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Research Article

Aspartate 338 contributes to the cationic specificity and to driver-amino acid coupling in the insect cotransporter KAAT1

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Abstract. To investigate the peculiar ionic specificity of KAAT1, an Na^+- and K^+-coupled amino acid cotransporter from Lepidoptera, a detailed analysis of membrane topology predictions was performed, together with sequence comparison with strictly Na^+-dependent mammalian cotransporters from the same family. The analysis identified aspartate 338, a residue present also in the other cotransporter accepting K^+ (CAATCH1), but absent in most mammalian transporters that have, instead, an asparagine in the corresponding position. Mutation of D338 in KAAT1 led either to non-functional transporters (D338G, D338C), or to an altered ionic selectivity (D338E, D338N), observable in uptake experiments and in electrophysiological properties. In particular, in D338E, the transport activity, while persisting in the presence of Na^+, appeared to be completely abolished in the presence of K^+. D338E also showed uncoupling between transport-associated current and uptake. The opposite mutation in the γ-aminobutyric acid transporter rGAT-1 (N327D) resulted in complete loss of function. In conclusion, aspartate 338 in KAAT1 appears to be important in allowing K^+, in addition to Na^+, to drive the transport mechanism, although other residues in different parts of the protein may also play a role in the complete determination of ionic selectivity.

Key words. KAAT1; amino acid transport; structure/function; ion selectivity; *Manduca sexta*.

The neutral amino acid cotransporter KAAT1, cloned from the intestine of the lepidopteran larva *Manduca sexta* [1], can drive the uptake of nutrients using either the Na^+ or the K^+ gradient [2]. While probably representing an adaptation to the peculiar physicochemical conditions of the intestine of this larva, this feature offers the possibility to investigate the structural determinants of ionic specificity in cotransporters. In fact, KAAT1 shares important structural and functional similarities with other members of the well-known family of Na^+- and Cl^--dependent cotrasporters, which are mainly involved in neurotransmitter reuptake [3]. The ability to use potassium is shared with another lepidopteran transporter, CAATCH1, which is very similar (90% identity) to KAAT1 [4]. The degree of identity with the mammalian members of this family, which are strictly sodium dependent, is instead much lower (about 35%). Clearly, the ability of the lepidopteran transporters to operate with potassium must be located in structural differences between the lepidopteran and mammalian transporters, and efforts to identify structural correlates of ionic specificity by mutating residues conserved in mammalian, but absent in insect transporters have already been attempted [5].

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Beside being strictly Na^+ dependent, the mammalian neurotransmitter transporters also show, in general, a very narrow selectivity for the organic substrate. Conversely, KAAT1 and CAATCH1, as most intestinal transporters, transport a wider spectrum of amino acids [1, 4]. Furthermore, recent data on the lepidopteran transporters [4, 5; our unpublished observations] suggest that the selectivity order for organic substrates may change according to the cation used to drive the process. For example, in CAATCH1 in the presence of Na^+, the transport-associated currents are larger with proline compared to threonine, while in the presence of K^+, the opposite is true [4]. In KAAT1, the selectivity sequence with K^+ is Met > Leu > Phe > Thr > Gly >> Pro, while with Na^+ it is Pro > Thr > Gly = Met > Leu > Phe [5].

These studies were performed by measuring amino acid uptakes or transport-associated currents in transporter-expressing *Xenopus* oocytes exposed to different ionic conditions. However, the interaction of ions with cotransporters may also be investigated considering other features of transporter activity in the absence of the organic substrate, namely the presteady-state and uncoupled currents, which are characteristic properties of most cotransporters from both vertebrates and invertebrates [6–11].

Different strategies may be followed when planning mutations aimed to investigate ionic specificity. In Liu et al. [5], nine divergent residues in KAAT1 were mutated back to the conserved form of the superfamily, but this multiple mutant was not functional. Other mutations were performed by these authors in KAAT1, based on effects observed in other transporters. Among these, Y147F showed very interesting results in terms of ionic selectivity alterations: while in the KAAT1 wild-type, the transport-associated currents for Met, Leu and Phe were larger in the presence of Na^+ than in the presence of K^+, in KAAT1 Y147F, the currents became larger in K^+ [5]. Shifts in efficiency between Na^+ and K^+ may also occur naturally: in wild-type KAAT1, the leucine transport-associated current in Na^+ is larger than in K^+ for membrane potentials more positive than –70 mV, while at more negative potentials, the opposite is true [1, 9].

Negatively charged residues may be expected to play an important role in defining cationic specificity, and the effect of the membrane potential mentioned above suggests that especially those residues located in the membrane-spanning domains may be involved in interaction with ions.

Therefore, we directed our attention to negatively charged residues located in the putative transmembrane domains of KAAT1, in an attempt to modify the wild-type selectivity through appropriate mutations.

Materials and methods

Site-directed mutagenesis

The mutants KAAT1 D338C, D338E, D338G, D338N and GAT-1 N327D were synthesized by PCR using the Quick Change Site-Directed Mutagenesis Kit (Stratagene) and the following primers:

D338C: 5′gattgtaacaactttgtgcacgttcacaagtttcttgtctggg 3′
D338G: 5′gattgtaacaactttgggcacgttcacaagtttcttgtctg 3′
D338E: 5′gattgtaacaactttggagacgttcacaagtttcttgtctg 3′
D338N: 5′gattgtaacaactttgaacacgttcacaagtttcttgtctggg 3′
N327D: 5′ggactccatcattgtttgctgcatcgactcctgcaccag 3′

DNA sequencing confirmed the mutations.

Oocyte expression of wild-type and mutated transporters

For uptake and electrophysiological experiments on KAAT1, a p-SPORT1 plasmid containing wild-type or mutant KAAT1 cDNA was *Not*1 digested, in vitro capped and transcribed using T7 RNA polymerase (Stratagene, La Jolla, Calif.). For the experiment on rGAT-1, cDNA encoding the rat GAT1 cotransporter was cloned into the pAMV-PA vector. After linearization with *Not*1, cRNA was synthesised in vitro in the presence of Cap Analog and 200 units of T7 RNA polymerase. All enzymes were supplied by Promega Italia (Milan, Italy).

Oocytes were isolated from mature *Xenopus laevis* female frogs and manually defolliculated after treatment with 1 mg/ml collagenase A (Roche, Basel, Switzerland) in the Ca^{2+}-free buffer OR II (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES/Tris pH 7.5) for 20–30 min at room temperature. Healthy oocytes were then selected and maintained at 17 °C in Barth's medium [88 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 0.33 mM $Ca(NO_3)_2$, 2.4 mM $NaHCO_3$, 10 mM HEPES/Tris pH 7.5] supplemented with 2.5 mM pyruvic acid and 50 mg/l gentamycin sulfate. The day after, oocytes were injected with 50 nl of the synthesized cRNA (12.5 ng/oocyte) using a manual Drummond injection system. Non-injected oocytes were considered as control.

Transport experiments

Amino acid uptake was measured 3 days after injection. Groups of eight to ten oocytes were incubated in 100 µl of uptake solution (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris pH 8) with [^{3}H] leucine (1000 KBq/ml), for KAAT1-induced uptake. For GAT-1, the uptake solution was at pH 7.6 in the presence of [^{3}H] γ-aminobutyric acid (GABA) (1000 KBq/ml). Then, oocytes were washed in ice-cold wash solution (100 mM choline chloride, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES/Tris pH 8) and dissolved in 250 µl of 10% SDS for liquid scintillation counting. Experiments in which the expression level of wild-type KAAT1 was less than five times above background were discarded.

In several figures, the KAAT1-mediated transport is plotted as the difference between the mean uptake measured in cRNA-injected oocytes and the mean uptake observed in non-injected oocytes.

