A Conserved Methionine Residue Controls the Substrate Selectivity of a Neuronal Glutamate Transporter.

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Running title: Substrate Specificity of Glutamate Transporter

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Glutamate transporters located in the brain, maintain low synaptic concentrations of the neurotransmitter by coupling its flux to that of sodium and other cations. In the binding pocket of the archeal homologue GltPh, a conserved methionine residue has been implicated in the binding of the benzyl moiety of the non-transportable substrate analogue threo-β-benzyl oxyaspartate. To determine if the corresponding methionine residue of the neuronal glutamate transporter EAAC1, Met-367, fulfils a similar role, M367L, M367C and M367S mutants were expressed in HeLa cells and Xenopus laevis oocytes to monitor radioactive transport and transport currents, respectively. The apparent affinity of the Met-367 mutants for D-aspartate and L-glutamate, but not for L-aspartate, was 10-20-fold reduced as compared to wild type. Unlike wild type, the magnitude of I_max was different for each of the three substrates. D-glutamate, which is also a transportable substrate of EAAC1, did not elicit any detectable response with M367C and M367S, but acted as a non-transportable substrate analogue in M367L. In the mutants, substrates inhibited the anion conductance as opposed to the stimulation observed with wild type. Remarkably, the apparent affinity of the blocker D,L-threo-β-benzyl oxyaspartate in the mutants was similar to that of wild type EAAC1. Our results are consistent with the idea that the side-chain of Met-367 fulfils a steric role in the positioning of the substrate in the binding pocket, in a step subsequent to its initial binding.

Glutamate transporters keep the synaptic concentrations of the neurotransmitter below neurotoxic levels and are key elements in the termination of the synaptic actions of the neurotransmitter. Glutamate transport is an electrogenic process (1,2) consisting of two sequential translocation steps: 1. cotransport of the neurotransmitter with three sodium ions and a proton (3,4) and 2. the countercurrent of one potassium ion (5-7). Glutamate transporters mediate two distinct types of substrate-induced steady-state current: an inward-rectifying or "coupled" current, reflecting electrogenic sodium-coupled glutamate translocation, and an "uncoupled" sodium-dependent current, which is carried by chloride ions and further activated by substrates of the transporter (8-10). Substrate analogues, such as TBOA (D,L-threo-β-benzyl oxyaspartate), can bind to the transporters but their extra bulk prevents them from being transported. These analogues are expected to "lock" the transporter in an outward-facing conformation and are not only competitive inhibitors of the two types of substrate-induced current, but also inhibit the basal sodium-dependent anion conductance (11,12). Moreover, when the membrane voltage is suddenly changed in the absence of substrate, sodium-dependent transient currents are observed (13). These transient currents presumably represent a charge-moving conformational change following binding/debinding of sodium. Addition of a transportable substrate enables transport and thereby converts the transient current into a steady-state current, whereas non-transportable analogues block the transients altogether (13).

A high-resolution crystal structure of a glutamate transporter homologue, GltPh, from the archean Pyrococcus horikoshii was published (14). It forms a trimer with a permeation pathway through each of the monomers, indicating that the monomer is the functional unit. This is also the case for the eukaryotic glutamate transporters (15-18). The membrane...
topology of the monomer (14) is quite unusual but is in excellent agreement with the topology inferred from biochemical studies (19-21). The monomer contains eight trans-membrane domains (TM) and two oppositely-oriented reentrant loops, one between domains 6 and 7 (HP1) and the other between domains 7 and 8 (HP2). TMs 1-6 form the outer shell of the transporter monomer whereas TMs 7 and 8 and the two reentrant loops participate in the formation of the binding pocket of Glt Ph (14,22). Importantly, many of the amino acid residues of the transporter, inferred to be important in the interaction with sodium (23,24), potassium (7,25) and glutamate (26,27) are facing toward the binding pocket.

