The Role of Cation Binding in Determining Substrate Selectivity of Glutamate Transporters*<sup>S</sup>

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Glutamate transport is coupled to the co-transport of 3Na<sup>+</sup> and 1H<sup>+</sup> and the countertransport of 1K<sup>+</sup>. However, the mechanism of how this process occurs is not well understood. The crystal structure of an archaeal homolog of the human glutamate transporters, GltPh, has provided the framework to begin to understand the mechanism of transport. The glutamate transporter EAAT2 is different from other subtypes in two respects. First, Li<sup>+</sup> cannot support transport by EAAT2, whereas it can support transport by the other excitatory amino acid transporters, and second, EAAT2 is sensitive to a wider range of blockers than other subtypes. We have investigated the relationship between the cation driving transport and whether the glutamate analogues, L-anti-endo-3,4-methanopyrrolinedicarboxylic acid (MPDC) and (2S,4R)-4-methylglutamate (4MG), are substrates or blockers of transport. We have also investigated the molecular basis for these differences. EAAT2 has a Ser residue at position 441 with hairpin loop 2, whereas the corresponding residue in EAAT1 is a Gly residue. We demonstrate that if the transporter has a Ser residue at this position, then 4MG and MPDC are poor substrates in Na<sup>+</sup>, and Li<sup>+</sup> cannot support transport of any substrate. Conversely, if the transporter has a Gly residue at this position, then in Na<sup>+</sup> 4MG and MPDC are substrates with efficacy comparable with glutamate, but in Li<sup>+</sup> 4MG and MPDC are poor substrates relative to glutamate. This Ser/Gly residue is located between the bound substrate and one of the cation binding sites, which provides an explanation for the coupling of substrate and cation binding.

Glutamate transporters, also termed excitatory amino acid transporters (EAATs)<sup>4</sup> (1–3), are essential for regulating extracellular L-glutamate concentrations to maintain dynamic signaling processes between neurons. Glutamate transport by the EAATs is coupled to the co-transport of 3Na<sup>+</sup> and 1H<sup>+</sup> and the countertransport of 1K<sup>+</sup> (4), which provides sufficient energy to generate a 10<sup>5</sup>-fold gradient of L-glutamate across the cell membrane. In addition to ion-coupled transport, the binding of Na<sup>+</sup> and L-glutamate to the transporter activates an uncoupled chloride conductance, which has been proposed to play a variety of roles in regulating neuronal excitability, enhancing the concentration capacity of the transporter (5, 6). Furthermore, binding of Na<sup>+</sup> permits a leak conductance that is also carried by chloride ions (7, 8). An understanding of the mechanism by which transporters utilize the energy derived from ion gradients to drive the movement of substrates against their concentration gradients is crucial to understanding how transporters work and how they regulate their cellular environment. In this study, we examined the relationship between the coupled cation and a series of substrates and substrate analogues. We have identified the structural basis that determines whether a compound acts as a substrate or a blocker.

Crystal structures of an archaeal homolog of glutamate transporters, GltPh, have recently been determined in the presence and absence of substrates and also the blocker DL-threo-β-benzoyloxyaspartate (TBOA) (9, 10), which have greatly enhanced our ability to address the question of how glutamate is transported. GltPh<sub>o</sub> is an Na<sup>+</sup>-dependent aspartate transporter that also has an uncoupled chloride conductance (11). GltPh<sub>o</sub> shares a remarkably high 37% amino acid sequence identity with the EAATs, with the degree of identity considerably higher in the substrate binding site (10). GltPh<sub>o</sub> is a homotrimer, with the three protein subunits coming together to form an extracellular facing bowl-shaped complex. Each protomer consists of eight transmembrane (TM) domains and two helix-turn-helix motifs or hairpin loops, termed HP1 and HP2. The substrate (aspartate) binding site is formed by residues in TM7 and TM8 and the tips of HP1 and HP2. Two putative Na<sup>+</sup> binding sites are in close proximity to the bound aspartate. The first is located beneath the aspartate molecule and is formed by backbone carbonyls of residues in TM7 and TM8 and a carbonyl group from Asp<sup>405</sup> in TM8. The second is located above the binding site and is formed by backbone carbonyls from HP2 and TM7 (Fig. 1A). In the crystal structure of GltPh<sub>o</sub> in complex with TBOA, the aspartate moiety of TBOA binds in the aspartate binding site, whereas the benzyl group props open HP2, preventing the formation of the second Na<sup>+</sup> site. This suggests that the mechanism of TBOA inhibition is a combination of being too bulky to pass through the transporter and also preventing formation of the second Na<sup>+</sup> site that is required for transport (9).
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EXPERIMENTAL PROCEDURES

Chemicals—All chemicals were obtained from Sigma unless otherwise stated. 4MG, threo-3-methylglutamate, and MPDC were obtained from Tocris (Bristol, UK).

