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Research Article

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Selective Loss of Calcium Permeability on Maturation of Reticulocytes

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ABSTRACT Calcium and sodium permeability of human reticulocytes have been studied and compared to mature erythrocytes. Mature erythrocytes had extremely low Ca^{2+} permeability which was less than 0.1% of values published for squid axon or HeLa cells. Calcium entry was markedly increased in reticulocyte-rich suspensions and the uptake was linearly related to the percentage of reticulocytes present. The data suggest that reticulocytes are 43-fold more permeable to Ca^{2+} than mature cells although their Ca^{2+} concentration is not increased. Sodium influx into reticulocyte-rich suspensions was also increased in direct proportion to the percent of reticulocytes present. Reticulocytes are sixfold more permeable to Na^+ than mature cells so the ratio of Ca^{2+}:Na^+ permeability falls by sevenfold as the reticulocyte changes to an erythrocyte. [^{3}H]Ouabain binding was increased in reticulocyte-rich cell suspensions and the correlation suggested a value of about 4,000 sites per reticulocyte compared with 362±69 per mature cell. Maturation of the human reticulocyte produces disproportionate changes in cation permeability and in particular a selective loss of Ca^{2+} permeability.

INTRODUCTION

Reticulocytes lose their ribosomes within 36 h after entering the peripheral blood, although this maturation time may be almost doubled in conditions of erythropoietic stress (1, 2). During the maturation process, reticulocyte volume decreases by 20–30% and several studies have shown that cytoplasmic hemoglobin and membrane lipids are decreased in the same proportion (3, 4). The two major lipids of the membrane, phospholipid and cholesterol, are both lost in parallel while changes in osmotic fragility confirm that membrane area is reduced (3). Major alterations in membrane transport occur during this maturation process. Reticulocytes possess a Na^+-dependent transport system for alanine and α-aminoisobutyric acid which disappears on cell maturation (5). Active transport of Na^+ is also lower in mature erythrocytes compared with reticulocyte-rich populations (6), although cation fluxes have not been correlated with reticulocyte numbers. In this study the fluxes of Ca^{2+} and Na^+ as well as the binding of [^{3}H]ouabain have been measured in reticulocyte-rich erythrocytes. Results show a major reduction in Ca^{2+} permeability on maturation of reticulocytes which is almost an order of magnitude greater than the Na^+ permeability changes.

METHODS

Materials. NaCl, KCl, sodium EGTA,[1] $SrCl_2$ were Analar grade from Fisher Scientific Co., Pittsburgh, Pa.; LiCl was from K and K Laboratories, Inc., Plainview, N. Y.; inosine, hexokinase, and glucose-6-phosphate dehydrogenase were from Boehringer, Mannheim, W. Germany; iodoacetic acid and sodium iodoacetamide were from Eastman Kodak Co., Rochester, N. Y.; ATP, $NADP^+$, and Tris-HCl were from Sigma Chemical Co., St. Louis, Mo.; and $^{45}CaCl_2$ and $^{22}NaCl$ were from New England Nuclear, Boston, Mass. [^{3}H]-Ouabain, 5 Ci/mM ethanol-benzene solution, New England Nuclear lot no. 184–196, was evaporated to dryness under a stream of nitrogen and redissolved in 20-fold excess of unlabeled ouabain to give a stock solution of 10 μM ouabain in water of activity 12.5 μCi/ml. The exact concentration of ouabain was confirmed by spectrophotometric analysis with a molar extinction coefficient of 1.54×10^4 at the absorption peak of 220 nm. (7). This stock solution was stable at 4°C for several months. Silica crucibles (high-form, 5 ml capacity) were from Thermal American

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[1] *Abbreviations used in this paper*: EGTA, ethylene glycol-bis(β-aminoethyl ether)*N,N,N',N'*-tetraacetate.

Fused Quartz Co., Montville, N. J.; standard solutions of Ca^{2+} (5–50 μM) were prepared by dilution of commercial 1,000 ppm Ca $(NO_3)_2$ (i.e., 25 mM) from Fisher Scientific Co. in a solution of 0.1 N HCl plus 10 mM $SrCl_2$.

High-reticulocyte blood donors. Patients were selected with peripheral blood reticulocytosis of 2.0–25% resulting from chronic autoimmune hemolytic anemia, pyruvate kinase deficiency hemolytic anemia, chronic gastrointestinal blood loss, or during hematinic therapy of a deficiency anemia. Patients with renal disease were excluded because of the inhibitory effects of uremic toxins on cation transport in the erythrocyte (8). Likewise, patients with liver disease, untreated megaloblastic anemia, or alcoholism were excluded because of their increased erythrocyte size and surface area (9). Sodium and Ca^{2+} influx were studied simultaneously in 8 of the 20 patients with reticulocytosis while 12 patients were studied with only one flux measurement.

Preparation of erythrocytes. Venous blood was collected into heparin and erythrocytes were separated from leukocytes by thrice washing at 4°C in a medium of composition 145 mM NaCl, 5 mM KCl, plus 20 mM imidazole Cl, pH 7.5. Care was taken to remove all buffy coat since both leukocytes and platelets contain large amounts of calcium. This basic medium was used for all incubations since the imidazole buffer does not associate with added Ca^{2+} ions.