Another important residue was identified when the crystal structure of GltPh in complex with TBOA was solved (22). This is a methionine residue found near the benzyl and aspartate moieties of the bound TBOA, which has been implicated in the binding of the benzyl moiety of the non-transportable substrate analogue (22). This methionine is located in the unwound part of transmembrane helix 7 and is conserved in the eukaryotic glutamate transporters, including the neuronal transporter EAAC1 (Met-367 in the rabbit form of EAAC1), also known as EAAT3. Here we ask the question if in the eukaryotic glutamate transporters this methionine also interacts with bound blocker and substrate by probing if mutation of Met-367 of EAAC1 results in changes of selectivity and apparent affinity of blocker and substrates.

Experimental Procedures

Generation and subcloning of mutants- The C-terminal histidine-tagged versions of rabbit EAAC1 (28,29) in the vector pBluescript SK' (Stratagene) was used as a parent for site-directed mutagenesis (30,31). This was followed by subcloning of the mutations into the histagged EAAC1, residing in the oocyte expression vector pOG1 (29), using the unique restriction enzymes NsiI and Stul. The subcloned DNA fragments were sequenced between these unique restriction sites.

Cell growth and expression- HeLa cells were cultured (32), infected with the recombinant vaccinia/t7 virus vTF7-3 (33) and transfected with the plasmid DNA harboring the WT or mutant constructs or with the plasmid vectors alone (32). Transport of D-[3H]-aspartate or other radiolabelled substrates was done as described (30). Briefly, HeLa cells were plated on 24-well plates and washed with transport medium containing 150 mM NaCl/5 mM KPi, pH 7.4. Each well was then incubated with 200 µl of transport medium supplemented with 0.4 µCi of the tritium labeled substrates and incubated for 10 minutes, followed by washing, solubilization of the cells with SDS and scintillation counting.

Expression in oocytes and electrophysiology- cRNA was transcribed using mMESSAGE-mMACHINE (Ambion), injected into Xenopus laevis oocytes, and maintained as described (24). Oocytes were placed in the recording chamber, penetrated with two agarose-cushioned micropipettes (1%/2M KCl, resistance varied between 0.5 and 3 mΩ), voltage clamped using GeneClamp 500 (Axon Instruments) and digitized using Digidata 1322 (Axon Instruments both controlled by the pClamp9.0 suite (Axon Instruments). Voltage jumping was performed using a conventional two-electrode voltage clamp as described previously (29). The standard buffer, termed ND96, was composed of 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 5 mM Na-HEPES, pH 7.5). In sodium titration experiments, the composition of the buffer was the same except for the NaCl which was increased to 130 mM. In these experiments (see Fig. 5), NaCl was replaced by equimolar concentrations of choline Cl. The composition of other perfusion solutions is indicated in the Figure legends. Offset voltages in chloride substitution experiments were avoided by use of an agarose bridge (1%/2M KCl) that connected the recording chamber to the Ag/AgCl ground electrode. Before the application of sulphydryl reagents, the oocytes were perfused with 30 ml of the same solutions used during the treatment with the sulphydryl reagent with the exception of TBOA (see below). Subsequently the flow was stopped and the sulphydryl reagent was added directly to the bath under voltage clamp (-25mV) at the final concentration indicated in the legend to Fig. 8. When the effect of TBOA on the inhibition by the sulphydryl reagents was tested, it was also directly added to the bath 1 min prior to the addition of the sulphydryl reagent. After a 5 min incubation with the sulphydryl reagent the oocytes were perfused with 15 ml of the same solution (without sulphydryl reagent), followed by another 30 ml of ND96. Currents induced by L-Glutamate were measured before as well as after the treatment with the sulphydryl reagent.

Data analysis- All current-voltage relations represent steady-state substrate- elicited net
currents \((\text{I}_{\text{buffer+substrate}} - \text{I}_{\text{buffer}})\) and were analyzed by Clampfit version 8.2 or 9.0 (Axon instruments). Because of the variability in expression level within and between different oocyte batches, the data have been normalized as indicated in the Figure legends. Kinetic parameters were determined by non-linear fitting to the generalized Hill equation using the build-in functions of Origin 6.1 (Microcal). For determination of apparent affinity for substrate, the current observed at saturating substrate concentrations (\(I_{\text{max}}\)) and \(K_{0.5}\) were allowed to vary and the value of \(n_H\) was fixed at 1. Because of the voltage dependence of \(I_{\text{max}}\), we defined the kinetic parameters at -100mV.

