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The Influence of Erythrocyte Maturity on Ion Transport and Membrane Lipid Composition in the Rat

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Summary

Significant relationships between ion transport and membrane lipid composition (cholesterol, total phospholipids and sphingomyelins) were found in erythrocytes of salt hypertensive Dahl rats. In these animals mean cellular hemoglobin content correlated negatively with Na^+-K^+ pump activity and Na^+ leak but positively with Na^+-K^+ cotransport activity. Immature erythrocytes exhibit lower mean cellular hemoglobin content (MCHC) than mature ones. The aim of the present study was to find a relationship between erythrocyte maturity, membrane lipid composition and ion transport activity in Wistar rats aged three months which were subjected to repeated hemorrhage (blood loss 2 ml/day for 6 days) to enrich circulating erythrocytes with immature forms. Immature and mature erythrocyte fractions in control and hemorrhaged rats were separated by repeated centrifugation. Hemorrhaged rats had increased number of reticulocytes but reduced hematocrit and MCHC compared to control rats. Immature erythrocytes of hemorrhaged rats differed from mature ones of control animals by elevated Na^+-K^+ pump activity, reduced Na^+-K^+ cotransport activity and increased Rb^+ leak. These ion transport changes in immature erythrocytes were accompanied by higher concentration of total phospholipids in their cell membranes. Membrane phospholipid content correlated positively with Na^+-K^+ pump activity and cation leaks but negatively with Na^+-K^+ cotransport activity. Moreover, they were also negatively related with MCHC which correlated negatively with Na^+-K^+ pump activity and Rb^+ leak but positively with Na^+-K^+ cotransport activity. Thus certain abnormalities of erythrocyte ion transport and membrane lipid composition detected in hypertensive animals might be caused by higher incidence of immature cells.

Key words

Reticulocytes • Immature erythrocytes • Mean cellular hemoglobin content • Na^+-K^+ pump • Na^+-K^+ cotransport • Na^+ leak • Rb^+ leak • Membrane phospholipids • Membrane cholesterol

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Introduction

Growing and/or proliferating cells change not only their morphology but also their function. Vascular smooth muscle cells are a good example because mature cells with contractile phenotype might dedifferentiate into the cells with synthetic phenotype which are characterized by reduced contractility, altered response to various vasoactive agents, abnormal cell calcium handling and different changes of receptors, ion transporters and intracellular signaling pathways (Matchkov *et al.* 2012, Misárková *et al.* 2016). There is increasing evidence that such pronounced changes can also be found during the maturation of human or rat erythrocytes.

Immature human erythrocytes, which are enhanced in the circulation e.g. during anemia of various origin, have lower mean cellular hemoglobin content (MCHC), increased K^+ content and augmented activity of the Na^+-K^+ pump and reduced activity of the Na^+-K^+

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cotransport in comparison with mature erythrocytes (Hentschel *et al.* 1986, Engelmann *et al.* 1990a). Repeated blood donations increased erythrocyte K^+ content and enhanced the activities of multiple transport systems such as K^+-Cl^- cotransport, Na^+-Li^+ countertransport or Na^+-K^+ pump, whereas this was not the case for Na^+-K^+-$2Cl^-$ cotransport system (Brugnara *et al.* 1993). K^+-Cl^- cotransport activity, which is responsible for volume reduction of reticulocytes, is also enhanced in immature erythrocytes of healthy humans (Brugnara and Tosteson 1987) or patients with sickle cell anemia (Canessa *et al.* 1987, Etzion *et al.* 1996, Quarmyne *et al.* 2011).

The enhanced activity of Na^+-K^+ pump was also demonstrated in immature sheep and pig erythrocytes and this activity decreased during *in vitro* maturation of these cells (Blostein *et al.* 1983, Lauf *et al.* 1984). Potassium turnover is also markedly enhanced in rat reticulocytes, both influx and efflux rates being approximately threefold increased compared to mature cells. The enhanced K^+ accumulation in reticulocytes can be attributed to the increased number of Na^+-K^+ pump units which are qualitatively identical to those of mature cells (Furukawa *et al.* 1981). Moreover, Mäirbaurl *et al.* (2000) demonstrated that enhanced Na^+-K^+ pump activity and augmented ouabain- and bumetanide-insensitive Na^+ leak decreased during *in vitro* maturation of rat reticulocytes. Finally, Ihrig *et al.* (1992) reported a progressive age-dependent reduction of ouabain-sensitive K^+ influx mediated by Na^+-K^+ pump in erythrocytes of Wistar rats between the 11^{th} and 19^{th} week of their age, whereas bumetanide-sensitive K^+ influx mediated by Na^+-K^+ cotransport moderately increased in the same age period.
Our previous study in Dahl rats (Zicha and Duhm 1990) reported decreased MCHC and very high Na^+-K^+ pump activity due to the augmented Na^+ leak in a subgroup of anemic salt hypertensive animals. Furthermore, we showed significant relationships between erythrocyte ion transport and membrane lipid composition in Dahl rats with salt hypertension in which MCHC correlated negatively with activity of the Na^+-K^+ pump and Na^+ leak and positively with activity of the Na^+-K^+ cotransport (Vokurková *et al.* 2005).

