EPITHELIAL TRANSPORT

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Steady-state kinetic characterization of the mouse B⁰AT1 sodium-dependent neutral amino acid transporter

Abstract The members of the neurotransmitter transporter family SLC6A exhibit a high degree of structural homology; however differences arise in many aspects of their transport mechanisms. In this study we report that mouse B⁰AT1 (mouse Slc6a19) mediates the electrogenic transport of a broad range of neutral amino acids but not of the chemically similar substrates transported by other SLC6A family members. Co-transport of L-Leu and Na^+ generates a saturable, reversible, inward current with Michaelis-Menten kinetics (Hill coefficient ∼1) yielding a $K_{0.5}$ for L-Leu of 1.16 mM and for Na^+ of 16 mM at a holding potential of −50 mV. Changing the membrane voltage influences both substrate binding and substrate translocation. Li^+ can substitute partially for Na^+ in the generation of L-Leu-evoked inward currents, whereas both Cl^- and H^+ concentrations influence its magnitude. The simultaneous measurement of charge translocation and L-Leu uptake in the same cell indicates that B⁰AT1 transports one Na^+ per neutral amino acid. This appears to be accomplished by an ordered, simultaneous mechanism, with the amino acid binding prior to the Na^+, followed by the simultaneous translocation of both co-substrates across the plasma membrane. From this kinetic analysis, we conclude that the relatively constant $[Na^+]$ along the renal proximal tubule both drives the uptake of neutral amino acids via B⁰AT1 thermodynamically and ensures that, upon binding, these are translocated efficiently into the cell.

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Introduction

Approximately 30 years ago an up-hill, Na^+-dependent transport system for neutral amino acids, later called B or B⁰, was identified and characterized in brush-border membrane vesicles (BBMV) of proximal kidney tubule and intestine [7, 9, 26]. This Na^+-dependent, broad-range uptake system for neutral L-amino acids was thought to be uniquely characteristic of epithelial systems [19] and to mediate the high-capacity, low-affinity transport of L-Phe, L-Ala, L-Leu and L-Gln in a Na^+-dependent, Cl^--independent manner [17]. Furthermore, a familial condition with urinary loss and decreased intestinal absorption of neutral amino acids named Hartnup disorder [23, 25] was proposed to be due to a defect in the system B⁰ transporter.

Recently, an orphan transporter belonging to the family of Na^+- and Cl^--dependent neurotransmitter transporters (SCL6) has been identified as a B⁰-type transporter and the gene product renamed B⁰AT1 [3]. Expression of mouse B⁰AT1 in *Xenopus laevis* oocytes induces Na^+-dependent, Cl^--independent transport of neutral L-amino acids with an apparent affinity in the low millimolar range that is analogous to previously characterized endogenous epithelial B⁰ systems [13, 21, 31]. In addition, as described for system B⁰, the mouse B⁰AT1 protein has been shown by immunofluorescence to be localized to kidney and small intestine brush border membranes ([12] and E. Romeo, unpublished observation).

The human B⁰AT1 gene orthologue has been localized by homozygosity mapping in Hartnup sibships to chromosome 5p15, and the *SLC6A19* mRNA shown to be expressed in kidneys and small intestine [12, 18, 24]. Furthermore, sequencing has verified the presence

of homozygote or compound heterozygote mutations in the *SLC6A19* gene of a large fraction of the tested Hartnup disorder cases, including the original Hartnup family [12, 24]. Taken together, the *SLC6A19* gene product B^0AT1 appears to be a major transporter of neutral amino acids in the small intestine and kidney.

In this study we report the detailed functional characterization of the neutral L-amino acid-induced current and its correlation with amino acid uptake in *Xenopus laevis* oocytes expressing mouse B^0AT1. As mentioned, system B^0 transport has been described previously in a wide variety of preparations including BBMV [7, 13, 16, 30], microperfused kidney proximal tubules [21] and cultured epithelial cells ([6, 11, 19] and Z. Ristic, S. Camargo, unpublished observations). Unfortunately, due to the confounding presence of multiple transport systems, the data from the various cell and tissue preparations have not always been in agreement. Furthermore, the specific functional characteristics of the *SLC6A19* gene product cannot be inferred from the behaviour of related SLC6 family members as they are too diverse. The SLC6A family members can be classified into subfamilies or clusters according to their phylogenetic relationships and their function as neurotransmitter [GABA (GAT), norepinephrine (NET), dopamine (DAT), serotonin (SERT)], osmolyte [taurine (TauT) and betaine (BGT1)], metabolite [creatine (CT1)], neutral amino acid (PROT, GlyT, SIT1), cationic and neutral amino acid (ATB^{0+}) and orphan transporters (for review see [4]). Although structurally related, these SLC6A family members vary widely not only in substrate selectivity, but also display differences at the level of their transport mechanism as indicated by differences in the type of currents mediated by the transporter, in the kinetic parameters, ion and pH dependence, stoichiometry and mode of translocation of substrates.

Materials and methods

Isolation and subcloning of mouse B^0AT1 cDNA

Total mouse kidney RNA was isolated with the RNeasy Mini Kit according to the manufacturer's protocol (Qiagen, Hombrechtikon, Switzerland). Total RNA (1 µg) was reverse transcribed (oligoDT primers) using the TaqMan Reverse Transcription Kit (Applied Biosystem, Roche, Penzberg, Germany). B^0AT1 cDNA was amplified by PCR using a proof-reading polymerase (Pfu, Promega, Madison, Wisc., USA) for 40 cycles (45 s 94°C, 1 min 55°C, 6 min 72°C). The primers for PCR were as described in [3]. The amplified PCR fragment was subcloned into a pBlueScript modified *Xenopus* expression vector (KSM) containing both the 5′- and 3′-untranslated regions (UTRs) of the β-globin gene. For expression in *Xenopus* oocytes the mouse B^0AT1-KSM plasmid was linearized with Xba 1 (Promega) and used as template for RNA synthesis from the T3 promoter (mMESSAGE mMACHINE, Ambion, Austin, Tex., USA).

Mouse B^0AT1 expression in *Xenopus* oocytes

Dissection of *Xenopus* ovaries, collection and preparation has been detailed elsewhere [15]. Defoliated stage V and VI oocytes were injected with 40–60 ng RNA and incubated for 3–12 days at 16°C in ND96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 5 mM HEPES, pH 7.4 adjusted with 1 M TRIS, and 50 mg/l tetracycline). Non-injected oocytes were used as controls.

