Neutralization of the Aspartic Acid Residue Asp-367, but Not Asp-454, Inhibits Binding of Na\(^+\) to the Glutamate-free Form and Cycling of the Glutamate Transporter EAAC1*  

Zhen Tao\(^1\), Zhou Zhang, and Christof Grewer\(^2\)  
From the Department of Physiology and Biophysics, University of Miami School of Medicine, Miami, Florida 33136  

Substrate transport by the plasma membrane glutamate transporter EAAC1 is coupled to cotransport of three sodium ions. One of these Na\(^+\) ions binds to the transporter already in the absence of glutamate. Here, we have investigated the possible involvement of two conserved aspartic acid residues in transmembrane segments 7 and 8 of EAAC1, Asp-367 and Asp-454, in Na\(^+\) cotransport. To test the effect of charge neutralization mutations in these positions on Na\(^+\) binding to the glutamate-free transport, we recorded the Na\(^+\)-induced anion leak current to determine the \(K_m\) of EAAC1 for Na\(^+\). For EAAC1\(_{WT}\), this \(K_m\) was determined as 120 mM. When the negative charge of Asp-367 was neutralized by mutagenesis to asparagine, Na\(^+\) activated the anion leak current with a \(K_m\) of about 2 mM, indicating dramatically impaired Na\(^+\) binding to the mutant transporter. In contrast, the Na\(^+\) affinity of EAAC1\(_{D367N}\) was virtually unchanged compared with the wild type transporter (\(K_m = 90\) mM). The reduced occupancy of the Na\(^+\) binding site of EAAC1\(_{D367N}\) resulted in a dramatic reduction in glutamate affinity (\(K_m = 3.6\) mM, 140 mM [Na\(^+\)]), which could be partially overcome by increasing extracellular [Na\(^+\)]. In addition to impairing Na\(^+\) binding, the D367N mutation slowed glutamate transport, as shown by pre-steady-state kinetic analysis of transport currents, by strongly decreasing the rate of a reaction step associated with glutamate translocation. Our data are consistent with a model in which Asp-367, but not Asp-454, is involved in coordinating the bound Na\(^+\) in the glutamate-free transporter form.

Excitatory amino acid carrier 1 (EAAC1)\(^3\) belongs to the excitatory amino acid transporter (EAAT) family, which consists of five members, EAAT1 (GLAST) (1), EAAT2 (Glt-1) (2–4), EAAT3 (EAAC1) (5), EAAT4 (6), and EAAT5 (7). The major function of glutamate transporters in the central nervous system is to remove glutamate from the synaptic cleft to prevent the glutamate concentration from reaching neurotoxic levels (8, 9). Glutamate/aspartate transporters move glutamate from the extracellular space into the cell against its own transmembrane concentration gradient by coupling transport to the downhill movement of Na\(^+\) and K\(^+\) ions across the membrane (5). The stoichiometry of this coupling is movement of 1 glutamate\(^-\), 3 Na\(^+\), and 1 H\(^+\) into the cell and 1 K\(^+\) out of the cell (9–12). According to this stoichiometry, glutamate transport is electrogenic with a total of two positive charges being translocated to the intracellular side during each transport cycle. Important questions that are still unresolved regard the translocation pathways and the location of binding sites of the substrate and the co- and countertransported ions. One breakthrough finding toward localizing the glutamate binding site was that an arginine residue (Arg-446) in EAAC1 is responsible for the coordination of the negatively charged \(\gamma\)-carboxylate group of glutamate (13). Replacement of this amino acid by cysteine changes EAAC1 from an acidic amino acid transporter to a neutral amino acid transporter indicating that Arg-446 is absolutely necessary for the binding of the negatively charged \(\gamma\)-carboxylate group of glutamate to EAAC1. Although this involvement of Arg-446 in glutamate binding was confirmed in the recently published crystal structure of a bacterial glutamate transporter homologue (14), GltPh, possible binding sites for the co-transported cations were not resolved in this structure. Because the mechanism of glutamate transport by EAAC1 is assumed to be based on charge compensation (15), it is likely that negatively charged residues in the transmembrane segments of the transporter contribute to the binding of these cations (16, 17). Several acidic amino acid residues are highly conserved in the transporter family (16). One of them, Glu-373 in EAAC1, was proposed to be responsible for proton binding (18). Furthermore, it was suggested that this amino acid residue is also involved in the K\(^+\) interaction with the transporter (19). In another report, Pines et al. (16) hypothesized that two aspartate residues of Glt-1 corresponding to Asp-367 and Asp-439 in EAAC1 may be involved in the binding and/or translocation of one or more of the cotransported cations. However, these authors did not show clear experimental evidence to support this hypothesis besides the fact that transporters with charge neutralizations in these positions were not functional.

Here, the requirement of aspartate residues Asp-367 and Asp-454 for the transport function of EAAC1 was studied in more detail. Both of these residues are highly conserved among members in the EAAT family (Fig. 1) and are localized in the transmembrane domain of the transporter, according to the GltPh structure (14). Charge neutralization of these two residues resulted in transporters that were still able to bind Na\(^+\), but did not catalyze glutamate transport. Whereas the D454N mutation had only little effect on the apparent affinity of Na\(^+\) binding to the empty transporter, EAAC1\(_{D367N}\) showed strongly decreased apparent affinity for this Na\(^+\) binding step. Mutation of the corresponding amino acid residue Asp-386 to asparagine (Fig. 1) in the related neutral amino acid transporter ASCT2 (alanine-serine-cysteine transporter 2), which has a much higher affinity to Na\(^+\) than EAAC1, resulted in a mutant transporter with a very similar behavior to that of EAAC1\(_{D367N}\); namely, the affinity for Na\(^+\) was dramatically decreased. Based on these results, we propose that Asp-367 in EAAC1, but not Asp-454, is
Na\(^{+}\) Binding to Glutamate Transporters

Asp-454 of EAATs and ASCTs belonging to the SLC1 (solute carrier 1) family.