Kinetics

Na^+ activation experiments and leucine kinetics were performed on KAAT1 wild-type, D338E and water-injected oocytes. The experiments were performed incubating oocytes for 5 min in the uptake solution. In Na^+ activation experiments, Na^+ concentration ranged from 0 to 130 mM and NaCl was osmotically replaced by choline chloride. The L-[^{3}H]-leucine concentration was 1 mM. In leucine kinetics, the L-[^{3}H]-leucine concentration ranged from 25 to 2000 µM (2500–6200 KBq/ml).

Kinetic parameters were calculated using a multiparameter, iterative, non-linear regression program (SigmaPlot; SPSS, Chicago, Ill.).

Electrophysiology

The classical two-electrode voltage-clamp was used to perform electrophysiological experiments (Geneclamp; Axon Instruments, Union City, Calif.). For KAAT1-expressing oocytes, the holding potential was kept at –60 mV and the typical protocol consisted of 200-ms voltage pulses spanning the range –160 to +20 mV in 20-mV steps; for experiments on rGAT-1, the holding potential (V_h) was kept at –40 mV, the voltage pulses were 400-ms long and the voltage range was from –120 to +80 mV. Four pulses were averaged at each potential; signals were filtered at 1 KHz and sampled at 2 KHz. Experimental protocols, data acquisition and analysis were done using the pClamp 8 software (Axon Instruments). Presteady-state currents were isolated by separation of the fast and slow component of the current relaxation by double exponential fitting, as described elsewhere [12]. The external control solution was composed as follows (in mM): NaCl 98, $CaCl_2$ 1.8, $MgCl_2$ 1, Hepes-free acid 5, at pH 7.6; in the other solutions, NaCl was replaced by tetramethylammonium chloride (TMACl), KCl and LiCl. The pH was adjusted to 7.6 by adding the corresponding hydroxide for each alkali ion and TMAOH for TMA^+ solution. In electrophysiological experiments, leucine, proline, threonine and methionine (0.5 mM), or GABA (100 µM) were added to induce transport-associated currents. The rGAT-1-specific blocker SKF89976A was used at a concentration of 30 µM.

Cell surface biotinylation of oocytes

The cell surface expression of GAT-1 wild-type and the mutant N327D was tested using a membrane-impermeant reagent, EZ-Link Sulfo-NHS-SS-Biotin (Pierce, Rockford, Ill.). Groups of ten oocytes were rinsed three times with ice-cold PBS, pH 8. The oocytes were then incubated on ice in 750 µl PBS containing EZ-Link Sulfo-NHS-SS-Biotin (1.3 mg/ml) for two successive 15-min periods. After labeling, the oocytes were rinsed briefly with ice-cold PBS and incubated in 50 mM glycine in PBS for 15 min on ice to quench the biotinylation reagent. The oocytes were then dissolved in lysis buffer (150 mM NaCl, 20 mM Tris/HCl, pH 7.6, 1% Triton X-100) by gentle shaking on ice for 40 min. The solubilized oocytes were centrifuged for 15 min at 13,000 g and the supernatants transferred to new tubes. The biotinylated proteins were recovered from the supernatant solution by adding 50 µl of ImmunoPure Immobilized Streptavidin beads (Pierce), and incubating for 1.5 h with gentle agitation on ice. The biotin-streptavidin-agarose bead complexes were washed three times by suspension in lysis buffer followed by centrifugation. The final pellets were resuspended in the appropriate sample buffer and boiled for 2 min before being loaded onto a 10% polyacrylamide gel for SDS/PAGE. The transporters were detected by Western blotting with affinity-purified antibody to GAT-1 at a 1:1,500 dilution, horseradish peroxidase-conjugated secondary antibody at a 1:10,000 dilution and enhanced chemiluminescence (Amersham, Chicago, Ill.).

Results

Membrane topology of KAAT1

The prediction of topology for a membrane protein may be very helpful when selecting mutations to perform for structure-function studies. However, the computational methods that predict transmembrane segments based on calculated hydrophobicity must only be considered as indications, and different methods often give different results, especially in ambiguous portions of the sequence. In the case of KAAT1, for example, the two published topologies [1, 5] are considerably diverse, and we therefore judged it worthwhile to compare various methods of topology prediction available on the web. The results, in terms of number of transmembrane domains (TMs), from the eight selected methods are shown in table 1: four predict 11 TMs, while the others predict 12 (or 13) TMs. The hydrophobicity profiles of those predicting only 11 segments show, however, an initial region of the sequence possessing a certain degree of hydrophobicity, though not sufficient to reach the threshold to assign it to a TM. Figure 1 shows a comparison between two of these methods and the topologies previously reported [1, 5]. Although all four models predict 12 TMs, the exact positions of the helices clearly differ considerably. Having decided to concentrate our attention on transmembrane amino acids bearing a negative charge, Figure 1 shows that there are 11 potential residues satisfying this requirement, although not all prediction methods are in agreement. Table 2, illustrating the degree of agreement of the different methods in locating these residues to TMs, shows that only D338 is assigned by all eight predictions.

Table 1. KAAT1 TM predictions.

Method	HMMTOP	TMHMM	TMAP	SOSUI	MEMSAT2	TMPRED	PREDTMR2	DAS
Reference	36	37	38, 39	40	41		42	43
Number of TM domains	12	11	11	12	12	13/12*	11	11

* The model with 13 TMs is preferred.