Expression of Transporters in Xenopus laevis Oocytes and Electrophysiological Recordings—cDNAs encoding the glutamate transporters were subcloned into the pOTV plasmid. Mutations were generated using the QuikChange site-directed mutagenesis kit from Stratagene, and all mutations were confirmed by DNA sequencing. The wild type and mutant transporters were linearized with SpeI and cRNA transcribed from the cDNA constructs with T7 RNA polymerase and capped with 5’-7-methylguanosine using the mMessage mMachine kit (Ambion Inc., Austin, TX).

Oocytes were harvested from X. laevis, as previously described, with all procedures in accordance with the Australian National Health and Medical Research Council guidelines for the prevention of cruelty to animals. 50 nl of cRNA was injected into defolliculated, stage V oocytes and incubated at 16 °C in standard frog Ringer’s solution (ND96; 96 mM NaCl, 2 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 5 mM HEPES, pH 7.53), supplemented with 2.5 mM sodium pyruvate, 0.5 mM theophylline, 50 µg/ml gentamicin. 2–5 days later, current recordings were made using the two-electrode voltage clamp technique with a Geneclamp 500 amplifier (Axon Instruments, Foster City, CA) using the pCLAMP 10 suite of programs and interfaced with a MacLab 2e chart recorder (ADI Instruments, Sydney, Australia) using the Chart software (ADI Instruments).

Current-voltage relationships were determined by subtraction of steady state current measurements in the absence of substrate (or blocker) during 100-ms pulses to membrane potentials from −100 to +60 mV (in 10-mV intervals) from current measurements in the presence of substrates and/or blockers.

Data Analysis—The analysis of kinetic data was carried out using the Prism software version 4. Current (I) as a function of substrate concentration ([S]) was fitted by least-squares analysis to Equation 1,

\[
I/I_{\text{max}} = [S]/(E_{50} + [S])
\]

(Eq. 1)

where \(I_{\text{max}}\) represents the maximal current, \(E_{50}\) is the concentration of substrate that generates a half-maximal current, and [S] is the concentration of substrate. Concentration-dependent inhibition of substrate-induced currents by blockers were fitted to Equation 2,

\[
I/I_{\text{max}} = 1 - ([B]/(IC_{50} + [B]))
\]

(Eq. 2)

where [B] represents the concentration of the blocker, and IC_{50} is the concentration of blocker that generates half-maximal inhibition. It has previously been demonstrated that 4MG, threo-3-methylglutamate, TBOA, and kainate are competitive blockers of EAAT2 (12, 14, 15), so \(K_i\) values were calculated from IC_{50} values using the Cheng-Prusoff equation (16).

RESULTS

Ionic Currents Mediated by EAAT1 and EAAT2 in Na^+ and Li^+—EAAT1 and EAAT2 show distinct differences in pharmacology and cation dependence of transport. First, we compare...
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The pharmacological actions of L-glutamate, MPDC, and 4MG on EAAT1 in Na\(^+\) and Li\(^+\).

A, representative current traces from cells expressing EAAT1 under voltage clamp at −100 mV. In the Na\(^+\)-based buffer, 30 μM glutamate, 30 μM 4MG, and 30 μM MPDC generate inward currents. In the Li\(^+\)-based buffer, 300 μM glutamate generates an inward current, but 300 μM 4MG and 300 μM MPDC generate greatly reduced inward currents. B, MPDC blocks a leak current in Li\(^+\). Concentration-dependent current-voltage plots for MPDC (10–5000 μM) and 500 μM TBOA (crosses) in the Li\(^+\)-based buffer. Current measurements are normalized to the current blocked by TBOA at +60 mV. C, MPDC inhibition of glutamate transport in Li\(^+\). Concentration-dependent current-voltage plots for MPDC (10–1000 μM) inhibition of 300 μM L-glutamate transport. The glutamate transport current blocked by TBOA is shown with crosses. The currents are normalized to the current generated by 300 μM L-glutamate in the absence of MPDC or TBOA. D, 4MG generates transport currents and blocks a leak current in Li\(^+\). Shown are concentration-dependent current-voltage plots for 4MG in Li\(^+\). E, 4MG inhibition of glutamate transport currents in Li\(^+\). Concentration-dependent current-voltage plots for 4MG (30–2000 μM) inhibition of 300 μM L-glutamate in Li\(^+\). Currents are normalized to the current generated by L-glutamate alone. For B–E, currents measured in the absence of substrates and/or blockers are subtracted from currents measured in their presence.