FIGURE 1. Sequence alignment of the highly conserved region around Asp-367 and Asp-454 of EAATs and ASCTs belonging to the SLC1 (solute carrier 1) family. The conserved aspartic acid residues Asp-367 and Asp-454 are shown in red.

EXPERIMENTAL PROCEDURES

Molecular Biology and Transient Expression—Wild-type rat EAAC1 and rat ASCT2 were subcloned into pBK-CMV (Stratagene), as described previously (15), and used for site-directed mutagenesis according to the QuikChange protocol (Stratagene, La Jolla, CA), as described by the supplier. The primers for mutation experiments were obtained from the DNA core lab, Department of Biochemistry at the University of Miami School of Medicine. The complete coding sequences of mutated clones were subsequently sequenced. Wild type and mutant transporter constructs were used for transient transfection of subconfluent human embryonic kidney cell (HEK293T/17, ATCC number CRL 11268) cultures using FuGENE 6 Transfection Reagent (Roche) according to the instructions of the supplier. Electrophysiological recordings were performed between days 1 and 3 post-transfection.

Electrophysiology—Substrate-induced transporter currents were recorded with an Adams & List EPC7 amplifier under voltage-clamp conditions in the whole cell current-recording configuration. The typical resistance of the recording electrode was 2–3 M\(\Omega\); the series resistance was 5–8 M\(\Omega\). Because the currents induced by substrate or cation application were small (typically <500 pA), series resistance (R\(_s\)) compensation had a negligible effect on the magnitude of the observed currents (<4% error). Therefore, R\(_s\) was not compensated. For the experiments in the forward transport mode, the extracellular solution contained (in mM): 140 NaCl, 2 CaCl\(_2\), 2 MgCl\(_2\), and 30 HEPES, pH 7.3. Na\(^{+}\) was replaced by NMG\(^+\) (N-methylglucamine) in Na\(^{+}\)-free solutions. Two different pipette solutions were used depending on whether mainly the non-coupled anion current (with thiocyanate) or the coupled transport current (with chloride or gluconate) was investigated (20). These solutions contained (in mM): 140 NaCl, 2 CaCl\(_2\), 2 MgCl\(_2\), and 10 HEPES (pH 7.4, KOH). Thiocyanate was used because it enhances glutamate transporter-associated currents and it allowed us to determine the effect of the Na\(^{+}\) concentration on the leak anion conductance mode (20).

For the electrophysiological investigation of the Na\(^{+}\)/glutamate exchange mode the pipette solution contained (in mM) 140 NaCl, 2 NaSCN, 2 MgCl\(_2\), 10 EGTA, and 10 HEPES (pH 7.4, NaOH) (21). It was shown previously that application of extracellular glutamate in the exchange mode results in a redistribution of glutamate binding sites within the membrane associated with permanent activation of an anion current (22, 23). This permanent anion current was used as a tool to study the behavior of mutant transporters in the exchange mode. The currents were low pass filtered at 3 kHz, and digitized with a digitizer board (Axon, Digidata 1200) at a sampling rate of 10–50 kHz, which was controlled by software (Axon Pclamp). All the experiments were performed at room temperature.

Rapid Solution Exchange and Laser-pulse Photolysis—Rapid solution exchange was performed as described previously (15). Briefly, substrates were applied to the EAAC1- or ASCT2-expressing cell by means of a quartz tube (opening diameter: 350 \(\mu m\)) positioned at a distance of ~0.5 mm to the cell. The linear flow rate of the solutions emerging from the opening of the tube was ~5–10 cm/s, resulting in typical rise times of the whole cell current of 20–50 ms (10–90%). Laser-pulse photolysis experiments were performed according to previous studies (15, 22). 4-methoxy-7-nitroindolyl-caged glutamate (24) (Tocris) in concentrations of 1–4 mM or free glutamate were applied to the cells and photolysis of the caged glutamate was initiated with a light flash (340 nm, 15 ns, excimer laser pumped dye laser, Lambda Physik, Göttingen, Germany). The light was coupled into a quartz fiber (diameter, 365 \(\mu m\)) that was positioned at a distance of 300 \(\mu m\) from the cell. The laser energy was adjusted with neutral density filters (Andover Corp.). With maximum light intensities of 500–600 mJ/cm\(^2\) saturating glutamate concentrations could be released, which was tested by comparison of the steady-state current with that generated by rapid perfusion of the same cell with a glutamate concentration that saturated the respective mutant transporter.

Data Analysis—Non-linear regression fits of experimental data were performed with Origin (Microcal Software, Northampton, MA) or Clampfit (pClamp8 software, Axon Instruments, Foster City, CA). Data points are given ± S.D. Pre-steady-state currents were fitted with sums of two exponential terms. Dose-response relationships of currents were fitted with a Michaelis-Menten-like equation, yielding \(K_m\) and \(I_{max}\). The glutamate-free transporter form has a relatively low affinity for extracellular Na\(^{+}\). Therefore, it was not possible to saturate the binding site on this transporter form within the [Na\(^{+}\)] concentration range accessible. For this reason, we estimate the error associated with the determined \(K_m\) values to be at least ±20%. This is especially relevant for the data on the D367N mutant transporter, for which it was not possible to reach half-saturating concentrations (the current-[Na\(^{+}\)] relationship was almost linear). Therefore, only a crude estimate of the \(K_m\) of this mutant for Na\(^{+}\) can be given.

