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Intracellular Calcium Content of Human Erythrocytes: Relation to Sodium Transport Systems

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Summary. To study the possible role of intracellular Ca (Ca_i) in controlling the activities of the Na^+-K^+ pump, the Na^+-K^+ cotransport and the Na^+/Li^+ exchange system of human erythrocytes, a method was developed to measure the amount of Ca embodied within the red cell. For complete removal of Ca associated with the outer aspect of the membrane, it proved to be essential to wash the cells in buffers containing less than 20 nM Ca. Ca was extracted by $HClO_4$ in Teflon® vessels boiled in acid to avoid Ca contaminations and quantitated by flameless atomic absorption. Ca_i of fresh human erythrocytes of apparently healthy donors ranged between 0.9 and 2.8 μmol/liter cells. The mean value found in females was significantly higher than in males. The interindividual different Ca contents remained constant over periods of more than one year. Sixty to 90% of Ca_i could be removed by incubation of the cells with A23187 and EGTA. The activities of the Na^+-K^+ pump, of Na^+-K^+ cotransport and Na^+/Li^+ exchange and the mean cellular hemoglobin content fell with rising Ca_i; the red cell Na^+ and K^+ contents rose with Ca_i. Ca depletion by A23187 plus EGTA as well as chelation of intracellular Ca^{2+} by quin-2 did not significantly enhance the transport rates. It is concluded that the large scatter of the values of Ca_i of normal human erythrocytes reported in the literature mainly results from a widely differing removal of Ca associated with the outer aspect of the membrane.

Key Words Na^+-K^+ pump · Na^+-K^+ cotransport · Na^+/Li^+ exchange · Ca depletion

Introduction

The first thorough analysis of the intracellular calcium content (Ca_i) of human erythrocytes was performed by Harrison and Long in 1968 [20]. These authors reported a mean value of 16 μmol/liter cells for the total Ca content of normal red blood cells. About 90% of the total Ca was removed by washing the cells in media containing 5 mM EDTA, indicating that only 1.6 of the 16 μmol/liter cells are located intracellularly, the remainder being bound to the outer aspect of the membrane. This finding demonstrated that one has to distinguish between total "cellular" Ca content, including intracellular Ca and a fraction associated with the extracellular half of the membrane, and the "intracellular" Ca embodied within the cell.

Since then, several studies on total red cell Ca content were performed reporting values ranging between 0.8 and 40 μmol Ca/liter cells. In studies in which the washing solutions were purified from Ca contaminations by prior passage over chelex-100 and/or in which Ca chelators were added to the washing solutions [3, 20, 26, 32, 43–45] lower Ca contents (0.8 to 19 μmol/liter cells) were obtained than in those where such precautions seemingly were not taken (6 to 40 μmol/liter cells, ref. 11, 12, 16, 31, 37–39). These findings indicate that the Ca concentrations in solutions nominally free of Ca, ranging between 0.2 and 10 μM, may cause a substantial contamination of the intracellular Ca by extracellular Ca. At a Ca concentration of 10 μM, for instance, the external high-affinity Ca binding sites (exhibiting binding constants of 6×10^4 [27] to 1.6×10^6 liter/mole [5]) would be saturated with Ca by 40 to 96%. If the capacity of these binding sites is taken to be 16 μmol/liter cells [27], about 6.4 to 15.4 μmol Ca/liter cells would be bound to the outer aspect of the membrane at 10 μM Ca in the medium. The conclusion to be drawn from these considerations is that many of the data reported in the literature for the total red cell Ca content probably include a considerable fraction of Ca not located intracellularly but rather associated with the outside of the membrane.

Most of the Ca present inside the cells is presumably bound to the inner aspect of the membrane [16, 26]. Binding sites include unspecific low-affinity sites (10^3 [33]), phospholipids, the Ca pump (10^6 [1]), the Na^+-K^+ pump (10^5 [4, 11]), proteins phosphorylated in the presence of ATP (6×10^7 [33]), and the resealing site (2×10^6 [33]) (binding constants given in liter/mole in parentheses). Cytosolic binding sites are calmodulin (2×10^6 [7]), ATP (10^4

[40]) and other Ca chelators such as 2,3-DPG (10^3 [6]). Possibly, a fraction of total intracellular Ca is stored in intracellular vesicles [24].

The cytosolic concentration of ionized Ca^{2+} in human red blood cells is maintained below 300 nM by the action of the ATP-dependent Ca pump; at higher concentrations the Ca^{2+}-dependent K^+ channel opens [21, 35, 41]. By an indirect method, an upper estimate of 400 nM for the free Ca^{2+} concentration in normal human erythrocytes was obtained by Simons [42]. Murphy et al. [30] and Lew et al. [25] reported values as low as 61 and 26 nM, respectively. Accordingly, ionized Ca^{2+} comprises only a small fraction of total intracellular Ca.