The activities of L-glutamate, MPDC, and 4MG on EAAT1 in Na\(^+\)- and Li\(^+\)-based buffers, and then we compare these properties with those observed for EAAT2.

Applications of MPDC or 4MG in Na\(^+\) to oocytes expressing EAAT1 generate inward currents of an amplitude comparable with that of L-glutamate (Fig. 2A), with K\(_{0.5}\) values of 22 ± 1 and 54 ± 17 μM, respectively (Table 1). These results are consistent with a number of previous studies (2, 12, 13). In a Li\(^+\)-based buffer, L-glutamate also generates concentration-dependent inward currents for EAAT1 but with an increased K\(_{0.5}\) of 365 ± 14 μM compared with 20 ± 3 μM in Na\(^+\) and a maximal current at −100 mV in Li\(^+\) of 15 ± 1% of that measured in Na\(^+\). The activity of MPDC on EAAT1 in Li\(^+\) shows striking differences compared with its activity in Na\(^+\). In Na\(^+\), MPDC generates transport currents with an I\(_{\text{max}}\) of 26 ± 2% of that for L-glutamate, but in Li\(^+\) MPDC does not generate transport currents (Fig. 2, A and B). At membrane potentials less than −60 mV, MPDC generates small concentration-dependent outward currents, and at positive membrane potentials, the current is inward (Fig. 2B). This current voltage profile is more akin to MPDC blocking a leak current. Co-application of increasing concentrations of MPDC with a fixed concentration of glutamate reduces the amplitude of the glutamate transport current (Fig. 2C). Thus, in Li\(^+\), MPDC acts as a blocker of glutamate transport by EAAT1, whereas in Na\(^+\) it is a substrate (Fig. 2, A and B). By way of comparison, application of the non-selective transporter blocker, TBOA, in Li\(^+\) generated a current-voltage profile similar to that of MPDC (Fig. 2B) and also blocked L-glutamate transport currents (Fig. 2C).

The actions of 4MG on EAAT1 in Li\(^+\) are more complex than that of L-glutamate or TBOA and MPDC and are likely to be a result of a mixture of currents. In Na\(^+\), glutamate and 4MG activate significant uncoupled chloride conductances, which are most easily observed as outward currents at positive membrane potentials (2, 17). In Li\(^+\), glutamate generates inward currents at membrane potentials up to +50 mV (Fig. 2C), which suggests that the substrate-activated anion current is reduced in Li\(^+\) (see supplemental material). In Li\(^+\), 4MG generates concentration-dependent inward currents of increasing magnitude at negative membrane potentials. However, instead of approaching zero current at positive membrane potentials, as observed for L-glutamate, larger inward currents are generated, with the current voltage plot showing a bell-shaped curve (Fig. 2D). The amplitude of the maximal inward current at positive membrane potentials is not as great as observed for MPDC and TBOA. Furthermore, co-application of increasing 4MG concentrations with a fixed L-glutamate concentration decreased the amplitude of the L-glutamate currents at negative potentials and increased the amplitude at positive potentials (Fig. 2E). These observations
TABLE 1
Kinetic parameters for EAAT1, EAAT2, and mutants in Na+ and Li+

| Ligand | EAAT1/Na+ | EAAT1G442S/Na+ |
|--------|-----------|----------------|
|        | K\text{m} | K\text{i} | I_{\text{max}} | K\text{m} | K\text{i} | I_{\text{max}} |
| Glutamate | 20 ± 2 \text{\mu M} | 100 | 36 ± 3 | 100 | 36 ± 3 | 100 |
| 4MG    | 54 ± 17 | 80 ± 5 | 17 ± 2 | 9 ± 1 | 17 ± 2 | 9 ± 1 |
| MPDC   | 22 ± 1 | 26 ± 2 | 13 ± 2 | 5 ± 0 | 13 ± 2 | 5 ± 0 |
| TBOA   | 2.8 ± 0.3 | 6.2 ± 0.4 | 1.7 ± 0.2 | 1.7 ± 0.2 | 1.7 ± 0.2 | 1.7 ± 0.2 |

