Characterization of mouse amino acid transporter B^{0}AT1 (slc6a19)

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The mechanism of the mouse (m)B^{0}AT1 (slc6a19) transporter was studied in detail using two electrode voltage-clamp techniques and tracer studies in the *Xenopus* oocyte expression system. All neutral amino acids induced inward currents at physiological potentials, but large neutral non-aromatic amino acids were the preferred substrates of mB^{0}AT1. Substrates were transported with $K_{0.5}$ values ranging from approx. 1 mM to approx. 10 mM. The transporter mediates Na^+–amino acid co-transport with a stoichiometry of 1:1. No other ions were involved in the transport mechanism. An increase in the extracellular Na^+ concentration reduced the $K_{0.5}$ for leucine, and vice versa. Moreover, the $K_{0.5}$ values and V_{max} values of both substrates varied with the membrane potential. As a result, $K_{0.5}$ and V_{max} values are a complex function of the concentration of substrate and co-substrate and the membrane potential. A model is presented assuming random binding order and a positive charge associated with the ternary [Na^+–substrate–transporter] complex, which is consistent with the experimental data.

Key words: amino acid transport, Hartnup disorder, neurotransmitter transporter family, transport mechanism.

INTRODUCTION

Transport of neutral amino acids across the apical membrane of kidney epithelial cells is thought to be carried out by a Na^+-dependent transport activity [1]. Closer inspection of reported K_m values, however, reveals significant heterogeneity: for phenylalanine, for example, K_m values between 1.5 mM and 10 mM were reported [2–5]; for leucine and valine, K_m values were reported of 0.2 mM and 0.8 mM respectively [6]; and for alanine, K_m values were found to vary between 0.4 and 8 mM [2,3,7]. Apart from the variability of the K_m values, all studies agree in other properties of the system. First, the transporter is electrogenic and can be driven by either a substrate gradient or an electrical gradient. Secondly, the Na^+–substrate co-transport stoichiometry appears to be 1:1. Thirdly, the K_m of the substrate decreases with increasing Na^+ concentration. Furthermore, it appears that all neutral amino acids tested are transported by the same system, because uptake of one neutral amino acid is inhibited by other neutral amino acids [8]. As a result, the transport activity is referred to as 'system B^0' to indicate its broad substrate specificity [9,10]. Despite the large variability of the reported K_m values, it appears that the early segments of the proximal tubule (S1 and S2) most likely have only one transport activity for neutral amino acids, whereas two additional transport activities can be found in the S3 segment [4,6,11]. One of these activities has a higher affinity than that in the proximal tubule; the other has a lower affinity. This pattern was observed for the transport of phenylalanine, alanine, serine, leucine and valine, consistent with the hypothesis that all amino acids are transported by the same activity.

In the intestine, heterogeneity has been observed for different reasons. Large neutral amino acids are transported by three different transport systems in the intestine, namely system B^0, system B$^{0,+}$ (an Na^+-dependent transporter for neutral and cationic amino acids) and system ASC (an Na^+-dependent transporter for mid-size neutral amino acids) [12–14]. Small neutral amino acids are, in addition, transported by PAT1 (proton–amino acid co-transporter 1) [15]. The activity of the intestinal system B^0, typically studied in preparations from jejunum, is very similar to the activity observed in kidney cortex [16–20]. Similar to the kidney, K_m values differ significantly between preparations from intestine. In an early study, K_m values were reported for alanine (9 mM), valine (5 mM) and leucine (4 mM) [18], whereas lower K_m values were reported in two later studies (between 0.4–0.7 mM for alanine, valine and leucine) [16,17]. In agreement with the characteristics of the transport activity from kidney cortex, it was found that increased Na^+ concentration reduced the K_m of the substrate.

Recently, we have identified a new member of the neurotransmitter family (slc6a19 or B^{0}AT1), which has all the hallmarks of system B^0, namely Na^+-dependence, electrogenicity and a broad substrate specificity including almost all neutral amino acids [10]. Furthermore, we and others [21,22] subsequently demonstrated that SLC6A19 constitutes a dominant transport activity for neutral amino acids in the kidney and intestine by showing that a malfunction of SLC6A19 results in severe neutral aminoaciduria, known as Hartnup disorder. Mouse slc6a19 is expressed in the outer cortex of the kidney and in the intestine [10], and therefore most likely corresponds to the transporter characterized in vesicle studies using kidney cortex or intestinal preparations. However, expression of SLC6A19 has also been reported in the S3 segment [22].

Given the reported heterogeneity of B^0-like activities, particularly in the kidney, the aim of the present study was to characterize B^{0}AT1 more extensively, to identify whether this transporter is indeed identical with the activity demonstrated in kidney cortex and jejunum brush border, and to define its substrate specificity more comprehensively.

MATERIALS AND METHODS

Oocytes and injections

Oocyte isolation and maintenance have been described in detail elsewhere [23]. For expression in oocytes, mouse (m)B^{0}AT1

Abbreviations used: NMDG(-Cl), *N*-methyl-D-glucamine (chloride); PAT1, proton–amino acid co-transporter 1.