Reticulocyte enumeration. During washing of erythrocytes and removal of buffy coat up to one-fourth of the reticulocytes may be lost. Therefore, the percentage of reticulocytes was always measured after washing. One drop of washed erythrocytes was mixed with three drops of autologous plasma and stained for 15 min with an equal volume of freshly-filtered 1.0% new methylene blue (10). The number of reticulocytes in 1,000 erythrocytes was counted.

Calcium influx. Washed erythrocytes were depleted of ATP by preincubation for 90 min in medium plus 1 mM iodoacetate and 10 mM inosine (11). The erythrocytes were then washed twice and added to prewarmed media of composition 145 mM NaCl, 5 mM KCl, 20 mM imidazole Cl, pH 7.5, plus 1.5 mM $^{45}CaCl_2$ (1 μCi/ml). Samples were taken after 15 min and after 1, 2, 3, and 4 h, and washed four times in cold 150 mM NaCl plus 1 mM Na EGTA. Each cell pellet was hemolyzed in 0.01 N NH_4OH, and part of the hemolysate was deproteinized with 6% (wt/vol) perchloric acid plus 1 mM Na EGTA and $^{45}Ca^{2+}$ in the supernate was measured by liquid scintillation counting. To convert the uptake of radioactive calcium from nanomoles per micromole Hb into nanomoles per milliliter cells, the mean corpuscular hemoglobin concentration was measured in each experiment both for the abnormal and normal cells. Na^+ influx was measured in cells depleted of ATP by the above procedure to find if the iodoacetate treatment had induced a generalized permeability increase. No increase in Na^+ influx was found.

Total cell calcium concentration. Calcium was measured by atomic absorption spectroscopy of a dry ashed sample of erythrocytes. Erythrocytes, which had been washed free of buffy coat were further washed four times at 4°C in 150 mM NaCl or 150 mM LiCl either with or without 1 mM Na EGTA. The final pellet of washed cells was hemolyzed with 6 ml of 0.01 N NH_4OH (verified Ca^{2+}-free), and 5 ml of the hemolysate was added to a silica crucible, slowly evaporated to dryness under an infrared lamp, and combusted at 500°C overnight in a muffle furnace. It was important that the sample was completely dry before combustion, since any trace of water led to uncontrollable bubbling when the sample was heated toward 500°C. The residue in each crucible was extracted with dilute acid by adding 3 ml of 0.1 N HCl plus 10 mM $SrCl_2$ to the crucible and shaking for 2 h at room temperature. The contents of the crucible were decanted into polypropylene tubes, and the iron oxide was allowed to settle. Calcium in the supernate was analyzed on a Varian Techtron 1200 Atomic Absorption Spectrophotometer (Varian Associates, Walnut Creek, Calif.) at 422.6 nm with acetylene fuel plus compressed air. Ca^{2+} contaminant in HCl-$SrCl_2$ solution (solvent blank) was 2 μM. A 1-ml aliquot of the hemolysate was taken for measurement of hemoglobin concentration, so that the Ca^{2+} present in each crucible could be expressed as nanomoles per milliliter of cells.

Precautions to exclude calcium contamination. Isotonic NaCl and LiCl contain 1–4 μM Ca^{2+} contaminant which was removed by filtering these solutions through a 8 × 1.5-cm column of Chelex-100 ion-exchange resin (Bio-Rad Laboratories, Richmond, Calif.). These solutions and others used in Ca^{2+} measurements were stored in polypropylene bottles since both glass and some polystyrene and polyethylene containers contribute small and variable amounts of Ca^{2+} to a solution. When Pyrex glassware was used, it was acid washed and kept immersed in deionized water until immediately before use, when it was again rinsed in deionized water. All crucibles had close-fitting lids, and after combustion the lids were dusted free of refractory brick dust before opening. Recovery studies of Ca^{2+} added to crucibles and dry ashed at 500°C established that no significant amounts of Ca^{2+} were retained by the silica crucibles. $^{45}CaCl_2$ (1.25, 2.5, 3.75, 5, and 74 nmol) was added to crucibles, 5 ml of an erythrocyte hemolysate was added and the contents were dry ashed and extracted in dilute acid as above. The recovery of ^{45}Ca was estimated by liquid scintillation counting and was 81, 85, 86, 98, and 101%, respectively, for each of the above amounts of added Ca^{2+}.

Sodium influx. This was measured from the uptake of radioactivity by cells incubated 10 and 20 min in media containing $^{22}NaCl$ as described by Wiley and Cooper (12). Media always contained glucose (10 mM) plus ouabain (50 μM).

ATP estimation. The cell suspension was deproteinized with 2 vol of 0.6 N-perchloric acid, centrifuged at 0°C, and the supernate adjusted to pH 6–7 with K_2CO_3. ATP in the neutralized extract was estimated spectrophotometrically by measuring the reduction of $NADP^+$ by a coupled hexokinase-glucose-6-phosphate dehydrogenase reaction.

