Effect of Adrenaline on Glucose Transport in Red Cells of *Rana balcanica*

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Abstract. The characteristics of 3-O-methyl-D-glucose (3-OMG) uptake by frog erythrocytes were studied. 3-OMG transport was increased by adrenaline. Although the transport is inhibited by phloretin, the lack of saturation kinetics suggests that a glucose transporter doesn't exist or that its affinity for glucose is extremely low. Frog *Rana balcanica* red cells suspended in an isotonic medium containing adrenaline enlarge rapidly to reach a new pH-dependent steady state volume. At pH 8.0, the cells swell less than at pH 7.3. This is explained by a differential pH effect on the two pathways controlling the movement of the cations: as pH becomes more acidic K^+ loss decreases. On the contrary as pH becomes more acidic Na^+ uptake increases. The increase in glucose transport after osmotic swelling and the inhibition of swelling-induced glucose transport by phloretin suggest that the glucose transport pathway in *Rana balcanica* erythrocytes may is a volume-activated channel.

Key words: Red cells — Adrenaline — pH — Glucose uptake

Introduction

In many cell types, catecholamines trigger the activation of membrane transport systems with the result that the cell undergoes a regulatory volume modulation response. Rainbow trout and frog red cells are particularly sensitive to high levels of circulating catecholamines occurring under stress conditions (hypoxia and after exhaustive exercise) inducing a rise in the Na^+ concentration inside the cells and a cell swelling (Baroin et al. 1984; Kaloyianni and Rasidaki 1996; Kaloyianni et al. 1997). The cell swelling seems to be coupled to Na^+-H^+ exchanger in *Rana balcanica* red cells (Kaloyianni et al. 1997, 1999). Na^+-H^+ exchanger contributes in many cells to a volume and/or pH regulatory capacity (Cala and Maldonado 1994). The effect also seems to be mediated by a rise in the Adenosine $3'; 5'$-cyclic monophosphate (cAMP) level, through the hormone interaction with membrane

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receptors of the β, α_1 and α_2 type in *Rana balcanica* red cells (Kaloyianni and Rasidaki 1996; Kaloyianni et al. 1997, 2000a, 2000b).

It has been shown that the red cells may play an important role in glucose metabolism, being involved in the biosynthesis of glutathione (Ellory et al. 1983), and in transport between organs (Christensen 1982). Regulation in sugar transport is brought about by a variety of factors: anoxia, exercise, insulin and catecholamines and its mechanism remains obscure (Simons 1983). Whitfield and Morgan (1973) have reported that catecholamines stimulate sugar transport in avian red cells. On the other side Gallardo et al. (1992) have suggested that catecholamines may alter the amino acid transport system carrier's ability by changing the internal sodium concentration in trout red cells. Catecholamine-treated frog red cells show an increased glucose catabolism as well as an increase in activity of the glycolytic enzyme pyruvate kinase (Kaloyianni et al. 1997, 2000a,b). Also Kirk et al. (1992) have shown that the membrane permeability to glucose increases with the increase of flounder red cell volume. Thus, it is possible that catecholamine induced increase in glucose permeability could be mediated by changes in red cell volume. It is also possible that changes in extracellular pH could affect the rate of glucose transport as in other cell types (Ismail-Beigi 1993).

This investigation describes studies designed to investigate if sugar transport is implicated in the regulation of cell volume and clarify the factors that influence glucose transport in *Rana balcanica* erythrocytes. We studied the influence of adrenaline, extracellular pH and cell swelling on the permeability of 3-O-methyl-D-glucose (3-OMG) of the frog *Rana balcanica* erythrocyte membrane. In addition, in order to investigate the regulatory behavior of the frog *Rana balcanica* red cell in relation to glucose transport and volume change which is possibly due to the function of Na^+-H^+ exchanger, it was of interest to examine the influence of pH and adrenaline on cellular volume and ion concentration.

Materials and Methods

Animals

Frogs (*Rana balcanica*) weighting 50–200 g were supplied by a local dealer after having been caught in the vicinity of Larisa. They were kept in containers in fresh water and used 1 week after arrival.

