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Different functional roles of arginine residues 39 and 61 and tyrosine residue 98 in transport and channel mode of the glutamate transporter EAAC1

Yani Zhu[a,b,c], Larisa A. Vasilets[d], Jian Fei[a,c,*], Lihe Guo[c], Wolfgang Schwarz[a,b,*]

[a]*Max-Planck Guest Laboratory at the Institute of Biochemistry and Cell Biology, CAS, 320 Yue Yang Road, Shanghai 200031, China*
[b]*Max-Planck-Institut für Biophysik, Marie-Curie Str. 15, 60439 Frankfurt/Main, Germany*
[c]*Laboratory of Molecular Cell Biology, Institute of Biochemistry and Cell Biology, CAS, 320 Yue Yang Road, Shanghai 200031, China*
[d]*Julius-Bernstein-Institut für Physiologie, Martin-Luther-Universität Halle-Wittenberg, Magdeburger Str. 6, 06112 Halle/Saale, Germany*

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Abstract

The excitatory amino acid transporter EAAC1 is an electrogenic Na^+- and K^+-gradient-driven transporter. In addition, the transporter mediates in the presence of Na^+ and glutamate an anion conductance uncoupled from the transport of the glutamate. The first two N-terminal domains, important for forming the conductance mode, are extracellularly bordered by positively charged arginine residues, R39 and R61, being completely conserved throughout the transporter family. Also the conserved tyrosine residue Y98 could be important for Cl^- conductance. We have investigated, by measurements of glutamate uptake and glutamate-induced currents, the effects of mutation of the arginines and the tyrosine to alanine. The mutation R39A hardly affects transport and channel mode. The mutation R61A, on the other hand, reduces the activity of transport but stimulates the channel conductance. In addition, the apparent K_m values for glutamate uptake and for the glutamate-activated current are reduced. Glutamate stimulation of current seems to be associated with a voltage-dependent step, and the apparent valence of charge moved during binding is reduced in the R61A mutant. The mutation Y98A leads to reduced function with reduced apparent K_m value for glutamate, and with strong reduction of the selectivity ration between NO_3^- and Cl^- of the conductance mode.

Keywords: *Xenopus* oocyte; Glutamate uptake; EAAC1-mediated current

1. Introduction

Transporters for the excitatory neurotransmitter glutamate play an essential role in the termination of synaptic transmission [1,2] and in the protection of cells from the neurotoxic effects of extracellular glutamate [1,3–5]. They belong to a family of secondarily active transporters that are driven by the electrochemical gradients for Na^+ and K^+ across the cell membrane. The uptake of glutamate is coupled to the co-transport of Na^+ and H^+ and the counter-transport of K^+ with a stoichiometry of 3 Na^+/1 H^+/1 K^+ per glutamate [6]. Hence, the transporter is electrogenic, generating an inwardly directed current carried by the two positive net charges. In addition, the transport protein can mediate an uncoupled anion conductance in the presence of Na^+ and glutamate [7,8].

In mammalian tissue, five isoforms have been identified and cloned: GLAST1 or EAAT1 [9], GLT-1 or EAAT2 [10], EAAC1 or EAAT3 [11], EAAT4 [12], and EAAC5 [13], which exhibit an identity in amino acid sequence of about 50% among each other. In our study, we investigated the function of

Abbreviations: SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TEVC, two-electrode voltage clamp; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; TEA^+, tetraethylammonium; TMA^+, tetramethylammonium; I–V, current–voltage; EAAC, excitatory amino acid carrier; WT, wild-type

* Corresponding authors. Wolfgang Schwarz is to be contacted at Max-Planck-Institut für Biophysik, Marie-Curie Str. 15, 60439 Frankfurt/Main, Germany. Tel.: +49 69 6303 339; fax: +49 69 6303 340. Fei Jian, Max-Planck Guest Laboratory at the Institute of Biochemistry and Cell Biology, CAS, 320 Yue Yang Road, Shanghai 200031, China. Tel.: +86 21 6431 5030; fax: +86 21 6433 1090.

E-mail addresses: schwarz@mpibp-frankfurt.mpg.de (W. Schwarz), jfei@sibs.ac.cn (J. Fei).

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wild-type and mutated glutamate transporters of mouse brain (mEAAC1). It has been suggested that this transporter forms oligomers [14] of most likely five subunits when expressed in the oocytes [15], and this pentameric structure may form the channel for uncoupled Cl^- permeation [16].