Creatine. This was measured in a neutralized perchloric acid extract of washed cells (13).

[^{3}H]Ouabain binding to erythrocytes. Binding of ouabain was measured essentially as described by Hoffman (14) and Wiley et al. (15). Washed erythrocytes were incubated with 20 nM [^{3}H]ouabain for 30 or 60 min after which cells were washed and 0.5-ml portions of packed cells were added to 15 ml Aquafluor (New England Nuclear) and counted after storage for 72 h in the dark. Another portion was taken for erythrocyte enumeration with the Coulter model ZB electronic particle counter (Coulter Electronics Inc., Hialeah, Fla.). Potassium influx was also measured to assess the fractional inhibition of the cation pump. ^{42}KCl was added to the basic incubation medium to give a final K^+ concentration of 5.6–7.2 mM and influx measured over a 1-h incubation. The total number of ouabain-binding sites per cell was equated to the number of [^{3}H]ouabain molecules bound per cell when there was 100% inhibition of active K^+ influx.

Statistics. Mean values ± 1 SD are shown unless otherwise noted. Regression lines and correlation coefficients were calculated by the method of least squares and differences between sample means analyzed by a t test.

TABLE I

Calcium Concentration of Normal and Reticulocyte-Rich Erythrocytes

Diagnosis	Reticulocytes	Washing medium	Mean calcium	SE	Range
	%		*nmol/ml cells*		
Normals	<1.0	NaCl	5.7	0.8	3.6–7.4
Normals	<1.0	NaCl + EGTA	6.5	1.4	3.1–11.2
Normals	<1.0	LiCl	5.2	1.1	2.1–8.9
Normals	<1.0	LiCl + EGTA	6.3	0.6	5.0–8.3
G. I. blood loss	11.4	NaCl + EGTA	3.7	0.6	2.7–5.7
Folate deficiency on therapy	5.6	NaCl + EGTA	2.7	0.2	2.3–3.2
Autoimmune hemolytic anemia	24	NaCl + EGTA	9.2	0.7	8.4–10.1
Autoimmune hemolytic anemia	25	NaCl + EGTA	9.3	0.2	9.0–9.6

Normal group was three males and three females and the results with different washing media were always obtained with paired observations on the same cells on the same day. Measurements on the four patients with elevated reticulocytes were obtained from quadruplicate samples.

RESULTS

Erythrocyte calcium concentrations. The mean calcium content of erythrocytes from six normal donors was 5.9±1.8 nmol/ml cells after four washes in a verified Ca^{2+}-free medium. Identical values were obtained whether the medium was isotonic NaCl or LiCl or whether the chelating agent, EGTA, was present or absent (Table I). The range of values obtained for replicate determinations on one individual is shown in Table I and usually fell within a two- or threefold range. The calcium content of erythrocytes from patients with a reticulocytosis (5.6–25%) did not differ significantly from normal (Table I)

Calcium exchangeability. Erythrocyte Ca^{2+} exchanged with isotopic Ca^{2+} added to a physiological medium in which the cells were incubated at 37°C. This exchange was complete by 5 min since the uptake of isotopic Ca^{2+} was the same in samples taken at 5, 10, 15 and 60 min. Concurrent measurements of $^{45}Ca^{2+}$ uptake by liquid scintillation counting plus total cell Ca^{2+} concentration by atomic absorption showed that isotopic Ca^{2+} exchanged with only a fraction of total cell Ca^{2+}. Two normals exchanged 15 and 40% of their total Ca^{2+}, while the patient with 25% reticulocytes showed an 88% exchange. This greater exchangeability of reticulocyte Ca^{2+} was studied by measurements of unidirectional Ca^{2+} influx.

Calcium influx into normal erythrocytes. Unidirectional ^{45}Ca influx was studied in erythrocytes depleted of ATP to inhibit the outward pumping of Ca^{2+} ions (11). To deplete the ATP the washed erythrocytes were preincubated with 10 mM inosine plus either 1 mM iodoacetate or 5 mM iodoacetamide. These inhibitors were equally effective in combination with inosine since ATP fell from 1.2 μmol/ml cells initially to less than 0.1 μmol/ml cells after 90 min (Fig. 1A). Cells were again washed and incubated without inhibitors in media containing 1.5 mM $^{45}Ca^{2+}$ ion. A rapid initial uptake of isotopic Ca^{2+} occurred within 15 min followed by a slower uptake which was linear between 15 min and 4 h (Fig. 1B). In cells depleted by inosine plus iodoacetate the initial uptake was 2.3 nmol Ca^{2+}/ml cells and the linear phase averaged 0.8 nmol Ca^{2+}/ml cells every hour to reach 5.4±1.5 nmol Ca^{2+}/ml cells after a 4-h incubation. Calcium uptake by cells depleted of ATP by incubation with inosine plus iodoacetamide was not significantly different to the values above (Fig. 1B). Analysis of total Ca^{2+} in one experiment confirmed a net increase in this cation.

