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Magnitude of calcium influx required to induce dehydration of normal human red cells

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Activation by $[Ca^{2+}]_i$ of Ca^{2+}-sensitive K^+ channels has long been known to cause dehydration of red cells suspended in low-K, plasma-like media. However, the fundamental question of the extent to which Ca influx must be increased to trigger dense cell formation in conditions likely to arise in the circulation has not been established. We report here that in ionophore permeabilized red cells, increasing Ca influx above 0.7 mmol/litre cells per h induces the formation of subpopulations of dehydrated cells within 1–2 hours. The presence or absence of glycolytic substrates had little effect suggesting that ATP depletion was not large enough to significantly inhibit the pump within that period. Below maximal dehydrating Ca influxes of about 1.2 mmol/litre cells per h, the trend was for the fraction of dense cells formed to remain steady in time. As Ca influx was increased, both the rate of dense cell formation and the fraction of dense cells formed increased. These results are analyzed in relation to mechanisms and to possible states of increased Ca^{2+} permeability in physiological and physiopathological conditions.

Introduction

Ca^{2+} permeabilization of red cells in vivo has been suggested to occur under normal and abnormal conditions, but was only convincingly demonstrated under certain abnormal conditions [1,2]. Shear stress in the microcirculation, and the process of normal red cell senescence were claimed to occur with states of increased Ca^{2+} permeability [3–6]. In sickle cell anaemia, the elevated red cell $[Ca^T]_i$ [7,8], mostly accumulated by inward transporting Ca^{2+} pumps within endocytic vesicles [9–11], documents past episodes of intracirculatory Ca^{2+} permeabilization with transient increases in $[Ca^{2+}]_i$. More recent evidence suggests that these transient $[Ca^{2+}]_i$ increases may also activate the K^+ channels of some cells and cause their dehydration [1,2]. This is important and a subject of intense research because such a mechanism may explain critical steps in the physiopathology of sickle cell anaemia, where the presence of dense and irreversibly deformed red cells (ISCs) is thought to participate in vasoocclusive episodes responsible for the main symptoms in this disease.

What has not been investigated yet is the more general and basic question of the precise level of Ca^{2+} permeabilization required to trigger dehydration of normal red cells through K^+ channel activation in plasma-like media. Aside from its intrinsic interest, this investigation represents a necessary 'normal cell calibration' for comparison with the results in abnormal red cells. The results

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presented here show that ionophore-induced Ca influxes representing less than 3% of the maximal Ca extrusion capacity of the Ca^{2+} pump can generate subpopulations of dense cells, as dense as ISCs, within one hour of Ca^{2+} permeabilization, that dehydration of all cells is readily obtained with only twice the threshold influx, and that below maximal response different cells show different degrees of dehydration and tend to remain partially dehydrated despite sustained Ca^{2+} permeabilization.

Materials and Methods

Blood was obtained from the blood bank (less than two weeks old) or from healthy volunteers by venipuncture into heparin-containing syringes. The cells were washed four times in a medium (solution A) containing (in mM): NaCl, 145; KCl, 3; Tris-HCl or Hepes (pH 7.6–7.7 at 37°C), 10; $MgCl_2$, 0.2; EGTA, 0.1. The buffy coat and topmost cell layer were removed after each wash. The remaining washed red cells were resuspended at a 10% haematocrit in solution A but with only 20 μM EGTA. When present, the external concentrations of glucose and inosine were 10 mM and that of iodoacetamide, 6 mM. The cell suspension was preincubated at 37°C for 15–30 min. Ionophore A23187 was added to this suspension from concentrated stock in ethanol or dimethyl sulfoxide (DMSO) to give the reported final concentrations. Two to five minutes after ionophore addition aliquots of the cell suspension were distributed into magnetically stirred tubes containing concentrated Ca^{2+} solutions to give the different intended final Ca^{2+} concentrations. Time zero for each condition corresponded to the initiation of the Ca influx, at the moment of mixing the Ca^{2+} permeabilized cells with Ca^{2+}. At the indicated times, 50 μl samples of each suspension were added to 1.5 ml nominal capacity plastic microfuge tubes containing 0.95 ml of solution A and 0.4 ml of phthalate oils, at room temperature, and rapidly spun for about 1 min at $14000 \times g$. It was important to perform the density separations at about 20°C since the viscosity of the denser phthalate oils is increased at lower temperatures and this reduces the fraction of cells which could traverse the oil within convenient spinning times. After the spin, the fluids and cells on top of the cell pellets were aspirated, the tube walls dried and cleaned with cotton swabs, and the cells in the pellet lysed in 1.4 ml of Drabkin's solution for haemoglobin measurements. The phthalate oils used were dibutylphthalate of relative density 1.042, through which all cells would spin, diethylphthalate of relative density 1.117–1.118, through which only cells that have lost over 36% of their original K content [3,12] would spin, and mixtures prepared ad hoc to give intermediate densities as illustrated in the experiment of Fig. 3. The haemoglobin in dense cell pellets was divided by the total haemoglobin in dibutylphthalate pellets from comparable samples in order to calculate the reported fractions of dense cells. This automaticaly corrects for lysis since the haemoglobin in the dense cells is referred to that in unlysed cells at each time.

