On the Mechanism of Shrinkage-induced Potassium Influx in Rat and Human Erythrocytes

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Abstract. The rates of ^{86}Rb influx into human and rat erythrocytes were studied in media of various tonicity. At sucrose concentrations below 0.3 mol/l, the ouabain-insensitive, furosemide-inhibited component of influx increased in rat but not in human erythrocytes; this may be explained by a rise in the rate of Na^+, K^+, Cl^-- and/or K^+, Cl-cotransport. An increase in osmolarity resulted in a reduction of this as well as of the ouabain and furosemide-insensitive component in rat erythrocytes. At the same conditions a drastic inhibition of Na^+, K^+-pump occurred both in rat and human erythrocytes. We failed to observe a lag-phase in the activation of the cotransport in rat erythrocytes; i. e. the process of activation parallels the shrinkage of cells. In rat erythrocyte ghosts, the shrinkage-induced stimulation of the cotransport was lost, and the direction of their osmotic reaction (inhibition of transport pathways) was similar to that in human erythrocyte ghosts. It is suggested that the mechanism of volume regulation of ion transport in intact cells involves a step of physical amplification via a change in interactions between the protein carcass and the lipid bilayer.

Key words: Volume regulation — Ion transport — Erythrocytes

Introduction

A number of cells are able to restore their volume changed by the action of factors tending to increase or decrease it. The mechanism underlying these reactions, called regulatory volume decrease (RVD) and regulatory volume increase (RVI), is induction of fluxes of sodium, potassium and chloride ions as the main osmotically active components of the cytoplasm. In most cases, specific ion transport systems (carriers and channels) activated by cell swelling or shrinkage could be identified (Grinstein et al. 1984; Eveloff and Warnock 1987). However, the attempts to

establish the nature of sensor of volume changes, the mechanism of amplification and signal transmission to the respective ion transport system of plasma membrane, have been unsuccessful as yet.

Recently we found that shrinkage of rat erythrocytes in hypertonic medium or isotonic medium containing valinomycin resulted in RVI via severalfold increases of Na_0^+/H_i^+ exchange and K_0^+, Cl_0^- (or Na_0^+, K_0^+, Cl_0^-)-cotransport (Orlov et al. 1987, 1989a). These carriers were identified also in human erythrocytes but were shown not to be activated by shrinkage. In this connection we believed that a detailed comparison of the ion transporting properties of human and rat erythrocyte membranes might be helpful in elucidating the molecular mechanism of signaling of osmotic potential changes, resulting in RVI. On approach to this problem is reconstruction of the volume-dependent regulation system of ion carriers using plasma membrane vesicles. Here we report the results obtained with resealed ghosts prepared from human and rat erythrocytes, and an analysis of the kinetics of osmotic shrinkage-induced potassium influx in rat erythrocytes.

Materials and Methods

Erythrocytes. Fresh donor blood and blood of 16–20 week-old female Kyoto-Wistar rats were used. The samples of heparinized blood (25–50 IU/ml) were kept on ice for no more than 1 hr. The erythrocytes were sedimented (2 000×*g*, 10 min), plasma and white blood cells were sucked off. The sediment was centrifuged twice using two to threefold volume of medium A, containing (in mmol/l): 150 NaCl, 5 sodium phosphate buffer, pH 7.4. All procedures were carried out at 0 – +4 °C. The packed erythrocytes were stored on ice for no more than 4–5 hrs.

Resealed ghosts. Resealed ghosts were prepared by two methods differing in the duration of hypotonic hemolysis and in the composition of the resealing medium.