Previously, we have performed functional analysis of chimeric transporters formed by EAAC1 and the ASCT1 transporter that accepts alanine, serine and cystein as substrate [17]. The results demonstrated that the N- and C-termini of mEAAC1, expressed in *Xenopus* oocytes, are essential for the formation of the uncoupled anion conductance mode. In the present study, we have mutated the positively charged arginine residue R39 or R61, bordering extracellularly the first two N-terminal putative transmembrane domains and conserved in all EAAT isoforms (Fig. 1), to alanine. Side-chain oxygen atoms from a serine and tyrosine residue have been shown by X-ray structural analysis to be involved in coordinating the Cl^- ion in ClC chloride channels [18]. As a possible candidate in EAAC1, we selected the tyrosine residue Y98, neighbouring Y99, which is conserved in all isoforms (Fig. 1). Here we investigated the effect of mutants with tyrosine deletion, Y98δ, and substitution by alanine, Y98A. We analysed transport function by measurements of glutamate uptake and EAAC1-mediated currents under voltage clamp. The results indicate that the mutated residues are indeed important for transport function. In particular, the mutation of R61 to alanine facilitates the formation of the channel mode and increases the affinity for glutamate. The amino acid at position 98 is critical for transporter function, and in the uncoupled conductance mode the selectivity between Cl^- and NO_3^- becomes reduced.

2. Materials and methods

2.1. Construction of point mutations R39A, R61A, Y98A, and Y98δ

Mutants of mEAAC1 with the point mutations R39A, R61A, and Y98A (R39A-EAAC1, R61A-EAAC1, and Y98A-EAAC1, respectively) and with the deletion of Y98 (Y98δ-EAAC1) were constructed by using recombinant PCR with Pfu DNA Polymerase (Sangon). The mutants were cloned into the pNWP vector [19], which was modified from the pNSK2. pNWP has a polyA$^+$ tail and ribosome binding sites of *Xenopus* oocytes to ensure high transcription in vitro and expression in the oocytes. The full-length sequences of the mutants were checked to make sure that no unexpected mutation occurred. The following primers were used (bold letters indicate the coding regions of single amino acids anticipated to be changed):

Full length primers:

5P:	5’-tca gtg aga tct atg ggg aag ccc acg agc-3’	
	*Bgl*II	mEAAC1(77-94)
3P:	5’-gag gtc gga tcc cta gaa ctg tga ggt ctg-3’	
	*Bam*HI	mEAAC1(1648-1631)

Mutation primers:

For R39A:

5P:	5’-gga gtc gtg gtt **gc**a gga cac agt gag-3’	
		mEAAC1(179-205)
3P:	5’-ctc act gtg tcc t**gc** aac cac gac tcc-3’	
		mEAAC1(205-179)

For R61A:

5P:	5’-gaa att ctg atg **gc**g atg ctg aag ctg-3’	
		mEAAC1(245-271)
3P:	5’-cag ctt cag cat c**gc** cat cag aat ttc-3’	
		mEAAC1(271-245)

For Y98A:

5P:	5’-gct gta gta tat **gc**t ttc tcc acc acc-3’	
		mEAAC1(356-384)
3P:	5’-ggt ggt gga gaa a**gc** ata tac tac agc-3’	
		mEAAC1(384-356)

For Y98δ:

5P:	5’-gct gta gta tat ttc tcc acc acc-3’	
		mEAAC1(356-384)
3P:	5’-ggt ggt gga gaa ata tac tac agc-3’	
		mEAAC1(384-356)

2.2. Expression of EAAC1 in Xenopus oocytes

Full-grown prophase-arrested oocytes were isolated from female frogs of *Xenopus laevis* and treated with 3

Fig. 1. Alignment of the sequence range of the EAAT family which includes the arginine residues 39 and 61 and the tyrosine residue 98 of the mEAAC1 sequence. Grey underlays indicate putative transmembrane domains.

or 1.5 mg/ml collagenase (GibcoBRL, Type I) for 2–4 h or overnight, respectively. EAAC1, either wild-type or the mutants R39A, R61A, Y98A or Y98δ, were expressed in oocytes by injection of the respective cRNAs (23 ng/oocyte). The highest degree of expression was obtained after 2 days of incubation at 19 °C. All experiments were conducted at room temperature (24–26 °C).

2.3. Voltage-clamp experiments

Electrophysiological experiments were performed on the oocytes with conventional two-electrode voltage clamp (TEVC) using Turbo TEC 05 or 10 amplifiers (NPI electronic) and IQV software (Schwarz and Vasilets, unpublished). For measuring EAAC1-mediated steady-state currents, membrane currents were recorded at the end of 200 ms voltage pulses from −150 up to +100 mV in 10 mV increments that were applied from a holding potential of −60 mV. The difference between the steady-state currents in the presence of extracellular glutamate (10, 25, 50, 100, 200 or 1000 μM) and in its absence was used as a measure of the EAAC1-mediated currents. The external test solutions were similar to ORi solution (see Solutions) but contained $BaCl_2$ instead of $CaCl_2$. When external Cl^- concentrations were changed, bath electrodes were separated via agar bridges containing 0.1% agar in 1 M KCl.

