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Role of a conserved glycine triplet in the NSS amino acid transporter KAAT1

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ABSTRACT

K^+-coupled amino acid transporter 1 (KAAT1) belongs to the NSS family of solute transporters and it is expressed in the midgut and in salivary glands of *Manduca sexta* larvae. As more than 80% of family members, KAAT1 shows a stretch of three glycines (G85–G87) that according to the structure of the prototype transporter LeuT, is located close to the access of the permeation pathway. In this work the role of the triplet has been investigated by alanine and cysteine scanning methods in protein heterologously expressed in *Xenopus laevis* oocytes. All the mutants were functional but the surface expression level was reduced for G85A and G87A mutants and unaffected for G86A mutant. All presented altered amino acid uptake and transport associated currents in the presence of each of the cations (Na^+, K^+, Li^+) that can be exploited by the wt. G87A mutant induced increased uncoupled fluxes in the presence of all the cations. Cross-linking studies, performed by the treatment of cysteine mutants with the oxidative complex Cu(II)(1,10-phenanthroline)$_3$, showed that limiting the flexibility of the region by covalent blockage of position 87, causes a significant reduction of amino acid uptake. Na^+ protected G87C mutant from oxidation, both directly and indirectly. The conserved glycine triplet in KAAT1 plays therefore a complex role that allows initial steps of cation interaction with the transporter.

1. Introduction

KAAT1 is an insect amino acid transporter member of the NSS family of solute carriers that groups Na^+-dependent neurotransmitter, amino acid and osmolyte cotransporters [1,2]. It shows high sequence identity with the structural prototype of the family, the bacterial transporter LeuT, in the amino acids residues that have been directly implicated in the transport process [3].

KAAT1 is characterized by peculiar functional properties: an electrogenic amino acid uptake activated by K^+, Na^+ and Li^+ at differing extents [4–6] and an amino acid selectivity influenced by the driving ion [7,8].

In recent years, we have taken advantage of the special features of KAAT1 to investigate the structural/functional relationships within the NSS family. Sequence comparisons and site-directed mutagenesis studies have allowed us to identify structural determinants of transport activity such as residues involved in Na^+ and K^+ interaction and in amino acids translocation [9–12].

The aim of this work has been to study the functional role of a highly conserved sequence, extending from the extracellular loop 1 (EL1) to the transmembrane domain 2 (TM2) of KAAT1 in which three consecutive glycines (Gly85–Gly87, Fig. 1A) are found. By homology modeling with LeuT and according to accessibility studies in other members of the NSS family as the serotonin transporter SERT and in the GABA transporter GAT1 [13,14], the glycine triplet is located at the extracellular side of the protein, close to one of the residues that participates in the external gate (Arg 30 in LeuT, Arg 76 in KAAT1, Fig. 1B). The degree of conservation in NSS transporters together with the structural localization suggests that the glycine triplet plays a relevant role in transport function. This region has already been studied individually in both GAT1 and SERT [15,16] with a similar approach. In GAT1, the residues between Asn77 and Ala81 (corresponding to Asn84 and Ala88 in KAAT1, Fig. 1A) have been mutated into alanine and cysteine and their accessibility has been analyzed by treatment with cysteine reactive compounds, as MTS reagents, in the context of the MTS insensitive C74A mutant. The N77C mutant showed lower activity compared to the wild type protein, but was stimulated by treatment with the cysteine modifying agent [2-(trimethylammonium)ethyl]methanethiosulfonate bromide (MTSET). The G78C and G79C mutants were functional and inhibited by MTSET treatment, whereas the G80C mutant showed a complete loss of GABA uptake, but still exhibited sodium-dependent

Abbreviations: KAAT1, K^+-coupled amino acid transporter 1; CuPh, Cu(II)(1,10-phenanthroline)$_3$; DTT, dithiothreitol; TMA, tetramethylammonium; EL, extracellular loop; TM, transmembrane domain

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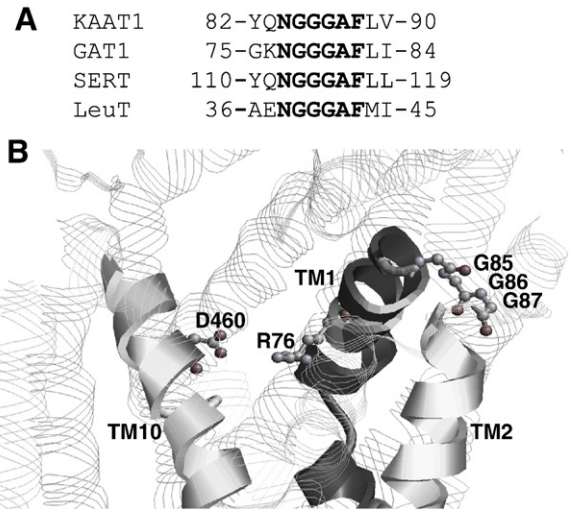

Fig. 1. A: alignment of KAAT1, GAT1, SERT and LeuT sequences extending from Tyr82 to Val90 (KAAT1 numbering). B: homology modeling of KAAT1 structure based on the three-dimensional organization of LeuT. The model shows the spatial relationships of the glycine repeat with the residues forming part of the putative external gate of the transporter (Arg76 and Asp460 of KAAT1). Gly85–87, Arg76 and Asp460 are depicted as ball and stick, TM1, TM2 and TM10 as ribbons, the rest of the structure as strands.

transient currents [15]. The results obtained by electrophysiological analysis of this last mutant were interpreted by a model for GAT1 transport cycle, in which the transition between two outward-facing conformations of the empty transporter is impaired by the mutation of Gly80. Consequently, in the absence of Na^+, the transporter is locked in a conformation from which it can only be released by GABA or by depolarization.