For the Na\(^{+}\) concentration dependence of the leak anion current, the data were corrected by subtraction for the nonspecific component of the current, which increases linearly with increasing [Na\(^{+}\)]. This nonspecific component was determined from non-transfected HEK293 cells. To compare the [Na\(^{+}\)] dependence of the Na\(^{+}\)-induced current and the inhibitor-induced current, Scheme 1 was used. In Scheme 1, \(T\) is the empty transporter, \(NT\) the Na\(^{+}\)-bound, anion conducting state, and \(NTI\) the inhibitor (I)-bound state. The anion current is proportional to the population of the Na\(^{+}\)-bound state (\(P_{NT}\)), and can be expressed as in the following equations.

\[
I \approx P_{NTI}(0) = \frac{[Na^+]^2}{[Na^+] + K_{Na^+}} (\text{absence of inhibitor}) \quad \text{(Eq. 1)}
\]

\[
I \approx P_{NTI}(0) - P_{NTI}(I) = -\frac{K_I}{[I] + K_I} \frac{[Na^+]}{[Na^+] + K_{Na^+}} + \frac{[Na^+]}{[Na^+] + K_{Na^+}} (\text{presence of inhibitor}) \quad \text{(Eq. 2)}
\]

Here, \(K_I\) and \(K_{Na^+}\) are the apparent dissociation constants of the inhibitor and Na\(^{+}\), respectively. It is clear that in the presence of satu-
Na\(^+\) Binding to Glutamate Transporters

RESULTS

[Na\(^+\)]-dependent Anion Leak Current—In addition to the glutamate-gated anion conductance (25), glutamate transporters exhibit a leak pathway for hydrophobic anions (22, 26), such as SCN\(^-\), to cross the cell membrane. Current associated with the anion leak is already present in the total absence of transported substrates, but requires the presence of extracellular Na\(^+\). By recording and analyzing this Na\(^+\)-induced leak current, the affinity of the glutamate-free form of EAAC1 for Na\(^+\) can be determined. This [Na\(^+\)]-dependent leak anion current was analyzed for both wild type and mutant transporters. The Na\(^+\)-induced anion leak current was determined either after applying a [Na\(^+\)] jump to an EAAC1 expressing cell, or by inhibiting the leak current by application of a competitive blocker of glutamate uptake, TBOA (DL-threo-\(\beta\)-benzyloxyaspartate) (27, 28) (summarized in Fig. 2).

As shown in Fig. 2A, application of 140 mM Na\(^+\) to an EAAC1-transfected cell in the presence of 130 mM intracellular SCN\(^-\) resulted in an inward current (−108 ± 35 pA, from 6 cells) caused by SCN\(^-\) outflow. When 140 mM Na\(^+\) was co-applied with 20 \(\mu\)M TBOA, only 13 ± 3% (n = 5) of the current in the absence of TBOA was observed (see Fig. 2B for a representative curve), showing that the majority (almost 90%) of the Na\(^+\)-induced current was specifically carried by EAAC1 and was not because of nonspecific background conductances. Fig. 2C shows a typical result obtained when 20 \(\mu\)M TBOA was applied in the background presence of 140 mM Na\(^+\). The apparent outward current is caused by inhibition of the Na\(^+\)-dependent anion leak current as demonstrated previously by us and others (15, 29). To further test the specificity of the Na\(^+\)-induced current, we applied 140 mM Na\(^+\) to non-transfected control cells (Fig. 2D). The average current induced in these cells was −9 ± 1 pA (from 7 cells). To directly test whether the Na\(^+\)-induced current is carried by SCN\(^-\), we determined the voltage dependence of the inward current. As shown in Fig. 2E, the inward current increased with increasingly negative membrane potentials (closed circles) and did not reverse within the voltage range tested. When the SCN\(^-\) concentration gradient was reversed, an outward current was observed at each voltage (Fig. 2E, triangles), with a current-voltage relationship that can be described by the Goldman-Hodgkin-Katz equation (30). Together, these results demonstrate that the EAAC1-containing membrane acts as a specific SCN\(^-\) electrode in the presence of Na\(^+\). In non-transfected control cells, only very little current was observed within the voltage range tested (Fig. 2E, open circles).

Na\(^+\) Affinity of the Glutamate-Free Transporter Form—Next, we applied the [Na\(^+\)] jump method to determine the \(K_m\) for Na\(^+\) binding to the glutamate-free form of wild type EAAC1. As shown in Fig. 3A (closed circles), the anion leak current increased with increasing [Na\(^+\)]. The \(K_m\) was estimated as 120 ± 20 mM (n = 3) after correction for the nonspecific component of the leak current (open triangles and dashed line, obtained from non-transfected cells). When the \(K_m\) was determined by using the inhibitor method, we obtained a value of 80 ± 20 mM (open circles). These results show that application of both methods allow us to determine the apparent affinity of the empty transporter binding site(s) for Na\(^+\). It should be noted that the data were fitted with a Hill coefficient of 1, assuming that only one Na\(^+\) ion binds to the empty transporter. Although using a Hill coefficient of 2 resulted in a worse fit, by evaluating the wild type data we cannot exclude the possibility that two of the three co-transported Na\(^+\) bind to the empty transporter form.