In the present paper an attempt is made to determine the amount of Ca embodied within the interior of normal erythrocytes (Ca_i) and not contaminated by Ca associated with external high-affinity Ca binding sites.

Intracellular Ca is known to inhibit a number of red cell ion transport systems, including the Na^+-K^+ pump [4], the Na^+-K^+ cotransport system [17] and the anion exchanger [29]. Accordingly, it could well be that the interindividually different activities of the Na^+-K^+ cotransport and the Na^+-Li^+ exchange system in human erythrocytes [9] may be related to the individual Ca content. Therefore, the relation between the individual Ca contents and the Na^+ transport rates was examined, as well as the potential effect of a Ca depletion on the transport systems.

Preliminary accounts of this work have appeared elsewhere [13, 14].

Materials and Methods

Materials

Ca contaminations of the plastic tubes, vessels, bottles and pipettes used [Teflon® (PFA or PTFE) and polypropylene] were removed by prior boiling in 3 N HCl and extensive washing. Teflon tubes and vessels were obtained from Reichelt, Heidelberg, and from Bohlender, Lauda-Königshofen. The water used for washing and preparing the solutions was distilled twice over quartz, its Ca content being below the detection limit of 10 nM.

Chelex-100 (Na-form, 200 to 400 mesh) was from BioRad, Munich, A23187, quin-2-acetoxymethylester, and fatty acid-free serum albumin were from Sigma, Deisenhofen, and all other chemicals were obtained from Merck AG, Darmstadt.

Methods

Blood was anaerobically drawn from antecubital veins of apparently healthy donors without using a tourniquet into plastic syringes and anticoagulated by 5 IU heparin/ml. Whole blood was analyzed for hemoglobin content (cyanmethemoglobin method) and hematocrit, and the mean cellular hemoglobin content (MCHC) was calculated. The blood samples were immediately centrifuged in Teflon tubes (10 ml, PTFE) for 5 min at 4°C (4500 × *g*). Plasma was removed for analysis of ionized calcium (measured together with plasma pH at 37°C using a Radiometer ICA 1, the values being corrected for a pH of 7.4) and total plasma Ca (atomic absorption).

The buffy coat was carefully aspirated together with the most upper layer of red cells. 2.5 ml of packed cells were washed seven times within 60 min at 4°C with a 10-fold excess of a 145 mM NaCl (suprapur) −5 mM glycylglycine (gly-gly) buffer (pH 7.4 at room temperature) in 30-ml Teflon vessels (PFA, inner diameter 25 mm, closed by screw caps). Glycylglycine was used as buffer because its Ca content was much lower than that of other buffers (e.g., morpholino propane-sulfonic acid, tris hydroxy-methyl-aminomethane or Na-phosphate). The calcium content of the NaCl-gly-gly buffer was lowered from 200 nM to less than 20 nM (usually 15 nM) by a prior passage over a chelex-100 column as described below.

Removal of Ca from the NaCl-gly-gly Buffer

The chelex-100 column was processed essentially as described by Blinks et al. [2]. Briefly, the resin was washed twice with 1 N NaOH, once with 1 N HCl, five times with water, twice with 1 N NaOH, once with 1 N HCl, and finally twice with water (room temperature). The pH of the resin suspension was then adjusted to 8.3 using 1 N NaOH previously passed over the resin. A 12-cm column was formed (inner diameter: 2 cm) and washed with about 200 ml of the 145 mM NaCl (suprapur) −5 mM gly-gly buffer until the pH of the eluate dropped to 7.4. Then about 1 liter of the buffer was collected and stored at 4°C in polypropylene bottles. The resin was subsequently regenerated as described above.

Perchloric Acid Extraction of Red Cells

The washed cells were suspended in the purified NaCl-gly-gly buffer at a hematocrit of 0.65 (0.5 to 0.7) and the hemoglobin concentration of the suspension was determined. In the *standard procedure*, six 300-µl aliquots were transferred to round-bottom Teflon tubes (8 ml, PFA, inner diameter 9 mm, closed by screw-caps). In three of the tubes the red cells were hemolyzed by adding 1 ml of water and vortexing. Subsequently, 3 ml of 1 N $HClO_4$ (suprapur) was added to each of the six tubes. The tubes were vortexed and left standing for 30 min. After 15 min of centrifugation at 4500 × *g* the supernatants were decanted into 2-ml Teflon vessels (PTFE) which were sealed using Parafilm. All procedures were carried out at room temperature.

Ca Determinations

The Ca content of the perchloric acid extracts was determined at 422.7 nm by flameless atomic absorption (Perkin Elmer 420, graphite cell HGA 76). Fifty-µl samples were injected into pyrolytic graphite cuvettes using the sample injector ASI. The Teflon vessels containing the extract were opened only for about 30 sec and closed thereafter because prolonged opening caused a substantial Ca contamination by air-borne Ca. Prior to the measurements the graphite cuvette was heated several times at 2700°C for 5 sec until the absorption reached a zero value. The samples

were heated to 180°C within 16 sec and dried at 180°C for 25 sec, heated to 1250°C within 90 sec and washed at that temperature for 20 sec. The extracts were finally atomized at 2650°C for 5 sec at a reduced gas flow and the maximum peak height of absorption was recorded. The inert gas used was purified nitrogen. Prior to the next measurement, the cuvette was heated at 2700°C for 5 sec to remove any residual Ca.

