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Na^+-dependent transport of anionic amino acids by preimplantation mouse blastocysts

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Negatively charged amino acids, such as aspartate and glutamate, were selected as substrates by low- and high-K_m components of mediated Na^+-dependent transport in preimplantation mouse blastocysts. These and other relatively small anionic amino acids with two carbon atoms between the negatively charged groups (or up to three carbon atoms when the groups were both carboxyl groups) interacted strongly with the low-K_m component of transport, whereas larger anionic amino acids interacted weakly or not at all. The low-K_m system was also stereoselective except in the case of aspartate. Moreover, transport was Cl^--dependent and slower at pH values outside the range 5.6–7.4. L-Aspartate, D-aspartate and L-glutamate each interacted strongly with the low-K_m component of transport with K_m values for transport nearly equal to their K_i values for inhibition of transport of one of the other amino acids. By these criteria, the low-K_m component of transport of anionic amino acids in blastocysts appears to be the same as the familiar system X_{AG}^- that is present in other types of mammalian cells. In contrast, the high-K_m component of transport in blastocysts preferred L-aspartate to L-glutamate, whereas the reverse is true for fibroblasts. Therefore, transport of anionic amino acids in blastocysts may occur via at least one process that has not been described in other types of cells. Roughly half of mediated glutamate and aspartate transport in blastocysts may occur via the high-K_m component of transport at the concentrations of these amino acids that may be present in uterine secretions.

Introduction

Several developmentally regulated amino acid transport systems have been delineated in preimplantation mouse conceptuses [1–17]. Although anionic amino acids do not interact strongly with these known systems, two of the systems receive some cationic and zwitterionic amino acids equally well as substrates [1,3]. Thus, the latter two systems, provisionally designated $B^{0,+}$ and $b^{0,+}$, do not fit well into the conventional classification scheme for amino acid transport systems. This scheme contains three broad classes of amino acid transport systems based on whether the systems prefer anionic, cationic or zwitterionic substrates [18,19]. In addition to systems $B^{0,+}$ and $b^{0,+}$, preimplantation conceptuses contain systems B and b^+ [4,9–11,13]. At least two forms of system b^+ have been identified, and they have been provisionally designated systems b_1^+ and b_2^+ [11,13]. Systems B and b^+ prefer zwitterionic and cationic substrates, respectively, so they fit into conventional classes. Nevertheless, their substrate selectivities are different from other known members of these classes [9,11,13]. Preimplantation conceptuses also contain forms of the better-known systems Gly, L and T which select for particular groups of zwitterionic amino acids in conceptuses [3,4,6,10,14] as well as in other tissues [5,19]. Since more than half of the known transport systems for cationic and zwitterionic amino acids in conceptuses were described for the first time in these cells, we wanted to determine whether the characteristics of transport of anionic amino acids are also novel in conceptuses.

Both Na^+-dependent and Na^+-independent systems for anionic amino acids have been well characterized in mammalian cells. The Na^+-dependent system X_{AG}^- selects as substrates anionic amino acids with three or fewer carbon atoms between the negatively charged groups, whereas the Na^+-independent system x_C^- selects substrates with three or more such carbons [20–

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22]. System x_C^- also transports L-cystine. Na^+-dependent L-aspartate uptake cannot be detected in preimplantation conceptuses until the 8-cell stage of development, and this transport activity becomes fully expressed in mouse blastocysts [7,13]. Little else is known, however, about aspartate transport in blastocysts. Moreover, mediated Na^+-independent L-glutamate transport becomes nearly undetectable in blastocysts [13]. Therefore, we characterized Na^+-dependent transport of anionic amino acids in preimplantation mouse blastocysts, and we compared the characteristics of this transport to the characteristics of system X_{AG}^-.

