Neuronal Glutamate Transporters Vary in Substrate Transport Rate but Not in Unitary Anion Channel Conductance*\(^5\)

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Excitable amino acid transporters (EAATs) not only sustain a secondary active glutamate transport but also function as anion-selective ion channels. The relative proportion of currents generated by glutamate transport or by the chloride conductance varies for each cloned EAAT subtype. For EAAT1, EAAT2, and EAAT3, the anion current is only a small component of the total transporter-associated current amplitude, whereas EAAT4 and EAAT5 transporters mediate predominately anion currents. We here demonstrate that the distinct current proportions are entirely due to differences in glutamate transport rates. EAAT3 and EAAT4 differ in unitary glutamate transport rates as well as in the voltage and substrate dependence of anion channel opening, but ion conduction properties are very similar. Noise analysis revealed identical unitary current amplitudes and similar absolute open probabilities for the two anion channels. The low glutamate transport rate of EAAT4 allows regulation of cellular excitability without interfering with extracellular glutamate homeostasis and makes this EAAT isoform ideally suited to regulate excitability in dendritic spines of Purkinje neurons.

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system. After the release from glutamatergic nerve terminals, glial and neuronal glutamate transporters remove glutamate from the synaptic cleft to ensure low resting glutamate levels and to prevent neuronal damage by excessive glutamate receptor activation. EAATs\(^2\) mediate two different transport processes; they are secondary active glutamate transporters and anion-selective channels (1). Different EAAT isoforms differ in the relative contribution of anion currents to the total transporter-mediated current (2–5). These differences have been interpreted as an indication that some EAATs play physiological roles as glutamate transporters and others play roles as glutamate-gated anion channels involved in the regulation of cellular excitability.

To obtain a better understanding of the functional basis of these differences, we compared the functional properties of two neuronal EAAT isoforms, EAAT3 and EAAT4. EAAT3 displays only modest anion currents in heterologous expression systems (2), whereas EAAT4 anion currents greatly exceed electrogenic transport currents (3). Here we demonstrate that the unitary current amplitudes and the absolute open probabilities of EAAT3 and EAAT4 are very similar. EAAT3 and EAAT4 anion channels differ in anion channel gating and glutamate dependence of the anion channel activation, two features that might be explained by a variable interaction between individual subunits of the multimeric transporter protein.

EXPERIMENTAL PROCEDURES

Heterologous Expression and Functional Characterization of EAAT3 and EAAT4—The coding region of hEAAT3 (GenBank\(^\text{TM}\) accession number NP_004161) was excised from pTLN2-hEAAT3 (provided by Dr. Matthias A. Hediger, University of Bern, Bern, Switzerland) (6) and subcloned into pcDNA3.1 (Invitrogen). The pcDNA3.1-rEAAT4 construct (GenBank\(^\text{TM}\) accession number NM_114454) was provided by Dr. J. Rothstein (Johns Hopkins University, Baltimore, MD). Point mutations were introduced into the wild type hEAAT3 cDNAs using the QuikChange mutagenesis kit (Stratagene). For each construct, two independent recombinants from the same transformation were examined and shown to exhibit indistinguishable functional properties. Transient transfection of tsA201 cells were performed as previously described (5). In some of the experiments, a stable inducible cell line was used (7), generated by selecting Flp-In-T-Rex cells (Invitrogen) after transfection with pcDNA5-FRT-To-rEAAT4.

Capped cRNA was synthesized from MluI-linearized pTLN2-hEAAT3 (6) and from Nhel-linearized pGEMHE-rEAAT4 (8) templates through use of MESSAGE machine kits (Ambion, Austin, TX). Injection and handling of oocytes were performed as described elsewhere (9). Oocytes expressing EAAT3 were usually measured 1 day after injection. To account for differences in expression levels, this period was increased to 4–5 days for EAAT4.

Electrophysiology—Standard whole cell patch clamp recordings were performed using an Axopatch 200B (Molecular Devices, Sunnyvale, CA) amplifier as described (5, 8). The cells were clamped to 0 mV for at least 10 s between test sweeps. In

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§ The abbreviations used are: EAAT, excitatory amino acid transporter; TBOA, l-threo-benzyloxyaspartate.
experiments measuring EAAT-associated noise, the pipettes were covered with dental wax to reduce their capacitance. More than 80% of the series resistance was compensated by an analog procedure resulting in calculated voltage errors of <5 mV. Standard solutions contained 140 mM NaSCN (standard NaSCN-based external solution) or NaCl (standard NaCl-based external solution), 4 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPES, pH 7.4, intracellular 115 mM NaCl (standard NaCl-based internal solution) or NaSCN (standard NaSCN-based external solution), 2 mM MgCl₂, 5 mM EGTA, 10 mM HEPES, pH 7.4. For the concentration dependences of glutamate (see Fig. 5), a given concentration of L-glutamate was added to the external solution.