Method 1. The method proposed by Smith and Lauf (1985) was used with the following modifications. Aliquots (5 ml) of washed packed erythrocytes were washed once more with twofold volume of medium K containing (in mmol/l): 150 choline chloride, 10 HEPES-Tris, pH 7.4 (2 000×*g*, 10 min). The packed erythrocytes were added drop by drop to 10-fold volume of medium L containing (in mmol/l): 17.5 choline chloride, 10 HEPES-Tris, pH 7.4, 0.1 EGTA. Lysis was carried out for 10 min under constant stirring on ice. After centrifugation (2 000×*g*, 10 min) the sedimented erythrocytes were decanted and the ghosts contained in the supernatant were sedimented by an additional centrifugation (20,000×*g*, 15 min). After removal of the hemolysate, the sediment was mixed with the resealing medium M (25 ml) containing (in mmol/l): 10 NaCl, 130 KCl, 10 HEPES-Tris, 0.15 $MgCl_2$, 0.1 EGTA and 2 ATP, pH 7.4 and shaken at 37 °C for 45 min. The resealed ghosts were sedimented (6 000 r.p.m., 25 min) and washed twice with 10-fold volume of medium K (6 000 r.p.m., 15 min) in an FP-9 centrifuge (Finland).

Method 2. The method proposed by Hoffman (1960) and Postnov et al. (1977) was used with the following modifications. An aliquot of washed erythrocyte suspension (3 ml, hematocrit 80% in medium A) was added drop by drop to 9 volumes of hypotonic medium containing (in mmol/l): 4 $MgCl_2$, 0.1 EGTA and 2 ATP (pH 7.0, +4 °C). Lysis was carried out for 1 min under stirring on ice; then, 3 ml of 1 mol/l KCl and 0.6 ml 1 mol/l NaCl were

added (to obtain a final concentration of the salts 90 mmol/l and 18 mmol/l , respectively), followed by incubation for 45 min at 37 °C in a shaker to complete the resealing. After centrifugation at 6 000 r.p.m. for 15 min, the dense sediment of erythrocytes was decanted; the lower-density sediment was resuspended in the supernatant, sedimented and washed with 4-fold volume of incubation medium B containing (in mmol/l): 140 NaCl, 3 KCl, 1 $MgCl_2$, 1 K_2HPO_4, 10 HEPES-Tris, 10 glucose and 0.1 EGTA, pH 7.4, under the same conditions of centrifugation, and immediately used in experiment. Method 2 of ghost preparation is characterized by a shorter time of lysis and by resealing without preliminary sedimentation of ghosts, i.e. in the presence of cytosol from lysed erythrocytes (cytosol dilution is approx. 15 times).

^{86}Rb influx. Erythrocytes of resealed ghosts (100 μl) prepared by Method 1 were introduced into 1 ml of cold medium B, incubated for 20 min at 37 °C and sedimented at 5 000 r.p.m. (5 min for erythrocytes, 8 min for resealed ghosts). In experiments with resealed ghosts prepared by Method 2, sedimentation was carried out without preincubation. Then, 0.4 ml of medium B containing 1–2 μCi/ml ^{86}Rb was added to the pellet. For the sake of identification of the specific transport systems, in some cases the incubation medium contained inhibitors of ion transport systems: 0.2 mmol/l ouabain or 0.2 mmol/l ouabain + 0.5 mmol/l furosemide. The same incubation medium supplemented with various concentrations of sucrose was used for osmotic shrinkage of erythrocytes and resealed ghosts. At fixed intervals, aliquots of the cell suspension were transferred into 1 ml of cold medium C containing 150 mmol/l choline chloride, and after centrifugation at 5 000 r.p.m. (5 min for erythrocytes and 8 min for resealed ghosts), the pellet was washed twice with medium C under the same conditions. The pellets were treated with 0.5 ml of 0.5% Triton X-100 and 0.5 ml of 10% TCA solutions. After centrifugation, 0.8 ml of the protein-free supernatant was transferred into Bray's solution (Bray 1960). The rate of ^{86}Rb influx was calculated as $V = A(a.m.t.)^{-1}$ where A is the radioactivity of m liters of cells (cpm), a is the specific radioactivity of ^{86}Rb related to the concentration of its analog, potassium (cpm/mmol), and t is the incubation time (hr).

The relative volume change of erythrocytes induced by the increase in osmolarity of the incubation medium was evaluated by light scattering as described elsewhere (Orlov et al. 1989a).

