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REGULATION OF THE ASC SYSTEM AND Na^+/K^+ PUMP ACTIVITIES IN BROWN TROUT (*SALMO TRUTTA*) HEPATOCYTES

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Summary

The present study investigates the regulation of Na^+/K^+ pump activity and alanine uptake in trout hepatocytes. Pump activity increased when cells were incubated in an amino-acid-free medium, while it was reduced in cells from fasted animals. Short-term exposure (3 h) to glucagon modified the activity of the pump in a complex seasonally dependent pattern: in experiments carried out in autumn and winter there was some inhibition, while in spring the pump was activated by this hormone.

Pharmacological modification of levels of two intracellular signal transducers, namely cyclic AMP and Ca^{2+}, always led to a reduction in pump activity. These experiments were conducted in May, when activation of the pump by glucagon exposure occurred. There is no apparent explanation for the mechanism by which this hormone modifies the activity of the pump. Glucagon also regulates the activity of system ASC (a Na^+-dependent amino acid carrier with short-chain neutral amino acids as preferred substrates). This regulation also showed a seasonally dependent pattern, although the pattern was opposite to that found for the regulation of Na^+/K^+ pump activity.

Key words: ASC system, ATPase, Na^+/K^+ pump, glucagon, adaptive regulation, fasting, hepatocytes, trout, *Salmo trutta*.

Introduction

System A is the main amino acid carrier involved in the concentrating Na^+-dependent uptake of short-chain neutral amino acids by the cells of higher vertebrates (for reviews, see Barker and Ellory, 1990; McGivan and Pastor-Anglada, 1994). This transport system carries methylated substrates (Christensen *et al.* 1965, 1967) and is *trans*-inhibitable (Gazzola *et al.* 1973) and pH-sensitive (Oxender and Christensen, 1963). Its activity is also regulated by fasting (Hayes and McGivan, 1982) and certain hormones (Fehlmann *et al.* 1979; Boerner and Saier, 1985) and is subject to adaptive regulation (Englesberg and Moffet, 1986).

Mechanisms for the long-term regulation of system A activity have been proposed for different cell types (Hardlogten and Kilberg, 1984; Moffet and Englesberg, 1984), suggesting that this carrier is synthesized *de novo* as a consequence of stimulation by insulin (Shotwell *et al.* 1983) or glucagon (Edmonson and Lumeng, 1980) or under nutritional control (Gazzola *et al.* 1981).

The activity of the Na^+/K^+ pump maintains the electrochemical gradient across cell membranes by pumping 2 K^+ into the cells and 3 Na^+ out of the cells against their respective concentration gradients at the expense of 1 molecule of ATP, thus maintaining the driving force for Na^+-dependent carriers. Different physiological situations show an apparent co-variation of system A and Na^+/K^+ pump activities (Bourdel *et al.* 1990; Martínez-Mas *et al.* 1993; Ruiz-Montasell *et al.* 1994), probably at the transcriptional level, by means of repression and negative control by a regulatory gene (Qian *et al.* 1989, 1991).

System ASC is another amino acid carrier of widespread occurrence, sharing most of its substrates with system A, as well as its Na^+-dependence. However, in higher vertebrate cells, the activity of system ASC is regulated neither by hormones (Christensen and Kilberg, 1987) nor by fasting (Fehlmann *et al.* 1979) and is not subject to adaptive regulation (Gazzola *et al.* 1981).

Because of the absence of system A in fish hepatocytes (Ballatori and Boyer, 1988; Canals *et al.* 1992), it is not surprising that the activity of a different short-chain neutral amino acid carrier is physiologically regulated. It has been shown that the activity of system ASC from trout hepatocytes is increased by fasting of the donor animal (Canals *et al.* 1992), *in vitro* amino acid deprivation (Canals *et al.* 1993) or insulin exposure (Canals *et al.* 1995). In the present study, we consider the possibility that the activities of the Na^+/K^+ pump and system ASC may be co-regulated under different experimental conditions, such as amino acid deprivation, fasting of the donor animal and the presence of glucagon.