Materials and Methods

Conceptuses were obtained as described previously (see, for example, Refs. 1, 6, 11) from sexually mature 8–11-week-old ICR mice (Harlan Sprague Dawley, Inc.). Mice were induced to ovulate and mate utilizing gonadotropins [23]. Eight-cell conceptuses were obtained from oviducts about 66 h after administration of human chorionic gonadotropin, and blastocysts were obtained from uteri about 94 h after administration of this hormone. Conceptuses were washed and stored for less than 6 h in Brinster's medium [24] in a humidified atmosphere of 5% CO_2 in air at 37 °C (pH 7.4). Transport was not observed to change in blastocysts during incubation for 6 h *in vitro*, although transport was faster in 8-cell conceptuses after they had become compacted (i.e., after individual cells had flattened against each other and were no longer spherical, see Results).

Conceptuses were incubated with a ^3H-labeled form of L-aspartate, D-aspartate or L-glutamate (Amersham) and one of several concentrations of various nonradioactive amino acids in phosphate-buffered media at 37 °C (pH 7.1; Refs. 1, 3, 4, 11 and see figure legends). In some cases, the pH was adjusted and buffered with Tris or ϵ-amino-*N*-caproate to values between 5.1 and 8.6 as described in the appropriate figure legends. Amino acid transport was frequently studied at substrate concentrations near 1 μM for reasons discussed more completely elsewhere [8,9] and because some of the K_m values for transport of anionic amino acids by blastocysts were near or below 10 μM (see Results). Incubations were short enough to estimate initial velocities of uptake (i.e., 5 min or less), and no saturable binding of amino acids to plasma membranes has been detected in preimplantation conceptuses (Refs. 8, 9, 11 and data not shown). The extracellular concentrations of amino acids seemed to remain virtually constant during the course of experiments as discussed previously [3,4]. All nonradioactive amino acids and other chemicals and hormones were purchased from Sigma Chemical Company or Behring Diagnostics except for D-cysteate which was a gift from Dr. Halvor N. Christensen [21]. After incubation with radiolabeled substrate, conceptuses were processed to determine how much of the substrate they had taken up [1]. Where appropriate, data were assessed statistically utilizing analysis of variance [25,26].

Results

Na^+-dependent L-aspartate uptake has not been detected in 2-cell conceptuses [7,13], and it was more than 10-fold faster in blastocysts than in either compacted or noncompacted 8-cell conceptuses (data not shown). For these reasons, Na^+-dependent transport of anionic amino acids was characterized thoroughly only in blastocysts. Moreover, in most of the experiments performed with 8-cell conceptuses, compacted and noncompacted conceptuses were not studied separately. Nevertheless, L-aspartate uptake was about 6-fold faster in compacted than in noncompacted 8-cell conceptuses (data not shown), thus reflecting the rapid increase in Na^+-dependent aspartate transport activity that occurs during this developmental period [7,13].

L-Aspartate transport by blastocysts was linearly proportional to the extracellular Na^+ concentration up to about 100 mM Na^+ (Fig. 1). Li^+ appeared to substitute somewhat for the Na^+ in stimulating aspartate uptake, and aspartate transport also appeared to be stimulated by Cl^-. Aspartate transport was not affected in a statistically significant manner by differences in pH in the range 5.6–7.4, although it was slower outside this range (Fig. 2). Na^+-dependent [^3H]aspartate transport was inhibited almost completely by anionic amino acids, but not by cationic or zwitterionic ones (Table I). Incomplete inhibition by L-glutamine (Table I) was probably due to contamination of this commercial preparation by a small amount of L-glutamate (Sigma Chemical Co.). Uptake of L-[^3H]aspartate was inhibited strongly by L-glutamate, L-cysteate, D-aspartate, L-cysteinesulfinate and nonradioactive L-aspartate and weakly by a D-cysteate preparation, but it was not inhibited by up to 1.0 mM of the larger anionic amino acids, L-aminoadipate and L-aminopimelate, or by D-glutamate, L-homocysteate and L-cystine (Fig. 3). N-Methylation of strong inhibitors greatly reduced or eliminated their ability to inhibit L-aspartate uptake (data not shown).

