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Kinetics and peculiarities of thermal inactivation of volume-induced Na^+/H^+ exchange, $Na^+,K^+,2Cl^-$ cotransport and K^+,Cl^- cotransport in rat erythrocytes

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The kinetics of the volume-dependent activation of Na^+/H^+ exchange, $Na^+,K^+,2Cl^-$-cotransport and K^+,Cl^--cotransport in rat erythrocytes was studied. The significant increase in the rate of Na^+/H^+ exchange is observed within 15 min after hypertonic shrinkage while the maximum transport rate is reached by 20 min. A delay of about 5 min was found in activation of $Na^+,K^+,2Cl^-$-cotransport, the maximum transport rate being reached 10 min after shrinkage. Activation of K^+,Cl^--cotransport by hypotonic swelling was registered within 10 min after cell swelling, with a simultaneous achievement of the constant transport rate. Preincubation of cells at 49°C has no effect on the basal Na^+/H^+ exchange and $Na^+,K^+,2Cl^-$-cotransport but suppresses the activation of these systems by osmotic shrinkage. On the contrary, the rate of K^+,Cl^--cotransport in isosmotic medium is raised 10-fold after preincubation at 49°C. The thermal treatment at 49°C blocks the activation of K^+,Cl^--cotransport by swelling. On the basis of the data on thermal denaturation of spectrin at the same temperature it was suggested that the cytoskeleton of erythrocyte membrane is involved in volume regulation of the ion-transporting systems and that the molecular mechanisms which underlie the activation of Na^+/H^+ exchange, $Na^+,K^+,2Cl^-$-cotransport and K^+,Cl^--cotransport are essentially different.

Introduction

Cells from a number of species are able to respond to environmental changes in osmolarity by an increase in the rates of the volume-sensitive ion fluxes. In most cases the specific transport systems activated by cell shrinkage and swelling have been identified. In many mammalian cells the osmotic shrinkage results in the activation of Na^+/H^+ exchange and $Na^+,K^+,2Cl^-$-cotransport while the osmotic swelling stimulates K^+,Cl^--cotransport [1,2]. Also in rat erythrocytes the volume-dependent regulation of the ion carries mentioned has been found [3,4].

Unlike a hormonal or electrical regulation the mechanisms of intracellular signalling involved in the volume-sensitive regulation of ion transport seem to be not mediated by the systems of second messengers known so far (cyclic nucleotides, internal calcium, hydrolysis products of polyphosphoinositides) for which an activation of the specific protein kinases serves as a terminal stage. Thus, for example, it has been shown that in various cell types the activity of Na^+,K^+,Cl^--cotransport, K^+,Cl^--cotransport and Na^+/H^+ exchange can be modulated by the cyclic nucleotides. No evidence was presented, however, that this signalling system is actually involved in the volume regulation of transport (see Refs. 2 and 5 for a review). There are indirect evidences for the involvement of some polyphosphoinositide derivatives in the shrinkage-induced activation of ion transport in rat erythrocytes. Upon hyperosmotic shrinkage of these cells the level of ^{32}P incorporation into the membrane polyphosphoinositides is drastically increased [4]. No such phenomenon was observed in human erythrocytes where shrinkage-induced ion transport was also not detected [4]. We failed also to find any shift in the phosphorylation level of the membrane proteins in rat erythrocytes after osmotic shrinkage [4].

Since not only erythrocyte volume but also cell shape is changed in anisotonic media, it seems plausible that

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a cell cytoskeleton may be involved in the volume regulation [2]. Some data support such a notion. In the gall-bladder epithelium cells of *Necturus* [7], rat liver slices [8] and peritoneal mouse macrophages [9] treatment with cytochalasin B, preventing a formation of actin microfilaments, leads to the complete blockage of the regulatory volume decrease under swelling. In human erythrocytes 1 mM cytochalasin B decreased the sensitivity of K^+,Cl^--cotransport to hyposmotic swelling [10].

It is known that the erythrocyte membrane possesses a kind of protein skeleton consisting of spectrin, actin and minor proteins (the bands 2.1, 4.1, 4.9) which control the interaction of spectrin and actin with the transmembraneous proteins (the band-3 protein and glycophorin) [11]. A method that makes it possible to affect the membrane carcass is the heat treatment of erythrocytes. Using the scanning microcalorimetry on human [12] and rat [13] erythrocytes it was shown that the first peak of a heat absorption is observed at 49–50°C, presumably as a result of spectrin melting [12,14].

