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Transport of Proline and Hydroxyproline by the Neutral Amino-acid Exchanger ASCT1

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Abstract. ASCT1 is a member of the glutamate transporter superfamily cloned from human brain and characterized as a Na^+-dependent neutral amino-acid exchanger, which displays substrate-induced chloride-channel activity and mediates concentrative transport of alanine. Initial studies in ASCT1-expressing *Xenopus laevis* oocytes showed that proline did not elicit measurable currents, in contrast to what occurred with alanine, serine or cysteine, suggesting that proline was not an ASCT1 substrate, although it induced the release of alanine from preloaded oocytes. Here, we have studied the uptake of proline and hydroxyproline by ASCT1-expressing oocytes in order to investigate the ability of ASCT1 to translocate these imino acids. The results demonstrate ASCT1-mediated proline transport that is Na^+-dependent, saturable, inhibited by the reported ASCT1 substrates as well as by hydroxyproline and can drive the imino acid against its concentration gradient. The apparent kinetic constants for the transport of alanine and the imino acids, obtained with oocytes from the same batch, showed maximal transport rate for proline and hydroxyproline to be half of that for alanine. However, $K_{0.5}$ for proline was 704 ± 86 μM, about three times higher than alanine $K_{0.5}$ (203.3 ± 36.4 μM), whereas hydroxyproline $K_{0.5}$ was 33.2 ± 4.3 μM, indicating that the hydroxylation on carbon 4 of proline strongly increases the affinity of ASCT1 for this proline derivative. In summary, the present work demonstrates for the first time the ability of ASCT1 to transport proline and hydroxyproline.

Key words: ASCT1-Exchanger — Proline — Hydroxyproline — *Xenopus laevis* oocytes

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Introduction

Amino-acid transport into mammalian cells is mediated by different transport systems with overlapping substrate specifities (Malandro & Kilberg, 1996; Palacin et al., 1998). One of these systems, termed ASC, is an ubiquitous system which mediates Na^+-dependent transport of small zwitterionic amino acids, excludes branched-chain amino acids, and was originally identified and characterized by Christensen's group (Christensen, Liang & Archer, 1967; Wheeler & Christensen, 1967; Kilberg, Handlogten & Christensen, 1981; Christensen, 1990). The ASCT1 cDNA was isolated from human motor cortex and hippocampus cDNA libraries (Arriza et al., 1993; Shafqat et al., 1993) and upon expression in *Xenopus laevis* oocytes, showed ASC system transport activity (Arriza et al., 1993). Structural homology studies indicate that ASCT1 is a member of the superfamily of Na^+-dependent transporters, which comprises anionic and zwitterionic amino-acid transporters (Palacín et al., 1998). Electrophysiological studies performed in ASCT1-expressing oocytes showed that ASCT1 is a Na^+-dependent neutral amino-acid exchanger that displays thermodynamically uncoupled substrate-gated chloride conductance (Zerangue & Kavanaugh, 1996). Previous results from our laboratory, from experiments also performed on oocytes, have demonstrated that ASCT1 mediates concentrative transport of alanine and suggest that it functions as a tertiary active transport mechanism where sodium would act as an allosteric modulator that is bidirectionally cotransported (Pinilla, Barber & Lostao, 2001a,b). In addition, studies using HeLa cells transfected with ASCT1 cDNA (Shafqat et al., 1993; Tamarappoo, McDonald & Kilberg, 1996) confirm the high affinity of ASCT1 for the transport of alanine, serine, cysteine and threonine, the insensitivity of mediated neutral amino-acid transport

with a decrease of pH from 7.5 to 6 and the ability of cysteate, glutamate and aspartate to inhibit alanine transport at acidic pH.