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in pGem-He-Juel [10] was linearized with SalI and transcribed *in vitro* using the T7 mMessage mMachine Kit (Ambion, Austin, TX, USA). Rat MCT1 was used as described previously [24]. Oocytes were injected with cRNA (23 ng) encoding mouse B^0AT1 or rat MCT1. Transport measurements and electrophysiological recordings were carried out after 3–8 days following injection.

Flux measurements

For each determination, groups of eight to ten cRNA-injected or non-injected oocytes were washed twice with 4 ml of ND96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM Hepes; titrated with NaOH to pH 7.4, unless indicated otherwise) before incubation at room temperature in a 5 ml polypropylene tube containing 90 μl of the same buffer supplemented with ^{14}C-labelled amino acids and different amounts of unlabelled substrate. Transport was stopped after different time intervals by washing oocytes three times with 4 ml of ice-cold ND96 buffer. Single oocytes were placed into scintillation vials, and lysed by addition of 200 μl of 10 % (w/v) SDS. After lysis, 3 ml of scintillation fluid was added, and the radioactivity was determined by liquid scintillation counting. To determine the dependence of transport activity on the Na^+ concentration, NaCl-ND96 (pH 7.4) was mixed with NMDG-Cl (*N*-methyl-D-glucamine chloride)-ND96 (pH 7.4; NaCl replaced by NMDG-Cl) in different proportions. It is worth noting that ND96 contains approx. 100 mM Na^+ due to the titration. Different pH values were adjusted by mixing Mes-buffered ND96 (5 mM Hepes replaced by 5 mM Mes) with Tris-buffered ND96 (5 mM Hepes replaced by 5 mM Tris base). To lower the intracellular pH, oocytes expressing B^0AT1 and MCT1 were incubated for 15 min in the presence of 30 mM lactate. Subsequently, uptake of [^{14}C]leucine (100 μM) was studied in the continued presence of 30 mM lactate. Since uptake of [^{14}C]leucine increased linearly with time for up to 20 min, uptake was determined using an incubation period of 15 min. For efflux, oocytes were injected with 50 nl of a 1:1 mixture of [^{14}C]leucine and 100 mM leucine. Following injection, oocytes were maintained for 20 min to allow an even amino acid concentration throughout the cytosol and healing of the injection site.

Electrophysiological recordings

Amino-acid-induced currents were analysed by two-electrode voltage-clamp recording. The recordings were performed with $1 \times$ LU and $10 \times$ MGU headstages connected to a Geneclamp 500B electronic amplifier (Axon Instruments, Union City, CA, U.S.A.). The output signal was amplified 10 times and filtered at 1 kHz. The analogue signal was converted into digital by a Digidata 1322A (Axon Instruments), and data were sampled at 10 Hz using pCLAMP software (Axon Instruments). The sampling rate was increased as appropriate in protocols where the holding potential was varied. Oocytes were chosen that had a membrane potential of less than -30 mV. Once a stable membrane potential was reached under current-clamp conditions, the amplifier was switched to the voltage-clamp mode, holding the oocytes at -50 mV unless indicated otherwise. To study voltage-dependence of the transporter, the holding potential was increased stepwise from -150 mV to 50 mV in 10 mV increments; the step length was 500 ms. The control solution was ND96, pH 7.4, unless indicated otherwise. A complete exchange of the buffer was accomplished in about 10 s. Variation of the extracellular pH or of the extracellular NaCl concentration was achieved as described in the Flux measurements subsection (above).

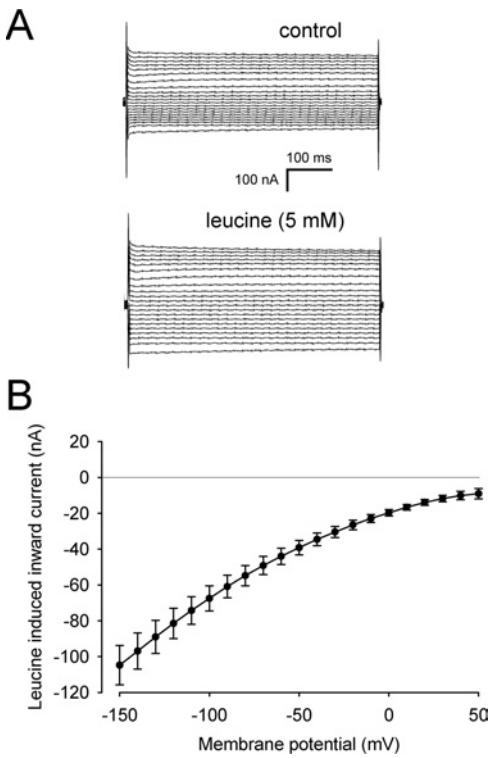

Figure 1 Voltage-dependence of leucine-induced inward currents

Oocytes expressing mB^0AT1 cRNA were incubated for 5 days and subsequently superfused with 5 mM leucine at a holding potential of -60 mV. After a stable inward current was established, the holding potential was changed between -150 mV and $+50$ mV in steps of 10 mV. (**A**) Original tracings obtained in the absence and presence of 5 mM leucine. (**B**) Resulting net leucine-induced currents as a function of the holding potential ($n = 10$ oocytes).