Blood sampling and preparation of red cells

Blood samples were taken into heparinized syringes by cardiac puncture from anaesthetized in 3-aminobenzoic acid ethyl ester (MS-222, 0.1 $g \cdot l^{-1}$) frogs. Immediately upon collection the blood was centrifuged (10 min, $500\times$ g) and the plasma and surface layer of white cells were removed by aspiration. The erythrocytes were washed three times with 0.1 mol/l NaCl. Subsequently, the cells were suspended in isotonic saline (300 mOsm/kg H_2O) of following composition (in mmol/l): 100 NaCl, 5.9 KCl, 1.25 $CaCl_2$, 2.4 MgSO4, 4.2 imidazole, 7.6 glycylglycine, 1.2 KH_2PO_4,

5 glucose, 3 pyruvate and 0.05 isobutylmethylxanthine (IBMX) at pH 7.3–7.4, at haematocrit of 50% and left overnight, at 5°C. This ensured that the cells were in a steady state with respect to volume and ion content and that they were not in a catecholamine-stimulated condition (Bourne and Cossins 1982). IBMX was added to the suspension to inhibit phosphodiesterase activity. The following day the red blood cells were again washed and resuspended in two different buffers (pH 7.3 and pH 8.0) at haematocrit of 30% at 25°C. The cells remained suspended in the respective pH for 60 min at 25°C. The experiments at pH 7.3 were performed in the previous mentioned saline. The alkaline saline (pH 8.0) contained (in mmol/l): 100 NaCl, 5.9 KCl, 2.4 $MgSO_4$, 20 H_3BO_3 and 5 glucose. The red cells were incubated with 10^{-5} mol/l adrenaline in the presence or absence of inhibitors (as indicated in the figures). *In vivo* measurements of blood pH of over 20 animals tested showed a value of 7.30 ± 0.04 at 25°C. The measurements were conducted by the use of a Radiometer Copenhagen ABL3.

Cell water content determination

After each incubation samples of 500 μl were taken for the determination of the water content. The blood samples taken for the cell water content determination were in the presence of phosphodiesterase inhibitor. The samples were centrifuged (2 min, $16{,}000 \times g$) in a pre-weighted Eppendorf tube and the supernatant and the topmost layer of the red blood cells were discarded. The cell pellet was then weighted (wet weight) and subsequently dried in 90°C incubator for 12 h. The difference between wet and dry weight represented the water loss of the red cells.

Separation of intra-and extracellular phase. Determination of intracellular Na^+ and K^+ concentrations

For the determination of intracellular concentrations of Na^+ and K^+ ions, 200 μl of the incubated cells suspension was withdrawn from the flask at the time 0 and 40 min, and gently layered over 0.2 ml of silicon oil (density 1.035 $g \cdot cm^{-3}$) that had been layered on top of 0.085 ml of 30% perchloric acid (PCA) in an Eppendorf tube. Tubes were immediately centrifuged for 1 min. The top layer was subsequently withdrawn and was considered to be the extracellular fluid and contents (Kaloyianni and Freedland 1990). The acid layer represented the intracellular contents of the cells with some (less than 5%) extracellular contamination (Zurrendorck and Tager 1974). Na^+ and K^+ ions were measured in the intracellular phase by the use of atomic absorption spectrophotometer (Perkin-Elmer model 2380). The results are given as mmol/l of red blood cells.

Preparation of the red cells for the transport studies

Cells were prepared for transport experiments by washing three times with an isoosmotic incubation medium containing (in mmol/l): 100 NaCl, 5.9 KCl, 1.25 $CaCl_2$, 2.4 $MgSO_4$, 4.2 imidazole, 7.6 glycylglycine and 1.2 KH_2PO_4. The washing procedure was adequate to remove intracellular glucose. The buffy coat was also removed

and the washed red cells were re-suspended at a haematocrit of 10 % in incubation medium in the presence of the inhibitors. The pH value of the medium was 7.3 except the experiments in which the effects of pH on glucose transport were studied. All experiments were carried out under air at 25 ℃. In swelling experiments, cells were suspended in hypotonic (or isotonic as controls) Na^+-free (high K^+) medium, the osmolarity of which varied down to 80 % of its normal physiological value. The isotonic medium consisted (in mmol/l) of: 106 KCl, 1.25 $CaCl_2$, 1.2 KH_2PO_4, 4.2 imidazole, 7.6 glycylglycine and 2.4 $MgSO_4$ (pH 7.3). The use of K^+ as the extracellular cation minimizes the regulatory volume decrease following hypotonic swelling.