Ca standard solutions were prepared from Ca Titrisol (Merck 9943) by dilution with the 1 N $HClO_4$ (suprapur) used for extraction of the cells and stored in polypropylene bottles.

The Ca content of the 1 N $HClO_4$ (suprapur) was about 40 nM, i.e., more than ten times lower then that of trichloracetic acid, HNO_3 and H_2SO_4 of the highest purity available. The standard curve for Ca was linear between 15 and 200 nM, encompassing the entire range of Ca concentrations in the perchloric acid extracts of normal erythrocytes. A 125-nM Ca standard gave an average absorption of 0.074 ± 0.010 (SD) after subtraction of the background value of 0.024 ± 0.007 resulting from the Ca present in the 1 N perchloric acid. The measurements on each extract were done in duplicates (mean deviation within the duplicates ± 5.3%). The standard curve (0, 62.5, 125 and 250 nM added Ca in $HClO_4$) was repeated after every fourth duplicate to take account of a possible deterioration of the graphite cuvette. The cuvettes were usually discarded after 60 measurements. Within the triplicate extracts of each, the hemolyzed and the nonhemolyzed cells, the standard deviation accounted to ± 6.4%. The triplicate of the hemolyzed cells, yielding about a 25% reduction of the absorption relative to the nonhemolyzed cells, served as an internal control. The red cell Ca contents calculated from the two triplicates corresponded to each other within ± 7.2%. Single values in the triplicates exceeding the other two by more than 20% were considered to contain Ca contaminations and were excluded. The data given in Results are mean values of the two triplicates.

The perchloric acid background value was subtracted from the absorption of the extracts, and the red cell Ca contents were calculated taking into account the standard curve, the dilution and the MCHC of the original cells.

Duplicate determinations of red cell Ca content of individual donors coincided within ± 9.7% when done on the same day, and within ± 7.9% when performed at subsequent days (Fig. 3). The recovery of Ca added to the cell suspensions prior to perchloric acid extraction was 96.5 ± 6.4%.

Determination of the Activities of Red Cell Na^+ Transport Systems

The activities of red cell cation transport systems were determined as previously described [9]. In brief, the Na^+-K^+ pump was assessed by measuring ouabain-sensitive Rb^+ uptake in 145 mM Na^+-5 mM Rb^+ media. Furosemide-sensitive Rb^+ uptake in the same media containing ouabain served as a measure of Na^+-K^+ cotransport. Na^+/Li^+ exchange was determined as phloretin-sensitive Li^+ uptake in isotonic 75 mM Mg-sucrose media (0.5 mM phloretin). The Na^+ and K^+ leaks were assessed by measurements of Na^+ and Rb^+ uptake in the presence of ouabain (0.2 mM) plus furosemide (0.5 mM). All media contained 5 mM glucose, 1 mM inorganic phosphate and 10 mM trishydroxy-methyl-aminomethane-morpholino-propanesulfonic acid buffer (pH 7.4 at 37°C). Transport rates and red cell Na^+ and K^+ contents refer to the MCHC of the original cells. The data for Na^+/Li^+ exchange and for Na^+-K^+ pump rates given in Fig. 5 and Table 3 are corrected for the interindividually different concentrations of Na^+ in cell water by assuming simple Michaelis-Menten kinetics and apparent K_m values of 10 mM intracellular Na^+ for both transport systems.

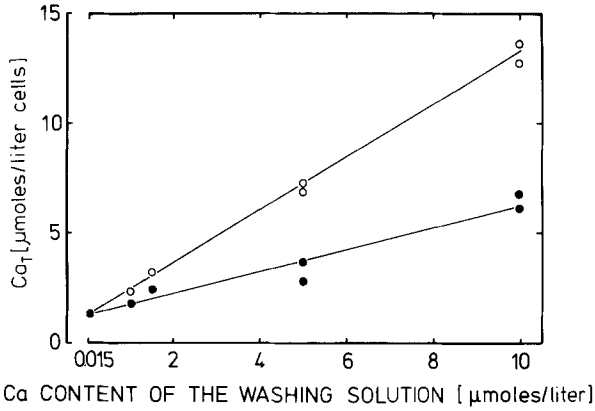

Fig. 1. Dependence of the total red cell Ca content (Ca_T) on the Ca concentration of the washing solution. Ca contents were determined according to the standard procedure on cells washed in the Na-gly-gly buffer passed over the chelex-100 column (about 15 nM Ca) containing increasing amounts of added Ca. The hematocrit of the cell suspensions extracted with $HClO_4$ was 0.5 to 0.7. Single values obtained in three experiments on red cells of one donor. Open circles: Uncorrected (assuming that the washing buffer is free of Ca). Filled circles: Corrected for the Ca contamination of the washing solution by subtracting $(1\text{-hct})hct^{-1} \cdot Ca_o$ from the measured values (hct = hematocrit of the cell suspension subjected to perchloric acid extraction)

Statistics

Statistical analyses were performed by unpaired Students *t*-test and linear regression analysis. *P* values exceeding 0.05 were considered not significant.