Components of Na^+-dependent L-aspartate uptake with low (≈ 8.4 μM) and high (≈ 460 μM) K_m values were detected in preimplantation blastocysts (Fig. 4). The high-K_m component of L-aspartate transport did not appear to be completely inhibited even by 40 mM L-glutamate, whereas D-aspartate inhibited mediated L-aspartate uptake virtually completely (Fig. 5). Moreover, a Hofstee plot for L-glutamate transport over a range of concentrations similar to the range used to study L-aspartate uptake (Fig. 4) revealed two compo-

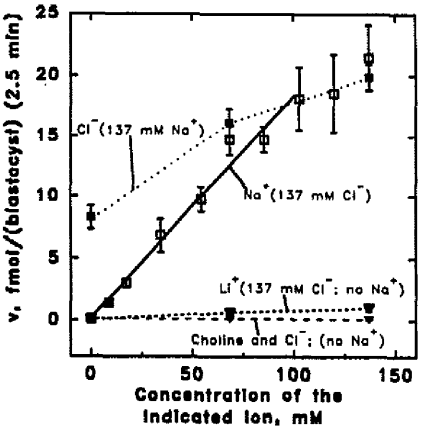

Fig. 1. Effect of various ions on L-aspartate uptake by preimplantation mouse blastocysts. Blastocysts were incubated with 1.5 μM L-[^{3}H]aspartate (27 Ci/mmol) and the indicated concentration of each ion for 2.5 or 5.0 min in medium buffered with 10 mM KH_2PO_4 adjusted to pH 7.1 with KOH. NaCl and LiCl were replaced with choline Cl to vary the concentrations of Na^+ or Li^+, and NaCl was replaced with Na acetate to vary the concentration of Cl^-. The concentrations of choline and Cl^- were varied simultaneously by replacing choline Cl with an equal osmolar amount of trimethylamine-*N*-oxide, a nonionic osmolite (e.g., Ref. 8). The mean uptake ±S.E. was calculated from 3–11 replicate determinations (approximately four blastocysts per determination) obtained in three or four independent experiments (when the S.E. is not shown it was within the symbol). Uptake was related linearly to the Na^+ concentration up to about 103 mM Na^+ (correlation coefficient 0.99), and it appeared to be stimulated significantly by both Cl^- and Li^+ ($P < 0.01$). I^- could not substitute for Cl^- in stimulating transport (data not shown).

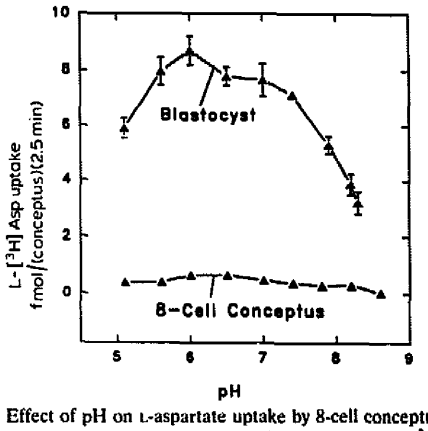

Fig. 2. Effect of pH on L-aspartate uptake by 8-cell conceptuses and blastocysts. Conceptuses were incubated with 0.74 μM L-[^{3}H]aspartate (27 Ci/mmol) for 2.5 min at 37 °C in medium composed of 10 mM ϵ-amino-*N*-caproate, 10 mM KH_2PO_4, 10 mM Tris-HCl, 127 mM NaCl and 1.0 mg/ml albumin ±20 mM L-leucine. The pH was adjusted to the indicated values with HCl or KOH. The results were the same regardless of whether 20 mM L-leucine was present, so these data were combined. The mean uptake ±S.E. was calculated from 5–11 replicate determinations (approximately five conceptuses per determination) obtained in three or four independent experiments. Uptake by blastocysts was slower outside the range of pH values 5.6–7.4 ($P < 0.01$).

nents of L-glutamate transport in blastocysts (data not shown). The K_m value for the high-K_m component was, however, about nine times higher for L-glutamate ($\approx$ 4100 μM) than for L-aspartate ($\approx$ 460 μM). At relatively low substrate concentrations, where transport was primarily via the low-K_m component (Table II), L-glutamate strongly and competitively inhibited L-aspartate uptake and vice versa. Similarly, D-aspartate strongly and competitively inhibited L-aspartate uptake via the low-K_m component of their transport (Table II).

