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Communication

ASCT-1 Is a Neutral Amino Acid Exchanger with Chloride Channel Activity*

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The ubiquitous transport activity known as system ASC is characterized by a preference for small neutral amino acids including alanine, serine, and cysteine. ASCT-1 and ASCT-2, recently cloned transporters exhibiting system ASC-like selectivity, are members of a major amino acid transporter family that includes a number of glutamate transporters. Here we show that ASCT1 functions as an electroneutral exchanger that mediates negligible net amino acid flux. The electrical currents previously shown to be associated with ASCT1-mediated transport result from activation of a thermodynamically uncoupled chloride conductance with permeation properties similar to those described for the glutamate transporter subfamily. Like glutamate transporters, ASCT1 activity requires extracellular Na^+. However, unlike glutamate transporters, which mediate net flux and complete a transport cycle by countertransport of K^+, ASCT-1 mediates only homo- and heteroexchange of amino acids and is insensitive to K^+. The properties of ASCT-1 suggest that it may function to equilibrate different pools of neutral amino acids and provide a mechanism to link amino acid concentration gradients.

The cellular transport of amino acids is mediated by multiple membrane proteins with diverse specificity and localization (for recent review see Ref. 1). Uptake of neutral amino acids is mediated by several transporters that display varying degrees of overlap. One such transport activity was first identified by Christensen and colleagues (2), who named it system ASC because of its preference for alanine, serine, and cysteine. This activity represents a major component of neutral amino acid transport in many tissues and cell lines (2–8). A cloned transporter exhibiting these properties, termed ASCT1 by Arriza *et al.* (9) and SATT by Shafqat *et al.* (10), was isolated from human brain and expressed in *Xenopus* oocytes. Recently a related neutral amino acid transporter was isolated from an adipocyte cell line (11) and from mouse testis (12). The predicted amino acid sequence of this transporter, termed ASCT2 (12), exhibits 57% identity with human ASCT1. Notably, ASCT1 and ASCT2 share approximately 40% sequence identity with the cloned glutamate transporters (13), indicating the existence of a large amino acid transporter gene family.

The fact that ASCT1 and ASCT2 are members of a gene family including glutamate transporters provides some insights into the structure and function of these molecules. System ASC-mediated uptake of glutamate, which occurs at low pH values, appears to reflect an alteration of the selectivity of the ASC transporter (7, 14). A similar phenomenon was described with both the cloned ASCT1 (15) and ASCT2 (12) transporters. Conversely, the canonical system ASC substrate L-cysteine was recently shown to be transported in a pH-independent fashion by the neuronal glutamate transporter EAAT3/EAAC1 (16). The neuronal glutamate transporter is likely to catalyze translocation of a molecule of cysteine or glutamate as a proton-thiolate or proton-carboxylate ion pair, respectively (16, 17). The overlapping substrate specificity of EAAT3 and ASCT-1 may reflect conserved elements of structure important for determining substrate selectivity in members of the neutral and acidic amino acid transporter subfamilies.

A surprising property found in all cloned glutamate transporters examined to date is a chloride conductance that is activated during transport (18, 19). The magnitude and direction of the chloride flux do not influence amino acid transport, and it has therefore been termed a thermodynamically uncoupled flux (18). In this work it is shown that, like glutamate transporters, ASCT1 mediates an analogous substrate-activated chloride conductance that is thermodynamically uncoupled from amino acid flux. However, a striking difference is observed between glutamate transporters and ASCT1 in terms of transport thermodynamics and cation flux coupling. Unlike glutamate transporters, which mediate a transport cycle that is completed by countertransport of potassium and results in net amino acid flux, ASCT1-mediated transport is shown to involve a symmetrical potassium-independent electroneutral exchange of neutral amino acids and sodium, such that the current activated during transport is carried only by chloride ions.

MATERIALS AND METHODS

Expression of ASCT1—Stage V–VI oocytes were obtained by partial ovariectomy of *Xenopus laevis* under tricaine (1 g/liter) anesthesia. Oocytes were defolliculated by collagenase treatment (1 mg/ml, Boehringer Mannheim) in calcium-free Ringer's solution. Capped mRNA was transcribed from linearized plasmids containing the coding region of ASCT1 (9), approximately 50 ng of RNA was injected into oocytes, and expression was assayed 3–5 days later by two-microelectrode voltage clamp recording and radiotracer flux measurements.