We chose the [Na\(^+\)] jump method to determine the Na\(^+\) affinities of transporters with charge neutralization mutations to amino acids in possible Na\(^+\) binding sites in the following experiments. Fig. 3B shows the relationship between the anion leak current and [Na\(^+\)] for EAAC1\(_{D367N}\) ([Na\(^+\)] up to 500 mM was used, note the 2.6-fold expanded concentration scale). This relationship was nearly linear within the [Na\(^+\)] range used (5–500 mM, Fig. 3B, straight dotted line). This means that a [Na\(^+\)] as high as 500 mM is still far from saturating the Na\(^+\) binding site(s) on EAAC1\(_{D367N}\). The data can also be fitted by the Hill equation (Fig. 3B, solid line), which allowed us to estimate the apparent \(K_m\) for Na\(^+\) binding to the transporter as 1.9 ± 1.5 mM (corrected for nonspecific currents, Fig. 3B, triangles). Although this value is more than 10 times higher than that of wild type EAAC1, it represents only a crude estimate because we were unable to use higher [Na\(^+\)] than 500 mM without damaging the cells. For EAAC1\(_{D367N}\), the nonspecific component was more prominent (42% of the specific current at 500 mM Na\(^+\), Fig. 3, B and F). In any case, the data clearly show that Na\(^+\) binds with a much lower affinity to EAAC1\(_{D367N}\) than to EAAC1\(_{WT}\), as demonstrated by the poor fit of the data with the wild-type \(K_m\) fixed to 120 mM (Fig. 3B, curved dotted line).
**Na\(^+\) Binding to Glutamate Transporters**

Next, we tested EAAC1\(_{D454N}\). As shown in Fig. 3C, Na\(^+\) application resulted in dose-dependent inward currents in EAAC1\(_{D454N}\)-expressing cells. The apparent \(K_m\) for the first Na\(^+\) binding to EAAC1\(_{D454N}\) was 90 ± 20 mM, a value statistically not significantly different from that of the wild type transporter. This result suggests that neutralization of Asp-454 has no effect on Na\(^+\) binding to the glutamate-free transporter form.

The leak anion current of the two mutant transporters extrapolated to saturating concentrations of Na\(^+\) was larger than that induced in EAAC1\(_{WT}\) (Fig. 3, A–C). This effect was not caused by increased cell surface expression of the mutant transporters. As shown in Fig. 1 of the supplementary information, cell surface expression (assayed by biotinylation of EAAC1 and subsequent Western blotting) was not increased by the D367N and D454N mutations compared with the wild type transporter. Thus, it is possible that these mutations alter the anion conductance properties of EAAC1. However, this effect was not further analyzed in this work.

ASCT2 is a neutral amino acid transporter (31, 32), which has an amino acid sequence closely related to those of EAATs. ASCT2 has a very high affinity for Na\(^+\) (~1 mM, see Fig. 3D and Ref. 33). The aspartic acid residue Asp-386 in ASCT2 corresponds to Asp-367 in EAAC1 (Fig. 1). To determine whether Asp-386 has the same effect to ASCT2 as Asp-367 to EAAC1, we mutated this amino acid residue to asparagine.

Asp-454 has no effect on Na\(^+\) binding to the glutamate-free transporter form. The D473N mutation, corresponding to D454N in EAAC1, the apparent dissociation constant of Na\(^+\) from the empty transporter was 5 ± 2 mM, showing that the effect of this mutation on Na\(^+\) binding is minor.

**Affinities for Amino Acid Substrates and Competitive Inhibitors**—The Glit-1 analog of EAAC1\(_{D367N}\) was previously shown to be defective in glutamate uptake (16). Consistent with this result, application of 100 \(\mu\)M glutamate to EAAC1\(_{D367N}\) (140 mM [Na\(^+\)]) did not result in any measurable current, either in the transport mode, or in the anion conducting mode (\(n = 10\)). However, after increasing the glutamate concentration to 5 mM, large inwardly directed anion currents were observed (−870 ± 400 pA, \(n = 5\); a typical current trace is shown in Fig. 4A), demonstrating that the mutant transporter can still bind glutamate and activate the substrate-dependent anion current. The \(K_m\) values for substrates of EAAC1\(_{D367N}\) and ASCT2\(_{D386N}\) are listed in Table 1. At 140 mM Na\(^+\), the \(K_m\) of EAAC1\(_{D367N}\) for glutamate was determined as 3.6 ± 0.8 mM (Table 1 and Fig. 4C). This value is about 500 times higher than that of EAAC1 wild type (7.2 ± 1.1 \(\mu\)M, Table 1). The \(K_m\) for glutamine of ASCT2\(_{D386N}\) was determined as 105 ± 10 \(\mu\)M at 140 mM NaCl (Table 1, Fig. 4G), which is 4 times higher than that of wild type ASCT2 (25 ± 1 \(\mu\)M, Table 1). These results show that the charge-neutralization mutation in position 367 strongly reduces the apparent affinity for the transported substrate. The \(K_m\) values for competitive inhibitors show a similar behavior. At 140 mM NaCl, the \(K_m\) of EAAC1\(_{D367N}\) for TBOA was 180 ± 100 \(\mu\)M (Table 1), which is about 35 times higher than that of wild type EAAC1. Under the same conditions, \(K_m\) of ASCT2\(_{D386N}\) for benzylserine, a competitive inhibitor of ASCT2 (33), was found to be 3.0 ± 1.2 mM (Table 1), 6 times higher than that of wild type ASCT2. These inhibitor data suggest that part of the effect of low occupancy of the Na\(^+\) binding site must be directly on substrate binding, and not only on conformational changes following binding that increase the affinity of the transporter for the substrate. These conformational changes, such as substrate translocation, are only possible in the substrate-bound
transporter, but not in the inhibitor-bound form of the protein. In contrast to the data obtained for EAAC1<sub>D367N</sub>, the <i>K<sub>m</i></sub> of EAAC1<sub>D454N</sub> for glutamate was virtually unchanged compared with the wild type transporter (Table I).

