J. Membrane Biol. 66, 235–247 (1982)

The Journal of
Membrane Biology

A Method for Estimating Free Ca within Human Red Blood Cells, with an Application to the Study of Their Ca-Dependent K Permeability

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Summary. Murphy, Coll, Rich and Williamson (*J. Biol. Chem.* **255**:6600–6608, 1980) described a null-point method for estimating intracellular free Ca in liver cells. They used digitonin to lyse the cells in solutions of varying Ca concentration. This method has been adapted for use with human red cells. The values found are about 0.4 μM Ca in fresh cells, and from 0.4 to 0.7 μM Ca in blood-bank cells, at pH 7.2 and 37 °C. These are likely to be overestimates, and the errors and limitations of the method are discussed. Red cells may be loaded with Ca by metabolic depletion in Ca-containing solutions. Such cells have an elevated K permeability, and the relationships between free Ca, total Ca and K permeability were investigated, using ^{86}Rb as a tracer for K. ^{86}Rb flux studies show that the affinity of the K channel for Ca is the same in cells as in resealed ghosts where intracellular Ca can be controlled with Ca buffers, but the rate of tracer equilibration is 3–6 times faster in ghosts than in cells.

Key words human red cells (or erythrocytes) · intracellular free Ca · digitonin · null-point · Ca-dependent K permeability (or K permeability)

Introduction

No method to estimate the free Ca in red cells has yet been described, except for the rather special case of the cell treated with the Ca ionophore A23187, when intracellular Ca can be set to any value by varying the external Ca concentration (Ferreira & Lew, 1976). It would be very useful to measure the free Ca in untreated cells, in order to investigate phenomena like the Ca-dependent K permeability of human red cells (Gardos, 1959), the role of Ca in the control of sugar transport in avian red cells (Carruthers & Simons, 1978) and the abnormalities of Ca metabolism in sickle cells (Bookchin & Lew, 1980).

In 1980, Murphy, Coll, Rich and Williamson made a notable advance by measuring the free Ca in hepatocytes by a null-point method. They found that the cells take up Ca when the plasma membrane is made freely permeable with digitonin, and the uptake varies with the initial extracellular Ca concentration in a manner which suggests that it is dependent on the initial gradient of Ca across the membrane. Intracellular free Ca is assumed to be equal to the external Ca concentration at which no uptake occurs. This method ought, in principle, to be applicable to any suspension of small cells, but could suffer from two disadvantages. Digitonin might affect internal organelles, although it seems only to dissolve the plasma membrane (Fiskum, Craig, Becker & Lehninger, 1980), and not to affect the Ca-regulating ability of mitochondria and endoplasmic reticulum (Becker, Fiskum & Lehninger, 1980). Secondly, the load of Ca or chelating agents imposed upon the cytoplasm might perturb its Ca-regulating mechanisms and thereby affect the result. Neither of these factors is likely to occur in the mammalian red blood cell, which lacks organelles, making these cells ideal for further investigation of the null-point method.

In their work with hepatocytes, Murphy et al. (1980) monitored extracellular Ca by spectrophotometry, with arsenazo III as an indicator. This method was found to be impracticable with red blood cells, as light scattering prevents continuous recording of extracellular Ca in cell suspensions. Instead, extracellular Ca was monitored continuously with a commercial Ca electrode. The electrode measurements are in agreement with the simple theory that digitonin hemolyzes the cells, causing the mixture of intracellular and extracellular solutions. Intracellular free Ca can be estimated (although not very accurately), and the effects of varying experimental conditions on the measurements were investigated. Free Ca was also measured in cells loaded with Ca by metabolic depletion in Ca-containing solutions. Such cells have a raised K permeability (Gardos, 1959). Accurate measurements of K movements in the steady state through the Ca-dependent pathway have only been made in ghosts

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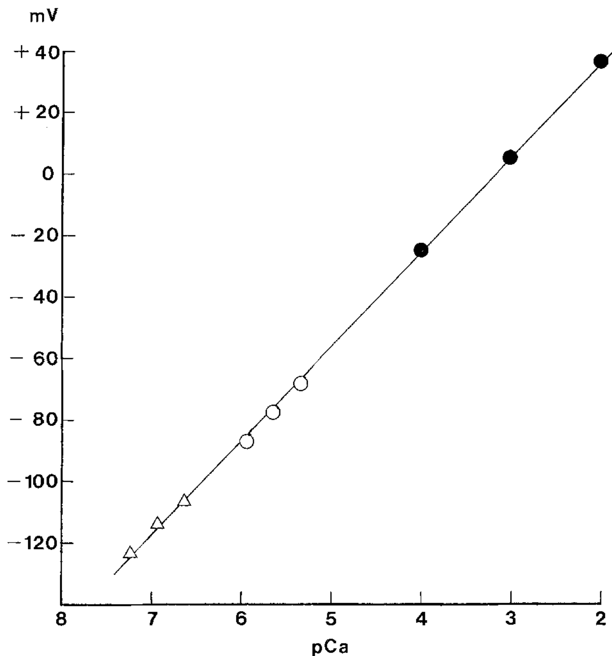

Fig. 1. Calibration of the Ca electrode at 37 °C, ionic strength 0.1 (KCl). Solutions contain unbuffered Ca (●), Ca/HEDTA buffers (○) or Ca/EGTA buffers (△). The abscissa is in *p*Ca units ($=-\log_{10}[Ca^{2+}]$), calculated as described in the text. The line $E(\mathrm{mV})=97.5-30.7\,p\mathrm{Ca}$ is a least-squares fit to the points

(Simons, 1976*b*), or in intact cells treated with A23187 (Lew & Ferreira, 1976), so it was interesting to compare ^{86}Rb transport (used as a tracer for K) in cells loaded with Ca by metabolic depletion with the earlier work.

A preliminary account of part of this work has been published (Simons, 1981).

Materials and Methods

Red Blood Cells and Ghosts

Blood was either fresh (drawn from the author and heparinized) or citrated, from the South London Blood Transfusion Centre. Bank blood was stored at 4 °C and used 5–14 days after being drawn. Red blood cells were isolated by centrifuging in a refrigerated centrifuge, and resuspending the cells with wash medium (150 mM KCl, 2 mM $MgCl_2$ and 2 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid) neutralized to pH 7.4 with KOH). This was done 3–5 times, and each time the topmost layer of cells, including any visible white cells, was removed. Cells were always washed free of externally bound Ca before measurements of free Ca or total Ca. This was done by resuspending the cells in wash medium supplemented with 1 mM EGTA (ethyleneglycol *bis*-(2-aminoethyl) tetra-acetic acid), centrifuging, then washing the cells twice more by resuspension in nominally Ca-free medium (*see below*) and centrifuging, to remove extracellular EGTA.

The acidity of citrated cells is not removed by simply washing them in buffered solutions. It was found necessary to titrate suspensions of blood bank cells to pH 7.6–7.7 at 37 °C with KOH, in order to obtain a final pH of 7.1–7.2 at 37 °C. This was done either after the initial removal of white cells, or, in the case of metabolically depleted cells, between the two incubations.

Cells were metabolically depleted and loaded with Ca in two stages. They were first incubated for 18–20 hr at 37 °C in wash medium supplemented with 1 mM EGTA, 10 µg/ml chloramphenicol and 20 µg/ml penicillin, then incubated for 2 hr at 37 °C in wash medium supplemnted with 5 mM 2-deoxyglucose, 5 mM iodoacetamide, from 0 to 1 mM $CaCl_2$, and ^{86}Rb (if required). Intracellular ATP was 5–10 µM after this procedure, when assayed by a fluorimetric method (Bergmeyer, 1965).

Resealed ghosts were prepared as described previously (Simons, 1976*a*), except that an extra 100 mM sucrose was incorporated internally, to bring the osmotic pressure to 300 ideal mosmole/liter.

Ca Electrode Measurements

These were made with an Orion 93-20-01 Ca electrode, a reference electrode filled with 4 M KCl saturated with AgCl, an Orion 811 pH/mV meter, and recorded with an LKB flat-bed chart recorder. Samples (usually 4 ml) were contained in a polypropylene "accuvette" (Coulter Electronics Ltd.) in a 37 °C waterbath, and the electrodes were equilibrated at 37 °C. The contents were stirred vigorously with pieces of paper clip sealed in polythene tubing and controlled by a submersible magnetic stirrer.