Results

Radioactive transport and transport currents by Met-367 mutants - The conserved methionine residue at position 367 of EAAC1 was mutated to residues with smaller or larger side-chains, such as cysteine and serine or phenylalanine, respectively and also to leucine, whose side-chain is roughly the same size as methionine. Upon expression of these mutants in HeLa cells transport of L-[\(^{3}\text{H}\)]-aspartate, L-[\(^{3}\text{H}\)]-glutamate and D-[\(^{3}\text{H}\)]-aspartate was reduced relative to EAAC1-WT (Fig. 1). Remarkably, transport is not decreased to the same extent for all three substrates. Thus, transport of L-[\(^{3}\text{H}\)]-aspartate by M367C, M367S and M367L was affected less than that of L-[\(^{3}\text{H}\)]-glutamate and D-[\(^{3}\text{H}\)]-aspartate (Fig. 1). This argues against a decreased expression level of the mutants, but rather points to a change in substrate selectivity.

In order to avoid the use of excessive amounts of radioactive substrate, necessary to determine \(K_m\) and \(V_{\text{max}}\) in the face of the relatively low transport levels of the mutants, we measured the transport currents in oocytes expressing these mutants to determine the apparent substrate affinity and the values of \(I_{\text{max}}\).

In oocytes expressing EAAC1-WT saturating concentrations of L-aspartate, L-glutamate and D-aspartate each induced currents of similar magnitude (Fig. 2B-D, Table I). With either EAAC1-WT or the mutants, these currents were neither observed in the absence of sodium (replacement by choline; data not shown) nor in the presence of GABA, which is not a substrate (Fig. 2A-D). The L-aspartate-induced transport currents in oocytes expressing these mutants showed relatively modest changes in \(K_m\), but with D-aspartate and L-glutamate, the \(K_m\) values were increased between 10-20-fold (Fig. 3 and Table I).

Transport currents in the presence of D-glutamate and TBOA- In EAAC1-WT, D-glutamate, induced steady-state transport currents which had a similar voltage dependence to those induced by L-glutamate (Fig. 4A). The currents induced by saturating concentrations of D-glutamate at -100mV were 80.7±3.4 % of those induced by L-glutamate (n=3). However, the \(K_m\) value of the D-isomer was 417.1±9.0 \(\mu\text{M}\) (n=3), which is 20-50-fold larger than that of L-glutamate, L-aspartate and D-aspartate (Table I). In the absence of substrates, EAAC1-WT also exhibited sodium dependent transient currents, which were inhibited by the non-transportable substrate analogue TBOA. The currents in the presence of sodium subtracted from those in sodium and TBOA are shown in the bottom level of Fig. 4A and the two sets of absolute currents used for the subtraction are shown in Fig. 4B. The transient currents are dependent on the expression of the transporter, since they were not observed with water-injected oocytes (Fig. 4A and B). Similar transient currents were also observed with M367C and with M367L, but were hardly detectable with M367S (Fig. 4A and B). In contrast to EAAC1-WT, D-glutamate did not induce any steady-state currents in oocytes expressing M367C, M367S or M367L, even though it was applied at a concentration of 5 mM (Fig. 4A). However, D-glutamate blocked the sodium-dependent transient currents of the M367L mutant (Fig. 4A), indicating that D-glutamate still could bind to the M367L transporters. The IC50 of M367L for D-glutamate was 2.78±0.18 mM (n=3). On the other hand, the transient currents by M367C could not be blocked by D-glutamate. The difficulty to detect sodium-dependent transient currents with M367S is not due to the inability of sodium to bind to the transporter. As stated above, the transport currents by M367S were sodium dependent. Remarkably, the half-maximal concentration of sodium required to
activate the currents of M367S was at least 2.5-fold higher than that of EAAC1-WT (24.9 ±2.7 mM), because even at 130 mM of sodium no saturation was reached with M367S (Fig. 5). An apparent lower affinity for sodium was also observed with M367C and M367L (Fig. 5).