Voltage Clamp Recording—During two-microelectrode voltage clamp recording (18), oocytes were perfused with frog Ringer's solution containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5. The chloride equilibrium potential was determined by measuring the reversal potential of endogenous calcium-dependent chloride channels following activation with A23187 (18). In experiments where $[Cl^-]$ was varied or replaced, equimolar anion substitution was employed, and recordings were made with the bath grounded via a 3 M KCl/agar bridge connected to a 3 M KCl reservoir containing a Ag/AgCl electrode.

Radiotracer Flux Measurements—Uptake of radiolabeled amino acid was measured under voltage clamp as described (20). Briefly, 300 μM [^{3}H]L-alanine (2 μCi/ml, Amersham Corp.) was superfused onto oocytes clamped at indicated membrane potentials for 90 s, followed by rapid washout and scintillation counting. Heteroexchange release of [^{3}H]L-alanine was measured after labeling the internal neutral amino acid pool by incubating cells with carrier-free [^{3}H]L-alanine (58 Ci/mmol; 10 μCi/ml) for 1 h followed by transfer into wells containing 500 μl of Ringer's solution for 60 s in the presence of various test amino acids or control Ringer's solution. The specific radioactivity of the internal pool
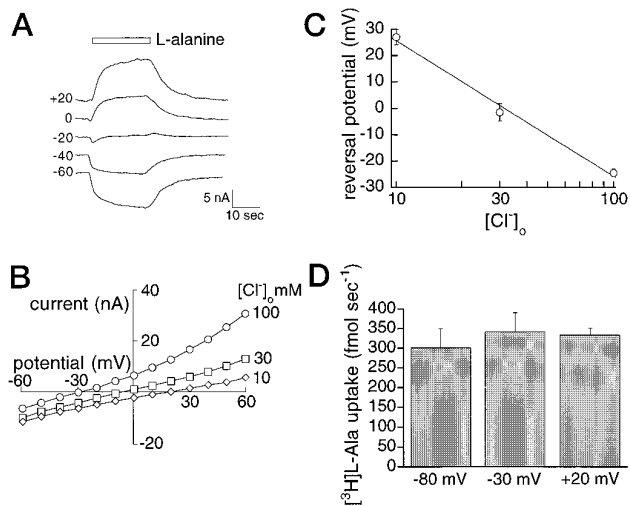
FIG. 1. **Currents during alanine flux mediated by ASCT1 result from activation of a thermodynamically uncoupled chloride conductance.** *A*, application of 300 μM L-alanine for a duration indicated by the *bar* to a representative oocyte voltage-clamped at indicated membrane potentials results in activation of a reversible electrical current. *B*, voltage dependence of currents in representative cells activated by L-alanine application in the presence of 10, 30, and 100 mM $[Cl^-]_o$. *C*, the alanine-induced current reversal potential is shifted 53 mV/log change in $[Cl^-]_o$; *points* represent mean $\pm$ S.E., $n = 5$. *D*, uptake of $[^3H]$L-Ala measured in voltage-clamped oocytes demonstrates that uptake is independent of membrane potential. *Bars* represent specific mean $\pm$ S.E.; $n = 3-4$.

was determined from the quantity of free intracellular ASCT1 substrate as described previously (16). Briefly, oocytes expressing ASCT1 were homogenized in ice-cold Ringer's solution (five separate pools of 10 oocytes/200 μl). Responses of voltage-clamped cells expressing ASCT1 to application of diluted homogenates were compared with the responses elicited by standard L-alanine solutions. No responses to application of cytosol were detected in oocytes not injected with ASCT1 RNA. The intracellular pool of ASCT1 substrate was equivalent to 1.2 $\pm$ 0.1 nmol alanine/oocyte ($n = 5$). Experiments were repeated with oocytes isolated from at least three different frogs. Efflux and influx magnitudes are expressed as ASCT1-specific values obtained after subtraction of values in matched control uninjected oocytes, which represented <5% of specific fluxes.

RESULTS

Thermodynamically Uncoupled Electrical Currents Associated with ASCT1 Transport—Application of L-alanine to voltage-clamped oocytes expressing ASCT1 resulted in activation of an inward membrane current at negative potentials that reversed and became outward at positive potentials (Fig. 1*A*). The reversal potential of the alanine-activated current (-21.1 ± 2.0 mV; $n = 4$) was not significantly different from the chloride equilibrium potential in these cells (-23.0 ± 1.3 mV; $n = 4$). Similar to glutamate transporter currents (18, 19), changing the concentration of chloride ion in the bath solution had a dramatic effect on the current. Varying $[Cl^-]_o$ shifted the reversal potential 52-mV/10-fold change in $[Cl^-]_o$, close to the prediction of the Nernst equation for a chloride-selective conductance (Fig. 1, *B* and *C*). Furthermore, substitution of Cl^- by gluconate in the Ringer's solution bathing the cell abolished the outward current at positive potentials up to +80 mV (not shown), suggesting that Cl^- ions carried the major part of the current activated by L-alanine superfusion.