Red cells were normally suspended at 4–5% hematocrit in nominally Ca-free medium containing 100 mM KCl, 0.5 mM $MgSO_4$ (both Johnson Matthey Specpure grade), 100 mM sucrose (B.D.H. Aristar grade) and 5 mM HEPES, neutralized to pH 7.2 at 37 °C with KOH (B.D.H. Aristar grade). This composition was chosen in order to be approximately isotonic with plasma, to have an ionic strength of 0.1 (convenient for work with Ca buffers) and to have a pH and free Mg concentration approximately equal to the normal intracellular values. Cell suspensions were hemolyzed by addition of digitonin (B.D.H. Analar grade, 10 mg/ml in ethanol), normally to a final concentration of 0.1 mg/ml. This is approximately equivalent to 6 µg/mg dry cell weight, comparable to the concentration of 5 µg/mg dry weight used by Murphy et al. (1980) with hepatocytes. Digitonin did not affect the Ca electrode in the absence of cells, nor did digitonin-induced hemolysis affect the pH of cell suspensions.

Electrode Calibration

Six solutions were used routinely, all made up to a final concentration of 90–95 mM KCl and 5 mM (K)HEPES, pH 7.2. Three contained unbuffered $CaCl_2$, at concentrations of 10, 1 or 0.1 mM (*p*Ca ($=-\log_{10}[Ca]$) = 2, 3 and 4, respectively). The other three contained Ca buffers: 2 mM Ca plus 3 mM HEDTA (N-hydroxyethyl ethylenediamine triacetic acid), 1 mM Ca plus 3 mM HEDTA and 1.5 mM Ca plus 3 mM EGTA. Under the convention used previously (Simons, 1976*a*, and subsequent publications), these last three solutions would have *p*Ca values of 5.46, 6.06 and 7.15, respectively, at 37 °C. Tsien and Rink (1980) have pointed out that if the pH electrode is calibrated in H^+ activity, these values should be revised to 5.36, 5.96 and 6.95, respectively. Figure 1 shows the fullest electrode calibration performed, in which several additional solutions were used. The fit to the equation $E(\mathrm{mV})=a+b\cdot p\mathrm{Ca}$ is marginally better if the revised *p*Ca values are used: the correlation coefficient is −0.9998, instead of −0.9995, and the slope is -30.7 ± 0.3 mV, instead of -29.4 ± 0.4 mV (theoretical slope −30.8 mV at 37 °C). Accordingly, re-

sults in this paper are presented in terms of the revised *p*Ca values for the Ca buffers, which represent a reduction of 0.20 in *p*Ca for EGTA, and 0.10 for HEDTA, over the values used previously.

In each experiment, the electrode was first calibrated with all six solutions, then measurements with cell suspensions were alternated with the adjacent calibration standards, to correct for electrode drift. There was no interference by H, K or Mg ions, in the range of concentrations used.

Some observations were made which indicate that the Ca electrode may not always display a linear response down to 10^{-7} M Ca, as it does in solutions well-buffered with respect to Ca. The Ca-free solution used in the electrode studies had a Ca content of 1 μM by atomic absorption spectrophotometry, but gave an electrode reading which corresponds to 2 μM Ca. Small amounts of Ca were added to this solution, and Fig. 2 shows the relationship between the electrode reading and the *p*Ca estimated by atomic absorption spectrophotometry. The results begin to deviate from the line below about 5 μM Ca (*p*Ca 5.3). When low concentrations of a 1:2 Ca/EGTA buffer are added to the nominally Ca-free solution, the electrode readings are again less negative then expected, and the discrepancy decreases as the EGTA concentration is increased. The deviations seen in Fig. 2 are not caused by any of the components of the solution, insofar as changing their concentrations does not affect the deviations. These findings suggest that deviations of electrode response from theory at low Ca levels occur in solutions poorly buffered with respect to Ca. This imposes a limitation on measurements of intracellular free Ca, such that any value obtained can only be an upper limit.

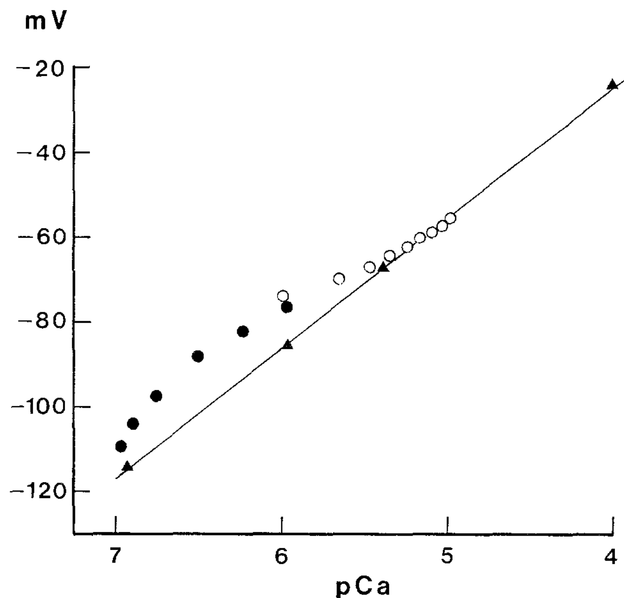

Fig. 2. Deviations from the calibration curve of the Ca electrode. (▲) – calibration using standards as in Fig. 1; (○) – addition of Ca to nominally Ca-free solution: *p*Ca assumed to be the logarithm of the Ca content measured by atomic absorption spectrophotometry; (●) – addition of 1:2 Ca/EGTA buffers to nominally Ca-free solutions: *p*Ca calculated from Ca and EGTA concentrations (EGTA concentration range 1.5–23 μM)

^{86}*Rb Fluxes*

^{86}Rb was used as a tracer for K movements through the Ca-dependent K channel. Rb movements are slightly faster than those of K under comparable conditions (Simons, 1976*b*), and evidence is presented below that *tracer* ^{86}Rb efflux is comparable to ^{42}K efflux from ghosts (Fig. 17).

Influx. Red cells were suspended at 5–10% hematocrit in a solution containing 150 mM KCl, 0.5 mM $MgSO_4$ and 5 mM (K)HEPES (pH 7.2 at 37 °C), ^{86}Rb was added (about 5 μCi/ml, 12 μM Rb), and the suspensions incubated at 37 °C. At suitable times between 0 and 40 min, 0.3-ml samples were transferred to 1.5 ml centrifuge tubes containing 0.5 ml silicone oil (Dow Corning 550, specific gravity 1.07) and 0.5 ml unlabeled medium. The tubes were immediately capped and centrifuged (Eppendorf 5414). The supernatant fluid was removed by suction, and the tip of the tube containing the cell pellet was cut off and counted in a Nuclear Enterprises 8311 γ-counter. With this technique the extracellular radioactivity contaminating the pellet is about 0.3% of the total. The rate constant of influx is given by the slope of a graph of $\log_e(1-$(counts in cells)/(counts in cells at equilibrium)) against time (Fig. 16). Internal counts at equilibrium were estimated by adding 10 μM valinomycin to the suspension, then centrifuging the cells through silicone oil (without unlabeled medium), and correcting for trapped extracellular radioactivity (about 1%). In a trial experiment, the ratio of ^{86}Rb, counts/ml, in/out, in the presence of valinomycin, was equal to the K concentration ratio, in/out, measured by flame photometry.

Efflux. Red cells were loaded with ^{86}Rb by incubation at 37 °C for 2 hr in suspensions containing 5 μCi/ml (12 μM Rb), then washed free of extracellular radioactivity by centrifuging and resuspension in ice-cold wash medium. Efflux was measured at 37 °C, with the cells suspended at 5–10% hematocrit in the same 150 mM K solution used for influx. One-milliliter samples were removed at suitable times between 0 and 40 min, centrifuged (20 sec in Eppendorf 5414) and 0.8 ml of supernatant was taken and counted. 0.8 ml portions of suspension were counted, and the fraction of counts remaining in the cells calculated. The rate constant of efflux is given by the slope of a graph of $\log_e$(fraction of counts remaining in the cells) against time (*see* Fig. 16). Resealed ghosts were loaded with ^{86}Rb at hemolysis, but in other respects efflux was measured in the same way as with cells.

Other Measurements

pH was measured with the Orion 811 meter and a combined glass/reference electrode. Ca was measured with a Pye Unicam SP9 atomic absorption spectrophotometer. Cells were extracted with 2% perchloric acid (B.D.H. Analar grade) in acid-washed polycarbonate tubes, centrifuged, and Ca measured in the supernatant. The number of cells in suspensions was measured with a model ZF Coulter counter. A mean cell volume of 80 fl was assumed in order to calculate the hematocrit.

Theory

Ferreira and Lew (1976) showed that red cell cytoplasm buffers Ca in a linear fashion – that is it behaves as a solution of a low affinity, high capacity buffer. This is confirmed in an experiment reported below.