Next, we tested whether a change in occupancy of the <i>Na</i><sup>+</i> binding site changes the apparent affinity of the mutant transporters for the substrate. When the <i>Na</i><sup>+</i> concentration was decreased to 50 mM, the <i>K<sub>m</sub></i> values of EAAC1<sub>D367N</sub> for glutamate increased to 8.3 ± 0.7 mM (Table I and Fig. 4, B and C). When [<i>Na</i><sup>+</i>] was raised to 300 mM, the <i>K<sub>m</sub></i> of EAAC1<sub>D367N</sub> for glutamate decreased to 1.8 ± 0.1 mM (Table I and Fig. 4D), indicating that increasing the population of the <i>Na</i><sup>+</i> <i>K<sub>m</sub></i> form resulted in an increase of glutamate affinity (Fig. 5A), as expected. We could not increase the <i>Na</i><sup>+</i> concentration to values that would saturate the <i>Na</i><sup>+</i> binding site. Therefore, we extrapolated the [<i>Na</i><sup>+</i>] dependence of <i>K<sub>m</sub></i> to that expected at the saturating concentration of 20 mM extracellular <i>Na</i><sup>+</i> (10-fold), yielding a <i>K<sub>m</sub></i> of about 40 mM. Although this value is still slightly higher (about 2 times) than that found for EAAC1<sub>WT</sub> (open triangles in Fig. 5A), this analysis suggests that D367N exchange reduces the glutamate affinity mainly because of the non-saturated <i>Na</i><sup>+</i> binding site on the empty transporter. Repeating the same experiments for ASCT2<sub>D386N</sub> (a typical current induced by glutamine application is shown in Fig. 4E), the <i>K<sub>m</sub></i> decreased when the [<i>Na</i><sup>+</i>]

### TABLE I

| Parameter | Wild type | D367N | D454N | Wild type | D386N |
|-----------|-----------|-------|-------|-----------|-------|
| <i>K<sub>m</sub></i> for substrates<sup>a</sup> at 50 mM NaCl (μM) | 380 ± 50 | 8300 ± 700<sup>b</sup> | 800 ± 90<sup>c</sup> | ND<sup>d</sup> | 240 ± 40<sup>d</sup> |
| <i>K<sub>m</sub></i> for substrates<sup>a</sup> at 140 mM NaCl (μM) | 14 ± 1<sup>c</sup> | 3600 ± 800<sup>c</sup> | 20 ± 4<sup>c</sup> | 25 ± 1<sup>c</sup> | 105 ± 10<sup>c</sup> |
| <i>K<sub>m</sub></i> for substrates<sup>a</sup> at 300 mM NaCl (μM) | ND | 1800 ± 100<sup>c</sup> | 180 ± 100<sup>c</sup> | 520 ± 100<sup>c</sup> | 900 (33) |
| <i>K<sub>m</sub></i> for inhibitors<sup>a</sup> at 140 mM NaCl (μM) | 7.2 ± 1.1 | 180 ± 100<sup>c</sup> | 180 ± 100<sup>c</sup> | 3000 ± 1200<sup>c</sup> |

<sup>a</sup> Apparent affinity for substrates. Glutamate was used for EAAC1 and glutamine for ASCT2.

<sup>b</sup> ND, not determined.

<sup>c</sup> Exchange conditions.

<sup>d</sup> Apparent affinity for inhibitors. TBOA was used for EAAC1 and benzylserine for ASCT2.

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### FIGURE 4

Determination of the apparent <i>K<sub>m</sub></i> values of substrates for EAAC1<sub>D367N</sub> and ASCT2<sub>D386N</sub>. Apparent affinities for substrates of EAAC1<sub>D367N</sub> and ASCT2<sub>D386N</sub> were determined by recording substrate-induced anion currents in the homoexchange mode (with <i>Na</i><sup>+</i> and substrate present on the both sides of the cell membrane) at different extracellular [<i>Na</i><sup>+</i>]. Typical anion currents induced by glutamate (for EAAC1<sub>D367N</sub>) and glutamine (for ASCT2<sub>D386N</sub>) are shown in A and E, respectively, with <i>V<sub>out</sub></i> = 0 mV. The apparent <i>K<sub>m</sub></i> values for glutamate activation of EAAC1<sub>D367N</sub> at 50 mM NaCl, 140 mM NaCl, and 300 mM NaCl are (in mM): 8.3 ± 0.7 (B), 3.6 ± 0.8 (C), and 1.8 ± 0.1 (D), respectively. The apparent <i>K<sub>m</sub></i> values of glutamine activation of ASCT2<sub>D386N</sub> at 50, 140, and 300 mM NaCl are (in μM): 242 ± 37 (F), 103 ± 9 (G), and 517 ± 99 (H), respectively.
Na\textsuperscript{+} Binding to Glutamate Transporters

than 300 m\textsuperscript{s} (see Fig. 4, B–D), as shown by the solid squares in Fig. 5B. We then estimated the \( I_{\text{max}} \) at each Na\textsuperscript{+} concentration by extrapolating the \( K_{\text{m}} \) versus [Na\textsuperscript{+}] curve shown in Fig. 5A. As illustrated in Fig. 5B, the \( I_{\text{max}} \) shows strong [Na\textsuperscript{+}] dependence. The data can be fitted by the Hill equation with a Hill coefficient of \( n = 1.9 \). The \( K_{\text{m}} \) for these Na\textsuperscript{+} ions was estimated as 43 \pm 11 m\textsuperscript{s}. Compared with wild type EAAC1, this \( K_{\text{m}} \) is about 2 times higher, suggesting that the main effect of the D367N mutation is to inhibit Na\textsuperscript{+} binding to the glutamate-free form of EAAC1, but not to the glutamate-bound form of the transporter.