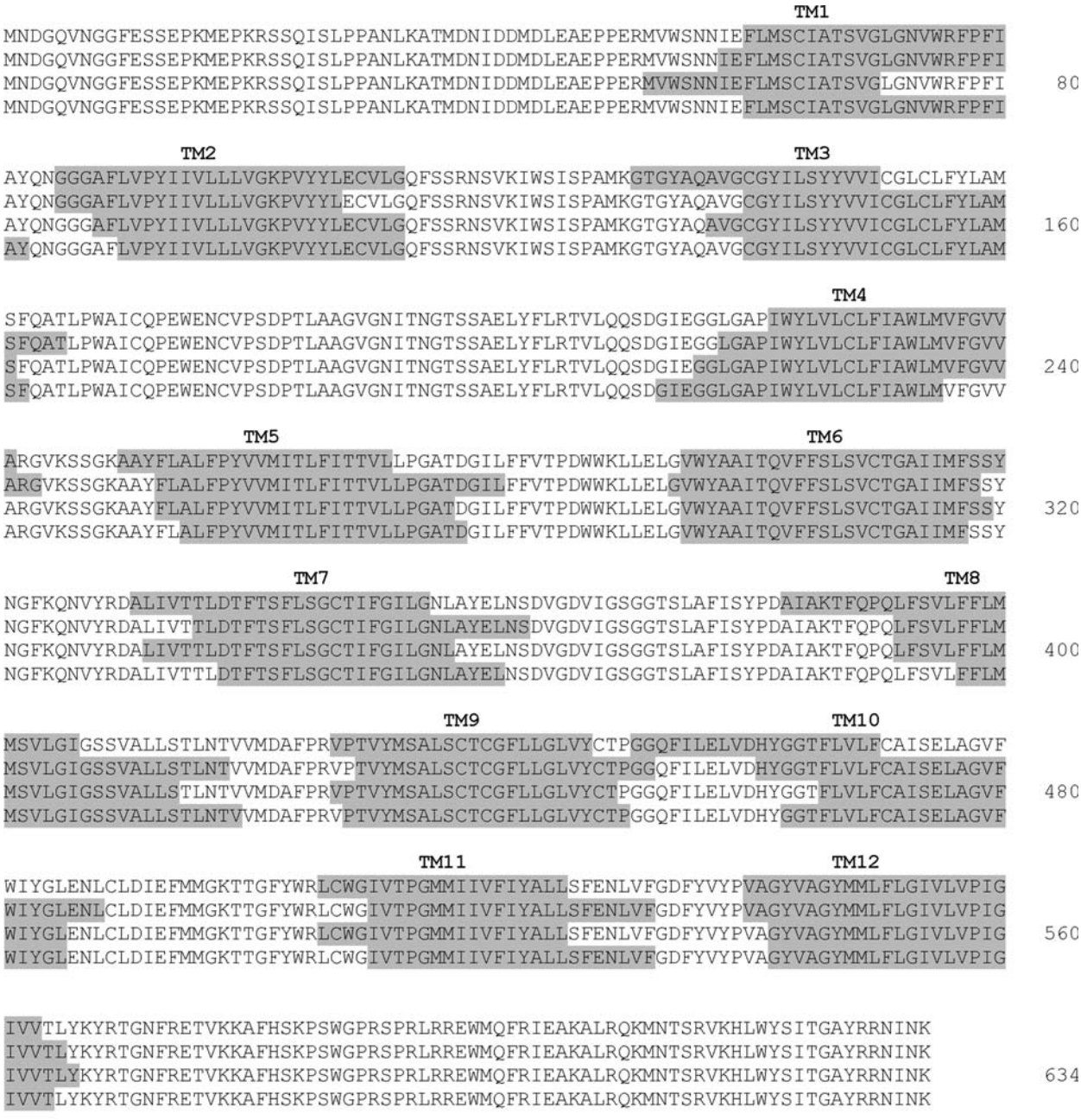

Figure 1. KAAT1 TM predictions obtained from Castagna et al. [1] (line 1, Kyte-Doolittle), from Liu et al. [5] (line 2), HMMTOP (line 3), and SOSUI (line 4).

Table 2. Assignment to a TM of specific negative residues by the indicated method.

Residue	Kyte-Doolittle [1]	[5]	HMMTOP	SOSUI	TMHMM	TMAP	TMPRED	MEMSAT2	Total
E59	no	yes	yes	no	no	no	yes	yes	4/8
E108	yes	no	yes	yes	no	no	no	no	3/8
E215	no	no	no	yes	no	no	no	no	1/8
D277	no	yes	no	yes	no	yes	no	no	3/8
D338	yes	yes	yes	yes	yes	yes	yes	yes	8/8
E359	no	yes	no	yes	no	no	no	no	2/8
E457	yes	no	no	no	no	no	no	no	1/8
D460	yes	no	no	no	no	yes	no	no	2/8
E475	no	yes	yes	yes	yes	yes	yes	yes	7/8
E486	no	yes	no	no	no	yes	no	no	2/8
E528	no	yes	no	yes	no	yes	no	no	3/8

Table 3. Residue correspondence between KAAT1 and other transporters of the Na^+/Cl^--dependent family.

Position in KAAT1	59	108	215	277	338	359	457	460	475	486	528
KAAT1 [1]	E	E	E	D	D	E	E	D	E	E	E
rGAT1 [13]	D	E	D	E	N	V	K	D	E	N	T
rSERT [14]	D	E	G	R	N	M	T	E	E	T	P
hDAT [15]	D	E	L	D	N	K	T	D	E	G	R

Other interesting observations arise when the amino acid sequence of KAAT1 is compared to those of the mammalian members of the Na^+/Cl^- dependent neurotransmitter Transporter family. Concentrating on the 11 positions listed in table 2, the corresponding residues in the three representative mammalian cotransporters rGAT1, rSERT and hDAT [13, 15] are identical or show a conservative E/D substitution in 4 instances (E59, E108, D460, E475), while in the remaining 7, different kinds of residues are present (table 3). E457 is predicted to be transmembrane only by the Kyte-Doolittle method [1], while the other methods, and the topologies of most mammalian transporters as well, assign this residue to the extracellular loop connecting the 9th and 10th TMs. Of the remaining 6, E215 is predicted to be transmembrane only by SOSUI in KAAT1, and the corresponding residue in mammalian cotransporters appears to be extracellular; E359 and E486 are predicted to be transmembrane by [5] and either the SOSUI or the TMAP method, while the corresponding residues in mammalian transporters appear to be extra or intracellular, similarly D277 and E528 are predicted transmembrane by [5], SOSUI and TMAP, but extracellular by the other methods, and in neurotransmitter transporters as well. Only D338 in KAAT1 is predicted to be transmembrane by all eight methods, and, furthermore, the residue in the corresponding position in most mammalian cotransporters of the family is an asparagine which is also predicted to be transmembrane.

From this analysis, we therefore gave priority to D338 as an interesting residue potentially involved in cation binding and selectivity.

Amino acid uptake

In figure 2 the ability of different mutated forms of KAAT1 to transport leucine at a concentration of 0.1 mM in the presence of Na^+ was compared to that of the wild type. The replacement of D338 with glycine, asparagine or cysteine, appeared to abolish leucine uptake, while the conservative substitution with a glutamate residue left significant uptake activity (about 50%) in this condition. We also tested the behavior of this last mutant in the presence of potassium and lithium, since both ions have been shown able to support amino acid uptake and transport-associated currents in KAAT1 [1, 16, 17]. The results presented in figure 3 showed that the amino acid uptake was completely abolished in K^+ and in Li^+, both at low (0.1 mM; fig. 3 A) and high (0.5 mM; fig. 3 B) leucine concentration. This observation was the

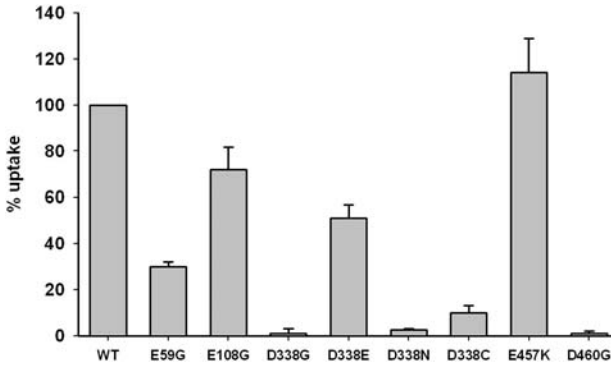

Figure 2. Leucine uptake at 0.1 mM concentration induced by the indicated mutants. Uptake is expressed as percent of the wild type (WT) and data are mean ± SE of eight to ten oocytes in at least three independent experiments.

first indication that the ionic selectivity of the transporter might have been altered.

In the presence of Na^+ and 0.5 mM leucine, the mutant exhibited a larger uptake than the wild type, which suggested a change in the apparent affinity of the mutated transporter for the amino acid. To test this hypothesis, leucine uptake as a function of leucine concentration was measured in oocytes expressing KAAT1 WT and D338E in the presence of 100 mM NaCl. The leucine kinetics shown in figure 4 indicated that the mutated transporter had a lower apparent affinity for the amino acid, the K_m value increasing from 55 ± 17 to 545 ± 201 μM (n = 3), while the Vmax value was more than twofold higher (34 ± 5 pmol/oocyte per 5 min) than in the wild-type protein (13 ± 2 pmol/oocyte per 5 min). These kinetic parameters explain why at high amino acid concentration, the mutant transport capacity was increased, whereas at low amino acid concentration it was reduced (see figs 3, 4). The apparent discrepancy between figures 3B and 4, considering the data at 0.5 mM leucine concentration, was due to the different incubation times. A long incubation time allows a better discrimination of the rates of transport processes; however, kinetics are usually carried out for a short incubation time. In kinetics experiments carried out over 60 min, the shapes of the two curves were not qualitatively different from those shown in figure 4 but the uptake by the mutant transporter at 0.5 mM leucine was approximately twofold higher than the uptake by the wild type (not shown).