**Effects of substrates and blockers on anion conductance-** Even though the voltage-dependence of the steady-state transport currents induced by substrates by the Met-367 mutants was similar to that by EAAC1-WT at negative voltages, only the latter exhibited small outward currents at positive voltages (Fig. 2A). Such outward currents reflect the substrate-induced anion conductance (8). In EAAC1-WT, replacing 20% of the external NaCl by NaSCN almost fully abolished the inward-rectification of the currents induced by L-aspartate, L-glutamate and D-aspartate (Figs. 2A and 6A). This is due to the fact that the permeability of SCN⁻ through the transporter-mediated anion conductance is around 70-fold higher than the permeability of Cl⁻ (34). This permeability is further enhanced by substrate (8). As a result the substrate-induced outward currents, reflecting the entry of the permeant anion at positive potentials, were markedly enhanced in the presence of SCN⁻ (Figs. 2A and 6A). Such currents were not induced by GABA, which does not interact with the transporter. The non-transportable analogue TBOA blocked the anion currents present in sodium medium (the subtraction of currents in the absence of blocker from those in its presence yields currents in the opposite direction), particularly at positive potentials (Fig. 6A). The blocker similarly suppressed the currents by M367C, M367S and M367L (Fig. 6B-D). However in the SCN⁻ containing medium, substrates had a different effect on the currents by the mutants than on those by EAAC1-WT (Fig. 6B-D). In the mutants these currents were stimulated at negative potentials, but at positive potentials they were suppressed by substrate, just as observed with TBOA (Fig. 6B-D). This indicates that, in the mutants, substrate blocks rather than stimulates the anion conductance. D-glutamate, which acted similar to TBOA on the transient currents of M367L (Fig. 4), also inhibited the anion conductance of this mutant but not of M367C and M367S (data not shown). In contrast to the markedly decreased apparent affinities of the mutants for L-glutamate and D-aspartate, the IC₅₀ values for TBOA were only modestly affected. These values were determined using the ability of the blocker to inhibit the sodium-dependent transient currents (Fig. 7A) and the anion leak currents in the presence of 20 mM of SCN⁻ (Fig. 7B). There was a 5-10 fold difference in the IC₅₀ values between the two assays, suggesting the possibility of the existence of more than one TBOA-bound state of the transporter. However, regardless of which assay was used, the apparent affinity for TBOA of some of the mutants was similar to that of EAAC1-WT. Using the inhibition of the sodium-dependent transient currents (Fig. 7A), the IC₅₀ values were (µM): 0.27±0.01, 0.81±0.12 and 0.34±0.07 for EAAC1-WT, M367C and M367L, respectively (n=3; the transient currents by M367S were too small for quantitative measurements). The IC₅₀ values for TBOA on the inhibition of the anion leak currents were (µM): 2.41±0.67, 1.73±0.09, 1.51±0.09 and 5.27±1.20 for EAAC1-WT, M367C, M367L and M367S, respectively (n=3).

**Sulfhydryl modification of M367C-** When oocytes expressing M367C were preincubated for 5 min in sodium containing medium in the presence of 8 mM of the membrane impermeant negatively charged sulfhydryl reagent MTSES, the glutamate-induced currents measured after washout of the reagent were inhibited by more than 70% (Fig. 8A). Such inhibition was neither observed with EAAC1-WT nor with M367L (Fig. 8A), suggesting that the inhibition of the transport currents of M367C is due to the modification of the introduced cysteine itself and not of an endogenous cysteine which became accessible upon mutation of Met-367. Similar results were observed with the positively charged impermeant sulfhydryl reagent (2-trimethylammonium) methanethiosulfonate (data not shown). The inhibition in the presence of sodium by MTSES of transport by M367C (Fig. 8A) was markedly diminished by either 5 mM or 200 µM of L-glutamate (Fig. 8B, first two bars from the left). On the other hand, concentrations lower than the Km for L-glutamate (Table I) did not protect (next two bars of Fig. 8B). Protection against the inhibition by MTSES was also observed by L-aspartate, but not by GABA (Fig. 8B), which is not a substrate of the transporter. Protection was also provided by the non-transportable analogue TBOA (Fig. 8B), but not by D-glutamate which was not capable to interact with M367C (Fig. 4). The inhibition by MTSES was also observed in the presence of lithium, which can replace sodium in EAAC1...
(24), and again protection was afforded by L-glutamate (Fig. 8B, the two bars from the right). On the other hand, very little inhibition by MTSES was observed in the presence of the inert choline, regardless of the presence or absence of L-glutamate (Fig. 8B).