Results

Intracellular Red Cell Calcium Content (Ca_i)

To remove Ca reversibly bound to the outer aspect of the membrane, red cells were washed several times in a 10-fold excess of a sodium-gly-gly buffer containing less than 20 nM Ca after passage over a chelex-100 column. The washings were performed at 4°C to minimize Ca extrusion from the cells by the ATP-dependent Ca pump. After five washings, a baseline Ca content was obtained which did not further decrease with subsequent washings. Therefore, the cells were washed seven times in the standard procedure.

The effect of the Ca concentration in the washing buffer on the total Ca content determined with a 50 to 70% red cell suspension is shown in Fig. 1.

Table 1. Ca_i is not affected by adding 1 mM EGTA to the washing buffer during the last three washings in the standard procedure

Donor	Sex	Ca_i (μmol/liter cells)	
		Washing solution − EGTA	Washing solution + EGTA
1	♂	1.28	1.28
2	♂	1.34	1.17
3	♂	1.70	2.00
4	♀	1.77	1.42
5	♂	2.19	2.07
6	♀	2.25	2.30
7	♂	2.33	2.00
Mean values (±1 SD)		1.84 (±0.43)	1.75 (±0.45)

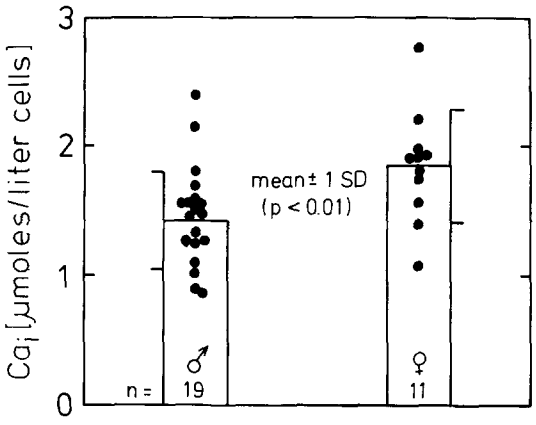

Fig. 2. Ca_i in 30 apparently healthy donors. The 19 male and 11 female individuals were matched with respect to age (39 ± 12 and 38 ± 13 years, respectively)

The upper curve depicts the results obtained assuming that all of the Ca present in the extracts appertains to the red cells. Obviously, the total Ca content calculated under this assumption increased from 1.3 to 13 μmol/liter cells upon elevation of the Ca concentration from 15 nM to 10 μM. When the Ca present in the washing buffer was subtracted (lower curve in Fig. 1) the red cell Ca content increased from 1.3 to 6.5 μmol/liter, indicating that the high affinity binding sites on the outside of the red cell membrane [27] can bind substantial amounts of Ca even at low extracellular Ca concentrations. The two regression lines of Fig. 1 converge at 15 nM Ca in the washing buffer, a concentration where the contribution of the contaminating Ca in the medium to total red cell Ca is less than 1%.

To exclude that a substantial fraction of the Ca associated with the erythrocytes washed in a medium containing 15 nM Ca is still bound to the outer side of the membrane, either 1 mM Mn^{2+}, Co^{2+} or Mg^{2+} was added to the washing buffer to displace extracellular Ca. Addition of the divalent cations did not reduce the Ca content (*data not shown*). Lowering the free Ca^{2+} concentration from 1.5×10^{-8} M to about 10^{-13} M by addition of 1 mM EGTA to the washing buffer (assuming an apparent dissociation constant $K' = 10^{-7.3}$ of the Ca-EGTA complex [35]), did not reduce significantly the red cell Ca content of seven donors (Table 1). Accordingly, essentially all Ca reversibly bound to the outer aspect of the red cell membrane is removed in the standard procedure.