2.4. Measurements of ^{3}H-glutamate uptake

For measurements of ^{3}H-glutamate uptake, about 10 oocytes were incubated in 200 μl of the respective glutamate-containing ORi solution for 10 min; within this time period uptake still shows linear time dependency. The content of ^{3}H-labeled glutamate (Amersham) was 19 or 38 kBq/200 μl. To determine nonspecific uptake, some experiments were performed in solution where all Na^+ was replaced by tetramethylammonium (TMA^+). To exclude oocytes with high membrane leakage, 1 mM sucrose with 18 μM ^{14}C-labeled sucrose (16 kBq/200 μl, Du Pont NEN) was added to the incubation medium [20].

2.5. Detection of membrane proteins

Two days after injecting cRNA, 30 oocytes were washed three times with OPBS (see Solutions) and labeled with 1 mg/ml Sulfo-NHS-Biotin (PIERCE) at 4 °C for 30 min. After washing with ORi once, the biotinylation was terminated with 10 mM glycine in ORi. Then the oocytes were lysed in HBO buffer (see Solutions) plus 1% Triton X-100, and were centrifuged at 150×*g* at 4 °C for 5 min to remove the yolk, followed by centrifuging at 15,000 rpm at 4 °C for 15 min to remove the insoluble pellet [21]. The lysates were precipitated with 12 μl of ImmunoPure Immobilized Streptavidin Gel (PIERCE) at

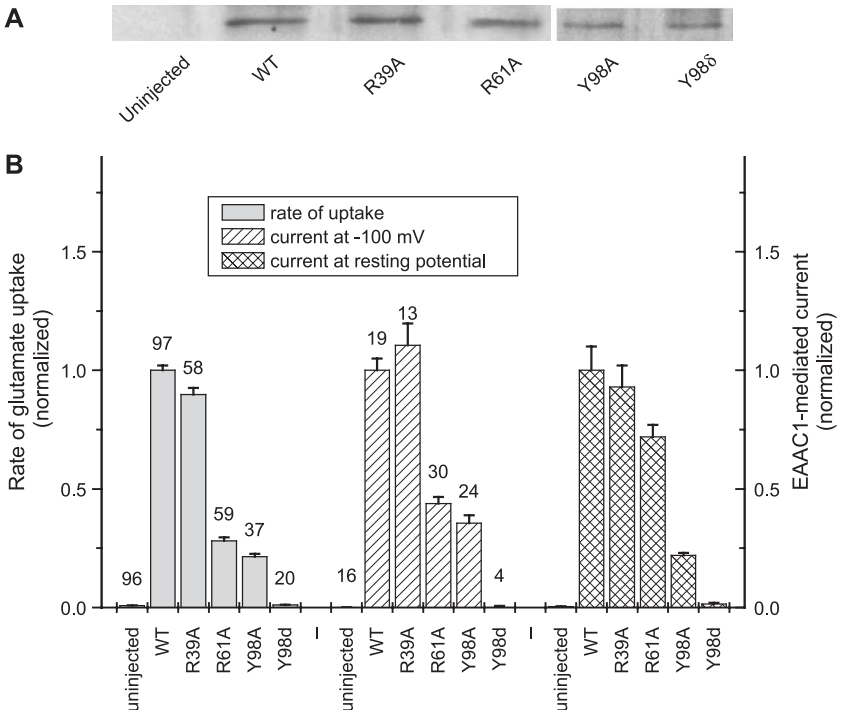

Fig. 2. (A) Expression of WT and mutant EAAC1 on oocyte surface membranes. (B) Rate of normalized glutamate uptake and normalized EAAC1-mediated current at −100 mV and at the respective resting potentials, which was (in mV) −47±3 (*n*=15) for uninjected oocytes, −13±1 (*n*=48) for wild-type, −3.7±1.3 (*n*=24) for R39A, −6.9±1.5 (*n*=29) for R61A, −19±1 (*n*=28) for Y98A, and −29±4 (*n*=4) for Y98δ (indicated as Y98d) transporters. Data from each batch of oocytes (5–10 cells per condition) were normalized to the respective average value of wild-type EAAC1. The glutamate concentration was 200 μM. Data represent averages±S.E. Values above columns represent number of measurements. For rate of uptake "one" corresponds to 442±21 fmol/s, for current at −100 mV to −833±84 nA and for current at resting potential to −113±16 nA.