Calculations, statistics and computer analysis

For electrophysiological recordings, data were averaged over at least seven oocytes. Oocytes expressing B^0AT1 displayed amino-acid-induced currents of the order of 30–80 nA. Non-injected oocytes, by contrast, displayed currents of 1–2 nA in response to the same amino acids. Currents of this size cannot be resolved against a noise of approx. ± 1 nA. Given that noise plus endogenous currents amount to 2–3 % of the B^0AT1-induced signal, no correction was attempted. Endogenous fluxes of labelled amino acids, by contrast, were significant. Therefore flux measurements were performed with eight to ten oocytes for each datum point, and the activity of the same number of non-injected oocytes was subtracted in each case. This indicates that endogenous transporter(s) are amino acid exchangers carrying fluxes, but not currents. Flux experiments and experiments involving electrophysiological recordings were performed three times.

Kinetic constants were derived by non-linear curve fitting using Origin7.0 software (OriginLab Corp., Northampton, MA, U.S.A.). To determine K_m and V_{max} values, the Michaelis–Menten equation [$v = V_{max}S/(K_m + S)$] or the Hill equation [$v = V_{max}S^n/(K_m{}^n + S^n)$] was used. Normal distribution of data was tested with InStat software (OriginLab Corp.).

RESULTS

Substrate specificity

Superfusion of mB^0AT1-expressing oocytes with leucine, at different holding potentials, increased inward currents (Figure 1A),

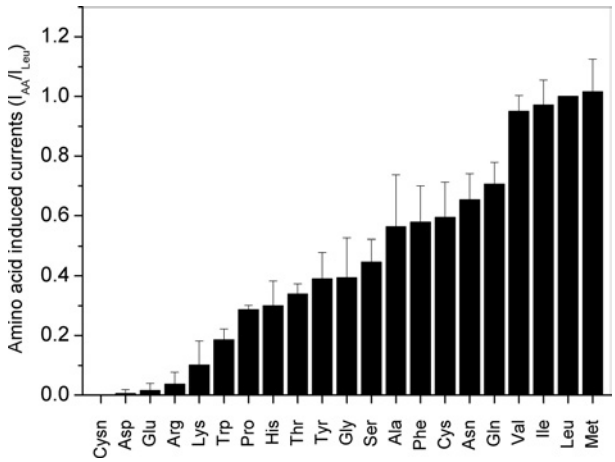

Figure 2 Substrate specificity of mouse B^{0}AT1

Oocytes expressing mB^{0}AT1 cRNA were incubated for 5–8 days. Subsequently, oocytes were held at a membrane potential of −50 mV and then superfused with ND96 (pH 7.4) alone or ND96 (pH 7.4) containing different amino acids at a final concentration of 5 mM, or 0.3 mM in the case of cystine. Each bar represents the mean ± S.D. of $n > 7$ oocytes from different batches for each amino acid. Data were compiled from different oocyte batches and normalized to the current induced by 5 mM leucine.

whereas outward currents remained unaltered. The net leucine-induced currents hence were inwardly directed at all tested holding potentials (Figure 1B), suggesting that they are coupled with substrate transport. All other neutral amino acids also induced inward currents in mB^{0}AT1-expressing oocytes (Figure 2). Large aliphatic neutral amino acids were the preferred substrates of the transporter, with leucine, isoleucine, valine and methionine inducing currents of comparable size. Slightly smaller currents were induced by glutamine, phenylalanine, tyrosine, cysteine and alanine (50–80 % of leucine). Glycine, proline, serine, histidine and tryptophan induced the smallest currents (20–30 % of leucine). Anionic amino acids (aspartate and glutamate), arginine and cystine did not induce currents in mB^{0}AT1-expressing oocytes. Lysine produced only small currents (approx. 10 % of leucine).

The observed transport velocities at a concentration of 5 mM coincided well with the K_m values of different amino acids. We tested five different amino acids, and found $K_{0.5}$ values of 1.1 ± 0.1 mM (leucine), 3.2 ± 0.2 mM (glutamine), 4.1 ± 0.3 mM (alanine), 4.7 ± 0.9 mM (phenylalanine) and 11.7 ± 1.6 mM (glycine) (Figure 3A). I_{max} was relatively similar for the five amino acids tested, ranging from −39.0 ± 4.9 nA (alanine) to −63.0 ± 7.6 nA (glycine) (Figure 3C). Hyperpolarization increased the affinity of mB^{0}AT1 for the different amino acids and increased I_{max} (Figures 3B and 3C). The similar I_{max} values suggested that the translocation of the empty transporter might be the rate-limiting step in the transport cycle, in which case, *trans*-stimulation of the transporter would be expected. Injection of a final concentration of 10 mM leucine, glutamine, histidine (substrates of mB^{0}AT1) or glutamate (not a substrate of mB^{0}AT1) into oocytes, however, did not enhance leucine uptake. Injection of 25 mM NaCl into oocytes even *trans*-inhibited leucine uptake (Table 1). The *trans*-inhibition could be relieved by co-injection of 25 mM NaCl plus 10 mM glutamine or histidine into the oocytes, but not by co-injection of 10 mM glutamate. Leucine itself did not relieve *trans*-inhibition exerted by NaCl.

