A K⁺/Na⁺ co-binding state: Simultaneous versus competitive binding of K⁺ and Na⁺ to glutamate transporters

Plasma membrane–associated glutamate transporters play a key role in signaling by the major excitatory neurotransmitter glutamate. Uphill glutamate uptake into cells is energetically driven by coupling to co-transport of three Na⁺ ions. In exchange, one K⁺ ion is counter-transported. Currently accepted transport mechanisms assume that Na⁺ and K⁺ effects are exclusive, resulting from competition of these cations at the binding level. Here, we used electrophysiological analysis to test the effects of K⁺ and Na⁺ on neuronal glutamate transporter excitatory amino acid carrier 1 (EAAC1; the rat homologue of human excitatory amino acid transporter 3 (EAAT3)). Unexpectedly, extracellular K⁺ application to EAAC1 induced anion current, but only in the presence of Na⁺. This result could be explained with a K⁺/Na⁺ co-binding state in which the two cations simultaneously bind to the transporter. We obtained further evidence for this co-binding state, and its anion conductance, by analyzing transient currents when Na⁺ was exchanged for K⁺ and effects of the [K⁺]/[Na⁺] ratio on glutamate affinity. Interestingly, we observed the K⁺/Na⁺ co-binding state not only in EAAC1 but also in the subtypes EAAT1 and -2, which, unlike EAAC1, conducted anions in response to K⁺ only. We incorporated these experimental findings in a revised transport mechanism, including the K⁺/Na⁺ co-binding state and the ability of K⁺ to activate anion current. Overall, these results suggest that differentiation between Na⁺ and K⁺ does not occur at the binding level but is conferred by coupling of cation binding to conformational changes. These findings have implications also for other exchangers.

Plasma membrane glutamate transporters from the solute carrier 1 (SLC1) family take up glutamate into cells against a concentration gradient (1–3). Glutamate uptake is energetically coupled to co-transport of three sodium ions and one proton in exchange for one K⁺ ion (1, 3). Because glutamate is thought to be transported in the negatively charged form (3–5), this stoichiometry predicts that two positive charges move into the cell with each glutamate molecule, giving rise to a coupled transport current (5, 6). Consistent with this prediction, transport current induced by glutamate application to glutamate transporter (excitatory amino acid transporter (EAAT))–expressing cells has been observed in many reports (7–10).

In previously proposed transport mechanisms, K⁺ binds to the transporter from the intracellular side after glutamate and Na⁺ have dissociated to the cytoplasm (2, 3, 11–13). After K⁺ binding, a conformational change takes place, relocating the glutamate-binding site to the extracellular side. This relocation reaction is independent of the Na⁺/glutamate translocation step (3), as is typical in exchange-type transporters. K⁺ binding from the extracellular face of the membrane induces relocation in the opposite direction, facilitating glutamate release by reverse transport (14, 15). Because Na⁺ and K⁺ have opposing effects, it was assumed that binding of these two cations is competitive (3, 16), with Na⁺ binding favoring glutamate binding and translocation and K⁺ binding favoring relocation of the glutamate-free transporter.

In addition to the coupled transport current, other current components have been observed in several EAAT subtypes. For example, a current carried by anions was activated by glutamate (1, 5) application to the transporter. This current is kinetically, but not thermodynamically, coupled to glutamate transport (1). The current hypothesis is that anion current is associated with distinct states that are populated by the transporter as it transitions through the transport cycle (1, 2, 8, 17). Pathways of anion permeation have been suggested in several reports (8, 18–21) based on molecular dynamics simulations and site-directed mutagenesis. It is assumed that an intermediate state along the translocation pathway plays a major role in anion permeation (21).

Although anion current is the most pronounced in the presence of transported substrates, smaller anion current is also observed without substrate (1). This anion current component was termed the leak anion current. Although the leak anion current requires the presence of sodium ions in the solution for the neuronal subtypes EAAT3 (8) and -4 (22), it is sodium-independent for the glial subtypes EAAT1 (23) and -2 (24). This finding suggests that subtle differences exist among the members of the SLC1 family with respect to the mechanism of anion conductance activation.

In most previous reports on kinetic modeling of the glutamate transporter anion conductance (23), it was assumed that...
the empty transporter (no Na\(^+\) and/or glutamate bound) as well as the K\(^+\)-bound transporter is unable to catalyze anion flow across the membrane (3, 16, 21). However, to our knowledge, the effects of K\(^+\) on anion conductance activation have not been explicitly experimentally tested. Here, we measured anion current in the presence of varying concentrations of extracellular Na\(^+\) and K\(^+\) ions for the neuronal glutamate transporter excitatory amino acid carrier 1 (EAAC1; the rat homologue of human EAAT3). Interestingly, our data show that external K\(^+\) is able to activate the glutamate transporter anion conductance, but only when extracellular Na\(^+\) is also present. These results suggest the existence of a state in which Na\(^+\) and K\(^+\) can bind simultaneously to the transporter, in contrast to previous hypotheses of these two cations acting in a competitive fashion (23). A kinetic model to explain the data includes a novel “co-binding” state, suggesting that at least one cation-binding site is poorly selective for Na\(^+\) versus K\(^+\).