It should be kept in mind that the changes in membrane lipid composition might considerably modify Na^+ and K^+ transport in erythrocytes as was demonstrated by Engelmann *et al.* (1990b, 1993). Sailaja *et al.* (2004) reported a reduction of cholesterol and phospholipid membrane content during maturation of human reticulocytes, whereas cholesterol-to-phospholipid ratio was increased in mature erythrocytes compared to reticulocytes. Moreover, fatty acid compositions of phosphatidylcholine and phosphatidylethanolamine as well as the rates of fatty acid esterification to these phospholipids were found to be changed progressively during erythrocyte *in vivo* aging. These changes were revealed by a comparison of "young" and "old" erythrocyte populations separated by density gradient centrifugation (Kunimoto *et al.* 1984).

The aim of our present study was to find a relationship between erythrocyte maturity, lipid composition of erythrocyte membranes and the activity of ion transporters in the rat. Density centrifugation was used to separate erythrocytes into fractions enriched with younger and older cells in control rats and in animals subjected to repeated hemorrhage in order to increase the fraction of immature cells in the circulating erythrocytes. In this study we compared immature erythrocytes of hemorrhaged rats with mature erythrocytes of control animals.

Methods

Animals

Eighteen male Wistar rats (Velaz, CR) aged three months were used in this study. Animals were housed under standard laboratory conditions (temperature 23±1 °C, 12-h light/dark cycle) and maintained on tap water and standard rodent chow ST 1 *ad libitum*. One half of the rats (experimental group) was subjected to repeated hemorrhage blood loss 2 ml per day for 6 consecutive days to enrich circulating erythrocytes with immature forms. On the day of the experiment, about 10 ml of blood was withdrawn from the abdominal aorta and anticoagulated by 200 IU of heparin (Léčiva, Prague, CR).

All procedures performed in experimental animals, which were approved by the *Ethical Committee of the Institute of Physiology of the Czech Academy of Sciences*, conform to the *European Convention on Animal Protection*.

Isolation of immature and mature erythrocytes

Hematocrit (15 min, hematocrit centrifuge 316, Unipan, Warsaw, Poland) and hemoglobin content (Merckotest Hemoglobin, Merck, Darmstadt, Germany) were determined from heparinized blood and used for calculation of mean cell hemoglobin content (MCHC,

mmol/l RBC). Thereafter blood was centrifuged (10 min, 2205 x g, 4 °C) and plasma and buffy coat were removed. Isolated red blood cells (RBC) from control and hemorrhaged rats were gently resuspended in isotonic choline chloride solution (155 mmol/l) and the immature RBC were separated by repeated centrifugation (10 min, 2205 x g, 4 °C) from the mature cells. 50 % suspensions of erythrocytes were prepared from the matured and immatured erythrocytes and used for subsequent determinations.

Counting of reticulocytes

Both whole blood and 50 % suspension of erythrocytes were mixed with 2 % brilliant cresyl blue and kept at room temperature for 15 min. The number of reticulocytes was expressed per 1000 erythrocytes which were counted under 100 x oil immersion.