| EAAT2/Na+ | EAAT254441G/Na+ |
|-----------|----------------|
|        | K\text{m} | K\text{i} | I_{\text{max}} | K\text{m} | K\text{i} | I_{\text{max}} |
| Glutamate | 18 ± 3 | 100 | 0.6 ± 0.1 | 100 | 0.6 ± 0.1 | 100 |
| 4MG    | 3.4 ± 0.2 | 0.8 ± 0.3 | 91 ± 10 | 3.4 ± 0.2 | 0.8 ± 0.3 | 91 ± 10 |
| MPDC   | 0.20 ± 0.02 | 0.6 ± 0.1 | 73 ± 4 | 0.20 ± 0.02 | 0.6 ± 0.1 | 73 ± 4 |
| TBOA   | 0.08 ± 0.01 | 0.26 ± 0.03 | 3.4 ± 0.2 | 0.26 ± 0.03 | 3.4 ± 0.2 |

| EAAT1/Li+ | EAAT254441G/Li+ |
|-----------|----------------|
|        | K\text{m} | K\text{i} | I_{\text{max}} | K\text{m} | K\text{i} | I_{\text{max}} |
| Glutamate | 365 ± 14 | 100 | 10 ± 2 | 100 | 10 ± 2 | 100 |
| 4MG    | 24 ± 6 | 0.34 ± 0.03 | 3.4 ± 0.2 | 0.34 ± 0.03 | 3.4 ± 0.2 |
| MPDC   | 3.3 ± 0.3 | 3.3 ± 0.3 | 3.4 ± 0.2 | 3.3 ± 0.3 | 3.4 ± 0.2 |

* K_{\text{i}} values for blockers were calculated from I_{\text{Cmax}} values using the Cheng-Prusoff equation, assuming competitive antagonism (12, 14).
* I_{\text{Cmax}} values for substrates are relative to L-glutamate.
* All values presented are the mean ± S.E. with n = 4–12.
* Applications of MPDC and 4MG in Na+ to oocytes expressing EAAT2 generate small transport currents that are 4.8 ± 0.4 and 2.8 ± 0.9% of that for glutamate. However, it is difficult to accurately measure their K_{\text{m}} values. We have presented K_{\text{i}} values for inhibition of glutamate transport as a measure of their relative affinities for the transporter.
* L-glutamate, MPDC, and 4MG have been reported to be blockers of EAAT2 (when measured at −60 mV), but if we measure MPDC- and 4MG-induced currents at −100 mV, they induce inward currents that are 4.8 ± 0.4 and 2.8 ± 0.9% of the current generated by L-glutamate, respectively (3.4). This suggests that at −100 mV, these compounds are not blockers but rather are poor substrates. Given the amplitudes of these currents, it is not possible to characterize their kinetics when acting as a substrate. The current-voltage plots for MPDC and 4MG in EAAT2 are bell-shaped (Fig. 3B) and show similarities to that observed for these compounds acting on EAAT1 in Li+ (Fig. 2D), suggesting that they also block a standing leak current. Co-application of MPDC and 4MG with L-glutamate causes concentration-dependent currents, with K_{\text{i}} values of 0.20 ± 0.02 and 3.4 ± 0.2 \mu M, respectively (Table 1). Second, L-glutamate cannot substitute for Na+ in supporting transport of L-glutamate (or other substrates) by EAAT2 (3.4). For additional details on the pharmacological differences between EAAT1 and EAAT2, see the supplemental material. Below we have exploited the pharmacological and ionic differences between EAAT1 and EAAT2 to better understand the molecular basis for ion coupling of transport and how this impacts on whether 4MG and MPDC are substrates, blockers, or poor substrates.