Assume a suspension of cells with hematocrit *h* (as a fraction), Ca_o = initial extracellular free Ca, Ca_i = intracellular free Ca, and Ca_T = total Ca as-

sociated with the cells. All cell concentrations are referred to the whole cell volume, to simplify the algebra. Then

$$\mathrm{Ca}_T = \mathrm{Ca}_i \left(1 + \sum_i \left(\frac{L_i}{\mathrm{K}_i + \mathrm{Ca}_i}\right)\right), \tag{1}$$

where L_i are ligand concentrations, with dissociation constants K_i

$$\mathrm{Ca}_T = \mathrm{Ca}_i \left(1 + \sum_i \frac{L_i}{\mathrm{K}_i}\right), \tag{2}$$

if $\mathrm{K}_i \gg \mathrm{Ca}_i$ and

$$\mathrm{Ca}_T = \frac{\mathrm{Ca}_i}{\alpha}, \tag{3}$$

where $\alpha = \left(1 + \sum_i \frac{L_i}{\mathrm{K}_i}\right)^{-1}$.

α is the fraction of Ca that is ionized. This analysis was given by Ferreira and Lew (1976). Now assume the cells are hemolyzed. The total Ca concentration is now

$$\begin{aligned}\mathrm{Ca}'_T &= (1-h)\,\mathrm{Ca}_o + h\,\mathrm{Ca}_T \\ &= (1-h)\,\mathrm{Ca}_o + h\,\mathrm{Ca}_i/\alpha.\end{aligned} \tag{4}$$

The buffering ligands are now present at concentrations hL_i, so the solution will behave as a linear Ca buffer, with the fraction of Ca that is ionized, α', given by

$$\left(\frac{1}{\alpha'} - 1\right) = h\left(\frac{1}{\alpha} - 1\right), \quad \text{or} \quad \alpha' = \frac{\alpha}{\alpha + h(1-\alpha)}. \tag{5}$$

The free Ca after hemolysis, Ca'_o, will be

$$\mathrm{Ca}'_o = \frac{\alpha(1-h)\,\mathrm{Ca}_o + h\,\mathrm{Ca}_i}{\alpha + h(1-\alpha)} \tag{6}$$

and the change in external free Ca $= \mathrm{Ca}'_o - \mathrm{Ca}_o$

$$= \frac{h(\mathrm{Ca}_i - \mathrm{Ca}_o)}{\alpha + h(1-\alpha)}. \tag{7}$$

A graph of the change in external free Ca against the initial external free Ca should have slope $\frac{-h}{\alpha + h(1-\alpha)}$ and intercept the abscissa where $\mathrm{Ca}_i = \mathrm{Ca}_o$. Figure 3 shows this theoretical graph, using values for the parameters close to those observed in practice. It also shows two possible deviations from theory. In the first case, the cells are assumed to contain additional tightly bound Ca, Ca_B, that is released by the detergent action of digitonin. This

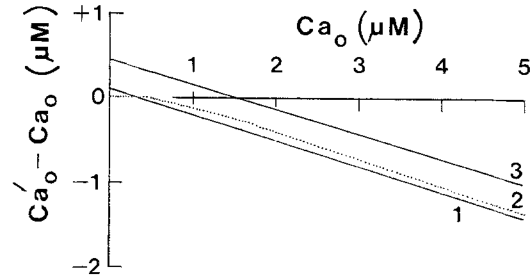

Fig. 3. Theoretical analysis of the null-point method for estimating intracellular free Ca. Line 1 is that predicted by theory (Eq. (7)], for values of $\mathrm{Ca}_i = 0.3\,\mu\mathrm{M}$, $h = 0.05$ and $\alpha = 0.125$. Line 2 (dotted) shows the effect of 10 μM EGTA in the extracellular fluid, assuming log K'_{Ca} for EGTA to be 6.9, and line 3 shows the effect of assuming the cells contain an extra 10 μM bound Ca that is released by digitonin

results in an upward shift of the line, such that the null-point becomes $\mathrm{Ca}_i + \alpha\,\mathrm{Ca}_B$. Clearly intracellular free Ca would be overestimated in this case. The second possibility considered is the presence of EGTA in the external solution. As will be seen, this is often necessary to obtain low initial values of Ca_o. Figure 3 shows the results of a numerical calculation of the effect of 10 μM EGTA on the graph. It is no longer a straight line, and the most serious effect is to suppress the positive part of the graph, at very low Ca_o.

Results

Binding of Ca by Hemolysates

The free Ca in cell suspensions was monitored with the electrode and Ca buffering curves were constructed by making successive additions of Ca (Fig. 4). In a suspension of intact cells, the free Ca is almost exactly equal to the added Ca. When the cells are hemolyzed by digitonin or by freezing and thawing, free Ca is reduced because of binding by cytoplasmic buffers. The linearity of the binding curve in the 0 to 150 μM Ca range is in agreement with the findings of Ferreira and Lew (1976), and justifies the method used to analyze the null-point data (Fig. 6). The Ca buffering power of the cytoplasm can be calculated from the slope of Fig. 4. The results for the hemolysates correspond to $\alpha = 0.096$ in intact cells, although this value is not very accurate because of the low hematocrit.

Estimation of Intracellular Free Ca

Figure 5 gives the records of an experiment to measure intracellular free Ca in a sample of blood-bank cells. Each time, small quantities of Ca or EGTA

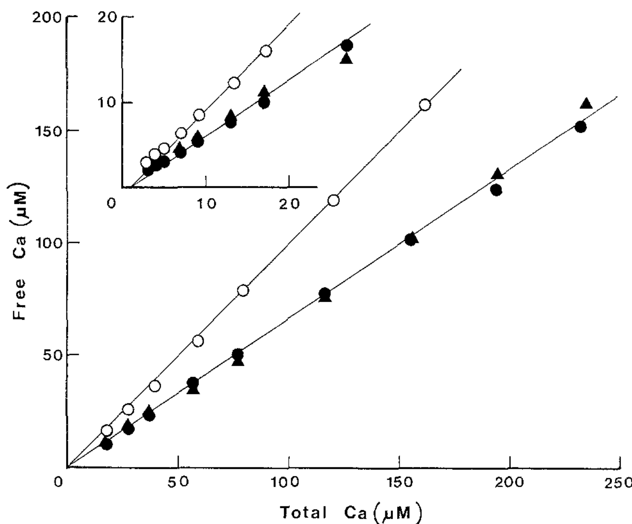

Fig. 4. Binding of Ca by suspensions of fresh human red blood cells. Cells were suspended in nominally Ca-free medium at 5.3% hematocrit at 37 °C, and the binding curves measured by making successive additions of Ca with (○) – intact cells, (●) – cells hemolyzed with 0.12 mg/ml digitonin and (▲) - cells hemolyzed by freezing and thawing. The inset shows an expansion of the scale near the origin. For the intact cells, the total Ca is calculated on the assumption that the available Ca occupies just the extracellular space, while it is assumed to occupy the entire volume in the hemolyzed suspensions. The lines are least-squares fits, and have slopes (○) 1.001±0.006, (●) 0.655±0.004 and (▲) 0.677 ±0.008, although the last two are combined for clarity

are added to the cell suspension in order to adjust the external free Ca, and the electrode potential is monitored continuously. A steady reading is obtained in 2-4 min (Ca_o), and digitonin is added. After another 2 min or so, a steady reading is again obtained (Ca'_o). These two readings are converted to concentrations in μM units, and plotted as a graph of $(Ca'_o - Ca_o)$ against Ca_o (Fig. 6*a*). A straight line is fitted to the points by the method of least squares, and gives a null-point of 0.45±0.13 μM Ca.

There are several errors associated with this measurement. Observations in the vicinity of the null-point require the presence of EGTA, and this distorts the graphical analysis of results, in particular suppressing increases of Ca_o when Ca_i is greater than Ca_o. (An example of this can be seen in Fig. 11, line *a*.) Because of this effect, not more than 5 μM EGTA was normally used. On the other hand, observations made at higher Ca levels are increasingly subject to error because of the logarithmic scale of the Ca electrode. There are also the possibilities of errors in the electrode calibration, or caused by the release of tightly bound Ca. An unexpected source of error was found: the degree of hemolysis produced by digitonin varied slightly, and this caused scatter in the null-point graphs. The records in Fig. 5 are part of a larger experiment in which the digitonin concentration and the hematocrit were varied. The results from the other experimental conditions are presented in Fig. 6 (*b*–*f*). The change in free Ca decreases as the digitonin concentration is reduced, because fewer of the cells are hemolyzed. The amount of hemoglobin released was measured in each case, and the slopes and intercepts of the lines relating $(Ca'_o - Ca_o)$ to Ca_o in Fig. 6 are plotted against the hemoglobin concentration in Fig. 7. If one assumes that substances buffering Ca are released from the cells in proportion to hemoglobin[1], the slopes should be equal to $\frac{-fh}{\alpha + fh(1-\alpha)}$ where f is the fraction of cells hemolyzed, and the other symbols are as in Eq. (7). Fig. 7(*a*) shows that this expression fits the results, within the errors of the measurements. Ca_i, however, should be independent of the degree of hemolysis, and this is confirmed in Fig. 7(*b*). The weighted mean gives $Ca_i = 0.50 \pm 0.09$ μM.

