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Influence of Hypo-osmolality on the Activity of Short-Chain Neutral Amino Acid Carriers in Trout (*Salmo trutta*) Red Blood Cells

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Abstract. The present study shows that in trout red blood cells the activity of some amino acid carriers, not directly involved in cell volume regulation, is affected by external osmolality. Glycine uptake has been used as the experimental approach because it was shown previously that it is effected by different carriers, namely the Na^+-dependent ASC and Gly systems, as well as the Na^+-independent asc and L systems.

An increase in the uptake through the Gly system and the two Na^+-independent carriers was found, while the ASC system appeared to be downregulated. Those systems whose activities were increased by hypo-osmolality did not share the mechanism by which this increase was obtained. Thus, the Gly system was sensitive to intracellular ionic changes, while the Na^+- independent systems were mechanically stimulated, as assessed by the iso-osmotic swelling caused by ammonium chloride.

On the other hand, a volume-sensitive transporter may be present in trout red blood cells, which is involved in the swelling-induced glycine movement, as can be deduced from the effect of some inhibitors such as pyridoxal phosphate, DIDS (4,4′-diisothiocyanate-stilbene-2,2′-disulfonic acid) and quinine.

Key words: Glycine uptake — Hypo-osmolality — Red blood cells — ASC system — Gly system — asc system — L system — Volume-sensitive channel — Trout (*Salmo trutta*)

Introduction

Amino acids cross cell membranes via a variety of transport systems, whose molecular specificity varies considerably (*see* White, 1985; Barker & Ellory, 1990; Berteloot & Maenz, 1990 and McGivan & Pastor-Anglada, 1994 for recent reviews on this field).

Studies on amino acid uptake by fish cells have largely been limited to taurine, GABA and β-alanine, because these amino acids are concentrated in fish cells and participate in cell volume regulation (Goldstein & Boyd, 1978; Fugelli & Thoroed, 1986; Fincham, Wolowyk & Young, 1987). Fugelli and Zachariassen (1976) have shown that flounder erythrocytes (*Platichthys flexus* L) concentrate threonine, serine, glutamate, glycine, alanine and lysine when fish are adapted to fresh water, indicating that they may play a role in cell volume control. Whether these changes are due to the activation of membrane carriers is not known, but most of these amino acids can be taken up through carriers such as the ASC, Gly, asc and L systems (Barker & Ellory, 1990).

Trout ASC system shows the kinetic characteristic of mammalian ASC carrier (Canals et al., 1992; Gallardo et al., 1992; Gallardo & Sánchez, 1993), but it is regulated by fasting (Canals et al., 1992), adaptive regulation (Canals et al., 1993) and insulin (Canals et al., 1995) unlike the ASC carrier present in mammalian cells (Barker & Ellory, 1990). The Gly system found in trout cells is very similar to that found in avian or mammalian cells, requiring two sodium and one chloride for every transported glycine (Gallardo & Sánchez, 1993).

In trout cells there are at least two functionally different Na^+-independent asc systems, both having as preferred substrates short-chain neutral amino acids, (Albi et al., 1994). Another Na^+-independent carrier for neutral amino acids, the L system is also present in fish cells (Gallardo & Sánchez, 1993; Gallardo, Albi & Sánchez, 1996) sharing its characteristics with the carrier present in mammalian cells.

Hypo-osmotic swelling also activates a transporter with the properties of an amino acid channel in skate erythrocytes. This transporter is capable of taking up α-,

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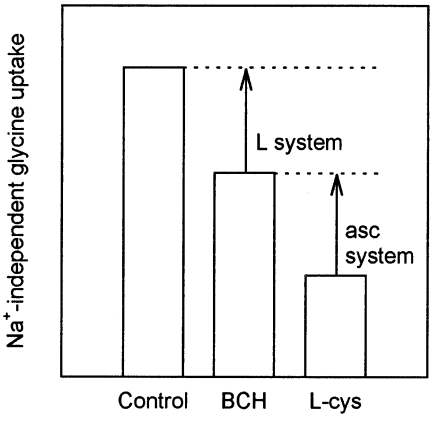

Fig. 1. The different routes of the Na^+-independent carrier-mediated uptake of glycine by trout red blood cells.