Discussion

Since about 97% of aspartate uptake by blastocysts can be attributed to the low-K_m component of transport at substrate concentrations near 1 μM (Fig. 4), the characteristics of aspartate transport depicted in Figs. 1–3 and Table I are primarily for the low-K_m component. These characteristics are similar to the characteristics of system X_{AG}^- in other types of mammalian cells [20]. A similar transport process has also been detected in both the apical and basal membranes of the human placental trophoblast [27,28]. In blastocysts, anionic L-amino acids with three or fewer carbon atoms between the anionic groups interact strongly with the low-K_m component of transport (Fig. 3). Although L-homocysteate has only three such carbons, its

TABLE I

Effect of various amino acids on L-aspartate uptake by blastocysts

Blastocysts were incubated with 1.5 μM L-[^{3}H]aspartate (27 Ci/mmol) and the indicated nonradioactive amino acid for 5 min at 37 °C in phosphate-buffered NaCl or this medium in which Li^+ or choline was substituted for Na^+. The mean uptake ±S.E. was calculated from 4–8 replicate determinations (approximately five blastocysts per determination) obtained in 3–5 independent experiments. Uptake was significantly slower in groups marked with double asterisks ($P < 0.01$)

Inhibitor (10 mM)	L-Aspartate uptake (% of control)
None	100.0 ± 17.3
L-Aspartate	0.6 ± 0.1 **
L-Glutamate	0.6 ± 0.1 **
D-Asparate	0.4 ± 0.0 **
L-Glutamine	55.1 ± 9.7
L-Alanine	78.7 ± 11.6
L-Leucine	82.9 ± 16.1
L-Lysine	97.0 ± 8.8
L-Tryptophan	85.0 ± 12.6
Taurine	90.7 ± 17.5
No Na^+ (Li^+)	3.1 ± 0.3 **
No Na^+ (choline)	0.3 ± 0.1 **

γ-sulfonate group apparently does not fit into the substrate receptor site as well as the γ-carboxyl group of L-glutamate. Larger anionic amino acids and D-glutamate also do not interact strongly with the low-K_m system in blastocysts (Fig. 3). The stereoselectivity for L- over D-cysteate may be greater than indicated by the data in Fig. 3 since the D-cysteate preparation may have been contaminated with L-cysteate [20]. In contrast, D-aspartate interacts with the low-K_m component of transport in blastocysts almost as strongly as L-aspartate does. Apparently, the β-carboxyl group of D-aspartate is recognized for transport at the subsite of the transporter that normally recognizes the α-carboxyl group of anionic L-amino acids, although such is not the case for the β-sulfonate group of D-cysteate [20]. The near equivalence of the K_m values for L-aspartate, L-glutamate and D-aspartate transport to their K_i values for competitive inhibition of transport of one of the other amino acids via the low-K_m component (Table II) supports the conclusion that they all are transported by system X^-_{AG} in blastocysts. Greater L-aspartate uptake at pH seven than at pH near five also