Transport studies

Uptake of 3-O-methyl-D[^{14}C] glucose was conducted according to the method described by Tse and Young (1990). Uptake was initiated by addition of the labeled substrate to the cell suspension (haematocrit 10 %). Final incubation contained ^{14}C-labelled 3-OMG at an activity of approximately 0.7 μCi·ml^{-1}. In the dose-response studies, a pre-incubation period of at least 1 h with phloretin or adrenaline preceded the uptake experiments. Fresh stock solutions of phloretin and adrenaline were prepared daily. Phloretin and adrenaline were dissolved in dimethyl sulphoxide (DMSO) and in saline of pH 3–4 respectively. The amount of DMSO added to the incubation did not affect the rate of 3-OMG uptake. Incubations were stopped at pre-determined times by transferring 0.2 ml of the cell suspension (haematocrit 10 %) to an Eppendorf tube containing 0.8 ml of ice-cold stop medium layered on top of 0.5 ml dibutyl pthalate. The stop medium containing 100 mmol/l NaCl, 4.2 mmol/l imidazole, 1.25 mmol/l KI, 1μmol/l $HgCl_2$ and 100 μmol/l phloretin was prepared daily. The tube was centrifuged immediately (10,000 × g for 10 s, at +4 ℃). The medium and oil were removed by suction, leaving the cell pellet at the bottom of the tube. After the inside of the tube had been carefully wiped, the cell pellet was lysed with 0.2 ml (30 %) PCA. The precipitates were removed by centrifugation (10,000 × g for 2 min) and 2.0 ml samples of protein-free supernatant counted. The radioactivity taken up by the cells was measured by liquid scintillation counting (Wallac model 1409), using the equation:

$$\text{Sugar uptake} = \frac{\text{Counts in cells}}{\text{Counts in whole suspension}} \times \times \frac{\text{Volume of “total” sample}}{\text{Cell volume which equilibrates with sugar}} \times \text{Sugar concentration}$$

Statistical evaluation

Statistical significance was assessed with Student's t-test applied to unpaired grouped samples. Changes in initial values as well as effects of additions at the various incubation time points were also statistically examined.

Results

The rate of 3-OMG uptake was tested at 10^{-9}–10^{-3} mol/l of adrenaline (results not shown). The rate of 3-OMG uptake for frog erythrocyte was stimulated by 10^{-4} mol/l adrenaline and at the 30 min of incubation the stimulation was maximal (Fig. 1). From the results it can be noted that 3-OMG uptake in the red cell under the effect of catecholamine was pH-independent. The entry of 3-OMG was 1.076 ± 0.17 at pH 8.0 and 1.082 ± 0.22 mmol/l red cell at pH 7.3. The rate of 3-OMG uptake into frog erythrocyte is shown in Fig. 2. The uptake is linear for at least 20 min and remained unchanged till the 3 h of incubation.

Fig. 3 shows the concentration-dependence of the initial rate of 3-OMG uptake. The rate of uptake rises linearly and saturation kinetics are not apparent at the concentration used 0.5–10 mmol/l which are close to the physiological ones. Maximal inhibition caused by 10^{-4} mol/l phloretin (Fig. 4). The inhibition constant I_{50} for phloretin was 4.1 ± 0.4 μmol/l.

Despite the pH independence of glucose uptake, the cell volume significantly changed when adrenaline was added to frog red cells in saline at pH 7.3 and at pH 8.0 (Fig. 5). From the results it can be noted that the volume response is pH-

