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On the Role of Na,K-ATPase: a Challenge for the Membrane-Pump and Association-Induction Hypotheses

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Abstract: The regulation of cellular ion levels has been an important issue of cell physiology since the beginning of the century. A special interest was focused on the monovalent ions which are involved in several cellular functions; in fact, the maintenance of high K^+ level inside the cells is one of the most basic life-phenomena. Regarding the regulation of monovalent ions in general, two opposing ideas emerged: one being the membrane theory and the other the sorption theory(ies). Today most scientists are familiar only with the membrane theory which involves the pump and leak hypothesis and only a few consider the predictions of the association-induction hypothesis which may be classified as one of the sorption theories.

In the regulation of monovalent ions the Na,K-ATPase is a key-molecule according to the membrane theory but not considered that important by the association-induction hypothesis. In this paper, we present two simple experiments which demonstrate the possible role of this molecule in the regulation of cellular Na^+, K^+ homeostasis and also disprove the pump and leak hypothesis.

LIVING CELLS usually maintain a high level of K^+ and low level of Na+ (HK-type). However, exceptions exist. Erythrocytes of certain species can have low K^+ and high Na+ (LK-type) and it is also known that there is a significant variation within the intracellular levels of K^+, either in the HK or the LK group (Evans, 1954; Dunham and Hoffman, 1971; Dunham, 1992; Hoffman and Tosteson, 1971). Nevertheless, levels of monovalent ions are tightly regulated in all cases (Ellory and Tucker, 1983).

According to the widely accepted membrane theory, the key mechanism of this regulation is based on the Na,K-ATPase molecule which "pumps" K^+ into, and Na+ out of the cell in an energy-dependent manner (Glynn, 1993; Skou, 1990). Besides that there are several other mechanisms which are believed to be involved in the regulation of K^+ and Na^+, like the Na^+-K^+-Cl^- co-transport, Na^+,Li^+-countertransport, Ca^{2+}-activated K^+ channel, Na^+-K^+

electrodiffusion. etc. (Alberts, 1989; Clark, 1988; Fujise, 1991; Willis, 1992). Depending on species and type (HK vs. LK) there is a wide variation of these proposed mechanisms in the case of erythrocytes.

Regarding monovalent ion-transport, the basic idea of the membrane theory is formulated in the pump and leak hypothesis (Alberts ***et al.***, 1989; Darnell ***et al.***, 1986; Glynn, 1993; Skou, 1990). This hypothesis states that there is a constant inward (Na^+) and outward (K^+) leakage of ions through the cell membrane which is compensated for by the continuous work of the Na,K-pump. As a consequence the steady state level of Na+and K^+ is maintained. The Na,K-pump has a specific inhibitor, ouabain, which can inhibit the pump.

The association-induction hypothesis predicts that the level of intracellular K^+ is regulated by the adsorption of the ion to intracellular proteins, and that Na+ ions are mostly excluded from the cell water, which has an altered solvency for Na+ (as well as for K+) compared to dilute solutions and because Na^+ cannot compete successfully under normal physiological conditions, against K^+ for the β- and y-carboxyl groups of intracellular cell proteins (Ling, 1984, 1992). The adsorption of K^+ needs an energy (ATP)-dependent extended conformation of intracellular proteins, which is also responsible for affecting intracellular water. According to the association-induction hypothesis ouabain would reduce the preference of proteins to adsorb K^+ ion, and does not link the effect of ouabain directly to the Na,K-pump.

In the experiments presented herein we tested the Rb+(K^+) uptake and leak of different types of mammalian erythrocytes (HK-LK). The experimental results are discussed with a critical analysis of the two opposing theories.

Materials and Methods

Heparinized human blood samples were drawn from healthy volunteers. Blood samples of different animal species were obtained from the experimental farm of the Pannon Agricultural University, Kaposvár, Hungary. The LK and HK sheep were of the Suffolk breed. 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 the products of Reanal, Hungary.

Experimental protocols:

Before incubation, blood samples were supplemented with 5 mM RbCl (plasma concentration) and an additional 5 mM glucose was added to provide sufficient energy source for the 6 h incubation period. Glucose levels were monitored at the end of incubations. At the start of incubation, the preparations were placed at 37°C in a water bath, and 0.5 ml samples were taken at chosen time-points for ion analysis. The samples were centrifuged for 1 min, and the plasma removed and saved. The pellets were spun for an additional 15 min at 13,000 g and any residual plasma was carefully removed. Based on the data of 0 hour Rb+ measurements the remaining plasma volume of the blood samples is around 10%. The pelleted cells were processed for ion measurement.

To study the leakage of Rb+ from erythrocytes, the cells were loaded with Rb^+ during incubation in their own plasma supplemented with 5 mM RbCl for 6 h (see above). The erythrocytes were briefly washed once in Hank's solution, before being incubated in Hank's

TABLE I. Cation content of erythrocytes of different species.

species	n	K		Na		H_2O	
		mmol/l	SD	mmol/l	SD	U/U	SD
human	10	131,3	±3,7	29,62	±5,95	2,09	±0,05
horse	8	143,2	±6,17	25,54	±4,18	1,82	±0,03
HK sheep	8	111	±4,6	45,48	±2,94	1,83	±0,02
LK sheep	8	29,24	±7,25	138	±8,87	2,01	±0,03

solution for an additional 6 h, with samples being taken at selected time-points for ion measurements.