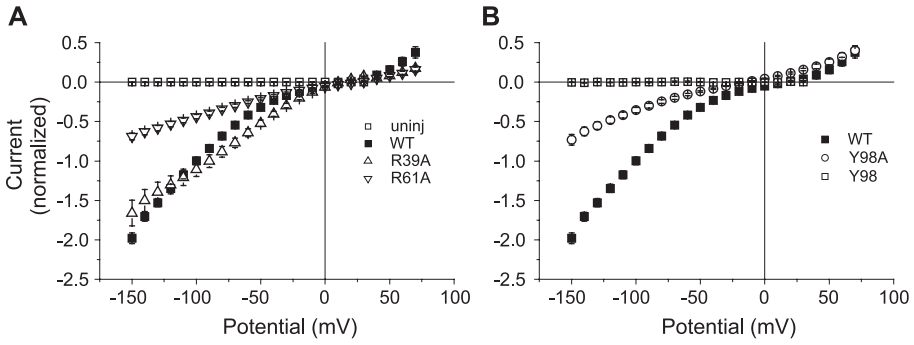

Fig. 3. Voltage dependence of current induced by 200 μM glutamate. (A) For uninjected oocytes (n=16), oocytes with wild-type EAAC1 (n=19), R39A-EAAC1 (n=13) and R61A-EAAC1 (n=30). (B) For oocytes with wild-type EAAC1 (n=16, for potentials above +30 mV, n=4), Y98A-EAAC1 n=20, for potentials above +30 mV, n=4) and Y98δ-EAAC1 (n=4). Data represent±S.E., and are normalized to the current of WT-EAAC1 at −100 mV.

4 °C with end-to-end rotation for 3–4 h. After washing the gels four times with PBS, the gels were mixed with Laemmli sample buffer, boiled for 5 min under reducing conditions, electrophoresed on a 10% polyacrylamide gel, and transferred to Hybond C nitrocellulose membrane. The membrane was subsequently blocked overnight with 10% non-fat dry milk in TBST buffer (see Solutions). The first strips were washed three times in TBST and incubated with anti-mEAAC1 C-terminus (462–523 aa) antibody (prepared by Inst. Biochem. and Cell Biol., Chinese Acad. Sci., 1:500) for 1 h at room temperature. After extensive washing, the membrane fraction was incubated for 1 h with anti-rabbit antibody (CNI, 1:10,000) conjugated with peroxidase, washed several times in TBST, and visualized using enhanced chemiluminescence detection system (Amersham).

2.6. Solutions

The standard solution (ORi) was composed of (in mM): 90 NaCl, 2 KCl, 2 $CaCl_2$ and 5 MOPS ((3-(*N*-morpholino)-propanesulfonic acid) adjusted to pH 7.4 with Tris). The OPBS contained (in mM) 0.65 KH_2PO_4, 90 NaCl, 3 Na_2HPO_4, pH 7.4, and the HBO solution 5 $MgCl_2$, 5 NaH_2PO_4, 100 NaCl, 10 KCl, 1 DTT, 1 PMSF, 20 Tris–HCl (pH 7.4), and the TBST buffer (in mM) 150 NaCl, 0.1% Tween 20, 50 Tris–HCl (pH 7.4).

3. Results

For EAAC1 it has been demonstrated that it can mediate a substrate-independent anion conductance in mammalian expression systems [22,23]. This current component can be blocked by DL-threo-β-bebzyloxyaspartic acid (TBOA), a highly selective inhibitor of EAATs. In the *Xenopus* oocytes, 1 mM TBOA completely inhibited the glutamate-dependent current in wild-type as wells as mutant EAAC1, and the glutamate-independent and TBOA-sensitive current amounted to less than 5% of the glutamate-dependent current.

3.1. Functional expression of wild-type and mutated EAAC1

Precipitation after biotinylation revealed that wild-type (WT) EAAC1 and the mutated glutamate transporters were expressed in the oocytes and targeted to the cell membrane to a similar extent (Fig. 2A). The function of the transporters was tested by measurement of uptake of ^{3}H-labeled glutamate and of glutamate-induced current (Fig. 2B). The data were normalized to the respective signal of the WT-EAAC1. Occasionally, batches of oocytes were obtained where even uninjected cells exhibited significant glutamate uptake or glutamate-induced current; in those cases, the endogenous components determined in uninjected cells were subtracted.

The mutation of the arginine residues and the tyrosine residue to alanine led to functional transporters. While R39A-EAAC1 exhibited only slightly reduced rate of glutamate uptake (by 10%), uptake by R61A- and Y98A-EAAC1 was significantly reduced by 72% and 79%, respectively. The deletion mutant Y98δ did not exhibit uptake though it was targeted to the membrane. Qualita-

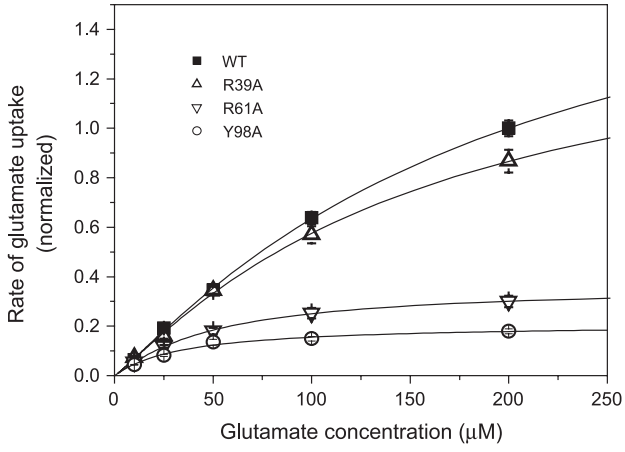

Fig. 4. Dependence of rate of uptake on glutamate concentration for wild-type EAAC1 (filled squares) and the mutant EAAC1 R39A (triangle up), R61A (triangle down) and Y98A (circles). Data represent averages of 20 oocytes per values±S.E. Lines are fits of Eq. (1) to the data with parameters listed in Table 1.