In the present work we compared the kinetics of the shrinkage-induced activation of Na^+/H^+ exchange, Na^+,K^+,2Cl^--cotransport and of the swelling-stimulated K^+,Cl^--cotransport in rat erythrocytes. The volume regulation of these transport systems was evaluated also after the thermal damage of the protein membrane skeleton.

Materials and Methods

Erythrocytes

Blood of 4–5-month-old white laboratory rats was used. The samples of blood containing heparin (25–50 IU/ml) were kept on ice for no more than 1 h. The erythrocytes were sedimented ($1000 \times g$, 10 min), plasma and the white blood cells were removed and the sediment was washed twice with a 2–3-fold volume of medium A, consisting of 150 mM NaCl, 5 mM phosphate buffer (pH 7.4) at the same conditions of centrifugation. All procedures were carried out at 2–4°C. The erythrocytes from several animals were mixed. The packed erythrocytes were stored on ice for no more than 1 h.

Heat treatment of erythrocytes

One volume of packed erythrocytes was introduced into four volumes of cold medium B containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, 1 mM Na_2HPO_4, 20 mM Hepes-Tris (pH 7.4) and incubated for 10 min at 37–49°C followed by cooling on ice and cell sedimentation.

When the ^{86}Rb-loaded erythrocytes (see below) were subjected to heat treatment, after loading the erythrocytes were cooled on ice and then incubated at 37–49°C with subsequent second cooling, centrifugation ($1000 \times g$, 10 min) and washing (twice) with cold medium B.

^{86}Rb influx

0.2 ml of incubation medium B warmed to 37°C and containing Rb (2–3 μCi/ml) was added to the sediment of erythrocytes. Osmolality was increased by addition of 300 mM sucrose. For the sake of identification of Na^+,K^+,Cl^--cotransport the incubation medium contained 1 mM ouabain or 1 mM ouabain + 10 μM bumetanide. Na^+,K^+,2Cl^--cotransport was determined as the ouabain-insensitive, bumetanide-inhibited component of ^{86}Rb influx. After termination of incubation 1.0 ml of cold medium A was added to the cell suspension, the erythrocytes were sedimented by centrifugation at 5000 rpm for 2 min followed by two washes of the pellet under the same conditions of centrifugation. The pellet was treated with 0.5 ml of 0.5% Triton X-100 and 0.5 ml of 10% trichloracetic acid (TCA). To determined radioactivity 0.8 ml of protein-free supernatant was transferred into 5 ml of scintillator.

^{22}Na influx

0.2 ml of the incubation medium B warmed to 37°C and containing ^{22}Na (2–3 μCi/ml) was added to the pellet. Osmolarity was increased by addition of 350 mM sucrose. To identify Na^+/H^+ exchange 1 mM ouabain or 1 mM ouabain + 10 μM ethylisopropylamiloride (EIPA) were used. Na^+/H^+ exchange was calculated as the ouabain-insensitive, EIPA-inhibitable component of Na influx. After termination of incubation at 37°C the reaction was stopped as described above.

^{86}Rb efflux

One volume of packed erythrocytes was mixed with four volumes of medium B containing additionally ^{86}Rb (4 μCi/ml) and incubated for 2 h at 37°C. Then, the suspension was cooled on ice, the cells were sedimented and washed two times with cold medium B. Aliquots (50 μl) of the packed, ^{86}Rb-loaded erythrocytes were mixed with 0.5 ml of cold medium B and the cells were sedimented (the third wash). 0.2 ml of medium B warmed to 37°C was added to the pellet. Osmolarity of medium was lowered by a decrease of the NaCl concentration to 80 mM. To identify K^+,Cl^--cotransport the incubation medium contained 1 mM ouabain + 10 μM bumetanide or 1 mM ouabain + 10 μM bumetanide + 1 mM furosemide. K^+,Cl^--cotransport was determined as the (ouabain + bumetanide)-insensitive, furosemide-inhibitable component of ^{86}Rb efflux. In some experiments (see Table I) for the sake of identification of K^+,Cl^--cotransport DIOA ([(dihydroindenyl)oxy]akanoic acid) was used. After termination of the incubation at 37°C, 1 ml of cold medium A was added to the suspension, the erythro-

cytes were sedimented and 0.8 ml of supernatant was transferred into 5 ml of scintillator. To determine the total radioactivity of the erythrocytes 0.5 ml of 0.5% Triton X-100 and 0.7 ml of a 10% TCA solution were mixed with a series of samples containing the loaded erythrocytes. After sedimentation of protein 0.8 ml of supernatant was transferred into 5 ml of scintillator.