Co-substrates and stoichiometry

Using [^{14}C]leucine, we recently showed a sigmoidal relationship between uptake activity and Na^+ concentration, suggesting that

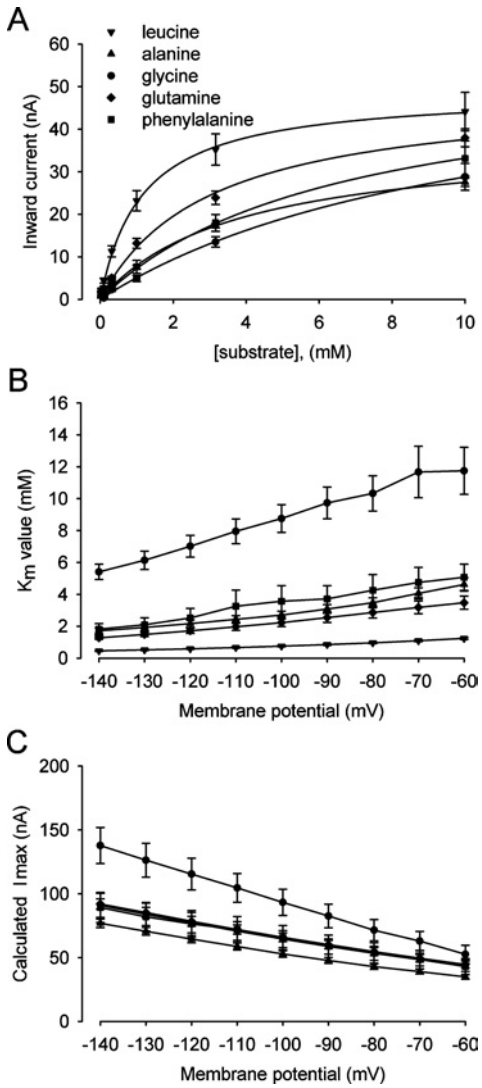

Figure 3 Amino-acid-induced currents as a function of substrate concentration

Oocytes expressing mB^{0}AT1 cRNA were incubated for 5 days. Subsequently, oocytes were superfused with ND96 (pH 7.4) alone or ND96 (pH 7.4) containing different amino acids (▼ leucine; ▲ alanine; ● glycine; ◆ glutamine; ■ phenylalanine) using concentrations of 0.1, 0.3, 1, 3 and 10 mM (**A**). After reaching steady-state currents, the holding potential was changed between −60 mV and −140 mV in steps of 10 mV to derive $K_{0.5}$ values (**B**) and I_{max} values (**C**) at different holding potentials. Each datum point represents the mean ± S.E. transport activity of $n = 17$–21 oocytes.

more than one Na^+ ion may be co-transported together with amino acid substrates [10]. Electrophysiological analysis of the Na^+-dependence at two different leucine concentrations (Figure 4; data for 30 mM leucine shown by closed squares, that for 5 mM leucine by closed circles), by contrast, resulted in a hyperbolic curve form, indicating the co-transport of only 1 Na^+ together with the substrate. As previously, we found a less-than-linear increase in transport activity at low Na^+ concentration, when using 0.1 mM [^{14}C]leucine (Figure 4, open circles). To investigate whether the sigmoidicity was caused by an inhibitory effect of NMDG-Cl, we determined [^{14}C]leucine uptake in transport buffer, in which half of the NaCl was replaced with different compounds. Under control conditions (100 mM NaCl), leucine uptake was 26 ± 7 pmol/15 min per oocyte. Replacing 50 mM NaCl with NMDG-Cl reduced transport activity to 14 ± 3 pmol/15 min,

Table 1 *Trans*-effect on leucine transport by intracellular Na^+ and amino acids

Oocytes were injected with mB^0AT1 cRNA or remained uninjected in the controls. After 5 days, [^{14}C]leucine uptake (100 μM) was determined in oocytes injected with 50 nl of water or 50 nl of a 100 mM solution of the indicated amino acid. Each datum point represents the mean ± S.D. transport activity of $n = 10$ oocytes. The transport activity of non-injected oocytes was subtracted (the experiment was repeated three times). Figures significantly different from the control are indicated by ** ($P < 0.01$) or *** ($P < 0.001$).

Injection (final concentration)	Uptake activity (pmol/15 min per oocyte)
None	61 ± 6
Water	68 ± 9
Gln (10 mM)	62 ± 11
His (10 mM)	56 ± 8
Glu (10 mM)	61 ± 8
Leu (10 mM)	48 ± 8***
NaCl (25 mM)	42 ± 9***
NaCl (25 mM) + Gln (10 mM)	60 ± 10
NaCl (25 mM) + His (10 mM)	62 ± 10
NaCl (25 mM) + Glu (10 mM)	41 ± 8**
NaCl (25 mM) + Leu (10 mM)	33 ± 5***