To measure the ionophore-induced Ca influx responsible for dense cell generation it was necessary to inhibit the Ca^{2+}-pump mediated fluxes. Pump inhibition was achieved by depleting red cell ATP by preincubation with inosine and iodoacetamide, as described previously [13]. The ATP-depleted cells were resuspended in similar conditions to those used for the normal-ATP cells except for the presence of ^{45}Ca (specific activity about 10^7 cpm/μmol). Ca influx and Ca-induced changes in density distribution were always measured in parallel in cells from the same batch, as illustrated in the experiment of Fig. 4. At selected times, 50 μl samples were delivered to microfuge tubes with solution A and dibutylphthalate oil as before but at 0°C to prevent loss of Ca from the cells through residual ionophore permeability (cell pelleting through dibutylphthalate is not reduced at low temperature even during short spins [14]. The Ca content of the cells was determined by measuring the ^{45}Ca activity in trichloroacetic acid extracts of the cell pellets [24,14].

Results

Preliminary experiments such as that of Fig. 1 indicated the range of ionophore and external Ca^{2+} concentrations required for submaximal dense cell formation using the density of diethylphthalate oil (1.117–1.118) as the cut-off point. The results in Fig. 1, with inosine-fed red cells from five different donors, are representative of

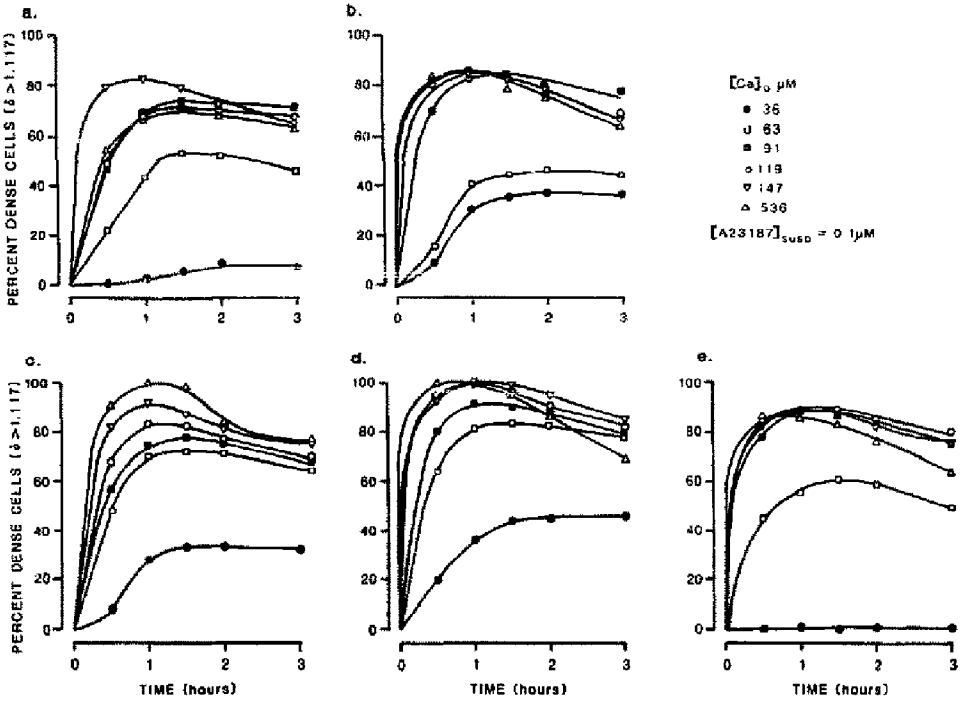

Fig. 1. Time course of Ca influx-induced dense cell formation in red cells from five different donors. Donor codes: a, SOA (blood bank); b, WN (blood bank); c, VLL; d, CJF; e, JTT.

six similar experiments. The ionophore concentration in the suspension used was 0.1 μM, equivalent to about 1 μmol/litre cells [15]. The external Ca^{2+} concentration range in which dense cells formed was 30–500 μM. At submaximal dense cell formation the fraction of dense cells levelled off in time and showed no tendency to rise progressively to maximal levels. The lowest measured steady dense cell fractions were reached in 90–120 min whereas the highest were attained in less than 30 min. The maximal fraction of dense cells formed comprised 