Determination of ion transport

Ion transport parameters were measured in the 50 % suspension of erythrocytes according to the protocol described in details by Bin Talib and Zicha (1992). Briefly, erythrocytes were washed three times with isotonic choline chloride solution (155 mmol/l) and hemolyzed by 6 % n-butanol with 1 % cesium chloride. Erythrocyte intracellular Na^+ (Na^+_i) content was determined using atomic absorption spectrophotometer (Solaar 969, Unicam, Cambridge, UK) and it was expressed in mmol/l RBC. Ion transport mediated by the Na^+-K^+ pump, Na^+-K^+-$2Cl^-$ cotransport and cation leaks were determined in erythrocytes that were washed three times with saline medium (in mmol/l: NaCl 140, glucose 5, morpholinepropanesulfonic acid 10, phosphoric acid 2.5, pH 7.4 at 37 °C, 310 mosmol/l). Washed erythrocytes were incubated in saline medium with 3.5 mmol/l RbCl for 30 min at 37 °C. Incubation media were nominally free of bicarbonate. Rb^+ was used as an analog of K^+ for its chemical and physical properties. Net Na^+ movements and unidirectional Rb^+ fluxes were assessed at intracellular Na^+ and extracellular Rb^+ (K^+) concentrations that were close to those found *in vivo*. Ouabain (5 mmol/l, Serva, Heidelberg, Germany) and bumetanide (10 μmol/l, Leo Pharmaceutical Product, Ballerup, Denmark) were used to inhibit Na^+-K^+ pump (ouabain-sensitive Na^+ net extrusion and ouabain-sensitive Rb^+ uptake) and Na^+-K^+-$2Cl^-$ cotransport (bumetanide-sensitive Na^+ net extrusion and bumetanide-sensitive Rb^+ uptake), respectively. Na^+ leak and Rb^+ leak were defined as residual fluxes resistant to both ouabain and bumetanide. Transport rates mediated by the above mentioned transport systems were expressed in mmol/l RBC/h (using mean cell hemoglobin content found in particular animals).

Analysis of membrane lipids

Erythrocytes in the 50 % suspension were washed three times with 0.9 % NaCl. Extraction of lipids was done according to Rose and Oklander (1965). Briefly, erythrocytes were lysed with distilled water for 30 min. Isopropanol was added during continuous stirring. After 60 min with occasional mixing, distilled chloroform was added and mixed. At the end of another hour, the mixture of erythrocytes, water, isopropanol and chloroform (1:1:11:7, v/v) was centrifuged at 785 x g for 15 min at 4 °C. The extracts were divided and evaporated under N_2 atmosphere. Dried extracts for the determination of the total phospholipids were dissolved in chloroform-methanol-0.9 % NaCl mixture (4:2:1, v/v). Phospholipid phosphorus from lower organic phase was mineralized to inorganic phosphorus which was measured colorimetrically at 820 nm and expressed in μmol phosphorus/g RBC (Rouser *et al.* 1970). In the other samples of dried extract the total cholesterol was estimated by diagnostic set (Bio-La-Test CHOL 150, Lachema, Brno, CR) and expressed in μmol/g RBC. Erythrocyte phospholipids were separated by two-dimensional thin layer chromatography into the individual phospholipid classes: phosphatidyl-ethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylinositol (PI) and phosphatidylserine (PS). Silica gel H (Merck, Darmstadt, Germany) as a slurry of 22.5 g in 62 ml water containing 2.5 g magnon (Merck, Darmstadt, Germany) was spread in 0.25 mm layer with a spreader (Desaga, Heidelberg, Germany) on glass plates (20 x 20 cm). Solvent mixtures were used according to the method of Rouser *et al.* (1970): chloroform-methanol-ammonia-water (70:25:4:1, v/v) and chloroform-methanol-acetone-acetic acid-water (70:12.5:17.5:10:4.5, v/v). Phospholipids were detected by the exposure of the plate to iodine vapor and the respective spots were scraped off. Phospholipid phosphorus was mineralized to inorganic phosphate, measured colorimetrically at 820 nm and expressed in μmol phosphorus/g RBC.

Statistical analysis

Results were expressed as means ± SEM and the statistical differences among experimental groups were

evaluated by Student t-test. Linear correlation analysis was used to test the association between ion transport parameters and lipid components of RBC membrane.

Results

Hemorrhaged rats had significantly increased number of reticulocytes, reduced hematocrit and decreased mean cellular hemoglobin content in comparison with control rats (Fig. 1). The immature erythrocytes in the hemorrhaged group differed from the mature cells of control rats not only by higher reticulocyte incidence (30.8±1.8 vs. 1.4±0.2 %, $p<0.001$) and decreased MCHC (4.75±0.08 vs. 5.06±0.07 mmol/l RBC, $p<0.01$) but also by the elevated activity of Na^+-K^+ pump (ouabain-sensitive Na^+ extrusion: 9.31±0.45 vs. 7.34±0.47 mmol Na^+/l RBC/h, $p<0.01$), reduced activity of Na^+-K^+ cotransport (bumetanide-sensitive Rb^+ uptake) and increased bumetanide-resistant Rb^+ leak (Fig. 2), whereas bumetanide-resistant Na^+ leak was not enhanced signifi-cantly (8.87±0.63 vs. 7.68±0.71 mmol Na^+/l RBC/h).