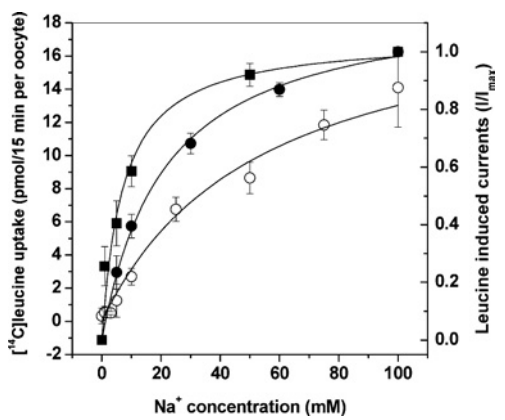

Figure 4 Leucine transport as a function of the Na^+ concentration

Oocytes were injected with mB^0AT1 cRNA or remained uninjected in the controls, and were incubated for 5–8 days. [^{14}C]Leucine uptake (100 μM; ○) was determined at Na^+ concentrations ranging from 0–100 mM (all pH 7.4). Each datum point represents the mean ± S.D. transport activity of $n = 10$ oocytes. The transport activity of non-injected oocytes was subtracted (the experiment was repeated three times). Using a two-electrode voltage clamp, leucine-induced currents [5 mM (●) and 30 mM (■)] were determined at Na^+ concentrations ranging from 0–100 mM (all pH 7.4). Each datum point represents the mean ± S.D. of $n = 5$ oocytes. The experiment was repeated with three different oocyte batches. Currents were normalized to the maximal current.

whereas replacement with choline-Cl resulted in a smaller reduction in the activity to 17 ± 2 pmol/15 min. Replacement with 100 mM sucrose resulted in an even smaller reduction in activity, to 25 ± 5 pmol/15 min. This result suggests that high concentrations of NMDG inhibit Na^+ binding in addition to the replacement effect, and suppress leucine uptake at low Na^+ concentrations. In order to clarify further the substrate stoichiometry, we investigated whether other ions participate in the transport mechanism of mB^0AT1. The pH-dependence showed an increase of transport activity with increasing pH (Table 2 and [10]), which would be compatible with a proton antiport. To discriminate between an allosteric pH-dependence and a catalytic pH-dependence, we co-expressed mB^0AT1 together with the H^+–monocarboxylate co-transporter MCT1. Incubation of oocytes with 25 mM lactate at an extracellular pH of 7.0 decreases the intracellular pH by approx. 0.8 pH unit, as shown previously

Table 2 Influence of ions on substrate-induced currents in mB^0AT1-expressing oocytes

Oocytes were injected with mB^0AT1 cRNA. After incubation for 5 days, oocytes were superfused with control solutions of different pH values and in ND96 where NaCl was replaced by sodium gluconate. Leucine-induced currents were determined under voltage-clamp (− 50 mV) and oocyte-endogenous currents arising from the buffer exchange were subtracted from the leucine-induced current in the same buffer. Each datum point represents the mean ± S.D. transport activity of at least $n = 7$ oocytes. Figures significantly different from the control are indicated by *** ($P < 0.001$).

Change of ion concentration	Leucine-induced currents (% of control)
Control (ND96), pH 7.4	100
Addition of 50 mM KCl	79 ± 3***
pH 5	53 ± 4***
pH 6	70 ± 9***
pH 7	95 ± 15
pH 8	106 ± 9
Replacement of Cl^-	102 ± 8

[24]. Uptake of [^{14}C]leucine, however, was unaffected by lactate-induced intracellular acidification (Figure 5A). Moreover, we found that the pH-dependence for leucine uptake was the same as for leucine efflux (Figure 5B). As a result, it appears that the pH-dependence of mB^0AT1 is caused by an exofacial modifier site, but not by any involvement of protons in the transport process. To disclose a possible K^+ antiport, we determined leucine-induced inward currents in the presence or absence of 50 mM extracellular KCl (Table 2). After subtracting the K^+-induced endogenous inward currents, leucine-induced currents were only slightly reduced by the presence of an elevated extracellular KCl concentration. As reported previously, mB^0AT1 was also not chloride-dependent (Table 2). In summary, it appears that only Na^+ ions and amino acid substrates are involved in the mB^0AT1 co-transport mechanism. To determine the Na^+–amino acid co-transport stoichiometry directly, we performed uptake experiments under voltage-clamp conditions. The uptake of [^{14}C]leucine by non-injected oocytes was subtracted in all experiments, whereas currents were used uncorrected because neutral amino acids did not induce inward currents in non-injected oocytes (< 2 nA). In $n = 7$ experiments, a stoichiometry of Na^+:amino acid of 0.7 ± 0.2 was obtained.

Mutual interactions between the substrate and co-substrate

The analysis of the co-transport stoichiometry indicates that mB^0AT1 is a 'simple' 1 Na^+–amino acid co-transporter. As pointed out in the Introduction, the kidney cortex transporter for neutral amino acids has been characterized by a decrease in the substrate K_m with increasing concentrations of the co-substrate. To investigate whether this is true for the cloned mB^0AT1, we determined the $K_{0.5}$ value for leucine at different NaCl concentrations (Figure 6). The $K_{0.5}$ value for leucine decreased from 4.6 ± 1.2 mM to 3.9 ± 1.1 mM to 1.1 ± 0.3 mM when the NaCl concentration was increased from 10 to 30 to 100 mM respectively. Also, the I_{max} was reduced by approx. 50 % when the extracellular Na^+ concentration decreased from 100 to 10 mM.