Although the current activated by alanine was voltage-dependent and reversed near the Cl^- equilibrium potential, flux of $[^3H]$L-alanine was dependent neither on the membrane potential nor the direction of the current (Fig. 1*D*). These data indicate that chloride ion flux is thermodynamically uncoupled from flux of alanine, similar to the phenomenon noted with
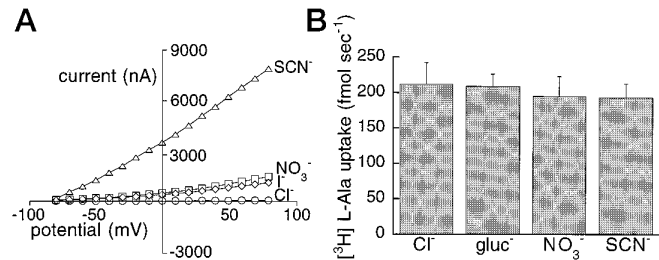

FIG. 2. **Selectivity sequence of ASCT1 anion conductance.** *A*, voltage dependence of currents induced by 300 μM alanine application to a representative oocyte expressing ASCT1 in the presence of Ringer's solution with various test anions substituted for Cl^-. A chaotropic conductance sequence was seen ($SCN^- > NO_3^{-Leu} > I^- > Cl^-$) *B*, ASCT-1-specific flux of $[^3H]$L-Ala measured at -30 mV ($n = 4$) is independent of external anion present or magnitude of anion flux.

cloned human glutamate transporters (18, 19). The chloride conductance activated during glutamate transport exhibits a chaotropic selectivity sequence $SCN^- > NO_3^- > I^- > Cl^-$ (18, 21). This sequence was also displayed by ASCT1 (Fig. 2*A*). Further supporting the notion that the movement of anions is uncoupled thermodynamically from movement of amino acid, $[^3H]$L-alanine uptake was not affected by the magnitude of the anion flux or the identity of the external anion (Fig. 2*B*). Superfusion of the canonical system ASC substrates L-serine and L-cysteine (9) similarly activated this anion current. With external SCN^- replacement, the outward current at 0 mV was activated with EC_{50} values of 38 ± 4, 53 ± 5, and 62 ± 22 μM, respectively, with L-alanine, L-serine, and L-cysteine ($n = 3-4$). The relative conductance of SCN^- ion through the channel activated by 1 mM alanine at 0 mV was approximately 60-fold greater than Cl^- ion. While this relative conductance is similar to that of the glutamate transporter EAAT1, the relative conductance of SCN^- through the calcium-dependent Cl^- channel endogenous to the oocyte is only 4–5.[1] This conductance difference and the lack of effect of typical Cl^- channel blockers on the conductance suggests that the anion conductance is associated with the transporter rather than an endogenous channel (18).

ASCT1 Mediates Sodium-dependent and Potassium-independent Electroneutral Exchange—The measured flux of $[^3H]$L-alanine, which was approximately 300 fmol s^{-1} in the experiment shown in Fig. 1, should result in a membrane current $\geq$29 nA if uptake involved stoichiometric translocation of at least one net positive charge during a transport cycle. However, the major current during transport of alanine results from an uncoupled chloride conductance, and at the chloride equilibrium potential no net current flows despite a large alanine flux (Fig. 1). These results suggest that alanine flux is electroneutral. This is also consistent with the voltage independence of $[^3H]$L-alanine flux (Fig. 1*D*).

One possible mechanism that could account for electroneutral flux would be a stoichiometric co- and countertransport of ions resulting in a zero net charge transfer during a transport cycle. For example, sodium is cotransported and potassium is countertransported by the structurally related glutamate transporters (22–24). Countertransport of a number of K^+ ions equal to the number of Na^+ ions cotransported with an alanine zwitterion would result in zero net charge transfer. However, varying $[K^+]_o$ under voltage clamp had no effect on the uptake of 300 μM $[^3H]$L-alanine. Uptake was unchanged by increasing $[K^+]_o$ from 0 to 50 mM (212 ± 30 fmol s^{-1} *versus* 219 ± 24 fmol s^{-1}, respectively; -30 mV; $n = 4$). Furthermore, raising $[K^+]_o$ in the absence of external alanine did not result in an increase