β-, and γ-amino acids as long as their molecular diameter is ≤6.3Å and they carry no net charge (Haynes & Goldstein, 1993). Moreover, the channel is also capable of transporting polyols and trimethylamines [the other two groups of organic osmolytes involved in cell-volume regulation (Goldstein & Davis, 1994)]. The inhibitors of the anion exchanger band 3 (García-Romeu, Cossins & Motais, 1991; Goldstein & Brill, 1991; Goldstein & Davis, 1994) and a variety of chloride channel inhibitors (Banderali & Roy, 1992; Jackson & Strange, 1993; Goldstein & Davis, 1994; Kirk, Ellory & Young, 1992) blocked the uptake through the channel stimulated by hypo-osmolality.

There are recent reports on the uptake of several short-chain neutral amino acids by fish cells (Ballatori & Boyer, 1988; Fincham, Wolowyk & Young, 1990; Canals et al., 1992; Gallardo, Planas & Sánchez, 1992; Canals, Gallardo & Sánchez, 1993; Gallardo & Sánchez, 1993 and Albi et al., 1994), but little is known about their properties. The present study shows the influence of a drop in the extracellular osmolality on the activity of several amino acid transport systems in trout RBC. It was carried out using glycine, since this amino acid can be taken up through several transport systems (Ellory, Jones & Young, 1981). Moreover, we have shown that most of them (Gly, ASC, asc and L system) are also active in trout blood cells (Gallardo & Sánchez, 1993). Some mechanisms that may be involved in their regulation are also discussed.

Materials and Methods

Brown trout (*Salmo trutta*) (weighing between 200 and 400 g) were obtained from fish farms (Medi Natural, Generalitat de Catalunya) in the Pyrenees, where they were maintained in open-water circuits, directly connected to a river.

(2^3H)-Glycine (47.5 Ci · $mmol^{-1}$) was obtained from New England Research Products (Bad Homburg, Germany) and all other chemicals were from Sigma (St. Louis, MO) except BCH (2-aminobicyclo(2,2,1)-heptane 2-carboxylic acid), which was from Fluka (Buchs, Switzerland) and used as directly provided by the supplier in its racemic form.

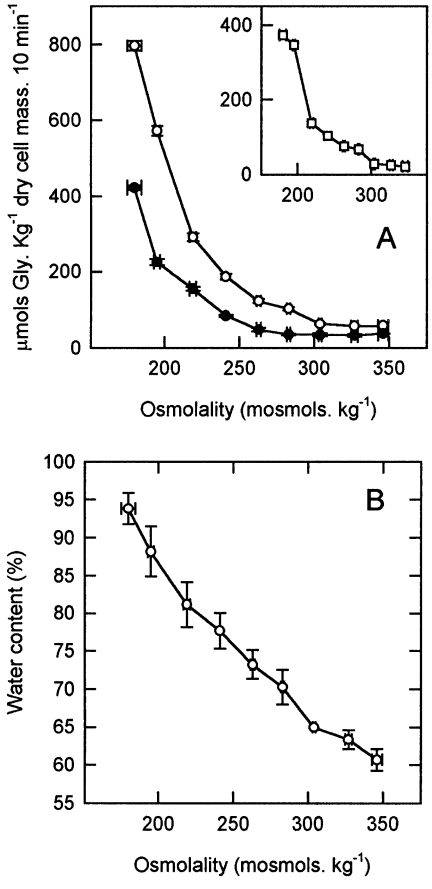

Fig. 2. The effect of osmotic stress on (*A*) 500 µM glycine uptake and (*B*) cell water content. The RBC were pre-equilibrated with a buffer containing either sodium or potassium as the main cation and an osmolality of 305 mosmol · Kg^{-1}. The incubation medium had a variable osmolality and either sodium or potassium as the main cation. The experiments started by mixing (1:3, v/v) the cells and the solutions. RBC were incubated for 10 min at 15°C in presence of glycine and amino-oxyacetic acid. Bars are standard deviation of the mean. (*A*) Glycine uptake: (○) total and (●) sodium-independent. The figure inserted shows the Na^+-dependent glycine uptake. In *A*, each point is the mean of 3 individual experiments. In *B*, each point is the mean of 12 individual experiments.

