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ARTICLE

Peter Bogner · Katalin Sipos · Andrea Ludány
Béla Somogyi · Attila Miseta

Steady-state volumes and metabolism-independent osmotic adaptation in mammalian erythrocytes

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Abstract Erythrocytes of various mammalian species – including human – maintain osmotic balance with the blood plasma (osmotic activity 270–310 mosmol). However, their intracellular levels of osmotically active ions (potassium, sodium, chloride, and hydrogencarbonate), water content and osmotic resistance deviate significantly. In the present report we study the relationship among intracellular water, potassium and sodium levels of the erythrocytes of various mammalian species and in the developing calf. In addition, the osmotic resistance, K^+ (Rb^+) uptake and the DPH fluorescence anisotropy of various erythrocytes and erythrocyte ghost membranes were correlated. The results show no statistically significant relationship between erythrocyte water content and $[K^+ + Na^+]$ levels or K^+/Na^+ ratios. The reversal of erythrocyte K^+/Na^+ ratios coincides with the decrease of steady-state ATP levels in the developing calf. The mobility of lipids within the hydrophobic inner layer of the plasma membrane relates closely to passive K^+ (Rb^+) uptake, and plays a significant role in regulatory volume changes.

Keywords Volume regulation · Erythrocyte · Na, K-ATPase · Membrane fluidity · Hemoglobin

P. Bogner · A. Ludány · A. Miseta (✉)
Department of Clinical Chemistry, University of Pécs, Faculty of Medicine, Ifjúság u. 13, 7624 Pécs, Hungary
E-mail: miseta@clinics.pote.hu
Tel.: +36-72-536001 ext. 5310
Fax: +36-72-536121

P. Bogner
Diagnostic Institute, University of Kaposvár, Kaposvár, Hungary

K. Sipos
Department of Biochemistry, University of Pécs, Faculty of Medicine, Pécs, Hungary

B. Somogyi
Department of Biophysics, University of Pécs, Faculty of Medicine, Pécs, Hungary

Introduction

The osmolality of blood plasma is maintained in the 270–310 mosmol range in various mammals. The major osmotic substances are sodium and potassium ions and their anions (chloride, hydrogencarbonate). In humans the reference ranges for plasma sodium and potassium are 136–145 mM and 3.6–5.4 mM, respectively. Glucose and urea are the only osmotically active metabolites present in the millimolar concentration range. The molar concentration of plasma proteins is low (1.33–1.82 mM), but they account for 8–12 mosmol colloid oncotic pressure.

Although the major plasma constituents of various mammalian species are similar, their erythrocytes contain various concentrations of water, potassium and sodium ions (Ellory and Tucker 1983; Miseta et al. 1992, 1993a, 1993b; Wheatley et al. 1994). Since more than 95% of the total erythrocyte protein content is hemoglobin, changes in erythrocyte water/protein levels are mirrored in the mean corpuscular hemoglobin concentrations (MCHC).

Hawkey et al. (1991) studied the main hematological parameters of more than 200 species belonging to 16 mammalian orders and found MCHC values in the 271–459 g/L range. Consequently, the steady-state water content of mammalian erythrocytes varies significantly.

Ideally, changes of the erythrocyte water content relate to the concentration of osmotically active molecules within the cell (Cameron et al. 1988, 1997; Colclasure and Parker 1991; Gary Bobo 1967; O'Neill 1999). However, this "null hypothesis" is confounded by the facts that, in addition to mobile cations and anions, the cytosol contains macromolecules (mostly hemoglobin), which display non-ideal osmotic behavior (Bogner et al. 1998; Zimmerman et al. 1995), and a number of osmotically active metabolic intermediates, which might interact with proteins and alter their osmotic behavior.

We described recently that the tightness of hemoglobin packaging (MCHC) relates to the hydrophilicity

of hemoglobins (Bogner et al. 1998). For example, the erythrocytes of camel and camelids (llama, guanaco, vicugna) contain about 45% hemoglobin, when compared to 25–35% in most other mammals (Bogner et al. 1998; Perk 1963). In addition, augmented hemoglobin hydrophilicity increases the osmotically non-responsive water fraction within erythrocytes (Bogner et al. 1998). The physiologically important consequence is that erythrocytes with high osmotically non-responsive water fractions tend to be more resistant to hyper- and hypohydration than others. The need for tolerating both hypo- and hyperhydration explains why desert animals evolved in a way such that their hemoglobins became rich in charged hydrophilic amino acid residues.