Reagents. NaCl, KCl, $MgCl_2$, Na_2HPO_4, NaH_2PO_4 - BDH (UK); choline chloride, EGTA (ethyleneglycol-bis(β-aminoethyl ether)-N,N'-tetraacetic acid), HEPES (N-(2-hydroxyethyl)piperazine-N'-2 ethane sulfonic acid), Tris (tris-(hydroxymethyl)aminomethane), Triton X-100, ATP Na_2, ouabain - Serva (FRG); furosemide - Sigma (USA). The stock solution of furosemide (250 mmol/l) was prepared using dimethylsulfoxide (Serva, FRG) as a solvent. ^{86}Rb was obtained from Amersham (UK).

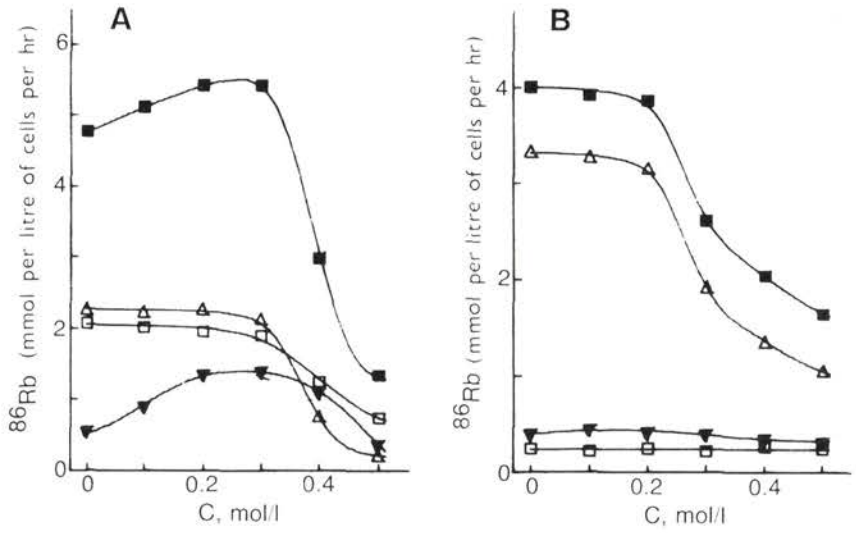

Figure 1. Effect of hypertonic shrinkage (addition of sucrose to isotonic medium) on the rate of ^{86}Rb influx in rat (*A*) and human (*B*) erythrocytes. 1 - total flux (■); 2 - ouabain-sensitive component (Δ); 3 - ouabain-insensitive, furosemide-sensitive component (▼); 4 - (ouabain + furosemide)-insensitive component (□). Incubation time with ^{86}Rb: 30 min. Results of a typical experiment.

Results

Three components can be distinguished in the total erythrocyte ^{86}Rb influx differing in their sensitivity to inhibitors: ouabain-sensitive Na,K-ATPase (OS), ouabain-insensitive, furosemide-sensitive Na^+, K^+, Cl^-- and/or K^+, Cl^-- cotransport (OIFS), and (ouabain+furosemide)-insensitive (OFI). Fig. 1 shows the dependence of the rates of ^{86}Rb influx into human and rat erythrocytes on the sucrose concentration in the incubation medium. In both cases, an increase of the sucrose concentration from 0.2–0.3 to 0.5 mol/l is accompanied by a two- to threefold decrease of the total ^{86}Rb influx; at low sucrose concentrations some stimulation of the influx is observed in rat erythrocytes only (Fig. 1*A*). In human erythrocytes, the inhibition of the total ^{86}Rb influx is accompanied by a decrease in the activity of Na,K-ATPase, while the ouabain-insensitive components of the ^{86}Rb influx are not changed significantly (Fig. 1*B*). At the same conditions, rat erythrocytes show a reduction of the OFI component along with an inhibition of the Na,K-ATPase (Fig. 1*A*). An increase of the sucrose concentration to 0.3 mol/l results in a more than twofold increase of the ^{86}Rb influx OIFS component in rat erythrocytes; presumably, this is responsible for the activation phase of the total ion flux (Fig. 1*A*).