Blood was obtained by caudal puncture, diluted with heparinized RPMI 1640 (Sigma), rinsed several times and left overnight at 4°C to eliminate a possible catecholamine effect. Red blood cells (RBC) were separated from mononuclear white cells by centrifugation with Histopaque-1077™ (Sigma), following the procedure suggested by the supplier, slightly modified because of the high viscosity of trout blood. Once separated, the RBC were rinsed four times in Cortland buffer (pH 7.4) (Houston et al., 1985) slightly modified (in mM) NaCl 141, KCl 3.5, $MgSO_4$ 1, NaH_2PO_4 3, $CaCl_2$ 1, pyruvic acid 2, HEPES 10, glucose 3, bovine serum albumin 0.3%. The osmolality was adjusted to 305 mosmol · Kg^{-1}, except when indicated. During this process, the RBC became depleted of amino acids (*data not shown*). In experiments using buffers of a different ionic composition, the whole rinsing procedure was performed using the final buffer.

The different final osmolalities were obtained by mixing the cell suspension with buffer of low osmolalities containing the labeled gly-

cine. Both cells and solutions were pre-equilibrated at 15°C, before the experiments were started, by mixing the RBC suspension with the glycine solution to obtain the desired concentrations or osmolalities and a final hematocrit of about 10%. (2^3H)-glycine (0.3 μCi for each ml of cell suspension) was added to the cold glycine solution before mixing with cells. 2 mM amino-oxyacetic acid (a transaminase inhibitor) was used throughout uptake experiments. Incubations were performed in a shaking bath at 15°C, using air as atmosphere.

The different Na^+-dependent uptakes were measured as the sarcosine-sensitive uptake (Gly system activity) and L-cysteine-sensitive uptake (ASC system). It was shown previously that these two Na^+-dependent systems account for all the Na^+-dependent uptake of glycine in trout RBC (Gallardo & Sánchez, 1993).

The measurement of the uptakes through the Na^+-independent carriers was more complex because no specific inhibitors for them are known in trout RBC (Albi et al., 1994). The uptake through the L system was measured as the BCH-sensitive Na^+-independent uptake, while the uptake through the asc system was assessed as the L-cysteine-sensitive Na^+-independent uptake minus the BCH-sensitive Na^+-independent uptake (*see* Fig. 1). The asc system activity was measured in this form because L-cysteine is able to inhibit the uptake of neutral amino acids through both the L and asc systems (Albi et al., 1994; Gallardo et al., 1996).

Glycine uptake was stopped by diluting with Cortland buffer at the same osmolality of incubation medium (1:9, v/v) containing a 10- to 50-fold excess of nonradioactive glycine, and rinsing the cells with this solution (1:9, v/v) three times. RBC were separated each time by centrifugation (810 × *g* for 8 min at 4°C). Cell suspensions were deproteinized by adding sufficient ice-cold perchloric acid to obtain a final concentration of 6%. A clear supernatant was obtained by centrifugation (1,825 × *g* for 20 min at 4°C). The radioactivity in this supernatant was measured in a liquid scintillation counter.

Cell water content was determined gravimetrically and intra/extracellular sodium measurements were performed by flame photometry following Mahé, García-Romeu & Motais (1985).

Curve fitting of experimental data was performed by computerized nonlinear regression analyses.