Discussion

Mutation of Met-367 of the neuronal glutamate transporter EAAC1, a conserved residue located in the unwound portion of TM7 (14), leads to changes in the substrate selectivity (Figs. 1-4, Table I). Moreover, the substrates inhibit the anion conductance in the mutants (Fig. 6B-D), in contrast to the stimulation observed in EAAC1-WT (Fig. 6A). It has been suggested that bound substrate can directly gate this anion conductance, which is not transported (Fig. 4A). However, this interaction is impaired and this influences the positioning of the substrate in the binding pocket. In the case of protomers B and C, the distances are smaller and the shortest distances range between 3.6-3.9Å. Thus also in GltPh the conserved methionine residue is not a molecular determinant for interaction with TBOA. We speculate that the broad specificity of TBOA amongst the glutamate transporters is due to the highly conserved residues which bind the two carboxyl groups and the amino group of the substrate and which also are involved in the binding of the aspartyl moiety of TBOA (Ref. 22).

Why is the substrate selectivity of the mutants altered? One possibility is that the interaction of Met-367 with the substrates is direct in at least one of the steps of the transport cycle. In the crystallized aspartate-bound GltPh conformation, the substrate and Met-311 are close, namely 3.6-4.0Å from the sulfur atom of the methionine residue to one of the α-carboxyl oxygens of the bound aspartate (pdb accession code 2nwl), dependent on which protomer is considered. It is possible that in the corresponding conformation of EAAC1, the distance between the substrate and Met-367 is somewhat shorter and is in fact direct. Alternatively, it is possible that the substrate and Met-367 become closer at a step after the initial binding in an intermediate conformation, which is reached during substrate transport. Consistent with this latter idea is the fact that the apparent affinity of the Met-367 mutants to TBOA, as opposed to that of the substrates, was basically not changed (Fig. 7). This is likely due to the fact that TBOA is unable to induce transport, whereas the substrate induces the conformational changes inherent in the transport process. It appears likely that the bulk of the methionine side chain imposes constraints on the way that the substrates can be accommodated in the binding pocket; in other words this residue is proposed to fulfill a steric role in the determination of the substrate specificity. In such a scenario, it is reasonable that in the mutants where the side-chain is shortened, namely M367S and M367C, this interaction is impaired and this influences the positioning of the substrate in the binding pocket. In the case of the M367L mutant, the side-chain is similar but not identical to that of EAAC1-WT, and perhaps this is the reason that M367L but not M367C can still interact with D-glutamate (Fig. 4A). However this interaction is imperfect and D-glutamate acts like a blocker which is not transported (Fig. 4A).
symmetry observed in the LeuT structure (38), a model of the inward-facing structure was generated (39) which was in excellent agreement with the subsequently solved structure of the structurally similar vSGLT (40). Based on the inverted repeats present in GltPh, a model for the cytoplasm-facing model form of this transporter has also been generated (Crisman, T.J., Qu, S., Kanner, B.I. and Forrest, L.R., Proc. Natl. Acad. Sci. USA, 10.1073/pnas.098570106). This model is in excellent agreement with a new, apparently inward-facing, structure of GltPh (Reyes, N., Ginter, C., and Boudker, O., Nature, 10.1038/nature.08616). Even though GltPh is predicted to function by the movement of the substrate-binding domain through the rest of the protein, it is nevertheless entirely possible that during this movement there is some repositioning of the substrate relative to the binding pocket, resulting in a direct contact of the conserved methionine with the substrate.