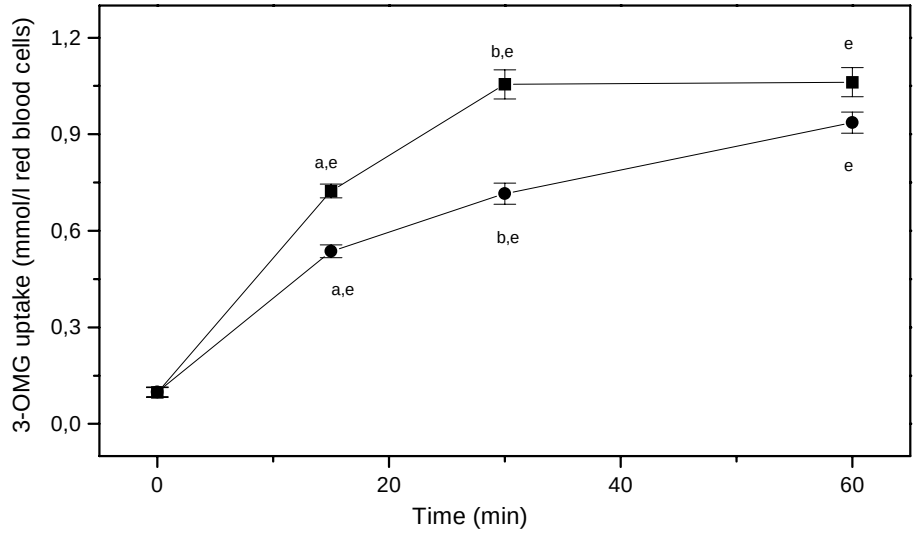

Figure 1. Time course of 3-OMG uptake by frog erythrocyte in the presence of 10^{-4} mol/l adrenaline. Upper curve, adrenaline values; lower curve, control values. The values are the mean of 8 measurements ± S.E.M. Each measurement was conducted with the blood of 2 or 3 animals to give a pool of cells. e, indicates statistically significant difference of the value at various time points from 0 time ($p < 0.001$). Values that share the same letter (a, b) indicate statistically significant difference of this value from the corresponding value at the same time point ($p < 0.001$).

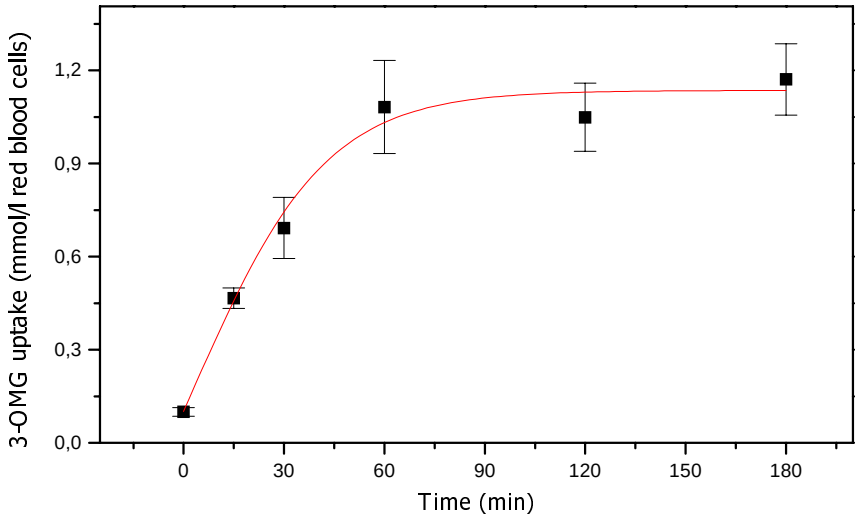

Figure 2. Time course of 3-OMG uptake by frog erythrocytes at extracellular concentration of 3 mmol/l 3-OMG. The values are the mean of 8 measurements ± S.E.M. Each measurement was conducted with the blood of 2 or 3 animals to give a pool of cells. Each separate value is significantly different from 0 time value ($p < 0.001$).