We also found an effect of leucine on the $K_{0.5}$ value of Na^+ (Figure 4). The $K_{0.5}$ for Na^+ fell from 58 ± 3 mM to 21 ± 2 mM to 7 ± 1 mM when the leucine concentration was raised from 0.1 to 5 to 30 mM respectively. Similar to the influence of Na^+ on I_{max}, maximal currents were also reduced by a low leucine concentration. For example, the I_{max} at 0.5 mM leucine amounted to 63 % of the I_{max} at 5 mM leucine (results not shown). In summary,

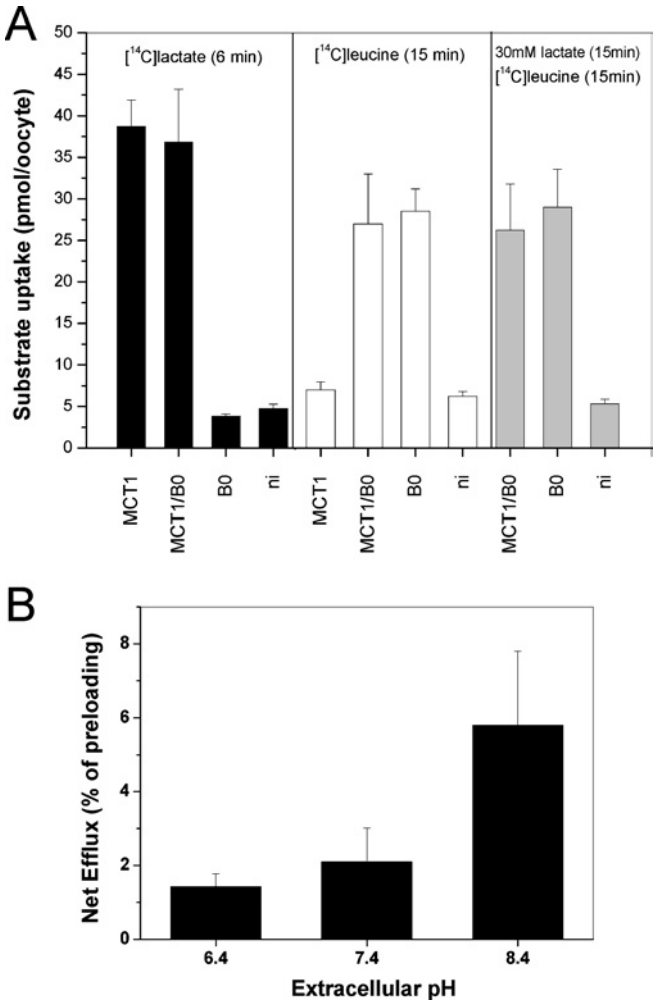

Figure 5 Influence of the pH on leucine transport via mB^{0}AT1

Oocytes were each injected with mB^{0}AT1 cRNA (23 ng), rMCT1 cRNA (10 ng), rMCT1 (10 ng) plus mB^{0}AT1 (23 ng) cRNA, or remained uninjected in the controls. (**A**) Uptake of L-[^{14}C]lactate (100 μM, left panel) and of L-[^{14}C]leucine (100 μM, middle panel) was studied in oocytes expressing rMCT1, mB^{0}AT1, rMCT1 plus mB^{0}AT1 and in non-injected oocytes to demonstrate active expression of the two transporters. To investigate the effect of intracellular acidification, [^{14}C]leucine uptake (100 μM, right panel) was studied after lowering the intracellular pH by incubation with 30 mM lactate. (**B**) Oocytes were injected with a final concentration of 10 mM [^{14}C]leucine. Subsequently, leucine efflux was studied at different extracellular pH measurements in the presence of 50 mM KCl to enhance efflux by depolarization. Each datum point represents the mean $\pm$ S.D. transport activity of $n = 5$ oocytes (the experiment was repeated three times). The transport activity of non-injected oocytes was subtracted. Results are from experiments performed 5–8 days after injection.

substrate and co-substrate can affect each other's K_m value, and both can limit I_{max}.

Effect of the membrane potential on kinetic parameters of amino-acid-induced currents

Leucine-induced currents were inwardly directed between -150 mV and $+50$ mV (Figure 1B). Although oocytes contain a total of 1 mM neutral amino acids, reversal of currents would only be expected at holding potentials above $+77$ mV, assuming a 1:1 Na^+–substrate co-transport stoichiometry and using the equation $[S_i]/[S_o] = ([Na_o]/[Na_i]) \times 10^{-(zF\Delta\Psi/2.3RT)}$ [where z is the number of charges, F is the Faraday constant (96 500 C $\cdot$ mol^{-1}), $\Delta\Psi$ is the difference in membrane potential, R is the universal gas constant

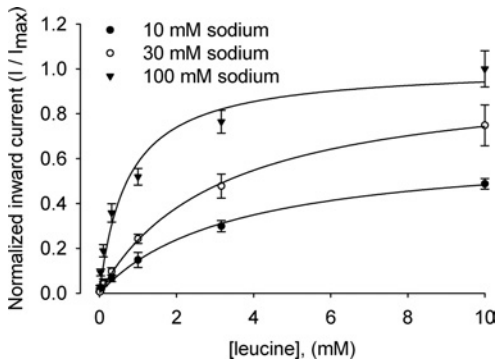

Figure 6 Interactions between substrate and co-substrate in mB^{0}AT1-expressing oocytes

Oocytes were injected with mB^{0}AT1 cRNA and incubated for 5 days. Subsequently, oocytes were held at a membrane potential of -50 mV and superfused with different concentrations of leucine in either ND96 (▼) or ND96 in which 90 mM NaCl (●) or 70 mM NaCl (○) were replaced by NMDG-Cl.

and T is the absolute temperature in Kelvin]. This confirms that substrate transport is coupled with Na^+ ions.