There are also alternative explanations for the substrate selectivity change of the Met-367 mutants. In the GltPh structure (2nwl), Several HP2 residues are close to Met-311. This is because HP2 has to pack against TM7, for the substrate translocation to take place. In the TBOA bound conformation (2nww), the bulky benzyl group of the blocker "pushes" HP2 away from the binding pocket (22). Therefore the HP2-TM7 interaction is already abolished in the TBOA bound conformation, explaining the relatively modest effects of the Met-367 mutants on the apparent affinity of the blocker (Fig. 7). The altered packing of HP2 against TM7, could affect the shape of the binding pocket depending on the nature of the introduced mutation, explaining the altered substrate selectivity. Yet another possible explanation for the altered substrate specificity is that in the substrate-bound GltPh structure (2nwl), M3-311 is very close to Asn-401 of TM8, the side-chain of which interacts directly with one of the β-carboxyl oxygen atoms of the bound substrate. Dependent on which methionine atom is considered, the distance to the amide nitrogen of Asn-401 is somewhat smaller than the 3.5Å required for a direct interaction. This could very nicely explain some of the substrate selectivity changes of the Met-367 mutants indirectly via Asn-451 (the EAA1 counterpart of Asn-401). The small effects of the mutations on the apparent affinity for TBOA could then be explained by the fact that TBOA interacts with the transporter at many more positions than the substrate and a single mutation would have a smaller effect on the IC50 for TBOA than on the EC50 values of the substrates. Because the side-chain of methionine is hydrophobic, Met-367 is not expected to interact with the amide group of Asn-451. Rather this hydrophobic side-chain is likely to play a steric/structural role in the binding pocket, and this is true for each of the three above mentioned scenarios.

The sodium-dependent transient currents are thought to reflect a charge-moving conformational change. These transient currents were readily observed with M367C and M367L, but not with M367S (Fig. 4A and B). While the reason for this is not clear, it is possible that the nature of the substituent at this position in the unwound part of TM7, is important for the ability to undergo this conformational change. The voltage dependence of sodium-dependent transient currents is a measure for the apparent affinity of the substrate-free transporter for sodium (13,41). The voltage-dependence of these transient currents by M367L and M367C is similar to those by EAAC1-WT (Fig. 4A and B). On the other hand, analysis of the sodium concentration dependence of the L-aspartate-induced currents by the M367C, M367S and M367L mutants indicates that the apparent affinity for sodium is reduced (Fig. 5). The explanation of this apparent discrepancy is probably that one or two sodium ions can bind before the substrate, but the binding of at least one sodium takes place after the substrate has bound (42,43). As we suggested previously (44) it is possible that the substrate itself participates in the liganding of this sodium ion, just as has been observed in the LeuT structure where one of the carboxyl oxygens of the substrate ligands one of the bound sodium ions (38). If because of the mutation at Met-367, the substrate has a slightly altered orientation, this could influence the apparent affinity of the sodium ion which binds after the substrate. Tl+ has been used as a Na+ surrogate to localize the sodium sites in GltPh and two such sites were identified (22). Because Tl+ does not support transport (22), it is uncertain that these sites represent genuine Na+ sites. However, our recent experiments support the idea that one of the two homologous Tl+ sites in EAAC1 and GLT-1 participates in an overlapping Na+ and K+ site (45). To understand how mutation Met-367 influences the apparent affinity of the substrate-loaded transporter for sodium, it will be necessary to determine the
location of all three Na⁺ sites in the eukaryotic glutamate transporters.

References

Table I- Kinetic parameters of the acid amino acid induced transport currents by EAAC1-WT and Met-367 mutants.

<table>
<thead>
<tr>
<th></th>
<th>L-glutamate</th>
<th>L-glutamate (SCN⁻)</th>
<th>L-aspartate</th>
<th>D-aspartate</th>
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<tr>
<td></td>
<td>Kₘ (µM)</td>
<td>EC₅₀/IC₅₀ (µM)</td>
<td>Kₘ (µM)</td>
<td>Iₘax (%)</td>
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<tr>
<td>WT</td>
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<td>15.0±2.1</td>
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<tr>
<td>M367C</td>
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<td>M367S</td>
<td>177.4±4.6</td>
<td>68.6±7.5</td>
<td>17.9±1.6</td>
<td>67.4±1.1</td>
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</table>

Kₘ values (µM) and maximal current (Iₘax) for each of the shown substrates were obtained by oocytes expressing WT and the indicated mutants. The currents at -100mV induced by various concentrations of the indicated substrate were measured in ND96-based medium, except for “L-glutamate (SCN⁻)”, where 20 mM NaCl of the ND96 medium was replaced by the same concentration of NaSCN and steady-state currents induced (EAAC1-WT)/inhibited (Met-367 mutants) by various concentrations of L-glutamate at +40mV were monitored and the EC₅₀ (EAAC1-WT) and IC₅₀ (mutants) were calculated (see also Fig. 6). Iₘax is expressed as a percentage of the current induced by saturating concentration of L-glutamate. Data analysis performed to determine the kinetic parameters is described under “Experimental Procedures”. The obtained parameters are based on data measured from at least three distinct experiments, and represent mean ± S.E.