Electrophysiological studies in oocytes demonstrated that 1 mM proline or glutamine did not induce inward currents in contrast to alanine, serine, cysteine, threonine or valine and thus, neither proline nor glutamine were designated as ASCT1 substrates (Arriza et al., 1993; Palacin et al., 1998). However, it has been reported that a lower concentration of proline in the extracellular medium (300 μM) was able to stimulate alanine efflux from preloaded ASCT1-expressing oocytes (Zerangue & Kavanaugh, 1996). Even though proline did not induce any chloride currents, we considered it of interest to explore the ability of ASCT1 to translocate this imino acid as well as its derivative hydroxyproline.

Materials and Methods

Expression of ASCT1 in *Xenopus laevis* Oocytes

ASCT1 cDNA, kindly donated by Dr. S. Amara (Vollum Institute, Oregon Health Sciences University, Portland, Oreg.), was linearized and *in vitro* transcribed (Pinilla et al., 2001b). ASCT1 cRNA (30 ng) was injected into stage VI *X. laevis* oocytes, as previously described (Lostao et al., 1994), and were maintained at 18°C in Barth's medium with 0.05 μg/μl gentamycin sulfate for 2–3 days prior to the experiments.

Radiotracer-uptake Experiments

Uptake experiments were performed in Na^+ buffer containing (in mM): 100 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES-Tris, pH 7.5. Groups of 8–10 oocytes were incubated at room temperature in 300 μl of the buffer containing L-[^{3}H]-alanine (specific activity 70.0 Ci/mmol), L-[^{3}H]-proline (specific activity 45.0 Ci/mmol), L-[^{3}H]-hydroxyproline (specific activity 15.0 Ci/mmol) or L-[^{3}H]-glutamine (specific activity 30.0 Ci/mmol) (NEN, Boston, MA) and the indicated concentrations of non-radiolabeled amino acid. After the incubation period, oocytes were washed four times with 4 ml choline buffer (same composition as Na^+ buffer but Na^+ was replaced with choline) at 4°C, and the ^{3}H content of each oocyte was determined by liquid scintillation counting. Experiments were performed at room temperature, around 20°C.

Results are expressed as the mean ± SE (pmol/oocyte per time) of 8–10 oocytes from the same batch. Figures show a representative experiment and similar results were obtained using at least 3 different batches of oocytes. L-Alanine, L-cysteine and 4-L-hydroxyproline were from Merck (Darmstadt, Germany); L-proline and L-serine were from Sigma-Aldrich (Madrid, Spain).

The apparent affinity constant ($K_{0.5}$) and the maximal velocity for saturating amino-acid concentration (V_{max}) were obtained by fitting the uptake values (V) to the equation:

$$V = V_{max}[S]/(K_{0.5} + [S]), \quad (1)$$

using the nonlinear fitting method in Sigma Plot 4 (SPSS Inc., Chicago, IL), where [S] is the substrate concentration. Amino-acid concentrations used to determine the kinetic parameters were from 0.03 to 1 mM. The ASCT1-mediated amino-acid uptake values were calculated as the difference between the ASCT1-expressing oocytes and the non-injected (NI) oocytes' uptake values, at each amino-acid concentration. Intracellular proline concentration was estimated assuming an intracellular space distribution of water of 176 ± 14 nl, as previously described (Pinilla et al., 2001b) and 390 nl (Bröer et al., 1999).

Results

In order to determine if ASCT1 was able to recognize proline as a substrate, we initially measured proline's ability to inhibit alanine uptake. Glutamine was also tested as a negative control, since it was reported that this amino acid does not elicit any inward current or stimulate the exit of alanine from preloaded oocytes. As shown in Fig. 1*A*, both proline and glutamine at 5 mM concentration inhibited the ASCT1-mediated transport of 100 μM alanine by 57% and 33%, respectively. However, as shown in Fig. 1*B*, proline but not glutamine was transported, and the ASCT1-mediated uptake of 1 mM proline (about 50 pmol/oocyte 30 min) was 3-fold lower than that of 1 mM alanine (about 160 pmol/oocyte 30 min). Because the uptake of the amino acids by NI oocytes is similar to the water-injected oocytes' uptake, in the rest of the experiments only the NI oocytes were used to measure the endogenous uptake of the tested amino acids.