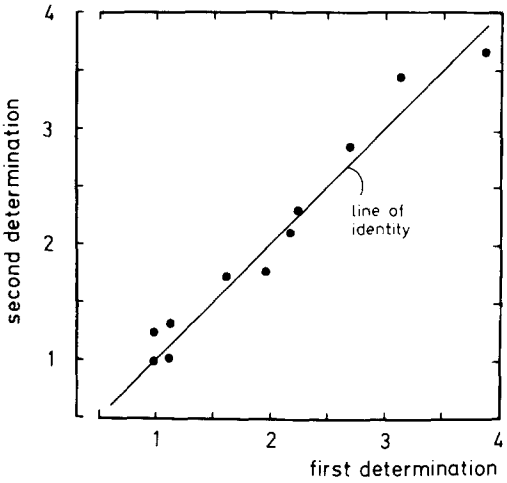

Fig. 3. Reproducibility of the determination of Ca_i (μmol/liter cells) in repeated determinations. The second determination was performed two weeks to fifteen months after the first determination (mean 5.2 months). The three individuals with the highest Ca_i were two women taking oral contraceptives and one woman suffering from an ovarian cyst. The mean reproducibility was ±7.9 (±5.9 SD) %

Ca_i in erythrocytes from 30 apparently healthy donors varied by a factor of three between 0.88 and 2.77 μmol/liter cells. Male subjects exhibited a significantly lower mean Ca_i (1.42 ± 0.38) than females taking no contraceptives (1.85 ± 0.44) (Fig. 2). In both groups, there was a tendency for an increase of Ca_i with age, the trends, however, being not significant.

The interindividually different values for Ca_i remained constant within ± 8% on repeated determinations over more than one year (Fig. 3).

There was a slight trend towards a lowering of Ca_i at higher values of total and ionized plasma Ca. However, the negative correlations seen between total and ionized plasma Ca and Ca_i were not significant (*data not shown*).

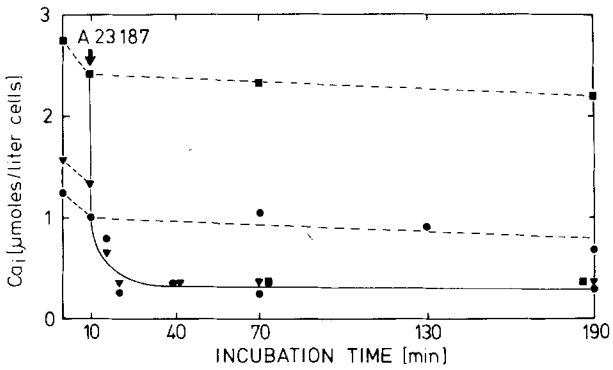

Fig. 4. Time-course of Ca_i depletion during incubation of red cells in a 140 mM Na-5 mM gly-gly buffer containing 5 mM glucose, 0.2 mM Mg, 1 mM EGTA, without (broken lines) and with (solid line) 10 μM A23187 added after 10 min (arrow) (pH 7.4, 37°C, hct 0.1). Different symbols refer to different donors

Depletion of Ca_i by EGTA and A23187

Incubation of erythrocytes with 1 mM EGTA for 60 min at 37°C reduced Ca_i by 10 to 15% in three donors. In 1 mM EGTA solutions additionally containing 10 μM A23187, Ca_i decreased by 83 to 90% of its original value within 60 min (Table 2). The amount of residual Ca_i was not significantly related to the initial Ca content in Table 2.

The time-course of Ca depletion by prolonged incubations with 1 mM EGTA at 37°C is shown in Fig. 4. Ca_i fell by 0.1 to 0.3 μmol/liter cells during the first 10 min of incubation in three donors. After 3 hr, Ca_i was reduced by about 20% in one specimen with high initial Ca_i, and by about 55% in another with low initial Ca_i (broken lines in Fig. 4). Upon addition of A23187, Ca_i dropped to a low value within 10 min, the residual Ca being largely independent of the initial Ca content. Extending the incubation time in the presence of EGTA and A23187 up to 3 hr (Fig. 4, solid line) or 24 hr (*not shown*) did not further reduce Ca_i. Lowering the extracellular pH to 6.6 did not enhance Ca depletion by EGTA plus ionophore. Residual Ca_i after 3 hr of incubation was not affected by increasing the ionophore concentration from 10 to 30 μM (*data not shown*). Removal of A23187 from the cells by washing with 1% albumin demonstrated that the residual Ca was not associated with the ionophore.

Relations between Ca_i and the Activities of Na^+ Transport Systems

The activities of the Na^+-K^+ pump, the Na^+-K^+ cotransport and the Na^+/Li^+ exchange system are plotted as a function of Ca_i in Fig. 5. Obviously, the