[1] This is true for digitonin-induced release of ATP and hemoglobin from pigeon red blood cells (evidence to be published elsewhere).

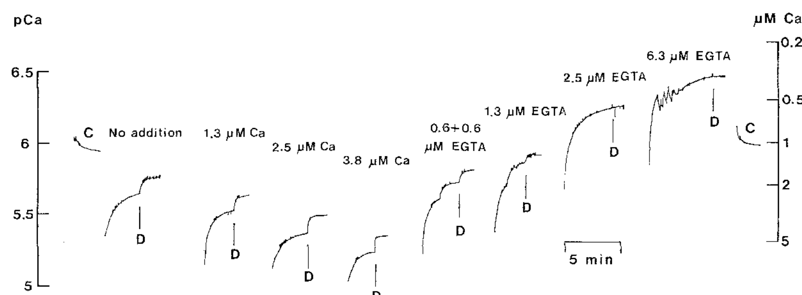

Fig. 5. Ca electrode recordings from an experiment to measure intracellular free Ca. Each trace gives the PD recorded by the electrode from a suspension of blood-bank cells in nominally Ca-free solution at 37 °C, 5% hematocrit, pH 7.2. The suspensions were made up with additions of Ca or EGTA, as small volumes of 1 mM solutions, and when a steady reading was obtained, digitonin was added (*D*) to a concentration of 125 μg/ml. Recordings of a Ca standard solution (*p*Ca 5.96) alternate with those of cell suspension, but these have been removed for the sake of clarity, except at the ends of the record (*C*). The violent oscillations in the record at the right-hand end are causes by a temporary failure of the stirrer

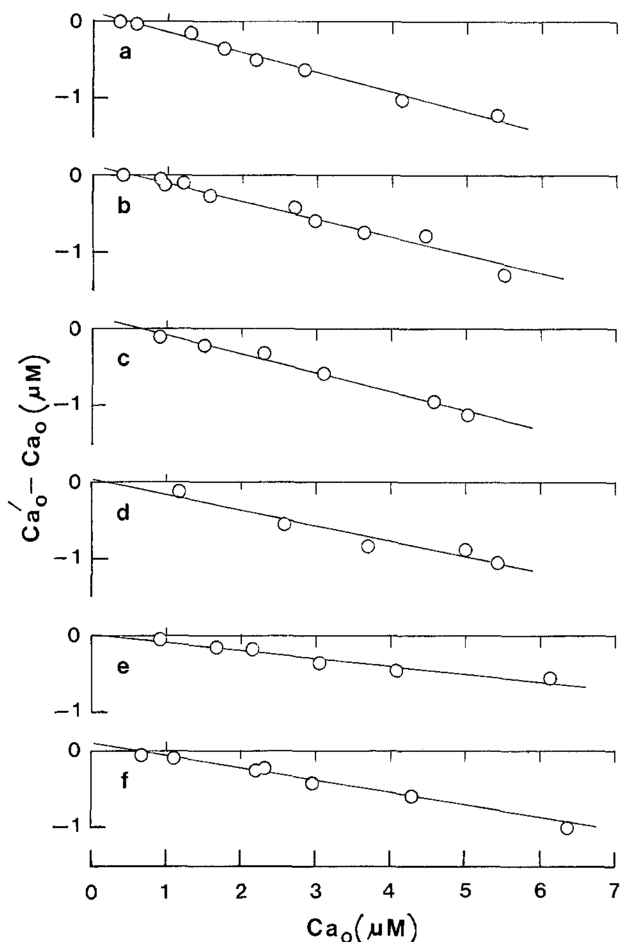

Fig. 6. Plots of digitonin-induced change in external Ca ($Ca_o' - Ca_o$) against initial Ca(Ca_o), for suspensions of blood-bank cells at 37 °C. The hematocrit and digitonin concentrations used were as follows: (*a*) to (*e*) were all 5% hematocrit, but with 125, 100, 75, 50 and 25 μg/ml digitonin, respectively, while (*f*) was at 2.5% hematocrit and 50 μg/ml digitonin. The original records for (*a*) are displayed in Fig. 5. The lines are least-squares fits to the points

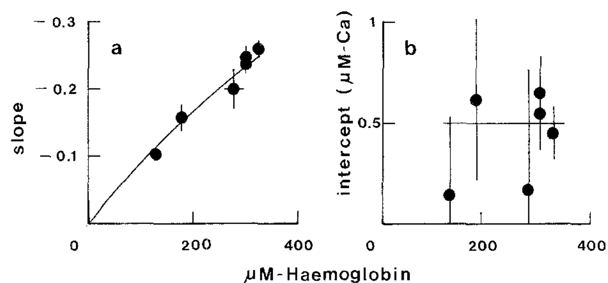

Fig. 7. Plots of (*a*) the slopes and (*b*) the intercepts on the abscissa, of the lines in Fig. 6, against the concentration of hemoglobin measured in the supernatant after centrifuging. The points are indicated ±1 SD. In (*a*) the line is drawn to fit the equation: slope $= -fh/(\alpha + fh(1-\alpha))$, with $h = 0.05$, $\alpha = 0.156$ and f (fractional hemolysis) assumed 1 for the right-hand point. In (*b*) a horizontal line has been drawn through the weighted mean value for Ca_i, 0.50 ± 0.09 μM

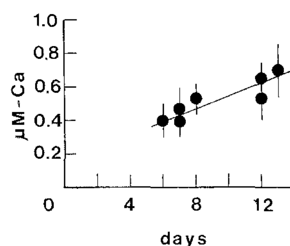

Fig. 8. Estimates of intracellular free Ca in blood-bank cells after storage at 4 °C for varying durations. Measurements were made on three different blood samples, and are shown ±1 SD. The line is a weighted least-squares fit (weighting by $(SD)^{-2}$)

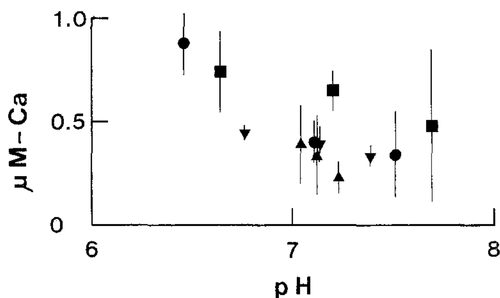

Fig. 9. Estimates of Ca_i made at different pH, all at 37 °C. The symbols refer to different batches of cells: (●, ■, ▾) blood-bank and (▲) fresh, and the results are given ±1 SD. pH was adjusted by the addition of HCl or KOH to the nominally Ca-free medium in which the cells were suspended

Measurements with Fresh Cells

Measurements were made with fresh human red blood cells on a number of occasions, but on only three of these were there sufficient observations, over a wide enough Ca range, to have confidence in the results. The values obtained, all using the author's blood, were 0.40 ± 0.06, 0.38 ± 0.13 and 0.29 ± 0.14 μM, weighted mean 0.38 ± 0.05 μM.

Ageing of Blood in Vitro

Figure 8 shows how the free Ca measured in blood-bank cells varied with the length of storage. It seems to increase with time, and this is confirmed by statistical analysis. A weighted regression line, fitted to the results, has a slope of $+0.0369 \pm 0.0085$ μM/day, which is significantly greater than zero ($0.005 < p < 0.01$, *t*-test, 5 degrees of freedom).