To evaluate the Na^+ activation of the amino acid transport, uptake from 1 mM leucine was measured as a function of Na^+ concentration. Figure 5 shows that leucine uptake was still Na^+ dependent and that the apparent K_{50Na^+} appeared to be increased in the mutant compared to the wild-type protein. Actually, the fitting to Michaelis-Menten curves was rather poor for the mutant. K_{50Na^+} ranged from 196 to 309 mM, an indication of a deep change in the interaction with Na^+ compared to the wild type (V_{max} 10 + 1 pmol /oocyte per 5 min and K_{50Na^+} 6 ± 3 mM) [1, 18]. A better fit could be obtained adding a diffusional component to the hyperbolic curve, but understanding the molecular mechanism underlying this behavior is difficult. Anyway, the enhanced transport capacity of the mutant observed in the experiments reported in figures 3B and 4 for a high leucine concentration was confirmed by the data shown in figure 5.

Transport-associated current

We then performed voltage-clamp experiments to investigate electrophysiologically the properties of the mutated transporters. Transport-associated currents elicited by the

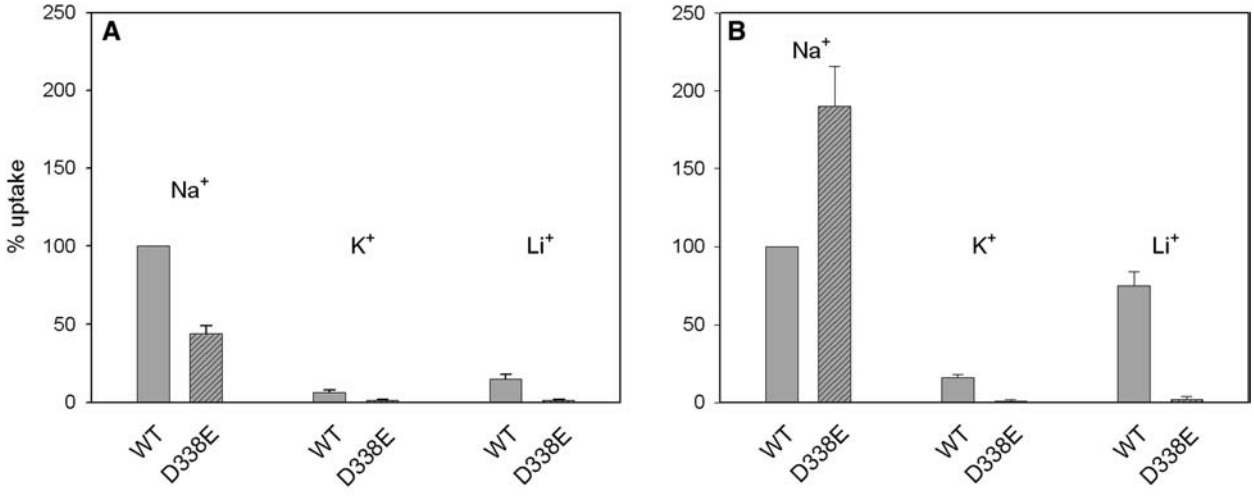

Figure 3. Wild-type (WT) and D338E KAAT1-mediated leucine uptake in the presence of different cations at 0.1 mM (*A*) and 0.5 mM (*B*) leucine concentration. Data are expressed as percent of the WT-mediated uptake in NaCl (100 mM), and are the mean ± SE of groups of eight to ten oocytes in a representative experiment. The lower values in K^+ are due to the fact that, in contrast to high Na^+ and high Li^+, oocytes are depolarized by the high-K^+ solution and, therefore, the electrical driving force is much lower.

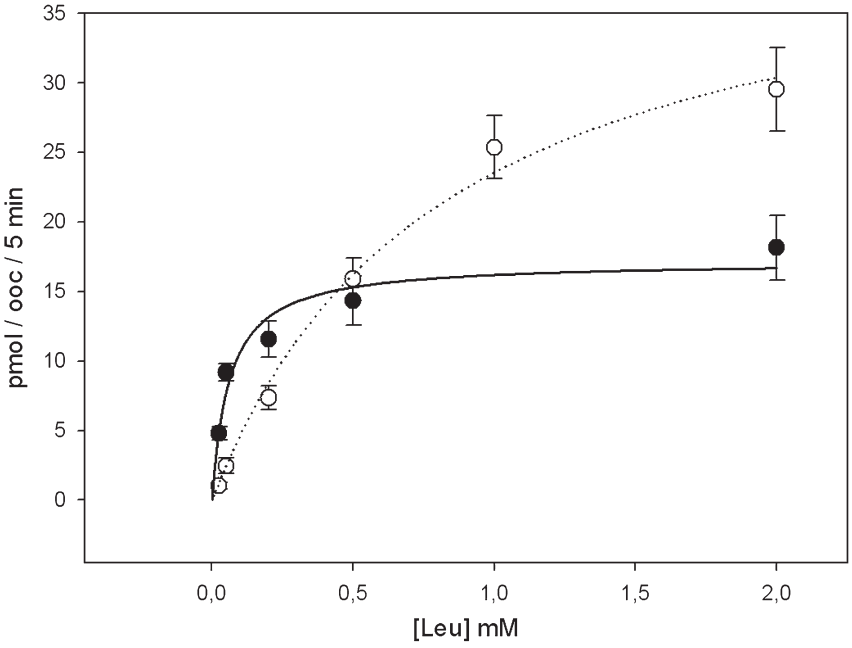

Figure 4. Kinetics of leucine uptake. Curves show wild-type (black circles) and D338E (white circles) KAAT1-mediated leucine uptake as a function of external leucine concentration, in the presence of 100 mM NaCl. Each pont is the mean ± SE of groups of 10–12 oocytes in a representative experiment.

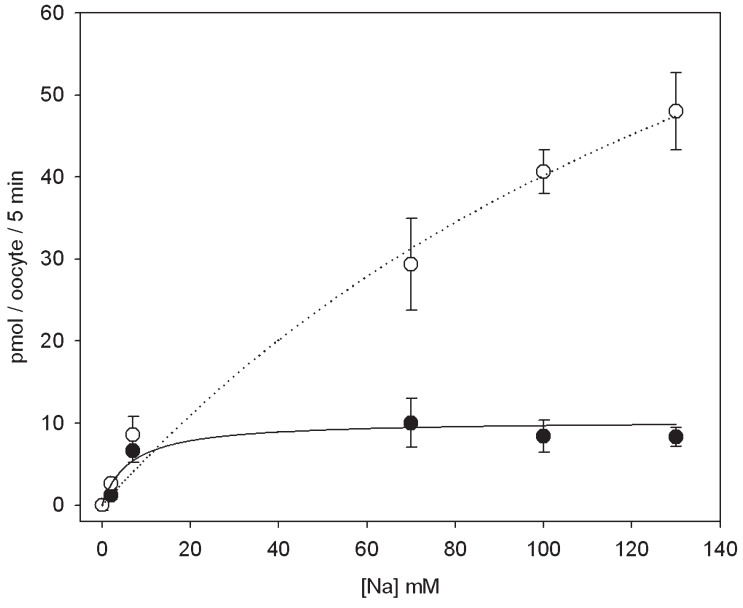

Figure 5. Na^+ activation of leucine uptake at 1 mM concentration. Curves show wild-type (black circles) or D338E (white circles) KAAT1-mediated leucine uptake measured at different Na^+ concentrations. NaCl was osmotically replaced by choline chloride. Data are the mean ± SE of groups of 10–12 oocytes in a representative experiment.

presence of various amino acids have been described for KAAT1, both in the presence of Na^+ and K^+ [1, 5]. These are shown in figure 6 for four amino acids: leucine, methionine, threonine and proline. In the wild type and in Na^+ solution, all four amino acids gave rise to significant currents, larger for proline and threonine, than for leucine and methionine. The last three amino acids were also able to elicit currents in the presence of K^+, while proline was not, confirming previous results [5]. The currents recorded in the two mutants D338E and D338N were always smaller than in the wild type, however, the degree of the reduction appeared to depend on the amino acid and on whether Na^+ or K^+ was present. Figure 6 shows that a reduced transport current was generated when leucine or

