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Functional properties of a newly cloned fish ortholog of the neutral amino acid transporter B^0AT1 (SLC6A19)

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ABSTRACT

The functional properties of an ortholog of the B^0AT1 (SLC6A19) amino acid transporter, cloned from the intestine of the sea bass *Dicentrachus labrax*, were investigated. The two-electrode voltage-clamp technique was applied to *Xenopus laevis* oocytes heterologously expressing the transporter in order to measure the currents associated with the transport process in different conditions. In particular the substrate specificity, the ionic requirements, and possible effects of pH were examined. Among the organic substrates, leucine, glycine, serine and valine generated the largest transport currents with apparent affinities in the lower millimolar range. The importance of Na^+ as the driver ion in the transport process is confirmed, although Li^+ is also capable to sustain transport, while K^+ is not. No evidence of a relevant role of Cl^- in the transport activity was found. Concerning the other two kinds of currents commonly found in electrogenic transporters, very fast pre-steady-state currents were detected in the absence of organic substrate, while lithium-specific leak currents were not observed. The comparison of these properties with those of the mammalian and insect orthologs may give interesting indication for future structure-function studies in this transporter subfamily.

1. Introduction

Absorption of amino acids in the animal intestinal tract and reabsorption in the kidney are accomplished by specific transport proteins located in the apical and basolateral membranes of the appropriate epithelial cells. Uphill intake of amino acids from the intestinal lumen is permitted by secondary active cotransporters in the apical membrane, such as the proton-coupled di- and tri-peptide transporter PepT1 (SLC15A1) (Daniel and Kottra, 2004; Bossi et al., 2011; Renna et al., 2011), and the Na^+- coupled neutral amino acid transporter B^0AT1 (SLC6A19) (Böhmer et al., 2005; Camargo et al., 2008). While PepT1 has been cloned and characterized in vertebrates and invertebrates, including bacteria and yeast (Daniel et al., 2006), the functional characterization of B^0AT1 is limited to mammalian orthologs (Kleta et al., 2004; Böhmer et al., 2005; Camargo et al., 2005) and to some insect transporters classified as B^0AT1-like, such as msKAAT1, msCAATCH1 and dmNAT1 (Bossi et al., 1999a; Feldman et al., 2000; Boudko et al., 2005; Bossi et al., 2007b; Miller et al., 2008).

The understanding of the functional properties of transporters responsible for nutrient absorption is obviously relevant under various points of view, such as the comprehension and modelling of the molecular transport mechanisms (Boudko, 2012), the assessment of their implication in pathological conditions (Seow et al., 2004; Bröer et al., 2005), or the establishment of optimal diet composition in animal farming (Edwards et al., 1999; Verri et al., 2011; Kwasek et al., 2012; Margheritis et al., 2012).

In connection with this last aspect, recently a fish ortholog from the sea bass *Dicentrachus labrax*, has been cloned (sbB^0AT1) (GenBank KC812315, Terova, in preparation).

In the present work we have studied the functional properties of sbB^0AT1 using electrophysiological methods in *Xenopus laevis* oocytes heterologously expressing the protein. The amino acid specificity, the ionic selectivity and the apparent affinity of the transporter were characterized by analyzing the transport associated current. Furthermore, we investigated the presence of two other kinds of current frequently observed in ion coupled cotransporters, namely the transmembrane "leak" or "slippage" current (Nelson et al., 2002; Andrini et al., 2008), and the intramembrane pre-steady-state current caused by the rearrangement of the transport protein and/or to the displacement of mobile ions in the membrane electric field (Hazama et al., 1997; Peres et al., 2004). These properties were compared to those of the mammalian and insect orthologs to highlight similarities and differences that could be useful for structure function studies.

2. Materials and methods

2.1. Oocyte preparation and mRNA injection

Xenopus laevis oocytes and RNAs were prepared as previously described in detail (Bossi et al., 2007a). The cDNA encoding for

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sbB^{0}AT1 was cloned from the gut of *Dicentrarchus labrax* (GenBank **AN. KC812315**; Terova, in preparation) and it was inserted into the pSPORT-1 vector as the cDNA coding for *ms*KAAT1 (Invitrogen, Milan, Italy, www.lifetechnologies.com). The cDNA of mouse collectrin kindly provided by Prof. Joseph Penninger (IMBA Institute of Molecular Biotechnology Vienna, Austria) was in pCDNA3.1(Camargo et al., 2008), the mouse B^{0}AT1, kindly provided by Simone Camargo (Institute of Physiology, University of Zurich) was into a pBlueScript modified Xenopus expression vector (KSM). For the expression in Xenopus oocytes the mouse B^{0}AT1-KSM plasmid was linearized with XbaI (Promega) and used as template for RNA synthesis from the T3 promoter. The other plasmids were linearized with NotI for sbB^{0}AT1 and msKAAT1 and with SmaI for mouse collectrin and synthesized from the T7 promoter, All cRNAs were transcribed in vitro in the presence of Cap Analog and 200 units of specific RNA polymerase. All enzymes were supplied by Promega Italia (www.promega.com, Milan, Italy). Oocytes were obtained from adult female *X. laevis* (Xenopus express, France, www.xenopus.com); the frogs were anesthetized in MS222 (tricaine methanesulfonate salt) (Sigma, Milan, Italy, www.sigmaaldrich.com) 0.10% w/v solution in tap water and portions of the ovary were removed through an incision on the abdomen. The oocytes were treated with collagenase Type IA (Sigma) 1 mg/mL in calcium-free ND96 for at least 1 h at 16 °C. After 24 h at 16 °C in modified Barth's saline solution (MBS), selected oocytes were injected with 25 ng of cRNA for sbB^{0}AT1, mB^{0}AT1 and mCollectrin, and 12,5 ng for msKAAT1 in 50 nl of water, using a manual microinjection system (Drummond Scientific Company, Broomall, PA, www.drummondsci.com). The oocytes were then incubated at 16 °C for 4–6 days in MBS before electrophysiological studies. The experiments were carried out according to the institutional and national ethical guidelines (permit no. 05/12).