In oocytes, glutamate transporter-associated currents were recorded by two-electrode voltage clamp using a CA1 amplifier (Dagan, Minneapolis, MN). Prior to electrophysiological testing, the oocytes were incubated for at least 5 h in a gluconate-based solution containing 96 mM sodium gluconate, 4 mM potassium gluconate, 0.3 mM calcium gluconate, 1 mM magnesium gluconate, 5 mM HEPES, pH 7.4. Voltage clamp experiments were started in a gluconate-based external solution. The oocytes were held at 0 mV, and the currents elicited by 100-ms voltage steps between −120 mV and +80 mV were filtered at 2 kHz (−3 dB) and digitalized with a sampling rate of 10 kHz using a Digidata AD/DA converter (Molecular Devices, Sunnyvale, CA). Glutamate uptake currents were measured by 100-ms current pulses (see Fig. 3), from subtracted traces using the fast Fourier transform routine (see Fig. 3, F and G) was subtracted. The resulting power spectra could be well fit with the sum of two Lorentzian components.

Data Analysis—The data were analyzed with a combination of the pClamp9 (Molecular Devices, Sunnyvale, CA) and SigmaPlot (Jandel Scientific, San Rafael, CA) programs. Current amplitudes were used without applying a subtraction procedure. Current-voltage relationships at various substrate concentrations were constructed by plotting isochronal current amplitudes determined 1 ms after the voltage step versus the membrane potential. Voltage dependences of the relative open probability (see Figs. 1 and 2) were determined by plotting the normalized isochronal current amplitude at +135 or −135 mV after 0.2 s prepulses to different voltages versus the preceding potential. Activation curves obtained in this manner were then fit with a single Boltzmann term plus a voltage-independent term (I(V) = Amp/[(1 + e^(-(V-V_{50}))/kV)] + P_{min}) (supplemental Table S1). Permeability ratios were calculated from reversal potential measurements using the Goldman-Hodgkin-Katz equation (10) as described elsewhere (5) (supplemental Fig. S1). Isochronal anion current amplitudes were measured at various concentrations at a given test potential to obtain the concentration dependence of anion channel activation by glutamate. The substrate dependences thus obtained were normalized to the maximum current amplitude, averaged after normalization, and fit with the Hill equation.

\[
I = \frac{I_{\text{max}} \cdot [\text{substrate}]^n}{[\text{substrate}]^n + K^*_D} + I_0
\]  

(Eq. 1)

The substrate-dependent \(I_{\text{max}}\) as well as the substrate-independent current amplitude \(I_0\) and the Hill coefficient \(n\) were determined as a fit parameter. For each membrane potential, apparent dissociation constants (\(K_D\) values) were determined on several cells and averaged. The Student’s t test was used for statistical evaluation. For comparisons between EAAT3 and EAAT4, cells and oocytes with comparable anion current amplitudes were used.

Noise Analysis—Current variances \(\sigma^2_n\) and mean current amplitudes \(\langle I \rangle\) were determined during the last 50 ms of 500-ms test steps to voltages between −155 mV and +105 mV. Changing the amount of data subjected to analysis (from 10 to 70 ms) did not significantly alter the variance. Current traces were sampled at 5 kHz and filtered using a Bessel low pass filter with a cut-off frequency of 1 kHz. For all experiments, the analysis was repeated after digital filtering with a Butterworth filter at 0.5 kHz, yielding similar results. The variance at the current reversal potential was subtracted as background noise.

Power spectra, \(S(f)\), were determined from current recordings upon voltage steps to −155 mV. Current traces were sampled at 50 kHz and filtered using a Bessel low pass filter with a cut-off frequency of 10 kHz. For each power spectrum, 30 traces were averaged, and the mean values were subtracted from individual recordings. Power spectra were computed from subtracted traces using the fast Fourier transform routine in Clampfit 9. The resulting spectra were averaged, and the power spectrum determined at the current reversal potential (see Fig. 3, F and G) was subtracted. The resulting power spectra could be well fit with the sum of two Lorentzian components.

\[
S(f) = \frac{S(0)}{1 + \left(\frac{f}{f_{\text{ss}}}\right)^2} + \frac{S(1)}{1 + \left(\frac{f}{f_{\text{ss}}}\right)^2}
\]  

(Eq. 2)

Lorentzian noise depends on the number of channels \(N_o\), the unitary current amplitude \(i\), and the absolute open probability \(p\),

\[
\sigma^2 = Ni^2p(1-p) + \sigma^2_0
\]  

(Eq. 3)

with \(\sigma^2_0\) being the background noise. Dividing the variance \((\sigma^2_{ss})\) by the mean current amplitude \(\langle I_{ss} = Npi \rangle\), after subtraction of the background noise, results as follows.