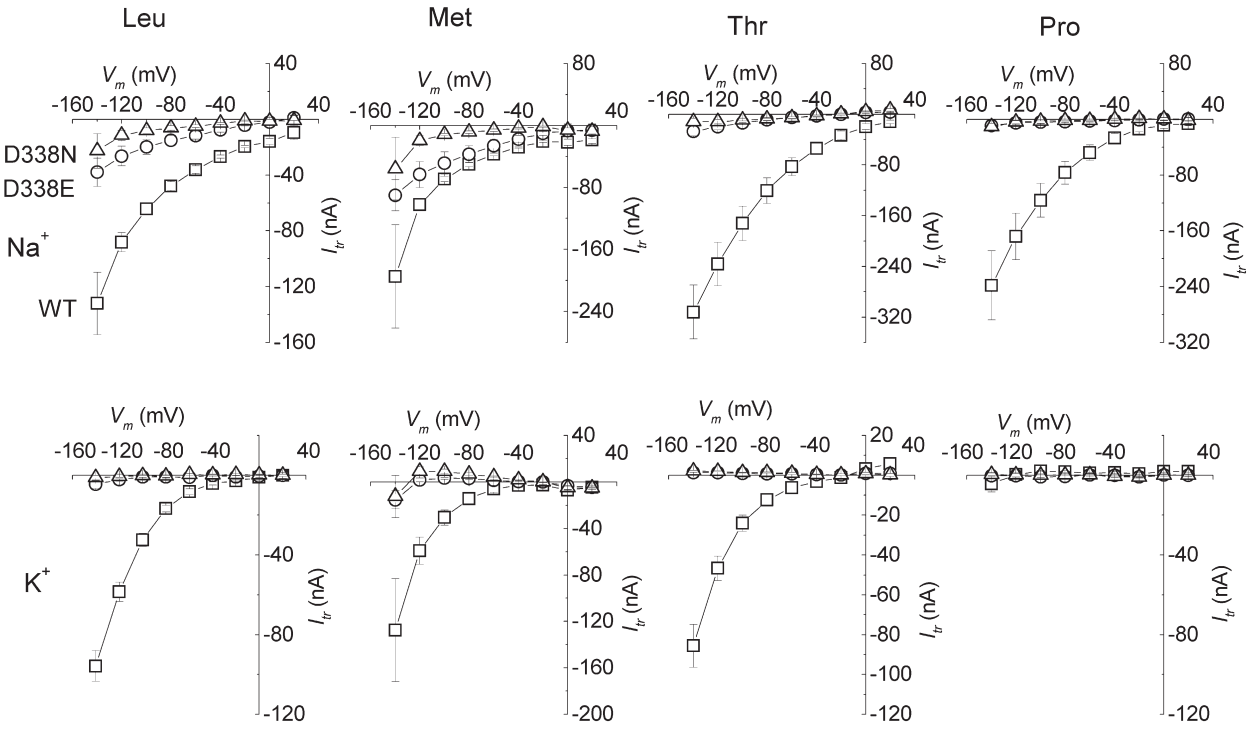

Figure 6. Voltage dependence of the current elicited by 0.5 mM of the indicated amino acids in wild-type and mutated KAAT1. In the top row, the currents were recorded in the presence of Na^+, while in the bottom row, K^+ was the predominant cation. Symbols represent wild-type (squares), and mutated (D338E circles, D338N triangles) KAAT1. Data are the mean ± SE from groups of 11–12 oocytes three batches.

methionine was added to the external solution, provided Na^+ was the main cation. No transport currents could be observed in the mutants for any of the tested amino acids when K^+ was the main cation. Clearly, these data were in agreement with the uptake results in showing the abolition of activity in K^+ and in pointing to a possible role of aspartate 338 in determining the ionic selectivity of the transporter. However, the reduction in transport current in Na^+ did not match the uptake data that showed, at the same 0.5 mM leucine concentration, no decrease with respect to the wild type (figs. 3B, 4).

Experiments to assess the effect of different leucine concentrations on the transport-associated current were also performed (fig. 7). The electrophysiological observations confirmed the reduction of the transport current in the mutant. Michaelis-Menten fitting of the data gave K_m = 21 µM and V_{max} = 74.9 nA for wild type, and K_m = 1.16 mM and V_{max} = 49.7 nA for D338E. Clearly, these results did not match the uptake data (fig. 4) in terms of V_{max}, while, in terms of affinity, the two sets of data were similar for both wild type and mutant.

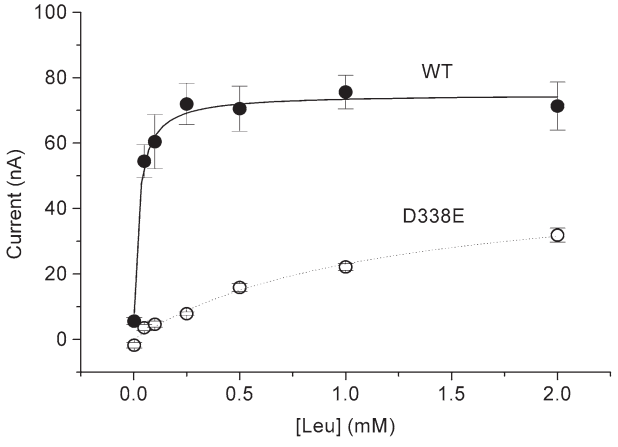

Figure 7. Leucine dependence of the transport-associated current, measured at −20 mV membrane potential for wild-type (WT) (filled symbols) and D338E (open symbols) oocytes. Data are the mean ± SE from two groups of four oocytes from the same batch.

Uncoupled current

Similar to several other cotransporters of the same family, KAAT1 displays uncoupled currents, i.e. transmembrane currents in the absence of organic substrates; these currents are particularly large when lithium is the predominant cation and the selectivity sequence is $Li^+ > Na^+ \approx Rb^+ \; K^+ > Cs^+$ [16]. The relatively high permeability to Li^+ is indeed a common feature of this kind of current in other cotransporters [19]. Figure 8 illustrates the ionic selectivity of the uncoupled current in KAAT1 wild type and in the mutated isoforms D338E and D338N. Interestingly, D338E retained the same ionic selectivity as the

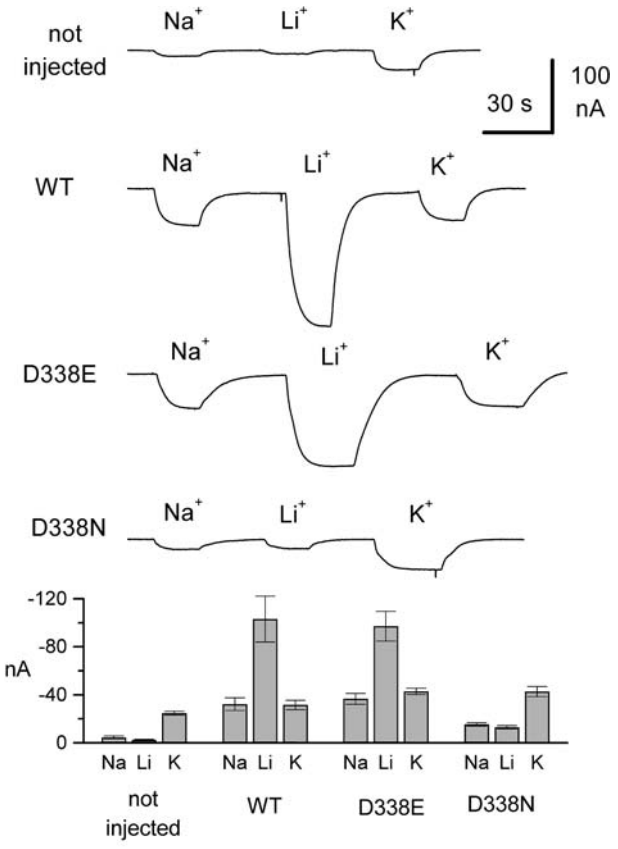

Figure 8. Ionic selectivity of the uncoupled current. Representative current traces from non-injected, wild-type and mutated KAAT1-expressing oocytes kept at $V_h = -60$ mV. The predominant cation between applications of alkali cations was TMA^+. The lower graph shows averages of the uncoupled current elicited by Na^+, Li^+ and K^+, as difference from the immediately preceding current in TMA^+. Mean ± SE from groups of six to seven oocytes from the same batch.

wild type, and also the same current amplitude, while D338N behaved more like non-injected oocytes in terms of ionic selectivity, although the current amplitudes for all three ions were significantly larger.