Effect of pH

Observations were normally made at pH 7.2. Figure 9 shows the effect of varying pH. A different

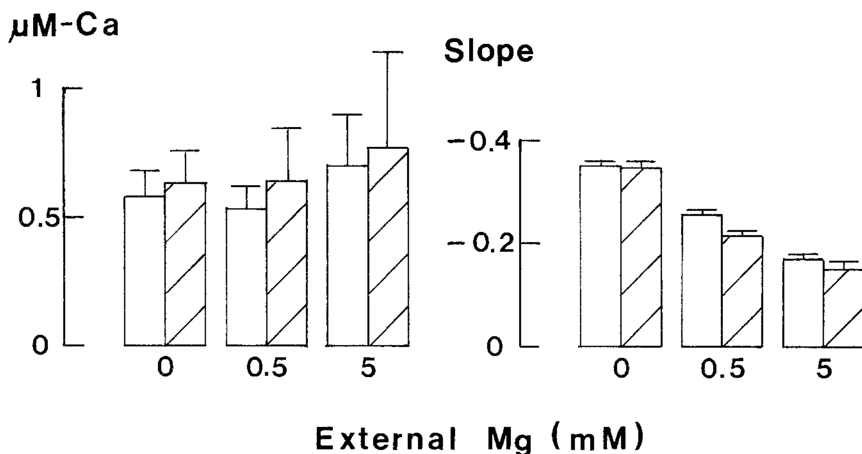

Fig. 10. Effect of varying Mg concentration. The histograms give the null-points and the slopes of the lines (+1 SD) from two experiments in which measurements were made at 0, 0.5 and 5 mM external Mg

symbol is used for each experiment, because of differences in intracellular free Ca between experiments. In every experiment, intracellular free Ca tends to decrease in alkaline solutions and increase in acid, which would be expected if hydrogen ions compete with Ca for the intracellular anions responsible for Ca buffering. It might be advantageous to put this on a quantitative basis. This is hard because of experimental scatter, but one way to do it is to consider each experiment separately, convert the Ca concentration to *p*Ca units, calculate a value for $\Delta p\mathrm{Ca}/\Delta\mathrm{pH}$, and take the weighted mean of these values. The result is $\Delta p\mathrm{Ca}/\Delta\mathrm{pH} = 0.20 \pm 0.02$.

Effect of Mg

Intracellular Ca was normally measured with 0.5 mM Mg in the external solution, close to the intracellular value of 0.4 mM estimated by Flatman and Lew (1980). Figure 10 gives the results of two experiments in which intracellular free Ca was also measured in solutions containing 0 and 5 mM Mg. It was not significantly altered by the changes in Mg, but there is a marked effect on the slopes of the null-point graphs, in the sense that the intracellular Ca buffers become less effective at buffering Ca after hemolysis when Mg is raised.

Cells Loaded with Ca

Figure 11 gives the results of an experiment in which cells were metabolically depleted, divided into five groups, and loaded with differing amounts of Ca. The graphs show that a true null-point is obtained with Ca-loaded cells, with increases in Ca_o as well as decreases when digitonin is added. This is probably because it is unnecessary to add EGTA to vary Ca_o when carrying out the null-point procedure with Ca-loaded cells. The results in Fig. 11 are analyzed by

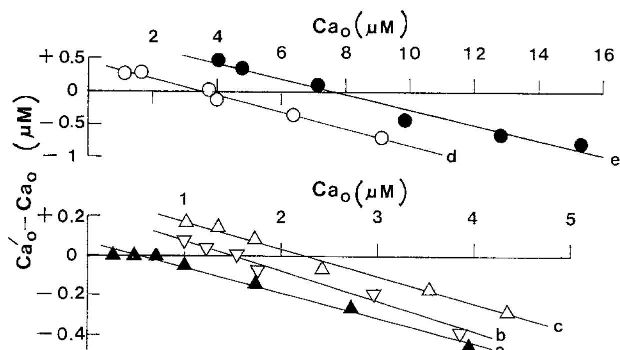

Fig. 11. Plots of digitonin-induced change in Ca($\mathrm{Ca}_o' - \mathrm{Ca}_o$) against initial Ca($\mathrm{Ca}_o$), for suspensions of Ca-loaded cells at 37 °C, 4.8 % hematocrit and pH 7.1. The scales of the two graphs are in proportion, and the lines are least-squares fits to the points. Further details are given in Table 1

fitting straight lines to the points, and the slopes of the lines and their intercepts on the abscissa are given in Table 1. Theory predicts that the slopes should be the same, and this is true within experimental error, but theory also allows α, the ionized fraction of cell Ca, to be calculated from the slopes. Total cell Ca should then be given by intracellular free Ca divided by α [Eq. (3)]. Table 1 compares these calculated values of total cell Ca with those actually measured by atomic absorption spectrophotometry. In every case, calculated Ca is less than measured Ca. The most likely explanation for this is that the cells contain tightly bound Ca that is not released by digitonin, so it does not participate in the electrode measurements.

Figure 12 shows the relationship between internal free Ca and total cell Ca (measured by atomic absorption spectrophotometry) in four experiments, including the one just described. The results are well-fitted by a straight line, corresponding to a ratio of total/free Ca of 4.7. In every experiment,

Table 1. The loading of red blood cells with Ca[a]

Cells	Symbol	External Ca or EGTA conc. while loading (mM)	Free Ca (μM) (from Fig. 11)	Slope (Fig. 11)	Total cell Ca (from Fig. 11) (μM)	Total cell Ca (μM) by atomic absorption
A	▲	0.5 EGTA	0.54 ± 0.13	-0.128 ± 0.008	1.6 ± 0.4	7
B	▽	0.1 Ca	1.50 ± 0.18	-0.152 ± 0.010	4.5 ± 0.5	12
C	△	0.2 Ca	2.24 ± 0.23	-0.137 ± 0.009	6.7 ± 0.7	16
D	○	0.5 Ca	3.56 ± 0.38	-0.128 ± 0.008	10.7 ± 1.1	27
E	●	1 Ca	7.7 ± 1.2	-0.116 ± 0.011	23.1 ± 3.7	39

[a] This table summarizes the results of an experiment in which blood-bank cells were metabolically depleted and loaded with different amounts of Ca. It gives the concentrations of Ca or EGTA in the suspension during Ca loading, the cell Ca content measured by atomic absorption spectrophotometry, and the results derived from the Ca electrode measurements on the cells, reported in detail in Fig. 11. They are expressed ± 1 SD

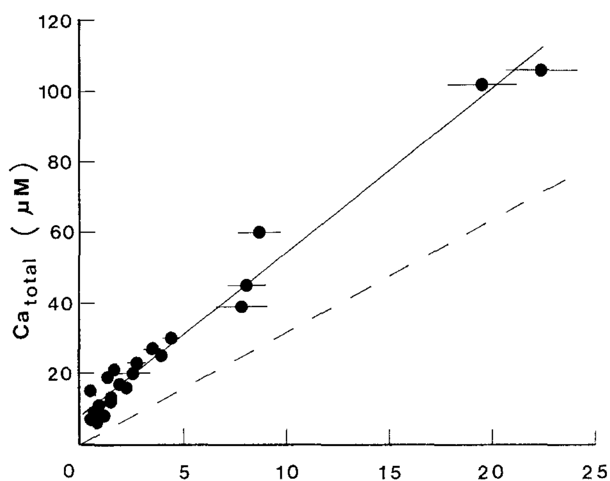

Fig. 12. Relationship between total Ca, measured by atomic absorption spectrophotometry, and free Ca, measured by the null-point technique at pH 7.1 or 7.2, in metabolically depleted red blood cells. The solid line is a least-squares fit to the points: Total Ca = 7.7 μM + 4.66 free Ca. In each experiment a value for $1/\alpha$ was calculated from the slopes of the null-point graphs. The individual mean values were 2.56, 3.00, 3.33 and 3.90. The broken line shows the overall average, representing a ratio of total/free Ca of 3.2

values of total Ca calculated from the slopes and intercepts of the null-point graphs were less than those actually measured. The average calculated value is given by the broken line, which represents a ratio of total/free Ca of 3.2. The difference between the two lines may represent tightly bound Ca.

Evidence in support of this hypothesis was often seen in electrode measurements with Ca-loaded cells. There was a rapid initial change in Ca_o after the cells were lysed with digitonin, followed by a slow upwards drift in the following 2–10 min. This happened in about half of the trials, and could represent the slow release of tightly bound Ca. When it occurred, the slow drift was ignored, and the initial rapid change in Ca_o was used to plot the null-point graphs.

Intracellular Ca Measurements with Resealed Ghosts

Another test of this technique for measuring intracellular free Ca is to apply it to resealed ghosts containing Ca buffers. This should also provide a test of the fidelity of the ghost preparations. Six batches of resealed ghosts were made, containing Ca/HEDTA buffers, and lacking ATP. Intracellular free Ca was estimated with the Ca electrode in exactly the same way as in experiments with intact cells. Figure 13 shows that straight lines fit the graphs of $(Ca_o' - Ca_o)$ against Ca_o extremely well. The inset compares the free Ca estimated by the null-point technique with the values calculated for the Ca buffers incorporated into the ghosts, showing excellent agreement.

The theory given above breaks down in this case, because the cells contain a high-affinity Ca buffer. An analytical solution is impossible, unless one assumes that the free Ca concentration is negligible compared with the bound Ca concentration, in which case the lines on Figure 13 can be predicted to have slopes of -1. The lines actually have slopes ranging from $-0.72(f)$ to $-0.91(a)$, and these values are reasonable if the simplifying assumption is not made.