Results

Anion conductance is activated by extracellular glutamate and Na\(^+\) but not by K\(^+\) in the reverse transport mode

To characterize anion-conducting properties of EAAC1, we first applied glutamate to the transporter in the forward transport mode in the presence of intracellular thiocyanate (SCN\(^-\)), an anion that was previously shown to be highly permeable. Whole-cell currents were measured from the EAAC1 transporter transiently expressed in HEK293 cells. As expected from previous results, large inward current was generated by glutamate application under these conditions (5) (data not shown). The whole-cell current was dominated by anion current because transport current (in the absence of intracellular SCN\(^-\)) was at least an order of magnitude smaller than anion current (22). Large anion currents were also seen under homoechange conditions when the intracellular solution contained saturating concentrations of Na\(^+\) and glutamate as well as SCN\(^-\) (Fig. 1A). Under these ionic conditions, no steady-state transport current is present (5, 8, 25, 26), and the current is purely carried by the anion conductance.

Under the same ionic conditions, whole-cell current was also observed after extracellular application of Na\(^+\), which is known to activate the leak anion conductance in EAAC1 (Fig. 1B and Refs. 17, 22, and 25). Finally, we measured currents under reverse transport conditions, upon application of extracellular K\(^+\), with saturating Na\(^+\) and glutamate internal concentrations (Fig. 1C). Currents recorded under these conditions were small and outwardly directed, indicating that they were caused by electrogenic reverse transport of glutamate but not by anion movement across the membrane.

Extracellular K\(^+\) and Na\(^+\) can bind simultaneously to the transporter to form a co-binding state

In the currently established transport mechanisms, the counter-transported K\(^+\) ion competes with co-transported Na\(^+\) for binding to the transporter (3, 5, 23, 26), i.e., either K\(^+\) binds, inducing relocation, or Na\(^+\) binds, inducing glutamate binding and translocation, but K\(^+\) and Na\(^+\) cannot bind at the same time. To test this hypothesis, external K\(^+\) was applied to the transporter in the presence of varying concentrations of extracellular Na\(^+\) (Fig. 2). If binding of the two cations were competitive, it would be expected that K\(^+\) inhibits the Na\(^+\) effect, which was measured using the Na\(^+\)-induced anion leak current. In contrast to this expectation, K\(^+\) enhanced the current at low concentrations (inward current in Fig. 2A, blue and red traces) but inhibited the current at high concentrations (inhibition of tonic inward current in Fig. 2A, green trace), with the largest anion current being observed at an intermediate [K\(^+\)] of 20 mM and [Na\(^+\)] of 120 mM (Fig. 2A, red trace). These results
are quantified in Fig. 2B, demonstrating a biphasic current 
\([\text{K}^+] / [\text{Na}^+]\) relationship. This biphasic relationship cannot be 
explained with competitive \([\text{K}^+] / [\text{Na}^+]\) binding models, illustrated in Fig. 2D (mechanisms 1 and 2). A calculation of predicted anion current according to competitive mechanisms, including either one \(\text{Na}^+\) binding step (simplified model) or two \(\text{Na}^+\) binding steps, is shown in Fig. 2C, red line, demonstrating purely inhibitory behavior of external \(\text{K}^+\). In contrast, the experimental data can be explained very well by including a \(\text{Na}^+ / \text{K}^+\) co-binding state (TKNa; Fig. 2, C and D, mechanisms

Glutamate transporter \(\text{K}^+ / \text{Na}^+\) co-binding state

Figure 2. Sodium and potassium can bind simultaneously to the glutamate transporter. A, anion current evoked by solution exchange from 140 mM Na\(^+\) to a combination of \(\text{K}^+ / \text{Na}^+\) concentrations indicated in the figure (bars on top). The intracellular solution contained 130 mM NaSCN and 10 mM glutamate. The transmembrane potential was \(V = 0\) mV. B, \([\text{K}^+] / [\text{Na}^+]\) dose dependence of the anion current shows biphasic current response, indicating the existence of a co-binding state with high anion permeability at intermediate \([\text{K}^+] / [\text{Na}^+]\) ratios. C, calculations of the current versus \([\text{K}^+] / [\text{Na}^+]\) relationship, based on two competitive binding models (red) and simultaneous (co-binding) models (black or blue for inclusion of TNa\(_2\)K state), illustrated in D. Here, T denotes the transporter in the apo form, which can either bind \(\text{Na}^+\) (TNa), \(\text{K}^+\) (TK), or both cations at the same time (TKNa and TNa\(_2\)K). The equations for competitive and simultaneous binding are listed under “Experimental procedures.” The simplified models contain only one \(\text{Na}^+\) binding step, whereas the more realistic binding models contain two \(\text{Na}^+\) binding steps. E, \([\text{K}^+] / [\text{Na}^+]\) dose dependence of the anion current shows biphasic current response. In contrast to the data shown in Fig. 2B, no glutamate was included in the pipette solution (see inset for ionic conditions). The voltage was 0 mV. F, in the presence of intracellular NMG-NO\(_3\) (130 mM; NMG is an inert cation with respect to EAAC1 binding), very little current was observed, and this current was independent of the \([\text{K}^+] / [\text{Na}^+]\) dose. Error bars represent S.D.
3 and 4, containing either one or two Na\(^+\) binding steps) in which K\(^+\) and Na\(^+\) can bind simultaneously to the transporter. From these simulations, we were able to estimate the relative anion current level of these three cation-bound states as TNa:TK:TNaK = 1.1:0.3 (1.8 for the TNa,K state; blue line).