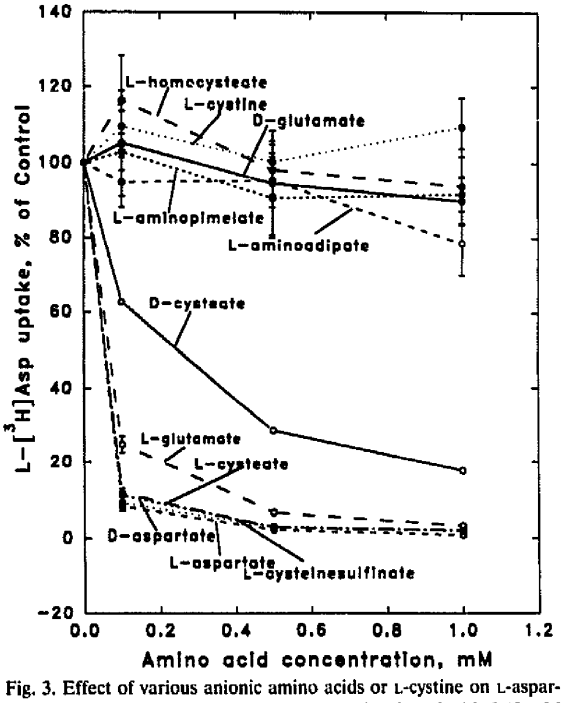

Fig. 3. Effect of various anionic amino acids or L-cystine on L-aspartate uptake by blastocysts. Blastocysts were incubated with 0.62 μM L-[^{3}H]aspartate (32 Ci/mmol), 4.0 μM nonradioactive L-aspartate and the indicated concentration of other nonradioactive amino acids for 5 min at 37 °C in phosphate-buffered NaCl. The mean uptake ± S.E. was calculated from four replicate determinations (approximately five blastocysts per determination) obtained in two or three independent experiments (one experiment and two determinations (S.E. not calculated) for L-cysteinesulfinate, L-cysteate and D-cysteate).

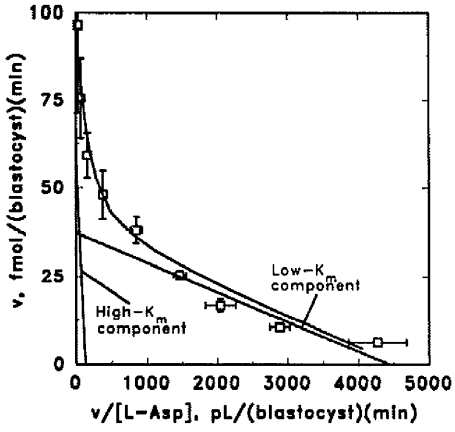

Fig. 4. Hofstee plot of Na^+-dependent L-aspartate uptake by blastocysts. Blastocysts were incubated with the indicated concentrations of L-aspartate for 2.5 min at 37 °C in medium composed of 10 mM KH_2PO_4, 137 mM NaCl, 1.0 mg/ml albumin and 10 mM L-tryptophan, L-arginine, L-leucine and glycine (pH adjusted to 7.1 with KOH). In some cases the Na^+ was replaced with choline, and Na^+-independent uptake was subtracted from total uptake in the presence of Na^+ to produce the data presented. The Na^+ salt of aspartate was used to vary the concentration of this amino acid in the presence of Na^+, so the concentration of NaCl was reduced in order to maintain a constant Na^+ concentration. This small variation in the Cl^- concentration (i.e., 134–137 mM) is unlikely to have had a significant effect on the values of the kinetic parameters (e.g., Fig. 1). The mean uptake ± S.E. was calculated from six replicate determinations (approximately four blastocysts per determination) obtained in three independent experiments. The data were assessed by the method of Spears et al. [36] assuming two Na^+-dependent transport systems were active. The values of the kinetic parameters derived in this way were $K_m \approx 8.4$ μM, $V_{max} \approx 37$ fmol blastocyst^{-1} min^{-1} and $K_m \approx 460$ μM, $V_{max} \approx 60$ fmol blastocyst^{-1} min^{-1} for the low- and high-K_m components, respectively. The curved line represents the combination of the two straight lines.

supports the conclusion that system X^-_{AG} transports L-aspartate in blastocysts (Fig. 2). The latter result also helps to rule out the possibility that transport of anionic amino acids in blastocysts is mainly via a system that normally transports zwitterionic amino acids but which transports anionic substrates when the system is protonated (see, for example, Ref. 19).