80 to 100% of the cells in samples from different donors. Increasing the external Ca^{2+} concentration increased both the rate of dense cell formation and the final fraction of dense cells. In comparable conditions, the fractions of dense cells varied somewhat among cells from different donors. In all, however, despite regular increments in external Ca^{2+}, the distribution of submaximal intermediate curves was such that few could be found in the middle range, suggesting that the range of Ca influx, from minimal to maximal effect, is rather narrow, and that the response to such influx is non-linear. The steepness of the response is illustrated in Fig. 2, drawn using the maximal dense cell fractions from each of the curves in Fig. 1.

Under conditions of rapid and high dense cell formation, the fraction of dense cells showed a variable tendency to decline with time (Figs. 1, 4a and 6, but see also Fig. 3), probably due to both time and Ca^{2+}-dependent lysis of ionophore-treated cells [16]. Lysis was allowed for in the present experiments (see Methods). Prelytic re-

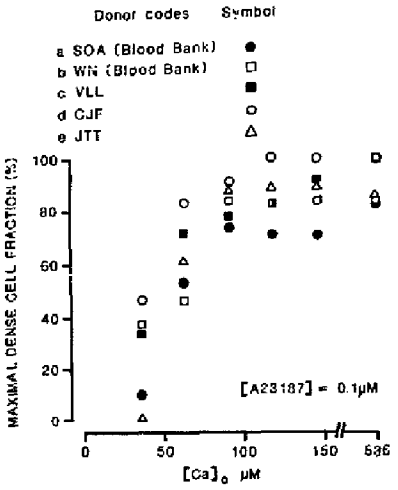

Fig. 2. Maximal dense cell fraction as a function of $[Ca^{2+}]_o$. Each point is from one of the curves in Fig. 1. Inset: donor codes (as in Fig. 1) and corresponding symbols.

swelling of some of the shrunken cells or activation of Na influx through the Na^+/H^+ exchanger by the increased $[Ca^{2+}]_i$ and the lowered internal pH within the shrunken cells may also contribute to explain the observed decline [12,17,18], but this was not investigated here.

Formation of dense cells in the experiment of Fig. 1 was followed with reference to a single arbitrary density cut-off point. In the experiment of Fig. 3 the Ca^{2+}-induced changes in cell density were measured for three density cut-off points. The results show that the dense cell fraction formed at each Ca influx is larger the lower the oil density used for separating dehydrated cells. This indicates the existence of intermediate density shifts which persist in time, suggesting that the increased Ca influx generates stable subpopulations of cells with different levels of dehydration.