Transient currents

The third type of current described in cotransporters, the transient 'presteady-state' currents elicited by voltage jumps in the absence of organic substrate, was also altered in the D338E and D338N mutants. This is shown in figure 9 as original current traces for representative oocytes (non-injected and expressing wild-type, D338E and D338N KAAT1) in the presence of different cations. The presteady-state currents were particularly evident in wild-type KAAT1 in Na^+ solution, especially when compared to the traces in TMA^+ solution. In Li^+ and in K^+, the transient currents were less prominent and possessed a different voltage dependence. Indeed, subtraction of the traces in TMA^+ solution has been used previously to quantify the voltage dependence and the kinetics of the underlying charge movement [9]. Both mutants displayed smaller transients that do not appeared to be strongly affected by the kind of bathing cation. Therefore, to analyze the presteady-state currents, we used the exponential peeling method, i.e. the currents relaxing after a voltage jump were fitted with two exponentials in order to separate the endogenous passive capacity of the oocyte membrane from the KAAT1-related charge movement. The results of this analysis are shown in figure 10, which shows that in the wild type, the amount of charge moving during the slower exponential decay was different, depending on the bathing ion. In particular, in the presence of Na^+, the displaced charge significantly exceeded that in TMA^+ and Li^+. The voltage dependence of the charge movement in Na^+ was sigmoidal with a half-charge voltage ($V_{1/2}$) of −86 mV. In the same conditions, the voltage dependence of the relaxation time constant was bell-shaped (fig. 10), with a maximum in the range −80 to −100 mV, matching V1/2, and in agreement with previous determinations [9]. In the two mutants D338E and D338N, the charge movement remained substantially the same irrespective of whether the main bathing cation was Na^+, K^+ or TMA^+ (fig. 10). Correspondingly, the slower time constant was substantially independent of voltage (fig. 10).

The mutant N327D of rGAT1 is not functional

A naive expectation from the sequence comparison between the KAAT1 and the mammalian neurotransmitter cotransporters would be that changing the asparagine residue in the position corresponding to D338 in KAAT1 might relax the strict Na^+ dependence of these transporters.

To verify this possibility, we mutated N327 of rGAT1 to aspartate. The mutated N327D rGAT1 was not functional, either when tested for radioactive GABA uptake (not shown), or in producing a transport-associated current (fig. 11 A). However, some transient current was still present at depolarized potentials and this was abolished when GABA (100 M) or the specific rGAT1 blocker SKF89976A (30 µM) were added to the bath, indicating that the mutated transporter was correctly inserted into the membrane, as confirmed by membrane protein biotinylation (fig. 11 B).

The lack of functionality of the mutated transporter prevented the possibility of studying possible alterations in ion selectivity. However, this result confirmed that the residue in position 327 of rGAT1 is crucial for activity.

Discussion

The cloning of KAAT1 in 1998 [1] represented a very interesting step forward in the study of structure-function relationships in cotransporters. The larvae of various lepidopteran species were already known [2] to possess in their intestine transporters that can energize the uptake of

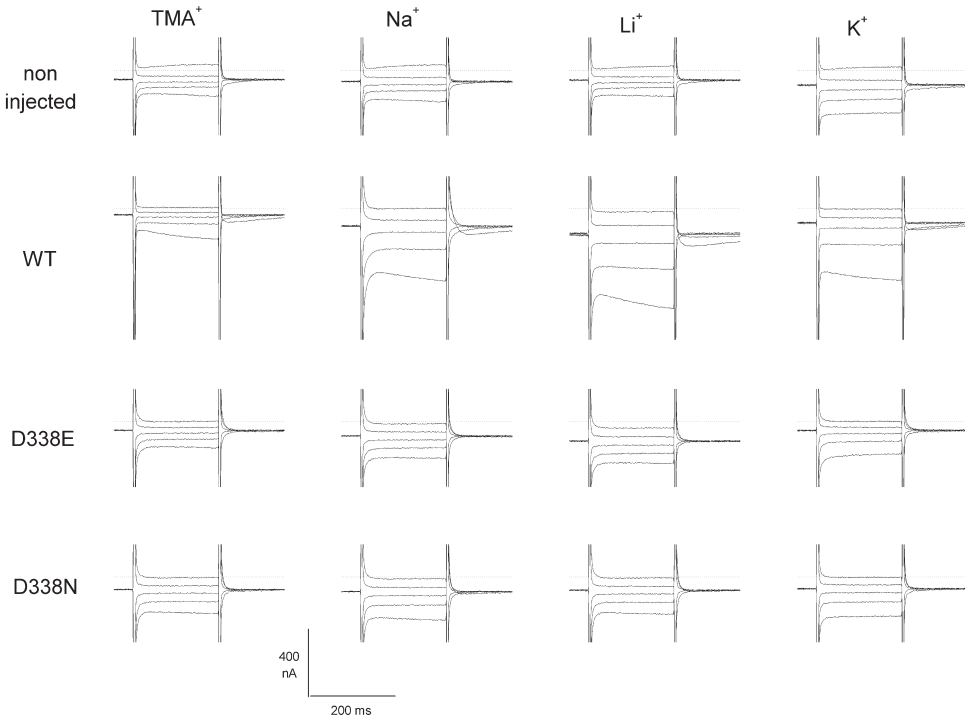

Figure 9. Presteady-state currents. Representative current traces in response to 200-ms voltage jumps from –160 to 0 mV (V_h = –60 mV) in solutions containing the indicated cation for non-injected, wild-type (WT) and mutated KAAT1 as indicated.