L-Amino acid uptake in *Xenopus* oocytes

Uptake experiments were performed 3–7 days after injection. The uptake solution contained: 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$ and 10 mM HEPES, pH 7.4 adjusted with TRIS. For each determination, groups of 5–10 oocytes were washed 3 times with uptake solution and then pre-incubated for 2 min at 22°C before addition of the appropriate uptake solution containing 0.1 or 1 mM of the indicated L-amino acid (0.2 µCi/ml of [^{3}H]L-amino acids). Uptake was stopped after 5 min of incubation at 22°C by washing 5 times with 3 ml ice-cold buffer. Individual oocytes were solublized in 2% SDS and the radioactivity determined by liquid scintillation counting. The mean radioactivity associated with non-injected control oocytes was subtracted from the mean radioactivity in expressing oocytes to obtain B^0AT1-mediated uptake (expressed as picomole min^{-1} per oocyte).

Electrophysiology: two-electrode voltage clamp

Kinetic characterization

The two-electrode voltage clamp technique was used for the recording of whole-cell currents from *Xenopus* oocytes. Microelectrodes (resistance ≤7 MΩ) were filled with 3 M KCl. Recordings were performed at room temperature 3–12 days after injection with B^0AT1 cRNA using a commercial two-electrode voltage-clamp (Oocytes Clamp OC725C, Warner Instruments, Hamden, Conn., USA) connected to data acquisition hardware (Digidata 11322A, Axon Instruments, Union City, Calif., USA). A computer running pClamp8 software (Axon instruments) was used to control the clamp, the valves switching the solutions and to record currents. Recordings were carried out as previously described [8]. Two basic protocols were used for generating steady-state recordings:

Continuous current recordings

Briefly, oocytes were tested at a membrane holding potential (V_h) of −50 mV, recorded data were low-pass filtered at 10 Hz and digitized at 20 Hz.

Current/voltage (I/V) recordings

Oocytes were clamped (V_h -50 mV) and step changes in V_h were applied (-120 to $+20$ mV in 20-mV increments). At each V_h four 80-ms pulses were averaged. Each substrate concentration tested was preceded by a control recording in the absence of substrate. Test solutions were always washed out with substrate-free solution for at least 2 min. Current records were low-pass filtered at 500 Hz and digitized at 20 μs/point.

For both protocols the standard perfusate buffer contained (mM): 100 NaCl, 2 KCl, 1.8 CaCl$_2$, 1 MgCl$_2$ and 10 HEPES, adjusted to pH 7.4 with 1 M TRIS base. Substrates were added to this solution at the indicated concentrations. For all studies the cells were first equilibrated with the indicated [Na$^+$] without substrate before exposure to the perfusate containing L-Leu. To study the Na$^+$-dependence of L-Leu-induced currents, Na$^+$ was replaced by equimolar N-methyl-D-glucamine (NMDG). For the ion-dependence studies, Na$^+$ was replaced by NMDG, or Li$^+$, and gluconate (Glc) was substituted for Cl$^-$ in the standard perfusate buffer composition as described above (i.e. salt, Mg^{2+}, Ca^{2+}, K$^+$ and HEPES buffer, pH 7.4). For experiments testing the pH-dependence of L-Leu-induced currents, the pH 5.5 and 6.5 perfusates were buffered with MES-TRIS base; the pH 8.0 perfusate was buffered with TRIS base-HEPES.

The measured substrate-induced steady-state current (I_s) is the difference between the maximal value recorded during a 30 s substrate super-fusion and the holding current in the absence of substrate. The amplitude of I_s varied depending on the time of measurement after cRNA injection and on the batch of oocytes. To control for variation in transporter expression the I_s data (V_h -50 mV) were normalized (I_{norm}) to the current at 10 mM L-Leu, 100 mM Na$^+$ (or as otherwise described in the text). To check for variability of the response during the experiment the current at 10 mM L-Leu, 100 mM Na$^+$ was measured at the beginning and at the end of each experiment. In all cases less than 10% of changes in response were observed.

Simultaneous voltage-clamp and [³H] L-Leu uptake

The steady-state currents from the continuous current protocol were measured in combination with the simultaneous measurement of radioactive tracer accumulation in individual oocytes as previously described [14]. Briefly, each cell was placed in a small perfusion chamber (volume 40 μl) and superfused continuously using a peristaltic pump at ~90 μl/min with the standard Na$^+$ perfusate buffer until the baseline stabilized (V_h -50 mV). L-Leu (2 mM, 0.2 μCi/ml [³H]L-Leu) was then added for 5–20 min, after which the L-Leu was washed out until the current returned to baseline. Cells were immediately removed from the chamber, washed in cold Na$^+$ uptake solution and

solublized in 2% SDS for scintillation counting. The L-Leu-induced current was measured as the difference between baseline current and the total current in the presence of L-Leu. Net charge transfer into the cells was obtained by integrating the L-Leu-induced current numerically. Charge was converted to a molar equivalent using Faraday's constant. The net positive charge (Q) transported across the plasma membrane was correlated with the corresponding L-Leu uptake ([³H]L-Leu content) for each oocyte.

Data analysis

Pooled data are shown as means $\pm$ SEM (n) where n represents the total number of cells tested. If error bars are not visible in the graphs, they are smaller than the symbols. Differences between sample means were tested for significance using Student's t-test for paired samples. $P < 0.05$ was considered significant. Experimental protocols were repeated at least twice. All calculations (linear as well as non-linear regression analysis) were performed using appropriate software (Prism v. 4.0, GraphPad, San Diego, Calif., USA).

Responses with respect to a variable substrate S were quantified as peak induced current (I_s) and fitted to a modified form of the Hill equation:

$$I_s = I_{max}[S]^H/([S]^H + (K_{0.5})^H) \tag{1}$$

where S is the substrate concentration, I_{max} the extrapolated maximum current, $K_{0.5}$ the concentration of substrate S giving a half-maximal response (i.e. the apparent affinity constant) and H the Hill coefficient ($H = 1$ describes the Michaelis-Menten equation).

Results

Uptake rates of L-Leu and L-Phe in B⁰AT1-expressing Xenopus oocytes are higher than for Gly

In an initial series of experiments we observed that reproducible B⁰AT1 neutral amino acid uptake measurements (at least twofold higher than uptakes in non-injected control oocytes) required the injection of large amounts of cRNA (40–60 ng) and expression for 3–7 days. Table 1 shows the results from radioactive tracer influx assays performed with the neutral amino acid substrates, L-Leu, L-Phe and Gly (0.1 and 1 mM for 5 min) as an initial characterization of the transport properties of B⁰AT1. During the assay there were no significant differences in specific uptake rates (total B⁰AT1-non-injected) for L-Leu and L-Phe, whereas the uptake rate for 1 mM Gly was 2 times lower. The absence of measurable glycine uptake at 0.1 mM suggested that Gly is transported with a lower apparent affinity than L-Leu and L-Phe.