**Population of the co-binding state does not require cycling of the transporter**

The possibility has to be considered that the reduction of the current at high [K\(^+\)]/[Na\(^+\)] ratios is caused by activation of reverse transport current, which is outwardly directed, thus canceling out the inwardly directed anion current. To test this possibility, we repeated the experiments shown in Fig. 2, A and B, but in the absence of intracellular glutamate. Under these ionic conditions, no reverse transport current can be activated, and the only observed current should be caused by the anion conductance. As shown in Fig. 2E, the anion current versus [K\(^+\)]/[Na\(^+\)] ratio relationship showed the same general behavior as in the presence of intracellular glutamate, with a maximum anion current observed at a ratio of 20 mM [K\(^+\)]/120 mM [Na\(^+\)] (Fig. 2E). In addition, very little current was observed in the absence of intracellular Na\(^+\) or K\(^+\) (replaced by NMG\(^-\); Fig. 2F), demonstrating that the current requires intracellular cations that interact with the transporter. These results suggest that cycling of the transporter in the reverse transport direction is not required for population of the co-binding state.

**The current induced by the Na\(^+\)/K\(^+\) co-binding state is carried by anions**

To determine the ionic basis of the current induced by the Na\(^+\)/K\(^+\) co-binding state, we determined the voltage dependence of the current under three experimental conditions to restrict the transporter to Na\(^+\)-, K\(^+\)/Na\(^+\)-, or K\(^+\)-bound states. For this purpose, 140 mM [Na\(^+\)], 120/20 mM [Na\(^+\)]/[K\(^+\)] (co-binding state), or 1/139 mM [Na\(^+\)]/[K\(^+\)] were applied in the external solution to the transporter in the presence of intracellular SCN\(^-\). Subsequently, the driving force for anion outflow was altered using a step in the membrane potential followed by observation of the current relaxation until the new steady state is reached. As illustrated in Fig. 3A, a voltage jump from 0 mV to a final value ranging from +60 to −100 mV (voltage protocol shown in Fig. 3A, top panel) resulted in a current rising rapidly within 5 ms, at which point the steady state is reached. The resulting current–voltage relationships (I–V curves) are shown in Fig. 3B and are consistent with negative voltages resulting in larger currents at all ionic conditions due to the increased driving force for SCN\(^-\) outflow. In the presence of SCN\(^-\) on both sides of the membrane (Fig. 3C: 130 mM inside and 140 mM outside), the reversal potential of the current induced by 120 mM Na\(^+\)/20 mM K\(^+\) is −1.5 mV, D, in the absence of intracellular SCN\(^-\), application of extracellular K\(^+\) (140 mM) induces reverse transport current (triangles), whereas insignificant reverse transport current is activated in the presence of Na\(^+\) only (black squares) or 120 mM Na\(^+\) in the presence of 20 mM K\(^+\) (circles; conditions of population of the co-binding state but without permeating anions). Error bars represent S.D.

**Glutamate transporter K\(^+\)/Na\(^+\) co-binding state**

![Figure 3](image-url)

*Figure 3. The inward current induced by the co-application of Na\(^+\) and K\(^+\) is caused by anion outflow.* A, typical current recordings as a function of the transmembrane potential (voltage jump protocol shown at the top) in the presence of 130 mM NaSCN and 5 mM glutamate internal and 120 mM Na\(^+\) and 20 mM K\(^+\) external (the anion was Mes\(^-\)); B, quantification of the currents shown in A (open circles) and after application of Na\(^+\) (filled squares) or K\(^+\) (open triangles) only. The open inverted triangles and diamonds represent anion current in response to glutamate application (homoexchange mode). All currents are given after subtraction of traces under the same condition in the presence of TBOA, except for the inverted triangle where the current in 140 mM Na\(^+\) was subtracted. C, in the presence of SCN\(^-\) on both sides of the membrane (130 mM inside and 140 mM outside), the reversal potential of the current induced by 120 mM Na\(^+\)/20 mM K\(^+\) is −1.5 mV. D, in the absence of intracellular SCN\(^-\), application of extracellular K\(^+\) (140 mM) induces reverse transport current (triangles), whereas insignificant reverse transport current is activated in the presence of Na\(^+\) only (black squares) or 120 mM Na\(^+\) in the presence of 20 mM K\(^+\) (circles; conditions of population of the co-binding state but without permeating anions). Error bars represent S.D.
Glutamate transporter $K^+$/Na$^+$ co-binding state

![Figure 4. Transient currents upon solution exchange between Na$^+$ and K$^+$ are consistent with existence of the co-binding state. Transient currents in response to solution exchange between Na$^+$ and K$^+$ and between K$^+$ and Na$^+$ (solution exchange protocol shown by bars at bottom) are shown. The intracellular solution contained 130 mM NaSCN and 10 mM glutamate. $V$ = 0 mV.](image)

shown in Fig. 4, transient current is present, manifesting itself as two symmetric inwardly directed current peaks between the steady-state current levels in the presence of Na$^+$ or K$^+$ only. This pre-steady-state, transient current supports the existence of an intermediate, co-binding state with large anion conductance in which Na$^+$ and K$^+$ are both bound. It also indicates that the co-binding state becomes transiently populated upon dissociation of Na$^+$/binding of K$^+$ as well as dissociation of K$^+$/binding of Na$^+$.