Although the osmotic resistance of camel erythrocytes is exceptional, other species shows remarkable variance as well (Coldman et al. 1970; Lang et al. 1998). However, the hemoglobins of non-camelid mammals are more similar in their hydrophilic properties. Therefore, differences in the osmotic resistance of their erythrocytes might be due to different plasma membrane properties.

The constancy of the cell volume under physiological conditions is generally thought to reflect a balance between solute influx and efflux, and is therefore critically dependent on the properties of the plasma membrane (Benga and Borza 1995; Crespo et al. 1988; Dunham 1992; Eitan et al. 1976; Nelson 1972; Miseta et al. 1995; Sarkadi and Parker 1991). For example, the plasma membrane monovalent cation permeability may be enhanced by the incorporation of polyoxyethylene-based non-ionic detergents (Miseta et al. 1995). In turn, enhanced monovalent cation permeability results in increased osmotic resistance in human erythrocytes.

In the present report we address three specific questions:

1. How does the erythrocyte steady-state water content relate to the concentrations of monovalent cations in various species?
2. What is the relationship between the erythrocyte water content and the K^+/Na^+ ratio under physiological conditions?
3. What is the relationship between the physicochemical properties of plasma membranes, passive potassium fluxes and the osmotic resistance of various erythrocytes?

Materials and methods

Materials

Heparinized human blood samples (100 IU/mL) were drawn from healthy volunteers. Heparinized blood samples (100–200 IU/mL) of different animal species (cat, sheep, pig, dog, rabbit, horse, rat, hamster, guinea pig, red deer, bovine, goat) were obtained from the experimental farm of the University of Kaposvár, Faculty of Animal Sciences, Kaposvár, Hungary. Baboon and camel blood samples were obtained from Pécs Zoo and Budapest Zoo, Hungary, respectively. Samples were placed on wet ice immediately after collection and kept there until use. Ouabain and RbCl were purchased from Sigma (St Louis, Mo., USA). All other chemicals were purchased from Reanal, Hungary.

Rb uptake measurements

Before incubation, heparinized blood samples were supplemented with 5 mM RbCl (plasma concentration) and 5 mM glucose was added to provide a sufficient energy source for the 8 h incubation period. Glucose levels were re-checked at the end of the incubations. Before starting, the incubation samples were divided into two equal volume aliquots. One aliquot was complemented with 1 mM ouabain. At the start of the incubation the preparations were placed at 37 °C in a water bath, and 0.5 mL samples taken at chosen time-points for ion analysis. The pelleted erythrocytes were processed for ion measurement as described below.

Measurements of erythrocyte K^+, Na^+, Rb^+ and water content

When collected for measurements of intracellular ions, the samples were centrifuged for 1 min, at 13,000×*g*, and the plasma removed and saved. The pellets were spun for an additional 15 min at 13,000×*g* at room temperature and any residual plasma was carefully removed. Centrifuged erythrocytes were weighed before being dried in a Savant SC-110 speed vacuum system (USA) until no further loss of water occurred (usually 6 h). Dry weights were subsequently measured, 0.8 mL of 1 M HCl was added to each sample, and incubated at room temperature on a rocker table for at least 24 h.

K^+ and Na^+ levels were measured in a flame photometer (Eppendorf EFOX 5070, Germany). Rb^+ levels were measured in a Varian AA-20 atomic absorption spectrometer (Varian Techtron, Australia). Erythrocyte ion concentrations were calculated based on sample ion concentration, dry weight and water content data, and dilution factors.

Measurements of erythrocyte ATP levels

Erythrocyte ATP concentrations were determined by the chemiluminescent firefly luciferin/luciferase assay system (ATP bioluminescence kit, Sigma) according to the method modified in our laboratory (Kõszegi 1988).

Osmotic resistance test

Blood samples were centrifuged at 4000×*g* at room temperature for 10 min, the plasma and buffy coat were carefully removed, and the erythrocyte pellet re-suspended in physiological saline at 50% hematocrit.

Samples (20 μL) of erythrocyte suspension were added to 8 mL of various osmolarity NaCl solution (50–300 mosmol in 25 mosmol increments). Samples were incubated at room temperature for 30 min, and centrifuged at 5000×*g* for 5 min. The 540 nm absorption of the supernatant was measured with a Perkin-Elmer Lambda 2 (USA) spectrophotometer. Complete hemolysis of the samples was induced by the addition of 0.1% Triton X-100 detergent, and the 540 nm absorption measured again.