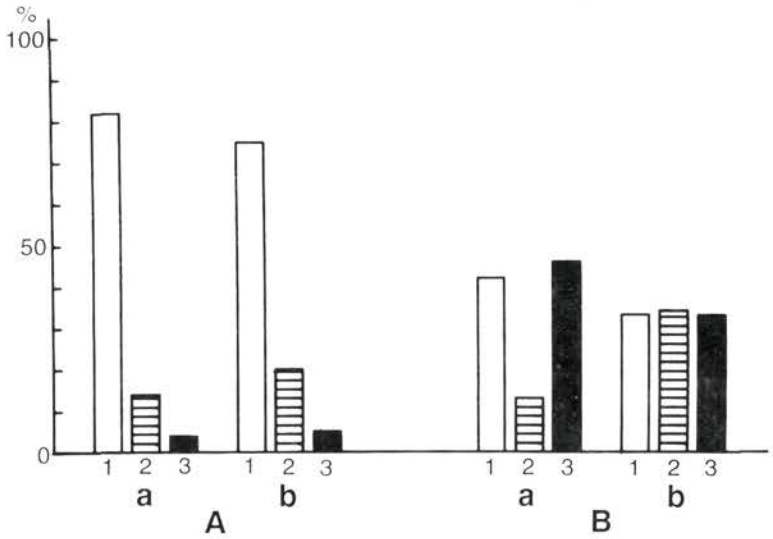

Figure 2. The relative contributions of the ouabain-sensitive (1), the ouabain-insensitive, furosemide-sensitive (2), and the (ouabain + furosemide)-insensitive (3) components to the total ^{86}Rb influx in human (*A*) and rat (*B*) erythrocytes in isotonic (*a*) and hypertonic (*b*) medium. Hypertonicity was achieved by addition of sucrose to a final concentration of 0.3 mol/l into the incubation medium. Incubation time with ^{86}Rb: 20 min. Data are means of three determinations ± S.E.M.

Further shrinking of cells results in a decrease of this component to the original level. For our subsequent studies of the influence of shrinkage on ^{86}Rb influx, the incubation medium containing 0.3 mol/l sucrose was used.

Fig. 2 illustrates the relative contributions of the components to the total ^{86}Rb influx in human and rat erythrocytes. In isotonic medium, the main contribution in human erythrocytes is that of the OS component, i.e. the sodium pump (Fig. 2*A*). In rat erythrocytes the OFI component is predominating (Fig. 2*B*). Osmotic shrinkage effectively remains without any effect on the ion transport components in human erythrocytes (Fig. 2*A*), but significantly raises the contribution of OIFS cotransport in rat erythrocytes (Fig. 2*B*).

The kinetics of ^{86}Rb accumulation in rat and human erythrocytes are known to be linear up to 60–120 min of incubation. Owing to this, in experiments illustrated in Figs. 1 and 2 the incubation time was limited to 30 min. In another series of experiments, the kinetics of volume change was compared with that of the modification of the ion transport systems after sucrose addition to rat erythrocytes.