Materials and methods

Animal and chemicals

Brown trout (*Salmo trutta* L.) (320±52 g, mean ± S.E.M., *N*=35) were obtained from a fish farm in the Pyrenees (Departament de Medi Natural, Generalitat de Catalunya,

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Bagà, Spain), where they were maintained in an open-water circuit directly connected to a river. Water temperature ranged between 7 and 15 °C.

All chemicals were of analytical grade from Sigma Co. (USA). L-[3-^{3}H]alanine (3.15 TBq mmol^{-1}) and rubidium ($^{86}Rb^+$) (37 GBq g^{-1}) were obtained from New England Nuclear (Germany).

Isolation of hepatocytes

Trout were anaesthetised in $NaHCO_3$-buffered MS222. The portal veins were cannulated and the liver was removed from the animal. Perfusion of the liver was continued in a Petri dish. Solutions for cell isolation were essentially as described by French *et al.* (1981), except that hyaluronidase was omitted and 5 mmol l^{-1} $NaHCO_3$ was used instead of 25 mmol l^{-1} $NaHCO_3$, because of the different gas mixture (99.5 % O_2:0.5 % CO_2) used to equilibrate all solutions before use. Final pH was adjusted to 7.6. Osmolality was determined using a micro-osmometer (model 3MO, Advanced Instruments, Inc. USA) and adjusted to 305 mosmol kg^{-1}. Following isolation, cells were suspended in a slightly modified Hanks' solution (in mmol l^{-1}): NaCl, 120; KCl, 5; $MgSO_4$, 1; $CaCl_2$, 2; KH_2PO_4, 0.44; Na_2HPO_4, 0.44; $NaHCO_3$, 5; Hepes, 10; glucose, 5; pyruvic acid, 2; unlabelled L-alanine, 0.15; and bovine serum albumin (BSA), 4 %. Final pH was adjusted to 7.6. The osmolality was adjusted to 305 mosmol kg^{-1} and the cells were left for 18 h at 4 °C to equilibrate with the medium.

The hepatocytes were subsequently rinsed four times in the Hanks' solution (as above except that L-alanine was omitted and the BSA concentration was 2 %). When K^+ was used instead of Na^+ as the main extracellular cation, all sodium salts were replaced by potassium salts. Cell viability was routinely assessed using Trypan Blue exclusion (Mommsen *et al.* 1994) and more than 95 % viability was obtained. Cell integrity over time was assessed by measuring lactate dehydrogenase (LDH) release (Mommsen *et al.* 1994); less than 5 % of initial intracellular LDH was found in the medium after 6 h of incubation. No loss of cell viability was observed in experiments in which Na^+ was replaced by K^+.

The DNA content of hepatocytes was measured as described by Buckley and Bulow (1987).

Alanine uptake experiments

Cells and solutions were pre-equilibrated at 15 °C and mixed. Experiments were carried out in a shaking bath at 15 °C. The activity of system ASC was measured as the Na^+-dependent uptake of L-alanine, since it has been shown previously that this is the only Na^+-dependent pathway for this amino acid in these cells (Canals *et al.* 1992). 2 mmol l^{-1} amino-oxyacetic acid (a transaminase inhibitor) was added simultaneously with L-alanine (labelled and unlabelled) and used throughout amino acid uptake experiments.

Tritiated L-alanine (150 μmol l^{-1} final concentration, 11.1 kBq ml^{-1} of final cell suspension) was taken up for 10 min, following the methods of Canals *et al.* (1992). Uptake was stopped by diluting the cells with Hanks' solution (1:9 v/v) containing 2 mmol l^{-1} unlabelled L-alanine. Cells were rinsed in this solution and centrifuged (1000 ***g*** for 5 min at 4 °C) three times. Hepatocytes were finally lysed using distilled water, and the radioactivity in the resulting solution was counted in a well-type liquid scintillation counter (Packard, UK).

Na^+/K^+ pump activity

Na^+/K^+ pump activity was monitored using radioactive rubidium ($^{86}Rb^+$, 25.9 kBq ml^{-1} final cell suspension), which replaces K^+ in the active transport of this cation into the cell (Resh *et al.* 1980). Temperature and general experimental conditions were as for L-alanine uptake. L-Alanine was not present unless indicated.