Time-course assays showed that transport of 350 μM proline mediated by ASCT1 increased linearly with time until close to 20 min (Fig. 2). From these data, a 10-min incubation period was chosen to perform the kinetic experiments.

The uptake of proline and alanine mediated by ASCT1 varied in a hyperbolic manner with their concentration from 30 μM to 1 mM (Fig. 3). These values were used to estimate the apparent kinetic constants for proline and alanine transport, which for the batch of oocytes in Fig. 3 were: $K_{0.5}$ = 672 ± 185 μM and V_{max} = 39 ± 6 pmol/oocyte/10 min for proline, and $K_{0.5}$ = 203 ± 36 μM and V_{max} = 76 ± 4 pmol/oocyte/10 min for alanine. Therefore, ASCT1 exhibited an affinity for proline three times lower than for alanine and half of the maximal transport rate for proline than for alanine. From 3 separate experiments, a $K_{0.5}$ value of 704 ± 86 μM for proline transport was calculated. Notice that the time-course assays for proline uptake were performed using a concentration of 350 μM, about half of the $K_{0.5}$ obtained for this imino acid; consequently, this apparent affinity constant could be slightly underestimated.

The uptake of 1 mM proline was abolished by the presence in the incubation medium of 5 mM serine, cysteine or alanine (Fig. 4*A*), further demonstrating the ability of ASCT1 to transport the imino acid. The transport of 300 μM alanine or proline was strongly reduced when 2 mM hydroxyproline was present in the assay medium (Fig. 4*B*), suggesting that this proline derivative is actually an ASCT1 substrate.

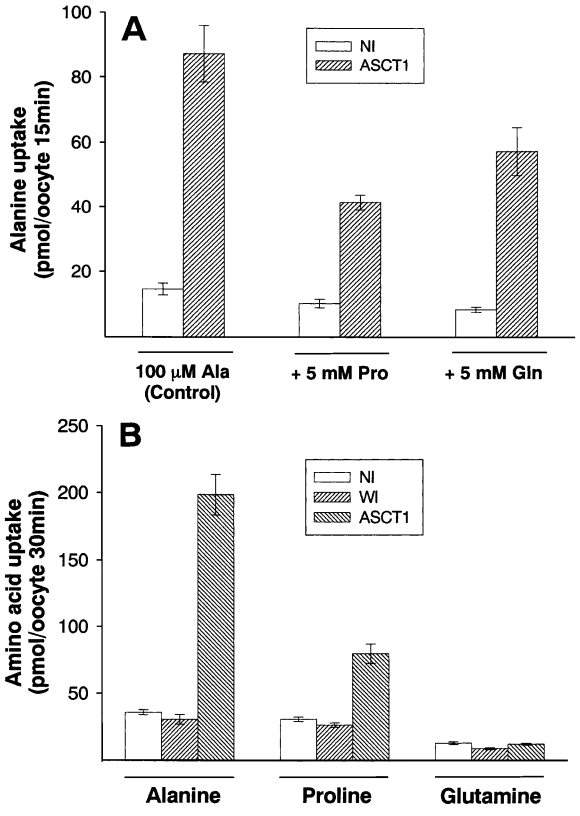

Fig. 1. Ability of ASCT1 to transport proline. (*A*) Inhibition of 100 μM alanine uptake by 5 mM proline or glutamine. (*B*) Uptake of 1 mM alanine, proline or glutamine. *NI*, non-injected oocytes. *WI*, water-injected oocytes; *ASCT1*, ASCT1-expressing oocytes.