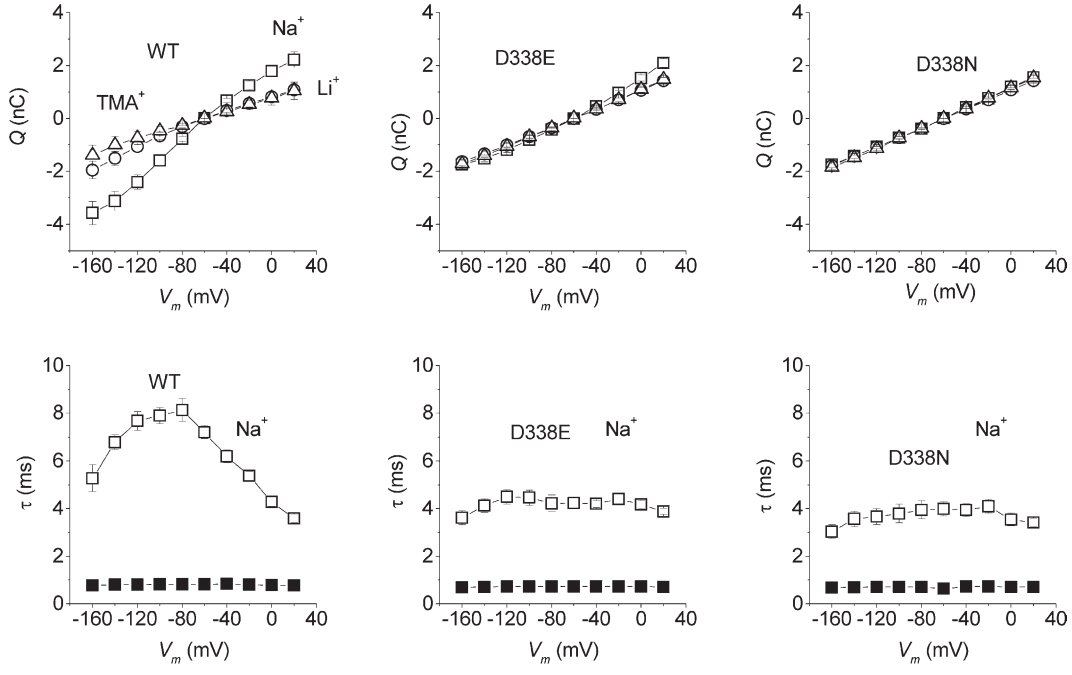

Figure 10. Analysis of presteady-state currents in wild-type (WT), D338E and D338N KAAT1. In the top row are Q/V curves obtained from integration of the peeled-off slow exponentials in the presence of the indicated ions (squares Na^+, circles Li^+, triangles TMA^+). In the lower row are the corresponding relaxation time constants of the presteady-state current in Na^+; filled squares are the time constant of the fast exponential (pooled from all conditions), indistinguishable from those derived by single exponential fitting in non-injected oocytes. Data represent mean values ± SE from 11 oocytes expressing WT KAAT1, 14 oocytes expressing D338E and 11 oocytes expressing D338N. Oocytes were pooled from four frogs.

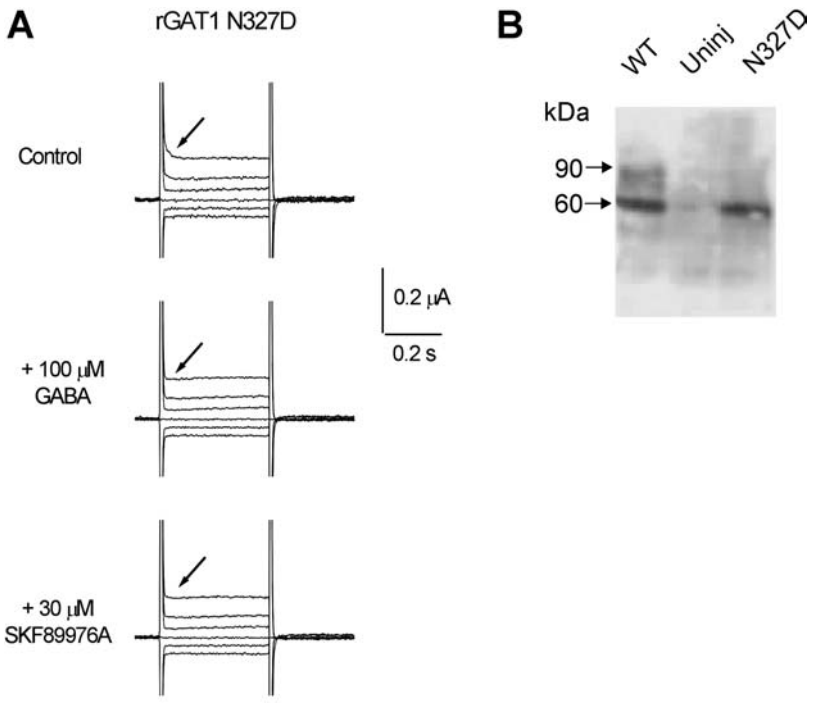

Figure 11. (*A*) Currents elicited by 400-ms pulses from –120 to +80 mV (in 40-mV steps, from $V_h = -40$ mV) in a representative oocyte injected with cRNA for rGAT1 N327D bathed in the indicated solutions. No change in steady-state level was observed upon addition of 100 µM GABA. Arrows indicate the slow transients present in control solution, which disappeared when GABA or the blocker SKF89976A were added. (*B*) Cell surface biotinylation of wild-type and N327D rat GABA transporter. Oocytes expressing the wild-type and the mutant transporters were labeled and processed as described in 'Materials and methods'. The three lanes represent the biotinylated samples of wild-type (WT), uninjected oocytes and N327D mutant. The positions of the molecular mass standards are indicated on the left.

amino acids using the K^+ gradient in place of the Na^+ gradient. This characteristic probably represents an adaptation to the peculiar ionic composition of the intestinal lumen of these animals, which is rich in potassium and poor in sodium [20, 21]. The surprising observation by Castagna et al. [1] was that KAAT1 displayed a significant sequence identity (≈35%) with the family of Na^+- and Cl^-- dependent neurotransmitter cotransporters. More recently, another protein, with different functional characteristics, but with the same peculiarity of being able to function with K^+, has been cloned from *M. sexta* [4]; this molecule, CAATCH1, shows 90% identity with KAAT1. Considering that all mammalian members of this family cannot be driven by potassium, at least in their physiological voltage range, investigating the structural determinants of the ionic selectivity in this family of cotransporters was of interest. This point has already been addressed in a recent work [5], in which the strategy was to construct a multiple mutant in which nine divergent KAAT1 residues were mutated back to the form conserved in the superfamily, or to mutate conserved residues found to be critical in the prototype of the family, the GABA transporter GAT-1. While the multiple mutant was non-functional, Y147F was found to strongly increase uptake, and to alter the amino acid and cation selectivity sequences.

Our strategy was based on preliminary identification of negatively charged residues located in the membrane electrical field. This choice was suggested by the observation that in KAAT1, the curves of the transport-associated currents as a function of voltage in Na^+ and in K^+ cross each other at about –70 to –80 mV [1, 9]. This means that for potentials more positive than this value, the current generated by Na^+ is larger than that generated by K^+, while at more negative potentials, the opposite occurs. In other words, the ionic selectivity is apparently voltage dependent, leading to the suggestion that the cotransported cation might interact with some (probably negatively) charged residue in the membrane electrical field. As shown in the Results, the predictions of membrane topology for KAAT1 by different methods were rather variable, and in only one case, that of aspartate 338, did all eight methods agree to place the residue in a TM. We have shown that mutations of this residue caused changes in KAAT1 function and, most interestingly, appeared to alter the cationic selectivity of the transporter. In the case of D338E (and to a lesser degree of D338N), the ability of potassium to activate amino acid uptake and transport-associated currents appeared to be abolished (figs. 3, 6).

In evaluating the results of the present paper, one must note that during uptake determinations, the membrane

voltage was not under control, and during transport activity it was presumably at about –20 to –30 mV because of the depolarizing action of the inward transport-associated current [12]. This fact must be remembered when comparing uptake data with electrophysiological observations, in which membrane voltage was controlled and may be moved to much more negative values.

Our results show that a negative residue in position 338 is crucial for transport: when the aspartate is substituted by glycine or asparagine no significant [^{3}H]-leucine uptake could be detected (fig. 2). Accordingly, substitution with glutamate still allowed leucine uptake at 0.1 mM concentration (≈ 50% reduction) provided that Na^+ was the major cation present. However, at higher amino acid concentrations, the uptake was enhanced in the mutated form. Leucine kinetics in wild-type and in D338E KAAT1 indicated an increase in the apparent K_m and V_{max} in the mutated protein, which could explain the observed effects. The electrophysiological data showed other interesting information, namely that in D338E and D338N and in the presence of Na^+, the reduction in current also occurred when methionine was the transported amino acid, while no current was generated by the mutants when threonine or proline were added (fig. 6). Most interestingly, the presence of aspartate in position 338 appeared to be essential for transport activity when K^+ was the dominant cation. In these conditions, both uptake (fig. 3) and electrophysiological data (fig. 6) showed no activity in the entire voltage range examined, and no differences among the four tested amino acids. The kinetics of leucine-associated currents in Na^+ confirmed the increase in the apparent K_m, but showed a decreased V_{max} in D338E (fig. 7).