Results

Figure 2*A* shows the effect of different osmolalities on the total glycine uptake. A decrease in buffer osmolality provoked a rise in both Na^+-dependent and Na^+-independent amino acid uptakes. The increase in the Na^+-independent uptake can be partially attributed to an increase in diffusion due to the rise in cell volume (Fig. 2*B*), but there was also an increase in the Na^+-independent-mediated uptake of glycine and a channel involved in volume regulation may also have been activated, as will be shown below. At the experimental times used in the present study there were no significant differences in the water content when cells were equilibrated in a buffer containing sodium or potassium, although this was not the case for longer times, in agreement with the results of García-Romeu et al. (1991).

Figure 3 shows that the activities of the ASC and Gly system were modified by changes in extracellular osmolality. The uptake through the Gly system rose if the buffer osmolality dropped, while in the same conditions the activity of the ASC system fell.

To determine whether the Gly system was the only

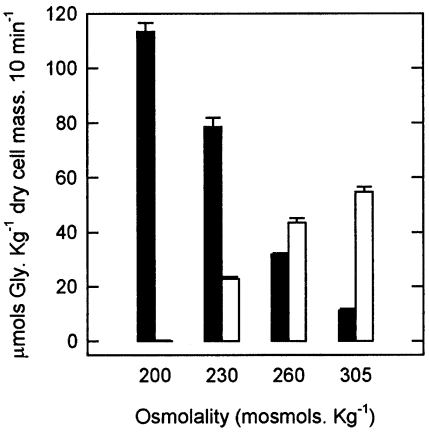

Fig. 3. The effect of an hypo-osmotic shock on the initial rates of 500 μM glycine uptake through the Gly and ASC systems. The RBC were equilibrated and incubated as described in Figure 2. (■) Uptake through the Gly system. (□) Uptake through the ASC system. Each point is the mean of 3 individual experiments. Bars are the standard deviation of the mean.

Na^+-dependent transporter involved in the rise of glycine uptake caused by the hypo-osmolality, advantage was taken of its strict selectivity for chloride, which cannot be replaced by acetate or sulfate (Gallardo & Sánchez, 1993). Figure 4 shows that the sarcosine-sensitive Na^+-dependent glycine uptake required chloride in both normo-osmotic and hypo-osmotic conditions. The β system (another Na^+-dependent amino acid transport requiring chloride, Fincham et al., 1987), was not involved in the glycine uptake in any condition, since β-alanine did not inhibit glycine uptake (*data not shown*). Thus, the Gly system seems to be the only Na^+-dependent amino acid carrier involved in the rise of glycine uptake due to hypo-osmolality.

Figure 5 shows the effect of hypo-osmolality on the Na^+-independent uptake of glycine through the L and asc systems. At 200 mosmol · Kg^{-1} the uptake through both carriers was 10 times higher than under normo-osmotic conditions. On the other hand, the apparently nonmediated uptake increased by a factor of 30 under the same experimental conditions (insert in Fig. 5).

Some of the apparently nonmediated uptake could be accounted for by a volume-sensitive transporter and could not be due to simple diffusion. To test this possibility, two kinds of experiments were carried out. First, the hypo-osmotically activated uptake of glycine through this carrier was tentatively inhibited with some of the main channel substrates, such as taurine, inositol, betaine and β-alanine. As shown in Fig. 6, no significant inhibition of the total Na^+-independent uptake was obtained, while under the same experimental conditions, both BCH and L-cysteine inhibited uptake. In the second experiment, inhibitors of the anion exchanger band 3 [pyridoxal phosphate (PLP), DIDS, 4-acetamido-4′-isothiocyanate-stilbene (SITS)], a chloride channel in-

