Thallium Ions Can Replace both Sodium and Potassium Ions in the Glutamate Transporter Excitatory Amino Acid Carrier 1[†]

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ABSTRACT: The excitatory amino acid carrier EAAC1 belongs to a family of glutamate transporters that use the electrochemical transmembrane gradients of sodium and potassium to mediate uphill transport of glutamate into the cell. While the sites of cation interaction with EAAC1 are unknown, two cation binding sites were observed in the crystal structure of the bacterial glutamate transporter homologue GltPh. Although occupied by Tl^+ in the crystal structure, these sites were proposed to be Na^+ binding sites. Therefore, we tested whether Tl^+ has the ability to replace Na^+ also in the mammalian transporters. Our data demonstrate that Tl^+ can bind to EAAC1 with high affinity and mediate a host of different functions. Tl^+ can functionally replace potassium when applied to the cytoplasm and can support glutamate transport current. When applied extracellularly, Tl^+ induces some behavior that mimics that of the Na^+-bound transporter, such as activation of the cation-induced anion conductance and creation of a substrate binding site, but it cannot replace Na^+ in supporting glutamate transport current. Moreover, our data show a differential effect of mutations to two acidic amino acids potentially involved in cation binding (D367 and D454) on Na^+ and Tl^+ affinity. Overall, our results demonstrate that the ability of the glutamate transporters to interact with Tl^+ is conserved between GltPh and a mammalian member of the transporter family. However, in contrast to GltPh, which does not bind K^+, Tl^+ is more efficient in mimicking K^+ than Na^+ when interacting with the mammalian protein.

The excitatory amino acid carrier 1 (EAAC1)[1] belongs to a family of sodium-driven glutamate transporters, which in humans has five known members (*1*−*6*). In the central nervous system (CNS), these transporters are responsible for uptake of the excitatory neurotransmitter glutamate from the synaptic cleft, thus contributing to the control of glutamate concentration in the synapse (*7, 8*). A loss of control of this glutamate concentration is believed to be correlated with some severe CNS disorders, such as amyotrophic lateral sclerosis (ALS), Huntington's disease, and Alzheimer's disease, and with the pathophysiology of brain insults (e.g., ischemia, hypoxia, hypoglycemia, epilepsy). Thus, it is important to understand the molecular mechanisms underlying glutamate transport by these proteins.

Mammalian glutamate transporters take up glutamate against a transmembrane concentration gradient by cotransporting three sodium ions into the cell and one potassium ion out of the cell down their own concentration gradient (*7, 9*−*11*). Moreover, one proton is cotransported with glutamate (*7, 12*). Therefore, the transport process is electrogenic; namely, two positive charges are moved into the cell in each transport cycle (*4*). Although the sites of cation interaction are not known for the mammalian transporters, a recent crystal structure of a bacterial glutamate transporter homologue, the aspartate transporter GltPh from *Pyrococcus horikoshii*, showed the protein in complex with two thallium ions (*13*). Tl^+ was used instead of Na^+ because of its robust anomalous scattering signal. On the basis of this crystal structure and functional studies, GltPh cotransports one aspartate molecule with two sodium ions, but it is independent of potassium (*13*). On the basis of results from competition experiments, it was proposed that the two binding sites of GltPh for Tl^+ are sodium binding sites. One of these two proposed sodium binding sites is in the vicinity but not in direct contact with the bound substrate and is covered by reentrant loop 2 (Na2 site). The other site (Na1 site) is located near the center part of transmembrane domain 8 (TM8), and the side chain of the highly conserved aspartate residue D405 (D454 in EAAC1) contributes to this site (*13*). However, in functional studies of mammalian EAAC1, no effect on Na^+ binding affinity was observed upon mutagenesis of D454 (*14*), while a significant effect on Na^+ affinity

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[1] Abbreviations: EAAC1, excitatory amino acid carrier 1; CNS, central nervous system; ALS, amyotrophic lateral sclerosis; GltPh, glutamate/aspartate transporter from *Pyrococcus horikoshii*; TBOA, DL-*threo*-β-benzyloxyaspartate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MeS, methanesulfonate; NMG^+, *N*-methylglucamine$^+$; SLC, solute carrier.

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would be expected if this residue is a ligand of the bound Na^+ ion. Another highly conserved aspartate, D367 of EAAC1, was proposed to also be involved in sodium binding (*14*), but no bound cations were observed in the crystal structure near this residue (*13*).

Due to these discrepancies in interpretation of the cation effects from structural and functional studies, which may be caused by use of the nonphysiological Tl^+ ion in the GltPh studies, we here investigate in detail the effects of Tl^+ on the mammalian glutamate transporter EAAC1.

MATERIALS AND METHODS

Molecular Biology and Transient Expression. Wild-type EAAC1 cloned from rat retina was subcloned into pBK-CMV (Stratagene) as described previously (*15*) and was 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 laboratory, Department of Biochemistry at the University of Miami School of Medicine. The complete coding sequences of mutated EAAC1 clones were subsequently sequenced. Wild-type and mutant EAAC1 constructs were used for transient transfection of subconfluent human embryonic kidney cell (HEK293T/17, ATCC no. CRL 11268) cultures by use of FuGENE 6 transfection reagent (Roche) according to the supplied protocol. Electrophysiological recordings were performed between days 1 and 3 posttransfection.