Calcium influx into reticulocytes. Calcium uptake is increased into erythrocytes from patients with a reticulocytosis. Cell suspensions with reticulocytes of 5.0 or 11.4% allowed approximately 6- or 10-fold greater Ca^{2+} uptake, respectively (Fig. 2). Similar uptakes were obtained for the same cells depleted of ATP by three different combinations of inhibitors: inosine plus iodoacetate, inosine plus iodoacetate plus cyanide, or inosine plus iodoacetamide. The combination of inosine plus iodoacetate was chosen to deplete cell ATP for all subsequent studies. The greater Ca^{2+} uptake by reticulocytes was confirmed with blood from a normal donor. The erythrocytes were washed free of buffy coat and fractionated by centrifugation into reticulocyte-rich and reticulocyte-poor fractions (16). Table II shows that the top fraction with 2.4% reticulocytes showed a twofold greater Ca^{2+} uptake than the reticulocyte-poor fractions.

Comparison of calcium and sodium influx. The large influx of Ca^{2+} into ATP-depleted reticulocytes suggests that the reticulocyte membrane is very permeable to this cation. Sodium influx was also measured in reticulocyte-rich cells for comparison with the Ca^{2+} fluxes. Fig. 3 shows that Na^+ influx was increased

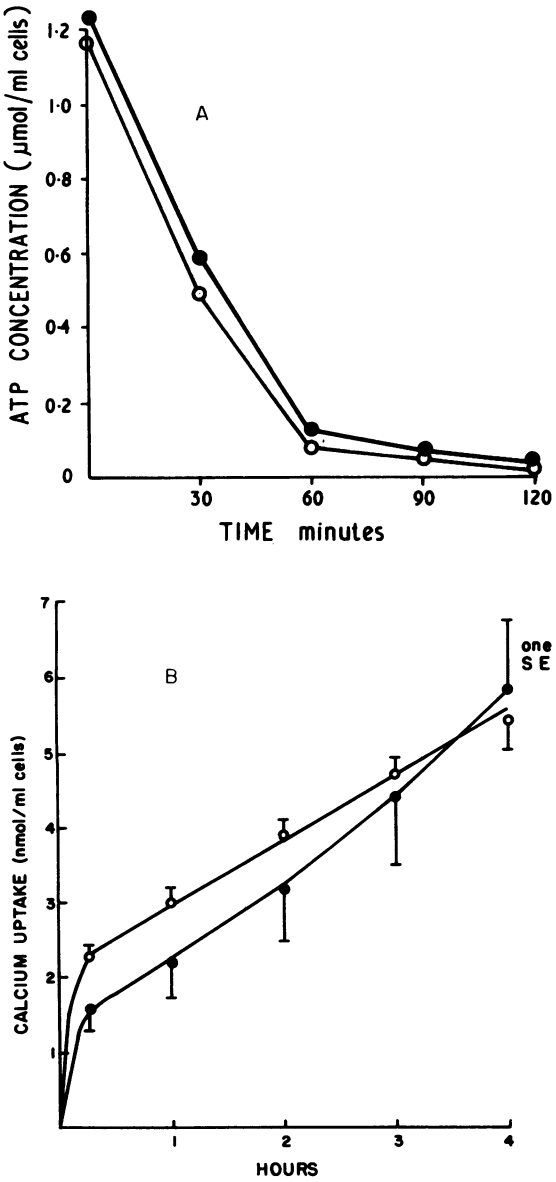

FIGURE 1 (A) ATP concentration of erythrocytes incubated at 37°C with 10 mM inosine plus 1 mM iodoacetate (○) or inosine plus 5 mM iodoacetamide (●). (B) Calcium uptake by erythrocytes depleted of ATP by either inosine plus iodoacetate (○) or iodoacetamide (●). Mean values are for 22 or 6 normal donors, respectively, and show±1 SE by the vertical bars.

in direct proportion to the percent reticulocytes present ($r = 0.88, P < 0.001$) and the influx was twofold normal for cells with 20% reticulocytosis. In contrast the Ca^{2+} uptake was elevated 11-fold normal in cells with 20% reticulocytosis (Fig. 3). A linear relation existed between Ca^{2+} uptake and percent reticulocytes ($r = 0.71$, $P < 0.01$) although the regression line did not pass through the mean for normal erythrocytes. Calcium uptake showed no correlation with erythrocyte creatine which was measured in 12 reticulocyte-rich cell suspensions as an index of the mean erythrocyte age. Sodium influx was measured into erythrocytes both before and after depletion of ATP with iodoacetate plus inosine. No increase in Na^+ influx was found in depleted cells in three separate experiments.

[^3H]Ouabain binding to reticulocytes. Reticulocytes bound more ouabain than normal cells and there was a direct relation between the number of ouabain molecules bound per cell and the percentage of reticulocytes present (Fig. 4). Eight patients were studied with reticulocytosis between 4 and 20%, and the number of binding sites per cell increased about twofold over this range. Seven normal subjects gave a value 362±69 for the mean number of ouabain-binding sites per erythrocyte. One subject (C. S.) gave repeated values of 320, 326, 278, 332, and 322 sites/cell at intervals over 2 yr and other subjects also gave reproducible results.