The membrane potential influenced both the $K_{0.5}$ value and I_{max} of amino acid transport via mB^{0}AT1. At more negative holding potentials (i.e. higher driving force), the $K_{0.5}$ values of both Na^+ and leucine decreased (Figures 3 and 7). However, the $K_{0.5}$ value of Na^+ remained at 16 mM at holding potentials more negative than -90 mV. By contrast, the I_{max} increased steadily in response to more negative holding potentials (Figure 7).

DISCUSSION

Physiological significance

We and others [21,22] have shown recently that mutations in the human B^{0}AT1 (SLC6A19) cause Hartnup disorder. This indicates that B^{0}AT1 is a major transport activity for neutral amino acids in the kidney and intestine. The results of the present study, together with previous studies, strongly suggest that B^{0}AT1 is the molecular correlate of the neutral amino acid transport activity characterized in kidney cortex (S1 and S2 segment) and jejunum: first, a Na^+ ion to substrate stoichiometry of 1:1 was confirmed. Secondly, substrate $K_{0.5}$ values are in the range of values reported previously (e.g. see [8]). Thirdly, the substrate $K_{0.5}$ value decreases with increasing Na^+ concentration. Fourthly, *in situ* hybridization [10] and immunohistochemistry [22] demonstrate expression of the transporter in the convoluted part of the proximal tubule (S1 and S2), but not in the pars recta (S3). Fifthly, the transporter is localized in the brush-border membrane on the apical side [22].

The two additional Na^+-dependent neutral amino acid transporters observed in the S3 segment of the proximal tubule remain to be identified. It is tempting to speculate that other members of the SLC6 family are also apical transporters, which is supported by the recent identification of SLC6A20 as the IMINO system [25,26]. The orphan transporter XT2 (SLC6A18), for example, is expressed at high levels in the kidney outer medulla ([27,28], and S. Kowalczuk and S. Bröer, unpublished work). XT2 is the closest homologue of B^{0}AT1, and has recently been suggested to be a glycine transporter [29]. However, mice nullizygous for XT2 also showed increased levels of other neutral amino acids in the urine. Elucidation of the transport activities of this orphan transporter will provide further insight into amino acid reabsorption in the kidney.

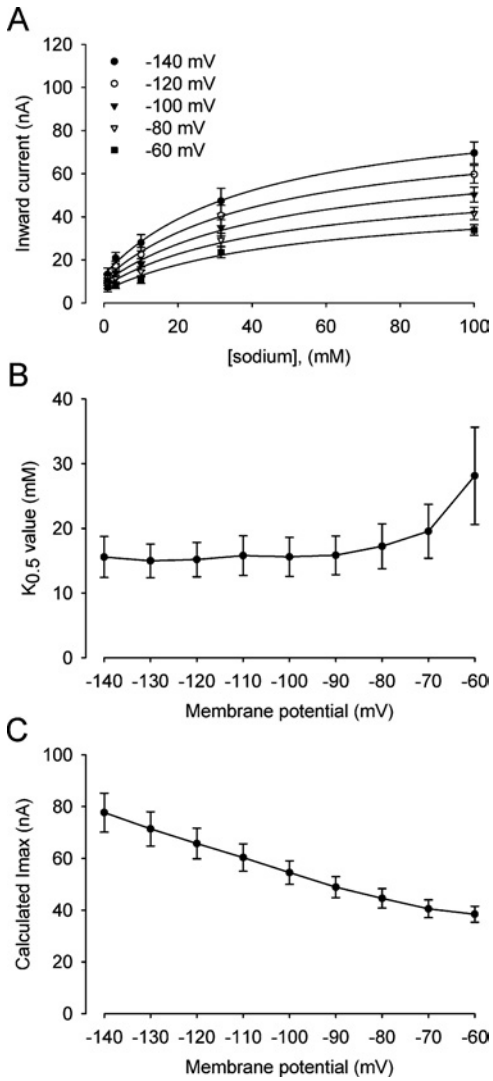

Figure 7 Kinetic parameters of mB^{0}AT1 activity as a function of the membrane potential and the extracellular leucine concentration

Oocytes were each injected with 23 ng of mB^{0}AT1 cRNA. (**A**) After incubation for 5 days, oocytes were superfused with ND96 (pH 7.4) or with ND96 where NaCl was substituted with NMDG-Cl to give the indicated concentration. Inward currents were induced by 5 mM leucine. The holding potential was changed from − 60 mV to − 140 mV in steps of 10 mV. Each datum point represents the mean ± S.E. of $n = 22$ oocytes. The calculated $K_{0.5}$ values (**B**) and I_{max} values (**C**) are plotted in relation to the holding potential.