Electrophysiology. EAAC1-mediated 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 MΩ; the series resistance was 5−8 MΩ. In general, EAAC1-mediated currents were small (typically <500 pA), so series resistance compensation had a negligible effect on the magnitude of the observed currents (<4% error). Therefore, series resistance was not compensated. For transport currents recorded in the presence of sodium, the extracellular solution contained 140 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, and 30 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.4; for transport current recording in the presence of Tl^+, the extracellular solution contained 20 mM (or otherwise indicated in text) TlF and 30 mM HEPES, pH 7.4 (Ca^{2+} and Mg^{2+} were not used, to avoid precipitation of their F^- salts; Ca^{2+} and Mg^{2+} have no effect on the function of EAAC1), adjusted to a total cation concentration of 140 mM with *N*-methylglucamine$^+$ (NMG^+). Two different pipet solutions were used: one solution contained 140 mM KCl, 2 mM $MgCl_2$, 10 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetracetic acid (EGTA), and 10 mM HEPES (pH 7.4/KOH); another one contained 140 mM TlF, 10 m EGTA, and 10 mM HEPES (pH 7.4/KOH). The latter solution was used to check whether Tl^+ can replace K^+ to mediate transport current.

For investigation of the effects of Tl^+ applied to the extracellular side, we used two methods to ensure outward-facing cation binding sites. In one method, intracellular K^+ was used to push the binding sites outward. In the second approach, we used high intracellular [Na^+] and [glutamate] (*16*), which pushed cation binding sites to outward-facing through reverse translocation. In this exchange mode (Na^+/glutamate homoexchange, or Na^+/glutamate/Tl^+ heteroexchange), the pipet solution contained 140 mM NaCl/NaSCN, 2 mM $MgCl_2$, 10 mM EGTA, 10 mM glutamate, and 10 mM HEPES (pH 7.4/NaOH). Thiocyanate was used because it enhances glutamate transporter-associated currents and allows detection of the EAAC1 anion-conducting mode (*17*). In the exchange mode, concentrations of glutamate are used on the intra- and extracellular sides that saturate their respective binding sites.

The currents were low-pass-filtered at 1−10 kHz (Krohn-Hite 3200), 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. Rapid solution exchange was performed as described previously (*15*). Briefly, substrates and/or cations (Na^+, Tl^+) were applied to the EAAC1-expressing cell by means of a quartz tube (opening diameter 350 μm) 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 30−50 ms (10−90%).

Data Analysis. Nonlinear regression fits of experimental data were performed with Origin (Microcal software, Northampton, MA) or Clampfit (pClamp8 software, Axon Instruments, Foster City, CA). Dose−response relationships of currents were fitted with a Michaelis−Menten-like equation, yielding K_m and I_{max}. Charge movement−voltage relationship is fitted to Boltzmann function, $Q(V_m) = Q_{min} + Q_{max}[1 + \exp(z_Q(V_Q - V_m)F/(RT))]^{-1}$ where F is the Faraday constant, R is the molar gas constant, and T is the temperature. V_Q is the midpoint potential of the charge movement, Q_{min} and Q_{max} are the minimum and maximum values of the charge movement, respectively, and z_Q is the apparent valence. Errors in kinetic parameters are given as standard deviation and were determined from at least four independent experiments from at least three different cells.

RESULTS

Tl^+ Interacts with EAAC1 with High Affinity. Application of Tl^+ to EAAC1-WT expressing cells (in the absence of glutamate) induced an anion current under conditions that favor outward-facing cation and substrate binding sites (140 mM NaSCN and 10 mM glutamate in the pipet solution; see Materials and Methods for details), as shown in Figure 1A. Only little current was observed upon Tl^+ application to nontransfected cells (Figure 1A, gray trace), indicating that the current is specific for EAAC1. The anion current was abolished when permeable anions were omitted from the intracellular solution. We have used this cation-induced anion current, which was previously also observed for Na^+ as the cation (*16, 18, 19*), as a tool to determine the apparent dissociation constant (K_{cation}) of the glutamate-free form of EAAC1-WT for Tl^+. As shown in Figure 1B, specific stationary anion currents increased with increasing Tl^+ concentration, saturating with an apparent K_{cation} of 1.8 ± 0.5 mM. EAAC1-specific anion currents were obtained by subtracting unspecific stationary currents, which were measured in control cells from the total currents (containing both specific and unspecific components) measured in EAAC-WT-transfected cells (Figure 1C, $n = 6$). Although a current

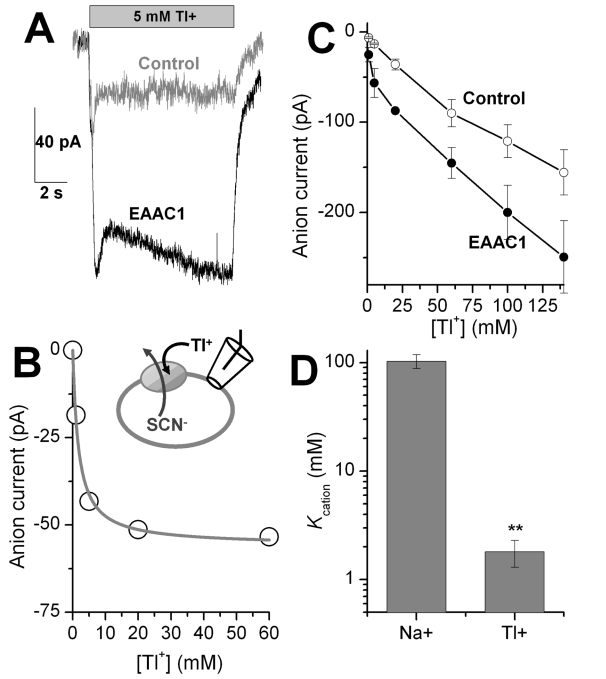

FIGURE 1: Tl^+ interacts with EAAC1 with higher affinity than sodium. (A) Typical traces of anion current (SCN^- efflux) induced by 5 mM Tl^+ in a control (nontransfected) cell (gray trace) and an EAAC1-WT expressing cell (black trace) in the absence of glutamate. (B) Tl^+ concentration dependence of specific anion currents carried by EAAC1-WT. The specific component of the current was determined by subtracting the Tl^+-induced stationary current measured in control (nontransfected) cells (○) from that measured in EAAC1-transfected cells (●), as shown in panel C. (D) Comparison of K_{cation} values determined for Tl^+ and Na^+ activation of anion current in the absence of glutamate. ** indicates statistical significance at the $p < 0.005$ level (Student's t test). All experiments were done under exchange conditions with 140 mM NaSCN and 10 mM glutamate in the pipet ($V = 0$ mV).