It should be noted that the rates of the rise and decay of the transient currents are most likely limited by the rate of the solution exchange. Thus, quantitative values for rate constants for cation interaction with EAAC1 cannot be determined from these data.

Despite existence of a K$^+$/Na$^+$ co-binding state, external K$^+$ functionally competes with Na$^+$ and glutamate

Next, we determined the concentration dependence of the effect of external K$^+$ on the rate of reverse transport in the presence of varying [Na$^+$]/[K$^+$] ratios or in the absence of Na$^+$. Reverse transport current was activated by application of extracellular potassium (Fig. 5A), in the presence of intracellular Na$^+$ and glutamate, introduced into the cell through the current-recording electrode. As illustrated in Fig. 5C, K$^+$ application induces reverse transport current at significantly lower concentrations in the absence of Na$^+$, with a Michaelis–Menten-like dose-response curve and a $K_m$ of 4.3 ± 1.1 mM (Fig. 5C). In contrast, reverse transport current was inhibited and did not become saturated in the simultaneous presence of Na$^+$ (Fig. 5B), indicating that Na$^+$ does, in fact, functionally compete with K$^+$ with respect to the transporter relocation reaction despite the existence of the Na$^+$/K$^+$ co-binding state. To further test this result, we determined reverse transport using a fluorescent glutamate efflux assay (Fig. 5D and Ref. 27). Here, K$^+$ activates glutamate efflux, increasing fluorescence of a genetically encoded glutamate sensor on the extracellular side of the membrane. The K$^+$ concentration dependence of the fluorescence was virtually identical with that of the reverse transport current, suggesting that our results are valid for both reverse transport current and glutamate efflux caused by reverse transport.

If a K$^+$/Na$^+$ co-binding state exists, it should also manifest itself in the cation dependence of the apparent affinity of the transporter for glutamate. To test this hypothesis, we measured the apparent dissociation constant, $K_{app}$, for glutamate as a function of the [K$^+$]/[Na$^+$] ratio. As shown in Fig. 6 and Fig. S1, $K_{app}$ value increased with increasing [K$^+$]/[Na$^+$] ratio. This result is expected because K$^+$ is thought to prevent the binding of organic substrate to the transporter. However, the increase of $K_{app}$ with the [K$^+$]/[Na$^+$] ratio is not monotonic but shows somewhat of a plateau at an intermediate [K$^+$]/[Na$^+$] ratio (Fig. 6A) in the same range at which the K$^+$/Na$^+$ co-binding state was detected. Such a plateau is not observed in the presence of Na$^+$ only (Fig. 6A) and is inconsistent with purely competitive Na$^+$/K$^+$ binding (Fig. 6B).

Cation selectivity of activation of the anion conductance in the co-binding state

Glutamate reverse transport by EAAC1 can be activated by application of extracellular K$^+$, Rb$^+$, and Cs$^+$ (23, 28, 29). Therefore, we tested whether the activation of the anion conductance by K$^+$ also shows relatively low selectivity for cations larger than K$^+$. Although 20 mM Rb$^+$-induced sizable anion current (Fig. 7B), it was smaller than the K$^+$-induced anion current (62 ± 12% relative to the K$^+$ response; Fig. 7C). In contrast, 20 mM Cs$^+$ activated very little anion current, less than 20% of the K$^+$ response (Fig. 7, A and C). These results suggest that the cation-induced anion conductance in the co-binding state prefers the physiological cation K$^+$ over the larger monovalent cations.

Co-binding states exist in EAAT1–3 subtypes

To test whether the co-binding state is specific for the glutamate transporter subtype EAAC1 or also observed in other subtypes, we tested co-application of Na$^+$ and K$^+$ to the glutamate transporters EAAT1 and -2. In contrast to EAAC1, anion conductance is not maximal at intermediate Na$^+$/K$^+$ ratios but rather increases with increasing K$^+$ concentration (Fig. 8A). Interestingly, in these subtypes, the anion conductance is larger in the presence of K$^+$ only compared with the presence of Na$^+$ only. Simulations of the anion currents for all three subtypes are shown in Fig. 8B using the simplified mechanisms 1 and 3, including the co-binding state, shown in Fig. 2D. The results of these simulations compare well with the experimental data and allow two major conclusions. 1) Na$^+$ and K$^+$ both bind with lower apparent affinity in EAAT1 and -2 compared with EAAC1 (estimated $K_m$ values shown in the legend of Fig. 8B). 2) The potassium-bound state is associated with anion conductance in EAAT1 and -2, but not in EAAC1, as demonstrated by
Glutamate transporter $K^+$/Na$^+$ co-binding state