The precise relation between cell ionophore concentration and ionophore-induced Ca influx was determined in the past for ATP-depleted cells suspended in high-K media, in conditions which prevented cell volume and ion composition changes [19,20]. A rough estimate of the likely Ca influx range in the experiment of Fig. 1 based on the equilibrium measurements suggests that dense cells may form when Ca influx is as low as 0.2–0.5 mmol/litre cells per h. This is far below the saturated Ca^{2+}-pump extrusion capacity (approx. 30 mmol/litre cels per h [21]), and only 4–10-times the physiological Ca influx (approx. 50 μmol/litre cells per h [22]). In conditions in which Ca^{2+}-permeabilized cells become dehydrated, the relation between cell ionophore concentration and Ca influx has not been investigated. Since the ionophore is a strict $Ca^{2+}/2H^+$ exchanger [23], and the proton concentration ratio increases in dehydrated cells [3,12,14,21,24,25], Ca influx and the final $[Ca^{2+}]_i$ level may be expected to be higher in dehydrated cells. It is therefore important to directly measure Ca influx in low-K media.

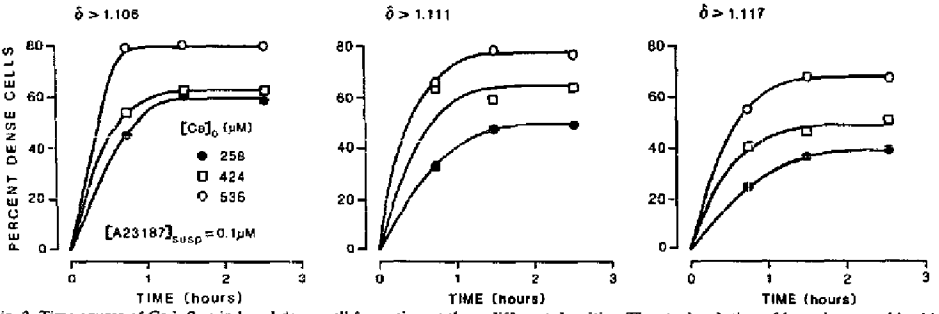

Fig. 3. Time course of Ca influx-induced dense cell formation at three different densities. The stock solution of ionophore used in this experiment (0.2 mM in ethanol) was about two years old and induced about 4-fold slower Ca (^{45}Ca) influxes into ATP-depleted cells than equivalent nominal concentrations of ionophore from the stocks used for the other experiments (not shown).