Erythrocyte ghost preparation and fluorescence emission anisotropy measurements

Erythrocyte ghosts were prepared by the method of Dodge et al. (1962). Isolated ghosts were labeled with DPH or TMA-DPH as

previously described (Miseta et al. 1995). Measurements were carried out with a Hitachi MPF-4 spectrofluorimeter (Japan), equipped with polarization accessories, at 37 °C using wavelengths of 360 nm and 425 nm for excitation and detection, respectively. Fluorescence anisotropy was calculated according to the equation $r = (I_{vv} - GI_{vh})/(I_{vv} + 2GI_{vh})$, where I_{vv} and I_{vh} are the fluorescence intensities measured with a vertical polarizer, and a vertically or horizontally mounted analyzer, respectively ($G = I_{hv}/I_{hh}$) (Donner and Stoltz 1985). For each sample the fluorescence was corrected for the scattering effect of unlabeled ghosts.

Isosmotic dehydration of human erythrocytes

Human erythrocytes were placed into a 300 mosmol mannitol solution at a hematocrit value of 10% at 4 °C. Samples (1.5 mL) were collected for at least 24 h. The frequency of sample taking was 2 h during the first 12 h but gradually decreased to 12 h towards the end of the incubation. Samples were processed for K^+, Na^+ and water measurements as described above.

Electrophoresis and Western blot analysis

Erythrocyte ghosts were dissolved in a 5× Laemmli sample buffer. Anti-AQP1 antibodies were a generous gift of Prof. Soren Nielsen (Aarhus, Denmark). The polyclonal antibody against aquaporin-1 was previously characterized (Nielsen et al. 1993).

Equal amounts (25 μg) of each protein sample were run on 12% polyacrylamide gel according to Laemmli (1970) with standard (100 V) voltage, then transferred to a nitrocellulose membrane using a semi-dry electrophoretic transfer method with a standard current (180 mA) for 45 min, and a buffer of 48 mM Tris, 39 mM glycine, 0.037% SDS, 20% methanol. The membrane was washed three times for 5 min in TBST (20 mM Tris-HCl, pH 7.4, 0.9% NaCl, 0.1% Tween 20), then blocked for 30 min in TBST containing 3% non-fat dry milk at room temperature with constant agitation. After blocking, the membrane was rinsed again with TBST, then probed with 1:2000 dilution of anti-AQP1 rabbit antiserum in TBST containing 1.5% non-fat dry milk. The reaction was carried out at room temperature with constant agitation for 1 h. After the reaction with the polyclonal antibody solution the membrane was washed again three times for 5 min with TBST and then reacted with 1:5000 dilution of anti-rabbit IgG (Sigma) conjugated with the enzyme peroxidase in TBST with 1.5% non-fat dry milk for 45 min at room temperature. After washing the membrane extensively in TBST, the immunoblots were developed using the ECL system.

Results

Monovalent cation levels and water content of mammalian erythrocytes

Potassium, sodium and chloride are the major osmotically active substances within erythrocytes. The concentration of plasma membrane non-permeable osmotically active substances (proteins, metabolites) is 4- to 6-fold higher within the erythrocyte when compared to plasma. Potassium and sodium concentrations of erythrocytes of 15 mammalian species are listed in Table 1. All species maintain 270–310 mosmol osmotic pressure in their blood plasma.

However, the water content of their erythrocytes differ remarkably. The water content of erythrocytes of most species fall into the 1.80–2.25 g water/g dry mass range. Erythrocytes of camels contain much less water (1.16 g water/g dry mass). These results show that the water content of various mammalian erythrocytes do not correlate significantly with $[K^+ + Na^+]$ concentrations (Fig. 1). Some of the LK-type erythrocytes (cat, sheep, dog) contain relatively high concentrations of monovalent cations, but their water content is not significantly higher when compared to other species.

We reported earlier that erythrocytes of the camel and camelids contain hemoglobins rich in charged amino acid residues (Bogner et al. 1998). Although both acidic (Asp, Glu) and basic (Arg, Lys, His) amino acids are present in higher numbers when compared to most other mammalian species, the net result is an increase of hemoglobin isoelectric point (p*I*) and hydrophilicity. The increase of the fixed (hemoglobin bound) positive charges results in reduced mobile monovalent cation levels. In addition, the increased hydrophilicity of camel hemoglobins allows closer packaging. Consequently, the net water content is reduced, while the osmotically non-responsive water fraction is increased.