Table 1 Net uptake rates for B^0AT1 transport of the neutral amino acid substrates, L-Leucine, L-phenylalanine and glycine. Data for the determination of uptake rates are pooled from three independent experiments as described in Materials and methods. Means $\pm$ SEM, $n = 16$–20

Amino acid	Concentration (mM)	Uptake (pmol min^{-1} per oocyte)
L-Leucine	0.1	1.56 ± 0.38
	1	4.28 ± 0.73
L-Phenylalanine	0.1	0.68 ± 0.09
	1	3.45 ± 0.42
Glycine	0.1	-0.04 ± 0.02
	1	2.23 ± 0.76

B^0AT1-mediated currents are evoked by neutral L-amino acids but not neurotransmitters, metabolites or osmolytes

To investigate the electrogenic transport properties of B^0AT1 we used the two-electrode voltage clamp on *Xenopus* oocytes. Figure 1a shows that perfusion of B^0AT1 expressing oocytes, clamped at -50 mV, with increasing concentrations of the neutral L-amino acid substrate L-Leu elicited saturable and reversible inward currents. These currents were dependent on both transporter expression and on the presence of Na^+ (data not shown). Under non-voltage clamp conditions, 10 mM L-Leu superfusion shifted the membrane potential resulting in a mean depolarization of 31 ± 5.8 mV ($n = 6$) (data not shown). No inward current or depolarization was observed for control non-injected control oocytes when superfused with L-Leu. Thus L-Leu evokes a net inward current, most probably due to the inward movement of positive charges via the mouse B^0AT1 transporter.

Since B^0AT1 belongs to the family of Na^+/Cl^--dependent neurotransmitter transporters and has been shown to transport a broad range of amino acid substrates [3], we determined whether it transports substrates of other SLC6 family members, specifically GABA, L-dopamine, L-DOPA, creatine, betaine and taurine. Addition of 10 mM L-Leu to oocytes clamped at -50 mV resulted in mean inward currents of 61.5 ± 9.6 nA ($n = 17$). None of the other tested SLC6 family substrates evoked currents in B^0AT1-expressing oocytes (Fig. 1b).

We then sought to determine the amino acid side-chain specificity of the B^0AT1 electrogenic response. Figure 1c shows that B^0AT1 responded to a broad range of amino acid substrates. Voltage-clamped B^0AT1-expressing oocytes (V_h -50 mV) were exposed to either 1 or 10 mM L-amino acids in 100 mM Na^+ (except L-Glu and L-Lys, which were tested at 1 mM only, and L-Tyr which was not tested) and the substrate-induced (I_s) current recorded. For each amino acid, the currents were normalized (I_{norm}) to 10 mM L-Leu-evoked currents and are shown ordered according to magnitude. With the exception of L-Arg, L-Lys and the acidic amino acids, all the amino acids tested induced currents in B^0AT1-expressing oocytes. The largest currents were observed for L-Leu, L-Ile, L-Met and

L-Cys (statistically indistinguishable). Comparing the ratio of the current measured at 1 and 10 mM substrate gives a qualitative measure of the transport affinity. The smallest difference between the 1 and 10 mM responses (the highest apparent affinity) was observed for the substrates that also showed the largest currents at 10 mM, namely L-Leu, L-Ile, L-Met, and L-Cys. The lowest apparent affinities were observed for L-Pro, L-Thr, L-His, Gly and L-Trp. B^0AT1 thus favours non-polar, large, bulky, branched or sulphur-containing amino acids over amino acids with short, unbranched or uncharged polar side chains. One exception to this generalization is the reduced ability of the large, aromatic, non-polar amino acid L-Trp to evoke currents (29% of L-Leu).

Kinetics of B^0AT1 substrate-induced currents are voltage dependent

The L-Leu concentration-dependence of the B^0AT1-mediated current was determined in the presence of 100 mM Na^+ for voltage-clamped oocytes (V_h -50 mV). To control for varying transporter expression levels, measured currents were normalized to the current induced by 10 mM L-Leu. Fitting the Hill equation to the data yielded a Hill coefficient of 0.51 ± 0.10, suggesting a single binding site for L-Leu. For further curve fitting we therefore assumed a Hill coefficient of 1 (Michaelis-Menten equation). Based on this the $K_{0.5}$ for L-Leu was calculated to be 1.16 ± 0.14 mM ($n = 17$) (Fig. 2a).

We next examined the voltage dependence of the L-Leu induced currents at 100 mM Na^+ for V_h between -120 and $+20$ mV (Fig. 2b,c and Table 2). At the various L-Leu concentrations the L-Leu-induced currents increased with more negative membrane potentials. Table 2 reports the values derived for I_{max} and $K_{0.5}$ vs. membrane potential showing that with hyperpolarization the I_{max} increased ~fourfold while the $K_{0.5}$ decreased ~sixfold from ~3 to 0.5 mM.

Figure 3a shows the response evoked by L-Leu (10 mM) with Na^+ as the variable substrate. Fitting the Michaelis-Menten equation to the data yielded a $K_{0.5}$ of 16.06 ± 1.93 mM ($n = 21$). The I/V relation for the Na^+ concentration dependence at 10 mM L-Leu is shown in Fig. 3b,c and Table 3 for V_h from -120 to $+20$ mV. For each $[Na^+]$ tested, NMDG-Cl was used to adjust the total ionic strength of the perfusate. Table 3 reports the values derived for I_{max} and $K_{0.5}$ showing that as for the L-Leu-evoked response, membrane hyperpolarization resulted in both a ~threefold increase in the Na^+-dependent currents and a ~fourfold decrease in $K_{0.5}$ from ~25 to 6 mM.