2.2. Electrophysiology and data analysis

Measurements of the currents generated by the transporter in controlled voltage conditions were performed using the two-electrode voltage clamp (TEVC) technique (GeneClamp, Molecular Devices, Sunnyvale, CA, USA) (Giovannardi et al., 2007). Intracellular glass microelectrodes, filled with KCl 3 M and with tip resistance between 0.5 and 4 MΩ were used. Agar bridges (3% agar in 3 M KCl) connected the bath electrodes to the experimental chamber. The holding potential (V_h) was −60 mV. Voltage pulses from −140 mV to +40 mV in 20 mV increments were applied for 200 ms. Data were analyzed using Clampfit 10.2 (Molecular Devices, www.moleculardevices.com), while statistics and figures were done with Origin 8.0 (originalLab Corp., Northampton, MA, USA, www.originlab.com). Transport currents were obtained by subtracting the current level in the absence of organic substrate from that in its presence. The "leak" currents through the transporter were investigated by comparing the membrane current needed to hold a constant potential in the presence of lithium with respect to sodium, taking as reference the current level in the presence of impermeant tetramethylammonium (TMA^+) (Bossi et al., 1999b; Hille, 2001).

Pre-steady-state currents were isolated by subtracting the traces in the presence of organic substrate from those in its absence, followed by a further subtraction of the residual steady level of the current at the end of the pulse (Mertl et al., 2008; Bossi et al., 2011). Analysis of the pre-steady-state currents was then performed by calculating the integrals of the isolated transients, to obtain the amount of moved charge (Q), and by fitting the current decay with a single exponential in order to obtain the relaxation time constant (τ).

2.3. Solutions

The oocyte culture and washing solutions had the following composition (in mM), calcium-free ND96: NaCl 96, KCl 2, $MgCl_2$ 1, HEPES 5, pH 7.6; ND96: NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8, HEPES 5, pH 7.6; MBS: NaCl 88, KCl 1, $NaHCO_3$ 2.4, HEPES 15, $Ca(NO_3)_2$ 0.30, $CaCl_2$ 0.41, $MgSO_4$ 0.82, sodium penicillin 10 μg/mL, streptomycin sulfate 10 μg/mL, gentamicin sulfate 100 μg/mL, pH 7.6. The external control solution during the electrophysiological recordings had the following composition (mM): NaCl 98; $MgCl_2$ 1;$CaCl_2$ 1.8, HEPES 5 mM, for pH 5.5 solution HEPES was substitute by MES and for the pH 8.5 by TAPS. The NaCl was totally or partially substituted by TMACl, KCl or LiCl and Na gluconate in ion dependence experiment. The final pHs were adjusted with NaOH, KOH, LiOH, TMAOH respectively. The substrates, dissolved in water, were added at the indicated concentrations. Experiments were conducted at room temperature (20–25 °C).

3. Results

3.1. Transport currents elicited by different substrates

In the initial experiments the functional expression of the sea bass B^{0}AT1 in the oocytes was investigated by recording the transport currents elicited by various substrates at constant membrane voltage (−60 mV). A representative recording of this kind of current is shown in Fig. 1A, while cumulative data averaged nine oocytes are shown in Fig. 1D. All the neutral amino acids tested elicited inward currents of comparable amplitude when applied at a concentration of 3 mM. On the contrary, no currents were generated by the charged amino acid glutamate nor by the dipeptide glycine-glutamine at the same concentration. These results are in substantial agreement with the observations on the renal form of B^{0}AT1 from mouse (Camargo et al., 2005), except for the effect of the imino acid proline that appears to generate currents of similar amplitude as leucine in the mouse transporter, but only produces a fast inward transient in the sea bass form. However the fast transient upon proline application can also be seen in non-injected oocytes (Fig. 1C), suggesting that it may be due to endogenous properties of the oocytes. The same sequence of substrates has been applied to another neutral amino acid transporter related to the SLC6A family, KAAT1 from the gut of the invertebrate *Manduca sexta* (Castagna et al., 1998). The representative record (Fig. 1B) shows distinct differences in comparison to B^{0}AT1 (Soragna et al., 2004), see for example the large currents elicited by proline and threonine and the small response to histidine. Finally we tested if the expression was influenced by the accessory protein collectrin as found for the mammalian orthologs, by investigating the effects of co-injection on the current recorded in oocytes expressing B^{0}AT1 and KAAT1 (Fig. 1E). As reported (Danilczyk et al., 2006) the transport current was increased five to six-fold in the oocytes co-expressing mouse B^{0}AT1 and mouse collectrin. This accessory protein was also able to significantly increase ($p > 0.01$) the activity of sbB^{0}AT1, but not that of msKAAT1.