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[1] J. I. Wadiche, E. M. Klamo, and M. P. Kavanaugh, unpublished observations.
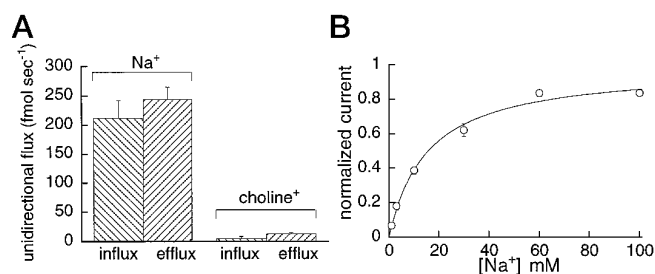
FIG. 3. **ASCT1 mediates one-for-one Na$^+$-dependent exchange.** *A*: *left*, the magnitude of unidirectional efflux induced by application of 300 μM unlabeled L-alanine to oocytes loaded with [^{3}H]L-Ala was not significantly different from unidirectional influx of 300 μM [^{3}H]L-Ala in other oocytes from the same batch. *Bars* represent mean ± S.E.; $n = 5$. Specific activity of the total intracellular substrate pool was determined as described under "Materials and Methods." *Right*, removal of sodium abolished both efflux and influx. *B*, [Na$^+$] dependence of currents induced by alanine transport. *Points* represent mean ± S.E.; $n = 4$. The *curve* represents least squares fit of data to $I/I_{max} = [Na^+]/[Na^+] + K_{0.5}$ with $K_{0.5} = 16$ mM.

in efflux of [^{3}H]L-alanine from preloaded cells (15.6 ± 2.3 fmol and 14.4 ± 1.7 fmol s^{-1} in the absence and presence of 50 mM [K$^+$]$_o$, respectively; $n = 5$). Neither did elevating external K$^+$ result in activation of a detectable anion current when SCN$^-$ was present in the bathing solution (data not shown). These results contrast with the properties of the glutamate transporters, in which efflux is stimulated by the presence of K$^+$ outside as a consequence of thermodynamically coupled K$^+$ countertransport (17, 22, 24). ASCT1-mediated currents and uptake of alanine were found to require the presence of Na^+_o, as reported previously (9, 10). Similar to ASCT2, which exhibited a hyperbolic dependence on [Na$^+$]$_o$ (12), alanine-induced currents mediated by ASCT1 were also well described by a Hill equation with $n = 1$ (Fig. 3B).

Another possible mechanism that could account for electroneutral uptake of [^{3}H]L-alanine involves electroneutral exchange of extracellular and intracellular amino acid pools. In order to investigate this possibility, unidirectional efflux of intracellular [^{3}H]L-alanine was measured during superfusion with unlabeled alanine and compared with the magnitude of the unidirectional influx. Superfusion of unlabeled alanine onto oocytes preloaded with [^{3}H]L-alanine induced a large release of radioactivity (Fig. 3A). Unidirectional influx, measured by superfusion of [^{3}H]L-alanine, was not significantly different from unidirectional efflux (Fig. 3A), indicating that an amount of amino acid was released equal to the amount of alanine taken up into the cell. Heteroexchange was examined by measuring release of intracellular [^{3}H]L-alanine induced by various extracellular amino acids (Fig. 4). Addition of 300 μM external alanine, serine, threonine, and cysteine resulted in release of large amounts of internal [^{3}H]L-alanine. Addition of equal concentrations of asparagine, proline, valine, and leucine resulted in smaller amounts of [^{3}H]L-alanine release relative to that induced by alanine.

DISCUSSION

The results of this study show that the ASCT1 transporter functions predominantly as a mediator of neutral amino acid exchange rather than of net uptake. In addition, transport is associated with a chloride channel activity that is thermodynamically uncoupled from amino acid transport. These conclusions are supported by several lines of evidence. The flux of [^{3}H]L-alanine was not measurably coupled to the membrane electric field (Fig. 1C) despite the apparent cotransport of Na$^+$ and lack of K$^+$ countertransport, which would be expected to result in electrogenic unidirectional flux. Related to this observation, unidirectional flux measured with [^{3}H]L-alanine oc-
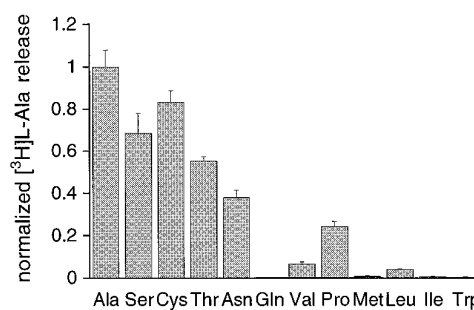