Calculations of the rates of the ion fluxes

The rates of ^{22}Na and ^{86}Rb influx were calculated as

$$V = \frac{A_2 - A_1}{am(t_2 - t_1)} \quad (1)$$

where A_1 and A_2 are the radioactivities in the sample (cpm) at fixed times t_1 and t_2, m is the amount of cells in the sample (liters), a is the specific radioactivity of ^{86}Rb related to the concentration of its analogue potassium (cpm/mmol), t_1 and t_2 are incubation times (h).

The rate of ^{86}Rb efflux was calculated as described above but in the expression a is the specific radioactivity of ^{86}Rb related to the intracellular concentration of its analogue potassium at the initial moment of incubation which was presumed to be 100 mM.

Reagents

NaCl, KCl, Na_2HPO_4, $MgCl_2$, $CaCl_2$ were from BDH; Hepes, Tris, choline chloride, Triton X-100, sucrose, glucose, ouabain were from Serva; furosemide was from Sigma; $^{86}RbCl$ and $^{22}NaCl$ were from Isotope. Bumetanide was a gift of Prof. J. Duhm (University of Munich, Munich, Germany). Ethylisopropylamiloride (EIPA) was synthesized by Ciba-Geigy (Basel, Switzerland, C6-P-35970); [(dihydroindenyl)oxy]akanoic acid (DIOA) was synthesized by E.J.C.

Results

Kinetics of activation of Na^+/H^+ exchange by hypertonic shrinking

Earlier it was shown that under isosmotic conditions the amiloride-sensitive sodium transport (Na^+/H^+ exchange) is quenched in rat erythrocytes [4]. In the present work this result is confirmed using a highly-specific inhibitor of Na^+/H^+ exchange, ethylisopropylamiloride (EIPA) [15] (Fig. 1, curve 1). As shown in Fig. 1 (curve 2), the activation of Na^+/H^+ exchange by osmotic shrinkage proceeds with a considerable delay: the significant increase of the Na^+/H^+ exchange rate is observed 10 min after shrinkage while a linear part of the curve is reached only after 10–12 min.

Kinetics of activation of $Na^+,K^+,2Cl^-$-cotransport by hypertonic shrinking

According to our previous data the activation of the ouabain-insensitive furosemide-inhibitable ^{86}Rb influx

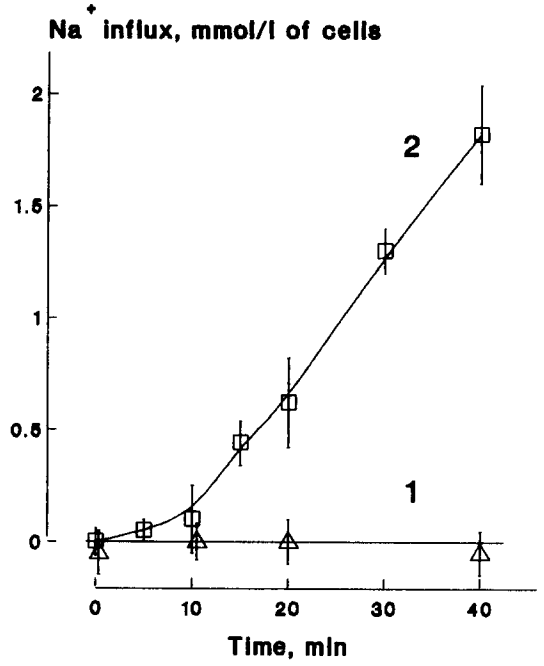

Fig. 1. The kinetics of the (ouabain + bumetanide)-insensitive, EIPA-inhibitable ^{22}Na influx (Na^+/H^+ exchange) in rat erythrocytes at isosmotic (curve 1) and hypertonic (curve 2) conditions. 1 – The medium contains 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 20 mM Hepes-Tris (pH 7.4), 5 mM glucose. 2 – The medium additionally contains 350 mM sucrose. Means ± S.E. obtained in three experiments are given.