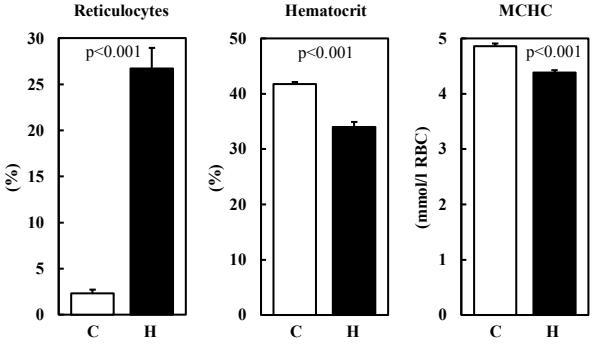

Fig. 1. Reticulocyte incidence, hematocrit and mean cell hemoglobin content (MCHC) in control (C) and hemorrhaged (H) rats. RBC – red blood cells.

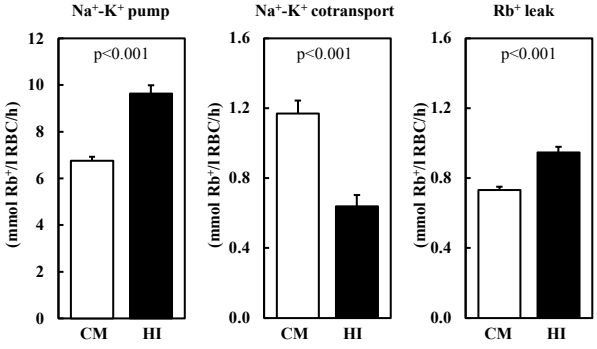

Fig. 2. Ion transport in mature erythrocytes of control rats (control-mature, CM) and immature erythrocytes of hemorrhaged rats (hemorrhaged-immature, HI).

The differences in ion transport parameters were accompanied by unchanged concentrations of total cholesterol, higher total phospholipids and reduced cholesterol-to-phospholipids ratio in immature compared to mature erythrocytes (Fig. 3). However, no significant differences in the membrane content of particular phospholipid classes were found between immature and mature erythrocytes (Table 1).

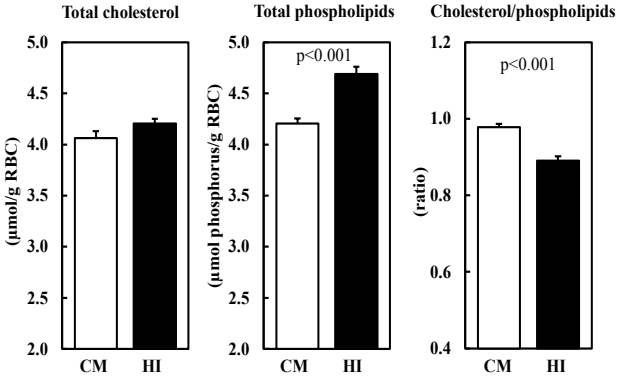

Fig. 3. Total cholesterol and phospholipids in mature erythrocytes of control rats (control-mature, CM) and immature erythrocytes of hemorrhaged rats (hemorrhaged-immature, HI).

There were no significant correlations of ion transport parameters with membrane cholesterol content, but membrane content of total phospholipids correlated positively with ouabain-sensitive Na^+-K^+ pump activity and negatively with bumetanide-sensitive Na^+-K^+ cotransport activity (Fig. 4). Membrane phospholipid content also correlated positively with ouabain- and bumetanide-resistant Na^+ leak and Rb^+ leak (Fig. 4). Phosphatidylserines were the only distinct phospholipid class which had the relationships to ion transport parameters similar to those reported above for total phospholipids (Fig. 5).

MCHC correlated highly significantly with cell Na^+ content ($r=0.800$, $n=33$, $p<0.001$) and it had negative relationships with membrane content of total phospholipids or phosphatidylserines ($r = -0.362$, $n=33$, $p<0.05$ and $r = -0.469$, $n=33$, $p<0.01$, respectively). There were significant negative correlations of MCHC with Na^+-K^+ pump activity but positive correlations of MCHC with Na^+-K^+ cotransport activity; negative relationship of MCHC with cation leaks was significant only for ouabain- and bumetanide-resistant Rb^+ leak (Fig. 6).

Table 1. Distribution of particular phospholipid classes in mature erythrocytes of control rats (CM) and immature ones of hemorrhaged animals (HI).