Figure 5. Na$^+$ decreases apparent affinity for extracellular K$^+$ for reverse transport. A, reverse transport current induced by extracellular K$^+$ application, measured in the absence of intracellular permeable anions (140 NaMes and 10 mM glutamate internal; $V = 0$ mV). B, reverse transport current as a function of the extracellular [K$^+$]/[Na$^+$] ratio (same ionic conditions as in A). C, similar experiment as in B but in the absence of Na$^+$. The $K_{m}$ for K$^+$ is $4.3 \pm 1.1$ mM, as determined by a fit to a Michaelis–Menten-like equation (red line). Na$^+$ was replaced by NMG$^+$. D, determination of glutamate release from EAAC1-transfected HEK293 cells using a fluorescent glutamate sensor (iGluSnFr). An increase in fluorescence indicates glutamate efflux from cells. The relative fluorescence (glutamate efflux) increases linearly with increasing concentration of external K$^+$ (balanced to 140 mM total cation concentration by Na$^+$). Images of cells expressing EAAC1 and iGluSnFr fluorescent sensor are shown in the inset. The images show the relative magnitude of fluorescence when various concentrations of K$^+$ and Na$^+$ are applied externally (from left to right: 20 mM K$^+$ and 120 mM Na$^+$, 50 mM K$^+$ and 90 mM Na$^+$, and 140 mM K$^+$ and 0 mM Na$^+$). Error bars represent S.D.

Figure 6. Dependence of apparent glutamate on the [K$^+$]/[Na$^+$] ratio provides evidence for an intermediate state in the presence of both cations. A, comparison of the $K_{m}$ for glutamate as a function of the [K$^+$]/[Na$^+$] ratio. B, predictions of the $K_{m}$ for glutamate as a function of the [K$^+$]/[Na$^+$] ratio according to Equations 1–3 (supporting Materials and Methods) and the co-binding models shown in Fig. 8 (blue, Na$^+$ only; red, K$^+$/Na$^+$ competitive; black, K$^+$/Na$^+$ co-binding). Error bars represent S.D.

the simulation parameters for relative anion currents listed in the legend of Fig. 8B. Together, these results suggest that the K$^+$/Na$^+$ co-binding state is present in all three transporter subtypes, but its effect on overall anion current is masked by the large conductance of the K$^+$-bound state in EAAT1 and -2.

Discussion

Here, we present evidence that Na$^+$ and K$^+$ can bind simultaneously to the glutamate transporter subtype EAAC1, forming a K$^+$/Na$^+$ co-binding state. This finding is in contrast to previously proposed transport mechanisms (3) in which either Na$^+$ binds to the transporter, resulting in glutamate binding and eventual translocation across the membrane, or K$^+$ binds to induce the reorientation of the binding site. Such a Na$^+$/K$^+$ exchange mechanism predicts that Na$^+$ and K$^+$ exclude each other from binding to the transporter, resulting in competitive binding. Our experimental results, showing biphasic concentration dependence of the Na$^+$/K$^+$ effect, cannot be explained with purely competitive binding but require that Na$^+$ and K$^+$ are bound to the transporter at the same time.

The evidence for the K$^+$/Na$^+$ co-binding state was obtained by analyzing the glutamate transporter anion conductance. This anion conductance was initially thought to be associated with Na$^+$-bound states of the transporter, either in the presence of glutamate, inducing a large anion conductance, or in the absence of glutamate in the form of a smaller leak anion conductance (8). However, this simplified model was later revised after it was shown for EAAT4 (30) that the anion conductance can be activated in several other states of the transport cycle, including the inward-facing, K$^+$-bound state that is responsible for K$^+$-induced relocation of the transporter. In addition, Amara and co-workers (24) showed that the glial glutamate transporter subtypes EAAT1 and -2 do not require Na$^+$ to acti-
Glutamate transporter K⁺/Na⁺ co-binding state

![Figure 7](image)

Cation selectivity of activation of anion conductance in the Na⁺/K⁺ co-binding state. A and B, Cs⁺ and Rb⁺ replacement of K⁺ was performed at 20/120 mM Cs⁺/Na⁺ (A) and Rb⁺/Na⁺ (B). Statistical analysis of currents relative to K⁺ is shown in C. In all experiments, the total cation concentration was 140 mM with 20 mM K⁺/Cs⁺/Rb⁺ balanced with 120 mM Na⁺. The error bars represent ±5D. The intracellular solution contained 130 mM NaSCN. The external anion was Mes⁻. The voltage was 0 mV.

![Figure 8](image)

The properties of the K⁺/Na⁺ co-binding state depend on the EAAT subtype. A, relative anion current induced by K⁺ application in EAAC1 (black), EAAT1 (red), and EAAT2 (green). Ionic conditions were the same as in Fig. 5. V = 0 mV. B, simulations of the anion current using the pre-equilibrium assumption according to the co-binding mechanism in Fig. 2D (Equation 5 under “Experimental procedures”). For EAAT1, the highest current [K⁺]/[Na⁺] ratio is 70/70 mM, but for EAAT2 it is 110/30 mM, and for EAAT3 (EAAC1) it is 20/120 mM. Error bars represent ±5D.

![Figure 9](image)

Proposed kinetic mechanisms including the K⁺/Na⁺ co-binding state (green). For simplicity, protonation is omitted. Anion-conducting states are indicated by the bar. The co-binding state is highlighted in green. A, anion-conducting states are intermediates in the transport cycle. B, anion-conducting states are in equilibrium with intermediates in the transport cycle.

dicate the leak anion conductance in contrast to the neuronal subtypes EAAT3 and -4. This finding further supported the idea that the anion conductance is present in many states of the transport cycle, including Na⁺-free states, and is modulated as the transporter moves through the transport cycle. Our data are consistent with these previous findings, showing that external K⁺ can also activate the leak anion conductance with larger potency than Na⁺ in the K⁺/Na⁺ co-binding state. This adds yet another state in the transport cycle that is permeable for anions. It will be important to explore how this conducting state fits with the structural models that predict anion movement across the membrane based on molecular dynamics and site-directed mutagenesis approaches.