Parallel experiments were carried out in which 0.1 mmol l^{-1} ouabain was added 15 min before the addition of $^{86}Rb^+$.

Uptake was stopped by rinsing the cells with Hanks' solution containing 0.1 mmol l^{-1} ouabain, and cells were processed as described above.

Statistical analysis

Comparisons between groups were performed using Student's *t*-tests.

Results

Basal Na^+/K^+ pump activity

The time course of $^{86}Rb^+$ uptake by trout hepatocytes is shown in Fig. 1. Uptake was linear for more than 30 min and this period was used for subsequent experiments. Na^+/K^+ pump activity was determined as the ouabain-sensitive uptake of $^{86}Rb^+$. After 30 min, 80 % of the labelled ion taken up by the cells entered through the pump.

Effect of short-term amino acid deprivation

Following a 3 h incubation of trout hepatocytes in a medium lacking amino acids, a significant increase was measured in the

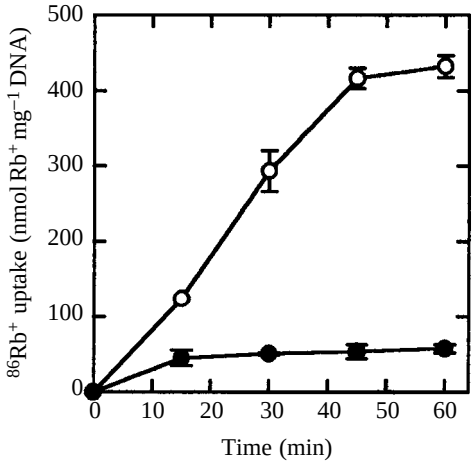

Fig. 1. Time course of $^{86}Rb^+$ uptake by trout hepatocytes. The hepatocytes were suspended in a Na^+-containing Hanks' solution for different periods either with (●) or without (○) addition of 0.1 mmol l^{-1} ouabain (see Materials and methods). Each point is the mean (±1 S.D.) of three individual experiments.

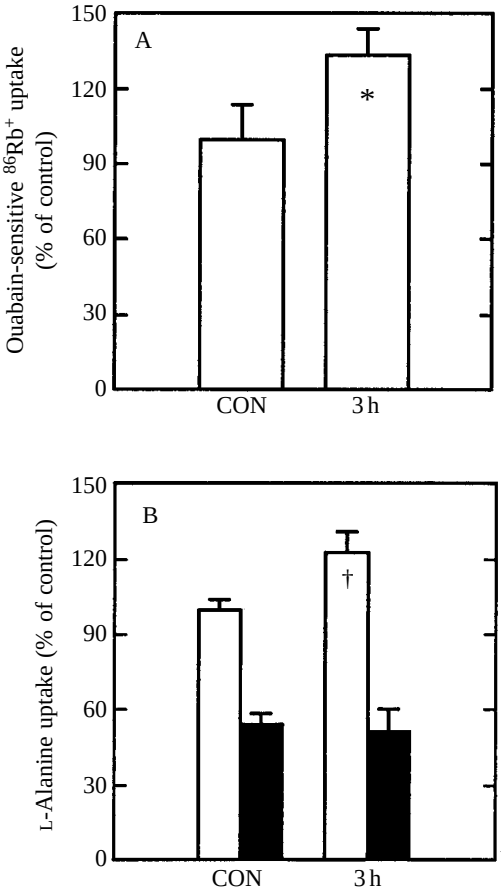

Fig. 2. Effect of *in vitro* amino acid deprivation on the rate of (A) ouabain-sensitive $^{86}Rb^+$ uptake and (B) L-[3-^{3}H]alanine uptake by trout hepatocytes. (A) Cells were incubated in a Na^+-containing medium for 30 min (CON) or 3 h in the absence of L-alanine. $^{86}Rb^+$ uptake was measured for 30 min starting at t=0 and t=2.5 h. Ouabain-sensitive $^{86}Rb^+$ uptake was measured as described in Materials and methods. An asterisk indicates a significant increase in ouabain-sensitive $^{86}Rb^+$ uptake due to adaptive regulation (P<0.001). (B) Cells were incubated in either Na^+-containing (open bars) or K^+-containing (filled bars) medium for 20 min (CON) or 170 min (3 h) in the absence of L-alanine. At these times, L-[3-^{3}H]alanine (150 μmol l^{-1}) and amino-oxyacetic acid (2 mmol l^{-1}) were added and the uptake was followed for 10 min. † indicates a significant increase in L-[3-^{3}H]alanine uptake (P<0.05) compared with the respective control value. Results are the mean + S.D. of nine individual experiments.