Phospholipids	CM	HI	Phospholipids	CM	HI
	Absolute values (µmol phosphorus/g RBC)			Expressed as a percentage of total phospholipids	
PE	0.80 ± 0.02	0.79 ± 0.05	*PE*	26.0 ± 1.2	24.5 ± 1.5
PC	1.61 ± 0.06	1.49 ± 0.08	*PC*	52.8 ± 3.1	46.0 ± 2.3
SM	0.35 ± 0.03	0.36 ± 0.02	*SM*	10.4 ± 0.6	10.8 ± 0.6
PS	0.31 ± 0.02	0.36 ± 0.03	*PS*	9.9 ± 0.8	11.2 ± 0.5
PI	0.03 ± 0.01	0.01 ± 0.01	*PI*	0.7 ± 0.2	0.4 ± 0.1

Values are means ± SEM. PE – phosphatidylethanolamines, PC – phosphatidylcholines, SM – sphingomyelins, PS – phosphatidylserines, PI – phosphatidylinositols, RBC – red blood cells.

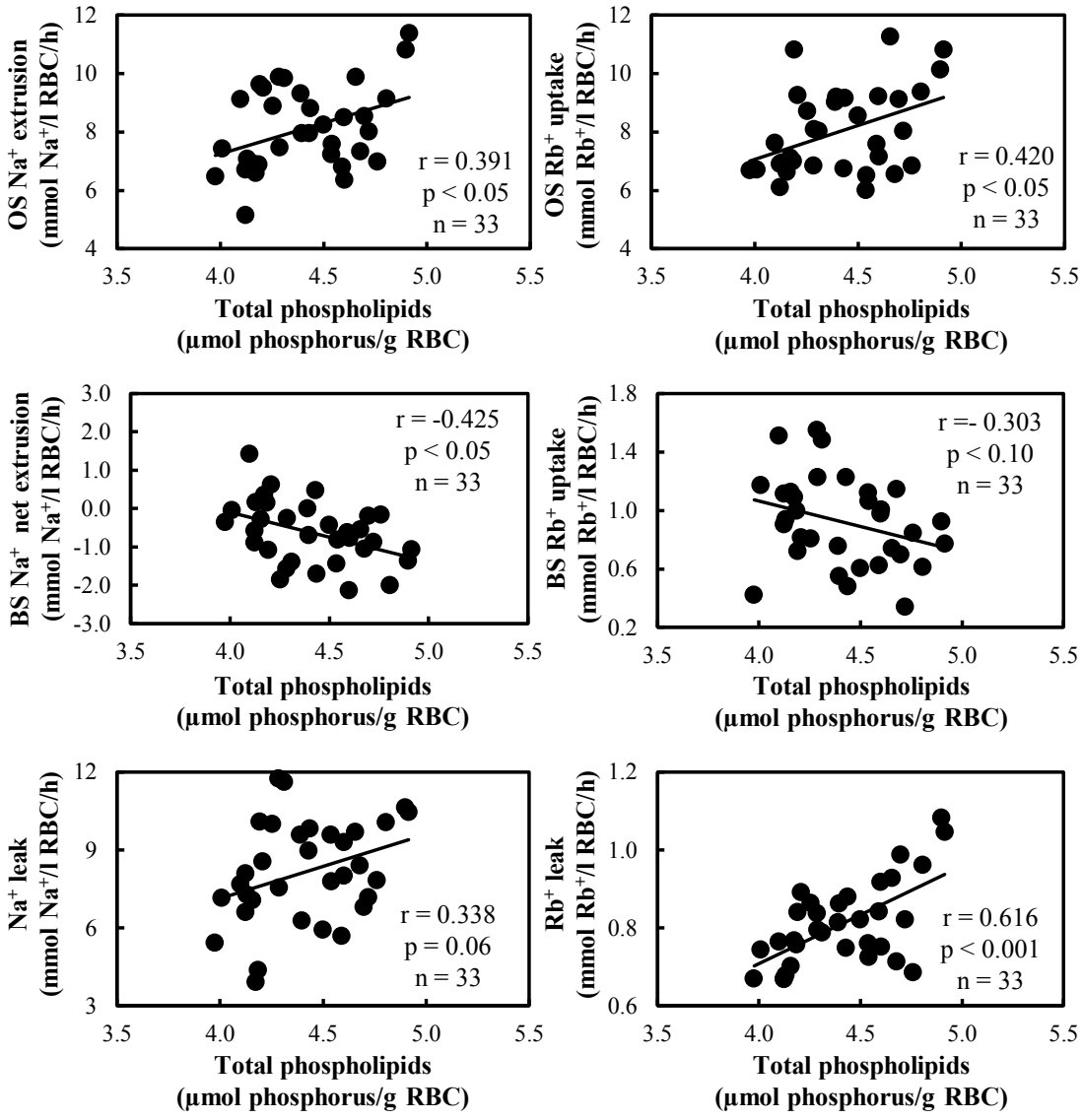

Fig. 4. Correlations of Na^+-K^+ pump activity, Na^+-K^+ cotransport activity and cation leaks with erythrocyte membrane content of total phospholipids in all studied erythrocyte populations.