Footnotes

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The abbreviations used are: TBOA, D,L-threo-β-benzylxoyaspartate; TM, trans-membrane domain; HP, hairpin loop; WT, wild type; MTSES, (2-sulfonatoethyl)methanethiosulfonate.
Figure Legends

**Figure 1.** Uptake of radioactive substrates by cells expressing Met-367 mutants of EAAC1. Uptake of D-[\(^3\)H]-aspartate (empty bars), L-[\(^3\)H]-aspartate (black bars), and L-[\(^3\)H]-glutamate (hatched bars) by HeLa cells expressing the indicated mutants was measured as described under “Experimental Procedures”. For each substrate, uptake was corrected for background uptake by cells transfected with the vector alone. For each substrate, uptake is shown as percentage of EAAC1-WT. Data is mean ± S.E of at least three different experiments each done in quadruplicate.

**Figure 2.** Substrate-induced steady-state currents by EAAC1-WT and mutants. Steady-state currents induced by 5mM of L-glutamate (circles), L-aspartate (squares), D-aspartate (triangles), or GABA (inverted triangles) are shown. As shown in Fig. 3, these are saturating concentrations for the substrates. Data were obtained from oocytes expressing either EAAC1-WT (A) or the indicated mutants (B-D) in a NaCl-based external medium (ND96), as described under “Experimental Procedures”. The membrane voltage was stepped from a holding potential of –25mV to voltages between –100 and +40mV in increments of +10mV. Each potential was held clamped for 250ms, followed by 250ms of a potential clamped at –25mV. Substrate-induced steady-state currents (I\(_{\text{substrate}} \text{– } I_{\text{ND96}}\)) from 210 to 240ms at each potential was averaged and normalized to the current induced by 5 mM L-glutamate at –100mV (I\(_{\text{norm}}\)). These currents were then plotted against the corresponding potential (V\(_{\text{m}}\)(mV)). Data are mean ± S.E of at least 3 repeats. The currents at -100mV induced by 5 mM of L-glutamate ranged from -470 to -1320nA in the WT, from -450 to -760 nA in M367C, from -150 to -580 nA in M367S, and from -350 to -680 nA in M367L.

**Figure 3.** Substrate concentration-dependence of the induced currents. Steady-state currents at -100mV, induced by various concentrations of L-glutamate (circles), L-aspartate (squares), or D-aspartate (triangles) obtained by oocytes expressing EAAC1-WT (A) and the indicated mutants (B-D). Currents were measured in ND96 as described under “Experimental Procedures”, using the same voltage protocol described in the Legend to Figure 2. Substrate-induced currents were normalized separately for each mutant (and WT) to the maximal current induced by L-glutamate (I/I\(_{\text{max}}\)). Data are mean ± S.E of at least three repeats.

**Figure 4.** Net currents by EAAC1-WT and mutants. Oocytes expressing EAAC1-WT or the indicated mutants were subjected to the same voltage jump protocol described in the Legend to Figure 2 in the NaCl-based buffer (ND96) as described under “Experimental Procedures”. A: Currents in ND96 were subtracted from those in the presence of ND96 and either 5mM L-glutamate (L-Glu), D-glutamate (D-Glu), or 10µM D,L-threo-β-benzyloxyaspartate (TBOA). B: Absolute (non-subtracted) currents are shown for the same oocytes depicted in (A). Please note that the subtraction procedure used is the opposite of that conventionally used to observe the TBOA-sensitive transient currents. This was done to allow a direct comparison with the transient currents of M367L visualized with D-glutamate. Traces shown are from oocytes representative of at least three. The dashed lines indicate 0 current.