3.2. Ionic dependence of transport currents

Previous studies on mammalian B^{0}AT1 (Böhmer et al., 2005; Camargo et al., 2005) indicated that, as in the other SLC6A family members, the presence of Na^+ is essential for transport. As illustrated in Fig. 2, this characteristic is confirmed in sbB^{0}AT1. In the total absence of Na^+ the transport current is abolished if the replacing ion is tetramethylammonium (TMA^+) or K^+, while it is still partially observable when Na^+ is substituted by Li^+ (Fig. 2A). We also investigated the possible involvement of Cl^-, performing experiments in which this anion was replaced by gluconate: as shown in Fig. 2B no significant changes in the sbB^{0}AT1 transport current were observed following this substitution, in agreement with the results reported by (Böhmer et al., 2005; O'Mara et al., 2006) on the mouse ortholog, similar results were obtained in the presence of a non saturating dose of substrate (500 μM leucine).

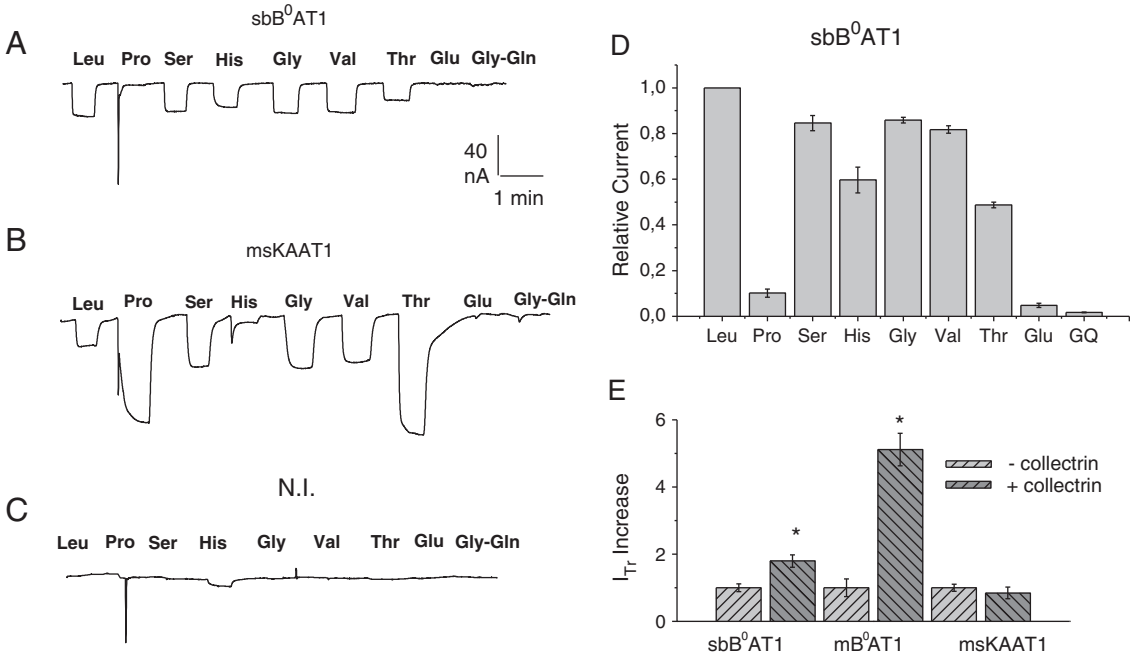

Fig. 1. Representative traces of the membrane currents induced by addition of the indicated substrates (all at 3 mM concentration) in three different oocytes expressing sbB^{0}AT1 (A), KAAT1 (B), or non-injected (C); the current and time scales are the same for the three traces; V_h was kept at −60 mV in all cases. sbB^{0}AT1 average currents ± SEM from 9 oocytes (three batches) (D), the responses to the indicated substrates were normalized with respect to leucine in each oocyte before averaging. (E) Collectrin effect reported as substrate transport current increase. Increment of transport currents at −60 mV induced by the co- injection of mouse collectrin are reported for the three transporters, the current of each protein was normalized to the mean current obtained from the oocytes injected with the transporter alone for each batch. Data from at least 2 batches and 10 oocytes. Substrate were leucine 10 mM for B^{0}AT1 and threonine 3 mM for msKAAT1.

3.3. Apparent affinity

The apparent affinity for Na^+ was calculated by fitting a Michaelis-Menten equation to dose-response relationships obtained measuring the transport current elicited at different potentials by 3 mM leucine in the presence of various external Na^+ concentrations (Fig. 3A). The values of the Na^+ concentration eliciting half of the maximal current at each potential ($K_{0.5}^{Na}$) are shown in Fig. 3B and are quantitatively and qualitatively in agreement with the results obtained in the mammalian B^{0}AT1 (Camargo et al., 2005). The apparent affinity for the organic substrate was similarly obtained for leucine (Fig. 3C). The values of the leucine concentration eliciting half of the maximal current at each potential ($K_{0.5}^{Leu}$) are shown in Fig. 3D and, again, are in good agreement with the results from the mouse B^{0}AT1.