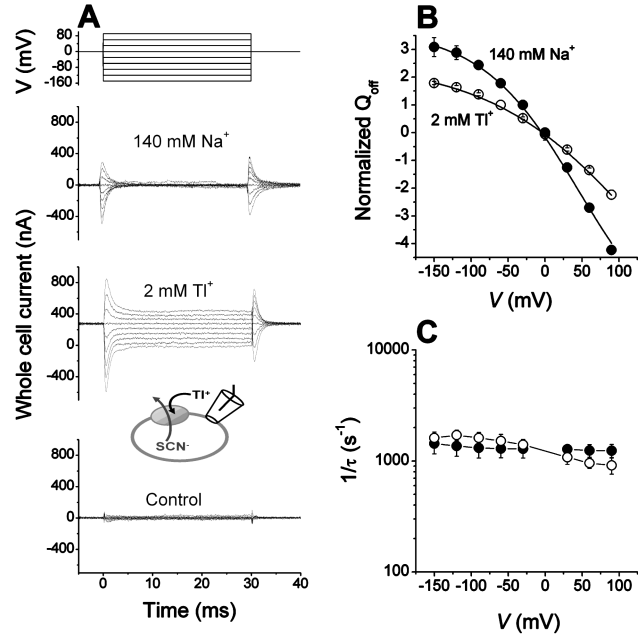

FIGURE 2: Tl^+ interaction with the glutamate transporter in the absence of glutamate is associated with transmembrane charge movement. (A) Time dependence of currents in response to voltage jumps (voltage protocol shown in the upper panel) in the presence of 140 mM Na^+ and 2 mM Tl^+. Control (nontransfected) cells did not respond to voltage jumps in the presence of Tl^+ (lower panel). The signals shown are the TBOA-sensitive components of the currents, obtained by subtraction of the signals in the absence and presence of TBOA (100 μM). (B) Voltage dependence of the normalized charge movement (obtained by integrating the transient component of the *off* current over time) in the presence of 140 mM Na^+ (●) and 2 mM Tl^+ (○, off charge movement). The lines represent calculations according to the Boltzmann equation with $z_Q = 0.38$ and $K_{cation} = 70$ mM at 140 mM Na^+ and $z_Q = 0.25$ and $K_{cation} = 0.6$ mM at 2 mM Tl^+. (C) Rate constants of the decay of the transient current (*on* current) induced by 2 mM Tl^+ (○) and 140 mM Na^+ (●). All experiments were done under exchange conditions with 140 mM NaMeS and 10 mM glutamate in the pipet. The low-pass filter used was 3 kHz, resulting in an effective time resolution of about 5 times the measured decay rates.

increasing almost linearly with [Tl^+] was also seen in control cells, the high-affinity component characteristic of the specific interaction of Tl^+ with EAAC1 was missing in the control-cell response (Figure 1C, ○). The exact origin of this unspecific current, which is most likely an anion current, was not determined here, but it could be induced either by the unspecific binding of Tl^+ to the membrane or by an unspecific effect of Tl^+ on EAAC1-independent anion conductances. Notably, Tl^+ interaction with EAAC1 is of much higher affinity than that with Na^+, with an apparent dissociation constant obtained from the sodium-induced anion current of 103 ± 14 mM (summarized in Figure 1D, $n = 4$), in agreement with previous results (*14*). Although Na^+ interacts with EAAC1 with lower affinity, the specific anion current (SCN^- in the pipet) induced by 140 mM Na^+, which is only about half saturating concentration, is −220 ± 10 pA ($n = 3$) which is about 2.5-fold larger than that induced by saturating Tl^+, indicating that the EAAC1−Tl^+ complex is less anion-conducting than the EAAC1−Na^+ complex.

Tl^+ Interaction with EAAC1 Is Associated with Charge Movement. A transient capacitive current was observed in response to voltage jumps applied to EAAC1-expressing cells in the presence of Na^+ and in the absence of glutamate (Figure 2A), consistent with previous reports (*20, 21*). This transient current was proposed to be caused by the electrogenic binding of sodium to the glutamate-free form of transporter (or a conformational change associated with it). In order to determine if the transporter displays the same behavior in the presence of Tl^+, we repeated the voltage jump experiment after replacing 140 mM Na^+ with 2 mM Tl^+. Permeable anions such as Cl^- or SCN^- were replaced with the impermeable anion methanesulfonate (MeS^-) to avoid contamination of the signal with anion current. Current traces specific for EAAC1 were obtained by subtracting currents recorded in the presence of TBOA from those in its absence (TBOA binds to EAAC1 in the presence of Tl^+, as discussed below). As shown in Figure 2A, transient currents were observed in addition to a steady-state current component, which was absent in the experiment performed in Na^+ solution and which was not further examined here. Both transient and steady-state currents were absent in nontransfected cells (Figure 2A, bottom panel).