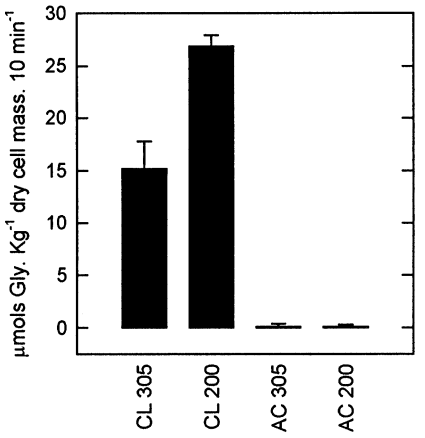

Fig. 4. The effect of hypo-osmotic shock (200 mosmol · Kg^{-1}) and substitution of chloride by acetate on the glycine uptake through the Gly system. RBC were equilibrated and incubated as described in Fig. 2. (305) Final osmolality of incubation medium: 305 mosmol · Kg^{-1}. (200) Final osmolality of incubation medium: 200 mosmol · Kg^{-1}. Each point is the mean of 3 individual experiments. Bars are the standard deviation of the mean.

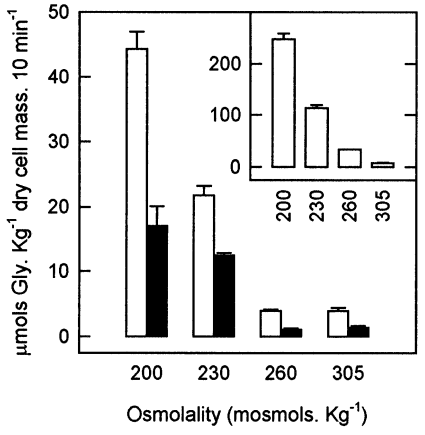

Fig. 5. The effect of hypo-osmotic shock on the Na^+-independent uptake of 500 µM glycine. RBC were equilibrated in a 305 mosmol · Kg^{-1} Na^+-free medium. The incubation buffer contained potassium as the main cation and had a variable osmolality. RBC were incubated as described in Fig. 2. (□) Uptake through the L system and (■) uptake through the asc system. The insert shows the apparently nonmediated Na^+-independent glycine uptake. Each point is the mean of 6 individual experiments. Bars are the standard deviation of the mean.

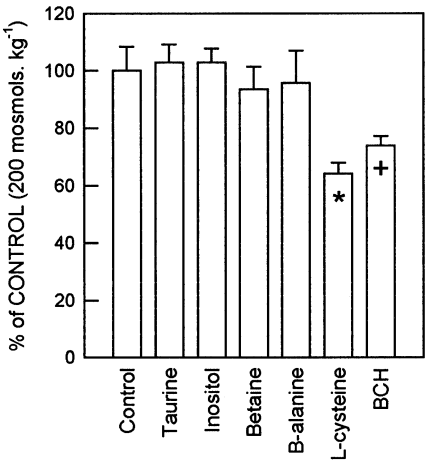

Fig. 6. Effect of volume-sensitive channel substrates on the Na^+-independent glycine uptake activated by hypo-osmolality. Cells were equilibrated and incubated as described in Fig. 5. 10 mM of taurine, inositol, betaine and β-alanine and 5 mM of L-cysteine and BCH were used. Symbols denote significant differences from 200 mosmol · Kg^{-1} without inhibitors: (*), $P \leqslant 0.005$. Each point is the mean of 3–6 individual experiments. Bars are the standard deviation of the mean.

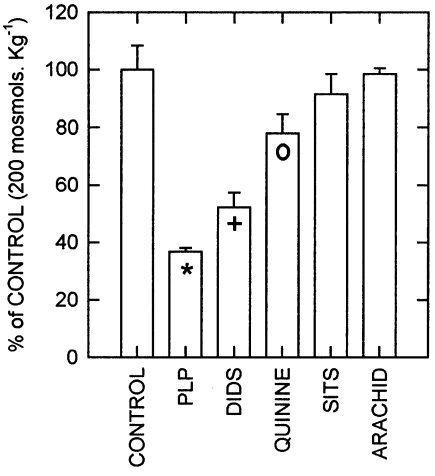

Fig. 7. Inhibition by 2 mM PLP, 0.1 mM DIDS, 1 mM quinine, 0.02 mM SITS and 0.05 mM arachidonic acid of the Na^+-independent glycine uptake activated by hypo-osmolality. Symbols denote significant differences from 200 mosmol · Kg^{-1} without inhibitors: (*), $P \leqslant 0.001$, (+), $P \leqslant 0.01$ and (o) $P \leqslant 0.05$. Values are means ± SE of 3 individual experiments.