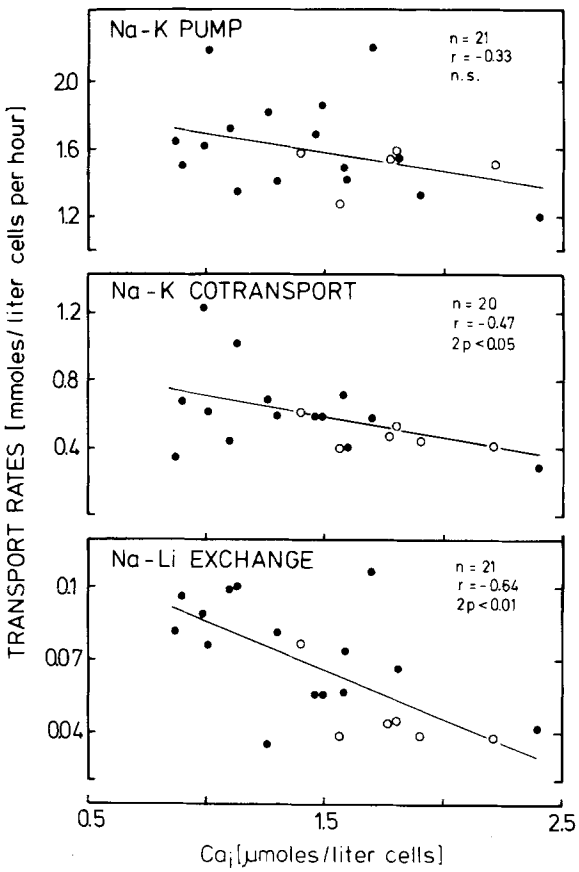

Fig. 5. Relations between the individual red cell Ca_i and the activities of three Na transport systems. For determination of the transport rates *see* Materials and Methods. ● males, ○ females

activities of the three transport systems fell by about 50% with Ca_i rising from 0.9 to 2.4 μmol/liter cells. The relation between Ca_i and the Na^+-K^+ pump just failed to be significant.

The rates of the inward Na^+ and Rb^+ leak determined in the presence of ouabain plus furosemide tended to decrease with rising Ca_i, the relations being not significant (*data not shown*).

Effect of Ca Depletion on Transport Rates

To examine whether the negative relations between Ca_i and the Na^+ transport rates shown in Fig. 5 are somehow causal, the effect of Ca depletion on the transport rates was investigated. Treatment with A23187 and EGTA caused a 56% reduction of Ca_i in the five donors studied in Table 3. The rates of the Na^+-K^+ pump, the Na^+-K^+ cotransport and the Na^+/Li^+ exchange, however, were not significantly altered by the Ca depletion.

The lack of an effect of Ca depletion on the

Table 2. Reduction of Ca_i (μmol/liter cells) by a 60-min incubation in a 140-nM Na-gly-gly buffer containing 1 mM EGTA without and with 10 μM A23187 (37°C, pH 7.4, 0.2 mM Mg, 5 mM glucose, hct 0.1)

Donor	Sex	Initial Ca_i	60-min Ca_i	Decrease (%)
1 mM EGTA				
1	♂	1.32	1.17	11%
2	♂	2.00	1.76	12%
3	♀	2.76	2.35	15%
1 mM EGTA + 10 μM A23187				
4	♂	1.08	0.13	88%
5	♂	1.12	0.19	83%
1	♂	1.25	0.21	83%
6	♀	1.94	0.29	85%
7	♀	2.30	0.22	90%

Na^+-K^+ pump, Na^+/Li^+ exchange and furosemide-sensitive Rb^+ uptake in Na^+ media was confirmed in experiments in which quin-2, an effective intracellular Ca chelator [28], was used to reduce the ionized Ca concentration within the cells (Table 3). Red cell ATP and 2,3-DPG contents were not significantly altered by Ca depletion with A23187 plus EGTA and treatment with 1.5 μM quin-2-acetoxymethylester, respectively (Table 3).

Relations between Ca_i and Red Cell Na, K and Hemoglobin Content

The red cell Na^+ and K^+ contents were positively related ($P < 0.01$) to Ca_i (Fig. 6). The mean cellular hemoglobin content fell with increasing Ca_i, indicating that the red cell water content increases with Ca_i.

Table 3. Effect of Ca depletion by A23187 and EGTA (40 min, hct 0.1) and intracellular Ca^{2+} chelation by quin-2 (90-min preincubation with 1.5 μM quin-2-acetoxymethylester, hct 0.01, according to [28]) on three Na^+ transport systems (transport rates in mmol/liter cells per hour, mean values ± SD from five experiments)

Ca content (μM/liter cells)	
Control	1.42 ± 0.24
A23187 and EGTA	0.63 ± 0.19
Na^+-K^+ pump[a]	
Control	1.82 ± 0.36
A23187 and EGTA	1.76 ± 0.29
Quin-2	1.94 ± 0.20
Na^+-K^+ cotransport[b]	
Control	0.60 ± 0.11
A23187 and EGTA	0.59 ± 0.11
Quin-2	0.60 ± 0.10
Na^+/Li^+ exchange[c]	
Control	0.074 ± 0.038
A23187 and EGTA	0.072 ± 0.034
Quin-2	0.075 ± 0.035
Na^+ content[d]	
Control	9.52 ± 2.60
A23187 and EGTA	10.60 ± 2.71
Quin-2	9.31 ± 1.93
ATP[e]	
Control	1.19 ± 0.07
A23187 and EGTA	1.20 ± 0.03
Quin-2	1.24 ± 0.10
2,3-DPG[e]	
Control	4.42 ± 0.80
A23187 and EGTA	4.90 ± 0.03
Quin-2	4.64 ± 1.17

[a] Ouabain-sensitive Rb^+ uptake in 145 mM Na-5 mM Rb.
[b] Furosemide-sensitive Rb^+ uptake in 145 mM Na-5 mM Rb.
[c] Phloretin-sensitive Li^+ uptake in 2 mM Li-Mg-sucrose.
[d] mmol/liter cells.
[e] mmol/liter cells, determined according to refs. [18] and [19].