The voltage dependence of the voltage jump-induced charge movement, Q, which was obtained by integrating the transient current over time, is shown in Figure 2B ($n = 5$). At 2 mM Tl^+, a concentration close to the K_{cation} for Tl^+, the charge movement starts saturating at very negative potential, but it is far from saturating at positive potentials, making a full Boltzmann-type analysis from fitting the data difficult. However, the data are consistent with a Boltzmann curve with the parameters $z_Q = 0.25$ and $K_{cation} = 0.6$ mM

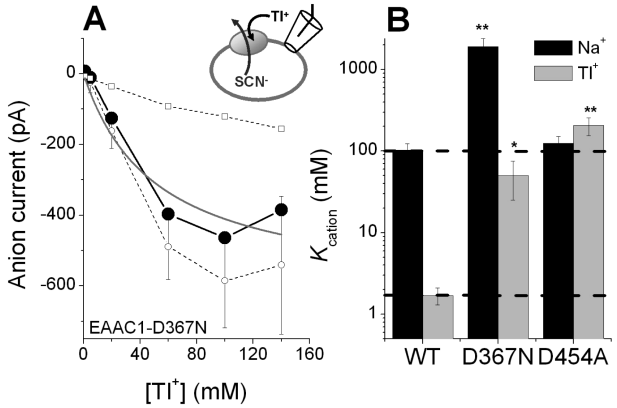

FIGURE 3: Differential effect of D367N and D454A mutations on affinities for Na^+ and Tl^+. (A) Tl^+ concentration dependence of specific anion currents in EAAC1-D367N-expressing cells (●). The specific component of the current was determined by subtracting the Tl^+-induced current measured in control (nontransfected) cells (□) from that measured in EAAC1-transfected cells (○). The solid line represents a calculation according to the Michaelis−Menten equation with $K_{cation} = 50$ mM. (B) Comparison of K_{cation} values obtained for Na^+ (black bars) and Tl^+ (gray bars) activation of anion current in EAAC1-WT and the two mutant transporters. ** and * indicate statistical significance at the $p < 0.005$ and 0.05 levels, respectively. All experiments were done under exchange conditions with 140 mM NaSCN and 10 mM glutamate in the pipet ($V = 0$ mV).

for Tl^+ association with EAAC1, as shown by the solid line in Figure 2B. For Na^+ as the cation, the data are consistent with previous reports (*20, 21*) with $z_Q = 0.38$ and $K_{cation} = 70$ mM. K_{cation} for Tl^+ is 3 times smaller than that obtained from the Tl^+ concentration jump experiment discussed above. This difference is not significant, mainly because of the lack of saturation of the charge movement at positive potentials, which leads to increased uncertainty of the parameters obtained from the Boltzmann analysis. With that said, comparison of both these two K_{cation} values for Tl^+ with the value for Na^+ indicates clearly that the affinity of EAAC1 for Tl^+ is much higher than that for Na^+.

At 2 mM Tl^+, the rate constants of the decay of the transient currents were 1700 ± 100 s^{-1} (Figure 2C, $n = 6$). Like in Na^+ (*16*), these rate constants were virtually voltage-independent (Figure 2C, $n = 6$).

Mutations of Two Conserved Acidic Amino Acid Residues Have Differential Effects on Tl^+ and Na^+ Affinity. It was previously shown that neutralization of D367, which is highly conserved among all members of the solute carrier 1 (SLC1) family, and which is located in the middle of transmembrane domain 7 (TM7), results in a dramatically decreased affinity of the transporter for Na^+, suggesting potential involvement of this residue in cation binding (*14*). Therefore, we performed the analogous concentration jump experiments with Tl^+ as the cation, in order to determine whether mutation of this residue has the same effect on Tl^+ affinity of the glutamate-free form of the transporter. As shown in Figure 3A, application of Tl^+ to an EAAC1-D367N-expressing cell induced [Tl^+]-dependent inward anion currents (SCN^- outflow). This inward anion current saturated at Tl^+ concentrations higher than 60 mM (Figure 3A, ●). The Tl^+-induced currents in EAAC1-D367N were significantly larger than those observed in control cells (Figure 3A, □). They were also much larger than the currents induced by Tl^+ in EAAC1-WT (60 mM Tl^+-induced anion current in EAAC1-WT was -53 ± 33 pA; 60 mM Tl^+-induced anion current in EAAC1-D367N was -400 ± 90 pA, which is 7-fold larger than that of wild type; see also Figure 1), consistent with previous results that showed larger Na^+-induced anion currents in EAAC1-D367N than in the wild-type transporter (*14*). The current−[Tl^+] relationship can be fitted with a Hill equation with $n = 3.6$ and $K_{cation} = 25 \pm 5$ mM. The Hill coefficient of $n = 3.6$ is somewhat unexpected, because there is only one Tl^+ binding site observed in the substrate-free form of GltPh. Therefore, and because Na^+ activates anion current in EAAC1-WT without apparent cooperativity, a Hill coefficient of $n = 1$ was expected. This deviation from $n = 1$ indicates that substrate-free EAAC1 may have more than one Tl^+ binding site. In any case, it is clear from comparison with the EAAC1-WT data (Figure 1) that Tl^+ induced anion current with a significantly lower affinity, which we estimated to be in the range between 25 and 50 mM (see solid line in Figure 3A calculated with $n = 1$, $K_{cation} = 50 \pm 25$ mM). Therefore, the affinity of the glutamate-free form of EAAC1-D367N for Tl^+ is decreased at least 14-fold compared to wild-type EAAC1, which is consistent with the 15-fold effect of the mutation on Na^+ affinity. To summarize, the D367N mutation has an equivalent inhibitory effect on both Na^+ and Tl^+ interaction with EAAC1.

A second acidic amino acid residue, D454, is also highly conserved among all SLC1 members and is located in the central region of TM8. In contrast to D367N, neutralization of this aspartate by mutation to asparagine had little effect on the sodium affinity of the glutamate-free form of the transporter (*14*). To further test this finding, we determined the Na^+ affinity of a transporter with a less conservative mutation, D454A, which in contrast to D454N has no side-chain oxygen that could potentially contribute a ligand to cation coordination. A K_{cation} value of about 130 mM was found (Figure 3B, black bars), confirming our previous results that showed minor effects of side-chain neutralization in this position on cation affinity. Next, we studied the effect of the D454A mutation on Tl^+ binding. In contrast to the results obtained for Na^+, K_{cation} for Tl^+ was dramatically reduced by the D454A mutation ($K_{cation} \sim 210$ mM; Figure 3B, gray bars), which is about 110-fold higher than that of wild-type EAAC1. All the mutagenesis results are summarized in Figure 3B. They suggest that mutations in position D454 have an inhibitory effect on Tl^+ interactions with the transporter, consistent with a recent study on GltPh (*13*), but only a minor inhibitory effect, if any, on Na^+ interaction.