hibitor (quinine) and a long-chain fatty acid (arachidonic acid) were used. Figure 7 shows that some of them, PLP, DIDS and, to a lesser extent, quinine, did inhibit the NA^+-independent uptake of glycine, while SITS and arachidonic acid did not.

Two possible mechanisms may be responsible for the effect of the hypo-osmotic treatment on the carrier-mediated uptake of glycine: chemical changes related to an alteration in the intracellular ionic strength and a mechanical membrane stress. Because ammonium sulfate can raise RBC volume without variation in the intracellular osmotic concentration (i.e., an iso-osmotic volume increase is induced), 50% of either sodium chloride or potassium chloride was replaced by ammonium chloride. As shown in Fig. 8*A*, a significant increase in cell volume was obtained due to the presence of this salt. Thus, it is a good method to determine whether the stimulation/inhibition of the different glycine uptake systems was related to any of the factors mentioned above.

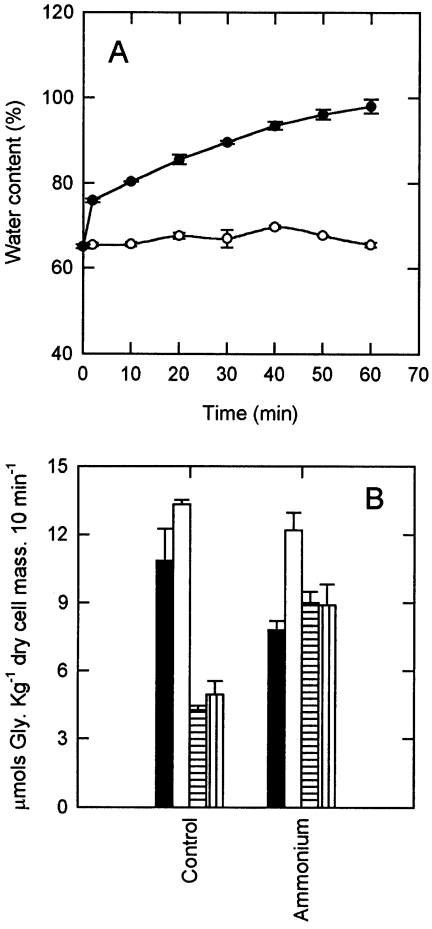

Fig. 8. Effect of 80 mM ammonium chloride on (*A*) water content and (*B*) the initial rates of 500 µM glycine uptake through the Gly, ASC, asc and L system. RBC were pre-equilibrated in a buffer containing sodium or potassium as the main cation and an osmolality of 305 mosmol · Kg^{-1}. The incubation medium contained sodium and potassium or ammonium as principal cation and an osmolality of 305 mosmol · Kg^{-1}. RBC were incubated for 10 mi at 15°C in the presence of glycine, aminooxyacetic acid and L-cysteine, sarcosine or BCH, as required for each experiment. (*A*) Time course of the RBC swelling induced by ammonium chloride. (○) Control. (●) In the presence of ammonium chloride. Each point is the mean of 6 individual experiments. Bars are the standard deviation of the mean. (*B*) Uptake through: Gly (■), ASC (□), L (□) and asc (□) and asc (□) systems. The measurement of glycine uptake through the different carriers was performed as described in Figs. 3 and 5. Each point is the mean of 3 individual experiments. Bars are the standard deviation of the mean.