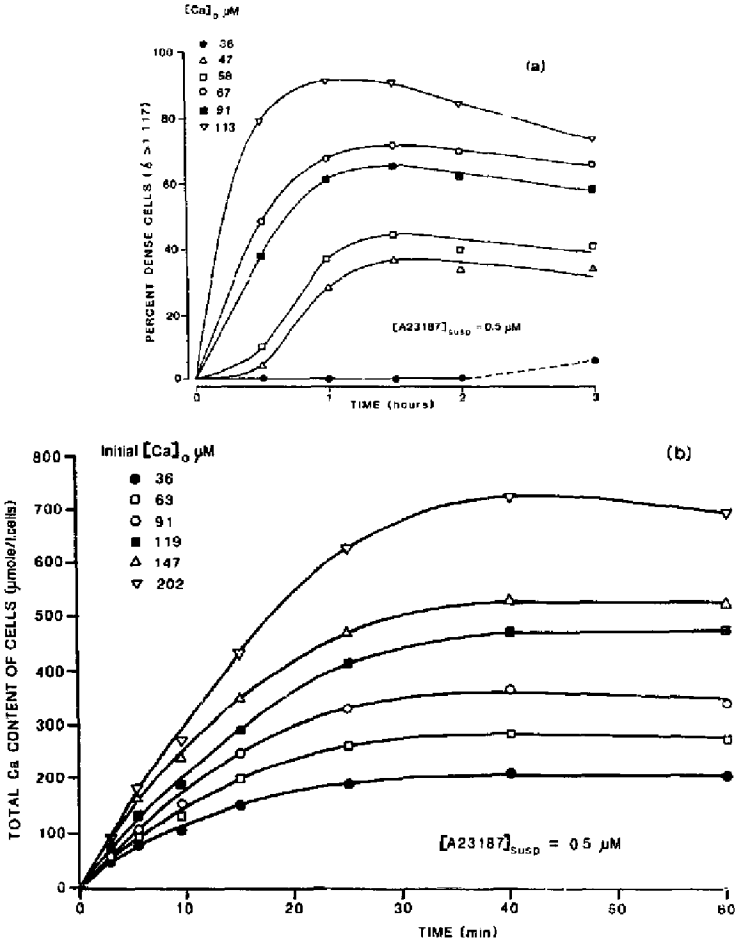

Fig. 4. Time course of Ca influx-induced dense cell formation (a), and Ca (^{45}Ca) influx into the same cells after ATP depletion (b).

This was done in the experiments illustrated in Figs. 4 and 5. Ca^{2+}-induced dense cell formation (Fig. 4a) and Ca influx (Fig. 4b) were determined in parallel in the same cells. Ca influx was measured after ATP depletion. Otherwise, the conditions were the same as those in the experiments shown in Fig. 1. The results in Fig. 4b report initial Ca influx values of between 0.7 and 1.2 mmol/litre cells per h for the range of Ca^{2+} concentrations spanning minimal to maximal levels of dense cell formation in the inosine-fed cells (Fig. 4a).

Ca influx was simultaneously measured in high-K and low-K conditions for comparison (Fig. 5). The ionophore and initial external Ca^{2+} concentrations were identical for both groups. At comparable external Ca^{2+} concentrations, both the initial Ca influx and the final cell Ca^{2+} con-

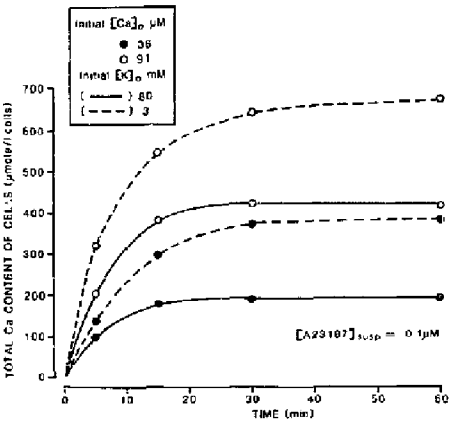

Fig. 5. Comparison between ionophore-induced Ca influx into ATP-depleted cells suspended in high-K (continuous line) or low-K (dashed line) media, at two different external Ca^{2+} concentrations.

tent were higher in the low-K conditions. Ca influx in low-K media was about twice that in equilibrium conditions. The detailed mechanism of these differences as well as the study of both Ca influx and cytoplasmic Ca buffering is outside the scope and aims of the present work, and will be reported separately. The relevant result here is that Ca influx in low-K media is at most only twice that in equilibrium conditions.

In two pilot experiments using ^{45}Ca, it was not possible to detect any significant increase in the mean total Ca content of dehydrated cells ($[Ca^T]_i$) exposed to ionophore induced Ca influx in any submaximal condition (not shown). With the precision of such measurements [14,24] $[Ca^T]_i$ must have increased by less than 1 μmol/litre cells. This means that the Ca^{2+} pump sustained a pump-leak balance with minimal total Ca gain in all but maximally permeabilized cells. This is in line with the low Ca influx found to be required to form dense cells, hardly a challenge to the Ca^{2+} pump in fed cells. With a Ca^{2+}/ATP stoichiometry of about 1:1 [26,27], the increased ATP consumption by the Ca^{2+} pump required to balance the ionophore-induced Ca influx, of up to about 1.5 mmol/litre cells per h, may however pose a small metabolic overload on the normally sluggish glycolytic metabolism of the red cells. The extent to which this may affect dense cell formation was investigated in the experiment illustrated in Fig. 6. The fractions of dense cells formed at different Ca influx in the absence of substrates, were compared with those formed in the presence of glucose or inosine. The protocol was otherwise identical to that of Fig. 1. The results show that the presence or absence of substrates has little effect on the range of Ca influx able to generate submaximal dense cell populations, and that, within the three hour period during which dense cell formation was followed, there was no late tendency for the fraction of dense cells to increase. There was a minor trend to form higher fractions of dense cells at comparable influx in glucose relative to inosine, and slightly more, in the absence of substrate relative to the conditions with glucose or inosine. This, and the stability of the submaximal fractions of dense cells formed, suggest that whatever the effect of substrate presence or absence might be, it occurs initially, and does not intensify within the three hour period explored.