activity of the Na^+/K^+ pump (Fig. 2A) and in the Na^+-dependent uptake of L-[3-^{3}H]alanine (Fig. 2B). In a previous study, it was shown that Na^+-dependent alanine uptake alone increased as a consequence of amino acid deprivation (Canals *et al.* 1993). It should be noted that the increase in the activity of the Na^+/K^+ pump was measured in the absence of amino acids in the medium, i.e. it did not result from an increase in the internal Na^+ concentration due to Na^+-dependent amino acid uptake.

Fasting

In previous studies, we demonstrated that fasting of the donor animal (for 21 days) induced an increase in the uptake of L-alanine through system ASC (Canals *et al.* 1992). Fig. 3A shows that, under similar experimental conditions of fasting, the basal activity of the Na^+/K^+ pump was inhibited.

A reduction in hepatocyte ATP levels because of fasting could be a possible explanation for the reduction in Na^+/K^+ pump activity; however, hepatocytes taken from fasted animals and subjected to amino acid deprivation as described above were sensitive to the presence of L-alanine. An increase in the activity of the pump was observed when L-alanine was present (Fig. 3B).

Glucagon

The presence of 20 pg ml^{-1} porcine glucagon for 3 h provoked seasonally dependent changes in both the activity of the Na^+/K^+ pump and the uptake of L-[3-^{3}H]alanine (Fig. 4).

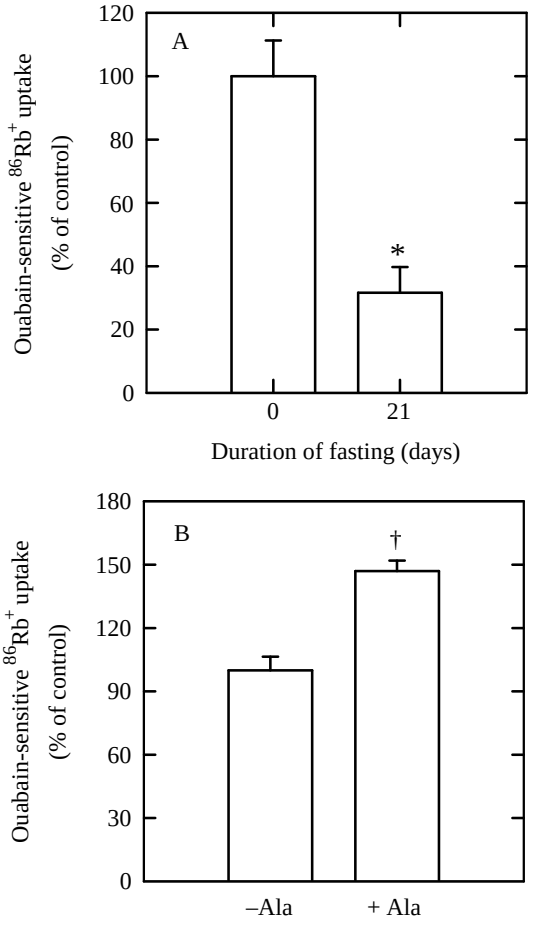

Fig. 3. Effect of fasting of the donor animal on the activity of the Na^+/K^+ pump in trout hepatocytes. (A) Cells were incubated in a Na^+-containing medium (with no L-alanine present) with $^{86}Rb^+$ for 30 min. (B) Hepatocytes taken from trout after 21 days of fasting were incubated in a Na^+-containing medium for 150 min in the absence of amino acids (–Ala) and in the presence of 150 μmol l^{-1} unlabelled alanine (+Ala). $^{86}Rb^+$ was then added and ouabain-sensitive $^{86}Rb^+$ uptake was followed for 30 min (see Materials and methods). * and † indicate significant increases in the uptake of $^{86}Rb^+$ (*P<0.001; †P<0.01). Values are the mean + S.D. of three individual experiments.