Table 1 Monovalent ion and water contents of mammalian erythrocytes

	n	K^+ (mM) (av ± SD)	Na^+ (mM) (av ± SD)	$K^+ + Na^+$ (mM) (av ± SD)	K^+/Na^+	H_2O[a] (av ± SD)
Cat	3	11.0 ± 0.1	187.3 ± 5.2	198.3	0.06	1.95 ± 0.04
Sheep	16	35.8 ± 2.7	157.7 ± 6.8	193.5	0.17	1.85 ± 0.07
Pig	6	165.6 ± 5.2	26.5 ± 1.9	192.1	6.26	1.85 ± 0.09
Dog	3	10.8 ± 1.23	180.4 ± 4.5	191.2	0.06	1.98 ± 0.03
Rabbit	6	154.8 ± 4.1	22.3 ± 1.4	177.1	6.90	1.99 ± 0.05
Horse	6	139.8 ± 4.7	31.7 ± 2.5	171.5	4.40	1.83 ± 0.05
Rat	5	141.2 ± 6.0	25.1 ± 2.1	166.3	5.62	1.96 ± 0.06
Hamster	5	140.0 ± 6.7	26.0 ± 2.6	166.0	5.35	2.09 ± 0.06
Human	14	142.5 ± 3.9	23.4 ± 2.8	165.4	6.08	2.09 ± 0.05
Guinea pig	3	130.6 ± 3.7	34.7 ± 1.1	165.3	3.76	2.19 ± 0.01
Red deer	3	122.8 ± 6.8	39.5 ± 4.35	162.3	3.15	1.85 ± 0.03
Baboon	6	133.5 ± 9.1	28.1 ± 5.7	161.6	4.92	2.25 ± 0.15
Bovine	5	41.6 ± 2.8	112.2 ± 5.3	153.8	0.36	1.86 ± 0.07
Goat	6	104.0 ± 5.0	48.0 ± 3.2	152.0	2.17	1.81 ± 0.14
Camel	3	69.7 ± 3.2	58.7 ± 2.8	128.4	1.12	1.16 ± 0.05

[a] g water/g dry mass

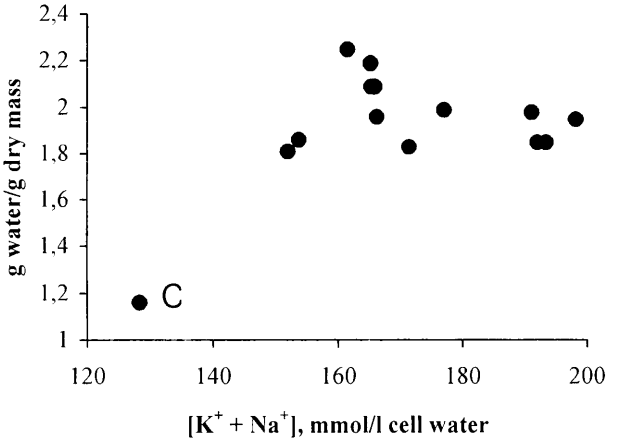

Fig. 1 The relationship between the monovalent cation concentrations $[K^+ + Na^+]$ and the water content of the erythrocytes of 15 mammalian species (see Table 1). Camel erythrocytes are indicated (*C*). No statistically significant correlation was detected between the two sets of parameters

The reversal of potassium and sodium concentrations does not result in a significant alteration of cellular water content, but relates to lower ATP levels in bovine erythrocytes

The erythrocytes of newborn calves are of the HK type. A reversal of the K^+ and Na^+ levels takes place after the 19th day of development (Fig. 2). We determined whether the reversal of the erythrocyte K^+/Na^+ ratio is associated with changes of $[K^+ + Na^+]$ and water content of the erythrocytes. The results showed no significant change in the erythrocyte $[K^+ + Na^+]$ levels or MCHC values following the complete reversal of intracellular K^+/Na^+ ratios (Fig. 2). The ATP concentrations of calf erythrocytes decrease in parallel with the decreasing K^+/Na^+ ratios ($r = 0.981$) (Fig. 2b). These results are in line with earlier observations indicating close positive correlation between the erythrocyte ATP levels and K^+/Na^+ ratios in various mammalian species and in individual Suffolk-type sheep (Miseta et al. 1993a).