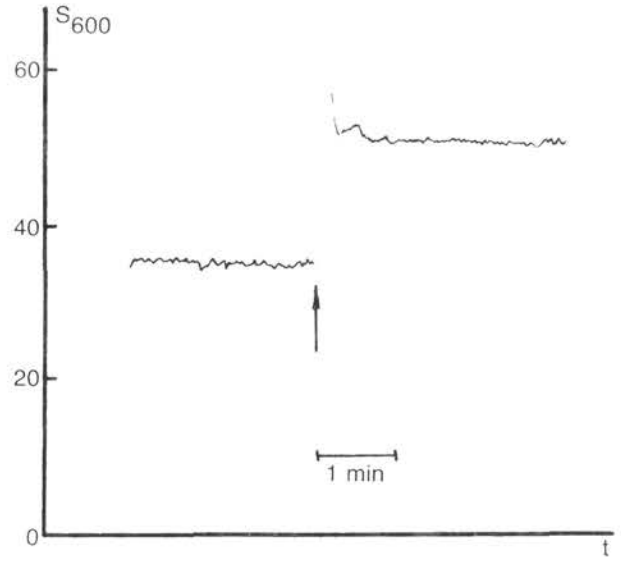

Figure 3. The kinetics of changes in light scattering by rat erythrocyte suspension following an increase in osmolarity of the incubation medium (addition of sucrose to a final concentration of 0.5 mol/l). S_{600} - relative intensity of the light scattering measured at 600 nm.

Judging by the light scattering (Fig. 3), the volume change is effectively instantaneous, i.e. the response to sucrose develops during an interval comparable with a cuvette mixing or with the value of the time constant of the spectrofluorimeter.

Fig. 4 shows the kinetics of ^{86}Rb influx in rat erythrocytes. As can be seen, only the rate of the OIFS component increases significantly upon osmotic shrinkage. Furthermore, a good linearity of the kinetic curve and its satisfactory extrapolation towards the point of intersection of the coordinates suggest that the process of shrinkage parallels stimulation of OIFS cotransport in rat erythrocytes.

Further studies were aimed at characterizing the volume regulation of ^{86}Rb transport in resealed ghosts. It should be noted that the process of ghost preparation results in changes of the relative contributions of the individual types of transport to the total ^{86}Rb influx (Figs. 2,5*A*). In particular, the OS component decreases two- to threefold, while the OFI component increases approx. 10-fold. In rat erythrocyte ghosts, the OS component is also drastically reduced as compared to intact cells. Moreover, in ghosts prepared by Method 1 we failed to detect this transport at all (Fig. 5*B*, *C*) and the contribution of the OIFS component was two- to threefold greater.

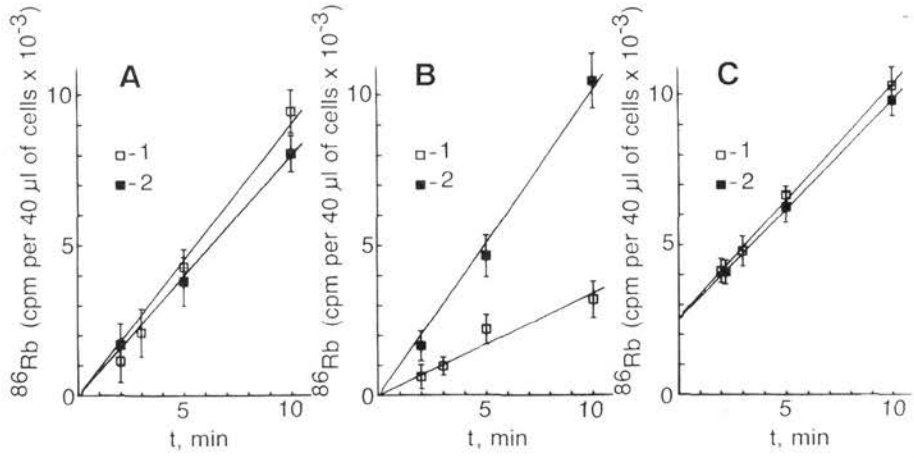

Figure 4. The kinetics of ^{86}Rb influx in rat erythrocytes in isotonic medium (□) and in hypertonic (■) medium containing 0.3 mol/l sucrose. *A*, the ouabain-sensitive component; *B*, the ouabain-insensitive, furosemide-sensitive component; *C*, the (ouabain + furosemide)-insensitive component. The intercept on ordinate in *C* reflects ^{86}Rb bound to the outer surface of erythrocytes. Data are means of three determinations ± S.E.M.