Amino Acid Substrate Binds to EAAC1 in the Presence of Tl^+. Under exchange conditions (with SCN^- in the pipet), application of glutamate induced an anion current when Tl^+ was the only extracellular cation present (Figure 4A). As expected, this glutamate-induced anion current was highly voltage-dependent, showing the typical $I-V$ profile previously observed in the presence of Na^+ for SCN^- outflow, with higher electrical driving force resulting in larger inward currents (Figure 4C). The currents were also dependent on glutamate concentration (Figure 4B). The K_m for glutamate at 20 mM Tl^+ was 33 ± 6 mM, which is about 2000 times higher than that at 140 mM Na^+. These results demonstrate that glutamate can bind to the Tl^+-bound form of EAAC1 but only with low affinity.

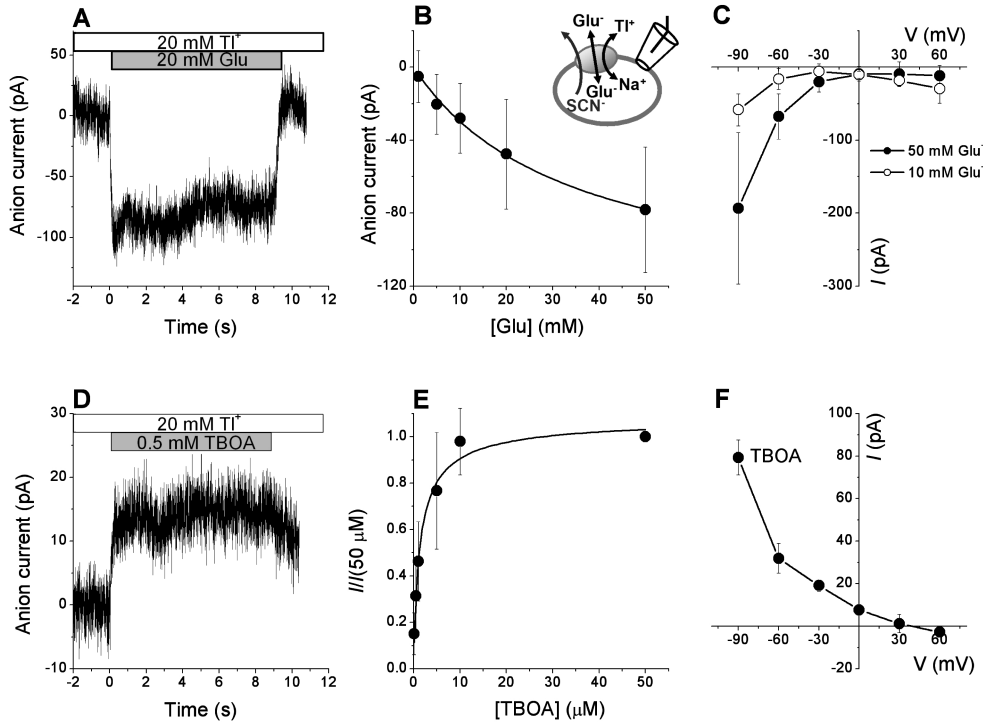

FIGURE 4: Amino acid substrates interact with the Tl^+-bound transporter. (A) Typical anion current induced by 20 mM glutamate at 20 mM Tl^+, $V = -60$ mV. (B) Determining the apparent dissociation constant (K_m) of Tl^+-bound EAAC1 for glutamate. Based on a fit of the Michaelis−Menten equation to the data (solid line), the K_m of EAAC1 for glutamate is 34 ± 6 mM at 20 mM Tl^+. (C) Voltage dependence of glutamate-induced anion currents at 20 mM Tl^+ and 10 mM glutamate (○) and 50 mM glutamate (●). (D) TBOA inhibits the leak anion current in the presence of 20 mM Tl^+ ($V = -60$ mV). (E) Determining the apparent dissociation constant (K_m) of EAAC1 for TBOA at 20 mM Tl^+ ($K_m = 1.4 \pm 0.4$ μM, $V = 0$ mV). (F) Voltage dependence of 500 μM TBOA-induced anion current at 20 mM Tl^+. All experiments were performed under exchange conditions with 140 mM NaSCN and 10 mM glutamate in the pipet.

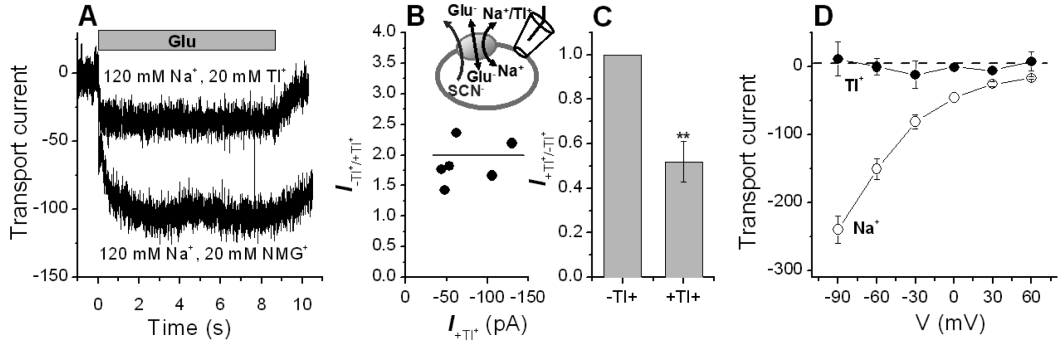

FIGURE 5: Tl^+ inhibits Na^+-mediated glutamate transport but does not support glutamate transport current. (A) Typical transport currents induced by 0.1 mM glutamate at 120 mM Na^+ and 20 mM Tl^+ (upper trace) or 120 mM Na^+ and 20 mM NMG^+ (lower trace). (B) Relative inhibition of the transport current by Tl^+ is independent of the magnitude of the expression level (5 cells). (C) Quantification and statistical analysis of the transport current inhibition (** indicates significance at the $p < 0.005$ level). (D) Voltage−transport current relationships at 140 mM Tl^+ (●, 20 mM glutamate) and at 140 mM Na^+ (○, 0.1 mM glutamate). All experiments were done in the forward transport mode with 140 mM KMeS in the pipet.