^{86}Rb Efflux from Ca-Loaded Cells

Figure 14 gives the results of two experiments in which ^{86}Rb efflux was measured from Ca-loaded, metabolically depleted red blood cells, over a range of internal Ca concentrations. ^{86}Rb efflux is expressed as a rate constant, and internal free Ca was estimated by the null-point technique. The rate of efflux seems to reach a limiting value above

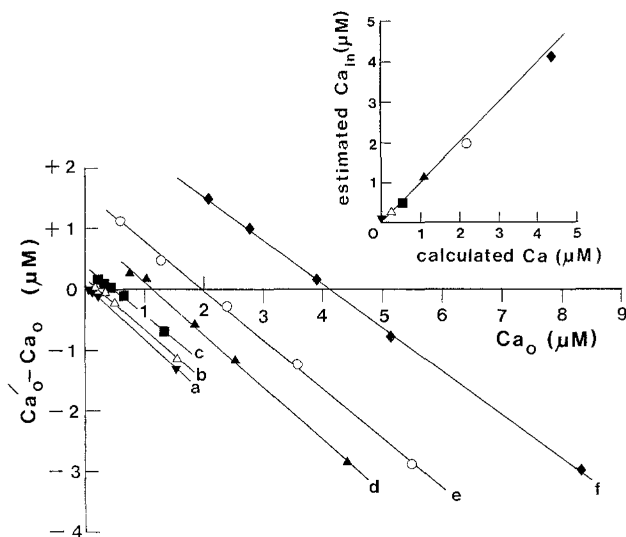

Fig. 13. Measurement of free Ca in resealed ghosts. The main graph shows plots of digitonin-induced changes in external Ca($Ca'_o - Ca_o$) against initial Ca(Ca_o), for suspensions of resealed ghosts at 6% hematocrit, 37 °C and pH 7.2 Ghosts were prepared by hemolysis of metabolically depleted blood-bank cells in solutions containing 3 mM HEDTA and 0(*a*), 0.3(*b*), 0.6(*c*), 1(*d*), 1.5(*e*) or 2 mM Ca(*f*), and resealed as described previously (Simons, 1976*a*). The inset is a plot of the null-points against the free Ca calculated for the Ca/HEDTA buffers incorporated into the ghosts. The line in the inset is the line of identity

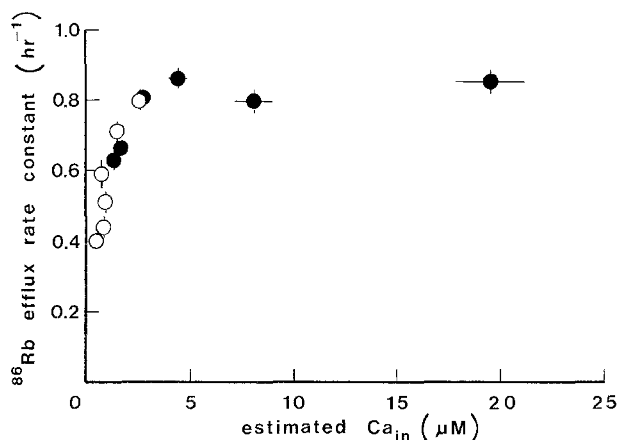

Fig. 14. ^{86}Rb efflux from Ca-loaded cells as a function of Ca_i. Blood-bank cells were metabolically depleted and loaded with varying amounts of Ca and with ^{86}Rb. The rate of ^{86}Rb efflux and internal free Ca were both measured at 37 °C and pH 7.2. The different symbols refer to two different experiments, and the results are given ±1 SD

2 μM Ca_i, and to be half-maximal at about 1 μM Ca_i. However, the lowest value seen (0.4 hr^{-1}) is much larger than the ouabain-insensitive ^{42}K efflux in fresh red blood cells (0.002 hr^{-1} in Table 2 of Simons, 1974), and may reflect an elevated K permeability caused by raised Ca levels in blood-bank cells after metabolic depletion.

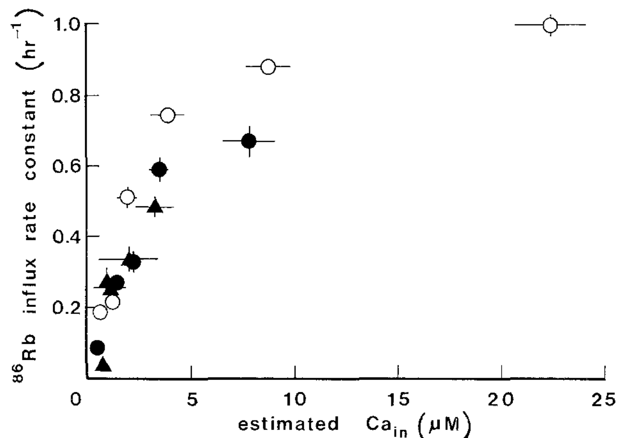

Fig. 15. ^{86}Rb influx into Ca-loaded cells as a function of Ca_i. Blood-bank (○, ●) or fresh (▲) red blood cells were metabolically depleted and loaded with varying amounts of Ca. The rate of ^{86}Rb influx and the internal free Ca were measured at 37 °C and pH 7.2. The results are given ±1 SD

^{86}Rb Influx into Ca-Loaded Cells

Figure 15 shows comparable experiments in which ^{86}Rb influx was measured with cells loaded with varying amounts of Ca. The influx seems to reach a maximum above 4 μM Ca_i, and to be half-maximal at around 2 μM Ca_i. Two of the experiments were with blood-blank cells, but the third used fresh, and in this case the ^{86}Rb influx into cells that were metabolically depleted, but not intentionally loaded with Ca, was 0.03 hr^{-1}, which seems a reasonable basal value.

Simultaneous Measurement of ^{86}Rb Influx and Efflux

In the experiments just described, influx or efflux were measured separately, on different batches of cells. The results suggest there might be differences between the curves relating ^{86}Rb influx or efflux to internal free Ca levels. This would be surprising, because ^{86}Rb is supposed to be a tracer for K, which is at equilibrium. The possibility of a discrepancy could be checked by measuring influx and efflux simultaneously. A more important reason for doing this lies in the frequent reports of heterogeneous behavior of K fluxes in red cells when the Ca-dependent K permeability mechanism is activated (Riordan & Passow, 1973; Lew & Ferreira, 1978).

Figure 16 gives the time course of ^{86}Rb influx and efflux, measured simultaneously, in batches of red cells adjusted to have low, intermediate and high levels of Ca. Cells were treated identically for influx and efflux measurements, apart from the inclusion of isotope in the final incubation, or the preincubation.

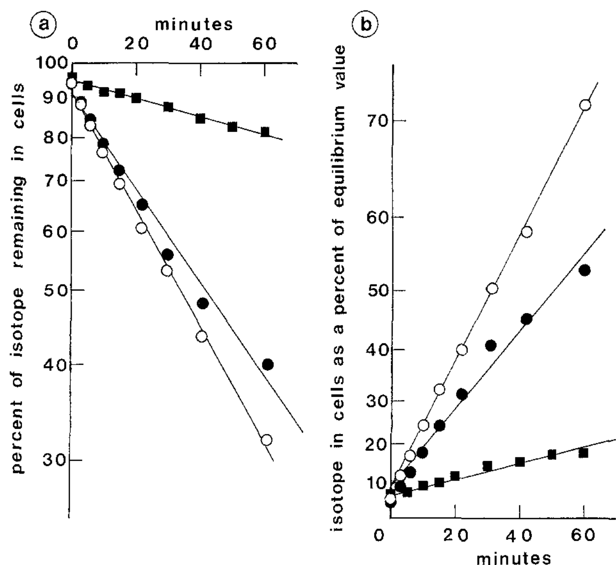

Fig. 16. Time course of (*a*) ^{86}Rb efflux and (*b*) ^{86}Rb influx out of and into metabolically depleted blood-bank cells at 37 °C. The cells were preincubated in solutions containing (■) 1 mM EGTA, (●) 0.2 mM Ca or (○) 2 mM Ca, and 150 mM K solutions were used throughout. Uptake or loss is shown on a logarithmic scale. The lines are least-squares fits to the points and have slopes (rate constants) of (■) 0.161 ± 0.006 (efflux), 0.146 ± 0.010 hr^{-1} (influx); (●) 0.86 ± 0.04 (efflux), 0.73 ± 0.04 hr^{-1} (influx); and (○) 1.06 ± 0.02 (efflux), 1.18 ± 0.02 hr^{-1} (influx)