For both substrates I/V relations became steeper in a concentration- and voltage-dependent manner (Figs. 2b,c and 3b,c; Tables 2 and 3). The derived I_{max} is approximated by the currents at the maximum substrate concentrations shown for both L-Leu and Na^+. Further, for both substrates, the slope of the change in I_{max} vs. V_h had virtually the same shape. The slopes were curvilinear
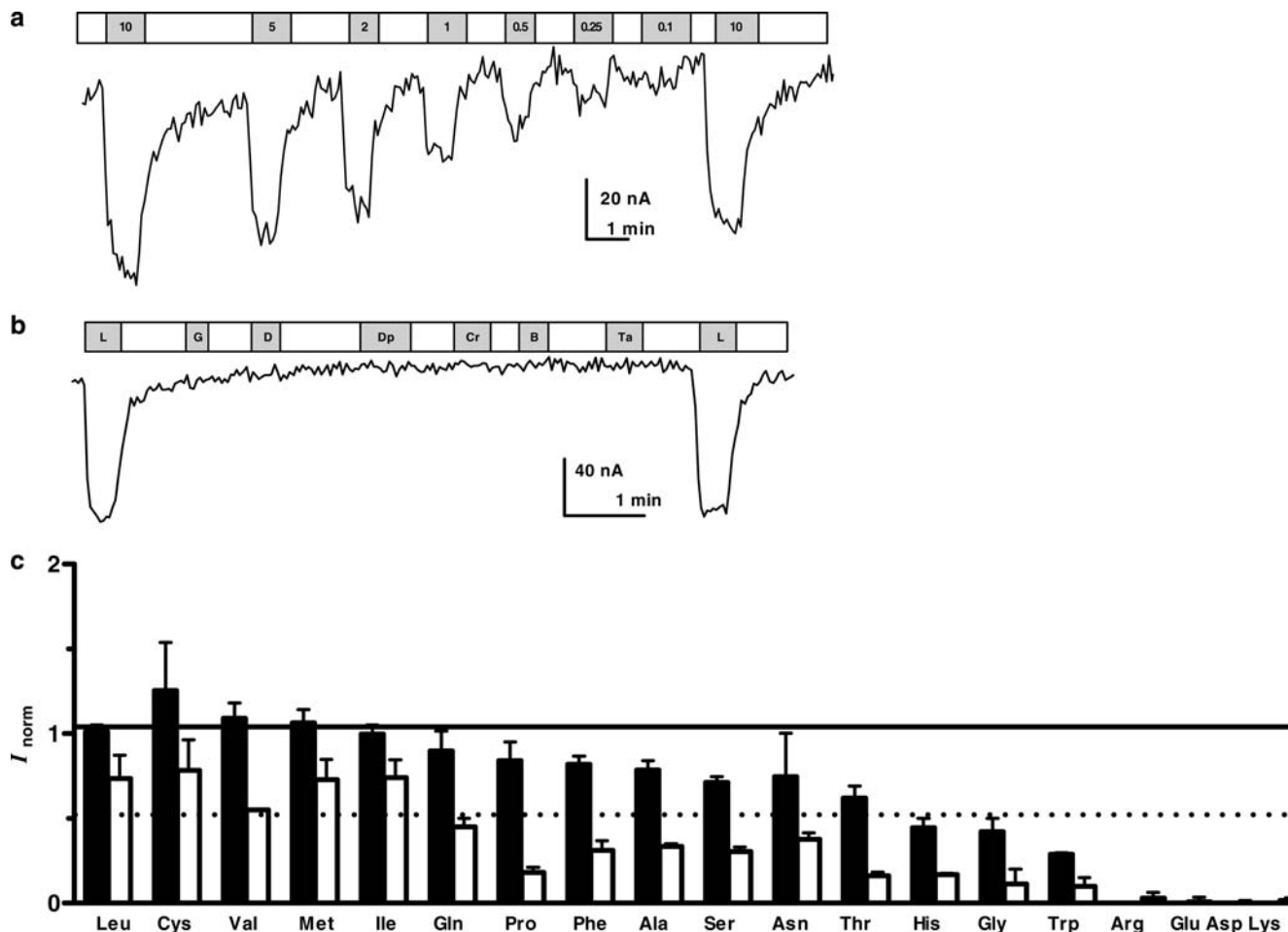
Fig. 1a–c Substrate specificity of electrogenic transport by B^0AT1. **a** Superfusion of *Xenopus* oocytes expressing B^0AT1 with increasing concentrations of L-Leu generates increasing inward currents. A representative current trace of a B^0AT1 expressing oocyte response to 0.1–10 mM L-Leu (as indicated) in standard perfusion buffer (100 mM Na^+) and voltage clamped at a holding potential (V_h) of −50 mV. **b** B^0AT1-expressing oocytes are not responsive to typical SLC6A transporter family substrates. Representative trace of the successive perfusion of a B^0AT1 expressing oocyte (under conditions as in **a**) with 1 mM GABA (*G*), L-dopamine (*D*), L-DOPA (*Dp*), 10 mM creatine (*Cr*), betaine (*B*), taurine (*Ta*) and L-Leu (*L*) (*n* = 4). **c** Amino acid side-chain specificity. Oocytes were perfused with 1 mM (*white bars*) or 10 mM (*black bars*) L-amino acid in standard perfusion buffer at a V_h of −50 mV. Means ± SEM from two experiments (*n* = 3–5 oocytes per experiment). Currents are normalized relative to 10 mM L-Leu-induced currents ($I_s = -70.4 \pm 3$ nA)
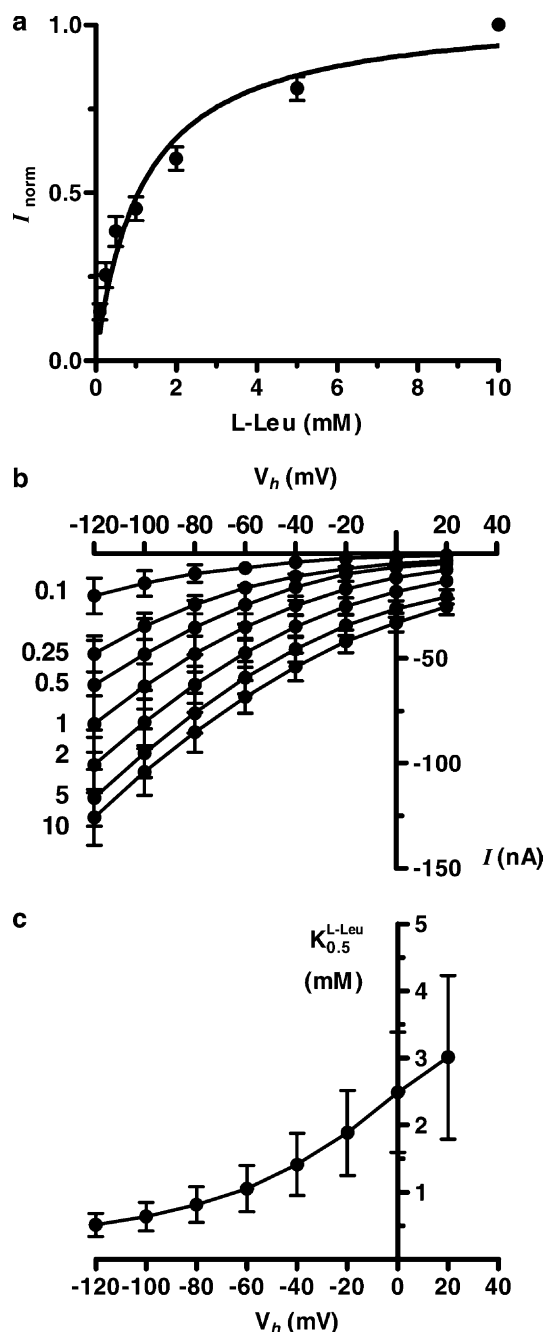
at positive V_h, became steeply linear at negative potentials and showed no rate-limiting behaviour at membrane potentials of up to −120 mV. For L-Leu the slope of the change in $K_{0.5}$ vs. V_h was linear from +20 to ∼−40 mV, at which point the slope began to flatten (Fig. 2c). In contrast, for Na^+ the slope of the change in $K_{0.5}$ vs. V_h was nearly linear at all the membrane potentials tested (Fig. 3c).