in rat erythrocytes takes place without a lag-phase, i.e., in fact simultaneously with the cell shrinking [16]. It is known, however, that in erythrocytes furosemide along with $Na^+,K^+,2Cl^-$-cotransport inhibits both K^+,Cl^--cotransport [10] and the anion transport via the band-3 protein [17]. In this connection in the present work a selective inhibitor, bumetanide [18], was used for the identification of $Na^+,K^+,2Cl^-$-cotransport. As can be seen from Fig. 2 the shrinkage-induced activation of $Na^+,K^+,2Cl^-$-cotransport to the maximum rate proceeds with a small delay: a significant increase in the rate of transport is observed by 5 min after cell shrinkage. Such a delay may by partially caused by the experimental conditions, i.e., the aliquots of medium containing ^{86}Rb and warmed to 37°C were added to erythrocytes kept at 2–4°C followed by incubation at 37°C. Therefore, in the initial time the local temperature of the erythrocytes might be in fact less than 37°C, thereby affecting the operation of the ion carriers.

Kinetics of activation of K^+,Cl^--cotransport by osmotic swelling

Recently, a new inhibitor (DIOA) has been synthesized which at 100 μM causes a complete blockage of the swelling-induced activation of ^{86}Rb efflux mediated by K^+,Cl^--cotransport. In human erythrocytes the NEM-stimulated K^+,Cl^--cotransport was inhibited by

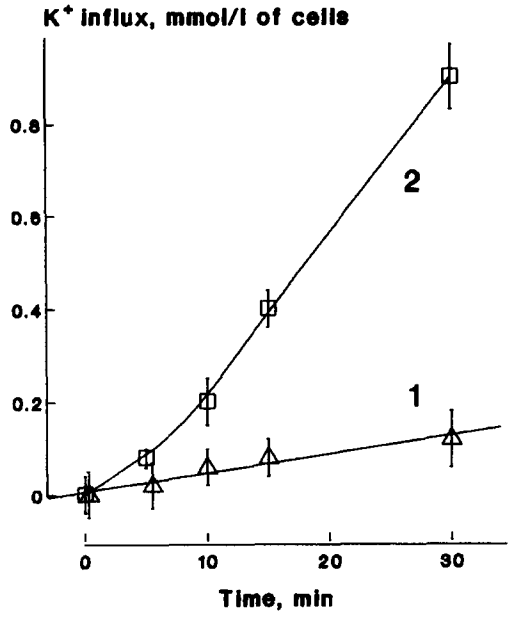

Fig. 2. The kinetics of the ouabain-insensitive, bumetanide-inhibitable ^{86}Rb influx ($Na^+,K^+,2Cl^-$-cotransport) in rat erythrocytes at isosmotic (curve 1) and hypertonic (curve 2) conditions. 1, The medium contains 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 20 mM Hepes-Tris (pH 7.4), 5 mM glucose. 2, The medium additionally contains 300 mM sucrose. Means ± S.E. obtained in three experiments are given.

DIOA and furosemide with a K_i of about 10^{-5} and 10^{-3}, respectively [10]. No experiment was reported however, on the comparison of the efficiency of these drugs in the volume-stimulated K^+,Cl^--cotransport. Such data obtained in rat erythrocytes are presented in Table I. It turned out that 1 mM furosemide and 100 μM DIOA are in fact equally effective in an inhibition of ^{86}Rb efflux from erythrocytes under hypotonic swelling. Subsequently, 1 mM furosemide was used for evaluation of K^+,Cl-cotransport.

TABLE I

Effect of furosemide and DIOA on ^{86}Rb efflux from rat erythrocytes

Data presented are means of three experiments ± S.E.

Conditions of assay	^{86}Rb efflux (mmol/l cells per 30 min)	
	Isosmotic medium	Hyposmotic medium
1. Control (in the presence of 1 mM ouabain and 10 μM bumetanide)	1.81 ± 0.05	3.52 ± 0.11
2. + Furosemide (1 mM)	1.52 ± 0.01	1.69 ± 0.02
3. + DIOA (100 μM)	1.45 ± 0.07	1.85 ± 0.10
4. Furosemide-inhibitable component	0.30 ± 0.07	1.83 ± 0.10
5. DIOA-inhibitable component	0.41 ± 0.05	1.67 ± 0.10

Fig. 3 shows the kinetics of activation of K^+,Cl^--cotransport by osmotic swelling. Up to 10 min from the beginning of cell incubation in hypotonic medium the rate of (ouabain + bumetanide)-insensitive, furosemide-inhibitable ^{86}Rb efflux is practically equal to that in isosmotic medium. Later, however, the rate of K^+,Cl^--cotransport is drastically increased reaching its maximum by 15 min.