Table 1
Fitted parameters of Eq. (1) for the data presented in Fig. 4 (rate of uptake), and Fig. 5 (current at −100 mV)

	For rate of uptake		For current at −100 mV		Fitted parameters for voltage dependence		
	R_{max}	K_m (μM)	R_{max}	K_m (μM)	K_m (0 mV) (μM)	z	$K_m^{+\infty}$ (μM)
Wild-type EAAC1	2.00	199	2.91	355	96	0.73	91
R39A mutant	1.54	160	3.05	440	97	0.77	81
R61A mutant	0.36	49	0.89	184	53	0.40	20
Y98A mutant	0.20	32	0.46	38	13	0.73	16

All data sets could be fitted with a Hill coefficient of m=1.11. The last two columns are fitted parameters of Eq. (2) to the data in Fig. 6.

tively similar effects of the mutations were detected with the steady-state glutamate-induced currents. The hatched bars in Fig. 2B show the currents recorded at −100 mV. The current mediated by R39A-EAAC1 only slightly deviated from that of WT-EAAC1. As already seen in the flux measurements, R61A and Y98A mutations led to a clear inhibition which amounted to 56% and 64%, respectively, and the Y98δ mutant did not generate any current. Since the flux measurements were not done under voltage clamp, Fig. 2B also shows for comparison with the fluxes steady-state currents (double-hatched bars) at the respective resting potentials, which were determined from current–voltage dependencies and are listed in the legend to Fig. 2. These data again show that R39A mutation hardly changed transporter-mediated current at this potential, while R61A-EAAC1 generated significantly reduced current (by nearly 30%) compared to WT-EAAC1 (P<0.001). This reduced degree of inhibition at resting potential compared to −100 mV is the result of less steep voltage dependencies of the I–V curves for the mutants compared to WT-EAAC1 (Fig. 3A); the voltage dependence among the two arginine mutants is nearly identical. The difference in effects of mutation on uptake and current may be an indication that the transport mode is more affected than the channel mode. The current mediated by Y98A-EAAC1 (Fig. 3B) exhibited with 78% the same degree of inhibition as the rate of uptake. On the other hand, we should realize that these estimates of current at resting potential are not as accurate as the flux measurement due to the small EAAC1-mediated currents which amounted for the mutants to only 30–100 nA.

3.2. Dependence on glutamate concentration of rate of uptake

Fig. 4 shows the dependencies of rate of glutamate uptake on glutamate concentration. The data were fitted by:

$$\text{Rate} = R_{max}\frac{[\text{glutamate}]^m}{[\text{glutamate}]^m + K_m^m} \quad (1)$$

with a common Hill coefficient of m=1.1. The other fitted parameters are listed in Table 1. As one could already expect from the flux data in Fig. 2B, the R_{max} value became only slightly reduced by the R39A mutation, but the R61A and Y98A mutations were much more effective. The same was obtained for the K_m values. The data for WT- and R39A-EAAC1 indicate that the 200 μM used in the experiments shown in Figs. 2 and 3 was not a saturating concentration for stimulating these two transporters. In experiments at 1 mM, we obtained uptake rates that were even slightly larger than the estimated R_{max} values for WT- and R39A-EAAC1. Dramatic increase in rate of glutamate uptake at mM concentrations was reported for an endogenous Na^+-coupled glutamate transporter that can occasionally be detected in the oocytes [24].

3.3. Dependence on glutamate concentration of glutamate-induced current

Fig. 5 shows EAAC1-mediated current at −100 mV for various glutamate concentrations. The concentration dependencies were fitted by Eq. (1) with m=1.1, and the other fitted parameters are listed in Table 1. The K_m values for stimulation of current by glutamate were considerably larger than those for uptake stimulation, except for the Y98A mutant. K_m values for WT- and the mutated EAAC1s were also determined for the other potentials and are shown in Fig. 6. The values were voltage-dependent, suggesting that glutamate binding is associated with a step in the transport cycle that involves charge movements. Clear

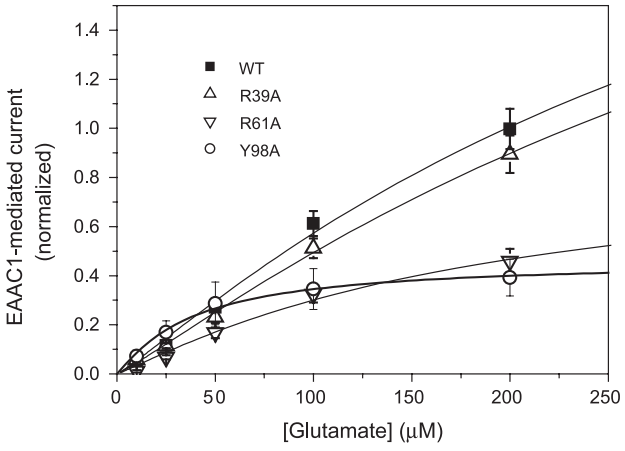

Fig. 5. Dependence of EAAC1-mediated current on glutamate concentration for wild-type EAAC1 (filled squares) and the mutant EAAC1 R39A (open triangles up) and R61A (open triangles down). Data represent the average values of 8–12 oocytes at −100 mV in the presence of glutamate. Lines are fits of Eq. (1) to the data with parameters listed in Table 1.