**Steady-state Properties**—As shown in Fig. 6A, no steady-state transport current was induced by application of 5 m\textsuperscript{M} glutamate to EAAC1\textsubscript{D367N} (glucuronate was the main intracellular anion, \( n = 10, 5 \) cells, \( V_{\text{hold}} = 0 \) m\textsuperscript{m}V). Because glucuronate does not permeate the EAAC1 anion conductance (34) and there was no driving force for anions present to cross the membrane (0 m\textsuperscript{m}V), only the coupled transport component of the current should be observed under these conditions. In contrast, application of 1 mM glutamate induced a significant steady-state inward transport current of \(-80 \pm 30 \) pA in EAAC1\textsubscript{WT} (Fig. 6B, \( n = 10, V_{\text{hold}} = 0 \) m\textsuperscript{m}V, KCl pipette solution). Although no steady-state transport current was induced in EAAC1\textsubscript{D367N} (Fig. 6C), there was a transient transport current present upon glutamate application (\(-35 \pm 12 \) pA, \( n = 7, \) Fig. 6A, arrow). This indicates that the reason why steady-state transport current could not be detected was not because there was no mutant transporter expressed in the HEK293T cells studied, but because the replacement of Asp-367 by asparagine has an inhibitory effect on the steady-state transport rate of EAAC1. In the presence of Cl\textsuperscript{−} instead of glucuronate, a small steady-state current was observed, indicating a permeability of EAAC1\textsubscript{D367N} for Cl\textsuperscript{−}.

We next tested whether increasing the driving force for glutamate uptake by hyperpolarizing the cell would overcome inhibition of the steady-state transport by EAAC1\textsubscript{D367N}. In Fig. 6D, glutamate-induced steady-state transport currents are plotted as a function of voltage. At \(-90 \) m\textsuperscript{m}V, about \(-9.0 \pm 4.0 \) pA coupled transport current was induced by applying a saturating concentration of 50 mM glutamate (Fig. 6E). In contrast, EAAC1\textsubscript{WT} catalyzed large steady-state transport currents at \(-90 \) m\textsuperscript{m}V (\(-310 \pm 25 \) pA). Together, these results indicate that steady-state uptake of glutamate is strongly impaired in EAAC1\textsubscript{D367N}, even when conditions were used that saturate the Na\textsuperscript{+} binding site on the glutamate-free transporter and the glutamate binding site.

**Kinetics and Voltage Dependence of Pre-steady-state Currents**—Whereas the glutamate-induced transient transport current in EAAC1\textsubscript{WT} decays within 30 ms (22), the transient phase of EAAC1\textsubscript{D367N} transport current decayed much more slowly with a time constant in the range of 200 ms (Fig. 6A). This current decay was slow enough that we were able to measure its decay kinetics by using rapid solution exchange (time resolution of 20–50 ms). The low rate of the decay of the transport current suggested that electrogenic processes, which are rapid in EAAC1\textsubscript{WT}, are slowed in EAAC1\textsubscript{D367N}. The transient transport current was also present when the experiment in Fig. 6A was repeated in the exchange mode (Fig. 6F), clearly demonstrating that this electrogenic...
process is associated with the glutamate translocation branch of the transporter.

We next measured the glutamate-induced anion current in the forward transport mode (KSCN internal). As shown in Fig. 7A, a steady-state anion current was observed under these conditions, which was about $-39 \pm 15$ pA ($n = 8$, 6 cells, $V_{hold} = 0$ mV). In contrast, EAAC1_D367N catalyzed on average steady-state whole cell current 12 times as large in the presence of saturating concentrations of glutamate (see Fig. 7B for a typical trace). This result was unexpected because for mutant glutamate transporters with impaired transport activity, for example, EAAC1_E373Q (18) and EAAC1_H295K (35), no steady-state currents can be detected in the forward transport mode with KSCN in the pipette solution. Before the steady-state was reached in EAAC1_D367N, a significant transient anion current component was observed, which consisted of two phases, a rising phase and a decaying phase. This transient current resembles that observed in wild-type EAAC1 (Fig. 7B), although it decays with a time constant ($350 \pm 140$ ms, $n = 5$; 5 mM glutamate) that is 40 times larger than that of the EAAC1_WT current decay. After increasing [glutamate] to 50 mM, a saturating concentration, the time constant for the decay phase of the current was $215 \pm 50$ ms. These results show that the relaxation rate constant for the current decay, $1/\tau_{decay}$, increases with increasing [glutamate], as already published for EAAC1 WT (15) and as expected for a glutamate-induced reaction that follows glutamate binding. $1/\tau_{decay}$ was voltage dependent, increasing with hyperpolarization of the cell (Fig. 7, C and D). The slope of the log(1/\tau) versus voltage relationship was determined as $(2.6 \pm 0.4) \times 10^{-7}$ mV for EAAC1_D367N. For EAAC1 WT, a slightly (statistically not significant) steeper voltage dependence was found ((3.8 \pm 0.9) \times 10^{-7}$ mV, Fig. 7D). The decaying phase of the transient anion current was previously assigned to glutamate translocation in EAAC1 WT. The slowed kinetics of this phase may indicate that glutamate translocation is slowed in EAAC1 D367N.