In SERT, residues from Tyr110 through Gly115 (corresponding to Tyr82 and Gly87 in KAAT1, Fig. 1A) were replaced by cysteine residues in a mutant lacking all the native cysteines known to react with MTS reagents. The G113C mutant retained full activity, the Q111C and N112C maintained partial activity, whereas Y110C, G114C and G115C mutants were inactive. Through analysis of the cysteine modification, Mao and co-workers concluded that in SERT Gln111 and Asn112 substitution decreased the transport rate, because the residues participate in steps of the transport cycle that are subsequent to the substrate binding and that involve serotonin translocation [16]. Summarizing, in the two papers the role assigned to the glycine residues was different suggesting that they might have a transport-specific task in each member of NSS family, that can be related to the substrate transported. In this context, the peculiarity of KAAT1 to be able to transport a rather wide spectrum of neutral amino acids with a potency order that depends on the driver cation [8,10], appears suitable to deeper investigate the role of this highly conserved sequence.

In the current work, the GGG sequence in KAAT1 has been analyzed by an alanine and cysteine scanning approach. Mutants were expressed in *Xenopus laevis* oocytes and functionally analyzed by radiolabeled amino acid uptake and electrophysiological measurements. Our data indicate that the glycine triplet is involved in KAAT1 EL1 flexibility required for the initial interactions with cations in the transport process.

2. Methods

2.1. Site-directed mutagenesis

KAAT1 mutants were obtained by PCR using high fidelity DNA polymerase *Pfu* (Promega).

DNA sequencing (Primm s.r.l. laboratories, Milan, Italy) confirmed the mutations.

2.2. Epitope tagging

To allow optical measurements of the surface expression of the transporter proteins, a FLAG epitope was inserted into the second extracellular loop, close to transmembrane domain 3 of KAAT1, in correspondence to the *PstI* site. The oligonucleotides encoding the FLAG epitope were ligated into the *PstI* site after annealing. DNA sequencing confirmed the correct FLAG construct.

2.3. Oocyte harvesting and selection

Oocytes were obtained from adult female *X. laevis* frogs and manually defolliculated after treatment with 1 mg/ml Collagenase A (Roche, Germany) for 30–40 min at RT in Ca^{++}-free ORII medium (82.5 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES/Tris, pH 7.5). Healthy V–VI stadium oocytes [17] were then selected for injection and maintained at 16 °C in Barth's solution (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 50 mg/l gentamicin sulfate and 2.5 mM sodium pyruvate.

2.4. Oocyte expression of KAAT1 wt and mutants

pSPORT-1 plasmid vector bearing KAAT1 wild type (wt) or mutants, and pAMV-PA plasmid vector for FLAG-KAAT1wt and relative mutants were linearized by *Not1* digestion. Corresponding cRNAs were *in vitro* transcribed and capped using T7 RNA polymerase.

Defolliculated oocytes were injected with 12.5 ng of cRNA dissolved in 50 nl of RNAse-free water via a manual microinjection system (Drummond). Before use, oocytes were maintained in Barth's solution at 16 °C supplemented as described above.

2.5. Chemiluminescence

Surface expression of the tagged transporters was detected using the monoclonal primary antibody anti-FLAG M2 (Sigma F3165, 1 μg/ml) and goat anti-mouse IgG secondary antibody labeled with HRP (Jackson ImmunoResearch Laboratories). Briefly, oocytes expressing different FLAG-KAAT1 isoforms, as well as non-injected oocytes, were washed twice for 5 min in ice-cold ND96 pH 7.6. These were then fixed with 4% paraformaldehyde in ND96 for 15 min, rinsed 3×5 min with equal volumes of ND96, and then incubated for 1 h in a 1% BSA-ND96 blocking solution (used in subsequent antibody incubation steps). Next the oocytes were incubated for 1 h in mouse anti-FLAG M2 1 μg/ml, washed 6×3 min in 1% BSA ND96, incubated for 1 h in peroxidase-conjugated goat anti-mouse IgG (HRP-IgG) 1 μg/ml, washed 6×3 min in 1% BSA-ND96 and then 6×3 min in ND96 alone. For the chemiluminescence readings, oocytes were transferred into a 96 well plate (Assay Plate White not treated flat bottom-Corning Costar) filled with 50 μl of SuperSignal Femto (Pierce). Luminescence was quantified with a Tecan Infinity 200 microplate reader. The plates were read not later than 5 min after the transfer of the first oocyte. The data were then acquired at least three times in 10 min and for each oocyte the mean of three readings was calculated. Results were normalized to the mean value of FLAG-KAAT1 wt for each batch and are plotted as relative chemiluminescence intensity.

2.6. Radiolabeled amino acid uptake

Amino acid uptake was evaluated 4 days after injection. Groups of 8–10 oocytes were incubated for 60 min in 120 μl of uptake solution (100 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes/NaOH, pH 8) with 0.1 mM [^{3}H]leucine (444 kBq/ml, specific activity 3.996 Tbq/mmol) or 0.1 mM [^{3}H]proline (592 kBq/ml, specific activity 2.775 TBq/mmol) (all from PerkinElmer Life Sciences). Alternatively, to evaluate uptake induced in the presence of lithium or potassium