B^0AT1 substrate-induced currents are Na^+-dependent and Cl^-- and H^+-sensitive

To analyse the cation and anion requirements for B^0AT1 for L-Leu-induced currents, oocytes were tested in standard perfusion buffer containing 100 mM NaCl or with either Na^+ or Cl^- replaced (V_h −50 mV). As shown previously by flux experiments, replacement of Na^+ with NMDG effectively reduces all inward currents to the levels found in non-injected controls [3]. However, unlike the flux assays, Li^+ was able to replace ∼40% of the L-Leu-induced Na^+ currents. In addition, substitution of gluconate for Cl^- resulted in a small but significant reduction of the inward current by ∼30% ($P < 0.05$) (see Fig. 4a).

To characterize further the mechanisms underlying the L-Leu-evoked currents, we examined the effect of [H^+] on currents. Figure 4b shows the data from oocytes tested in standard perfusion buffer adjusted to either pH 8, 7.4, 6.5 or 5.5 and containing 10 mM L-Leu (V_h −50 mV). Increasing external [H^+] ∼100-fold from pH 7.4 to pH 5.5 suppressed the L-Leu-induced current by ∼50%. Currents at pH 8.0 were approximately the same as those at pH 7.4.

◄

Fig. 2a–c Steady-state characteristics of B⁰AT1-expressing oocytes as a function of [L-Leu] and holding potential. **a** Steady-state concentration/response relationship for L-Leu at −50 mV. Data were derived from the set of experiments as shown in Fig. 1a. I_{norm} is the L-Leu-evoked current for each concentration of L-Leu normalized to the 10 mM L-Leu in the presence of 100 mM NaCl. The *continuous line* is the non-linear regression fit of the Michaelis-Menten equation to the data (Eq. 1, modified Hill equation with $H = 1$). Means ± SEM, $n = 17$. **b** Steady-state concentration/response relationships for L-Leu at various V_h. Oocytes were clamped at variable voltages (+20 to −120 mV) in the absence or presence of L-Leu (mM L-Leu as indicated). I is the L-Leu-evoked current subtracted from the current observed in the absence of L-Leu. The *continuous line*s are the non-linear regression fit of the Michaelis-Menten equation to the data (Eq. 1, modified Hill equation with $H = 1$). Means ± SEM, $n = 11$. **c** Dependence of the L-Leu concentration eliciting a half-maximal response ($K_{0.5}$) on V_h. The I_{max} to V_h relationship was qualitatively the same as that of the 10 mM L-Leu current as shown in Fig. 2b. Absolute values for the derived $K_{0.5}$ and I_{max} are given in Table 2

approached that depended on [H⁺]. When the data were normalized, all curves superimposed, indicating that pH did not influence the intrinsic voltage-dependence of the transport cycle (Fig. 4d).
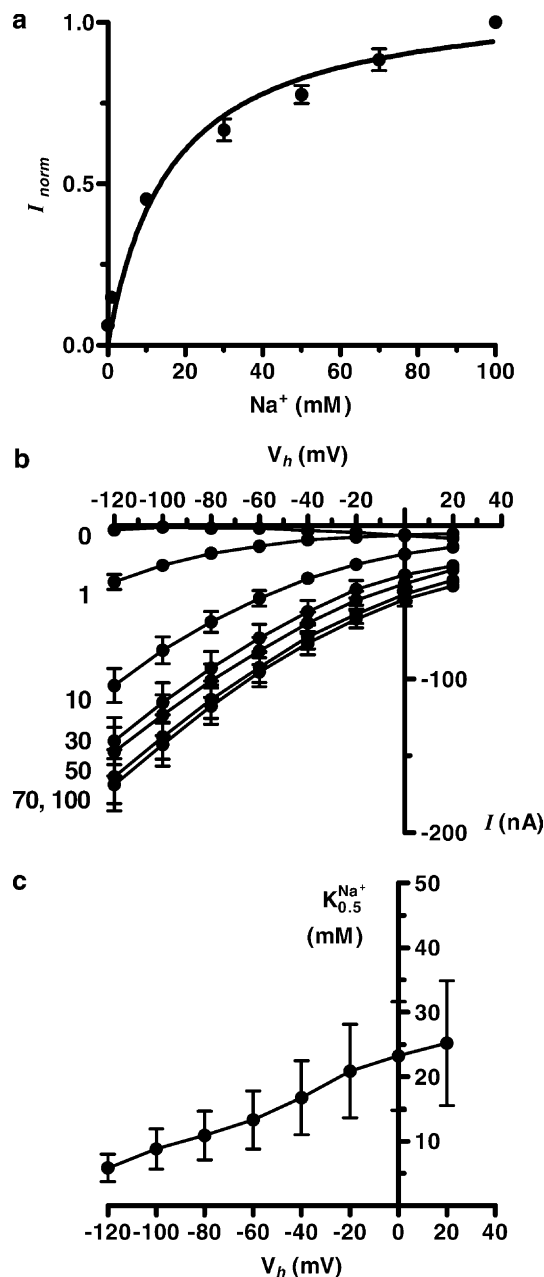
One Na⁺ is transported per neutral amino acid

When the modified Hill equation (Eq. 1) is fitted to the Na⁺ concentration-dependence data shown in Fig. 3b, the derived Hill coefficient is less than one (0.90 ± 0.38) indicating a stoichiometry of Na⁺ to L-Leu transport of at least 1. However, characterization of B⁰AT1 using a flux assay to determine L-Leu transport as a function of [Na⁺] has yielded a Hill coefficient of 1.5 ± 0.2 [3]. Since the Hill coefficient is an indication of the cooperativity of substrate binding and, at best, gives an indirect estimate of the lower limit of binding sites [33], we sought to determine the transport stoichiometry directly.