Temperature dependence of Na^+/H^+ exchange and $Na^+,K^+,2Cl^-$-cotransport

As we established earlier a preincubation of rat erythrocytes at 49–52°C has no effect on the magnitudes of the ouabain-insensitive, furosemide-inhibitable ^{86}Rb influx in isosmotic conditions and of the ouabain-insensitive, amiloride-inhibitable ^{22}Na influx induced by the electrochemical proton gradient. In contrast, heating in this temperature range suppresses an increase in the rates of both types of transport under the hypertonic shrinkage [6,14]. The data of the present study obtained with more selective inhibitors, i.e., bumetanide ($Na^+,K^+,2Cl^-$-cotransport) and EIPA (Na^+/H^+ exchange) are in accordance with the previous results. A rise in temperature of the preincubation medium to 49°C has no effect on the activity of Na^+/H^+ exchange (Fig. 4, curve 1) and of

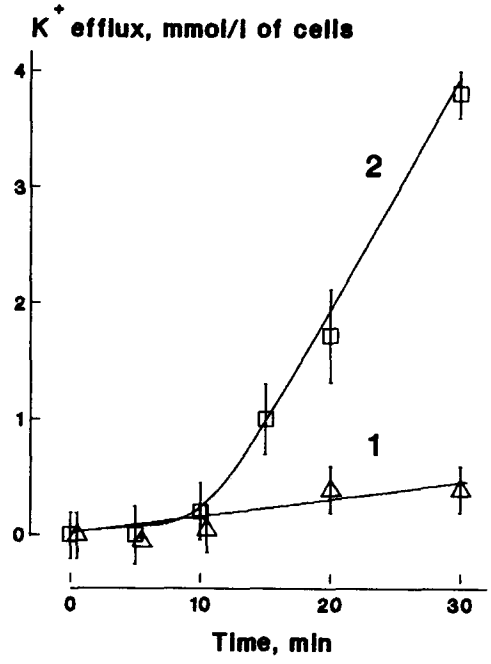

Fig. 3. The kinetics of the (ouabain + bumetanide)-insensitive furosemide-inhibitable ^{86}Rb efflux (K^+,Cl^--cotransport) from rat erythrocytes at isosmotic (curve 1) and hyposmotic (curve 2) conditions. 1, The medium contains 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 20 mM Hepes-Tris (pH 7.4), 5 mM glucose. 2, The medium contains 80 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 20 mM Hepes-Tris (pH 7.4), 5 mM glucose. Means ± S.E. obtained in three experiments are given.

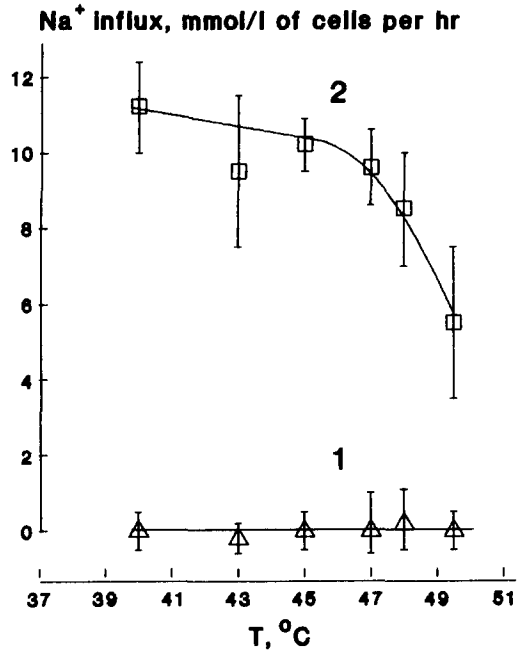

Fig. 4. The temperature dependence of the Na^+/H^+ exchange rate. Erythrocytes were preincubated in the medium at the temperatures indicated for 10 min; then, the cells were cooled to 2–4°C and placed into isosmotic (curve 1) or hypertonic (curve 2) medium at 37°C, containing ^{22}Na followed by a registration of the (ouabain + bumetanide)-insensitive, EIPA-inhibitable ^{22}Na influx (Na^+/H^+ exchange). The rate of Na^+/H^+ exchange in hypertonic medium (curve 2) was calculated under the full activation by shrinkage (between the 15th and 30th min from the beginning of incubation with ^{22}Na). Data presented are means of four determinations ± S.E.