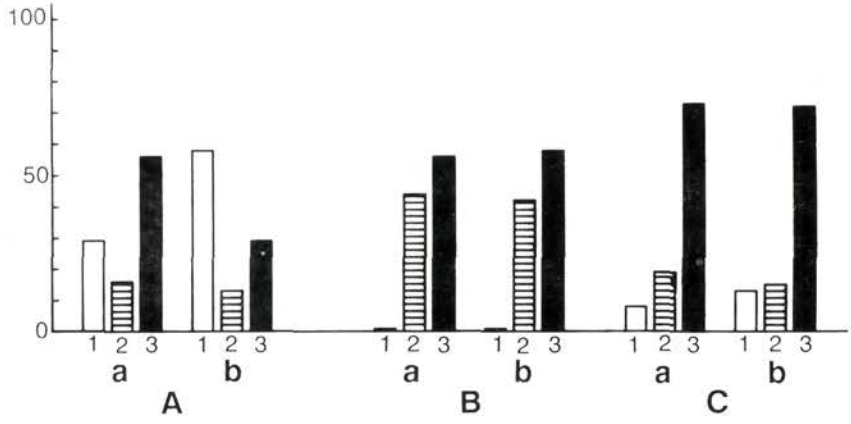

Figure 5. The relative contributions of ouabain-sensitive (1), the ouabain-insensitive, furosemide-sensitive (2), and the (ouabain + furosemide)-insensitive (3) components to the total ^{86}Rb influx in resealed ghosts of human (*A*) and rat (*B*, *C*) erythrocytes. *a*, isotonic medium; *b*, hypertonic medium containing 0.3 mol/l sucrose. Resealed ghosts were prepared by Method 1 (*A*, *B*) and 2 (*C*). Incubation time with ^{86}Rb: 20 min. Data are means of three determinations ± S.E.M.

Table 1. The effects of osmotic shrinkage (sucrose 0.3 mol/l) on the total ^{86}Rb influx rates in human and rat erythrocytes and their ghosts (mmol/l cells/hr).

Incubation medium	Erythrocytes		Ghosts		
	human	rat	human	rat	
			1	1	2
Isotonic	3.29±0.15	4.62±0.14	1.40±0.07	0.84±0.01	0.59±0.04
Hypertonic	1.91±0.08	5.56±0.17	0.31±0.02	0.26±0.02	0.15±0.02

Each value is the mean of three determinations ± S.E.M. Ghosts prepared by the Method 1 (1) and Method (2).

A comparison of the osmotic responses of intact erythrocytes and their ghosts is presented in Table 1. Shrinkage induced by addition of 0.3 mol/l sucrose affects the total ^{86}Rb influx in human and rat erythrocytes in an opposite manner (decreasing and increasing it, respectively). As was mentioned above (Fig. 1), in human erythrocytes the reduction of the influx is due to a decrease of the OS component (Na,K-ATPase), whereas the increase of the influx in rat erythrocytes is connected with the activation of the OIFS component. In resealed ghosts, the difference between the two species is abolished and the volume regulation of the total ^{86}Rb influx is directed towards a reduction of its rate in both types of cells. However, in rat erythrocyte ghosts, the individual components are equally inhibited by shrinkage, and therefore their contributions are not changed. On the other hand, in human erythrocyte ghosts the extent of the inhibition increases in the sequence OS (two- to threefold) < OIFS (approx. 5-fold) < OFI (8-fold), and as a result, their contributions to the total influx are substantially changed (Fig. 5).

Discussion

The results of the present work show that under severe shrinking (at sucrose concentrations exceeding 0.2–0.3 mol/l) there is a sharp reduction in the activity of the sodium pump in human and rat erythrocytes as well as in their ghosts. It seems that such regulation is not limited to erythrocytes only since it was observed earlier in axonal synaptic endings (Arvanov and Airapetyan 1980), and there it was explained by a drop in the number of exposed (functioning) Na,K-ATPase molecules due to an increased folding of the membrane surface. It should be noted however that volume regulation of the sodium pump is not universal phenomenon. For example, in carp erythrocytes where shrinkage stimulates Na^+/H^+exchange similarly as in rat erythrocytes, the activity of Na,K-ATPase remains constant (Orlov et al. 1989b). As shown in Figs. 1 and 2 and in other reports (Duhm and Gobel 1984a,b;