Potassium influx. The ouabain-sensitive (active) component of K^+ influx was increased for the eight patients with reticulocytosis (2.28±0.83 μeq/ml cells per h) when compared with normals (1.46±0.20 μeq/ml cells per h; $P < 0.01$). However there was no difference in ouabain-insensitive K^+ influx between the two groups (0.47±0.17 μeq/ml cells per h for patients and 0.46±0.10 μeq/ml cells per h for normals).

DISCUSSION

The influx of Ca^{2+} into human erythrocytes can only be adequately studied under conditions which inactivate the outward Ca^{2+} pumping. Selective inhibition of the pump is difficult to achieve since ruthenium red, La^{3+} ions, or chlorpromazine[2] block not only the outward pumping of Ca^{2+} but also the passive inward movement of this cation. Rapid depletion of ATP however inhibits the Ca^{2+} pump and allows a measurable uptake of Ca^{2+} ions (11). Circumstantial evidence suggests that this uptake measures the passive Ca^{2+} permeability of energy-replete cells. First, cells pretreated with two different metabolic inhibitors (iodoacetate and iodoacetamide) gave the same values for Ca^{2+} uptake (Figs. 1B and 2) which suggests that Ca^{2+} entry resulted from ATP depletion and not from a membrane effect of the inhibitor to increase Ca^{2+} permeability. Second, Na^+ influx into ATP-depleted cells was about the same as for ATP-rich cells which agrees with a previous study (17) and shows that iodoacetate or iodoacetamide pretreatment does not increase passive permeability to cation. A rapid initial uptake of 1–2 nmol ^{45}Ca/ml cells occurs within the first 5 min of incubation of erythrocytes with isotopic Ca^{2+}. This

[2] Wiley, J. S., and C. C. Shaller. Unpublished observations.

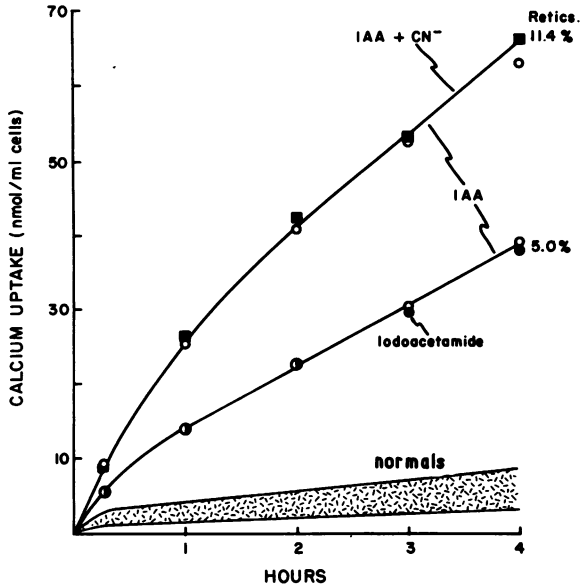

FIGURE 2 Calcium uptake by reticulocyte-rich erythrocytes, with either 11.4% reticulocytes (Retics.) (upper curve) or 5.0% reticulocytes (lower curve). Cells were depleted of ATP by preincubation with inosine plus iodoacetate (○), inosine plus iodoacetamide (●), or inosine plus iodoacetate plus cyanide (■). Uptake for normal erythrocytes ±2SD is shown by the cross-hatching.

initial uptake is the same for either ATP-rich or ATP-depleted cells and its rate is one to two orders of magnitude faster than the subsequent uptake of Ca^{2+} by ATP-depleted cells. Such rapid kinetics suggest that the initial uptake is a distinct process from transmembrane Ca^{2+} movement and may represent an exchange of membrane ^{40}Ca with ^{45}Ca in the medium. The slow uptake of Ca^{2+} between 15 min and 4 h can be shown with either ^{45}Ca or total Ca^{2+} analysis and measures transmembrane movement. Values for this flux confirm the very low Ca^{2+} permeability of erythrocytes, shown by Schatzmann and Vincenzi

TABLE II
Calcium Uptake by Reticulocyte-Enriched Normal Erythrocytes

Fraction	Reticulocytes	Ca^{2+} uptake
	%	*nmol/ml cells/4 h*
Top	2.4	22.1
Middle	0.8	9.2
Bottom	0.3	8.5

Washed erythrocytes were fractionated by centrifugation at 28,000 *g* for 60 min at 30°C. Cell ATP was then depleted by incubation for 90 min in media containing inosine plus iodoacetic acid. Finally Ca^{2+} uptake was measured over a 4-h incubation in saline media containing 1.5 mM $^{45}Ca\ Cl_2$.