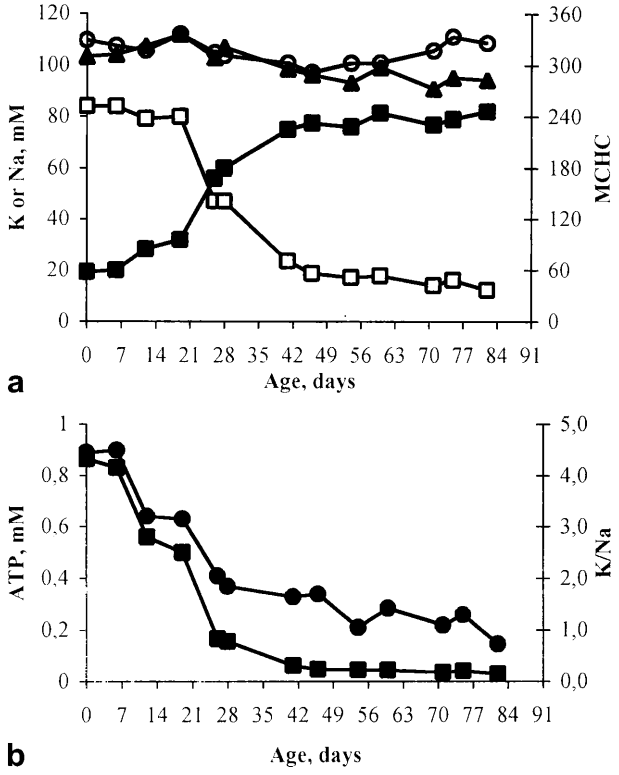

Fig. 2 **a** Erythrocyte K^+ (*empty squares*), Na^+ (*filled squares*), $K^+ + Na^+$ (*triangles*) levels, and MCHC values (*circles*) in the developing calf following birth. The reversal of K^+/Na^+ ratios between the 19th and 40th days is not followed by significant changes in the $[K^+ + Na^+]$ level and MCHC values. **b** Erythrocyte ATP levels (*circles*) and K^+/Na^+ ratios (*squares*) in the developing calf. Changes of the K^+/Na^+ ratio and ATP levels correlate ($r = 0.981$)

Isosmotic dehydration of human erythrocytes in mannitol solution suggests that 33% of the water molecules are not associated with monovalent cations

When placed into isosmotic mannitol solution, potassium and sodium leak out from the erythrocytes. We used intact human erythrocytes to study if the amounts of lost monovalent cations correlated with the loss of water. In this media, human erythrocytes remain intact at 4 °C for at least 24 h. We found that the decrease in cellular water and $[K^+ + Na^+]$ levels showed an excellent positive linear correlation ($r = 0.995$) (Fig. 3). However, when plotting the water content of cells against the corresponding $[K^+ + Na^+]$ levels, the amount of cell

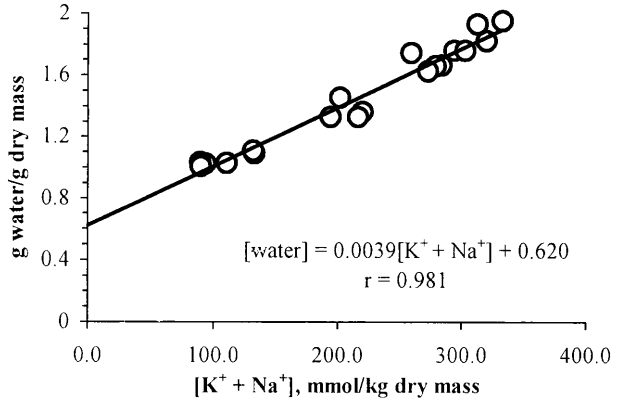

Fig. 3 The relationship between $[K^+ + Na^+]$ levels and water content during isosmotic dehydration of the human erythrocyte. Extrapolation of data indicates that 33% or 0.62 g water/g dry mass belongs to non-plasma membrane permeable osmolytes (proteins, metabolites)

water removed appears to be much less than anticipated, based on the intracellular concentrations of monovalent cations. Indeed, the curve predicts that 0.62 g water/g dry mass, or 33% of the total erythrocyte water, remains

within the erythrocyte after the (hypothetical) complete removal of potassium and sodium ions and their counter anions. Since the hemoglobin concentration in mature human erythrocytes is 4–6 times higher than the concentration of plasma proteins, the contribution to internal osmotic pressure is expected to be proportionally higher, ca. 40–70 mosmol, or 1/7th–1/4th of the total osmotic activity. Consequently, the actual osmotic contribution of hemoglobin (proteins) within the erythrocyte is about twice as much as anticipated. Accordingly, accurate adjustments in cell volume may be carried out by moving hydrated small ions, but hemoglobin plays a significant role in retaining water within the erythrocytes.

Rb^+ uptake, plasma membrane fluorescence anisotropy and the osmotic resistance of mammalian erythrocytes

The Rb^+ (K^+) uptake of the erythrocytes of 11 species (human, horse, hamster, rat, guinea pig, rabbit, sheep, goat, bovine, swine, camel) were studied in the presence or absence of 1 mM ouabain at 37 °C during an 8-h interval. Heparinized blood samples were analyzed with a CellDyn 3500 analyzer (RBC, Hb, Htc, MCV, MCHC, Price Jones distribution pattern), and the blood samples complemented with 5 mM RbCl. Figure 4 shows the correlation between the Na^+,K^+-ATPase-independent Rb^+ uptake and the fluorescence anisotropy of DPH-labeled erythrocyte ghosts. Exponential curve fitting resulted in a somewhat stronger correlation between the membrane fluorescence anisotropy and the Na^+,K^+-ATPase-independent Rb uptake ($r = 0.932$), when compared to linear fitting ($r = 0.905$).

