, it becomes necessary to omit substrates from the media during Benz2 loading and subsequent incubations, whenever the maintenance of high internal ATP levels is important. It may be convenient to pretreat the cells in ways which increase the initial ATP, like incubating them with inosine, adenosine or inosine + pyruvate + phosphate [21,22], a procedure known to raise the ATP levels far above normal. An important precaution would be to wash all substrates away before attempting Benz2 loading. Other than substrates, the Na^+ and Ca^{2+} pumps may contribute to ATP hydrolysis. At saturating external K^+ concentrations, ATP consumption by the Na^+ pump may become significant and the addition of ouabain of some advantage in certain experiments. ATP consumption by the Ca^{2+} pump may cause rapid depletion if internal Ca^{2+} is increased (using A23187) near saturation levels for the Ca^{2+} pump.

At physiological internal Ca^{2+} levels or below, the pump-leak turnover rate, as determined in Benz2-loaded cells, is about 50 μmol/l cells in plasma and about 30 μmol/l cells in plasma-like media [41]. This may allow 3–6 h of experimentation at 37 °C without meaningful ATP depletion. When higher than physiological Ca^{2+} levels are desired, minimal depletion may be achieved by a very brief treatment of the cells with appropriate concentrations of A23187 and Ca^{2+}, perhaps at a temperature lower than 37 °C. In any case, it is clear that measurements of cell ATP ought to accompany those of cell Ca^{2+} in all transport studies involving Ca^{2+}-pump-mediated fluxes when Benz2 ester is being used. The extent to which any such precautions may be needed in other cells ought to be established for each particular case; omission of glucose and use of pyruvate as substrate, for instance, may be generally adequate.

Finally, formaldehyde may be used experimentally to deplete red cells of ATP (as in the experiment of Fig. 4c) and as an alternative to iodoacetamide, in order to identify specific effects of these poisons, other than ATP depletion.

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