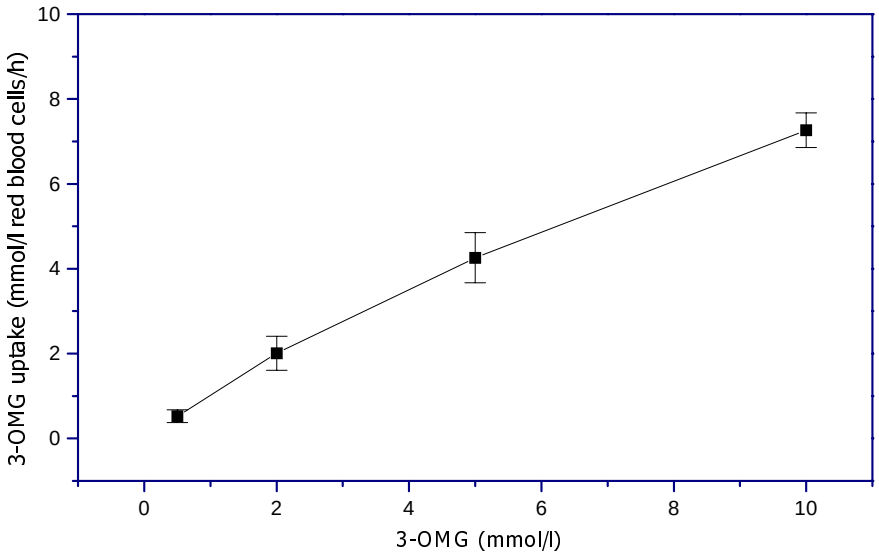

Figure 3. Concentration dependence of 3-OMG uptake by frog erythrocytes. The values are the mean of 8 measurements ± S.E.M. Each measurement was conducted with the blood of 2 or 3 animals to give a pool of cells.

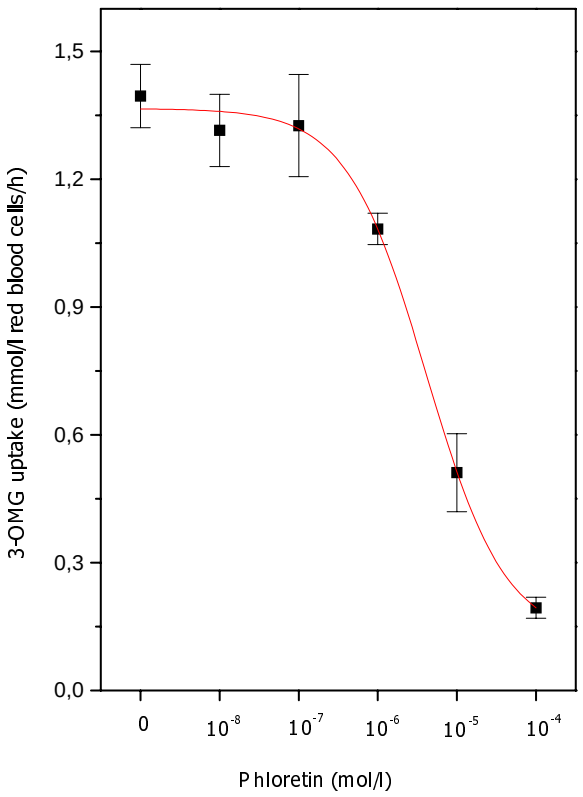

Figure 4. Inhibition of 3-OMG uptake in red cells by phloretin. The values are the mean of 8 measurements ± S.E.M. Each measurement was conducted with the blood of 2 or 3 animals to give a pool of cells.

dependent; at pH 8.0 the cells enlarge less and reach a new steady state of lower value than at pH 7.3. The latter is in accordance with results of Borgese et al. (1987) on trout red cells. It should be also pointed out that the initial value of the two cell batches was slightly different. This is due to the osmotic effect associated with the chloride shift induced by the difference in hemoglobin ionization at the two pH values.

Fig. 6 illustrates the pH dependence of net Na^+ influx and K^+ loss measured for 40 min after hormonal stimulation, in the same batch of cells. The net K^+ loss increases with pH. The net Na^+ influx shows a pH dependence with a maximum at pH 7.3. Specifically, at pH 7.3 Na^+ uptake is greater (3.34 mmol/l red cells) than at pH 8.0 (1.42 mmol/l red cells) at the time of incubation. On the other side, K^+ loss is less (14.3 mmol/l red cells) at pH 7.3, compared to 18.6 mmol/l red cells at pH 8.0. As previously reported, the Na^+ uptake is mediated by a Na^+-H^+ exchanger which is activated by adrenaline. In the absence of amiloride, Na^+ accumulated rapidly in the cells, in the presence of amiloride, the accumulation of Na^+ was very slow almost inert (Fig. 6). It was also observed that ouabain had no appreciable