Interestingly, the ability of cations to activate the anion conductance was found to vary in the three major subtypes of glutamate transporters, EAAT1–3. Although all three subtypes showed evidence for the anion-conducting K⁺/Na⁺ co-binding state, K⁺-activated anion current in EAAT-1 and -2 in the absence of Na⁺ but not in EAAC1 (rat homolog of EAAT3). These results suggest subtle differences in the way the anion conductance operates between the subtypes, consistent with results on differences in Na⁺ dependence of the anion conductance (24). These differences may be interesting to explore and may be linked to the different microenvironments of these transporters in their particular location of expression, which varies among the subtypes (22–24).

We propose revised kinetic mechanisms for cation interaction with the glutamate transporter as shown in Fig. 9. These mechanisms include anion-conducting states either as intermediates in the transport cycle or as distinct states branching off intermediates in the transport cycle as has been proposed previously (20, 21, 31). Both mechanisms are consistent with the experimental data.

1) The mechanisms account for the increase of anion conductance at intermediate Na⁺/K⁺ concentrations when the co-binding state is formed and its decrease at high K⁺ concentrations (in the subtype EAAC1) when the K⁺-bound state is formed. This K⁺-bound state makes the transporter competent for reverse transport. 2) The mechanisms explain the transient formation of an anion-conducting state as K⁺ is displaced by extracellular Na⁺ or K⁺ associates after Na⁺ dissociation (Fig. 4). 3) The existence of the co-binding state is consistent with the dependence of the apparent Km for glutamate as a function of the [K⁺]/[Na⁺] ratio (Fig. 6). Here, a “plateau” in affinity is observed at intermediate [K⁺]/[Na⁺] ratios, which would not be predicted in the absence of a K⁺/Na⁺-co-binding state.
Our results provide further insight into the mechanism of selectivity of cation-binding sites of glutamate transporters. Although it was previously suggested that a cation-binding site involving Asp-454 (Na1 site in GLT1) can be an overlapping Na+/K+ site (32, 33), it was assumed that K+ binding to this site results in relocation of the glutamate-free transporter at the exclusion of Na+ binding. In this mechanism, three cation-binding sites exist (Na1, -2, and -3) and can be occupied by three sodium ions. Alternatively, one of the cation-binding sites is occupied with K+ in the Na+- and glutamate-free transporter, most likely the Na1 site. In contrast to this mechanism, our results suggest that occupation of the Na1 site with K+ would still allow Na+ binding to one or both of the other two sites. Therefore, our results are consistent with the Na1 site having poor selectivity for monovalent cations. It can be speculated that the Na+ in the co-binding state binds either at the Na2 site observed in crystal structures (34) or the Na3 site, which was proposed based on site-directed mutagenesis results (35) and molecular dynamics simulations (36). If the cation-binding site is not very selective, how can the transporter discriminate between Na+ and K+? We hypothesize that the discrimination between these two cations does not occur during the binding step but rather when the conformational change(s) associated with the alternative access transport mechanism occurs. In such a model, relocation of the glutamate-free transporter can only occur when K+ or larger monovalent cations such as Rb+ or Cs+ are bound, but it cannot occur (or occurs at a much lower rate) when Na+ is bound or Na+ and K+ are both bound.

In a second potential mechanism, four separate cation-binding sites exist, one selective for K+ and larger cations and three selective for Na+ and, potentially, Li+. Evidence for this type of mechanism was provided by valence mapping analysis, predicting a K+-binding site that overlaps with the binding site for the organic substrate (37, 38). Such a mechanism would be attractive because binding of the substrate promotes Na+ binding to the transporter. If K+ bound to the substrate-binding site would impose partial substrate-like features, it could be hypothesized that K+ may promote Na+ binding as well, resulting in the K+/Na+ co-binding state.

In another antiporter, the Na+/Ca2+ exchanger, it was proposed, based on functional data, that the binding of Ca2+ and at least two Na+ ions is purely competitive, namely that these cations exclude each other from binding to the exchanger (39). In contrast to this observation, a crystal structure of an archaeal Na+/Ca2+ exchanger, NCX_Mj, showed electron density in four cation-binding sites, one presumably the Ca2+-binding site and three potential Na+–binding sites (40). The stoichiometry of this exchanger was shown to be 3:1 Na+:Ca2+ (41). This potential Na+/Ca2+–bound state is similar to the co-binding state observed here for the glutamate transporter. Results from simulations based on this NCX crystal structure (42) as well as later crystal structures in the presence of Na+ and Ca2+ only, however, suggested that the Ca2+ site is occupied by Na+ in the absence of Ca2+ and presence of Na+ (43). Therefore, it is likely that the initial crystal structure (40) is a mixture of Ca2+-bound and Na+-bound states rather than evidence for a Na+/Ca2+ co-binding state. As in the sodium calcium exchanger, mutagenesis experiments, molecular dynamics simulations, and structural evidence will be necessary to clarify the exact structural mechanism of cation binding and competition in glutamate transporters.