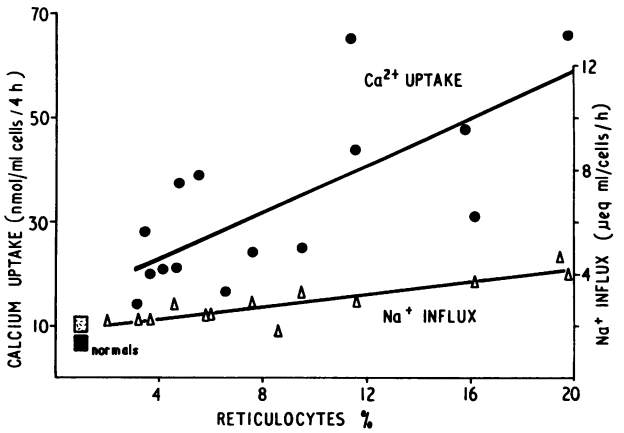

FIGURE 3 Comparison of Ca^{2+} uptake with Na^+ influx in cells with reticulocytosis from 2 to 20%. Regression lines have been fitted by the method of least squares with $r = 0.71$ for Ca^{2+} uptake and $r = 0.88$ for Na^+ influx. Mean for normals ±1 SD are shown for Na^+ influx (upper rectangle) and Ca^{2+} uptake (lower rectangle).

(18) for fresh cells stored at 4°C in plasma and by others for ATP-depleted cells at 37°C (11, 19).

The main finding is that reticulocytes have a Ca^{2+} permeability which is many fold greater than that of mature erythrocytes. Both Na^+ and Ca^+ influx were increased in the presence of reticulocytosis but while Na^+ flux increased 2-fold, the Ca^{2+} flux increased 11-fold for cells with 20% reticulocytosis. Extrapolation of these values suggest that reticulocytes have a 6-fold greater Na^+ influx and 43-fold greater Ca^{2+} uptake than mature cells. Two factors contribute to this difference between reticulocytes and mature cells; first, the permeability to Ca^{2+} per square micrometer of membrane, and second, the membrane surface area. Although the membrane area cannot be directly measured, a relative estimate has been provided by the

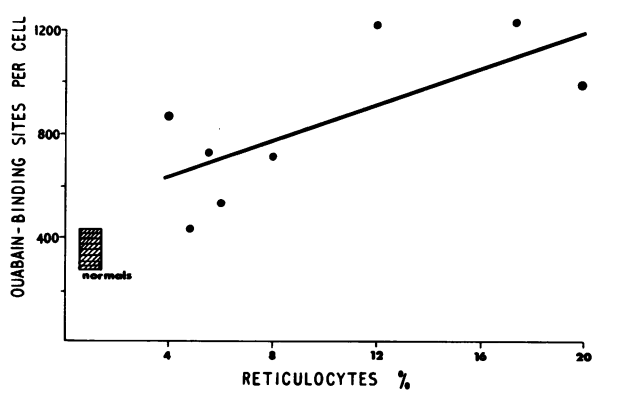

FIGURE 4 Correlation between ouabain binding sites per cell and percent reticulocytes in the suspension. Regression line has been fitted by the method of least squares with $r = 0.70$. Mean for normals ±1 SD is shown in the shaded rectangle.

lipid analyses of Shattil and Cooper (3). Cell membranes of reticulocytes lose 35% of their cholesterol and phospholipid on maturation which probably accompanies a decrease in membrane surface area of similar magnitude. Reticulocyte volume also decreases about 25–30% on maturation so that the membrane area of reticulocytes may be approximately 10% greater than that of mature cells when compared on a unit volume basis. Although this estimate is only approximate it does show that the many-fold reduction in cation fluxes on maturation of the reticulocyte can be attributed largely to a decrease in membrane permeability. The ratio of Ca^{2+}:Na^+ fluxes is quite independent of relative membrane area. The Ca^{2+}:Na^+ flux ratio falls by a factor of about 7 as the reticulocyte changes to an erythrocyte so that maturation of the reticulocyte leads to a selective loss of Ca^{2+} permeability.

Several lines of evidence suggest that reticulocytes lose their high Ca^{2+} permeability on maturation rather than during erythrocyte aging. First, the Ca^{2+} influx correlates well with percentage of reticulocytes (Fig. 3) but this flux does not correlate with erythrocyte creatine which reflects the mean cell age (13, 20). Second, when erythrocytes are separated by centrifugation into fractions of varying ages, the fraction with old cells showed the same Ca^{2+} uptake as the bulk of the red cell population. The mechanism by which Ca^{2+} permeability is selectively lost is not known although it may relate to the disappearance of membrane proteins specific to the reticulocyte (21). Absolute values for the Ca^{2+} influx may be calculated from the Ca^{2+} uptake of ATP-depleted erythrocytes (5.4 nmol/ml cells per 4 h) assuming a surface area of 134 μm^2 and mean cell volume of 85 fl (22). For mature cells the flux is 2×10^{-5} pmol/cm^2 per s and for reticulocytes about 1×10^{-3} pmol/cm^2 per s, although the latter value is approximate and depends on the assumption that reticulocytes and mature cells have the same membrane area per unit volume. This assumption is only approximate since reticulocytes are more osmotically resistant than mature cells which indicates a slightly greater surface area-to-volume ratio of these more immature cells (3). Whatever the exact values, it is clear that erythrocyte Ca^{2+} permeability is many orders of magnitude less than for squid axon, HeLa cells, or ascites tumor cells where flux values about 0.1 pmol/cm^2 per s have been found (23–25).