DPH-dependent fluorescence anisotropy is known to represent the state of the hydrophobic core of biological membranes. TMA-DPH is a more hydrophilic derivative, which incorporates into the outer parts of lipid bilayers. No significant correlation between the anisotropy of TMA-DPH-labeled erythrocyte ghosts and the Na,K-ATPase-independent Rb^+ uptake was detected (data not shown).

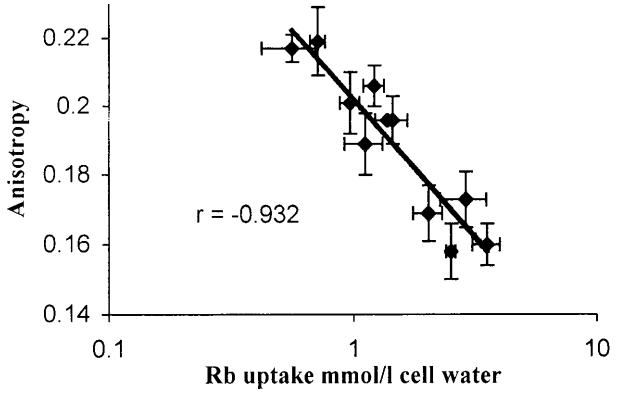

Fig. 4 The relationship between the fluorescence anisotropy of DPH-labeled erythrocyte ghosts and the Rb^+ uptake of erythrocytes in the presence of 1 mM ouabain in 11 mammalian species. The results suggest a good negative exponential relationship between the two sets of parameters ($r = -0.932$)

Osmotic fragility was characterized by the osmolality of the hypotonic NaCl solution where 50% of the erythrocytes lysed. The osmotic fragility and the Rb^+ uptake of the erythrocytes of 11 species (see above) were correlated. The results indicate a good positive correlation between osmotic fragility and Rb^+ uptake ($r = -0.547$) (Fig. 5). However, camel erythrocytes show extreme osmotic resistance, but rather average Rb^+ uptake. This is not surprising, since the increased osmotic resistance of camel erythrocytes is due to increased hemoglobin hydrophilicity, rather than to plasma membrane properties (Bogner et al. 1998). Therefore the correlation between the osmotic fragility of erythrocytes and Rb^+ uptake is further improved if data from camel erythrocytes are omitted ($r = -0.852$).

The above described correlations among Rb^+ uptake, DPH fluorescence anisotropy and osmotic resistance predict a good negative correlation between DPH fluorescence anisotropy and osmotic fragility. Indeed, the DPH fluorescence anisotropy of erythrocyte ghosts correlates negatively with the osmotic fragility of erythrocytes ($r = -0.912$).

Western blot analysis of aquaporin 1 in erythrocyte membranes

Anti-rat polyclonal antibody was used to detect aquaporin (type 1 isoform – Aqp1p) in erythrocyte membranes. The aim of this study was to reveal the possible relationship of Aqp1p to osmotic fragility. We loaded

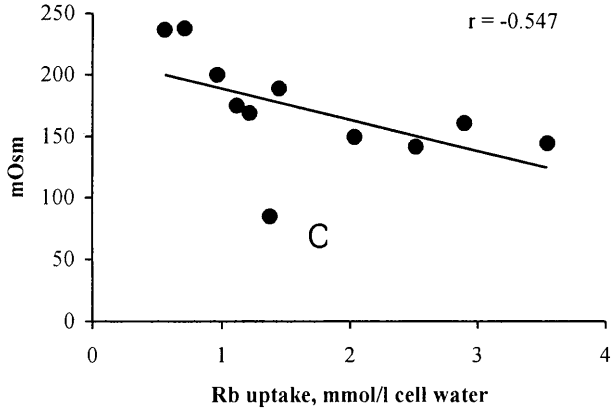

Fig. 5 The relationship between the ouabain-resistant Rb^+ uptake and osmotic fragility of erythrocytes of 11 mammalian species. The osmolarity of the NaCl solution in which 50% hemolysis was observed is displayed on the *y*-axis. The results suggest a relationship between the two sets of parameters ($r = -0.547$). Erythrocytes of the camel (*C*) are extremely resistant to osmotic lysis despite their average Rb^+ uptake. The correlation coefficient improves to $r = -0.852$ if data for the camel erythrocytes are omitted