Orlov et al. 1988d; Orlov et al. 1989a), a moderate shrinking (sucrose concentrations $\leq$ 0.3 mol/l) induces two- to threefold activation of the OIFS component of ^{86}Rb influx in rat but not in human erythrocytes. It is well documented that in human erythrocytes this component is mediated by an electroneutral system providing a symport of Na^+, $K^+$and Cl^-, i.e. Na^+, K^+, Cl^--cotransport (Dunham et al. 1980; Chipperfield 1981). In bird erythrocytes the stoichiometry of Na^+: K^+: $Cl^-$is 1:1:2 (Haas and Schmidt 1985). In human erythrocytes this stoichiometry presumably is not rigorous, and only the electroneutrality principle of the transfer is strictly fulfilled (Canessa et el. 1986). There is no reliable evidence for the existence of a similar system in rat erythrocytes. It is known however that larger concentrations of furosemide and other loop diuretics are able to partially inhibit the Na^+-independent K^+, Cl^--symport, i.e. K^+, Cl^--cotransport, in addition to Na^+, K^+, Cl^--cotransport. In human erythrocytes, K^+, Cl^--cotransport is activated by swelling, high hydrostatic pressure and addition of SH-reagents, e.g. N-ethylmaleimide (see for review Bernhardt et al. 1988). The type of cotransport stimulated by shrinkage of rat erythrocytes remains still unknown, and further studies are needed for its classification. The OFI component of ^{86}Rb influx may be mediated by simple diffusion of the ion across the membrane or by K^+, Cl^--cotransport that, as mentioned above, is not completely inhibited by furosemide. The latter suggestion is further supported by the finding that upon severe shrinkage of rat erythrocytes (sucrose concentrations > 0.3 mol/l) there is a decrease in both the OFI and the OIFS components (Fig. 1). In contrast, in human erythrocytes these components are not affected by shrinkage. In the absence of an additional treatment human erythrocytes unlike those of rat exhibit K^+, Cl^--cotransport only in fractions enriched with reticulocytes (Brugnara and Tosteson 1987).

While considering the mechanism of volume regulation of the transport it has been supposed that the amplification of a volume change signal may involve increase in concentrations of the well known secondary messengers (cAMP, diacylglycerol, Ca^{2+}) followed by a chemical modification of proteins due to the activation of the respective protein kinases, e.g. A, C or (Ca^{2+}-calmodulin)-dependent forms. In support of this hypothesis data have been provided on stimulation of various ion transport systems by natural or synthetic activators of protein kinases. It should be noted, however, that neither of the messengers must be considered as an obligatory element of amplification in volume regulation. In particular, both rat and fish erythrocytes exhibit osmotic activation of Na^+/H^+exchange (Borgese et al. 1986, Orlov et al. 1987, 1989a) but in the former case the exchange is stimulated by protein kinase C activator TPA (Orlov et al. 1988b) while in the latter one activators of protein kinase A (cAMP or β-adrenergic receptor agonists) are operative (Orlov et al. 1989b; Borgese et al. 1986; Orlov et al. 1988c). Furthermore, TPA is equally effective in stimulating Na^+/H^+exchange in human and rat erythrocytes (Orlov et al. 1988b; Postnov et al. 1988), but as already mentioned, shrinkage activates

this exchange in rat erythrocytes only. Intracellular calcium is involved in RVD of lymphocytes and Ehrlich ascites tumor cells (Grinstein et al. 1982; Hoffman 1985) due to the opening of potassium and chloride channels. On the contrary, activation of ion carriers is not dependent on the concentration of external or internal calcium (Orlov et al. 1988c). Finally, specific phosphorylation of membrane proteins used as a reliable test of activation of protein kinases A and C was not affected by shrinkage of rat erythrocytes in isoosmotic (Orlov et al. 1987) or osmotic (Orlov et al. 1988a, 1989a) conditions.