FIG. 4. **Amino acid heteroexchange mediated by ASCT1.** The ASCT1-specific release of radioactivity from cells preloaded with [^{3}H]L-Ala induced by addition of 300 μM indicated amino acid to the external Ringer's solution was normalized to the release induced by 300 μM L-alanine. *Bars* represent mean ± S.E.; $n = 4$–6.

curred at a rate higher than would be predicted from the measured electrical currents if a stoichiometric excess of positive charge were translocated with alanine. The current that did flow during transport was due to a thermodynamically uncoupled Cl$^-$ flux (Fig. 1B). As with chloride flux associated with glutamate transport (18), the direction of chloride ion flow did not affect ASCT-1-mediated alanine flux (Fig. 1). Finally, measurement of unidirectional radiotracer fluxes in the same group of cells showed that influx and efflux rates were equal, indicating an obligate exchange mechanism similar to a model of system ASC function proposed by Christensen (25).

ASCT1- and ASCT2-mediated fluxes are sodium-dependent (9, 10, 12). Utsunomiya-Tate *et al.* (12) found a Hill coefficient of 1 in examining the [Na$^+$] dependence of ASCT2, consistent with interaction of one Na$^+$ ion with the transporter. The same result was obtained with ASCT1 (Fig. 3). Because the reversal potential of the alanine transport current closely followed the Nernst equilibrium reversal potential for Cl$^-$ ion (Fig. 1C), an insignificant flux of net charge due to movement of Na$^+$ and amino acid is likely to occur. It is possible therefore that a symmetrical exchange of one Na$^+$ ion and a zwitterionic amino acid occurs during a transport cycle. This stoichiometry differs from glutamate transport, which involves cotransport of multiple Na$^+$ ions with glutamate (17, 26, 27). In further contrast to glutamate transporters, ASCT1-mediated flux is unaffected by K$^+$. This result is in accord with an alternating access transport model in which K$^+$ is countertransported in a distinct kinetic step to complete a transport cycle (22); this step would be circumvented in an exchange process. Although it is associated with an uncoupled chloride current, amino acid exchange mediated by ASCT1 is electroneutral in the strict thermodynamic sense. A similar conclusion about the electroneutrality of system ASC was reached in studies of human fibroblasts (8) and red blood cells (28), although in contrast to the present results, an increased rate of uptake at hyperpolarized potentials was reported (8).

An obligatory exchange mode of operation for ASCT1 has several implications for its physiological roles. This transport mode effectively catalyzes diffusional amino acid mixing without expending energy from the sodium electrochemical gradient. ASCT1 does not mediate net amino acid flux, but because it can catalyze heteroexchange it may effectively induce net transport of a particular substrate when a transmembrane concentration gradient for another one of its substrates exists. Intercellular flows of amino acids could be mediated in this manner. For example, abundant expression of ASC in red blood cells (6, 29) might serve as an efficient mechanism to facilitate interorgan amino acid fluxes (30, 31). The ASCT1 transporter would also in principle be well suited to play a role in vectorial transepithelial flux if it were paired with an appropriately

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segregated concentrative transporter with overlapping substrate specificity.

The present results also have structure-function implications for both the acidic and neutral amino acid transporters. ASCT1 flux, which involves K^+-independent one-for-one amino acid exchange, stands in marked contrast to flux mediated by the structurally related glutamate transporters, which countertransport K^+ (17, 22–24) and mediate a much smaller amount of amino acid exchange (15). Recently, a conserved amino acid residue (Glu-404) in the glutamate transporter Glt-1 was shown to be involved in binding K^+, and conservative mutations in this residue resulted in conversion of glutamate transport from net flux to obligate exchange (32). This residue is found within a highly conserved region of the glutamate transporters involved in binding the non-transported competitive antagonist kainate (33), and the residue corresponding to Glu-404 in Glt1 is Gln-386 in ASCT1, suggesting a possible structural basis for the marked difference in transport mechanisms between these transporter subtypes. Further studies on the role of this residue and the region surrounding it will be of interest.

The mechanism by which activation of an uncoupled anion conductance occurs during transport (18, 19, 21, 34) is at present unknown. One hypothetical mechanism involves an "open channel state" arising from the generation of an anion-selective site at some stage during permeation of the amino acid through the transporter (18). The present results tend to confirm the association of an anion-conducting state with an amino acid-bound state rather than another state such as in the potassium countertransporting limb (22) of the glutamate transport cycle. The conservation of the anion selectivity sequence in both ASCT1 and the glutamate transporters ($SCN^- > NO_3^- > I^- > Cl^-$) (18, 21) also suggests that key features of the permeation pathway are likely to be conserved in these molecules despite differences in amino acid selectivity and kinetic mechanism.

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