Next, we tested the effect of TBOA in the presence of Tl^+. Application of 0.5 mM TBOA to EAAC1-WT at 20 mM Tl^+ induced an apparent outward current (Figure 4D). This current was strongly voltage-dependent, with a larger current observed at negative membrane potentials (Figure 4F). Consistent with previous results obtained in the presence of Na^+ (*15*), these data indicate that the outward current is due to inhibition of the leak outflow of SCN^- induced by the sole presence of Tl^+ (see also Figure 1). By measuring the current at different TBOA concentrations (Figure 4E), the apparent inhibition constant (K_i) of EAAC1-WT for TBOA was determined as 1.4 ± 0.4 μM at 20 mM Tl^+, which is similar to that at 140 mM Na^+ (1.4 ± 0.3 μM). On the basis of the data mentioned above, TBOA binding is almost unaffected by the replacement of Na^+ with Tl^+ in extracellular solution, while glutamate binding is strongly affected.

Tl^+ Inhibits Na^+-Dependent Transport Current. Next, we checked the effect of Tl^+ on Na^+-dependent transport current when it coexists with Na^+ in the extracellular solution. Transport current induced by 1 mM glutamate was measured under forward transport conditions with KMeS in the pipet and at 120 mM extracellular Na^+ (Figure 5A; 20 mM NMG^+ was added to adjust the total cation concentration to 140 mM). In the absence of Tl^+, the transport current induced by 1 mM glutamate at 0 mV was 130 ± 30 pA. After 20 mM NMG^+ was replaced with 20 mM Tl^+, the current was 60 ± 37 pA, which is less than half that with NMG^+ in

solution (Figure 5A). The relative inhibition of this current was independent of the absolute size of the transport current (Figure 5B) and is quantified in Figure 5C.

When glutamate was applied to the cells in the presence of 20 mM Tl^+ but in the absence of extracellular Na^+, no transport currents were observed, while Na^+ catalyzed large transport currents in the same cells (Figure 5D). Together, these results suggest that Tl^+ inhibits Na^+-dependent transport current but that it cannot support glutamate transport by itself.

Tl^+ Can Replace Intracellular Potassium to Mediate Transport Current. So far, our data indicate that Tl^+ mediates effects similar to Na^+ when present in the extracellular solution but that it cannot replace sodium to mediate transport current. Because Tl^+ is traditionally viewed as a K^+ congener (*22*), due to its size, we wanted to know whether Tl^+ can replace potassium in the intracellular solution in catalyzing the cation-driven transporter relocation and, ultimately, to mediate transport current. Whole-cell transport current induced by 0.1 mM extracellular glutamate was recorded at 140 mM extracellular Na^+ and 140 mM intracellular Tl^+ (without K^+). The extracellular anion was MeS^- and the intracellular anion was F^-, both of which do not permeate the anion conductance (*16, 17*). At 0 mV the transport current was small but inward-directed (Figure 6A), as expected for the forward transport ionic conditions used in the experiment. No current was observed under the same conditions in nontransfected cells (Figure 6A,B). With an increased electrical driving force at −60 mV membrane potential, the steady-state current in the presence of Tl^+ was -25 ± 7 pA ($n = 5$), which is significant over background but smaller than the K^+-induced current of -170 ± 45 pA (Figure 6B, $n = 6$). The transport current−voltage relationship is shown in Figure 6C and shows exponential increase of the current with increasingly negative membrane potential. The Tl^+-dependent transport current is specific for EAAC1, as it can be blocked by coapplication of extracellular TBOA (Figure 6B).

To further test the ability of Tl^+ to induce the cation-induced relocation reaction, we determined whether anion current is induced by extracellular glutamate application in the presence of intracellular Tl^+ (forward transport conditions). It was shown previously that steady-state anion current is observed only when an intracellular cation is present that supports relocation, such as K^+, whereas intracellular choline$^+$ did not support anion current (*15*). Consistent with this result, anion current was induced by extracellular glutamate in EAAC1-expressing cells with Tl^+ as the sole intracellular cation (25 ± 7 pA, Figure 7A), whereas no such currents were seen in nontransfected cells. This anion current was outward, as expected, as it is caused by SCN^- inflow. Voltage dependence of the current is shown in Figure 7B and is consistent with an anion current, as no reversal of the current was observed, even at very negative potentials (infinite inward-directed SCN^- concentration gradient). Together with the results on Tl^+-induced transport current, these data suggest that Tl^+ can replace K^+ to catalyze the cation-dependent relocation reaction of glutamate transporters, consistent with the predicted role of Tl^+ as a K^+ congener.