The effect of ouabain was studied on human erythrocytes. The inhibitory effect on Na,K-ATPase was studied either in the first 6 hours during the Rb+loading period or in the second 6 hours when Rb+ was no longer present (leakage period). For inhibition, 1 mM ouabain was added prior to incubation.

The results depicted in Figures 1 and 2 are representative examples. Intraspecies differences in the rate of Rb+uptake occur; however, the rank of Rb+uptake between the different species and the uptake-leakage characteristics of the erythrocytes are highly reproducible.

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

The centrifuged pellets were weighed gravimetrically before being vacuum-dried in a Savant SC-110 speed vacuum system (USA). Dry weights were subsequently measured. 0.8 ml of 1M HCl was added to each sample, and incubated at room temperature on a rocker table for > 24 h.

K^+ and Na+ levels were measured with a flame photometer (Eppendorf EFOX 5070, Germany), and Rb+ levels with a Varian AA-20 atomic absorption spectrometer (Varian Techtron, Australia). Ion concentrations were calculated after correction for dilution factors, and were based on water content data. Water content is expressed as g waterlg dry weight (u/u).

Results

The physiological levels of K^+ and Na^+ in the erythrocytes examined are shown in Table I. Human, horse and HK-type sheep erythrocytes contain high levels of K^+ and much less Na^+ intracellularly; LK-type sheep show an opposite distribution of monovalent ions. As mentioned previously, there are significant differences between the K^+, Na^+ content of HK-type erythrocytes.

The uptake and leakage of Rb+ by the different erythrocytes is depicted in Figure 1. During the 6 hour incubation period the cells were incubated in plasma containing 5 mM

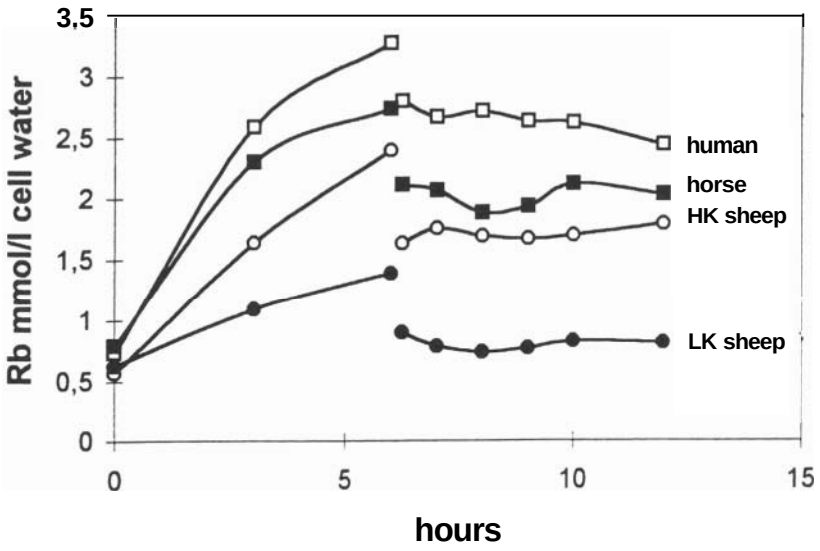

FIGURE 1. The uptake and release of Rb+ by erythrocytes of different species. Human (❒) horse (■), HK sheep (O), LK sheep (●) erythrocytes were incubated in the presence of 5 mM Rb+ in the first 6 hours, then they were transferred to Rb^+-free Hanks solution for the next 6 hours.

Rb^+. Human erythrocytes accumulated Rb+ to the greatest extent followed by horse, HK-type sheep and LK-type sheep erythrocytes.

After the 6 hour incubation period the cells were washed once in Rb^+-free Hank's solution which contained K^+ instead of Rb^+. This wash results in a quick drop in the Rb+ content of the samples which corresponds mostly to the extracellular Rb+ and the amount is basically equal to that of 0 hour Rb+ content of the samples. During the following 6 hours the cells were incubated in Rb^+-free Hank's solution. None of the cells released a significant amount of the Rb+ that had been accumulated through the first 6 hour incubation period.

The role of ouabain in Rb+ (K^+) transport was tested only on human erythrocytes. The experimental design was similar to that shown in Figure 1. One mM ouabain was added either to the cells in the uptake period or during the leakage period or both. As can be seen in Figure 2, 1 mM ouabain significantly reduced the uptake of Rb+ ions in the first 6 hours. However, the presence of ouabain during the "leakage" period (i.e. during the incubation in Rb^+-free Hank's solution) does not have any affect on the Rb+ level of the erythrocytes. If the uptake was reduced by ouabain, the Rb+ level also stayed steady in the presence of ouabain afterwards.