$Na^+,K^+,2Cl^-$-cotransport (Fig. 5, curve 1) under isosmotic conditions. However, a similar treatment decreases by 60% the increase of the Na^+/H^+-exchange rate caused by cell shrinkage (Fig. 4, curve 2). At the same conditions an increment in the rate of $Na^+,K^+,2Cl^-$-cotransport is fully abolished (Fig. 5, curve 2).

Thermal activation of K^+,Cl^--cotransport

Fig. 6 displays the dependence of the rate of K^+,Cl^--cotransport on the temperature of the preincubation medium. A dramatic increase in the basal activity of the carrier can be seen at the narrow temperature range from 47 to 49°C (Fig. 6, curve 1). We failed to observe any influence of hypertonic swelling on the rate of K^+,Cl^--cotransport in the erythrocytes preincubated at 49°C (Fig. 6, curve 2). The kinetics of K^+,Cl^--cotransport in rat erythrocytes under isosmotic conditions after a preincubation at 49°C was linear up to 20 min (Fig. 7).

Discussion

It is known that after the transfer of a number of animal cells devoid of a cell wall, e.g., erythrocytes, into hypo- or hypertonic medium their swelling or shrinking proceeds almost instaneously [4], due to a high permeability to water of the plasma membrane [19]. The present study showed that, in spite of the rapid volume change, the activation of ion carriers in rat erythrocytes by shrinkage and swelling is characterized by a consideralbe delay. These results are in accordance with data on a delay of volume-dependent activation of $Na^+,K^+,2Cl^-$-cotransport in avian erythrocytes and K^+,Cl^--cotransport in human, rabbit and rat erythrocytes (for review, see Refs. 2,20). The most rapidly activated under osmotic shrinkage is $Na^+,K^+,2Cl^-$-cotransport for which the delay in stimulation to the maximum level does not exceed 5 to 10 min (Fig. 2). As was already mentioned such a lag-phase may by partially explained by a delay in mixing and heating of the erythrocytes at 37°C. However, this is unlikely to be the reason for the more prolonged delays in activation of Na^+/H^+ exchange by shrinkage (Fig. 1) and K^+,Cl^--cotransport by swelling (Fig. 3). The activation delays may be interpreted on assumption of the existence in the membrane of a specific sensor, receiving information on the volume or shape change with involvement of the chemical reactions requireing some time to proceed. Support to this suggestion comes from the data obtained in studies of the effect of the phosphoprotein phosphatase inhibitor,

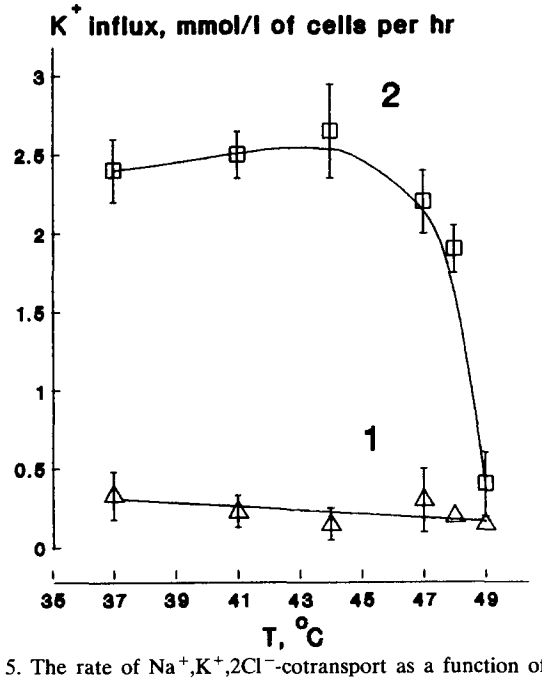

Fig. 5. The rate of $Na^+,K^+,2Cl^-$-cotransport as a function of temperature of the preincubation medium. Erythrocytes were preincubated in isosmotic medium at the indicated temperatures for 10 min. Then, the cells were cooled to 2–4°C, placed into isosmotic (curve 1) or hyposmotic (curve 2) medium at 37°C containing ^{86}Rb and the ouabain-insensitive, bumetanide-inhibitable ^{86}Rb influx ($Na^+,K^+,2Cl^-$-cotransport) was registred. Data presented are means of three determinations ± S.E.