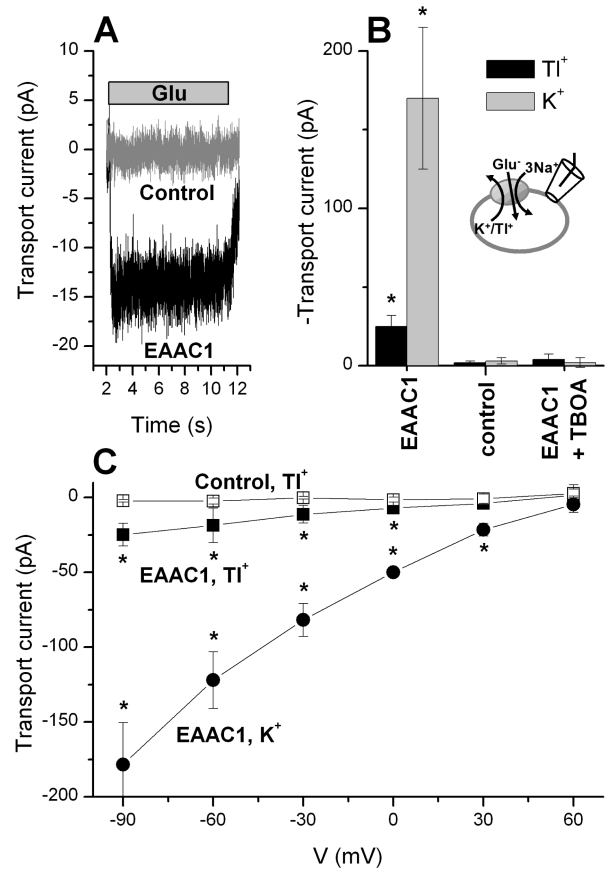

FIGURE 6: Intracellular Tl^+ supports glutamate transport. (A) Typical transport current generated by application of 1 mM glutamate (bar) in the presence of 140 mM intracellular Tl^+ in EAAC1-expressing and nontransfected control cells ($V = 0$ mV). The counteranion was fluoride, which does not generate anion current. (B) Quantification of transport currents in the presence of intracellular K^+ (gray bars) and Tl^+ (black bars) and their inhibition by 100 μM TBOA. (C) Voltage dependence of glutamate-induced transport currents in the presence of intracellular K^+ (●) and Tl^+ in EAAC1-expressing (■) and control cells (□). * indicates statistical significance with respect to control at the $p < 0.05$ level, as determined by two-way ANOVA.

DISCUSSION

In this paper, we studied the effects of thallium ions on the functional properties of the mammalian glutamate transporter EAAC1. The major conclusion is that Tl^+, which is typically used as an analogue of potassium with higher atomic weight (*22*), can replace both K^+ and Na^+ in EAAC1 and can mediate partial function (by replacing Na^+) or full function (by replacing potassium) of this protein.

Tl^+ Can Replace Intracellular Potassium to Mediate Transport Current. When Tl^+ was used to replace potassium at the intracellular side of the membrane, a small but significant voltage-dependent inward current was observed, which was most likely mediated by electrogenic inward glutamate transport coupled to the countertransport of Tl^+. Although this transport current, which was inhibited by the competitive inhibitor TBOA, was about 7-fold less than when potassium was used to drive countertransport, the data indicate that Tl^+ can replace intracellular K^+ to support the cation-dependent relocation process and, thus, transport current. This result is in line with previous reports on the ability of Tl^+ to act as a K^+ congener in transport proteins, most notably in the Na/K-ATPase (*22, 23*). Moreover, the

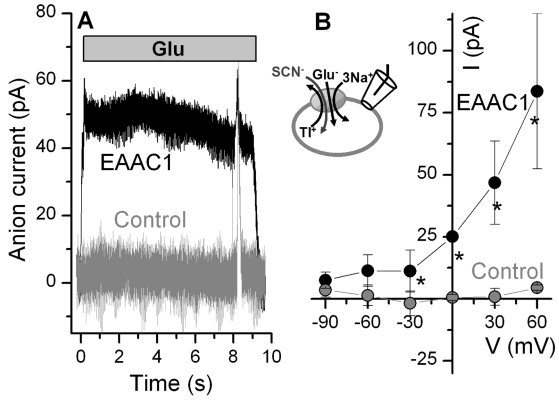

FIGURE 7: Extracellular glutamate induces anion current in the presence of intracellular Tl^+. (A) Typical anion current recordings (SCN^- influx) from EAAC1-transfected and control (nontransfected) cells in the presence of 1 mM glutamate (application time indicated by bar) and 140 mM intracellular Tl^+ ($V = 0$ mV). (B) Voltage dependence of glutamate-induced anion currents. The experiments were done under forward transport conditions, as illustrated in the inset. * indicates statistical significance with respect to control at the $p < 0.05$ level, as determined by two-way ANOVA.

ionic radii of Tl^+ and K^+ are very similar to each other ($r =$ 1.40 Å for Tl^+, $r = 1.33$Å for K^+), supporting the hypothesis of a specific interaction of Tl^+ with the EAAC1 K^+ binding site. However, Tl^+ is less efficient than potassium in driving relocation, resulting in a significantly reduced turnover rate in the presence of Tl^+. In previous work by Jahr and coworkers (*24*), Cs^+ was found to be able to replace intracellular K^+ and support glutamate transport but at a much lower rate. Together with our data on Tl^+ interaction with the glutamate-free transporter, these results indicate that the cation specificity of the K^+ binding site of glutamate transporters is low. This is not unusual for transport proteins, as the serotonin transporter, which belongs to the SLC6 family, is also potassium-dependent, but the function of potassium can be replaced by a proton (*25*).

Tl^+ Interaction with the Glutamate-Free Form of EAAC1 Resembles That of Na^+ but Is of Higher Affinity. When applied to the extracellular side of the membrane, Tl^+ induces functional behavior in EAAC1 that resembles that of the Na^+-bound transporter. However, despite some similarities in function, there are also differences in the properties. First, Tl^+ binds with about 50-fold higher affinity to EAAC1 than Na^+. Second, the amplitude of the anion current induced by a nearly saturating concentration of Tl^+ (20 mM) is much smaller than that induced by a half-saturating concentration of Na^+ (140 mM). The exact reason is unknown, but three possibilities (separate from each other or in combination) can be considered: (1) The conformations of EAAC1 induced by Tl^+ binding or Na^+ binding are different, raising the possibility that the pathways for the anion in these two cases are different. (2) The bigger bound Tl^+ ion obstructs the anion-conducting pathway. (3) Tl^+ is in the anion pathway and is involved in binding of the anion, but due to its different ionic properties the anion binding is altered. Although our data do not allow distinction among these possibilities, it has to be considered that, compared to Na^+, Tl^+ has a bigger ionic radius ($r = 0.95$ Å for Na^+) and is a highly polarizable ion. Thus, differential function of the Tl^+-and Na^+-bound transporters is highly plausible.