Discussion

The Intracellular Ca Content of Human Erythrocytes

The calcium content of normal human erythrocytes is much lower than in all other cell types because red cells of healthy donors contain very little [24] or no [36] intracellular organelles storing Ca. Most of the Ca embodied within human erythrocytes is thought to be bound to the inner side of the membrane [16, 26]. Accordingly, a determination of the total intracellular Ca content (Ca_i) seems to be a good estimate of Ca binding to the inner aspect of the membrane of intact cells.

It is well known that the extracellular aspect of human erythrocytes exhibits high-, medium- and low-affinity binding sites with a total capacity of about 500 μmol/liter cells [27]. Therefore, the cells were washed seven times with a 10-fold excess of a NaCl-gly-gly buffer purified using chelex-100 (containing less than 20 nM Ca), a procedure that proved to be effective in displacing almost all of the Ca reversibly bound to the outer membrane. Lowering the Ca in the washing solution to 10^{-13} M by adding 1 mM EGTA did not further reduce the red cell Ca content significantly (Table 1). A similar result was obtained by Wiley and Shaller [44]. On the other hand it was observed that adding 1 to 10 μM Ca to the washing solution (a range which covers the Ca concentrations usually present in isotonic solutions of analytical grade) caused an up to sixfold increase in apparent Ca_i even when the Ca contamination present in the medium of the cell suspensions sub-

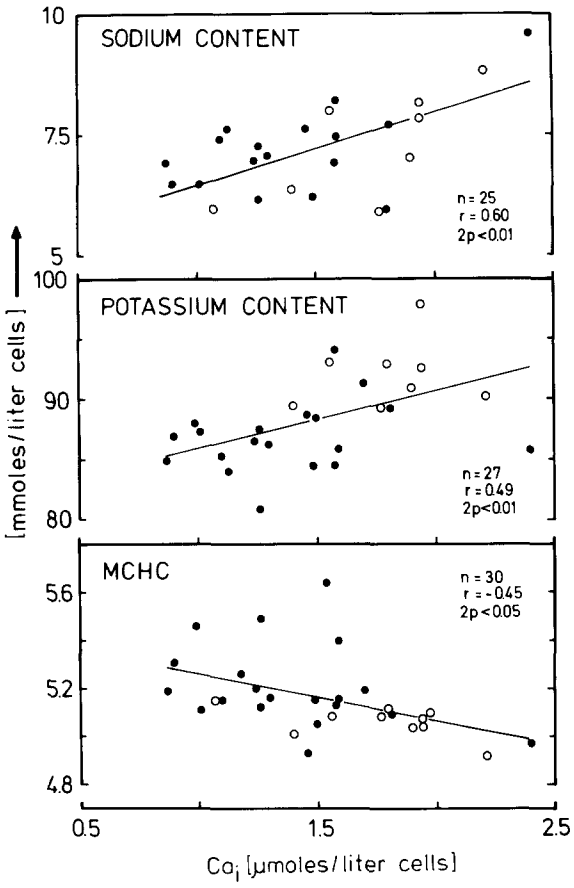

Fig. 6. Relations between Ca_i and red cell Na^+, K^+ and hemoglobin contents. MCHC units are expressed in mmol hemoglobin tetramer per liter cells. ● males, ○ females

jected to perchloric acid extraction was considered (Fig. 1). It seems safe to conclude, therefore, that a noteworthy fraction of the scatter of the Ca_i values reported in the literature is due to *i*) incomplete removal of Ca from the outer aspect of the membrane, and *ii*) Ca present in the extracellular volume of the cell suspensions extracted.

A second aspect is the signal-to-noise ratio and sensitivity of the method applied to quantitate Ca in the red cell extracts. The use of pyrolytic graphite cuvettes in the method of flameless atomic absorption employed in the present experiments resulted in a 10-fold increase of the Ca signals recorded as compared to ordinary graphite cuvettes. Thereby it became possible to determine Ca concentrations in the extracts (20 to 250 nM) with an analytical error below 10% (*see* Materials and Methods).