Discussion

The present results offer a first characterization of the conditions required for submaximal Ca-induced dehydration. The main findings are (i) that for dense cells to form within 1–3 h of Ca permeabilization, sustained increases in Ca influx of about 0.7 mmol/litre cells per h are required, (ii) that maximal dehydration is elicited when Ca influx is increased further to about 1.2 mmol/litre cells per h, and (iii) that as Ca influx is increased within this range both the rate of dehydration and the fraction of cells within each density fraction increase. At submaximal Ca influx each dense cell fraction formed would tend to remain stable in time. This means that despite uniform increases in Ca influx, different cells dehydrate initially to different extent and then tend to remain in a partially dehydrated state.

The dehydration process investigated here represents a complex multistep reaction [26]. The sequential steps in Ca^{2+}-induced red cell dehydration are: (i), increased Ca influx, (ii) increased $[Ca^{2+}]_i$, (iii) activation of Ca^{2+}-sensitive K^+ channels, (iv), net loss of K, largely with an accompa-

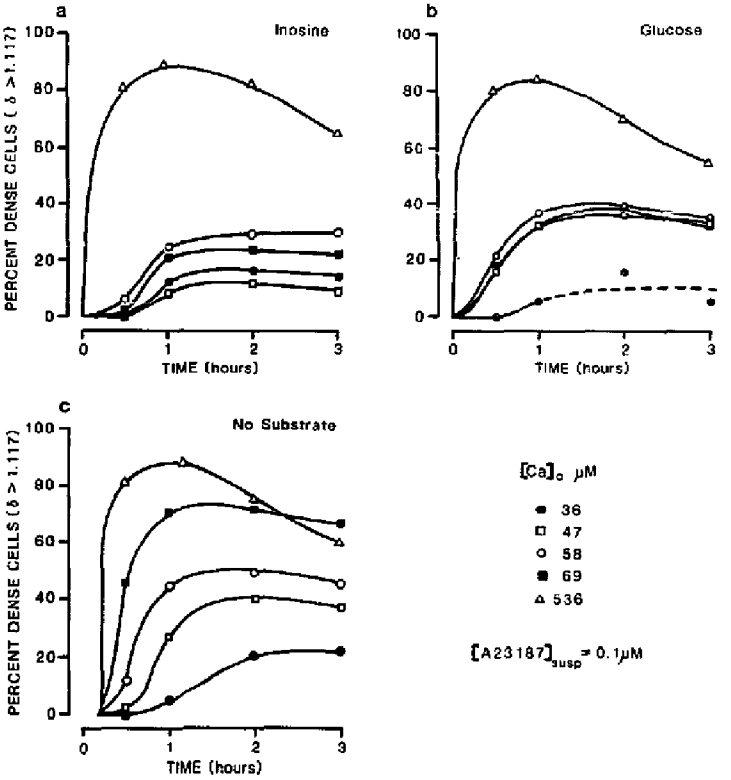

Fig. 6. Effect of the absence (c) or presence of glycolytic substrates (glucose b) or inosine (a)) on the time course of Ca influx-induced dense cell formation at five different external Ca^{2+} concentrations.

nying diffusible anion, but partly also in exchange for protons [3,12], and (v), loss of a slightly alkaline, hypertonic fluid [3,12]. We may now attempt to interpret the present results in terms of the information they provide on some intermediate steps of the dehydration process, as it may occur in physiological conditions.