Involvement of D367 and D454 Residues in Cation Binding. Neutralization of the highly conserved acidic amino acid residue D367 by mutation to asparagine (D367N) has been shown to decrease the affinity of Na^+ for the glutamate-free form of EAAC1 (*14*). Therefore, it was hypothesized that D367 contributes, directly or indirectly, to a cation binding site, which is deeply buried in the transmembrane domain of the transporter. Here, we show that the affinity of Tl^+ for the glutamate-free form of transporter is also decreased by D to N mutation in position D367, by about the same factor as Na^+ affinity is decreased. This result indicates that D367 in EAAC1 is involved in both Na^+ and Tl^+ binding. In the crystal structure of GltPh (*13*), no electron density assignable to a bound Tl^+ ion was observed near D312 (D367 in EAAC1). This could mean that the effect of the D367 mutation on cation binding is indirect. Two other possibilities have to be considered: (1) On the basis of the proposed stoichiometry of GltPh, two sodium ions (instead of three in EAAC1) bind to the transporter. Therefore, one cation binding site is not conserved between the two proteins. While D367 may contribute to this nonconserved binding site in EAAC1, this site is not present in GltPh. (2) D312 of GltPh may contribute to a third sodium binding in GltPh, but the affinity of this binding site for thallium ions is so low that no Tl^+ can be observed in the crystal structure. While further studies will be necessary to resolve this issue, the results presented here are compatible with the hypothesis of an additional cation binding site near D367 that is not observed in GltPh.

In the GltPh crystal structure (*13*), a Tl^+ ion was observed with the side chain of D405 as one of the ligands (D454 in EAAC1). Electron density from this bound Tl^+, as well as Na^+ binding to one of the two cation binding sites, was reduced when D405 was mutated to asparagine, indicating a destabilization of the bound cation by the mutation of the Na1 site of GltPh. In our previous work (*14*), the analogous D454N in EAAC1 mutation was shown to have little effect on sodium binding affinity of the glutamate-free form of the transporter, making a direct contribution to Na^+ binding unlikely. Here, we studied another, less conservative mutant in the same position, D454A. While EAAC1-D454N had unchanged affinity for Na^+ compared to the wild-type transporter, the D454A mutation had a moderate effect on Na^+ affinity (less than 3-fold decrease in affinity). However, a dramatic effect on Tl^+ affinity was observed for the D454A mutation (about 100-fold reduction in affinity). These data strongly indicate that, like D405 in GltPh, D454 in EAAC1 contributes a ligand to a Tl^+ binding site. However, the relatively small effect of the mutations on Na^+ binding is more ambiguous and argues against an important contribution of the EAAC1 cation binding site analogous to the GltPh Na1 site to Na^+ binding. Therefore, the possibility has to be considered that the GltPh Na1 site is predominantly a potassium binding site in the mammalian transporters.

Tl^+-Bound Transporter Binds TBOA and Glutamate but Does Not Support Glutamate Transport. Our data show that, like Na^+, Tl^+ supports binding of the competitive inhibitor TBOA. The affinity of EAAC1 for TBOA is similar in the Tl^+-bound and in the Na^+-bound transporter. In contrast, glutamate binding to the transporter, while still possible in the presence of Tl^+, occurs with dramatically reduced affinity in comparison to Na^+ binding. This result is consistent with

data on aspartate affinity of GltPh, which is about 100-fold higher in the presence of Na^+ than in the presence of Tl^+ (*13*). Therefore, it appears that the nature of the bound cation has a strong impact on the affinity and, most likely, the geometry of the substrate binding site. In a previous study it was shown that the nature of the amino acid substrate affects the affinity of the transporter for Na^+ (*21*). Moreover, it was demonstrated that the affinity for the amino acid substrate is different for Na^+ and Li^+-bound transporters (*26*). Our data on reduced glutamate interaction with the Tl^+-bound transporter add to the results from these reports, indicating that the bound amino acid substrate and the cation (presumably in the Na2 site) interact with each other.

In contrast to extracellular Na^+, extracellular Tl^+ cannot support glutamate transport, even when Tl^+ is present at saturating concentrations. This result is consistent with data published on aspartate transport by GltPh, which is also inhibited in the presence of Tl^+ (*13*). This suggests that the conformational changes most likely associated with substrate translocation and transport are dramatically slower in the Tl^+-bound form or that they do not occur at all.

In conclusion, our data demonstrate that Tl^+ can interact with the mammalian glutamate transporter EAAC1 with high affinity. When applied intracellularly, it can replace K^+ to mediate substrate transport. In contrast, Tl^+ is a less efficient congener for Na^+, which it can replace to mediate cation-dependent and glutamate-dependent anion currents but which it cannot replace to mediate glutamate transport. Two mutations to conserved amino acid residues potentially contributing to cation sites (D367N and D454A) mediate differential effects on Tl^+ and Na^+ affinity. The mutagenesis results indicate that while D367 is important for general cation affinity, D454 may contribute to coordinating Tl^+ (and possibly K^+), but not Na^+. Overall, our data highlight the conserved nature of the Tl^+ effect on bacterial and mammalian glutamate transporters but also show differences due to the ability of the mammalian transporters to transport potassium, which can be efficiently replaced by Tl^+.

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