3.4. Effects of pH

We also tested the effects of pH, since the activity of the mouse transporter was shown to be reduced by acidity and slightly increased by alkalinity (Böhmer et al., 2005; Camargo et al., 2005) as for the insect transporter KAAT1 (Peres and Bossi, 2000). Our results are shown in Fig. 4 and qualitatively confirm the previous findings, although quantitatively the effects of pH on sbB^{0}AT1 are smaller, and the effect of acidity appears more like a shift of the curve toward more negative potentials.

3.5. Leak lithium current

An interesting distinct feature exhibited by many ion-coupled cotransporters is the existence of transmembrane currents in the

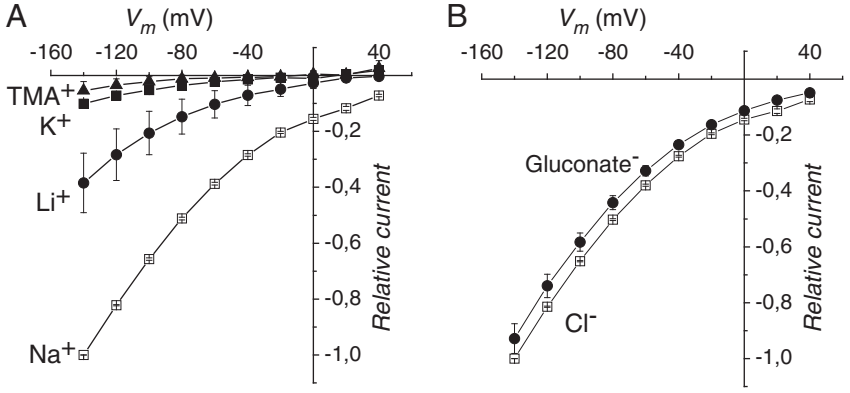

Fig. 2. Ionic dependence of transport currents. A: I–V relationship of the current elicited by 3 mM leucine in the presence of different cations, as indicated. B: Replacement of Cl^- with gluconate has no effect on the transport current elicited by 3 mM leucine. In both plots, currents were normalized to the value in Na^+ at −140 mV for each oocyte before averaging. Data are means ± SEM from 11 oocytes (three batches) in A, and from 10 oocytes (three batches) in B.

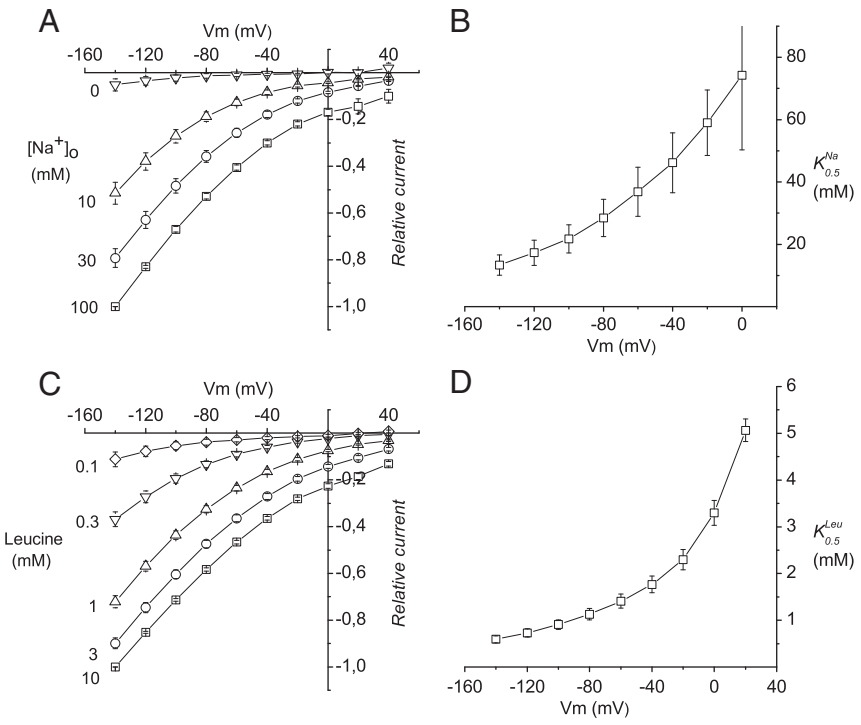

Fig. 3. Apparent affinities. A: I–V relationships elicited by leucine 3 mM in the presence of the indicated Na^+ concentrations (TMA^+ substitution, data from 5 oocytes, three batches). B: voltage dependence of the Na^+ concentration eliciting half of the maximal current at each potential ($K_{0.5}^{Na}$). C: I–V relationships elicited by the indicated leucine concentrations in the presence of 100 mM Na^+ (data from 8 oocytes, two batches). D: voltage dependence of the leucine concentration eliciting half of the maximal current ($K_{0.5}^{Leu}$). $K_{0.5}^{Na}$ and $K_{0.5}^{Leu}$ values were obtained by fitting a Michaelis–Menten equation to the dose–response graphs from the data in A and C respectively. In A and C the data are normalized to the current value at −140 mV in the highest variable substrate concentration.