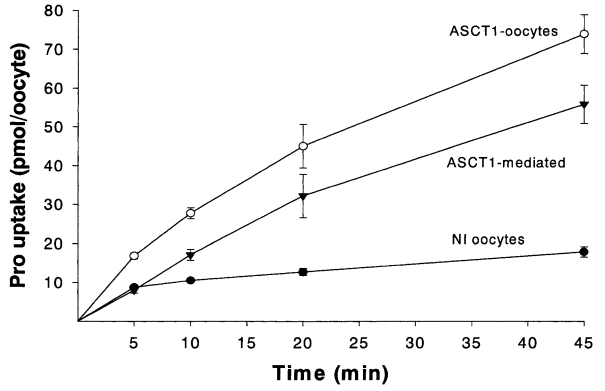

Fig. 2. Uptake of 350 μM proline: time-course analysis. The ASCT1-mediated proline uptake was calculated as the difference between the uptake values of the ASCT1-expressing oocytes and the non-injected oocytes at each time period.

This was confirmed by direct measurements of 100 μM hydroxyproline uptake by ASCT1-expressing oocytes (Fig. 4*B* inset). Moreover, the inhibition of alanine uptake by 2 mM hydroxyproline was higher (85%) than the inhibition by 2 mM proline (30%) and a similar degree of inhibition was obtained when the alanine or proline concentration was 1 mM and the hydroxyproline concentration was 5 mM (*data not shown*). These results indicate a higher affinity of ASCT1 for hydroxyproline than for proline.

Interestingly, proline uptake by NI oocytes was inhibited by cysteine, but not by serine, alanine (Fig. 4*A*) or hydroxyproline (Fig. 4*B*), and alanine transport was not inhibited by proline or hydroxyproline (Fig. 4*B*). Therefore, it seems that different endogenous transport systems mediate alanine and proline transport in stage VI *X. laevis* oocytes.

Kinetic studies such as those in Fig. 3 were performed to compare alanine and hydroxyproline ASCT1-mediated transport using oocytes from the same batch (Fig. 5). The estimated apparent kinetic constants in the experiment in Fig. 5 were: $K_{0.5}$ = 23 ± 5 μM and V_{max} 118 ± 4 pmol/oocyte/10 min for hydroxyproline, and $K_{0.5}$ = 118 ± 35 μM and V_{max} = 196 ± 17 pmol/oocyte/10 min for alanine. Therefore, ASCT1 exhibited a four times higher affinity and a 40% lower V_{max} for hydroxyproline than for alanine. From 4 separate experiments, a $K_{0.5}$ of 33.2 ± 4.3 μM for hydroxyproline transport was calculated.

Cytosolic accumulation of proline was obtained when oocytes were incubated for 1 hour with different proline concentrations. Thus, in the representative experiment shown in Fig. 6, the ASCT1-mediated uptake of 5, 30 or 300 μM proline was measured for 1 hour of incubation, 3 days after injection of the oocytes. The uptake values were 9.2, 29.7 and 210.4 pmol per oocyte, which correspond to cytosolic concentrations of 52.3, 168.8 and 1195.5 μM, calculated assuming an intracellular space distribution of water of 176 nl, which are about 10, 6 and 4-fold higher than the external proline concentration, respectively. Proline cytosolic accumulation was also obtained using 390 nl as intracellular space distribution of water (23.6, 76.2 and 539.5 μM for 5, 30 and 300 μM extracellular proline concentration, respectively). As proposed, this uphill influx of proline would be coupled to the downhill efflux of other amino-acid substrates of ASCT1 in the obligatory exchange activity of the transporter (Van Winkle 1999). In fact, high cytosolic concentrations of these amino-acid substrates of ASCT1 have been reported (Taylor & Smith 1987), which would account for the downhill efflux.

Discussion

Previous studies with ASCT1-expressing oocytes had shown that 1 mM alanine induced current in voltage-clamped oocytes, whereas 1 mM proline or glutamine did not (Arriza et al.,1993). On the other hand, heteroexchange experiments had demonstrated that 300 μM proline was able to elicit exit of alanine from