Figure 8*B* shows the effect of the presence of ammonium chloride on the activities of the ASC, Gly, asc and L system at 305 mosmol · Kg^{-1}. Only the uptake through the Na^+-independent carriers clearly increased, while the activity of the ASC system was not affected (or slightly depressed) and the activity of the Gly system was clearly depressed.

On the other hand, the activities of both Gly and ASC systems are dependent on the transmembrane sodium gradient, their activities being raised when the sodium gradient increases (Kristensen & Folke, 1986 and King & Gunn, 1989). Accordingly, the changes in the ratio extra/intracellular sodium content and in the sodium in/out concentration ratio after hypo-osmotic treatment by trout RBC were analyzed. As can be seen in the Table, when the osmolality fell, the total cell sodium content rose, but the intracellular sodium concentration decreased and, finally, the sodium gradient rose.

Calcium could be involved in the cell volume regulation process, and in the present study, such an involvement was also tested for the uptake of glycine. The intracellular calcium concentration was raised by means of the calcium ionophore A23187 (0.1 µM). The results were as follows: (i) there was no effect on the Na^+-independent uptake of glycine (*data not shown*) (ii) both Na^+-dependent systems were affected; the activity of the ASC system was depressed, while the activity of the Gly system was increased (Fig. 9).

Discussion

The properties of amino acid carriers other than their kinetic aspects are scarcely known (Barker & Ellory, 1990) and this is more evident concerning fish cell transporters. The present study shows that the activity of several short-chain neutral amino acid carriers from trout RBC (ASC, Gly, asc and L systems), measured through glycine uptake, was sensitive to changes in the extracellular osmolality and, under zero-trans conditions, a net increase in the amount of the amino acid taken up was obtained. However, not all the amino acid was incorporated through those carriers; some was probably taken up through a volume-sensitive channel. The nonmediated uptake was also increased by the lower osmolality of the extracellular medium.

Concerning the Na^+-dependent systems, there is the intriguing question of the opposite effect of cell volume change in their activities. At first glance, a direct relationship with changes in the transmembrane Na^+ gradient can be ruled out. The increase in the gradient should be accompanied by a rise in the activity of both systems, but this was not the case and some other mechanisms must be involved. Further, involvement of the β system was also ruled out because β-alanine failed to inhibit the uptake of glycine under either normo- and hypo-osmotic conditions.

On the other hand, the two Na^+-independent carriers tested, the L and asc systems, apparently showed a rise in their activities as a consequence of the change in the external osmolality. However, this increase could be explained by the activation of a volume-dependent channel.

Although our results suggest the presence of this channel in trout RBC, further studies are needed to confirm this, because of the properties of such entities. Namely, they show scarcely any saturability and broad

Table 1. Water content, sodium extra- and intracellular and sodium in/out concentration of trout RBC undergoing regulatory volume after hypo-osmotic swelling

Medium osmolality	Water content (%)	Sodium levels			Sodium in/out concentration ratio
		Intracellular		Extracellular	
		nM	mmol · Kg^{-1} d.w.	mM	
200	81.3 ± 4.4	32.5 ± 4.4	140.9 ± 19.0	107	0.303
250	70.6 ± 0.9	44.9 ± 7.9	107.9 ± 19.1	130	0.346
305	65.0 ± 0.8	58.6 ± 8.1	108.8 ± 15.0	160	0.366

The osmolality of incubation medium was measured as mosmol · Kg^{-1}. Results are expressed as the mean ±SD for $n = 6$ individual experiments.