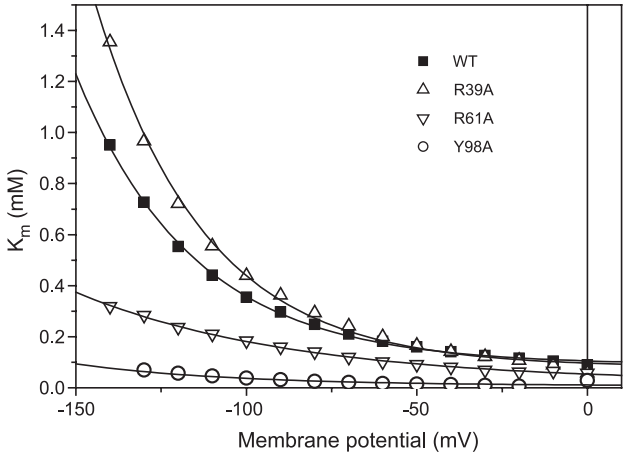

Fig. 6. Voltage dependence of apparent K_m values for stimulation of EAAC1-mediated current by glutamate for WT-EAAC1 (filled squares) and the mutants R39A- (open triangles up), R61A-EAAC1 (open triangle down) and Y98A-EAAC1 (circles) calculated from the glutamate dependence of the EAAC1-mediated current at different membrane potentials according to Eq. (1). The lines represent fits of Eq. (2) to the data. The fitted parameters are listed in Table 1.

voltage dependence became apparent only at very negative potentials; in the voltage range above −50 mV, on the other hand, only slight voltage dependence was apparent suggesting a description by the sum of a voltage-independent K_m value $K_m^{+\infty}$ and an exponential component:

$$K_m = K_m(0\ \text{mV})e^{\frac{-zFV}{RT}} + K_m^{+\infty} \quad (2)$$

z is the effective valence of the moved charge, K_m (0 mV) the voltage-dependent contribution to the apparent K_m value at 0 mV, $K_m^{+\infty}$ the voltage-independent contribution; F, R and T have their usual meaning. The apparent K_m value at 0 mV is K_m (0 mV)+$K_m^{+\infty}$. The fitted parameters are listed in Table 1. The mutation R39A only slightly affected the K_m value; increased values at very negative potentials may be attributed to the slightly increased effective valency z leading to more pronounced voltage dependence. The K_m values for R61A-EAAC1, on the other hand, were strongly reduced over the entire potential range, which is due to reduced K_m (0 mV), $K_m^{+\infty}$ as well as effective valency z. The K_m values for the Y98A-EAAC1 were even more strongly reduced, which could be attributed to reduced K_m (0 mV) and $K_m^{+\infty}$. The fitted K_m values at the respective resting potentials were of similar magnitude as those for the rate of uptake (compare with Table 1).

3.4. Effect of partial replacement of Cl^- by NO_3^-

In the channel mode, EAAC1 allows anions to cross the membrane along their electrochemical gradient with higher permeability for NO_3^- than for Cl^- [7]. Hence, when externally 90 mM NaCl was replaced by $NaNO_3$, an increased outward current could be detected resulting from the elevated inward movement of negative charges (Fig. 7). Increased outward current was detected for the wild-type as well as the mutant EAAC1s. The reversal potential in the NO_3^- solution was shifted in the negative direction (see insets in Fig. 7): for WT-EAAC1 and R39A-EAAC1 by about 80 mV from +25 mV (Fig. 7A), for R61A-EAAC1 by