To investigate the Na⁺:L-Leu coupling ratio for B⁰AT1 we correlated the influx of radio labelled L-Leu with the net positive charge transported across the plasma membrane for each individual oocyte (V_h −50 mV). Figure 5a shows the L-Leu uptake as a function of the inward charge for each oocyte. A linear regression could be fitted to the data and the linear correlation for individual oocytes indicated that for the assay conditions (transport time and L-Leu concentration) transport of charge and, by implication, Na⁺, and L-Leu are coupled ($r^2 = 0.84$). The linear regression of the correlated data yielded a Q/L slope of 1.3 ± 0.08 ($n = 44$), which, given the distribution of the points,

Since B⁰AT1 transporters display voltage-dependent behaviour we investigated the effects of pH changes on the I/V curves at saturating L-Leu and Na⁺. Figure 4c shows that as pH decreased the current also decreased, indicating that a maximum transport rate was

Table 2 L-Leu-evoked currents (I_{max}) in B⁰AT1-expressing oocytes at 100 mM Na⁺ and holding potentials (V_h) from −120 to +20 mV. Means ± SEM, $n = 11$ ($K_{0.5}$ L-Leu concentration eliciting half-maximal current)

V_h (mV)	−120	−100	−80	−60	−40	−20	0	20
I_{max} (nA)	-129 ± 10.27	-108.2 ± 9.05	−89.87 ± 7.97	−73.56 ± 6.99	−60.22 ± 6.25	−48.68 ± 5.61	−40.6 ± 5.47	−33.00 ± 5.30
$K_{0.5}$ (mM)	0.51 ± 0.17	0.638 ± 0.21	0.814 ± 0.27	1.054 ± 0.34	1.413 ± 0.46	1.884 ± 0.63	2.489 ± 0.90	3.01 ± 1.22

Fig. 3a–c Steady-state characteristics of B^0AT1-expressing oocytes as a function of $[Na^+]$ and V_h. **a** Steady-state concentration response for Na^+ at -50 mV. Data were derived from a set of experiments in which $[Na^+]$ was varied as indicated (mM Na^+) in 10 mM L-Leu. I_{norm} for each $[Na^+]$ is the current observed in the presence of 10 mM L-Leu subtracted from the current recorded in the absence of L-Leu and normalized to the 10 mM L-Leu evoked currents in 100 mM NaCl. The *continuous line* is the non-linear regression fit of the Michaelis-Menten equation to the data (Eq. 1, modified Hill equation with $H=1$). Means $\pm$ SEM, $n=21$. (**b**) Concentration/response relation for Na^+ as the variable substrate at different holding potentials. Oocytes were clamped at different $V_h(-120$ to $+20$ mV) and currents recorded in the absence or presence of variable Na^+ concentrations (as above) with 10 mM L-Leu. I is the current observed in the presence of 10 mM L-Leu subtracted from the current recorded in the absence of L-Leu at each $[Na^+]$. The *continuous lines* are the non-linear regression fit of the Michaelis-Menten equation to the data (Eq. 1, modified Hill equation with $H=1$).Means $\pm$ SEM, $n=8$. **c** Dependence of $K_{0.5}$ for Na^+ on V_h. The I_{max} to V_h relationship was qualitatively the same as that of the 100 mM Na^+ current as shown in Fig. 3b. Absolute values for the derived $K_{0.5}$ and I_{max} are given in Table 3

(1 mM L-Leu, or 10 for mM Na^+) concentrations of the respective co-substrate. The rationale for the experimental design and interpretation is described more thoroughly in the discussion. Briefly, a simultaneous model predicts that the ratio $K_{0.5}/I_{max}$ for the variable substrate will depend on the concentration of a fixed co-substrate. In contrast, a consecutive carrier model predicts that increasing co-substrate concentration would result effectively in the competition of both substrates for the transporter, such that the absolute value of $K_{0.5}$ would increase proportionally to co-substrate concentration and the ratio $K_{0.5}/I_{max}$ thus remain constant. We found a variable $K_{0.5}/I_{max}$ ratio for both Na^+ and L-Leu and an inverse relationship between co-substrate concentration and $K_{0.5}$ indicating that Na^+ and L-Leu are simultaneously, rather than consecutively, transported.

In addition, the I_{max} for L-Leu was dependent on $[Na^+]$, varying from ~40 to 100 nA when L-Leu was titrated in 10 vs. 100 mM Na^+. In contrast, when Na^+ was titrated in 1 vs. 10 mM L-Leu, I_{max} for Na^+ was independent of [L-Leu] and only the $K_{0.5}$ for Na^+ was concentration dependent. Therefore, the $[Na^+]$ was rate-limiting while the presence of saturating Na^+ was sufficient to drive the transporter at maximal velocity regardless of the [L-Leu]. Taken together these data are consistent with the model that the amino acid binds B^0AT1 before the binding of Na^+ and the subsequent simultaneous translocation of the two co-substrates across the plasma membrane.

supports a stoichiometry of 1:1 for Na^+:L-Leu co-transport.

Na^+ binds last in an ordered model for simultaneous translocation of co-substrates

The mechanism of co-transport and the order of substrate binding were determined by analysing the saturation kinetics for both Na^+ and L-Leu as a function of co-substrate concentration (Fig. 5b,c). To distinguish between a consecutive, a simultaneous or a random carrier transport model, and to determine the order of substrate binding, I_{max} and $K_{0.5}$ for both L-Leu and Na^+ were determined at near-saturating (10 mM L-Leu, or 100 mM for Na^+) and at sub-saturating

Discussion

Substrate selectivity of B^0AT1

Absorption of neutral amino acids from the lumen of the small intestine and the kidney proximal tubule is the primary physiological function of the B^0AT1

Table 3 Na$^+$-dependent, L-Leu-induced currents in B^{0}AT1-expressing oocytes at 10 mM L-Leu and V_h between -120 and $+20$ mV: I_{max} and $K_{0.5,Na}$. Means $\pm$ SEM, $n=8$