D338E may cause a general reduction in affinity for transported ions rather than a change in selectivity. However, at very negative membrane potentials, which correspond to the physiological conditions of the larval intestine, K^+ is much more efficient than Na^+ in inducing leucine uptake and transport-associated currents [1, 22]. A general reduction in affinity for transported ions would be expected to have proportional effects on both Na^+ and K^+. The results of figure 3 and 6 show, instead, a much stronger uptake and current reduction in K^+ than in Na^+.

These observations, therefore, indicate that D338E had lost the ability to use K^+ as a co-ion, and had become Na^+ selective, like most members of the Na^+/Cl^--dependent family neurotransmitter transporter.

However, the peculiar physiological characteristics of the mutated protein, namely reduced transport-coupled current in Na^+ and a parallel increase in the amino acid flux at leucine concentrations ≥ 0.5 mM, indicated a deep change in the transport mode of the transporter. The coupling between the driver ion and the organic substrate was modified so that an enhanced amino acid flux did not correspond to an increased transport-associated current. Leucine transport was still strongly Na^+ dependent (fig. 5), which suggests a possible double role of Na^+ as driver and/or activator, which is not transported but must interact with the protein to allow substrate binding and translocation. In other words, Na^+ could be mainly a low-affinity activator in the mutant D338E.

The mutated protein apparently had reduced affinities for leucine and Na^+ but greater amino acid transport capacity (figs. 4, 5). High transport capacity and relatively low affinity are features commonly shared by uniporters that mediate passive amino acid uptake in absorptive epithelia.

The alterations in the transport mechanism indicate that D338 may not be the unique determinant of ionic selectivity. The cation selectivity and the coupling mechanism probably require the cooperation of several residues, and an important future research objective will be to find a multiple mutant lacking the K^+ dependence, while maintaining the proper Na^+-coupled amino acid transport. The data presented here indicate that aspartate 338 is one of the residues involved both in cation selectivity and coupling.

These findings suggest some speculations on the nature of substrate selectivity and transport mechanism by cotransporters. In order to perform uptake (or to generate transport current) the right combination of three elements, namely (i) transporter, (ii) ion(s) and (iii) organic substrate, must apparently occur, i.e., in order to reach the functional state, the three elements must all interact together in the correct way. One cannot say in general that 'KAAT1 is able to transport amino acids in the presence of either Na^+ or K^+' as, in fact, proline is transported by wild-type KAAT1 only in presence of Na^+ (fig. 6) [5]. Therefore, the ionic selectivity is not a pure property of a particular transporter, but also depends on the particular inorganic and organic substrates to be transported.

Transporters appear to be versatile proteins, not only can they display 'channel-like' behavior in the absence of organic substrate (revealed by the uncoupled currents), but they have different functions depending on the interacting molecules and ions that may act both as substrates and modulators [23, 24]. In addition, the data presented here show that a single conservative mutation causes a partial uncoupling of Na^+ and leucine fluxes so that a cotransporter, characterized by a relatively tight coupling between organic and inorganic substrates, becomes more similar to a uniporter. This suggests that membrane transporters have in their structure the potential to perform transport processes with different mechanisms, as supported by several experimental results for other cotransporters [19, 25–27], and in line with the concept that the boundaries between ion channels, ligand-gated receptors and neurotransmitter transporters are weak [28, 29].

Further effects on cation selectivity may be observed by analyzing the behavior of the two other kinds of currents generated by KAAT1 in the absence of organic sub-

strates: the presteady-state current and the uncoupled current [9]. As shown in figure 8, the usual selectivity sequence for this kind of current, i.e. $Li^+ > Na^+ > K^+$ [16], was maintained in the D338E mutant, but it was altered in the D338N form, in which the Li^+ and Na^+ currents were strongly reduced, although they remained significantly higher than in oocytes not expressing the transporter. While relating these effects with those on the transport-associated current described above is not easy, the observations on the uncoupled currents were in line with a participation of aspartate 338 in the cationic selectivity filter of the transporter.

Substitution of aspartate 338 with either glutamate or asparagine also altered the transient, presteady-state currents elicited by voltage jumps. Transient currents could still be observed in the mutated forms, but they appeared to have lost their normal ion dependence. In KAAT1 and other members of the family, these currents are strongly Na^+ dependent [9, 30], and in KAAT1, Li^+ and K^+ are also able to elicit transients (with different voltage dependence) [9, 16]. The results reported here (figs. 9, 10) show that the transients exhibited by D338E and D338N could not be altered by substituting Na^+ with Li^+ or K^+, and they remained even when TMA^+ was the predominant cation. D338E and D338N may offer an undifferentiated binding possibility to the tested cations (including TMA^+) allowing similar, but smaller, transient currents. In any case, this result indicates that these mutations have altered the capacity of the transporter to recognize different cations, in line with the previous observations.

We have seen that all mammalian members of the family have an asparagine in the corresponding position of KAAT1 D338. In the serotonin transporter rSERT, asparagine 368 is part of a series of residues that have been carefully studied in terms of effects on transport and substrate accessibility [31, 32]. Indeed, mutation of this asparagine to aspartate, i.e. N368D, only slightly reduced transport activity, although substitutions with isoleucine or leucine strongly impaired rSERT activity. Based on accessibility experiments, the conclusion of the authors was, however, that transmembrane domain 7 might be 'involved in propagating conformational changes caused by ion binding, perhaps as part of the translocation mechanism,' more than representing the actual site where ion interaction takes place. Our observation that mutating N327 of the GABA transporter rGAT-1 to aspartate resulted in abolition of the activity, although disappointing with respect to the simple expectation of inducing the capacity to use potassium as a driving ion, is somehow different, but is in line with the suggestion that the amino acid in this position of the sequence is crucial for activity. Clearly this residue cannot be considered the primary determinant of ionic selectivity for mammalian cotransporters, but it may contribute in combination with other residues to define this characteristic.

An important difference between KAAT1 and mammalian neurotransmitter transporters concerns turnover rates. Compared to rGAT-1, glutamate and glycine transporters, or to monoamine transporters which have turnover rates in the range 5 to 20 s^{-1} [30, 33, 34], KAAT1 has a definitely larger transport current per unit displaced charge, implying faster turnover rates, up to 150 s^{-1} [9]. These characteristics correspond to the different relaxation time constants of the presteady-state currents, which are in the range 40–150 ms for rGAT-1 and 4–10 ms for KAAT1. Thus, one might speculate on a possible correlation between stricter ionic selectivity and lower turnover rates. In an exclusively Na^+-selective transporter, one might expect that the height of the energy barrier limiting the access of ions to the binding site should be such as to render it almost unreachable by other cations, at least at physiological potentials. Of course, Na^+ should be able to to overcome this barrier; however, the low inward rate of charge movement measured in rGAT-1 (10–25 s^{-1}) indicates that this event is somewhat sporadic, considering that the frequency of diffusion-limited collisions with the transporter is expected to be six to seven orders of magnitude larger [35]. Thus, the low turnover rate of these transporters might be related to the necessity to exclude access to K^+ ions. Conversely, when the ionic selectivity must be relaxed, as in KAAT1 and CAATCH1, the access energy barrier will have to be lowered to allow entry of K^+ ions and, as a consequence, the entry rate will become faster. In the D338E mutant, the reduction in transport-associated current seemed to contrast with the high leucine V_{max}, which is usually related to the number of transporters and to their turnover rate. This discrepancy is probably a consequence of the separation of Na^+ and amino acid movements through the transporter. Aspartate 338 is not only important for the cation selectivity but is also crucial for the cotransport mechanism, i.e. the coupling between the organic substrate and ionic flux.

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