It has been previously speculated that the glutamate-induced anion conductance of glutamate transporters plays a role in modulating the excitability of neuronal cells (44). However, evidence of inhibitory function by activation of the anion conductance has only been obtained for EAAT5 (45), which is associated with a large anion conductance relative to the other subtypes. Information about the physiological effects of the anion conductance in the absence of glutamate is currently lacking. The increase of the anion conductance in the K+/Na+ co-binding state is intriguing because it is maximal close to extracellular K+ concentrations that can be reached during neuronal stimulation (46), in particular upon repetitive stimulation ([K+] ~ 10 mM). The decay to steady-state [K+] takes place on the time scale of seconds (47). Therefore, it is possible that the leak anion conductance remains activated after glutamatergic signal transmission is complete, providing a potential for sustained inhibitory feedback. In addition, our transient current data (Fig. 4) show that the transporter passes through the anion-conducting co-binding state upon dissociation of K+ and association of Na+, a transition potentially occurring under conditions of cycling under neuronal glutamate uptake events, although the population of this state would be rather short-lived. Finally, data in Fig. 6 show that the co-binding state has an effect on apparent glutamate affinity of the transporter. Thus, extracellular K+ may be able to modulate glutamate affinity in ways that differ from the purely competitive mechanism. However, the determination of the exact physiological implications of the proposed co-binding mechanism will remain a challenge.

In summary, we have demonstrated that binding of Na+ and K+ to the glutamate transporter from the extracellular side of the membrane is not purely competitive but that Na+ and K+ can bind at the same time to EAAC1, forming a co-binding state. This co-binding state conducts anions, showing a higher conductance than the Na+- and K+-bound states, respectively, adding further evidence for the hypothesis that the transition through the transport cycle modulates the anion conductance, which may be present to a varying extent in most transporter states. Furthermore, these findings are important because they suggest that the mechanism of cation interaction with glutamate transporters is more complex than previously hypothesized, raising the possibility that at least one cation-binding site has poor selectivity for Na+. This site is most likely already present in the absence of glutamate bound to the transporter. In addition, selectivity for cations may be imposed by conformational changes of the transporter linked to cation binding rather than by the initial binding event itself. Therefore, current kinetic transport mechanisms should be revised to incorporate the new findings and the Na+/K+ co-binding state.

**Experimental procedures**

**Cell culture and transfection**

HEK293 cells (American Type Culture Collection, catalog number CRL 1573) were cultured as described previously (48).
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Cell cultures were transiently transfected with WT EAAC1, EAAT1, or EAAT2 cDNAs inserted into a modified pBK-CMV expression plasmid using jetPRIME transfection reagent according to the protocol supplied by the manufacturer (Polyplus-transfection). Before electrophysiological measurements, cells were incubated for 24–30 h after transfection.

Electrophysiology

Currents associated with glutamate transporters were measured in the whole-cell current recording configuration. Whole-cell currents were recorded with an EPC7 patch-clamp Amplifier (ALA Scientific, Westbury, NY) under voltage-clamp conditions (4). The resistance of the recording electrode was 2–3 MΩ. Series resistance was not compensated because of the small whole-cell currents carried by EAAC1, EAAT1, and EAAT2. The composition of the solutions for measuring forward transport currents was 140 mM NaMes/KMes, 2 mM Mg(Mes)$_2$, 10 mM EGTA, 10 mM HEPES, and 5 mM NaSCN (other ions as described above).

In cation selectivity experiments, extracellular solutions contained 130 mM NaSCN in anion current mode or 130 mM Mes$^-$ in the absence of NaSCN. In anion current experiments, extracellular Mes$^-$ was replaced by SCN$^-$. To lock the transporter in translocation equilibrium states, homoexchange conditions were employed, with saturating concentrations of 5 mM glutamate, 140 mM Na$^+$, 20/120 mM K$^+$, 139/110 mM K$^+$/Na$^+$, 2 M Mg(Mes)$_2$, 2 M Ca(Mes)$_2$, and 10 mM HEPES in the extracellular solution, adjusted to pH of 7.4. The pipette solution contained 130 mM NaSCN in anion current mode or 130 NaMes, 2 mM Mg(Mes)$_2$, 10 mM EGTA, 10 mM HEPES, and 5 mM glutamate, adjusted to pH 7.4 with methanesulfonic acid.

Voltage jumps

Voltage jumps (−100 to +60 mV) were applied to perturb the electrogenic glutamate translocation equilibrium. To determine EAAC1-specific currents, control currents were recorded in the presence of 200 μM extracellular DL-threo-β-benzyl-oxyaspartic acid (TBOA) and subtracted from the glutamate-induced currents. Capacitive transient compensation and series resistance compensation of up to 80% were employed using the EPC7 amplifier. Nonspecific transient currents were subtracted in Clampfit (Molecular Devices).

Steady-state current simulations

We used equations derived for pre-equilibrium conditions (Equations 1–4) to simulate two different Na$^+/K^+$ binding models, the solution of which was calculated by Mathematica software (Wolfram) with current calculated using Excel (Microsoft).