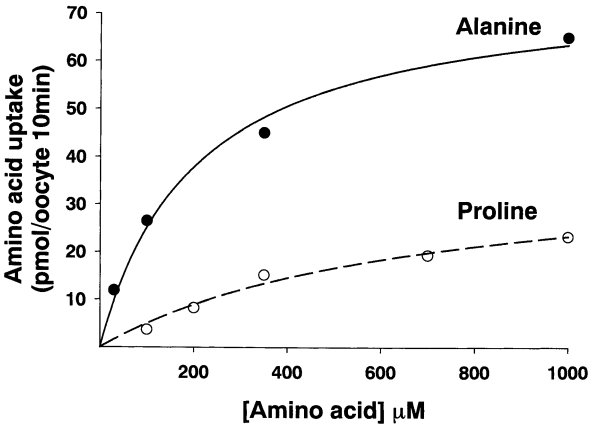

Fig. 3. ASCT1-mediated transport of proline and alanine as a function of the amino acids' concentrations (from 30 to 1000 μM). Uptake by non-injected and ASCT1-expressing oocytes was measured after 10 min incubation in oocytes from the same frog donor. The values in the figure (difference between the uptake values of the ASCT1-expressing and the non-injected oocytes) were fitted to Eq. 1 in Materials and Methods to calculate the apparent kinetic constants. For alanine transport, V_{max} = 76 ± 5 pmol/oocyte · 10 min and $K_{0.5}$ = 203 ± 36 μM and for proline V_{max} = 39 ± 6 pmol/oocyte · 10 min and $K_{0.5}$ = 672 ± 185 μM. The lines were obtained using the indicated kinetic constants.

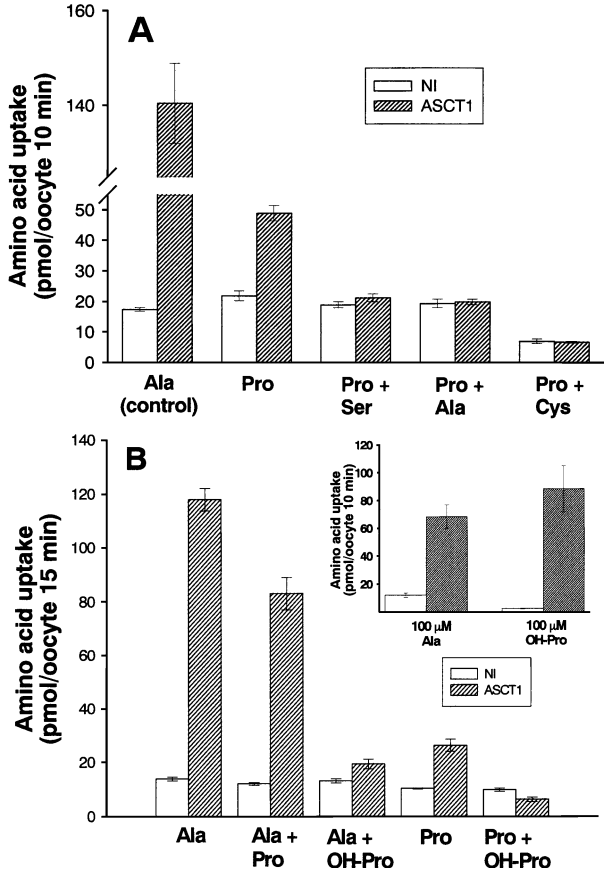

Fig. 4. Inhibition profile of proline uptake. (*A*) Inhibition of 1 mM proline uptake by 5 mM serine, alanine or cysteine. Uptake of 1 mM alanine was performed as control. (*B*) Inhibition of 300 μM alanine uptake by 2 mM proline or hydroxyproline, and of 300 μM proline uptake by 2 mM hydroxyproline. *Inset:* comparative uptake of alanine and hydroxyproline. *NI*, non-injected oocytes; *ASCT1*, ASCT1-expressing oocytes.

preloaded oocytes, which was 20% of that induced by 300 μM alanine, but glutamine did not (Zerangue & Kavanaugh, 1996). None of these studies, however, showed whether proline was actually transported by ASCT1. Here we demonstrate that ASCT1 translocates proline and hydroxyproline to the inside of the oocytes and that while glutamine is not transported, it inhibits ASCT1-mediated alanine uptake at high concentrations, suggesting that some glutamine binding to the exchanger may occur.