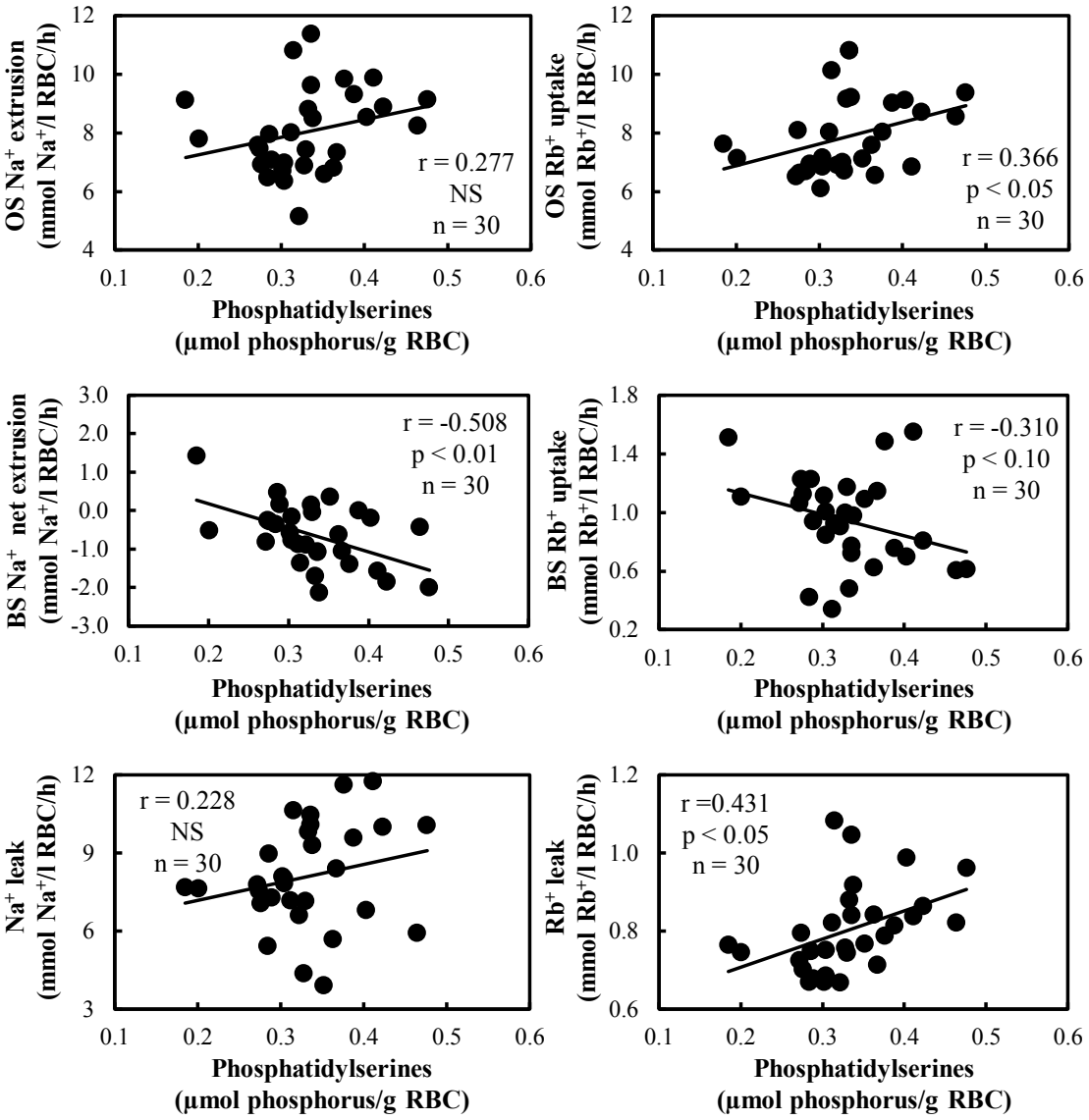

Fig. 5. Correlations of Na^+-K^+ pump activity, Na^+-K^+ cotransport activity and cation leaks with erythrocyte membrane content of phosphatidylserines in all studied erythrocyte populations.