absence of organic substrate. These "leak" or "slippage" currents (Nelson et al., 2002; Andrini et al., 2008) possess the peculiar characteristic of being best observed when lithium is the permeating ion (Mager et al., 1996; Bossi et al., 1999b; Zhou et al., 2006). In the rappresentative recording of Fig. 5A an oocyte expressing sbB^0AT1 was initially bathed in the control, Na^+-containing, solution, giving rise to an holding current of about −22 nA at −60 mV. As expected, replacement of sodium with TMA^+ caused a reduction of this current to about zero. When a solution containing lithium as the main cation was perfused on the oocyte, a small inwardly directed current (about −9 nA, definitely smaller than in sodium) developed. This sort of effect is normally seen in oocytes not expressing any transporter, and it is ascribed to the passage of lithium through the endogenous channels of the oocyte (Bossi et al., 1999b). Indeed lithium is normally less permeant than sodium in ionic channels (Hille, 2001). This oocyte was clearly expressing the transporter, as evident from the inward transport current generated by the addition of 3 mM leucine in the presence of sodium in the final part of the record. Therefore this result indicates that sbB^0AT1 does not show lithium leak currents. As a positive control, we repeated the same kind of experiment on oocytes expressing the insect transporter KAAT1: the representative trace of Fig. 5B shows that in this case the holding current in the presence of a high external lithium solution is much larger than when sodium is present (arrows). This observation, that confirms previous results (Bossi et al., 1999a, 1999b), indicates the existence of a leak pathway in the transporter. We can conclude therefore that sbB^0AT1 does not offer evidences to be able to carry such kind of current.

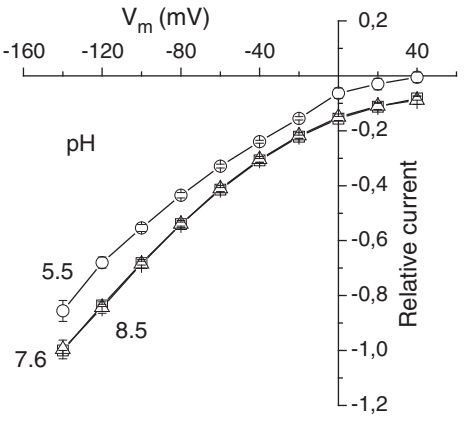

Fig. 4. Effects of pH on transport currents. The transport currents elicited by 3 mM leucine were measured in the same oocyte at the three indicated pH values. Currents were normalized at the value at −140 mV and pH 7.6 (squares) before averaging. Data are means ± SEM from ten oocytes (three batches).

3.6. Pre-steady-state currents

Generally, the occurrence of pre-steady-state currents in ion-coupled cotransporters can be easily detected during a voltage-clamp experiment as capacitative-like transients with relatively slow decline time constant (τ) after a voltage step. In most transporters these transient disappear upon addition of the transported substrate, that is, pre-steady-state and transport- associated currents are mutually exclusive (Peres et al., 2002, 2004). The interpretation of these observations is that the intramembrane charge movement causing the pre-steady-state currents represents the initial steps of the transport cycle that can be completed only when the organic substrate is present. The addition of the organic substrate then produces a conversion of the capacitative-like pre-steady-state currents in the resistive-like transport currents (Peres et al., 2002).

In the previous electrophysiological studies on the mouse B^0AT1 (SLC6A19) (Kleta et al., 2004; Böhmer et al., 2005; Camargo et al., 2005) there is no mention of pre-steady-state currents. Indeed visual inspection of the current traces obtained using voltage steps protocols,

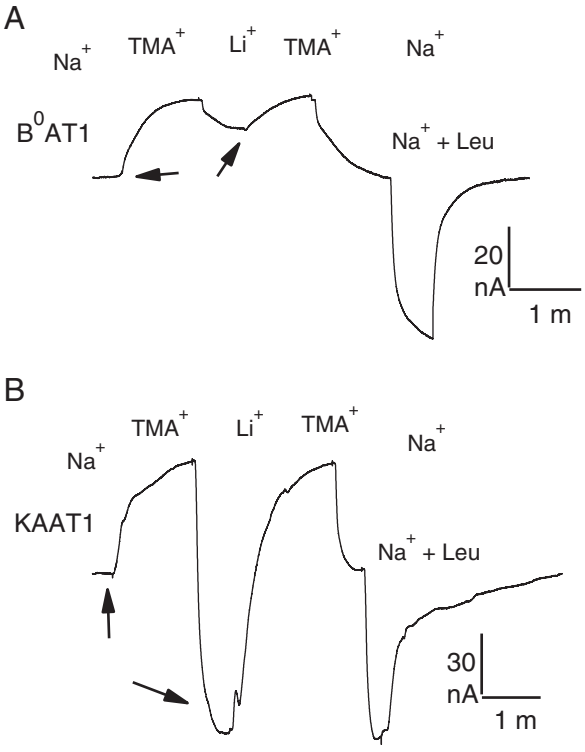

Fig. 5. Absence of lithium-specific leak current in sbB^{0}AT1. Sample recordings of transmembrane current in two oocytes expressing sbB^{0}AT1 (top) or KAAT1 (bottom) at $V_h = -60$ mV and exposed to the indicated external solutions. As in non-injected oocytes, in sbB^{0}AT1 the inward current in the presence of Li^+ is smaller than in Na^+ (arrows), while in KAAT1 the opposite occurs, indicating the presence of a leak current through the transporter. The response to leucine (3 mM) at the end of the recordings demonstrates that both oocytes were expressing the respective transporters. Analogous results concerning sbB^{0}AT1 were observed in three other oocytes from two batches.