The interindividual variability of Ca_i, already earlier noticed [12, 20, 32], was confirmed in the present study. The absolute values obtained (0.9 to 2.8 μmol Ca/liter cells) are comparable to values reported for cells washed in buffers purified using chelex-100 or containing Ca chelators [3, 20, 43–45]. The question arises, however, whether the Ca contents determined after removal of extracellular Ca are true measures of "native" intracellular Ca of the circulating erythrocytes. An increase of the red cell Ca content, e.g., could occur during the separation of the cells from plasma by centrifugation. Conversely, a Ca extrusion could be mediated by the ATP-dependent Ca pump in the course of the several washings in media virtually free of Ca. The latter process was minimized by lowering the temperature to 4°C during the washing procedure. Furthermore, Ca could leave the cells by other mechanisms. Since neither of these possibilities can be excluded with certainty the Ca contents obtained after removal of extracellular Ca are considered to be the best estimate of the "native" intracellular Ca content.

The individual Ca_i remained constant over more than one year (Fig. 3). Further studies are required to elucidate which reason(s) may be responsible for the interindividual variability of Ca_i, for the higher Ca_i observed in females as compared to males (Fig. 2) and for the tendency of Ca_i to increase with age. It appears unlikely that the insignificant negative relations found between total and ionized plasma Ca and Ca_i somehow play a causal role.

Ca Depletion In Vitro

Incubation of erythrocytes at 37°C in solutions containing EGTA (ionized Ca $< 10^{-13}$ M) results in a reduction of the red cell Ca content by 20 to 50% within 3 hr, most of which occurs during the first 10 min of incubation (Fig. 4). This Ca loss is probably the result of Ca extrusion by the ATP-dependent Ca pump. As the concentration of ionized Ca^{2+} decreases, some Ca will be released from intracellular Ca binding sites. Ca extrusion by the pump almost ceases, however, when ionized Ca^{2+} within the cells is lowered to such an extent that the Ca pump in its low-affinity and velocity state cannot further expel Ca (*see* Fig. 4 and ref. 25).

Upon permeabilization of the red cell membrane to Ca by addition of the ionophore A23187 Ca_i fell by 60 to 90% within 10 min (Fig. 4 and ref. 25). This indicates that the concentration of ionized Ca^{2+} was lowered by the ionophore below the value established by the Ca pump, thereby allowing a substantial release of Ca from intracellular binding sites. The possibility cannot be excluded, however, that treatment with A23187 plus EGTA mainly releases Ca originating from intracellular vesicles [24]

or from a minute fraction of cells with abnormally high Ca.

Ten to 40% of Ca_i could not be displaced by treatment of the cells with A23187 and EGTA, even during prolonged incubation with high concentrations of the ionophore. The residual Ca is thus probably embedded at inaccessible Ca binding sites that do not exchange with cellular ionized Ca^{2+} or have extremely high Ca affinities.

Ca_i and the Activities of Na^+ Transport Systems

It is well known that intracellular Ca can affect the activity of a multitude of transport pathways of human erythrocytes, including the Na^+-K^+ pump [4], the Na^+-K^+ cotransport system [17] and the anion exchanger [29] which are inhibited by raising cell Ca, as well as a K^+ channel [23] and an amiloride-sensitive Na^+ transport pathway [15] which are activated by intracellular Ca. Accordingly, the possible role of differences of Ca_i in the interindividual variability of the activity of the Na^+-K^+ cotransport and the Na^+/Li^+ exchange system [9] was investigated. Indeed, negative correlations were found between Ca_i and Na^+/Li^+ exchange, Na^+-K^+ cotransport and Na^+-K^+ pump activities (Fig. 5). This observation may be related to findings on red cells of essential hypertensive patients, indicating that a reduced Ca binding to the inner aspect of the membrane [8, 34] and a tendency towards a reduced Ca_i [14] may be associated with an accelerated Na^+/Li^+ exchange and Na^+-K^+ cotransport [14].

Red cell Na^+ and K^+ contents rose with Ca_i (Fig. 6), thereby causing an osmotically obliged uptake of water with concomitant reduction of the hemoglobin concentration as reflected by the fall of the mean cellular hemoglobin content. The association of a high cellular Na^+ content with a low activity of the Na^+-K^+ pump in cells with high Ca_i (Fig. 5) corresponds to the pump leak concept. The high K^+ content in cells with a low pump activity, in contrast, is difficult to reconcile with this concept. Possibly, the steady-state red cell K^+ content is more related to the activity of the Na^+-K^+ cotransport system [10] than to that of the Na^+-K^+ pump.

Lauf [22] has found a several-fold elevation of chloride-dependent K^+ transport in Ca-depleted low K^+ sheep red cells. In the present work neither Ca depletion by A23187 and EGTA nor intracellular Ca buffering by quin-2 did affect the activities of Na-K cotransport and the other two Na transport systems of human erythrocytes. Therefore, the observed relations between Ca_i and the transport rates are probably not causal. In view of the above-mentioned uncertainties regarding the origin of the released calcium, however, it cannot be excluded that the depletion procedure does not remove calcium from binding sites with functional significance for the Na^+ transport systems.

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