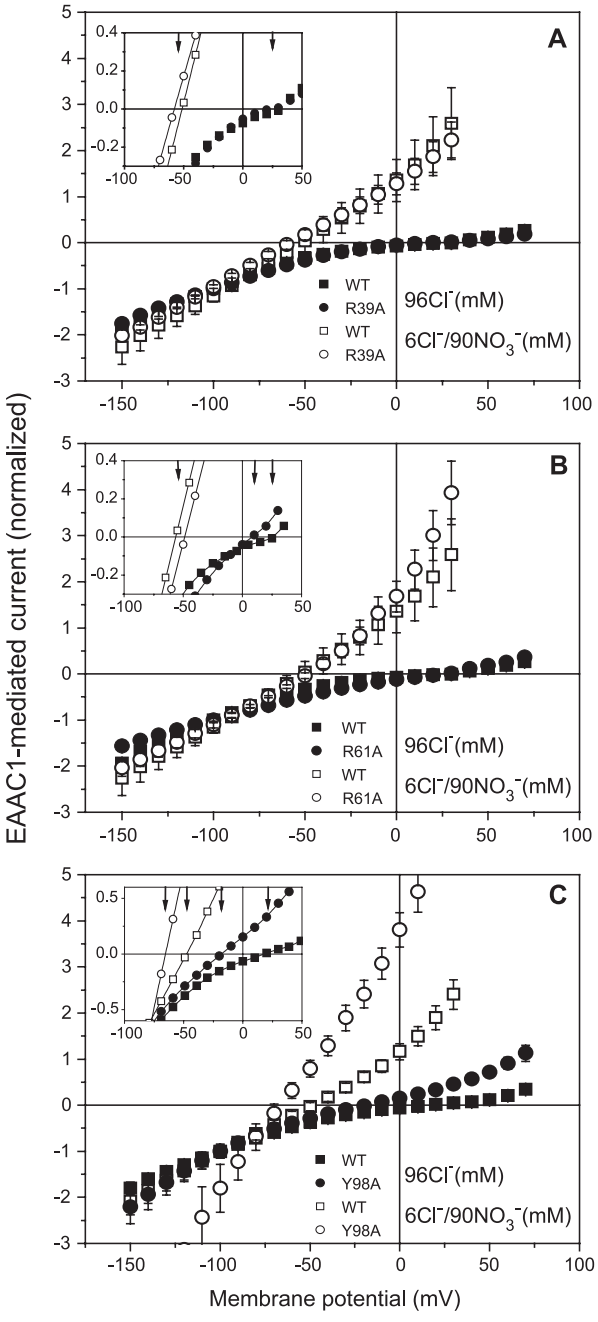

Fig. 7. Voltage dependence of current induced by 200 μM glutamate in 96 mM Cl^- (filled symbols) or 6 mM Cl^-/90 mM NO_3^- (open symbols) solution for oocytes with wild-type EAAC1 (squares) and mutants (circles). (A) For oocytes with wild-type EAAC1 and R39A-EAAC1, (B) with wild-type EAAC1 and R61A-EAAC1, and (C) with wild-type EAAC1 and Y98A-EAAC1. Data for wild-type EAAC1 in panels A and B are the same. Data were normalized to the respective current in Cl^- solution at −100 mV. The values represent averages of n=6–16 measurements±S.E.

about 65 mV from +10 mV (Fig. 7B), and for Y98A-EAAC1 by only 50 mV from −15 mV (Fig. 7C).

4. Discussion

It has been suggested that EAAT3 (equivalent to EAAC1) operates as an oligomeric assembly in native tissue as well as when expressed in *Xenopus* oocytes, and in particular a pentameric assembly has been proposed [15]. It has been speculated that this pentameric structure forms the pathway for uncoupled Cl^- conductance while transport may be mediated by the monomeric protein. We have shown previously [17] that replacement of the first 70 N-terminal amino acids of EAAC1 by the corresponding domain of ASCT1 (93 amino acids) nearly completely abolishes the formation of the uncoupled Cl^- conductance. This domain includes the first transmembrane domain and part of the second. Since EAAC1 and ASCT1 show the highest degree of diversity within the N-terminal cytoplasmic part, one might speculate that these domains are involved in monomer interaction and formation of the multimeric Cl^- channel-forming assembly. The two transmembrane domains are externally bordered by the positively charged arginine residues R39 and R61 (see Fig. 1) that are conserved among all EAAT isoforms. In particular, the motif around R61 (LM**R**ML) is conserved throughout. Arginine residues have been shown by X-ray structure analysis to participate in the formation of the selectivity filter of ClC chloride channels [18]. It has been shown that a tyrosine residue is involved in coordinating the Cl^- in the ClC channels [18]; the tyrosine Y98 conserved among the EAAT transporters (see Fig. 1) might be a possible candidate in EAAC1. Our results show that mutation of these residues to alanine still leads to expression of functional transporters, though with altered characteristics.

The R39A mutation has no pronounced effect on any of the analysed parameters, neither on rate of uptake and current nor on glutamate sensitivity and anion selectivity. In contrast, rate of transport becomes drastically reduced on R61A and Y98A mutations at 200 μM glutamate (Fig. 2) despite the fact that the K_m values become reduced simultaneously from 200 to 50 and 30 μM, respectively (Table 1). Also the EAAC1-mediated current and the corresponding K_m values are reduced over the entire potential range in both mutants. The effect of this mutation on the K_m values determined from current measurements becomes more apparent with more negative potential which can be attributed to a reduced effective valency z (Table 1) of the charges moved during a step associated with the glutamate binding. A possible explanation could be that the negatively charged glutamate has to pass an access channel sensing a fraction of the electrical field. Replacement of R61 by alanine would then reduce the apparent length of the access channel. Slight voltage-dependent glutamate binding, though not as pronounced as found in our experiments, have been reported previously [25]. Glutamate binding and voltage-dependent Na^+ binding have been demonstrated to occur sequentially [26], and it has been shown that the apparent K_m value for glutamate decrease with increasing Na^+ concentration [25]. Our results, on the other hand, show that hyperpolarisation, which would favour Na^+ binding, leads to an increase in the K_m value for glutamate.