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**FIGURE 3.** The pharmacological actions of L-glutamate, MPDC, and 4MG on EAAT2 in Na+ and Li+. A, representative current traces from cells expressing EAAT2 under voltage clamp at −100 mV. In the Na+-based buffer, the currents generated by 30 \mu M 4MG and 30 \mu M MPDC are substantially reduced compared with that of 30 \mu M L-glutamate. In the Li+-based buffer, all compounds (300 \mu M) block a small intrinsic leak current. B, MPDC is a poor substrate for EAAT2 in Na+. Shown are concentration-dependent current-voltage plots of MPDC (1–300 \mu M) in the Na+-based buffer. Current measurements are normalized to the current at −100 mV generated by 1 mM L-glutamate in Na+. C, 4MG is a poor substrate for EAAT2 in Na+. Shown are concentration-dependent current-voltage plots for 4MG (1–300 \mu M) in the Na+-based buffer. Currents are normalized to the maximal current generated by L-glutamate at −100 mV.
Molecular Basis for an Active Transport Complex—In HP2 of EAAT2 there is a Ser residue at position 441, and the corresponding residue in EAAT1 is a Gly residue. Zhang et al. (18) have demonstrated that mutation of the Ser to a Gly in the rat equivalent of human EAAT2 allows the transporter to use Li\(^+\) to drive transport and also reduces the affinity for the EAAT2-selective blocker, dihydrokainate. In the crystal structure of Glutamate transporter of 1.7 (near the third carbon of aspartate) and the second Na\(^+\) ion binding site (9) (Fig. 1A). The proximity of this residue to both the substrate and cation binding site prompted us to investigate whether this difference between EAAT1 and EAAT2, which is likely to influence differences in cation selectivity (18), is also responsible for determining whether MPDC and 4MG are substrates, blockers, or poor substrates and also how the driving cation influences these properties.

Application of \(L\)-glutamate to oocytes expressing EAAT2S441G generates inward currents at \(-100\) mV (Fig. 4). The \(K_{\text{m}}\) for \(L\)-glutamate generated currents is 0.6 ± 0.1 \(\mu M\), which is 33-fold lower than for EAAT2 (Table 1). The maximal current generated by the mutant was also reduced compared with EAAT2, with maximal currents in the 15–50 nA range, compared with 100–250 nA for EAAT2. In contrast to EAAT2, MPDC and 4MG are transported by EAAT2S441G with an efficacy similar to that for \(L\)-glutamate (Fig. 4 and Table 1). Thus, this mutation changes the pharmacology of 4MG and MPDC from being poor substrates to substrates of similar efficacy to \(L\)-glutamate and generates a transporter with a phenotype very similar to that of EAAT1.

We also investigated the reverse mutation in EAAT1, G442S. Glutamate generates similar transport currents in EAAT1G442S compared with that of EAAT1 (Fig. 4), with a \(K_{\text{m}}\) of 36 ± 3 \(\mu M\) and maximal currents similar to that of EAAT1. The reverse mutation switched the pharmacology of MPDC and 4MG to that observed for EAAT2. The \(I_{\text{max}}\) values for MPDC and 4MG were reduced to 4.7 ± 0.2 and 9 ± 1% of the \(L\)-glutamate \(I_{\text{max}}\) respectively, as opposed to 26 ± 2 and 80 ± 5% for EAAT1 (Table 1). In comparison, the equivalent relative \(I_{\text{max}}\) values for EAAT2 are 4.8 ± 0.4 and 2.8 ± 0.9%, respectively. Thus, the Ser for Gly difference is crucial in determining pharmacological differences between EAAT1 and EAAT2.

The EAAT2S441G Mutation Alters Na\(^+\) Coupling and the Mechanisms of Action of 4MG and MPDC—It appears that the mechanisms of action of MPDC and 4MG are dependent upon the cation driving transport and also the nature of the amino acid residue in HP2 at position 442 in EAAT1 and the corresponding residue in EAAT2. To investigate further this link between the driving cation and whether an active transport complex is formed, we investigated how changing the driving cation in the mutant transporters influences the mode of action of \(L\)-glutamate, MPDC, and 4MG. First, although Li\(^+\) cannot substitute for Na\(^+\) in allowing \(L\)-glutamate transport by the wild type EAAT2 (Fig. 4), Li\(^+\) can support \(L\)-glutamate transport by EAAT2S441G, generating a current at \(-100\) mV that is 38 ± 1% of that generated in Na\(^+\). As mentioned above, in Na\(^+\), MPDC and 4MG are substrates of the EAAT2S441G mutant, but if the driving cation is changed to Li\(^+\), 4MG and MPDC revert to being transport blockers (Fig. 4), with \(K_{\text{m}}\) values for the block of \(L\)-glutamate transporter of 1.7 ± 0.5 and 0.34 ± 0.03 \(\mu M\), respectively (Table 1). For the reverse mutant EAAT1G442S, Li\(^+\) cannot support transport of \(L\)-glutamate, 4MG, or MPDC, which is similar to that observed for EAAT2 in Li\(^+\). Thus, the Ser for Gly difference in HP2 is responsible for the differences in pharmacology between EAAT1 and EAAT2 and also how the driving cation influences the pharmacology.