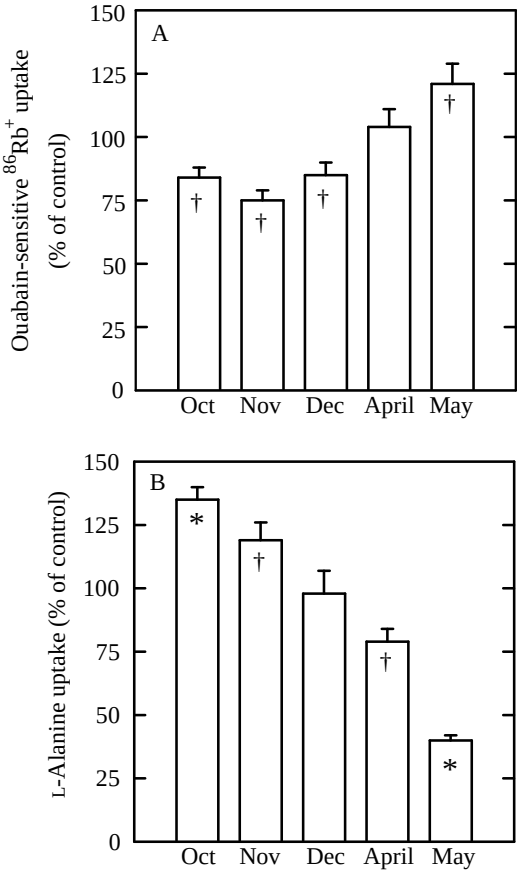

Fig. 4. Seasonal variation in the regulation of Na^+/K^+ pump activity (A) and L-alanine uptake (B) by porcine glucagon ($20\,pg\,ml^{-1}$) in trout hepatocytes. Cells were incubated in a medium containing porcine glucagon for 3 h. (A) Effect of glucagon exposure on ouabain-sensitive $^{86}Rb^+$ uptake. (B) Effect of glucagon exposure on the rate of L-[3-^{3}H]alanine uptake. In both A and B, values are expressed as a percentage of the respective control uptake. * and † indicate a significant difference from the respective control value (100 %) (*P<0.001; †P<0.01). Each value is the mean + S.D. of three experiments.