The ASCT1-mediated uptake of proline is always lower than that of alanine when the experiments are performed under the same conditions. This decrease can be explained not only by an increase in the affinity constant of ASCT1 for proline compared to that for alanine, but also by a reduction (around 50%) of proline V_{max}. Since a similar number of transporters is present in the plasma membrane of the oocytes, the decrease in V_{max} could be related to slower conformational changes of ASCT1 when the imino acid proline is being transported. In relation to these results, classical studies on the ASC system performed in pigeon erythrocytes demonstrated that proline is transported by this system, but interacts differently with it (Koser & Christensen, 1971). Based on those studies it was suggested that proline may hold the protein in the inward-facing conformation and therefore, slow the overall transport process (Van Winkle, 1999).

A peculiar characteristic of ASCT1 function, similar to excitatory amino-acid transporters (EAAT), is that it allows a substrate-gated uncoupled flux of chloride. Since 1 mM proline did not elicit measurable chloride currents (Arriza et al., 1993), the present data suggest that ASCT1 displays different responses concerning anion conductance depending on the transported amino acid. Also, an asymmetric influence of proline on the functionality of the ASC system has been reported (Koser & Christensen, 1971; Van Winkle, 1999): proline influences the stoichiometry of transport of other amino acids with Na^+ in opposite ways, depending upon whether they are on the inside or the outside of the cell. All these findings seem to indicate that the substrates of ASCT1 behave differently in the transport mechanism.

As it occurred with alanine (Pinilla et al., 2001a,b), an ASCT1-mediated transport of proline against its gradient is measured and in control (100 mM Na^+) medium at 5, 30 and 300 μM concentrations, which approximately correspond to proline concentration in human cerebrospinal fluid (CSF) (Kruse, Reiber & Neuhoff, 1985; Phang, Yeh & Scriver, 1995) and in plasma (Phang et al., 1995).

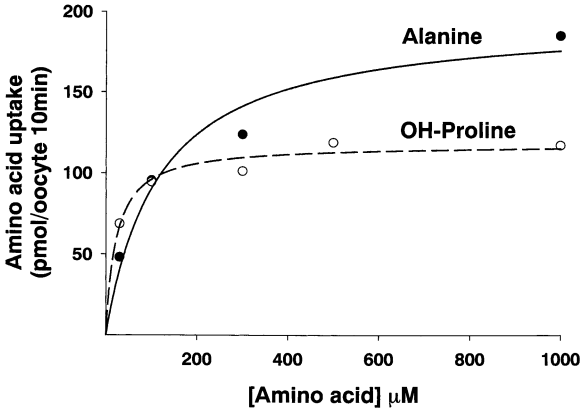

Fig. 5. ASCT1-mediated transport of hydroxyproline and alanine as a function of their concentrations (from 50 to 1000 μM). The calculated values for the apparent kinetic constants (as in Fig. 3) were: for alanine transport, V_{max} = 197 ± 17 pmol/oocyte · 10 min and $K_{0.5}$ = 118 ± 35 μM and for hydroxyproline transport, V_{max} = 118 ± 4 pmol/oocyte · 10 min and $K_{0.5}$ = 23 ± 5 μM. The lines were obtained using the indictated kinetic constants.