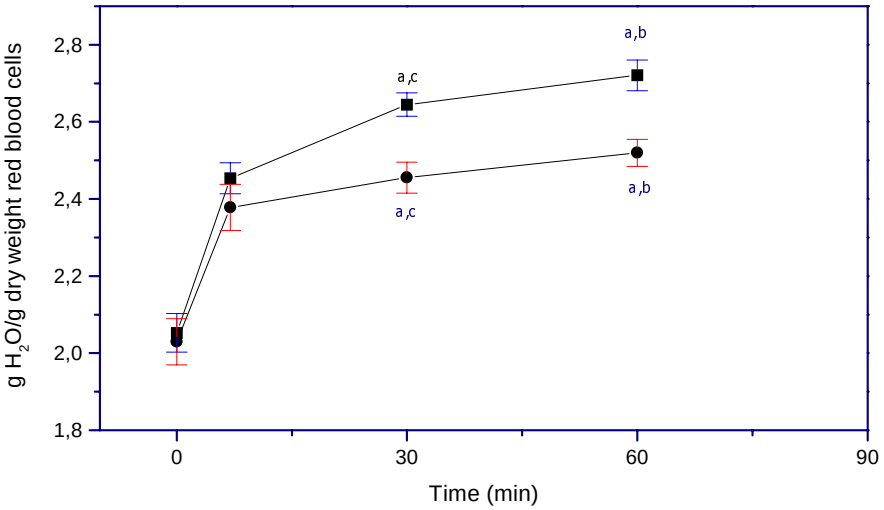

Figure 5. Time course of cell volume changes after stimulation of frog red cells by adrenaline (10^{-4} mol/l), at pH 7.3 (upper curve) and pH 8.0 (lower curve). The values are the mean of 8 measurements ± S.E.M. Each measurement was conducted with the blood of 2 or 3 animals to give a pool of cells. a, indicates statistically significant difference of the value at various time points from 0 time ($p < 0.005$). Values that share the same letter (b, c) indicate statistically significant difference to each other ($p < 0.005$).

effect on the time course or on the magnitude of volume changing at either pH 7.3 or pH 8.0 (results not shown).

Osmotic swelling also caused a clear increase in the total 3-OMG uptake in frog erythrocytes. The phloretin sensitive flux was also increased (Fig. 7).

Discussion

The rate of sugar transport in frog *Rana balcanica* red cells is low, (Fig. 2) as has been reported in other studies on fish (Tiihonen et al. 1995) and avian (Simons 1983) erythrocytes. Concentration dependence of the rate of uptake rises linearly and saturation kinetics are not apparent over the concentration range used (Fig. 3). The latter suggests lack of a glucose transporter or extremely low glucose affinity for the transporter. Similar findings were reported for fish erythrocytes (Tiihonen et al. 1995). Therefore we could suggest that the 3-OMG transport across the frog erythrocyte occurs by simple diffusion.

10^{-4} mol/l adrenaline stimulated 3-OMG uptake by frog erythrocyte (Fig. 1). Since adrenaline causes enlargement of the red cell (Fig. 5), we checked if the volume changing affects glucose uptake. The results showed that in hypotonic conditions the rate of 3-OMG uptake was also increased (Fig. 7). The results indicate that the cells react similarly by increasing 3-OMG uptake in both treatments: when they