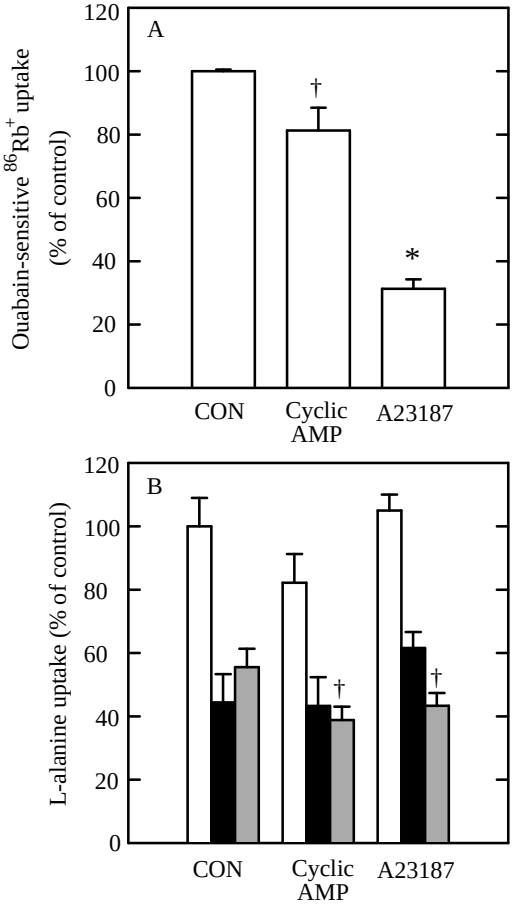

Fig. 5. Effect of short-term (3 h) cyclic AMP ($1\,mmol\,l^{-1}$) and A23187 ($0.1\,\mu mol\,l^{-1}$) exposure on the rate of ouabain-sensitive uptake of $^{86}Rb^+$ (A) and L-[3-^{3}H]alanine uptake (B) by trout hepatocytes. (A) Hepatocytes were incubated with $10^{-3}\,mol\,l^{-1}$ Br-cAMP plus $5\times10^{-5}\,mol\,l^{-1}$ IBMX (Cyclic AMP) or A23187. (B) L-Alanine uptake: total uptake (open bars), Na^+-dependent uptake (system ASC) (filled bars) and Na^+-independent uptake (hatched bars). Each value plotted is the mean + S.D. of six experiments. Values are expressed as a percentage of control uptake. * and † indicate significant differences from the control values (*P<0.001; †P<0.01).

However, these changes occurred in opposite directions. For hepatocytes obtained in October, glucagon induced a significant increase in Na^+-dependent uptake of L-[3-^{3}H]alanine, but a significant reduction in the Na^+/K^+ pump activity. For hepatocytes obtained in December, glucagon did not affect Na^+-dependent L-[3-^{3}H]alanine uptake, but the Na^+/K^+ pump was inhibited. For hepatocytes obtained in May, the Na^+/K^+ pump was activated, while the uptake of L-[3-^{3}H]alanine was inhibited.

To investigate the involvement of a signal transduction system in the effect of glucagon on the activity of the Na^+/K^+ pump and L-[3-^{3}H]alanine uptake, intracellular levels of cyclic AMP and Ca^{2+} were increased by the addition of 8-bromo-cyclic AMP (Br-cAMP) plus 3-isobutylmethylxanthine (IBMX), to prevent cyclic AMP degradation, or the addition of A23187 (an ionophore that facilitates Ca^{2+} entry into cells) to the incubation medium (Fig. 5). These hepatocytes were obtained in May.

Na^+-independent uptake of L-[3-^{3}H]alanine was significantly reduced when levels of cyclic AMP were raised by the addition of Br-cAMP plus IBMX, while the activity of system ASC (Na^+-dependent uptake) was unaffected (Fig. 5B). When the concentration of intracellular Ca^{2+} was raised by the addition of A23187, again only Na^+-independent uptake of L-[3-^{3}H]alanine was affected (there was significant inhibition).The activity of system ASC showed a tendency to increase, but the change was not statistically significant. Na^+/K^+ pump activity was inhibited by both Br-cAMP and A23187 (Fig. 5A).

Discussion

Previous reports on trout hepatocytes have demonstrated regulation of system ASC by fasting (Canals *et al.* 1993) and

insulin exposure (Canals *et al.* 1995). In the present study, regulation of system ASC activity by glucagon is also shown to occur. In addition, evidence that the activity of the Na^+/K^+ pump is modified by fasting and glucagon exposure is also presented.

There is convincing evidence that mammalian system A and Na^+/K^+ pump activities are co-regulated during *in vitro* amino acid deprivation by a repression–derepression mechanism. Previous studies (Moffet and Englesberg, 1984; Englesberg and Moffet, 1986; Quian *et al.* 1991) have suggested the involvement of a regulatory gene producing an inactive repressor that is converted into an active repressor of the synthesis of both system A and the α_1 subunit of the Na^+/K^+ pump in the presence of a substrate of system A.

The present data provide evidence for the regulation of both the Na^+/K^+ pump and system ASC in response to short-term amino acid deprivation. As shown in Fig. 2B, the activity of system ASC was increased by 3 h of incubation in the absence of amino acids. This process depends on protein synthesis (Canals *et al.* 1993). Under the same experimental conditions, the activity of the Na^+/K^+ pump also increased (Fig. 2A), even though there was no Na^+-dependent uptake of amino acid to alter the transmembrane gradient. Thus, the present data suggest co-regulation of system ASC and the Na^+/K^+ pump, as found previously for system A.