Discussion

Erythrocyte maturation is closely associated with the changes of cell volume which is decreasing during the cell life. Therefore, a repeated centrifugation can be used to separate more dense mature and older erythrocytes from the lighter fraction containing younger and immature forms of erythrocytes. In both human and rat erythrocytes the cell volume plays a critical role in the control of Na^+-K^+-$2Cl^-$ cotransport activity because cell shrinkage accompanying cell maturation/ageing, which is characterized by increased MCHC, is always associated with the enhanced activity of this cotransport to counteract these unfavorable cell volume change (Duhm and Göbel 1984a,b). In fact, if rat reticulocytes are exposed to hypertonic medium to induced cell shrinkage, their low activity of Na^+-K^+-$2Cl^-$ cotransport is enhanced eight-fold, indicating the presence of expressed molecules of this transporter in the membrane of immature red blood cells (Mäirbaurl *et al.* 2000). This explains why in our experiments the mature erythrocytes display higher activity of bumetanide-sensitive Na^+-K^+-$2Cl^-$ cotransporter compared to immature ones. The reticulocytes or immature erythrocytes are characterized by higher cell volume which is controlled by another ion transporter K^+-Cl^- cotransport the activity of which is decreasing with cell maturation (Brugnara and Tosteson 1987, Canessa *et al.* 1987).