such as in Fig. 6, does not reveal the usual slowly-decaying transients in the absence of substrate, nor obvious changes in the time course of the capacitive transients following addition of the organic substrate (Fig. 6A and B). However, when the current traces in saturating substrate concentration (Fig. 6B) are subtracted from the corresponding traces in the absence of substrate (Fig. 6A), the result discloses the existence of very fast transient currents both at the "on" and at the "off" of the voltage pulse (Fig. 6C). These transients may be analyzed as explained in the Method section, allowing the calculation of the amount of charge displaced in the membrane electrical field (Q), and of the time constant of decline (τ). These data are shown respectively in Fig. 6D and E as average values from several oocytes. Finally, Fig. 6F shows that, as requested for an intramembrane charge movement, the amount of charge moved in the same oocytes when the membrane voltage is changed from -140 to $+40$ mV is substantially identical to the amount moved in the opposite direction when the voltage is changed from $+40$ to -140 mV.

4. Discussion and conclusion

4.1. Comparison with mammalian B^{0}AT1 and KAAT1

The substrate selectivity of sbB^{0}AT1 shows only slight differences with respect to the mouse ortholog previously studied. These are limited to proline that does not elicit significant currents, and to histidine that is transported with an efficiency similar to leucine (while in the mouse shows a 50% reduced current). The differences in comparison to KAAT1 are more marked, since in the invertebrate transporter proline is actually the best transported amino acid (together with threonine), and histidine is not transported, see Fig. 1 and (Soragna et al., 2004).

Co-expression of mouse collectrin roughly doubled the transport current generated by sbB^{0}AT1. This effect is analogous, although definitely smaller, to that observed in the mouse B^{0}AT1, but no effect of collectrin was seen in msKAAT1.

These results encourage further investigations in this direction, although species-specific collectrin is clearly required to obtain more significant effects. However, the expression of sbB^{0}AT1 alone was normally quite good, at difference to that of the mouse ortholog, and definitely sufficient to electrophysiologically characterize the transporter.

In general, all these differences might be exploited in future structure-function studies aimed to understand the structural bases of protein interaction, substrate translocation and selectivity.

4.2. Cationic specificity

As evident from Fig. 2A, the transport activity is strongly Na^+ dependent, confirming the findings in the mammalian ortholog (Camargo et al., 2005). However, KAAT1 is known to be able to use also the K^+ and, to some extent, the Li^+ gradient to energize the transport activity (Castagna et al., 1998; Bossi et al., 1999a; Vincenti et al., 2000). Lithium can also partially power up the activity of the mouse B^{0}AT1 (Camargo et al., 2005). Our results show that in sbB^{0}AT1 no transport current is generated by K^+, while a modest activity can be produced by Li^+.

It is known that the ability to work with K^+ represents a functional adaptation of the *Manduca sexta* intestinal transporters KAAT1 and CAATCH1, because of the peculiar diet of this larva, rich in K^+ (Castagna et al., 1998) and it is not a common characteristics of insect NAT transporters (Boudko et al., 2005). An important role in conferring the ability to work with K^+ in these transporters has been attributed to the presence of an aspartate residue in position 338. In fact, all the other eukaryotic members of the family, unable to use potassium, have an asparagine residue in the corresponding position (Fig. 7). Furthermore, mutation of aspartate 338 to asparagine in KAAT1 was shown to affect the potassium sensitivity in KAAT1 (Mari et al., 2004). The lack of transport activity in K^+, exhibited by sbB^{0}AT1, may then be related to the presence of an asparagine in position 310. As noted previously (Mari et al., 2004), it is likely that other residues should be involved as well in determining the cationic selectivity, since for example the bacterial Tyrosine transporter TyT, and other insect NATs have been reported to be unable to work with potassium in spite of the presence of an aspartate in the same position (Boudko et al., 2005; Quick et al., 2006; Meleshkevitch et al., 2009) (Fig. 7).

4.3. Chloride dependence

The members of the SLC6A family show different sensitivity to chloride, ranging from strong dependence to no sensitivity (Betté et al., 2008). Substitution of external chloride with gluconate does not seem to significantly affect the transport activity of sbB^{0}AT1, as shown by the results of Fig. 2B. Again, KAAT1 is only partially inhibited in the absence of chloride, while contrasting results were reported for the mouse ortholog, from no effect (Böhmer et al., 2005; O'Mara et al., 2006), to partial reduction of the transport current (Camargo et al., 2005).