For the simplified co-binding mechanism (mechanism 3), the following equations were used.

\[
[T] + [TK] + [TNa] + [TNaK] = 1 \quad (\text{Eq. 1})
\]

\[
\frac{[TK][Na]}{[TNaK]} = N_1 \quad (\text{Eq. 2})
\]

\[
I_{[Na]} = \frac{[Na][T]}{[TNa]} = K_N \quad (\text{Eq. 3})
\]

\[
I_{[TK]} = \frac{[TK][Na]}{[TNaK]} = K_N \quad (\text{Eq. 4})
\]

\[
I_{total} = I_T + I_{[Na]}[TNa] + I_{[TK]}[TK] + I_{[TNaK]}[TNaK] \quad (\text{Eq. 5})
\]

For the two-Na$^+$-ion co-binding mechanism (mechanism 4), the following equations were used.

\[
[T] + [TK] + [TNa] + [TNa_2] + [TNaK] = 1 \quad (\text{Eq. 6})
\]

\[
\frac{[TK][Na]}{[TNa]} = K_{N_1} \quad (\text{Eq. 7})
\]

\[
\frac{[TK][Na]}{[TNaK]} = K_N \quad (\text{Eq. 8})
\]

\[
\frac{[TK][Na]}{[TNa]} = K_{N_2} \quad (\text{Eq. 9})
\]

\[
I_{total} = I_T + I_{[Na]}[TNa] + I_{[TNa_2]}[TNa_2] + I_{[TK]}[TK] + I_{[TNaK]}[TNaK] \quad (\text{Eq. 10})
\]

For the simplified competitive binding mechanism (mechanism 1), the following equations were used.

\[
[T] + [TK] + [TNa] = 1 \quad (\text{Eq. 11})
\]

\[
\frac{[Na][T]}{[TNa]} = K_N \quad (\text{Eq. 12})
\]

\[
\frac{[K][T]}{[TK]} = K_K \quad (\text{Eq. 13})
\]

\[
I_{total} = I_T + I_{[Na]}[TNa] + I_{[TK]}[TK] \quad (\text{Eq. 14})
\]

For the two-Na$^+$-ion competitive binding mechanism (mechanism 2), the following equations were used.

\[
[T] + [TK] + [TNa] + [TNa_2] = 1 \quad (\text{Eq. 15})
\]

\[
\frac{[Na][T]}{[TNa]} = K_{N_1} \quad (\text{Eq. 16})
\]

\[
\frac{[K][T]}{[TK]} = K_K \quad (\text{Eq. 17})
\]

\[
\frac{[Na][T]}{[TNa]} = K_{N_2} \quad (\text{Eq. 18})
\]

\[
I_{total} = I_T + I_{[Na]}[TNa] + I_{[TNa_2]}[TNa_2] + I_{[TK]}[TK] \quad (\text{Eq. 19})
\]

In these equations, $[T], [TNa], [TNa_2], [TK],$ and $[TNaK]$ are defined as the relative transporter concentrations in the apo, Na-bound, K-bound, and Na- and K-bound states. Relative steady-state anion currents for each state were adjusted to best represent the experimental data: $I_T = 0, I_{TNa} = 1.1, I_{TNa_2} = 1.1,$
$I_{\text{TNaK}} = 3$, and $I_{\text{TK}} = 0$; the two equilibrium constants used here were $K_{\text{Na}} = K_{\text{Na2}} = 50 \text{ mm}$ and $K_{\text{K}} = 4 \text{ mm}$. Although these values result in a current versus dose curve that describes the experimental data well, they are not expected to be unique solutions for the system of equations.

**Cellular glutamate efflux measured by extracellular glutamate-binding sensor**

EAAC1 transporter was co-expressed with iGluSnFR (27) extracellular glutamate-binding sensor in HEK293 cells as a system for measuring glutamate efflux via fluorescence intensity changes. The sensor is bound to the outside of the cell membrane and contains a glutamate-binding component that responds rapidly to extracellular glutamate concentration changes. The external glutamate concentration is reported via a fluorescence intensity change. This method specifically isolates the transport function of EAAC1 in response to various external conditions and eliminates the variable of anion conductance.

All imaging experiments utilized a live-cell flow-through imaging chamber (Warner Instruments, Series 20) together with an inverted fluorescence microscope (Zeiss Axiovert 25). The fluorescence filter set used was FITC, which was obtained from Omega. Bath buffer solution contained NaMes and KMes equaling a combined concentration of 140 mM, 2 mM Ca(gluconate)$_2$, 2 mM Mg(gluconate)$_2$, and 10 mM HEPES, pH 7.3 using NaOH. 140 mM NaMes solution was used to initially wash the remaining culturing medium from the cell surface and in between each test solution to act as a benchmark for changes in fluorescence. Solutions were passed through the imaging chamber for 30–45 s before recording an image. For each experiment, the exposure and other imaging features were set as constant and for an appropriate fluorescence intensity below saturation. Images were recorded after each solution exchange and then analyzed using ImageJ software. ImageJ was used to quantify the fluorescence intensity of 5–10 cells per image. The loci were held constant when comparing images in an experiment. The relative fluorescence change ($\Delta F/F$) was calculated as follows.

$$\frac{\Delta F}{F} = \frac{F_{\text{final}} - F_{\text{initial}}}{F_{\text{initial}}} \quad (\text{Eq. 21})$$

where $F_{\text{initial}}$ was the fluorescence intensity of the image taken after passing the initial solution directly prior to the test solution and $F_{\text{final}}$ was the fluorescence intensity of the same point in the image taken following the test solution.

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