okadaic acid, on the swelling-induced K^+,Cl^--cotransport. It was established that okadaic acid inhibits the induction of this type of transport by swelling in human erythrocytes [21]. The activation delay of the swelling-stimulated K^+,Cl^--cotransport in rabbit [22] and dog [23] erythrocytes was prolonged by okadaic acid. It can be suggested that the lag-phase in the activation by swelling of K^+,Cl^--cotransport is caused by a process of dephosphorylation of a hypothetical substrate associated with activation of this carrier. It should be stressed, however, that, as was noted in the Introduction, the information on involvement of the protein phosphorylation in the regulation of the ion-transporting systems in erythrocytes by shrinkage is negative rather than positive.

Hypotonic lysis of cells is known to disrupt the continuity of the erythrocyte membrane skeleton and to alter the composition of their polyphosphoinositides [24]. In rat erythrocytes even very mild hemolysis results in a loss of the stimulation by shrinkage of the furosemide-sensitive ^{86}Rb transport measured in resealed ghosts [25]. Similar experiments on human erythrocytes revealed a loss of sensitivity of K^+,Cl^--

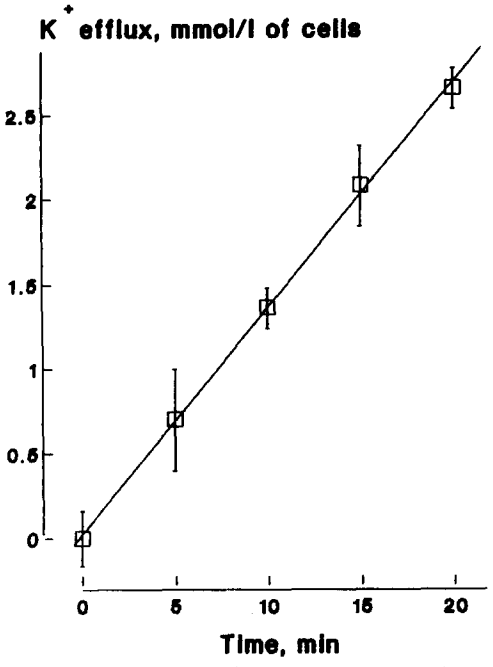

Fig. 7. The kinetics of the (ouabain + bumetanide)-insensitive, furosemide inhibitable ^{86}Rb efflux (K^+,Cl^--cotransport) from rat erythrocytes preincubated for 10 min at 49°C. The medium contained 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 20 mM Hepes-Tris (pH 7.4), 5 mM glucose. Data are means of three determinations ± S.E.

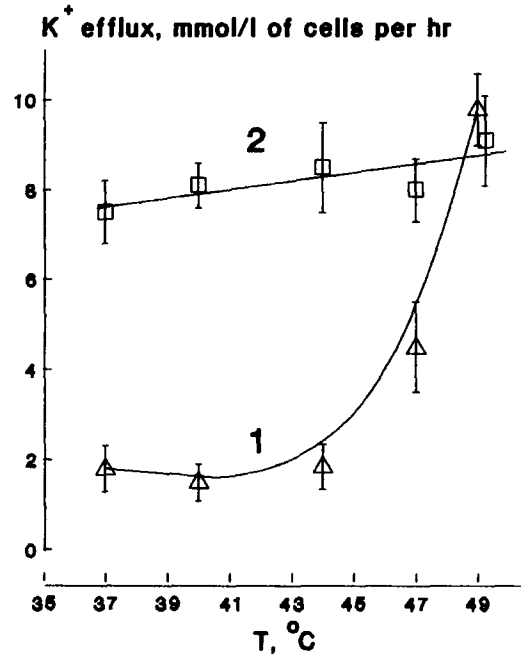

Fig. 6. The rate of K^+,Cl^--cotransport as a function of temperature of the preincubation medium. Erythrocytes were loaded with ^{86}Rb in isosmotic medium at 37°C during 2 h. Then, the cells were preincubated for the additional 10 min in the presence of ^{86}Rb in the temperature range 37–49°C. After termination of preincubation the cells were cooled to 2–4°C, placed in isosmotic (curve 1) or hyposmotic (curve 2) medium at 37°C and the (ouabain + Bumetanide)-insensitive, furosemide-inhibitable ^{86}Rb efflux (K^+,Cl^--cotransport) was registred. The rate of K^+,Cl^--cotransport was calculated in the interval between the 15th and 30th min after the beginning of incubation. Data are means of four determinations ± S.E.