To our surprise, we found that the K_m values determined previously for glutamine and phenylalanine by us [10] are significantly lower than those determined by amino-acid-induced currents in the present study. Owing to the low expression level of mB^{0}AT1, we could not evaluate uptake of labelled amino acids at concentrations above 3 mM. As a result, we might have underestimated the transport rate at higher concentrations in our earlier study. Moreover, kinetic parameters vary with the prevalent electrochemical gradient, rendering it difficult to compare $K_{0.5}$ values between studies unless they are determined under identical conditions (Na^+ gradient, substrate gradient and holding potential). Variability of kinetic parameters is also introduced by unstirred layer effects [30]. The apparent concentration of substrates at the binding site can be significantly lower when unstirred water layers act as diffusion barriers. For example, much higher K_m values were reported in studies using intact tissues [18] compared with studies using vesicles or oocytes, where the unstirred layers are much smaller. It is therefore likely that the lower K_m values reported here and in vesicle studies [8] are closer to the true affinity of the substrate-binding site. The derivation of kinetic constants also requires that transport is measured under zero-*trans* conditions. In vesicles, zero-*trans* conditions are present at the beginning of the experiment, but substrates quickly accumulate during the experiment. In oocytes, zero-*trans* conditions cannot be obtained due to the presence of intracellular amino acids, but these remain largely unchanged during the experiment. The charge:flux ratio of 0.7 indeed suggests that approx. 30 % of amino acid uptake occurs by the way of an exchange process. This appears plausible, because the combined intracellular concentration of all neutral amino acids in oocytes is approx. 1 mM [31].

No heterogeneity of the B^0-type activity has been reported in the intestine, which is supported by the observation that an oral loading of tryptophan in Hartnup disorder patients does not increase its plasma levels [32]. The mechanistic properties of the B^0-type transport activity in the intestine [12–14,16–20] fits well with properties of the cloned mouse B^{0}AT1, as reported here. As a result, it appears likely that B^{0}AT1 is the only transport activity for large neutral amino acids in the small intestine, whereas smaller amino acids are also transported by ASCT2 [13] and PAT1 [15].

Mechanism

Our data suggests that mB^{0}AT1 is a Na^+–amino acid co-transporter that operates with a 1:1 co-transport stoichiometry. We found a mutual influence between substrate and co-substrate: increasing concentrations of Na^+ reduced the $K_{0.5}$ value for leucine and increasing concentrations of leucine reduced the $K_{0.5}$ for Na^+. Furthermore, low concentrations of both Na^+ and leucine reduced the V_{max} value for the transport process. In an ordered binding mechanism, the maximum velocity is determined by the (co)substrate that binds last to the transporter [33], because diffusion of the first ligand to its binding site is usually much faster than turnover of the transporter. As a result, there will always be a sufficient number of partially loaded transporters, and V_{max} can be reached by providing excess amounts of the second ligand. Given that V_{max} is affected by both, i.e. the concentration of leucine and Na^+, we suggest that the binding order of Na^+ and amino acid is random. No information is currently available to deduce the binding order on the cytosolic face of the transporter. An electrogenic Na^+–amino acid co-transport mechanism may involve either a positively charged carrier–substrate complex or a negatively charged empty carrier. Because of the small currents associated with expression of mB^{0}AT1 in oocytes, we did not attempt to resolve pre-steady-state currents, which could reveal charges associated with the transporter. Instead, we used a kinetic model developed by Geck and Heinz [34], which applies to the mechanism of B^{0}AT1 and uses kinetic parameters to discriminate between both scenarios. This model predicts for an empty carrier with a nominal charge of − 1 that both the apparent $K_{0.5}$ values of the substrate and co-substrate increase at more negative membrane potentials. The alternative model, proposing a positively charged ternary complex, by contrast, predicts a decrease of the apparent $K_{0.5}$ at more negative membrane potentials, as observed in our experiments. The alternative model, which we could verify with our experimental data, suggests that the most significant charge movements in mB^{0}AT1 are associated with the fully loaded transporter. It must be pointed out that the nominal charges in this model may reflect the combined movement of several charged residues during the transport cycle. The charged ternary complex also explains the lack of *trans*-stimulation, because outward

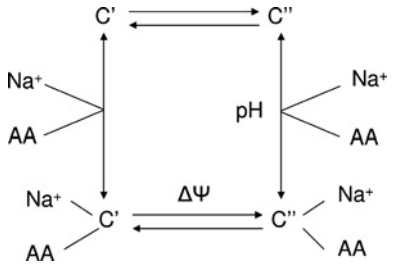

Figure 8 Kinetic scheme of the mouse B^0AT1 transporter

The kinetic model shows four states: the empty carrier and the fully loaded carrier with binding sites facing the cytosol (') or the extracellular space (''). The carrier itself is nominally uncharged. For simplicity, random binding/unbinding is assumed to occur on both sides of the membrane, although functional data to support this is only available for exofacial side. Steps are marked that are affected by changes of the pH or the membrane potential. AA, amino acid.

movement of the loaded transporter is opposed by the membrane potential. *trans*-Inhibition by Na^+ is caused by accumulation of partially loaded transporters on the inside, which are transport-incompetent. Co-injection of substrate saturates the substrate-binding site and allows the transporter to undergo exchange.

The kinetic model depicted in Figure 8 summarizes the results of the present study, and suggests random binding of substrate and co-substrate and a positively charged carrier–substrate–co-substrate complex.

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