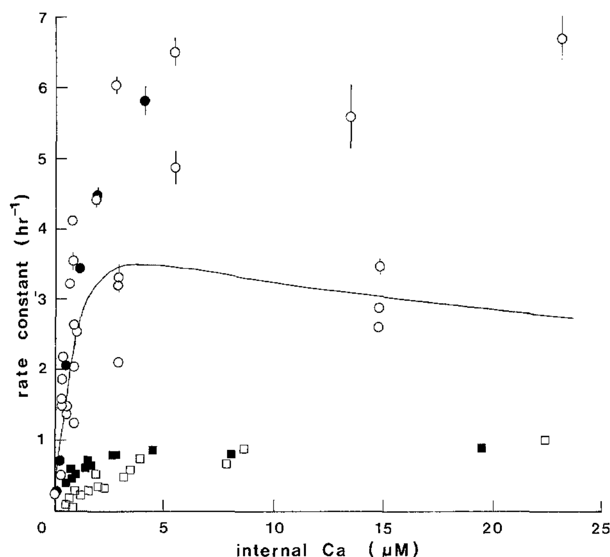

Fig. 17. Comparison between the Ca-dependence of the rate constants for ^{86}Rb influx (□) and ^{86}Rb efflux (■), taken from Fig. 14 and 15 and measured with intact cells; with those for ^{86}Rb efflux from the resealed ghosts of Fig. 13 (●) and from nine other experiments with resealed ghosts (○). The rate constants for efflux from resealed ghosts are shown ± 1 SD. The line shows the relationship between ^{42}*K efflux* from resealed ghosts and Ca_i, and is taken from Fig. 1 of Simons (1976*b*).

The results show that the appropriate logarithmic plots of intracellular isotope against time are linear, both for influx and efflux, up to 70% equilibration at the highest Ca level. They do not show flux heterogeneity of the type described previously (Lew & Ferreira, 1978). The rate constants for influx and efflux, given in the Figure legend, are in good agreement for each of the three types of cells. This is further evidence against flux heterogeneity, because cells with a higher permeability will tend to gain more isotope during loading, then lose it faster during efflux, so efflux rate constants would be larger than influx rate constants. These results make it likely that any difference between the Ca-activation curves of Fig. 14 and 15 arises through experimental variation.

Comparison of ^{86}Rb Fluxes in Intact Cells and Resealed Ghosts

The relationship between ^{42}K efflux and Ca_i has been investigated in detail with resealed ghosts (Simons, 1976*a, b*). In this paper ^{86}Rb is used as a tracer for K in experiments with intact cells. Figure 17 compares the Ca dependence of ^{86}Rb fluxes in intact cells with the ^{86}Rb efflux from the resealed ghosts used in the experiment illustrated in Fig. 13, together with nine other experiments with resealed ghosts, from earlier work. In the ghost experiments, internal and external K concentrations are both 100 mM, while with intact cells, internal K is about 100 mmole/liter cells (150 mmole/liter cell water), and external K is 150 mM. Experiments with resealed ghosts show that the ^{42}K efflux rate constant is virtually unaffected by varying the K concentration in the 0 to 200 mM range (Simons, 1976*b*). The comparison in Fig. 17 shows a marked difference between the rate constant for ^{86}Rb efflux at saturating levels of Ca, with the rate 3 to 6 times faster in the ghosts than in cells. There is considerable variation between the rates in the different experiments with resealed ghosts. The reason for this is not known: it was not seen to such an extent in experiments with ^{42}K (Simons, 1976*a*), so it may be caused by the use of ^{86}Rb as a tracer. Whatever the explanation, the difference between the rates in the cells and the ghosts is quite clear. However, there is little difference between the Ca concentration required for half-maximal stimulation of ^{86}Rb movements: it is about 1 μM in either case.

Discussion

Measurement of Intracellular Free Ca

The results described in this paper represent the first attempt to measure intracellular free Ca in human red blood cells. They are inevitably subject to con-

siderable uncertainty. The measurements take 40 to 60 min on each batch of cells, which makes it impossible to follow rapid changes in Ca_i. This is mainly caused by the slowness of the electrode response. The use of the Ca electrode brings with it doubts about its calibration. The electrode response appears to depend on the Ca buffering power of the solution, such that the free Ca is likely to be overestimated in poorly buffered solutions (Fig. 2). This would have the effect of causing the free Ca values reported in this paper to be overestimates, with the effect worst at the lowest concentrations. The magnitude of the error is uncertain, because the calibration error in suspensions of cells and hemolyzed cells is unknown. It should not be assumed that this difficulty could be avoided by the use of an indicator dye like arsenazo III. Light scattering precludes its use in cell suspensions, and it is reported to be bound avidly by cell constituents (Beeler, Schirbeci & Martonosi, 1980).

Mammalian red blood cells contain no Ca-sequestering organelles. Digitonin makes the membrane freely permeable, and allows mixing of intracellular and extracellular fluid. The change in Ca_o should in theory be proportional to the difference between Ca_o and Ca_i, and observations are in agreement with this. A null-point exists at which Ca_o and Ca_i are equal. Unfortunately, with normal cells, EGTA has to be added to the suspension to reach free Ca concentrations in the vicinity of the null-point. This distorts the results, greatly reducing the increase in Ca_o that should occur when Ca_o is less than Ca_i. If the use of EGTA is avoided, the null-point has to be estimated by extrapolation, and this leads to relatively large errors at low concentrations of Ca_i. It is noteworthy that Murphy et al. (1980) failed to see any distortions of null-points in their work with hepatocytes, even though they used EGTA. This may be because the change in free Ca when hepatocytes are treated with digitonin is not just caused by the mixing of intracellular and extracellular fluids, but also includes the movement of Ca into and out of organelles.

The measurements of Ca_i might also be overestimates if digitonin releases tightly bound Ca from the membrane. This is unlikely because digitonin fails to release a substantial fraction of the total Ca in the cells (Table 1 and Fig. 12). This Ca must be bound to the cell membranes. Porzig and Stoffel (1978) have also shown that saponin, a molecule with a very similar action to digitonin, actually induces a small *increase* in the number of high-affinity Ca-binding sites on the red cell membrane under isotonic conditions.

The values for intracellular free Ca reported in this paper – 0.4 μM in fresh red cells, and 0.4 to 0.7 μM in flood-bank cells – are substantially higher than those reported for other cells, for example 0.10 μM for squid axons (Baker, 1976), 0.19 μM for hepatocytes (Murphy et al., 1980) and 0.12 μM for lymphocytes (Pozzan, Rink & Tsien, 1981). This would be surprising if it were true, as all cells must depend on their Ca pumps to maintain a low intracellular free Ca, and the human red cell has an exceedingly active Ca pump (Schatzmann, 1975). 0.4 μM Ca should be regarded as an upper limit, because of the errors involved. Increases in H ion concentration tend to increase the null-point Ca in red cells, although increases in Mg do not, within experimental error. The converse was seen by Murphy et al. (1980) in liver cells. There is no obvious explanation for the difference in findings. Changes in the composition of the external solution should only be able to affect Ca_i if the internal solution changes as well: for red cells this is true of H but not Mg ions. If the measured value of Ca_i is affected by changes that occur after the cells are lysed with digitonin, then it is not a true null-point determination.

Buffering Power of Red Cell Cytoplasm and Distribution of Cell Ca

Ferreira and Lew (1976, 1977) studied Ca binding in human red cells by rendering the membrane selectively permeable to Ca (and Mg) with A23187. They found binding to be linear in Ca concentration, up to about 1 mM free Ca, with a slope, α (the fraction of cell Ca that is ionized) = 0.2 to 0.45. ATP-depletion had no significant effect on this value, which was measured in the presence of 1 mM external Mg, and probably a higher internal free Mg concentration. The results in this paper agree in that Ca binding is linear up to a concentration of about 150 μM, but suggest that the slope varies considerably with the conditions. Direct measurement of binding in a hemolysate of fresh red cells in 0.5 mM-Mg medium gives $\alpha = 0.10$ (Fig. 4). α can also be calculated from the slopes of null-point graphs. Blood-bank cells give a value of 0.16 in 0.5 mM-Mg (Figs. 6 and 7), although Ca-binding is much stronger in 0-Mg ($\alpha = 0.10$) and weaker in 5 mM-Mg solutions ($\alpha = 0.26$; calculated from Fig. 10). On the other hand, estimates of α are in the range of 0.26 to 0.39 with metabolically depleted blood-bank cells (Fig. 12) and 0.09 to 0.10 with fresh cells, in a 0.5 mM-Mg medium. Although these measurements are rather indirect, the variations in different conditions are probably too large to be explained by chance.