Fasting is detrimental to animal survival and some animals adopt energy-saving strategies in these circumstances. In poikilothermic animals, such mechanisms have attracted considerable attention, leading to the concept of hypometabolism (Foster and Moon, 1991). The activity of the Na^+/K^+ pump is considered to be energetically expensive and can be down-regulated under fasting conditions. This has been shown to occur in mammalian skeletal muscle cells (Nishida *et al.* 1992; Matsamura *et al.* 1992) and gill cells from tilapia (*Oreochromis mossambicus*) (Jürss *et al.* 1984) or trout (*Oncorhynchus mykiss*) (Nance *et al.* 1987). The present study provides evidence that this mechanism also operates in *Salmo trutta* liver cells. This reduction in the activity of the Na^+/K^+ pump in trout hepatocytes is reversible: addition of L-alanine to the incubation medium resulted in an increase in $^{86}Rb^+$ uptake as a consequence of the uptake of Na^+ linked to amino acid entry. However, amino acid uptake through system ASC is enhanced in cells from fasting trout (Canals *et al.* 1992), as for system A in higher vertebrate cells (Hayes and McGivan, 1982). This indicates that there is no global co-regulation of these two activities and that the mechanisms operating during amino acid deprivation of cells in culture and during fasting of the donor animal are likely to be different.

The absence of co-regulation is further supported by the effects of glucagon. This hormone showed either inhibitory or stimulatory effects on Na^+/K^+ pump activity that were seasonally dependent and reversed relative to the effects on the amino acid carrier: maximal stimulation of system ASC activity by glucagon was coincident with maximal inhibition of Na^+/K^+ pump activity by this hormone. These observations suggest a more complex regulation pattern.

The inhibitory effects of glucagon on system ASC activity found in the present study have not been observed for the regulation of system A activity by this hormone in mammalian cells, where only up-regulation has been described (McGivan and Pastor-Anglada, 1994). In a previous study, Inui and Ishioka (1983) found only stimulatory effects of glucagon on the uptake of α-aminoisobutyric acid by eel (*Anguilla japonica*) liver and muscle cells, possibly because seasonality was not considered.

Most of the effects of glucagon are mediated by cyclic AMP, at least in cells from higher vertebrates (McGivan *et al.* 1981), and Ca^{2+} is involved in cyclic-AMP-independent effects of glucagon (Wakelman *et al.* 1986). Two pharmacological modifiers of signal transduction systems were used in the present study, selected because of their ability to change the activity of system A in mammalian cells (Woodlock *et al.* 1989; Goldstone *et al.* 1983; Cheeseman, 1991). They have also been implicated in the actions of glucagon.

The modifiers used inhibited the activity of the Na^+/K^+ pump, particularly in the case of the Ca^{2+} ionophore A23187. However, only the Ca^{2+} ionophore had a slight (although not significant) effect on system ASC activity. At the time that these experiments were carried out (using May animals), glucagon had nearly opposite effects on system ASC (inhibition) and Na^+/K^+ pump (stimulation) activities measured separately. Increasing cyclic AMP levels inhibited Na^+/K^+ pump activity and had no effect on Na^+-dependent amino acid uptake. This result is in agreement with the lack of effect of β-agonists on the uptake of L-alanine by trout hepatocytes (P. Canals and J. Sánchez, unpublished data), despite the presence of β-adrenoceptors in these cells (Moon *et al.* 1988).

There are two possible explanations for this situation. First, there may be a seasonally dependent change in the action of cyclic AMP on the activities measured in the present study. Second, glucagon may use more than one second messenger in trout hepatocytes. However, a glucagon-dependent increase in intracellular Ca^{2+} levels can only partially explain the observed hormonal effects for December fish (lack of effect on system ASC, inhibition of the Na^+/K^+ pump). There is no clear explanation available for the glucagon-mediated increase in the activity of the pump or inhibition of the amino acid uptake in May animals.

In summary, these results indicate that, although system ASC has a similar role in fish to that of system A in mammalian cells and although their activities are both regulated by fasting and hormones, there appear to be substantial differences between them, such as the demonstrated lack of co-regulation of the activities of the Na^+/K^+ pump and system ASC.

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