cotransport to osmotic swelling [26] and a change of the response of this system to *N*-ethylmalemide [27]. To further investigate a role of the protein membrane carcass in the volume-dependent regulation of ion transport we used a method of thermodenaturation of spectrin at 49–50°C developed by Brandts et al. [12].

It can be seen from Figs. 4 and 5 that preincubation of erythrocytes at 49°C has no effect on the basal activity of the carriers capable of activation under shrinkage. In contrast, there is a considerable decrease in the Na^+/H^+ exchange activation and a full blockage of the $Na^+,K^+,2Cl^-$-cotransport stimulation caused by hypertonic medium. Unlike the ion-transporting systems induced by shrinkage, K^+,Cl^--cotransport at the isosmotic conditions is drastically increased after the thermal treatment, the carrier activation by the subsequent swelling being completely abolished (Fig. 6). Judging by the linearity of the transport kinetics in cells which underwent the thermal treatment, the activation of K^+,Cl^--cotransport by heating is irreversible for at least 20 min (Fig. 7).

Thus, the data obtained support the notion that the membrane skeletion proteins (or other minor proteins denaturated at the same temperature) are involved in regulation of the Na^+/H^+ exchange and Na^+,K^+, $2Cl^-$-cotransport stimulated under shrinkage of rat erythrocytes. A similar suggestion can be made with

respect to K^+,Cl^--cotransport induced by the cell swelling. The alternative explanation in the last case is a thermal activation of phosphoprotein phosphatase, e.g., as a result of the irreversible denaturation of some endogenous inhibitor. However, it is known that okadaic-acid-sensitive protein phosphatase type 1 is regulated by heat-stable inhibitors [28].

The present results demonstrate the diverse effects of heating on basal activities of various ion-transporting systems capable of stimulation under cell shrinkage or swelling: the rates of Na^+/H^+ exchange and Na^+,K^+,Cl^--cotransport remain constant while the rate of K^+,Cl^--cotransport is sharply increased. This observation is consistent with the data on an opposite response of Na^+/H^+ exchange and K^+,Cl^--cotransport to the same factor, e.g., to a change in intracellular concentration of magnesium, a loading with Li^+ or SCN^-, addition of okadaic acid (for a review, see Ref. 2). The reason for this diversity is unclear.

As was mentioned above, K^+,Cl^--cotransport induced by swelling was found both in rat and human erythrocytes [3,29,30]. On the contrary, the shrinkage-induced Na^+/H^+ exchange and $Na^+,K^+,2Cl^-$-cotransport are present in rat but absent in human erythrocytes [4]. From this it was concluded that the mechanisms responsible for amplification of a signal and its transmission to the ion transport systems activated by shrinkage ($Na^+,K^+,2Cl^-$-cotransport and Na^+/H^+ exchange) are identical for these two types of transport. The following data obtained in the present study are not in accordance with such a suggestion.

(1) In rat erythrocytes the kinetics of activation of the Na^+/H^+ exchange by osmotic shrinkage differs from that of the $Na^+,K^+,2Cl^-$-cotransport.

(2) Preincubation of erythrocytes at 49°C results in a full inability of the $Na^+,K^+,2Cl^-$-cotransport to be activated by shrinkage while under the same conditions the shrinkage-induced activation of the Na^+/H^+ exchange is reduced by 60% only.

(3) In erythrocytes of trout (*Salma truta*) osmotic shrinkage leads to a several-fold stimulation of the Na^+/H^+ exchange but not of the $Na^+,K^+,2Cl^-$-cotransport (Orlov and Hanninen, data not shown).

Thus, our results make it possible to suggest that the proteins of membrane skeleton can play a role of the sensor in a process of activation of ion carriers both by swelling and shrinkage. In addition, the mechanisms of intracellular signalling involved in activation of the Na^+/H^+ exchange and the $Na^+,K^+,2Cl^-$-cotransport under cell shrinkage seem to be essentially different. Further studies are needed for identification of these mechanisms.

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