To test whether the rates obtained by rapid solution exchange were not limited by the speed of the solution exchange, we performed laser
photolysis of caged glutamate to release free glutamate at the cell surface in the microsecond time range, as shown in Fig. 7, E and F. As in the rapid solution exchange experiments, two phases of the current were observed, a rising phase and a decaying phase (Fig. 7E). The time constant of the decaying phase was similar to the one determined by rapid solution exchange, \( \tau_{\text{decay}} = 180 \pm 50 \) ms \((n = 5)\). In contrast the rising phase was significantly faster \((\tau_{\text{rise}} = 2.1 \pm 0.5 \) ms\) than in the rapid solution exchange experiment. \( \tau_{\text{rise}} \) was about 4–6 times the value observed for EAAC1\(_{\text{WT}}\) at saturating concentrations of glutamate (Fig. 7G). At the glutamate concentration used for the concentration jump (about 0.8 mM), the glutamate binding site of EAAC1\(_{D367N}\) is still far from being saturated. We estimated that only 19% of transporters were in the bound state at this concentration. Thus, it is likely that the rising phase of the anion current was slowed because of the unfavorable pre-equilibrium of the glutamate binding step, being shifted mainly to the unbound form. To test this idea, we performed control experiments with the wild type transporter at a glutamate concentration (about 2 \(\mu\)M) that yields a similar distribution of glutamate-bound and glutamate-free states as in the D367N experiment. The time constant of the rising phase was 4.1 ± 0.1 ms, demonstrating that the slow rise of the EAAC1\(_{D367N}\) anion current was mainly caused by the inability to saturate the glutamate binding site. It should be noted that it is technically not possible to photolytically generate glutamate concentrations required to saturate EAAC1\(_{D367N}\), because the high concentration of caged compound necessary would generate large inner filter effects (the majority of the light is already absorbed at the surface of the optical fiber and does not reach the cell).

Finally, we determined the voltage dependence of the rate constant of the anion current rise (Fig. 7G). For EAAC1\(_{D367N}\), 1/\( \tau_{\text{rise}} \) slightly decreased with increasing membrane potential. The slope of the \( \log (1/\tau_{\text{rise}}) \) versus voltage relationship was \(-1.9 \times 10^{-3}\) mV. In contrast, \( \log (1/\tau_{\text{rise}}) \) increased with increasingly positive voltage for EAAC1\(_{\text{WT}}\) at both saturating and non-saturating glutamate concentrations (Fig. 7G) with slopes of \(1.7 \times 10^{-3}\) mV for both glutamate concentrations used.

**DISCUSSION**

In this report we studied the effects of charge neutralization of two highly conserved acidic amino acid residues in EAAC1, Asp-367 and Asp-454. Both of these amino acid residues are localized in hydrophobic segments of the transporter sequence and, according to the recently published x-ray structure of the bacterial glutamate transporter GltPh (14), reside in a position close to the center of the lipid bilayer. The two central new findings of this study are that replacement of Asp-367 with asparagine leads to: 1) a dramatic increase of the apparent \( K_m \) for Na\(^+\) binding to the empty transporter, and 2) slowed kinetics of glutamate translocation and transport. The D367N mutation has only a minor influence on binding of Na\(^+\) to the glutamate-bound transporter form (Figs. 4 and 5). In contrast to D367N, the D454N mutation has very little effect on the affinity of the empty transporter for Na\(^+\), suggesting that Asp-367, but not Asp-454 contributes to the sodium binding site on the unloaded EAAC1 protein. We confirmed our observations by investigating the effect of the analogous charge neutralization mutation in ASCT2, which has a much higher affinity for Na\(^+\) than EAAC1. After the corresponding amino acid residue Asp-386 in ASCT2 was mutated to asparagine, the apparent \( K_m \) for Na\(^+\) to this transporter was raised from the micromolar range to about 200 mM (Fig. 2). These data strongly indicate that both Asp-367 in EAAC1 and Asp-386 in ASCT2 are involved in binding of Na\(^+\) to the empty transporter.

**Effect of the D367N Mutation on Substrate Binding and Na\(^+\) Binding to the Glutamate-bound Form Is Indirect**—It is known from wild-type EAAC1 that the occupancy of the Na\(^+\) binding site on the empty transporter strongly influences substrate binding to the transporter (22). The substrate binding site is only formed when this Na\(^+\) binding site is occupied. In agreement with this idea, the apparent \( K_m \) values for substrates/inhibitors of EAAC1\(_{D367N}\) were strongly increased because the Na\(^+\) binding affinity was decreased. The apparent \( K_m \) for glutamate of EAAC1\(_{D367N}\) at physiological conditions (140 mM NaCl, pH 7.3) was increased more than 700 times compared with that of the wild type transporter (Table 1). At the same conditions, the \( K_m \) for the inhibitor TBOA of EAAC1\(_{D367N}\) was increased about 25 times compared with that of wild type (Table 1). ASCT2\(_{D386N}\) showed similar behavior with respect to the \( K_m \) values for the substrate glutamine and the inhibitor benzylserine (Table 1).

Like in wild type EAAC1, decreasing [Na\(^+\)] to 50 mM led to an increase in the apparent \( K_m \) of EAAC1\(_{D367N}\) for glutamate, whereas increasing [Na\(^+\)] to 300 mM led to a decrease in \( K_m \) (Table 1). However, even at 300 mM extracellular [Na\(^+\)], the Na\(^+\) binding site is only occupied at a maximum of 13% of transporters. Therefore, we expect that if it was possible to increase [Na\(^+\)] even further to saturating values the apparent \( K_m \) for glutamate of EAAC1\(_{D367N}\) would be close to that of the wild type transporter. Based on these data, it is likely that the effect of the D367N mutation on the glutamate binding site is indirect, which is in agreement with the x-ray structure of GltPh (14), showing that the glutamate binding site is located at a distance of about 11–12 Å from the aspartate side chain in position 367.

EAAC1 binds extracellular Na\(^+\) ions sequentially, with at least one Na\(^+\) binding to the glutamate-loaded form of the transporter (22). The physical location of the binding site for the second Na\(^+\) on EAAC1 is still unknown. The apparent \( K_m \) for Na\(^+\) binding to the glutamate-bound form of EAAC1\(_{D367N}\) is 43 m\(\text{M}\), whereas that of the EAAC1 wild type is in the range of 20 m\(\text{M}\) (Fig. 5). Therefore, the effect of the mutation on binding of this second Na\(^+\) ion is much less than that on binding of the first Na\(^+\). These results suggest that this second Na\(^+\) binding site is not in direct proximity of Asp-367.