**DISCUSSION**

In this study, we have utilized MPDC, 4MG, and Li\(^+\) as molecular probes of the substrate and Na\(^+\) binding sites and have exploited the subtle pharmacological and amino acid sequence differences between EAAT1 and EAAT2 to investigate how substrate and ion transport are coupled. For EAAT1, Li\(^+\) can partially substitute for Na\(^+\) in driving the transport of \(L\)-glutamate but not transport of MPDC. MPDC changes from being a substrate in Na\(^+\) to being a blocker in Li\(^+\). The amino acid sequences of EAAT1, EAAT2, and Glutamate transporter of 1.7 are closely related in the substrate and ion binding regions (Fig. 1), but there is a Ser residue in EAAT2 within the loop region between HP2a and HP2b, whereas the corresponding residue in Glutamate transporter of 1.7 and the other EAATs is a Gly. This Gly for Ser difference has previously been investigated using the rat equivalent of human EAAT2 (19), and it was found that the S440G mutation reduced the affinity of the EAAT2-selective blocker, dihydrokainate, and also allowed Li\(^+\) to drive transport. Two previous studies have also demonstrated that the affinity of Na\(^+\) for the transporter is dependent upon the substrate being transported (20, 21), which provided hints of a link between substrate/blocker interactions and Na\(^+\) binding. These observations provide us with the tools to investigate the specificity of the coupling process and why compounds such as MPDC and 4MG are very good substrates for EAAT1 and are either blockers or poor substrates of...
EAAT2. The results presented in this study show that if the transporter has a Gly residue at position 441/442, then Na\(^+\) can drive transport of MPDC or 4MG, but in Li\(^+\) they are blockers. Conversely, if the residue is a Ser, then in Na\(^+\), MPDC and 4MG are either blockers or very poor substrates (3–5% of the transport activity of l-glutamate; Table 1), and in Li\(^+\), transport of all substrates is severely impaired. Thus, the combination of the residue at position 441/442 and the structure of the ligand influences cation specificity, and this in turn determines whether a compound is a blocker or a substrate.

A question that arises from these results is whether the differences in activity of MPDC and 4MG can be attributed to differences in cation selectivity at all three cation binding sites or just one or two of the sites. Since a single point mutation can bring about the change in activity of these compounds, the simplest interpretation of the data is that the changes can be attributed to just one of the three cation binding sites. So which Na\(^+\) site is likely to be responsible for the switch in the coupling process? In the crystal structure of L-aspartate bound to GltPh, the second Na\(^+\) ion is coordinated by backbone carbonyls of Ser\(^{349}\), Ile\(^{350}\), and Thr\(^{352}\) from HP2 and Thr\(^{208}\) from TM7a (9). The Ser/Gly residue in HP2 that is responsible for conferring differences in MPDC action and cation coupling (Gly\(^{354}\) in GltPh) lies between the bound aspartate molecule and the second Na\(^+\) site and provides a plausible link between cation binding and whether an active transport complex is formed. In the study by Boudker et al. (9), it was demonstrated that TBOA prevents the formation of the second Na\(^+\) site by restricting the movement of HP2. Thus, a plausible explanation for the results in this study is that if the transporter has a Ser in the HP2 loop, then the HP2 movements may be more restricted than if a Gly residue is present. These differences in the degree of movement of HP2 may impact on the specificity of cation coupling and, as a consequence, the pharmacological actions of compounds such as MPDC and 4MG.

Other residues have also been implicated in binding of Na\(^+\) (22–25), but none of these additional sites are likely to be in close proximity to the Ser/Gly residue investigated in this study. However, one of the important differences between GltPh and the EAATs is that l-glutamate transport by the EAATs is coupled to the co-transport of three Na\(^+\) ions (4), whereas in GltPh only two Na\(^+\) binding sites have been located (9). Until we have a complete picture of the three Na\(^+\) sites in a human glutamate transporter, we can only infer that it is the second Na\(^+\) site, as defined by Boudker et al. (9), which is directly linked to the determination of pharmacological activity and coupling to the transport process.

In summary, we have demonstrated that a single amino acid difference between EAAT1 and EAAT2 is responsible for determining differences in cation selectivity between the transporters and that the specificity of cation interactions determines whether compounds, such as 4MG and MPDC, are substrates or blockers.

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