Focussing on the K_m values for current stimulation by glutamate at resting potential yields that channel and transport mode exhibit similar sensitivity in wild-type, R39A, and Y98A-EAAC1. The transport in the R61A-EAAC1, on the other hand, shows higher affinity for glutamate than the current mode (see Table 1). This suggests that the mutation R61A counteracts the formation of the uncoupled channel mode or reduces channel conductance. Opposing effects on transport and channel mode have also been observed in an I421C mutant by application of sulfhyhryl reagent which impacts the transport but not the channel mode [27], and by application of Li^+, which can replace Na^+ only in the aspartate-coupled transport mode [28].

Similar conclusions as outlined above are obtained from comparison of measured current and current calculated from glutamate uptake. Based on the stoichiometry with two positive charges translocated across the membrane per glutamate and the assumption that under the experimental condition, no significant electrically silent glutamate exchange is involved, the transport-associated current can be calculated. The wild-type transporter would generate a current of 84 nA. This is only slightly less than the 92 nA actually detectable at the resting potential, indicating that at this potential, only a small fraction of the total current is mediated by the uncoupled conductance mode. Similar results are obtained for R39A-EAAC1. In R61A-EAAC1, the glutamate-coupled current calculated from the rate of uptake would amount to only 24 nA. Compared to the measurable current at the resting potential of 70 nA, this means that now even more than 60% of the total current can be attributed to uncoupled conductance. In absolute values, this means that glutamate transport associated current drops from 84 to 24 nA on mutation of R61 to alanine while the channel mode associated current increases from 8 to 46 nA at the resting potential. In the Y98A-EAAC1, rate of uptake and current are reduced to the same extent as compared to the wild-type. This indicates that Y98 is important for proper function. The shift in reversal potential into the negative potential range, nevertheless, shows that the relative contribution from the channel mode to the current has increased. For the mutation of the corresponding tyrosine to phenylalanine in EAAT1 (Y127F), on the other hand, a loss of all glutamate-dependent current has been demonstrated [29].

Since the channel mode exhibits higher permeability P_{NO_3} for NO_3^- than P_{Cl} for Cl^-, substitution of Cl^- by NO_3^- revealed the expected shift of reversal potential into

negative direction (Fig. 7). On the basis of the Goldman–Hodgkin–Katz equations for the currents carried by Cl^- and NO_3^-:

$$I_{Cl} = F^2 \frac{E}{RT} \left(\frac{[Cl]_i \exp(-EF/RT) - [Cl]_o}{\exp(-EF/RT) - 1} \right) P_{Cl}$$

$$I_{NO_3} = F^2 \frac{E}{RT} \left(\frac{[NO_3]_i \exp(-EF/RT) - [NO_3]_o}{\exp(-EF/RT) - 1} \right) P_{NO_3}$$

and I_t the current contribution of the transport mode at the reversal potential, for simplicity expressed by:

$$I_t = -F^2 \frac{1}{(e^{+EF/RT} + 1)} P_t$$

permeability ratios of P_{NO_3}/P_{Cl} can be estimated from the reversal potentials as shown in Fig. 7 with:

$$I_{Cl} + I_{NO_3} + I_t = 0.$$

For intracellular Cl^- activity a value of 30 mM was assumed [30]. For the wild-type and R39A-EAAC1 a permeability ratio P_{NO_3}/P_{Cl} of about 50 is obtained. The mutation of R61A reduces the selectivity P_{NO_3}/P_{Cl} to about 25, and for Y98A the ratio even decreases to 8.

In conclusion, the two arginine residues (R39, R61) bordering the two first N-terminal transmembrane domains extracellularly and the tyrosine residue (Y98) are not essential for expression of the EAAC1, but they interfere with transport and channel function. In particular, R61 seems to play a critical role in glutamate affinity, rate of glutamate translocation and channel formation. While transport is reduced in the mutant EAAC1, channels current is increased with decreased selectivity for NO_3^- over Cl^-. Deletion of Y98 results in complete loss of function, and mutation to alanine reduces transport. The anion selectivity of the channel mode is dramatically decreased supporting the view that tyrosine residues may be involved in anion coordination as has been shown for Cl^- channels [18]. The results support the view that the transport mode involves different conformational changes than in the channel mode as has been also suggested for EAAT1 [31]. Altered balance between current and transport has also been reported for members of the GABA-transporter family (see Ref. [32]).

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