**The Asp-367 Mutation Eliminates Steady-state Glutamate Transport**—Mutation of Asp-367 to asparagine eliminated glutamate transport activity. This finding is in agreement with results from a previous study by the Kanner group showing that any mutation of the analogous Asp-398 in Glk-1 results in a transporter that cannot take up glutamate (16).

The steady-state transport data were confirmed by pre-steady-state kinetic experiments, showing a dramatic reduction in the rates of specific partial reactions of EAAC1\(_{D367N}\). For example, we identified one partial reaction (associated with the decay of the anion current) with a time constant of 220 ms. However, the process associated with this time constant is not rate-limiting for steady-state transporter cycling, which is limited by another, even slower partial reaction. The ratio of the steady-state and the pre-steady-state components of the anion current of about 0.15 (details of this method are described in Ref. 15) allowed us to estimate the rate constant of this rate-limiting process as 1.6 s\(^{-1}\). Thus, steady-state transport is slowed by about a factor of 50 compared with EAAC1\(_{\text{WT}}\), accounting fully for our inability to measure glutamate-induced transport current in this mutant carrier. At this point, it is not entirely clear which partial reactions are slowed by the D367N mutation. However, they are unlikely to be associated with glutamate binding and Na\(^+\) binding. This interpretation is based on the rapid rise of the glutamate-induced anion current (Fig. 7E), occurring with a time constant similar to that of EAAC1\(_{\text{WT}}\). It is well established for EAAC1\(_{\text{WT}}\) that binding of glutamate and Na\(^+\) are necessary to generate
this anion current (22). We can speculate that the reactions that are slowed are associated with structural changes of the transport protein, such as glutamate translocation. In the wild-type transporter, the decaying phase of the anion current was proposed to be associated with the glutamate translocation step (15, 29). If this is also the case in EAAC1D367N, glutamate translocation would be dramatically slowed by neutralization of this residue.

**Implications for the Mechanism of Glutamate Transport**—Involvement of Asp-367 in Na\(^+\) binding to the empty transporter suggests that the binding site for Na\(^+\) is located deeply buried in the transmembrane segment of EAAC1. In the GltPh structure (14), there are no obvious ion entry and exit pathways to this location. Therefore, the question can be asked: how does the Na\(^+\) ion get to this position? We suggest that the Na\(^+\) entry pathway is blocked off in the GltPh structure (substrate-bound form) by the bound acidic amino acid. Therefore, Na\(^+\) is locked into its binding site in the presence of substrate. This "locked-in" state is analogous to the "occluded" state that has been proposed for the Na,K-ATPase. Existence of such a Na\(^+\)-occluded state for the glutamate transporter is supported by previous pre-steady-state kinetic data, showing that there is a fixed sequence of first Na\(^+\) binding, and subsequent glutamate binding to the transporter. Furthermore, Na\(^+\) is also locked into its binding site by competitive inhibitors of glutamate transport and by negative voltage.

In the absence of transported substrate and Na\(^+\), it is likely that a more open conformation is adopted that allows access of Na\(^+\) to its binding site from the extracellular side of the membrane. Evidence for significantly different conformations of the Na\(^+\)-bound and the Na\(^-\)free forms comes from a study using site-directed fluorescence labeling, showing that large fluorescence changes of EAAT3 labeled in position 430 are associated with Na\(^+\) binding, as well as glutamate binding to the empty transporter form (36). Furthermore, cysteine-scanning mutagenesis analysis of amino acid residues in re-entrant loop 2 of Glt-1 also indicated a conformation change after Na\(^+\) binding to the substrate-free transporter because several positions in this loop could not be modified by hydrophilic methanethiosulfonate reagents when Na\(^+\) was present, but could be modified in its absence (37). Although this more open conformation may allow easier access of Na\(^+\) to its buried binding site, it is likely that a hydrophobic barrier needs to be crossed during binding, because the binding process is associated with transmembrane charge movement. Therefore, Na\(^+\) traverses part of the transmembrane electric field while accessing its binding site (38).

Taking all these observations together with our proposal of Na\(^+\) binding close to Asp-367, a structural picture emerges of Na\(^+\) and glutamate interaction with the transporter, as shown in Fig. 8. We assume that Asp-367 is negatively charged in EAAC1. This assumption is reasonable because we were unable to modify transporter activity with dicyclohexylcarbodiimide, either in the absence or presence of Na\(^+\) (data not shown). Because dicyclohexylcarbodiimide preferentially reacts with protonated carboxylates (39), it is likely that Asp-367 is not protonated. Lack of the negative charge in position 367 results in a severe reduction of the glutamate transport rate, even with a fully occupied Na\(^+\) and glutamate binding site, indicating that counterbalancing the positive charge of Na\(^+\) with the negative charge of the carboxylate ligand is important for transport, but not for access of Na\(^+\) and the substrate to their binding sites.

According to the physical location of the Na\(^+\) binding site proposed here in the GltPh structure (14), amino acid side chains other than Asp-367 might be also involved in Na\(^+\) binding. Interestingly, a buried hydrophilic patch of amino acid side chains in the vicinity of Asp-367 also involves two threonine residues in transmembrane 3, histidine 295 in transmembrane 6, and Asn-365 in transmembrane 7. All these amino acid residues are highly conserved in the mammalian members of the glutamate transporter family and such polar and charged side chains are known to participate in Na\(^+\) coordination in previously characterized Na\(^+\) binding sites (40).

In contrast to Asp-367, Asp-454 appears to be not involved in binding of Na\(^+\) to the empty transporter. It remains to be determined if negative charge on Asp-454 is required for transporter function. Preliminary data indicate that EAAC1D454N is functionally similar to the wild-type transporter.

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Na\(^+\) Binding to Glutamate Transporters

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