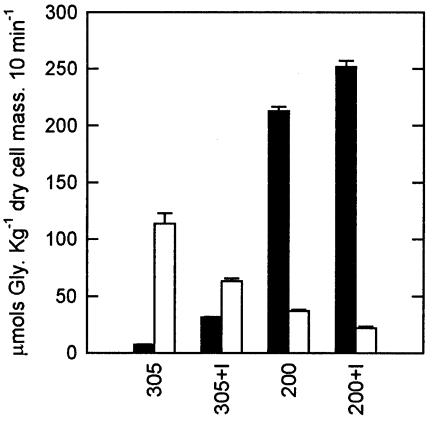

Fig. 9. Effects of hypo-osmolality and a calcium ionophore (A23187, 0.1 µM) on the initial rates of 500 µM glycine uptake through the Gly and ASC systems. RBC were pre-equilibrated and incubated as described in Fig. 3. (■) Uptake through the Gly system. (□) Uptake through the ASC system. (I) ionophore added. (305) Final osmolality of incubation medium 305 mosmol · Kg^{-1}. (200) Final osmolality of incubation medium 200 mosmol · Kg^{-1}. Each point is the mean of 3 individual experiments. Bars are the standard deviation of the mean.

substrate specificity (Kirk et al., 1992) as in the present study. The only data in favor of its involvement are those obtained from the inhibition caused by DIDs. PLP and quinine, but not directly by the inhibition by substrates such as β-alanine, etc. Moreover, when this lack of inhibition was obtained, both BCH and L-cysteine were able to clearly depress the uptake of glycine, confirming the involvement of the L and asc systems in the movement of this amino acid due to the lowering in the extracellular osmotic concentration.

The hypo-osmotic shock has two main consequences for cells: there is an increase in cell volume, with the corresponding increase in the mechanical stress on cell membranes, and there is a change in the concentration of some components in the intracellular compartment, with respect to the extracellular compartment (Hoffmann & Simonsen, 1989). Iso-osmotic cell swelling can help to ascertain whether the changes observed in the activities of glycine carriers can be explained by either mechanical stress or changes in ion composition (Goldstein & Brill, 1990). The results obtained here show that only the activity of the Na^+-independent systems was directly sensitive to the mechanical stress induced by swelling on the membrane.

Calcium has been implicated in the activation of volume-sensitive movements in Ehrlich ascites cells or in RBC from skate (Lambert, 1985 and Leite & Goldstein, 1987). Moreover, in skate RBC, these calcium movements seem to be related to an activation of the phosphoinositide cycle (Goldstein, 1989). Despite the different behavior of the two Na^+-dependent systems with respect to cell swelling, they share their sensitivity to Ca^{2+}. Changes in cell Ca^{2+} concentrations, provoked by the presence of A23187, modified the activities of both the ASC and Gly systems and the direction of the change was the same as that observed when cells were hypo-osmotically swollen. However, it remains unclear whether Ca^{2+} induces the variation of activities due to the hypo-osmotic shock in trout RBC, because Ca^{2+} and hypo-osmolality showed additive effects in the present study. It has recently been reported that suppression of extracellular Ca^{2+} did not suppress the volume-regulatory dependent taurine efflux in trout hepatocytes (Michel et al., 1994), suggesting that it is not the primary signal, but an additional modulator of these movements. In any case, the observed effects due to Ca^{2+} were specific for the Na^+-dependent systems.

The present study shares some results with those obtained by Fincham et al. (1987). These authors suggest that the concentrative Na^+-dependent systems should be downregulated under hypo-osmotic conditions to prevent the uptake of amino acid, facilitating the recovery of cell volume. This explanation can be extended to the present findings on the activity of the ASC system, but not to the Gly system. It should be noted that its concentrative properties are lower than those of the ASC system (Gallardo & Sánchez, 1993).

With reference to the Na^+-independent uptake, those authors also obtained an increase in the uptake of short-chain neutral amino acids (Fincham et al., 1987), which was attributed to the existence of a volume-activated channel. However, its selectivity for these amino acids raises doubt about whether this channel is the same as that described as a poorly substrate-selective channel in other cells types (Kirk et al., 1992) or whether it is a different entity. If the later were the case, results of the present study could indicate that glycine is taken up through this channel under hypo-osmotic conditions, although the insensitivity of such channel to long-chain amino acids leaves the door open to our initial suggestion that both L and asc systems are activated by a drop in the external osmolality.

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