The concept of a "normal" cell Ca^{2+} concentration is imprecise since cell Ca^{2+} depends upon several factors such as ambient Ca^{2+} levels and the number of saline washes before dry ashing of erythrocytes. Previous studies have reported mean values for erythrocyte Ca^{2+} of 16 and 15 nmol/ml cells (26, 27). A wide range of individual values from 5 to 22 nmol/ml cells was observed by Harrison and Long (26) who found that inclusion of EDTA in the washing medium reduced mean erythrocyte Ca^{2+} to only 2 nmol/ml cells. The present results gave a mean erythrocyte Ca^{2+} of 5.9±1.8 nmol/ml cells whether EGTA was present in the Ca^{2+}-free washing medium or not. The discrepancies between these studies may in part relate to the small Ca^{2+} contamination (about 1–2 μM) always present in saline washing media of previous studies. This Ca^{2+} may associate with phosphate anions leaking from the cells during washing so that insoluble calcium phosphate forms on the cell surface and is included in the cell Ca^{2+} analysis. Whatever value is accepted for erythrocyte Ca^{2+}, the analyses in this study employed consistent methods so it is valid to conclude that reticulocyte-rich (5–25%) erythrocytes have the same total cell Ca^{2+} concentration as mature cells. However, Ca^{2+} of reticulocytes can exchange more completely with isotopic $^{45}Ca^{2+}$ in the external medium, presumably because of the greater Ca^{2+} permeability of reticulocyte membranes.

Reticulocytes possess an increased number of cation pump sites which have been quantitated in this study by the [^{3}H]ouabain binding technique. Pump sites were increased about three-fold in cells with 20% reticulocytosis which extrapolates to a value of about 4,000 sites for each reticulocyte. This value is 11-fold higher than the pump site numbers found for mature erythrocytes of 362±69 sites/cell. Estimates for the ouabain-binding sites in mature human erythrocytes range between 228 and 1,200 sites/cells (28, 29), with most studies finding between 250 and 500 sites/cell (30–32). Active K^+ influx into reticulocyte-rich cells is also increased above normal although the stimulation of flux is less than expected from the increment in pump sites. This may be explained by the low normal or subnormal concentrations of Na^+ in the reticulocyte (6).

Control of cytoplasmic Ca^{2+} concentration is an important function of all cell types. Low cytoplasmic Ca^{2+} concentrations may be achieved by accumulation of this cation within organelles such as the sarcoplasmic reticulum of muscle, the dense granules of blood platelets, or mitochondria of various cells. The human erythrocyte lacks organelles and the low Ca^{2+} content of this cell is maintained by a calcium pump which extrudes this ion from the cytoplasm to the cell exterior (18). This calcium pump is an ATPase, which is active at the micromolar Ca^{2+} concentrations normally present in human erythrocytes (33, 34). Energy requirements by this calcium pump normally are only a negligible fraction of ATP production from glycolysis. The 43-fold decrease in Ca^{2+} permeability which occurs on maturation of the reticulocyte thus effectively limits the energy needed by the mature cell for Ca^{2+} homeostasis. Any failure by the mature cell to acquire

this low Ca^{2+} permeability will lead to cell destruction, which is well illustrated by the association of hemolytic anemia with a congenital erythrocyte Ca^{2+} leak (35).