V_h (mV)	-120	-100	-80	-60	-40	-20	0	20
I_{max} (nA)	-172.2 ± 10.56	-153.7 ± 10.97	-129.9 ± 10.04	-108.7 ± 8.99	-89.23 ± 8.34	-73.38 ± 7.70	-58.59 ± 6.75	-47.76 ± 6.04
$K_{0.5}$ (mM)	5.87 ± 2.12	8.81 ± 3.11	10.89 ± 3.78	13.31 ± 4.50	16.73 ± 5.74	20.87 ± 7.25	23.22 ± 8.425	25.21 ± 9.66
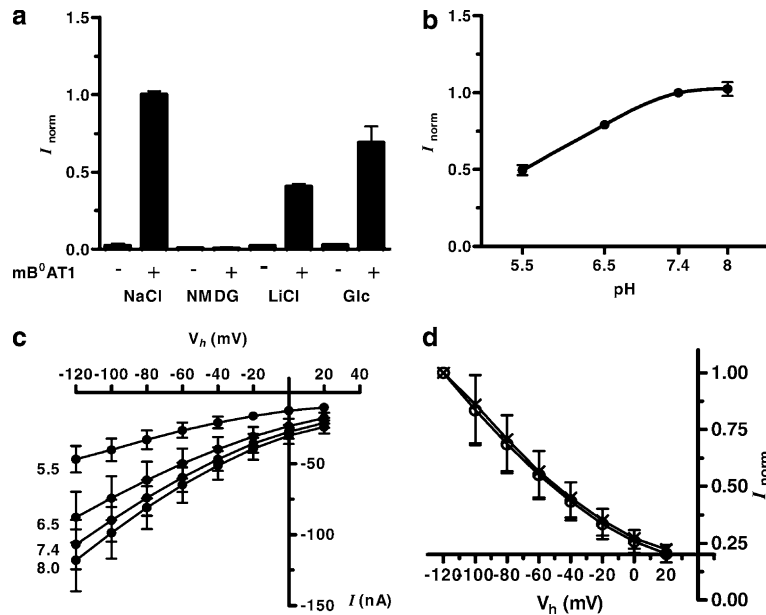
Fig. 4a–d Ion and pH dependence of L-Leucine-evoked inward currents. **a** Ion dependence of L-Leu-evoked inward currents. B^{0}AT1-expressing and non-injected control oocytes were tested with 10 mM L-Leu at a V_h of -50 mV in standard perfusion buffer containing either 100 mM NaCl, or with Na$^+$ replaced by 100 mM NMDG or Li$^+$, or with Cl$^-$ replaced by 100 mM gluconate (*Glc*). Currents were normalized (I_{norm}) to the mean current for L-Leu at 100 mM NaCl ($n=7$–16). **b** Steady-state currents as a function of buffer pH. Oocytes were voltage clamped to -50 mV and equilibrated with a control solution adjusted to the test pH before applying 10 mM L-Leu at the same pH. Currents were normalized (I_{norm}) to the mean current for L-Leu at pH 7.4. Means $\pm$ SEM, $n=7$. **c** Voltage dependence of steady-state currents as a function of buffer pH. After equilibration in buffers at the test pH without or with 10 mM L-Leu, B^{0}AT1-expressing oocytes were clamped to holding potentials from -120 to $+20$ mV. For each pH the traces generated in the absence of L-Leu were subtracted from the 10 mM L-Leu traces. Means $\pm$ SEM, $n=8$. In **b** and **c**, SEM values smaller than the *symbol* size are not shown. **d** I/V relationship for the normalized L-Leu evoked currents in pH 8.0 vs. pH 5.5 buffer. The I/V data for pH 8 and for pH 5.5 were normalized to the respective current at -120 mV and superimposed. *Open circles* I_{norm} pH 8; *crosses* I_{norm} pH 5.5

transporter. Indeed, when Na$^+$-dependent L-amino acid induced currents are analysed in *Xenopus* oocytes expressing mouse B^{0}AT1, the amino acid specificity is similar to that of B^0 as defined previously in studies using BBMV, cell cultures or micro-dissected tubules (see Introduction). Our results are also in close agreement with the results of radioactive tracer uptake competition experiments in oocytes expressing mouse B^{0}AT1 [3]. Although B^{0}AT1 accepts a broad spectrum of neutral amino acids it does not transport the amino acid derivatives or metabolites, GABA, L-DOPA, taurine, creatine, betaine or dopamine, even though several of these compounds, for example L-DOPA, are structurally very similar to efficiently transported amino acids. In considering both the previous competition experiments carried out by Bröer's group and the current work, it appears that several properties of the substrate influence the relative affinity: (1) presence and spacing of the

charged amino and carboxyl groups, (2) configuration of the α-carbon (L not D), (3) net neutral charge and (4) size, geometry and to an extent hydrophobicity of the side-chains.

The first three criteria are probably required for transport; the lack of one or more of them could explain the non-responsiveness of the transporter to some of the substrates tested (e.g. dopamine and creatine lack the COO$^-$ group and in GABA the COO$^-$ and NH$_3^+$ groups are separated by three CH$_2$ groups), but, apparently, are not sufficient for substrate uptake. For example, the amino acid dopamine precursor L-DOPA does not evoke inward currents. The size and geometry of the amino acid side-chain play a role in substrate recognition and selectivity. In general amino acids containing large, branched, sulphur-containing and/or β-carbon-substituted side-chains are favoured over those with smaller, unbranched side-chains. Specific

a

b

c

Fig. 5a–c Stoichiometry and order of binding. **a** B^0 AT1 co-transports one Na^+ per leucine. Oocytes were equilibrated in standard perfusion buffer at -50 mV before superfusion with 2 mM Leu ([³H]L-Leu at 0.2 μCi/ml). L-Leu-evoked net positive charge transported across the plasma membrane (Q) was calculated by integration over time (5–20 min) of the inward current, and correlated with the corresponding L-Leu uptake ([³H]L-Leu content) for each oocyte. *Filled circles*: B^0AT1-expressing oocytes, $n = 44$; *open circles*: non-injected controls, $n = 9$. The *continuous line* is the linear regression to the data points (Q/L slope $= 1.3 \pm 0.08$, $r^2 = 0.844$) and the *broken lines* indicate the 95% confidence interval for the fit. **b** Na^+ steady-state kinetics as a function of [L-Leu]. Data were derived from sets of experiments in which [L-Leu] was either 1 or 10 mM, while [Na^+] was varied (0, 5, 10, 30, 50, 70, 100 mM). The same oocytes were tested at each concentration of L-Leu. The *continuous line* is the non-linear regression fit of the Michaelis-Menten equation to the data (Eq. 1, modified Hill equation with $h = 1$). Means $\pm$ SEM, $n = 12$. **c** Steady-state kinetics of L-Leu-evoked currents as a function of [Na^+]. Data were derived from experiments in which [Na^+] was either 10 or 100 mM, while [L-Leu] was varied (0, 0.25, 0.5, 1, 2, 5, 10 mM). The same oocytes were tested at each [Na^+]. The *continuous line* is the non-linear regression fit of the Michaelis-Menten equation to the data (Eq. 1, modified Hill equation with $H = 1$). Means $\pm$ SEM, $n = 7$

Interaction of B^0AT1 with ions

The $K_{0.5}$ for Na^+ derived from this study (16 mM) is in close accord with a previous report characterizing system B^0 activity in Caco-2 cells [19]. This value is lower than the value determined for mouse B^0AT1 by radioactive tracer influx experiments (54 mM) [3]. However, given our data indicating that the Na^+ $K_{0.5}$ is dependent on both L-Leu concentration and on membrane potential, a higher $K_{0.5}$ for Na^+ would be expected for the radioactive tracer influx studies since the latter were made using low [L-Leu] (100 μM) under non-voltage clamped conditions. A substrate-independent, Na^+-dependent current is also associated with B^0AT1 expression. This current is not saturable at increasing concentrations, indicating channel-like, as opposed to carrier-mediated activity (data not shown). Interestingly, uncoupled Na^+ leak pathways have been described for many of the Na^+-dependent transporters including GAT [10], SERT [1, 20] and DAT [28]. Contrary to previous radioactive tracer influx experiments [3] in the absence of Na^+, Li^+ was able to substitute partially for Na^+ in generating inward currents (40% of I_{Na}). However, as shown in radioactive tracer influx experiments [3, 24] and as expected for B^0-type transport, L-Leu-evoked B^0AT1 inward currents were not Cl^--dependent. Although, as for the newly characterized proline transporter, SIT1 [32], Cl^- substitution (by gluconate) decreased the L-Leu induced current by 31% ($P < 0.05$) indicating that the transporter activity is sensitive to external Cl^-.