According to structure-function studies on this matter (Forrest et al., 2007; Zomot et al., 2007), the difference between chloride-sensitive and bacterial homolog, insensitive members of the family is due to the lack, in the former, of intrinsic negative charges in the vicinity of the Na^+-binding site(s). Particularly, the presence of two glutamate residues in position 287 and 290 (Fig. 7) in the structure of the SLC6A prototype transporter LeuT (Yamashita et al., 2005) is considered the structural determinant of its chloride-independence. On the other hand, Cl^--dependent transporters, such as GAT1 and SERT1 (Forrest et al., 2007; Zomot et al., 2007) have neutral amino acids in the

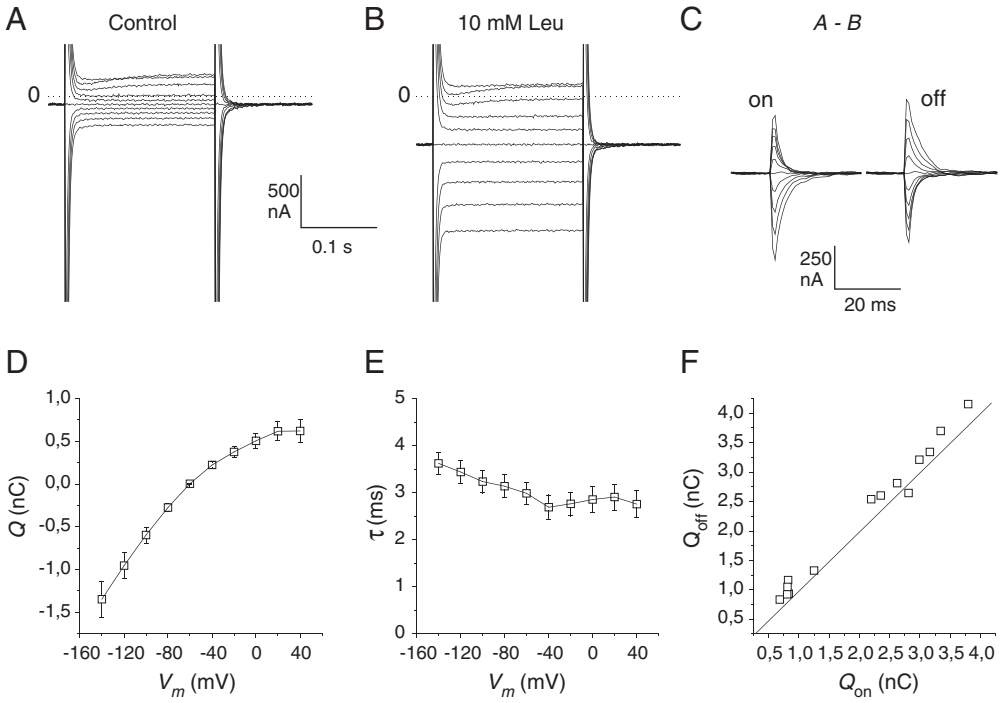

Fig. 6. Isolation and analysis of pre-steady-state currents. A: Records of membrane currents elicited by 200 ms long voltage steps from −140 to +40 mV in 20 mV intervals from a −60 mV holding potential (V_h), in a representative oocyte. B: Currents in the same oocytes upon addition of a saturating amount of leucine. C: Currents resulting from the subtraction of the traces in B from those in A, followed by the zeroing of the remaining steady-state currents (note the different scales). D: Voltage dependence of the intramembrane charge movement obtained by integration of the pre-steady-state transients as in C. E: Time constant of decay obtained by fitting the transients with single exponentials. Data in D and E are means ± SEM from fourteen oocytes (four batches). F: Correspondence of the amount of charge moved during the "on" and "off" transients from the same fourteen oocytes; the straight line represents equality between Q_{on} and Q_{off}.

corresponding position, consequently requiring the presence of a mobile negative charge, supplied by a chloride ion, for their correct functioning.

Analysis of the peptide sequence of KAAT1 and sbB^{0}AT1 shows that the cavity of the Cl^- binding site formed by residues such as tyrosine, serine, and asparagine, is conserved in all members of the family. The observed weak dependence of KAAT1 has been attributed to the presence in a nearby position of another negative amino acid (i.e. aspartate 338), also fundamental for the activity in potassium, which could partially contribute the required negative charge; however, sbB^{0}AT1 and other NATs transporter (mammalian B^{0}AT1 and B^{0}AT2, IMINO, and others), that are Cl^--independent, do not appear to have such negative residues in the adjacent regions of the sequence.

No straightforward explanation is therefore available for the virtually chloride-independent behavior of sbB^{0}AT1. It is possible, as suggested for the IMINO transporter (Bröer, 2006), that the chloride sensitivity depends on the substrate concentration, becoming minimal at saturation, but this is not the case of sbB^{0}AT1, or that these transporters may use a static chloride ion to stabilize their structure (Bröer, 2008).

Yet another hypothesis that could be considered is the indication that in mouse B^{0}AT1 the organic substrate may bind first, followed by Na^+ (Camargo et al., 2005). Commonly, in other transporters, the binding of the organic substrate is believed to occur after the ion interaction (Parent et al., 1992; Peres et al., 2004; Miszner et al., 2007). However, if the opposite order occurs in sbB^{0}AT1, it is possible to speculate that the organic substrate may participate in creating the binding pocket for the subsequent binding of Na^+, providing perhaps a stabilizing negative charge through its carboxyl end. This possibility would imply that a different mechanism might be adopted by the NAT transporters compared to NTT to return to the outward facing position (Forrest et al., 2007; Zomot et al., 2007).