It also is unlikely that the high-K_m component of Na^+-dependent L-aspartate transport in blastocysts can be attributed to weak interaction of aspartate with a system for cationic or zwitterionic amino acid transport. This high-K_m component was detected in the presence of 10 mM L-tryptophan, L-arginine, L-leucine and glycine (Fig. 4) which together inhibit all known systems for transport of cationic and zwitterionic α-amino acids in preimplantation conceptuses [1,3,4,6,9–11]. A similar Hofstee plot (not shown) was also obtained for D-aspartate transport, and D-aspartate completely inhibited mediated L-aspartate uptake in blastocysts (Fig. 5). Therefore, both L- and D-aspartate ap-

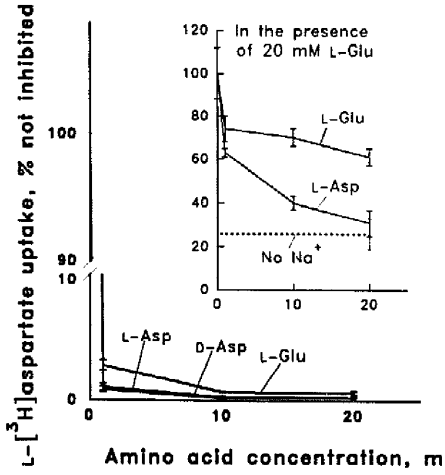

Fig. 5. Interaction of L-glutamate with the high-K_m component of L-aspartate uptake in blastocysts is weaker than interaction of aspartate with the high-K_m component. Blastocysts were incubated with 1.5 or 3.7 (inset) μM L-[^{3}H]aspartate (27 Ci/mmol) and the indicated concentration of a nonradioactive amino acid for 5 min at 37°C in phosphate-buffered NaCl. Experiments depicted in the inset were performed while 20 mM L-glutamate was already present (i.e., uptake was via a component of transport that was relatively resistant to inhibition by L-glutamate). Uptake in phosphate-buffered choline Cl is indicated by the horizontal line in the inset. The mean uptake ± S.E. was calculated from 4–12 replicate determinations (2–5 blastocysts per determination) obtained in 2–4 independent experiments. It can be calculated from the values of the kinetic parameters in Table II that greater than three times more mediated L-[^{3}H]aspartate uptake occurs in the presence of 20 mM L-glutamate than would be anticipated if uptake were only via the low-K_m component of transport. Therefore, the transport depicted in the inset was mainly via the high-K_m component of transport. For the high-K_m component (inset), inhibition by nonradioactive L-aspartate was significantly greater than by L-glutamate ($P < 0.01$).

pear to be taken up by the high-K_m, Na^+-dependent component of anionic amino acid transport in blastocysts. In contrast, 40 mM L-glutamate did not fully inhibit mediated L-aspartate uptake via the high-K_m component in blastocysts (Fig. 5), and the K_m value for L-glutamate transport via the high-K_m component was nine times higher than it was for L-aspartate (see Results). Therefore, glutamate does not appear to be transported as well as aspartate by the high-K_m component. Moreover, L-aspartate completely inhibited all mediated Na^+-dependent L-glutamate uptake (data not shown). These results are in contrast to results with fibroblasts where only one component of Na^+-dependent L-aspartate transport was detected but high- and low-K_m components of Na^+-dependent L-glutamate uptake were present [20]. Therefore, the high K_m component of Na^+-dependent anionic amino acid transport in preimplantation blastocysts may differ from high-K_m components in other cells.

Interestingly, the K_m value for glutamate uptake via the high-K_m component of transport exceeds the K_m value for aspartate transport by roughly the same amount (i.e., about 9-fold) as the glutamate concentration exceeds the aspartate concentration in rabbit uterine secretions [29]. If the concentrations of aspartate and glutamate in the uterine secretions of mice are similar to the concentrations in rabbits, then the high-K_m system would take up nearly equal amounts of glutamate and aspartate in mouse blastocysts *in situ*, whereas the low-K_m system would function mainly (e.g., about 85%) for glutamate uptake. At the concentrations of glutamate and aspartate apparently present in uterine secretions on day 3 post coitum (i.e., about 3.5 and 0.27 mM, respectively [29]), and at K_i values for glutamate that are about equal to its K_m values for uptake via each system, approximately 70% of mediated aspartate uptake would be via the high-K_m system (Fig. 4). Moreover, the concentrations of glutamate and aspartate in uterine secretions are near or below their K_m values for the high-K_m system, and the

TABLE II

Values of kinetic parameters for L-aspartate, D-aspartate or L-glutamate transport and competitive inhibition of transport via the low-K_m component of transport in blastocysts