It should be noted that all the measurements reported here, together with those of Ferreira and

Lew (1976, 1977) actually refer to the rapidly exchangeable fraction of cell-bound Ca, which may include membrane-bound Ca as well as cytoplasmic Ca. The distribution and exchangeability of red cell Ca is a confused and uncertain subject. Harrison and Long (1968) found that saline-washed fresh human red cells contain on average 16 μmole Ca/liter cells, but four washes with a solution containing 5 mM of a strong chelating agent reduces this to about 1.8 μmole/liter cells. They also found that the Ca content of ghosts obtained by hypotonic hemolysis is the same as that of intact cells, within experimental error. This has been widely misinterpreted to mean that all of the cell Ca is bound to the membrane, which neglects the increase in Ca binding caused by a reduction in ionic strength. In the present work, the cells were always washed once in a solution containing 1 mM EGTA. This was necessary in order to have a reasonably low basal level of Ca_o, but inevitably leads to the loss of an unknown quantity of "loosely bound" Ca. Prolonged washes with EGTA were not carried out, because it would not be desirable to lose too much Ca from Ca-loaded cells. The experiments with Ca-loaded cells show that not all the cell Ca participates in the redistribution when digitonin is added (Fig. 12). The nonparticipating fraction may be interpreted as "tightly bound" Ca, but if this is correct, the binding must be loose enough for the amount to vary with the Ca content of the cells. Most of the Ca in fresh red cells is not exchangeable with ^{45}Ca (*see*, for example, Bookchin & Lew, 1980), and it is possible that this might be the same fraction.

Dependence of Potassium Permeability on Intracellular Ca

Most of the work on the Ca-dependent K channel has involved measurements of net K loss from red cells suspended in low-K solutions. In these experiments, the K fluxes change continuously with time, and it is not possible to estimate permeabilities. Measurements of ^{42}K fluxes under exchange conditions have been made in energy-depleted resealed ghosts containing Ca buffers (Simons, 1976*a, b*) and in A23187-treated intact cells (Lew & Ferreira, 1976, 1978). In resealed ghosts, there is a high affinity for Ca (half-maximal velocity at 0.4 μM Ca_i with 0-Mg), and a maximum rate of about 3.5 hr^{-1}, while in A23187-treated cells, there is a low affinity for Ca (half-maximal at 1 mM Ca_i) in the presence of metabolic substrates and a low ionophore concentration, but a high affinity otherwise (depleted cells or a high ionophore concentration), and the maximum velocity varies between about 4 hr^{-1} and 10 hr^{-1} in the different conditions. The present results, in which ^{86}Rb is used as a tracer, show that metabolically depleted intact cells still have a high affinity for Ca in the absence of A23187 (half-maximal at about 1 μM Ca_i with about 0.5 mM free internal Mg), but the maximum rate of exchange is very low, about 1 hr^{-1}. This low rate is not caused by cell heterogeneity, nor does it seem to be due to the use of ^{86}Rb as a tracer. The most likely explanation is that the Ca-dependent K permeability mechanism does not have a fixed maximum rate of transport. It might be variable, dependent on such factors as ghosting the cells, or treating them with A23187. A physical explanation might be variations in the mean duration of opening of a channel, and the recent observation of "single-channel" K conductances in human red cell membranes (Hamill, 1981) may be pertinent. Further support for this suggestion comes from the observations of pharmacological effects of propranolol (Simons, 1980) and reducing agents (Garcia-Sancho, Sanchez & Herreros, 1979) on the maximum rate of transport.

The free Ca measured in fresh red cells (0.38 μM) is not consistent with the upper limit estimated earlier (0.25 μM, Simons 1976*a*) from the requirement that intracellular free Ca should not be sufficient to stimulate the K permeability above its resting value. That estimate depended upon the assumption that the properties of the Ca-dependent K channel are the same in fresh red cells as in resealed ghosts, which seems most unlikely on the evidence now available. It should also be noted that the figure of 0.38 μM is equivalent to 0.30 μM Ca in the earlier work, because of the revised ionized Ca values used in this paper.

I thank the Medical Research Council of Great Britain for financial support, the Director of the South London Blood Transfusion Centre and Professor M.W.B. Bradbury for help in supplying blood, and Professor P.F. Baker, Dr. V.L. Lew, Dr. R.J. Naftalin and Dr. T.J. Rink for helpful advice and suggestions.

References

Baker, P.F. 1976. The regulation of intracellular calcium. *Symp. Soc. Exp. Biol.* **30**:67–88

Becker, G.L., Fiskum, G., Lehninger, A.L. 1980. Regulation of free Ca^{2+} by liver mitochondria and endoplasmic reticulum. *J. Biol. Chem.* **255**:9009–9012

Beeler, T.J., Schirbeci, A., Martonosi, A. 1980. The binding of arsenazo III to cell components. *Biochim. Biophys. Acta* **629**:317–327

Bergmeyer, H.U. 1965. Methods of Enzymatic Analysis. p. 555. Second printing. Academic Press, New York and London

Bookchin, R.M., Lew, V.L. 1980. Progressive inhibition of the Ca pump and Ca:Ca exchange in sickle red cells. *Nature (London)* **284**:561–563

Carruthers, A., Simons, T.J.B. 1978. Calcium and ionophore A23187 stimulate sugar transport in pigeon red cells. *J. Physiol.* (*London*) **284**:49P

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* (*London*) **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. pp. 53–91. J.C. Ellory and V.L. Lew, editors. Academic Press, London

Fiskum, G., Craig, S.W., Becker, G.L., Lehninger, A.L. 1980. The cytoskeleton of digitonin-treated rat hepatocytes. *Proc. Natl. Acad. Sci. USA* **77**:3430–3434

Flatman, P.W., Lew V.L. 1980. Magnesium buffering in intact human red blood cells measured using the ionophore A23187. *J. Physiol.* (*London*) **305**:15–30

Garcia-Sancho, J., Sanchez, A., Herreros, B. 1979. Stimulation of monovalent cation fluxes by electron donors in the human red cell membrane. *Biochim. Biophys. Acta* **556**:118–130

Gardos, G. 1959. The role of calcium in the potassium permeability of human erythrocytes. *Acta Physiol. Hung.* **15**:121–125

Hamill, O.P. 1981. Potassium channel currents in human red blood cells. *J. Physiol.* (*London*) **319**:97*P*–98*P*

Harrison, D.G., Long, C. 1968. The calcium content of human erythrocytes. *J. Physiol.* (*London*) **199**:367–381

Lew, V.L., Ferreira, H.G. 1976. Variable Ca sensitivity of a K-selective channel in intact red cell membranes. *Nature* (*London*) **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. *Curr. Top. Membr. Transp.* **10**:217–271

Murphy, E., Coll, K., Rich, T.L., Williamson, J.R. 1980. Hormonal effects on calcium homeostasis in isolated hepatocytes. *J. Biol. Chem.* **255**:6600–6608

Porzig, H., Stoffel, D. 1978. Equilibrium binding of calcium to fragmented human red cell membranes and its relation to calcium-mediated effects on cation permeability. *J. Membrane Biol.* **40**:117–142

Pozzan, T., Rink, T.J., Tsien, R.Y. 1981. Intracellular free Ca^{2+} in intact lymphocytes. *J. Physiol.* (*London*) **318**:12P–13P

Riordan, J.R., Passow, H. 1973. The effect of calcium and lead on the potassium permeability of human erythrocytes and erythrocyte ghosts. *In:* Comparative Physiology. pp. 543–581. L. Bolis, K. Schmidt-Nielsen and S.H.P. Maddrell, editors. North-Holland Publishing Co., Amsterdam

Schatzmann, H.J. 1975. Active calcium transport and Ca^{2+}-activated ATPase in human red cells. *Curr. Top. Membr. Transp.* **6**:125–168

Simons, T.J.B. 1974. Potassium:potassium exchange catalysed by the sodium pump in human red cells. *J. Physiol.* (*London*) **237**:123–155

Simons, T.J.B. 1976*a*. The preparation of human red cell ghosts containing calcium buffers. *J. Physiol.* (*London*) **256**:209–225

Simons, T.J.B. 1976*b*. Calcium-dependent potassium exchange in human red cell ghosts. *J. Physiol.* (*London*) **256**:227–244

Simons, T.J.B. 1980. Effect of propranolol on calcium-dependent potassium permeability of red cell membranes. *J. Physiol.* (*London*) **300**:35P–36P

Simons, T.J.B. 1981. Measurement of free Ca^{2+} in red blood cells. *J. Physiol.* (*London*) **318**:38P–39P

Tsien, R.Y., Rink, T.J. 1980. Neutral carrier microelectrodes for measurement of intracellular free calcium. *Biochim. Biophys Acta* **599**:623–638

Received 5 October 1981