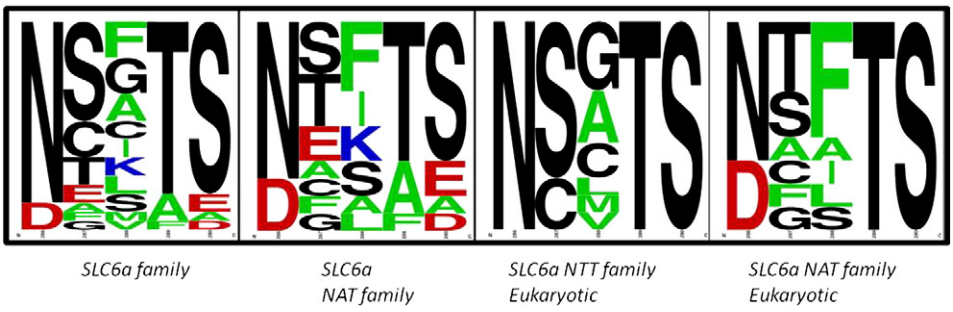

Fig. 7. Sequence logo representation of the chloride binding site in the SLC6A family, according to Zomot et al. (2007) and Forrest et al. (2007). The height of a given letter (amino acid residue) represents its frequency of occurrence at that particular position. The columns represent (from left to right): i) the main members of the SLC6A family from mammals, insects and bacteria, and including both neurotransmitter (NTT) and nutrient amino acid (NAT) transporters; ii) NATs from mammals, insect and bacteria; iii) eukaryotic NTT only; iv) eukaryotic NAT only.

4.4. Apparent affinity

As shown in Fig. 3, the apparent affinities are in the order of 10–60 mM for Na^+ and 1–5 mM for leucine, in good agreement with the values measured in the mouse ortholog (Böhmer et al., 2005; Camargo et al., 2005). Interestingly, the $K_{0.5}$ values for leucine increase at more positive potentials, confirming the observation performed in several other transporter that this parameter is inversely related to the time constant of decline of the pre-steady-state currents (see Fig. 6E).

4.5. pH effect

Differently from the mammalian ortholog, we observed only a modest effect of pH on the transport activity of sbB^{0}AT1. No clear physiological reasons for such difference are apparent, and this observation may be useful for identifying the site of interaction of protons (Camargo et al., 2005). Again, in the SLC6 family different sensitivities to pH can be found, ranging from strong dependence to a complete insensitivity. Some results suggest an involvement of residue 10.45 (generic numbering (Beuming et al., 2006)) in the pH dependence. A serine residue is present in this position (483) in human and mouse B^{0}AT1 while the cloned fish orthologs have instead an alanine (A482). The pH independent members of NTTs, have in this position a positively charged arginine, but they become pH sensitive when the arginine is substituted by glutamate in rGAT1 (Forlani et al., 2001) or by threonine in hSERT1 (Cao et al., 1998). It is likely therefore that the non polar alanine in sbB^{0}AT1 is responsible for the reduced pH sensitivity in this ortholog.

4.6. Transporter leak current

This transporter property, observed in solute carrier protein in the presence of lithium (Mager et al., 1996; Bossi et al., 1999b; Zhou et al., 2006; Andrini et al., 2008) was not previously investigated in the mouse ortholog. As shown in Fig. 5, our experiments failed to reveal the presence of a leak current in sbB^{0}AT1. In contrast, and as already reported (Bossi et al., 1999a, 1999b) a large lithium-induced current was seen in oocytes expressing the invertebrate KAAT1. It will be interesting to check for the presence of this current in the mouse B^{0}AT1 to understand whether this is a difference between vertebrate and invertebrate forms, and possibly to investigate the structural bases of this characteristic. The different order of substrate binding hypothesized for this transporter (see above) might also be relevant to this aspect.

4.7. Pre-steady-state current

The sbB^{0}AT1 pre-steady-state currents detected in this work are small and fast, and this is the reason why they are not easily detected in the original recordings. The graphs plotted in Fig. 6D and E suggest that the voltage range over which the charge movement occurs is rather wide, extending in the negative direction well beyond the voltages that can be experimentally explored. However, saturation of the Q-V curve occurs at positive potentials, and the τ-V curve shows a decline that is consistent with right half of a shallow bell-shaped relationship, as expected for the movement of charges between two states (Mager et al., 1996). Finally, the amount of charge moved during the "on" and "off" transitions is equal (Fig. 6F)„ These observations satisfy the two properties required for recognizing the intramembrane nature of the charge movement : i) the amount of charge moved by the "on" voltage step must be moved back to the original position by the "off" voltage step (i.e. the integrals of the "on" and "off" current transients must be equal), ii) the voltage dependence of the moved charge should show saturation at extreme positive and negative membrane potentials (Mager et al., 1998).

In conclusion, the electrophysiological characteristics of the transport processes of the sea bass form of B^{0}AT1 highlight similarities and differences with respect to the mammalian and insect counterparts. While the analysis of the transport efficiency of the different amino acids and the effects of pH may be useful for the design of optimal diets in aquaculture, the peculiarities in cationic selectivity and in the effects of chloride might be exploited for structure-function studies, especially in comparison with the orthologs from mammalian and insect species.

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