It was also suggested that volume regulation involves a change in the contents of some ion-transporting systems in plasma membrane due to splitting off or incorporation of intracellular vesicles (Grinstein and Rothstein 1986). However, it should be noted that these processes are absent in non-nuclear mammalian red cells.

We found that in rat and carp erythrocytes the shrinkage-induced increase in $Na^+$and K^+influx is partially inhibited after two-hour incubation with relatively large concentrations of pertussis toxin (10–20 μg/ml) indicating a possible involvement of G_i-protein in volume regulation (Orlov et al. 1989b). Nevertheless, the experiments with ADP-ribozylation did not confirm such a model (Pokudin et al. 1990).

Thus, the mechanism of signal transmission based on a chemical modification of proteins, as widely recognized for hormonal cellular regulation, presumably is not operative in the reactions of volume regulation of the ion transport. It may be suggested that in the latter case the mechanism of the so-called "physical amplification" are operative (for details see Konev 1987). According to this idea, osmotic shrinkage via a changed interaction between the membrane protein carcass and the lipid bilayer modulates a level of strain inherent to the structural organization of membranes in intact cells; this in turn results in a modification of the functional activity of several ion transport systems.

The following data may be cited to support this hypothesis.

1. In rat erythrocytes, the volume-dependent regulation of the rates of Na^+/H^+exchange and Na^+, K^+, Cl^-- and/or K^+, Cl^--cotransport is abolished after preincubation of the erythrocytes at 50 °C when alterations of interprotein interactions in the membrane carcass occur (Orlov et al. 1988d; Gurlo et al. 1991).
2. In mouse peritoneal macrophages, RVD induced by activation of Na^+, K^+, Cl^--cotransport is abolished after a damage of the cytoskeleton by cytochalasin B (Galkin and Khodorov 1988).
3. As shown in the present work, there is a rapid change of red cell volume in response to sucrose addition. A rapid shrinkage is in agreement with a high permeability to water of the plasma membranes in red cells of various species (Macey 1979). It follows from Figs. 3 and 4 that the activation of the OIFS ion flux proceeds without any delay, i.e. in fact simultaneously with the cells shrinking (Figs.

3,4); this in a good accordance with the hypothesis on the physical principle of the signal transmission.

4. Even slightest alterations of the structural organization of the erythrocyte membrane during very mild hemolysis and preparation of resealed ghosts leads to leveling of interspecies differences in responses of the total ^{86}Rb influx to osmotic shrinkage, and in particular of its OIFS component: in rat erythrocyte ghosts the volume-dependent stimulation of this component disappears (Table 1, Fig. 5). Moreover, such a damage to membranes of human erythrocytes results in the appearance of volume-dependent inhibition of both ouabain-insensitive components of ^{86}Rb influx, whereas in rat erythrocyte ghosts osmotic shrinkage induces a reduction of the OIFS component instead of its stimulation observed in intact cells. Previously it was also established that the alteration of the membrane organization during the preparation of resealed ghosts from human erythrocytes is accompanied by a change in the response of K^+, Cl^--cotransport to N-ethylmaleimide and hypotonic swelling (Smith and Lauf 1985; Brugnara et al. 1988).

Summarizing the data described above it seems plausible to suggest that the integrity of the cytoskeleton controls the discrete ion transport systems (especially, this is relevant to the sodium pump in rat erythrocytes) as well as their reaction to osmotic shrinkage. It was shown earlier that shrinkage of rat or, to a lesser extent, human erythrocytes leads to a drastic decrease in the ratio of tri- to diphosphoinositides, and that the metabolism of this class of phospholipids is strongly dependent on the structural state of membranes (Orlov et al. 1989a). The problem of the relations between volume-dependent structural changes, the polyphosphoinositide metabolism and the activation of phospholipase A_2 (for review on the last item see Grinstein and Rothstein 1986) remains open.

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