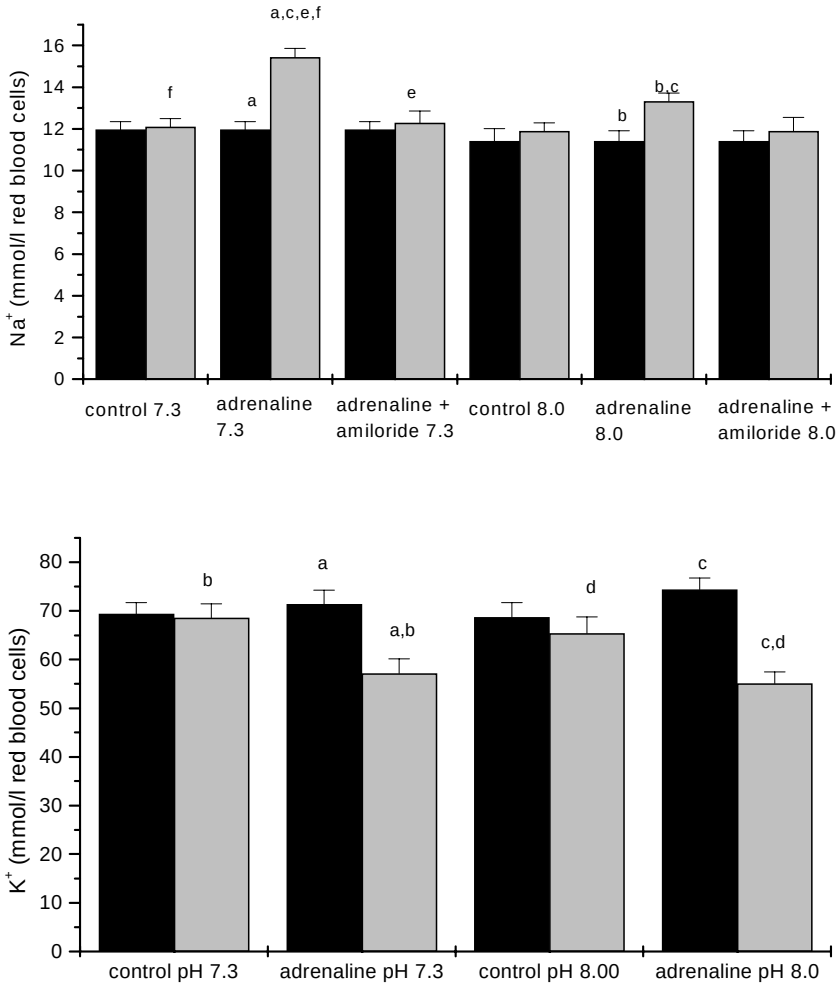

Figure 6. Changes in cation content after incubating *Rana balcanica* red cells for 40 min in the presence of adrenaline (10^{-4} mol/l), and adrenaline together with amiloride (0.5 mmol/l). Black columns, values at 0 time; grey columns, values at 40 min incubation time. The values are the mean of 8 measurements ± S.E.M. Each measurement was conducted with the blood of 2 or 3 animals to give a pool of cells. Na^+ values: the values that share the same letter indicate statistically significant difference to each other: a, $p < 0.001$; b, $p < 0.009$; c, $p < 0.01$; e, $p < 0.0002$; f, $p < 0.05$. K^+ values: the values that share the same letter indicate statistically significant difference to each other: a, $p < 0.005$; b, $p < 0.02$; c, $p < 0.0001$; d, $p < 0.03$.

are suspended in the presence of adrenaline, or in hypotonic medium. The latter implies that sugar transport is related to volume changes, since both adrenaline and hypotonicity induce volume changes in *Rana balcanica* red cells.