samples from 10 species on polyacrylamide gels and carried out Western blot analysis as described in Materials and methods. Note that the lack of reaction might either be due to lack of antibody specificity, or to the low copy number of aquaporin in erythrocytes. Good cross-reactions between the anti-rat AQP1-ab and erythrocyte membrane proteins of rat, bovine, rabbit and sheep were found (Fig. 6). For example, we found that rat and sheep erythrocyte ghosts samples yield equally strong signals. Assuming that sheep Aqp1p reacts with the rat anti-AQP1-ab at 100% or less effectively, sheep erythrocytes must contain equal or higher numbers of Aqp1p. However, the osmotic resistance of sheep erythrocytes is much less when compared to rat erythrocytes. Consequently, no correlation could be established between the quantity of AQP1 and osmotic fragility.

Discussion

The physical rules of dilute aqueous solutions are widely used in cell physiology. However, most scientists agree that a correction must be applied, because the interior of cells is not an ideal dilute aqueous solution (Augutter and Wheatley 2000; Bogner et al. 1998; Cameron et al. 1988; Kellermayer et al. 1986; Miseta et al. 1991). Unfortunately, the term, ''bound water'' is dependent on the physical characteristics of water and the measurement method.

Mature mammalian erythrocytes lack cellular organelles and are densely packed with hemoglobin (typically more than 95% of the total protein content is hemoglobin). While the overall homology similarity of hemoglobin molecules of 16 mammalian species belonging to different orders is more than 80% (Bogner et al. 1998), erythrocyte volumes, shapes, water concentrations (MCHC), monovalent cation levels and transport mechanisms show remarkable variation (Ellory and Tucker 1983; Evans 1954; Hawkey et al. 1991; Miseta et al. 1993a; Wheatley et al. 1994). Since the discovery was made of the Glu-Val change in the sixth position of the HBB chain in human hemoglobin, it is clear that a single amino acid replacement might dramatically alter hemoglobin packaging, shape, water and electrolyte levels, as well as the physiological properties of the erythrocyte (Ingram 1957).

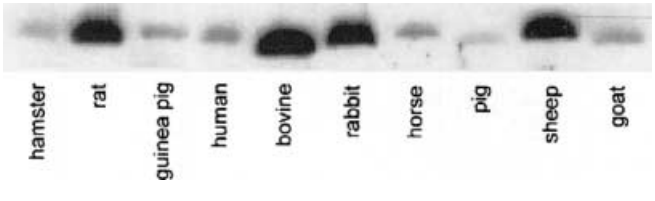

Fig. 6 Western blot analysis of the plasma membrane proteins of various erythrocytes. Samples were loaded according to decreasing osmotic resistance (*left to right*). Anti-rat AQP1 monoclonal antibody was used, and gave strong signals with rat, bovine, rabbit, and sheep samples, representing various osmotic resistances. Data suggest no relationship between AQP1 levels and the osmotic resistance of the erythrocytes of these species

Steady-state erythrocyte volumes in different species

In the present report we describe that, although the $[K^+ + Na^+]$ and water levels show significant variation in various mammalian erythrocytes, no statistically significant relationship could be established between these parameters (Table 1). This observation complements the earlier evidence indicating that it is not the electrolyte levels but the hydrophilicity of hemoglobin molecules that determines the steady-state erythrocyte volumes (Bogner et al. 1998).

Differences in the K^+ and Na^+ concentrations between the plasma and the erythrocyte are linked to the consumption of chemical energy via Na,K-ATPase (Kazennov et al. 1998; Skou 1990). When inhibited by the cardiac glycoside ouabain, the intracellular K^+ level decreases, while the Na^+ and water content increase in HK-type erythrocytes (Dunham and Hoffman 1971; Palma et al. 1994). Therefore the decrease of intracellular K^+/Na^+ ratios is believed to be directly associated with swelling. Based on our data, no significant correlation between the water content and K^+/Na^+ ratios of various erythrocytes could be established. This is in line with the observation that there is no statistically significant difference in the erythrocyte water content of HK and LK sheep (Miseta et al. 1993a).

Also in agreement with earlier results, the reversal of the erythrocyte K^+ and Na^+ levels in the developing calf correlates with ATP levels. Reduced ATP levels in LK-type erythrocytes may relate to increased ATP degradation by other metabolic pathways (glutathione synthesis) or to increased channeling of glycolytic metabolites towards the pentose phosphate shunt or 2,3-DPG synthesis.