It is possible to estimate the pump-leak steady-state value of $[Ca^{2+}]_i$ in the Ca influx range where submaximal dense cell fractions were generated. Ca efflux through the Ca^{2+} pump in the physiological $[Ca^{2+}]_i$ range, far below saturation kinetics, was found to be adequately described by the equation [22]

$$\text{Ca efflux} = A \cdot ([Ca^{2+}]_i)^2$$

In pump-leak steady-state, by definition,

$$L = A \cdot ([Ca^{2+}]_i)^2$$

where L represents the inward leak flux of Ca. If L' represents the ionophore-induced Ca influx in which dense cells are formed, and L and $[Ca^{2+}]_i$

the physiological Ca influx and $[Ca^{2+}]_i$ level, respectively, we may estimate $[Ca^{2+}]'_i$, the increased $[Ca^{2+}]_i$ expected in the measured L' range from

$$[Ca^{2+}]'_i = (L'/L)^{1/2} \cdot [Ca^{2+}]_i$$

With values of L', L and $[Ca^{2+}]_i$ of 0.7 to 1.2 and 0.05 mmol/litre cells per h, and 10 to 30 nM, respectively, as obtained from Ref. 22 and from the present results, the predicted increased $[Ca^{2+}]_i$ would be in the range 40 to 150 nM. This means, for instance, that in cells with physiological $[Ca^{2+}]_i$ levels around 10 nM, and in the present experimental conditions, minimal dehydration may be triggered by a $[Ca^{2+}]_i$ rise to about 40 nM, and maximal dehydration by a further $[Ca^{2+}]_i$ rise to about 50–60 nM. The threshold $[Ca^{2+}]_i$ values and the $[Ca^{2+}]_i$ range to maximal response compare to the lower values found to activate K^+ channels in inside-out vesicles, in Mg^{2+}-containing resealed ghosts and in intact cells under different experimental conditions [21,27–32]. The observed cell heterogeneity of the dehydration response precludes any direct assessment of the kinetics of K^+ channel activation. However, the narrow activation range of Ca influxes and estimated $[Ca^{2+}]_i$ values suggests a steep response of the K^+ permeability to $[Ca^{2+}]_i$.

The increase in Ca influx caused by polymerization of hemoglobin S in sickle cell anaemia red cells, was estimated previously in Benz-2-loaded discocytes, suspended in autologous plasma, and exposed to rapid and complete deoxygenation under argon in a tonometer [9]. The values found varied from 60 to 200 μmol/liter cells per h. These represent mean values in the cell population, and could be much higher in some cells than in others. Since the Ca influx range from minimal to maximal dense cell formation in normal red cells is so narrow, there is a distinct possibility that sickling can generate a sufficiently high Ca influx in some cells, which even if it persists for brief periods at a time, may lead to cumulative dehydration. If, as recent experiments suggest [2], only 5–30% of sickle cell anaemia red cells become highly permeable to Ca during each sickling pulse, and if most of the measured mean Ca influx is into such cells, $[Ca^{2+}]_i$ may easily reach values shown to have maximal dehydrating effects in normal cells.

There is a characteristic tendency for the fractions of dense cells to level off in time at submaximal Ca influx, before the cells had been maximally dehydrated. This means either that the dehydration process has been halted or that it has been balanced by oposing hydrating processes. The overall effect resembles a kind of all or none response [33–36]. Although in principle many diverse factors may cause such a response, the steep $[Ca^{2+}]_i$-dependence of the K^+ permeability discussed above suggests that minor timed reductions in $[Ca^{2+}]_i$ may offer the simplest explanation for the observed effects, and ought to be considered first in subsequent studies. The reasons why some cells dehydrate more than others, and some not at all, may be due to differences in pump-leak steady-state $[Ca^{2+}]_i$ among cells due to differences in pumping, or to coordinated differences in the $[Ca^{2+}]_i$ thresholds of the channels belonging to different cells [34,35,37,38]. Further work is needed to resolve these options.

In conclusion, uniform increases in Ca influx within the narrow range 0.7–1.2 mmol/litre cells per h cause minimal to maximal dehydration of normal human red cells suspended in plasma-like media. The dehydration response to Ca^{2+} permeabilization is therefore extremely steep and would seem to occur within the estimated range of $[Ca^{2+}]_i$ from 40 to 150 nM. At submaximal Ca influx the dehydration response is heterogeneous, either all or none or transient.

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