Discussion

The regulation of monovalent ion transport in erythrocytes is among the most thoroughly studied problems of cell physiology. In fact, these cells offer an excellent model because of their relatively simple metabolic and structural features. A unique characteristic of erythrocytes is their monovalent ion-polymorphism, mostly found in carnivores and ruminants (Ellory and Tucker, 1983; Evans, 1954; Hoffman and Tosteson, 1971; Miseta *et* al., 1992,

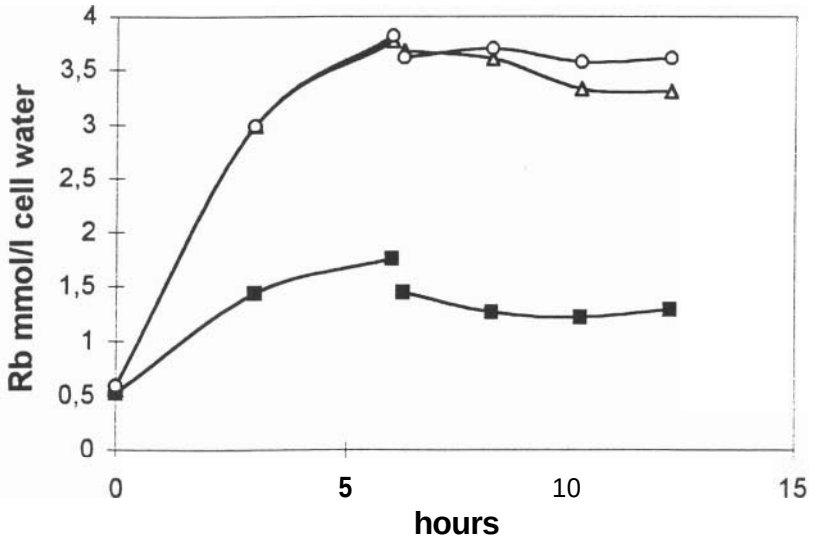

FIGURE 2. The uptake and release of Rb+ by human erythrocytes in the presence of 1 mM **ouabain in the first 6 hours (■), or the second 6 hours (O). Control cells (A) were not exposed to ouabain during the incubation periods. (For details see** *Materials and Methods).*

1993a,b). Regarding the Na^+, K^+ transport in the HK-LK erythrocytes a vast amount of work has been done in the last 40 years, and the characterization of the different pathways revealed significant inter- and intra-species diversity (Dunham and Blostein, 1976; Ellory and Tucker, 1983; Fujise ***et al.,*** 1991). Nevertheless, it seems to be established that the number and/or activity of Na,K-ATPase shows correlation with the steady state levels of Na+ and K^+ although this correlation is by no means perfect (Miseta ***et*** al., 1993a; Palma ***et al.,*** 1992).

The central role of the Na,K-ATPase in ***regulating*** intracellular Na^+, K^+ levels is generally accepted, along with the idea of pump and leak principle. Based on the observation presented here, this idea is rather controversial.

We have demonstrated in the different erythrocytes that the Rb^+ uptake (which ion is widely used as a K^+ analogue) during a 6 hour incubation period and the leakage of Rb+ during the next 6 hours is ***not equal.*** Irrespective of the HK-LK type, erythrocytes take up Rb+ at a faster rate than it is released. Based on this experimental fact we doubt that the widely accepted hypothesis, i.e. the pump and leak hypothesis could explain the observed phenomenon.

On the other hand, these results can be partly explained by the association-induction hypothesis, if we consider intracellular Rb+to be in an adsorbed state. Similarly to K+, Rb+ was leaking relatively slowly from erythrocytes (Bogner ***et al.,*** 1996).

The uptake of Rb+is significantly reduced by the addition of ouabain as predicted by the membrane-pump hypothesis. Nevertheless, even that fraction of Rb^+ which has been accumulated ***in the presence*** of ouabain shows similar non-leaking behavior during the incubation in Rb^+-free medium. Ouabain has no, or virtually no, effect in the second ("leaking")6 hours of the experiment which — in our view — contradicts the prediction of the association-induction hypothesis since ouabain should modify the adsorption of Rb^+,

thus an increased leakage could occur. Alternatively, one might argue that Rb+ gets adsorbed in the first 6 hours of the experiment where ouabain in fact reduces the intracellular level of Rb^+. Later the amount of the adsorbed quantity does not change. But why does ouabain not have any effect once the ion is in an adsorbed state?

It is interesting to note that, in different mammalian species, the rank of activity of Na,K-ATPase in isolated erythrocyte ghosts (measured by Palma *et* al., 1992) and the rank of Rb^+ uptake in the erythrocytes we tested show a very good correlation.

Considering the experimental data discussed above we conclude that, in erythrocytes, Na,K-ATPase seems to be involved in the uptake of Rb+ (K^+) and ouabain decreases this uptake significantly (if one accepts that the ourbain-inhibitable activity/function belongs to the Na,K-ATPase). However, the steady state distribution of Rb+ is not affected further by the Na,K-ATPase/ouabain. Since no leakage occurs (with/without ouabain), Rb+ is most probably in an adsorbed state. As a consequence, it seems very likely, that the steady state level of Rb+ is defined primarily by adsorption.

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