Mature erythrocytes of all species are exposed to oxidative and mechanical stress while carrying out their physiological function. Although some authors questioned if the loss of water and increasing density are good markers of erythrocyte aging (Beutler 1984), Waugh et al. (1992) combined different techniques for the unambiguous isolation of aged erythrocytes and found that progressive dehydration and volume decrease are characteristic features of erythrocyte aging. Earlier, we showed that these changes are associated with the decrease of K^+ and Cl^- content, while Ca^{2+} and SO_4^{2-} increased (Cameron et al. 1993). No statistically significant difference was observed in Na^+, Mg^{2+} and phosphorus levels.

In the present report, we used an older method, i.e. the incubation of erythrocytes in a plasma membrane impermeable mannitol solution, to obtain data about erythrocyte osmotic homeostasis. We found that 33% of erythrocyte water is resistant to the removal of potassium

and sodium ions and their counter anions in humans (Fig. 3).

Earlier determinations of osmotically inactive water fractions in erythrocytes were based on the exposure of erythrocytes to solutions of various osmolarity (Bogner et al. 1998; Ling and Negendank 1970). Under these circumstances, some loss of monovalent cations (which also depends on the osmolarity of the media) cannot be avoided. Such results underestimate osmotically inactive water fractions.

Metabolism-independent monovalent cation movement and regulatory volume changes in various mammalian erythrocytes

Although the osmotic contribution of hemoglobin is more than anticipated, monovalent cations and anions contribute some two-thirds of the total osmotic activity within the human erythrocyte. In the case of hypotonic challenge, the upper limit of volume change is the beginning of hemolysis and this limit is also known as osmotic resistance or fragility.

In the present report we show a very good correlation between the DPH fluorescence anisotropy and passive, i.e. ouabain-resistant, K^+ (Rb^+) uptake (Fig. 4). This observation suggests that the K^+ (Rb^+) uptake is significantly affected by the fluidity of the cell membrane. In addition, increased K^+ (Rb^+) uptake is closely associated with increased osmotic resistance (Fig. 5).

Earlier, Coldman et al. (1970) showed that the amounts of various membrane lipid components, especially phosphatidylcholine, may be correlated with osmotic fragility in different mammalian erythrocytes ($r = -0.950$). Kirk (1977) demonstrated a good positive relationship between total K^+ uptake and membrane phosphatidylcholine content in erythrocytes of nine mammalian species ($r = 0.765$). Miseta et al. (1995) showed that the incorporation of Brij series detergents affected the membrane fluidity of human erythrocytes, and that these changes were closely associated with increased K^+ and Na^+ permeabilities and osmotic resistance. It appears that the swift swelling of the erythrocytes in hypotonic solutions is immediately followed by the loss of mobile cations. However, the speed of loss of hydrated monovalent cations and their anions is critical in preventing further swelling and lysis. The evidence listed above suggests that this property is dependent on the lipid composition and physicochemical properties of the inner hydrophobic plasma membrane layer of erythrocytes.

Fernandes and Dewey (1994) reported the significance of bumetanide-inhibitable K^+ transport in erythrocytes of mice that carried a gene for resistance to osmotic lysis (*rol*). In these cells the total K^+ (Rb^+) uptake is increased by 56% compared to control cells. This increase has been linked mainly to the 4.5-fold increase of bumetanide-sensitive co-transport flux rate. Interestingly, the Na,K-ATPase activity (ouabain-inhibited flux) is also increased by almost 50%, but could not entirely compensate for the increased plasma membrane permeability. Therefore, the steady-state K^+ level was significantly lower when compared to the control cells. We did not see a relationship between the Aqp1p levels and erythrocyte osmotic resistance.

According to our results, the steady-state volume of mammalian erythrocytes is dependent on hemoglobin primary structure and hydrophilicity and no correlation was found between the $[K^+ + Na^+]$ levels and erythrocyte water content.

In contrast, adjustment to changing plasma osmolarity is carried out by two metabolism-independent factors:

1. The ouabain-resistant monovalent cation transport is a major contributor to the osmotic resistance of erythrocytes, and relates to the fluid state of plasma membrane inner layers.
2. Naturally, the more protein-bound water within the erythrocyte, the less is the net water movement in hypo- or hyperosmotic media. This property relates to hemoglobin hydrophilicity, and explains the osmotic behavior of the camel erythrocyte, which is extremely resistant to osmotic lysis but has rather average plasma membrane K^+ (Rb^+) permeability.

Although we were unable to find a correlation between the Aqp1p levels and the osmotic fragility of various erythrocytes, it is possible that the regulation of the aquaporin pathway differs in various species, and that these functional differences may affect osmotic adaptation.

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