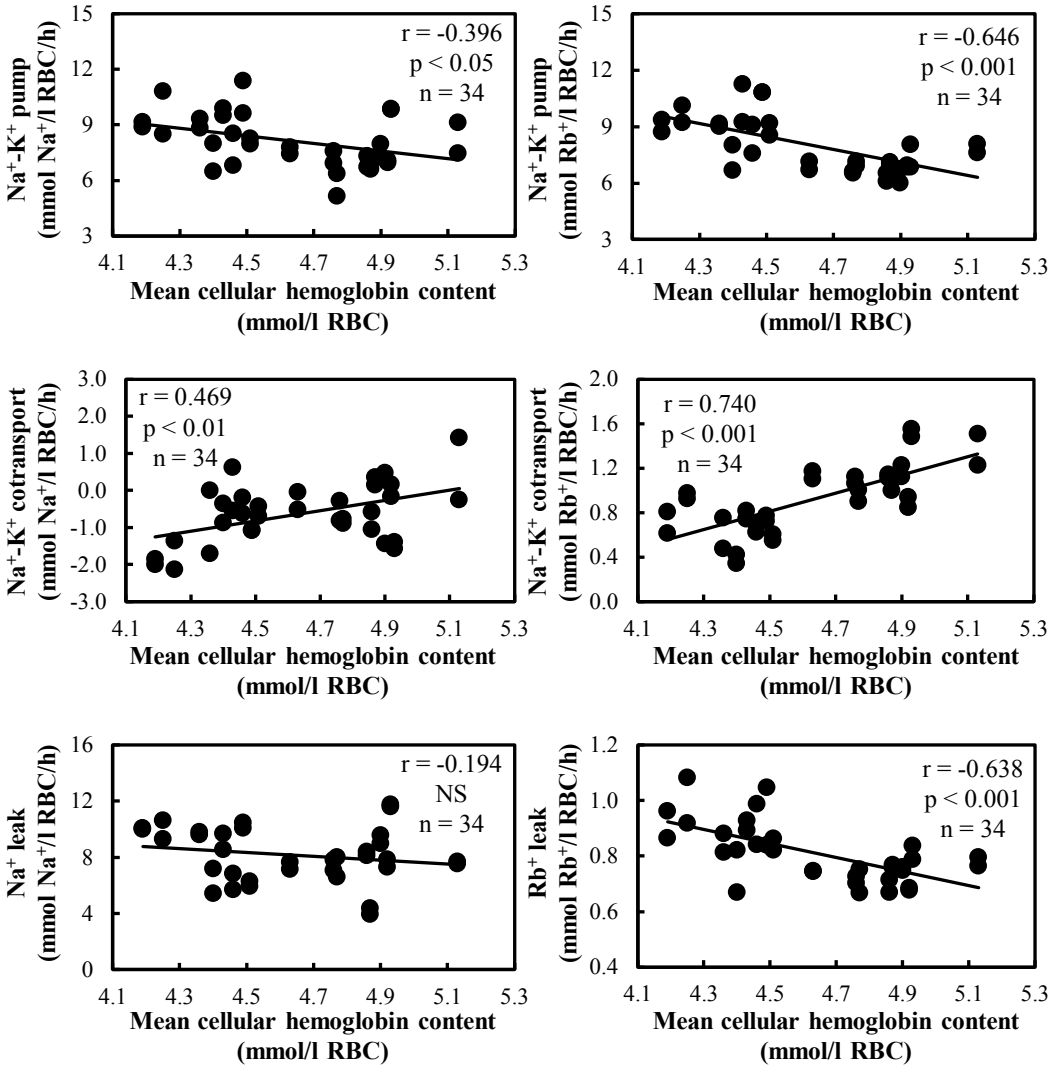

Fig. 6. Correlations of Na^+-K^+ pump activity, Na^+-K^+ cotransport activity and cation leaks with mean cell hemoglobin content in all studied erythrocyte populations.

Na^+-K^+ pump activity is elevated in rat reticulocytes (Furukawa *et al.* 1981, Mäirbaurl *et al.* 2000) and it decreases during cell maturation both *in vivo* and *in vitro*. One of the reason for the enhanced Na^+-K^+ pump activity might be a functional demand to keep pace with augmented Na^+ leak (Mäirbaurl *et al.* 2000) due to a possible increase in the passive membrane permeability in reticulocytes. The mechanism by which the balance between enhanced Na^+ entry and Na^+ extrusion is reestablished in reticulocytes, is the increased number of Na^+-K^+ pump sites on enlarged cell surface. Furukawa *et al.* (1981) reported three times greater number of ouabain biding sites per unit volume of rat reticulocytes compared to mature erythrocytes. Our data also indicate increased Na^+-K^+ pump activity and enhanced Rb^+ leak in immature rat erythrocytes as compared to mature ones.

The above mentioned ion transport changes can also reflect the age-dependent changes of membrane lipid composition. Engelmann *et al.* (1993b) demonstrated that Na^+-Li^+ countertransport is accelerated in hyperlipidemic patients and maximal activity of this transporter is positively related to red cell phosphatidylcholine but negatively to sphingomyelin membrane content. The important changes of ion transport mediated by Na^+-Li^+ countertransport or furosemide-sensitive cotransporter(s) might be induced by fatty acid exchange in phosphatidylcholine of erythrocyte membrane (Engelmann *et al.* 1990b). Molecular species of membrane phosphatidylcholine and phosphatidyl-ethanolamine contribute to the interindividual variability of Na^+-Li^+ countertransport in normal human population (Engelmann *et al.* 1993a) as well as in patients with hyperlipidemias (Engelmann *et al.* 1992).

Our study revealed certain changes in plasma membrane lipid composition which occur during erythrocyte maturation. As far as the membrane

cholesterol or phospholipid content is concerned, our present data from rat erythrocytes are in a good agreement with the changes disclosed during maturation of human reticulocytes (Sailaja *et al.* 2004).

Although we failed to find significant differences in particular phospholipid classes between immature and mature erythrocytes, the measured ion transport parameters had significant relationships to total membrane phospholipid content and this was also true for phosphatidylserines. It should be noted that there was a complex interrelationship between mean cell hemoglobin content, membrane phospholipids and ion transport which reflected the process of rat erythrocyte maturation.

Our previous study performed in salt hypertensive Dahl rats (Vokurková *et al.* 2005) demonstrated similar dependence of particular ion transport parameters on mean cell hemoglobin content as our present study, suggesting the important contribution of immature cells to ion transport abnormalities reported in salt hypertensive animals. Less clear is the contribution of phospholipid alterations because phosphatidylserines had a major impact in this study, whereas sphingomyelins were important in our previous study of Vokurková *et al.* (2005).

Our data suggest that certain abnormalities of ion transport and membrane lipid composition detected in erythrocytes of hypertensive animals might be caused by higher incidence of immature cells hemolytic anemia in salt hypertensive Dahl rats is a typical example (Luckhaus *et al.* 1982, Zicha and Duhm 1990). Another important factor contributing to ion transport alterations in hypertension might be related to the coincidence of various forms of dyslipidemia that might affect membrane lipid composition, membrane fluidity and the lipid environment of ion transporters located in the cell membrane (for details see Zicha *et al.* 1999).

In conclusion, it is evident that the maturation of circulating rat erythrocytes is accompanied by characteristic changes in the activity of principle ion transporters (such as Na^+-K^+ pump and Na^+-K^+-$2Cl^-$ cotransporter) and passive membrane permeability as well as by the changes of membrane content of total cholesterol and total phospholipids.

Conflict of Interest

There is no conflict of interest.

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