The values of K_m and V_{max} were determined as described in the legend of Fig. 4 but at substrate concentrations of 251 μM or lower, so only the low-K_m component of transport was discernible. K_m values are shown when an amino acid is listed as its own inhibitor. When a S.E. value is shown, the mean value ± S.E. was calculated from three replicate determinations. The K_i values were calculated from the K_m values for Na^+-dependent substrate transport in the presence (K_{app}) or absence (K_m) of inhibitor (i.e., K_i = [inhibitor]/($(K_{app}/K_m) - 1$)). At the substrate and inhibitor concentrations utilized, 63% or more of the uptake (usually more than 82% of the uptake) of both substrates and inhibitors was via the low-K_m component of transport as determined from the values of the kinetic parameters for the amino acids calculated as described in the legend of Fig. 4. It was not possible to isolate completely transport via the low-K_m component because each of the three amino acids interacted with both the low- and the high-K_m components, although with widely different strengths (see Results). For these reasons, it can be calculated that the values in the table may be as much as 50% higher than their actual values. Nevertheless, the values represent principally transport via the low-K_m component and are consistent with the interpretation that all three amino acids are transported by the same low-K_m transport process. Similar K_i values (not shown) can be calculated utilizing data in Fig. 3, a substrate concentration of 4.6 μM and the formula, $K_i = (v_i/(v - v_i)) \cdot (K_m \cdot [I]/([S] + K_m))$, where v = substrate influx, v_i = substrate influx in the presence of inhibitor, [I] = inhibitor concentration and [S] = substrate concentration (Refs. 2–4). At a substrate concentration of 4.6 μM, it can be calculated from data in Fig. 4 that about 96% of mediated aspartate transport is via the low-K_m component

Substrate	V_{max} (fmol blastocyst^{-1} min^{-1})	K_i or K_m (μM)		
		L-aspartate	L-glutamate	D-aspartate
L-Aspartate	40 ± 4	9.0 ± 1.6	21	22
L-Glutamate	45	7.3	10	–
D-Aspartate	46	–	–	13

concentrations change as much as 5-fold during preimplantation development [29]. Such changes in the concentrations of glutamate and aspartate could lead to similar changes in the rates of their uptake via the high-K_m system in blastocysts.

Highly concentrative, Na^+-dependent transport of anionic amino acids in blastocysts but not in unfertilized eggs [13] may help account for the higher levels of aspartate and glutamate in blastocysts than in eggs [30]. Conversion of some glutamate to glutamine could also help account for the higher levels of glutamine in blastocysts [30]. Na^+-dependent transport of anionic amino acids in liver appears to be located preferentially in perivenous hepatocytes [31,32]. The glutamate taken up by perivenous hepatocytes may be converted to glutamine in order to help reduce the NH_4^+ concentration in blood to levels acceptable for the systemic circulation [31–33]. Greater glutamate uptake could be needed by preimplantation conceptuses at the blastocyst stage of development because more NH_4^+ is generated at this stage due to more rapid protein turnover (see for example, Ref. 34). We are currently studying amino acid metabolism in mouse conceptuses at various stages of preimplantation development. These studies should help determine not only whether glutamate is taken up and converted to glutamine in blastocysts, but also whether glutamine is taken up and converted to glutamate in 1-cell conceptuses. Release of glutamate from conceptuses could occur more easily at the 1-cell stage where the activity of a system similar to the Na^+-independent system x_C^- is about 10-times higher than at later stages of preimplantation development [13]. Release of glutamine-derived glutamate via system x_C^- in 1-cell conceptuses could contribute to the mechanism by which glutamine helps these conceptuses remain developmentally competent [13,35]. Regardless of their possible physiological functions, at least one of the mediated Na^+-dependent components of L-aspartate transport in blastocysts may not have been detected previously in other mammalian cells (see above). Therefore, as for transport of cationic and zwitterionic amino acids, some of the transport of anionic amino acids in preimplantation conceptuses may occur via a process that is novel to these cells.

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