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REFERENCES

1. Stryckmans, P. A., E. P. Cronkite, G. Giacomelli, L. M. Schiffer, and H. Schnappauf. 1968. The maturation and fate of reticulocytes after in vitro labeling with tritiated amino acids. *Blood.* **31:** 33–43.
2. Ganzoni, A., R. S. Hillman, and C. A. Finch. 1969. Maturation of the macroreticulocyte. *Br. J. Haematol.* **16:** 119–135.
3. Shattil, S. J., and R. A. Cooper. 1972. Maturation of macroreticulocyte membranes in vivo. *J. Lab. Clin. Med.* **79:** 215–227.
4. Come, S. E., S. B. Shohet, and S. H. Robinson. 1974. Surface remodeling vs. whole-cell hemolysis of reticulocytes produced with erythroid stimulation or iron-deficiency anemia. *Blood.* **44:** 817–1830.
5. Wise, W. C. 1976. Maturation of membrane function: transport of amino acid by rat erythroid cells. *J. Cell. Physiol.* **87:** 199–211.
6. Bernstein, R. E. 1959. Alterations in metabolic energetics and cation transport during aging of red cells. *J. Clin. Invest.* **38:** 1572–1586.
7. Lauf, P. K., B. A. Rasmusen, P. G. Hoffman, P. B. Dunham, P. Cook, M. L. Parmelee, and D. C. Tosteson. 1970. Stimulation of active potassium transport in LK sheep red cells by blood group-L-antiserum. *J. Membr. Biol.* **3:** 1–13.
8. Smith, E. K. M., and L. G. Welt. 1967. A reversible defect of membrane ATPase in the red cells of uremic patients. *Clin. Res.* **15:** 51. (Abstr.)
9. Cooper, R. A., and J. H. Jandl. 1968. Bile salts and cholesterol in the pathogenesis of target cells in obstructive jaundice. *J. Clin. Invest.* **47:** 809–822.
10. Dacie, J. V., and S. M. Lewis. 1975. Practical Haematology. J. & A. Churchill Ltd., London. 5th edition. 79.
11. Lew, V. L. 1971. On the ATP dependence of the Ca^{2+}-induced increase in K^+ permeability observed in human red cells. *Biochim. Biophys. Acta.* **233:** 827–830.
12. Wiley, J. S., and R. A. Cooper. 1974. A furosemide-sensitive cotransport of sodium plus potassium in the human red cell. *J. Clin. Invest.* **53:** 745–755.
13. Griffiths, W. J., and M. Fitzpatrick. 1967. The effect of age on the creatine in red cells. *Br. J. Haematol.* **13:** 175–180.
14. Hoffman, J. F. 1969. The interaction between tritiated ouabain and the Na-K pump in red blood cells. *J. Gen. Physiol.* **54:** 343s–350s.
15. Wiley, J. S., J. C. Ellory, M. A. Shuman, C. C. Shaller, and R. A. Cooper. 1975. Characteristics of the membrane defect in the hereditary stomatocytosis syndrome. *Blood.* **46:** 337–356.
16. Murphy, J. R. 1973. Influence of temperature and method of centrifugation on the separation of erythrocytes. *J. Lab. Clin. Med.* **82:** 334–341.
17. Hoffman, J. F., and F. M. Kregenow. 1966. The characterization of new energy dependent cation transport processes in red blood cells. *Ann. N. Y. Acad. Sci.* **137:** 566–576.
18. Schatzmann, H. J., and F. F. Vincenzi. 1969. Calcium movements acoss the membrane of human red cells. *J. Physiol. (Lond.).* **201:** 369–395.
19. Kregenow, F. M., and J. F. Hoffman. 1972. Some kinetic and metabolic characteristics of calcium-induced transport in human red cells. *J. Gen. Physiol.* **60:** 406–429.
20. Griffiths, W. J., and E. J. Lothian. 1969. Erythropoiesis, red-cell creatine and plasma aldolase activity in anaemia in the rabbit and man. *Br. J. Haematol.* **17:** 477–484.
21. Koch, P. A., F. H. Gardner, and J. R. Carter, Jr. 1973. Rec cell maturation: loss of a reticulocyte-specific membrane protein. *Biochem. Biophys. Res. Commun.* **54:** 1296–1299.
22. Canham, P. B., and A. C. Burton. 1968. Distribution of size and shape in populations of normal human red cells. *Circ. Res.* **22:** 405–422.
23. Blaustein, M. P., and A. L. Hodgkin. 1969. The effect of cyanide on the efflux of calcium from squid axons. *J. Physiol. (Lond.).* **200:** 497–527.
24. Borle, A. B. 1969. Kinetic analysis of calcium movements in Hela cell cultures. I. Calcium influx. *J. Gen. Physiol.* **53:** 43–56.
25. Cittadini, A., A. Scarpa, and B. Chance. 1973. Calcium transport in intact Ehrlich Ascites tumor cells. *Biochim. Biophys. Acta.* **291:** 246–259.
26. Harrison, D. G., and C. Long. 1968. The calcium content of human erythrocytes. *J. Physiol. (Lond.).* **199:** 367–381.
27. Lichtman, M. A., and R. I. Weed. 1972. Divalent cation content of normal and ATP-depleted erythrocytes and erythrocyte membranes. *Nouv. Rev. Fr. Hematol.* **12:** 799–813.
28. Erdmann, E., and W. Hasse. 1975. Quantitative aspects of ouabain binding to human erythrocyte and cardiac membranes. *J. Physiol. (Lond.).* **251:** 671–682.
29. Gardner, J. D., and T. P. Conlon. 1972. The effects of sodium and potassium on ouabain binding by human erythrocytes. *J. Gen. Physiol.* **60:** 609–629.
30. Ellory, J. C., and R. D. Keynes. 1969. Binding of tritiated digoxin to human red cell ghosts. *Nature (Lond.).* **221:** 776.
31. Hoffman, J. F. 1973. Molecular aspects of the Na^+, K^+-pump in red blood cells. *In* Organization of Energy-Transducing Membranes. M. Nakao and L. Packer, editors. University Park Press, Baltimore. 9–21.
32. Lauf, P. K., and C. H. Joiner. 1976. Increased potassium transport and ouabain binding in human Rh^{null} red blood cells. *Blood.* **48:** 457–468.
33. Ferreira, H. G., and V. L. Lew. 1976. Use of ionophore A23187 to measure cytoplasmic Ca buffering and activation of the Ca pump by internal Ca. *Nature (Lond.).* **259:** 47–49.
34. Schatzmann, H. J. 1973. Dependence on calcium concentration and stoichiometry of the calcium pump in human red cells. *J. Physiol. (Lond.).* **235:** 551–569.
35. Wiley, J. S., and F. M. Gill. 1976. Red cell calcium leak in congenital hemolytic anemia with extreme microcytosis. *Blood.* **47:** 197–210.