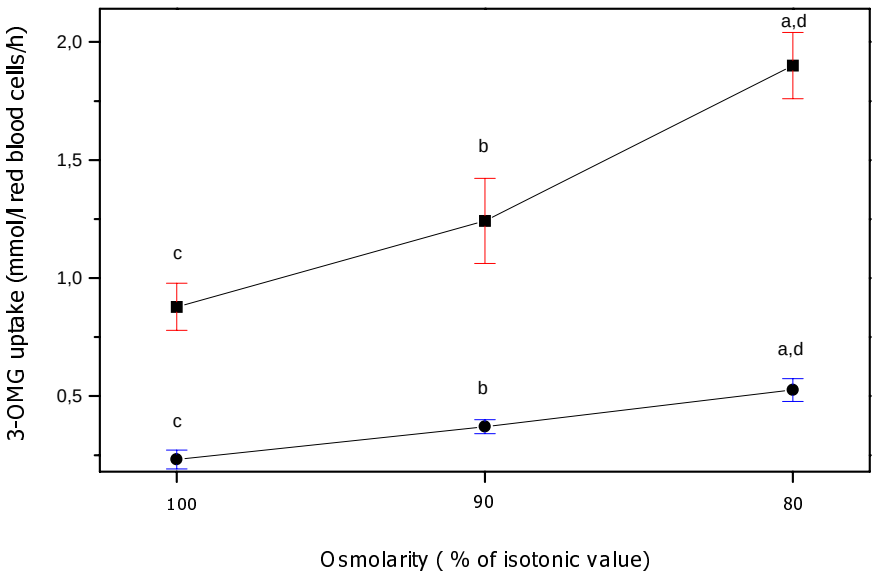

Figure 7. Effect of osmolarity on the initial rate of 3-OMG uptake by frog erythrocytes. Upper curve, control values; lower curve, values in the presence of phloretin. The osmolarity of the media varied down to 80% of its isotonic value (100%). The values are the mean of 4 measurements ± S.E.M. Each measurement was conducted with the blood of 2 or 3 animals to give a pool of cells. a, indicates statistical significance difference of the value at various time points from 100% value ($p < 0.001$). Values that share the same letter (b, c, d) indicate statistically significant difference to each other ($p < 0.001$).

Glucose transport is sensitive to phloretin which binds at the external surface of the transporter (Krupka 1985). The inhibition of glucose transport by phloretin (Fig. 4) may be the result of its interaction with the membrane skeleton or the specific inhibition of glucose transporter by phloretin. The percentage inhibition of transport by phloretin was 74% – after osmotic swelling at 80% osmolarity – and 71% in isotonic medium (Fig. 7). These similar values indicate that transport in both isotonic and hypotonic medium occurs *via* the same pathway. Furthermore, due to the changes in glucose transport at different osmolarity medium, 3-OMG uptake may be mediated by a mechanism similar to the catecholamine-induced cell swelling. Therefore, the results lead us to suggest that sugar transport probably occurs *via* a volume-activated channel. The latter is activated under stress conditions, resulting in increased uptake of glucose in the red blood cells. In agreement with our results Kirk et al. (1992) reported that glucose transport in flounder erythrocytes is also mediated *via* a volume-activated channel that resembles the volume-activated chloride channel. Also, Tse and Young (1990) have reported for fish red blood cells, that the catecholamines-induced changes in glucose transport activity possibly are a secondary consequence of cell volume changes.

Rana balcanica red cell swelling is due to Na^+-H^+ exchanger activation which is subject to an intracellular pH-dependent control. The water movements involved in swelling are linearly related to net movement of Na^+ and K^+ (Fig. 6). This observation is explained by the differential pH effect on the two regulatory processes, i.e. Na^+-H^+ exchange and K^+ leak (Fig. 6). In interval of pH 8.0–7.3, K^+ loss decreases as the pH becomes more acidic. From the results we could suggest the volume sensitive K^+ fluxes in frog erythrocytes which is in accordance with results in rainbow trout red cells (Borgese et al. 1987). On the other hand amiloride sensitive Na^+ uptake increases as the pH becomes more acidic (Fig. 6). From the results it can be suggested that at alkaline pH, the Na^+-H^+ exchanger is less activated while the K^+ pathway is more activated compared to pH 7.3; it was expected since this exchanger is activated when the medium conditions become acidic. Therefore, the results show the different pH dependence of Na^+ and K^+ permeability, while glucose transport is pH independent. On the other hand, both entries of sugar as well as of Na^+ are volume-dependent processes. We could therefore suggest that glucose may be transported across erythrocyte membrane by co-transport mechanism with the Na^+ ions after adrenaline treatment.

Furthermore, studies on avian erythrocytes demonstrate that catecholamine stimulation of sugar transport is associated with a lowering of intracellular ATP levels (Whitfield et al. 1973). The regulatory role of ATP in glucose transport has been also referred in human red cells. According to Kaloyianni and Baker (1998), ATP modulates glucose transport in human erythrocytes. Glucose transport in frog erythrocytes may also be affected by ATP, since catecholamines influence glucose uptake as well as the ATP consuming enzyme of glycolysis pyruvate kinase (Fig. 5, Kaloyianni et al. 2000a).

In conclusion, the present results show that although transport is inhibited by phloretin, the lack of saturation kinetics for transport over the concentration range suggests that a glucose transporter does not exist. The increase in transport after osmotic swelling suggests the glucose transport pathway being a volume-activated channel. The glucose transport is mediated by a mechanism dependent on the catecholamine-induced cell swelling. In addition, cell volume increase is pH-dependent and affects the Na^+-H^+ exchanger and K^+ pathway. The possible relation of Na^+-H^+ exchanger activation to glucose uptake after adrenaline treatment remains to be elucidated.

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