**Figure 5.** Sodium concentration dependence of L-aspartate-induced currents mediated by Met-367 mutants. Steady-state currents induced by 5mM L-aspartate at –100 mV mediated by oocytes expressing EAAC1-WT (squares), M367C (circles), M367L (triangles), and M367S (inverted triangles) were recorded at the indicated sodium concentrations (choline substitution, as detailed in “Experimental Procedures”) using the same voltage protocol described in the Legend to Figure 2. For each mutant and also for EAAC1-WT, currents are normalized to those measured at a sodium concentration of 130mM sodium (I/I\(_{\text{max}}\)). Data are mean ± S.E of at least three repeats.

**Figure 6.** Substrate-modulated anion conductance by EAAC1-WT and Met-367 mutants. Steady-state currents induced by 5mM L-glutamate (circles), L-aspartate (squares), D-aspartate (triangles), GABA (inverted triangles), or by 10µM D,L-threo-β-benzyloxyaspartate (TBOA) (diamonds) in oocytes expressing either EAAC1-WT (A) or the indicated mutants (B-D), using the same voltage protocol described in the Legend to Figure 2. Currents were measured in ND96-based external medium, where
20 mM NaCl was replaced by an equimolar concentration of NaSCN. Prior to chloride substitution, 5mM L-glutamate induced currents were measured (not shown). Data were normalized to the glutamate-induced currents obtained at -100mV in the SCN⁻-free media, and plotted against the holding potential. Values are mean ± SE of three to five repeats.

Figure 7  Concentration dependence of the inhibition of charge movements and anion conductance by TBOA. Blockade of charge movements (A), and anion conductance (B) by TBOA in oocytes expressing EAAC1-WT (squares), M367C (circles), M367L (triangles), or M367S (inverted triangles) using the same voltage protocol described in the Legend to Figure 2. Charge movements obtained in ND96, were calculated by adding the current–time integrals during the “off”-charge movements elicited by stepping back from -100 to -25mV to those where the voltage was stepped back from +40 to -25mV. The fraction of charge movement blocked at each TBOA concentration was normalized to the charge movement blocked by 5 µM TBOA (Q/Qmax). The ranges of the charge movements were (nC): 2.0-7.5 (EAAC1-WT); 1.5-8.0 (M367C); 2.4-9.4 (M367L). In the case of M367S the moved charge ranged from 0.3 -0.8 nC, too small to perform reliable titrations (A). The steady-state currents inhibited by the indicated TBOA concentrations at +40mV were measured in the 20mM SCN⁻-containing external medium as described in the Legend to Figure 6 (I), were normalized to those inhibited by 5 µM of TBOA (Imax). Data are mean ± S.E of at least four repeats.

Figure 8. Inhibition of L-glutamate-induced currents in oocytes expressing M367C by MTSES. Currents in ND96 induced by 5mM L-glutamate by oocytes expressing EAAC1-WT and the indicated mutants at -100mV were measured before and after bath application of 8 mM of MTSES in ND96 for 5 min. (A). In the case of M367C, the preincubation with MTSES was done under the conditions indicated below the bars (B). The details of the treatment with MTSES are given under Experimental Procedures. TBOA was used at a final concentration of 10 µM. The currents induced by 5 mM L-glutamate after the MTSES-treatment are shown as the percentage of those recorded before the treatment (control). Data are mean ± S.E., n=3 or larger.)
Figure 1

Transport of $^3$H-substrate (% of WT)

- M367C
- M367S
- M367L
- M367F

Substrates: D-Asp, L-Asp, L-Glu
Figure 2

- ○ L-Glutamate; □ L-Aspartate; ▲ D-Aspartate; ▼ GABA
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

L-Glu-induced currents (% of control)

A

WT
M367L
M367C

B

ND96+L-glu (5mM)
ND96+L-glu (200µM)
ND96+L-glu (50µM)
ND96+L-asp (5mM)
ND96+L-asp (200µM)
ND96+D-glu (5mM)
ND96+TBOA
ND96+GABA (5mM)
Ch96+L-glu (5mM)
Ch96
Li96
Li96+L-glu (5mM)
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