B^0AT1 substrate-induced currents were highly sensitive to both pH and voltage. The correlation between lowering the pH from 7.4 to 5.5 and a ~50% reduction in the L-Leu-evoked currents closely mirrors the reported impact of pH on L-Leu uptake [3]. Mechanistically, H^+ may interact with the transporter to

interactions between transporter and side chain also play a role since there is some advantage accorded to non-polar amino acids (Met, Val), which would be expected to interact with a hydrophobic pocket over polar amino acids (Asn, Thr) for which such an environment would be unfavourable. This may account for the inability of L-DOPA, which contains two polar OH groups, to interact with the transporter. The weak response of mouse B^0AT1 to L-Trp contrasts somewhat with earlier results from endogenously expressed transporters [16, 19, 30]. Further, human B^0AT1-expressing oocytes do not transport L-Cys, L-His, L-Pro and Gly efficiently, suggesting species-specific effects on amino acid affinity of the B^0AT1 transporter [12].

compete directly with Na^+ at the binding site. Alternatively protonation of transporter residues may prevent substrate translocation allosterically. The impact of membrane potential on transporter kinetics is a gauge of which steps of the transport mechanism it modulates. This transporter property is highly heterogeneous among Na^+-coupled transporters. For mouse B^0AT1, membrane potential affected both the $K_{0.5}$ and the derived I_{max} for both co-substrates strongly, suggesting that both the apparent substrate binding affinity and translocation rate are influenced by membrane potential. This is not surprising as phenomenological parameters such as apparent substrate affinity are necessarily functions of all the transition rate constants associated with the transport cycle.

Coupling of B^0AT1 transport

The stoichiometry of transport was assessed by direct comparison of the amount of charge transferred with the L-Leu uptake for the same oocyte. This method yielded a slope of 1.3 Q/L-Leu transported that, taken together with a Hill co-efficient of less than 1, is consistent with a 1:1 stoichiometry. The presence of uncoupled currents, such as seen for the majority of the SLC6 family, can confound the precise determination of transport stoichiometry. In the case of B^0AT1, there is preliminary evidence that this transporter may indeed facilitate uncoupled currents; however, without a specific inhibitor we were unable to determine the contribution of this putative leak current to B^0AT1 transport stoichiometry.

A simple model for B^0AT1 transport function

In humans, the kidney glomerulus filters $\sim$300 μmol amino acids/min, $\sim$80% of which is reabsorbed by the proximal tubule (for review see [22, 27]) that expresses high levels of B^0AT1 [12] (E. Romeo unpublished observations). Several characteristics of the B^0AT1 transport mechanism influence its amino acid reabsorption efficiency strongly. First, the low substrate binding affinity allows B^0AT1 to bind a large class of (neutral) amino acids selectively, partially compensating for the low plasma concentration of individual amino acids while promoting rapid transporter dissociation rates. Second, the 1:1 coupling stoichiometry between the Na^+ current and the L-Leu flux determines the thermodynamic upper limit to its concentrative capacity that might, to some extent, be counteracted by the presence of uncoupled conductive pathways. We have indeed seen indications that the B^0AT1 transporter may provide pathways for Na^+, H^+, and/or Cl^- that are not thermodynamically coupled to amino acid transport.

A third set of important transporter characteristics are the type of co-transport and the order of substrate binding. Along with the macroscopic kinetic parameters (i.e. $K_{0.5}$ and I_{max}) these determine how Na^+ and amino acid concentrations impact on the maximum turnover rate of B^0AT1. These two transport features can be investigated by analysing the saturation kinetics for both Na^+ and L-Leu as functions of co-substrate concentration. Various alternative models have been used to describe the transport mechanism of two-substrate transport systems like B^0AT1. The two most important of the proposed mechanisms are the simultaneous model and the consecutive model of co-transport. Our data do not address a third model, the single-file model, which has been used to describe a channel-like transporter mechanism [2]. A simultaneous mechanism postulates that both of the substrates bind to the transport protein before translocation and release occur. In the consecutive model, a binding site for the second substrate at the original *cis* side of the membrane is available only after translocation of the first substrate to the *trans* side of the membrane. Delivery of the second substrate reconstitutes the original form of the transporter. Cleland [5, 34] has shown that these two mechanisms can be distinguished experimentally by determining the relationship of $K_{0.5}$ and I_{max} at varying co-substrate concentrations. In the simultaneous model, where both substrates are postulated to be translocated together, the $K_{0.5}$ of the variable substrate should decrease with increasing co-substrate concentration and therefore the ratio $K_{0.5}/I_{max}$ will vary. Alternatively, the consecutive carrier model predicts that increasing co-substrate concentration results in the competition of both substrates for the transporter such that $K_{0.5}/I_{max}$ ratio is constant, i.e. the rate of change of the $K_{0.5}$ and I_{max} is proportional over a range of co-substrate concentrations. Comparing $K_{0.5}$ and I_{max} for both L-Leu and Na^+ at saturating and at sub-saturating concentrations of the respective co-substrate showed a variable $K_{0.5}/I_{max}$ ratio and an inverse relationship between co-substrate concentration and $K_{0.5}$ for both Na^+ and L-Leu consistent with a simultaneous mechanism of transport.

The same experimental protocol also predicts the order of substrate binding [29]. Assuming that the rate of dissociation of a substrate from a transporter protein is much greater than the rate of translocation, the maximal rate of turnover, or I_{max}, will depend on the concentration of the second substrate to bind. With only a small amount of the transporter complexed to the first substrate, a saturating amount of the second substrate will stimulate the maximal velocity of transport. In contrast to I_{max}, the $K_{0.5}$ for both substrates will depend on the amount of the respective co-substrate. For B^0AT1, I_{max}, but not $K_{0.5}$ for Na^+, was independent of the L-Leu concentration. In contrast, both $K_{0.5}$ and I_{max} for L-Leu were Na^+ dependent, leading to the conclusion that Na^+ binds last. Therefore, in the proximal tubule the realization of the maximum turnover rate by B^0AT1 depends on the relatively constant $[Na^+]$ and not on the rapidly diminishing amino acid concentration.

In summary, our working model for B^0AT1 transport is that one Na^+ binds to the already formed transporter-amino acid complex. Subsequently, B^0AT1 translocates

both co-substrates simultaneously across the plasma membrane by an electrogenic mechanism. The $[Na^+]$, which remains relatively constant throughout the proximal tubule, drives the transport thermodynamically and assures that the transporter with bound amino acid turns over at a high rate. In this context, the relatively low affinity of B^0AT1 for individual amino acids allows for the selective reabsorption and rapid intracellular release of neutral amino acids over the large decrease in substrate concentrations encountered descending the length of the kidney proximal tubule.

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