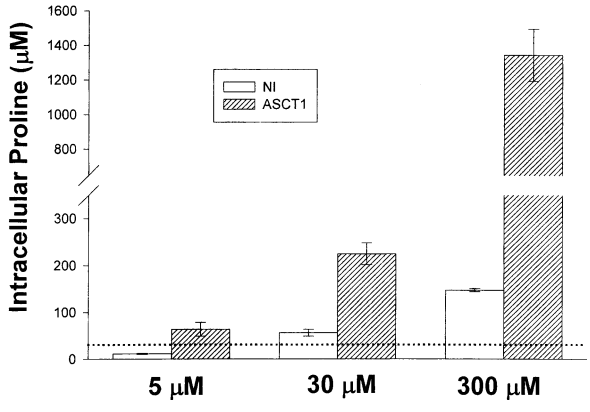

Fig. 6. Intracellular accumulation of proline into ASCT1-expressing oocytes. Oocytes, three days after injection of ASCT1 cRNA, were incubated for 1 hour with 5, 30 or 300 μM proline. Cytosolic concentration was calculated from uptake values both in ASCT1-expressing and NI oocytes by assuming a space distribution of water in the oocytes of 176 nl, as indicated in the text. The dotted line indicates 30 μM concentration. *NI*, non-injected oocytes; *ASCT1*, ASCT1-expressing oocytes.

Proline transport is inhibited by the typical substrates of ASCT1, cysteine, serine and alanine, and also by hydroxyproline. This proline derivative inhibits ASCT1-mediated transport of alanine more strongly than does proline, suggesting that it may also be an ASCT1 substrate with higher affinity for the transporter. Effectively, kinetic studies of hydroxyproline and alanine uptake performed in oocytes from the same batch show that ASCT1 exhibits higher affinity for hydroxyproline than for alanine or proline. In contrast, hydroxyproline V_{max} is lower than alanine V_{max} and similar to that for proline. These data demonstrate that the hydroxylation on C4 of proline increases the affinity of ASCT1, a property also described for the ASC system in pigeon erythrocytes and rabbit reticulocytes (Koser & Christensen, 1971; Thomas & Christensen, 1970). Also hydroxyproline transport by the ASC system measured in isolated rat hepatocytes was about six-fold greater than the rate of proline transport (Kilberg et al., 1981; Van Winkle, 1999).

Activity of the ASC system has been demonstrated in basolateral membrane of Caco-2 cells and so has expression of ASCT1 mRNA in this cell line and human small intestine (Howard et al., 2000). Also, our recent Western blot results indicate that ASCT1 protein is clearly detected in homogenates of Caco2 cells (*unpublished data*). Since hydroxyproline is one of the most abundant amino acids in collagen (Adams & Frank, 1980), it could be suggested that, after cytosolic accumulation inside the enterocytes mainly via the apical IMINO transporter (Palacín et al., 1998), ASCT1 may contribute to the intestinal absorption of dietary hydroxyproline by mediating the exit from the enterocytes to the blood across the basolateral membrane. The ubiquitous presence of ASCT1 at the plasma membrane would mediate the entry of this imino acid into the cells where it would be degraded to glyoxylate and pyruvate, serving as energy source by entering into the Krebs cycle (Phang et al., 1995).

A neuromodulatory role of proline has been shown in the mammalian central nervous system (Fremeau, Caron & Blakely, 1992), including potentiation of glutamate transmission in some synapses (Cohen & Nadler, 1997). Recently, inmunocytochemical analysis of ASCT1 expression performed in developing rat brain shows that ASCT1 is localized primarily to regions that rely on glutamate as a major neurotransmitter, both on neurons and on astrocytes (Weiss et al., 2001). Proline uptake in axon terminals seems to be mainly mediated by the brain-specific transporter PROT (Fremeau et al., 1992; Velaz-Faircloth et al., 1995). Regarding proline transport in glial cells, although it has been reported that proline uptake in isolated glioblastoma cells is mainly carried out by system A and only a minor proportion by the ASC (Zafra, Aragón & Giménez, 1994), both the high glutamine concentration in CSF (546 μM; Kruse et al., 1985) and the low total concentration of ASCT1 substrates in CSF (about 120 μM; Kruse et al., 1985) probably diminishes the effective role of system A to transport proline in favor of ASC for glial Na^+-dependent proline uptake. Therefore, we suggest that the ASCT1 exchanger may participate in maintaining the homeostasis of proline levels in the glutamatergic synapses where ASCT1 is localized.

In summary, our results demonstrate for the first time that L-proline and 4-L-hydroxyproline are transported by ASCT1 and that hydroxylation of

proline on C-4 strongly increases the affinity of ASCT1 for the imino acid.

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