\[
\frac{\sigma^2_{ss} - \sigma^2_0}{I_{ss}} = i(1-p)
\]  

(Eq. 4)

To determine the unitary current amplitudes and the absolute open probabilities of EAAT3 and EAAT4, we employed a variation of stationary variance analysis (11, 12). EAAT3 and EAAT4 anion channels display a voltage-dependent rectification of the unitary current amplitude (see Fig. 1). To account for this voltage-dependent conductance, the instantaneous current amplitude was measured by extrapolating the current amplitude back to the moment of the voltage jump; this extrapolation was done by fitting a biexponential function to the time dependence of the current relaxation. The instantaneous current is proportional to the unitary current ampli-
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RESULTS

Voltage-dependent Gating of EAAT3- and EAAT4-associated Anion Currents—We expressed hEAAT3 and rEAAT4 glutamate transporters heterologously in tsA201 cells and measured currents through whole cell patch clamp experiments. Electrogenic glutamate uptake was abolished by dialyzing cells with a K+ free internal solution (8). Using Cl− or NO3− as main permeant anions, EAAT3-associated anion currents were quite small, and we therefore used SCN− (2, 5) as the main anion on one membrane side and Cl− on the other. Under these conditions, whole cell currents in cells expressing EAAT3 and EAAT4 were significantly larger than those in untransfected tsA201 cells, in the absence as well as in the presence of glutamate (Figs. 1 and 2). Complete substitution of anions with glutamate− resulted in negligible current amplitudes (at 0 mV, 0.2 ± 0.04 nA (n = 5) after substitution of internal and external anions with glutamate in the presence of 500 µM L-glutamate). Moreover, application of 200 µM L-threo-β-benzoxaspartate (TBOA) (13) reduced the current amplitudes to values close to background levels (Fig. 1). These results demonstrate that, under our experimental conditions, EAAT3 and EAAT4 whole cell currents predominantly represent transporter-associated anion currents (5, 7, 8).

Both transporter isoforms conduct anions in the absence of glutamate. Application of glutamate increases the amplitudes and changes the time and voltage dependence of anion currents (Figs. 1 and 2). With SCN− on the internal membrane side, glutamate augments EAAT3 and EAAT4 anion current amplitudes to a similar extent (Fig. 1, B and E). With the anion distribution reversed, increases of glutamate-induced current amplitudes were much smaller for EAAT3 than for EAAT4 (Fig. 2, B and E).

EAAT anion channels exhibit time- and voltage-dependent current relaxations upon voltage steps. Under all of the tested conditions, the anion currents of both the EAAT3 and EAAT4 isoforms rise instantaneously upon voltage steps to negative and positive potentials (Figs. 1 and 2). With SCN− being the main internal anion, hyperpolarizing voltage steps elicit time- and voltage-dependent current decays of EAAT3 and EAAT4 anion currents. This time-dependent decay was more pronounced in the presence than in the absence of glutamate (Fig. 1, A and D). Gating of EAAT3 anion channels is modified by permeant anions (5, 8). The gating of EAAT3 and EAAT4 anion channels is completely different when studied with external SCN− and internal Cl− (Fig. 2, A and D) than at the inverted anion distribution. At Clint/SCNext, there is a fast time-dependent and voltage-dependent deactivation of EAAT4 currents at
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FIGURE 2. Voltage and substrate dependence of EAAT3- and EAAT4-associated anion currents in cells dialyzed with Cl⁻-based solutions. A and D, representative current recordings from cells expressing EAAT3 (A) or EAAT4 (D). B and E, mean isochronal current-voltage relationships for EAAT3 (B) and EAAT4 (E). The experiments were performed in the absence (closed symbols, \( n = 12 \) and \( n = 6 \), respectively) and the presence (open symbols, \( n = 12 \) and \( n = 6 \), respectively) of 500 \( \mu \)M L-glutamate. The solid lines represent current-voltage relationships determined with untransfected tsA201 cells in the presence of 500 \( \mu \)M L-glutamate. The open symbols represent current amplitudes and absolute open probabilities (open symbols, \( n = 9 \) for EAAT3; \( n = 7 \) for EAAT4) of 500 \( \mu \)M L-glutamate.

positive voltages, whereas EAAT3 shows a depolarization-induced activation in the absence or presence of glutamate. The depolarization-induced relative current increase of EAAT3 was augmented by glutamate, whereas current deactivation of EAAT4 was less pronounced in the presence of glutamate than in its absence. Voltage dependences of relative open probabilities were determined by plotting normalized isochronal current amplitudes at a fixed test step versus the preceding potential (Figs. 1C and F, and 2C and F). These activation curves can be described well by Boltzmann functions for both isoforms and both ionic conditions (supplemental Table S1). The activation curves of EAAT3 and EAAT4 are similar for SCN⁻/Cl⁻, but there are prominent isofrom-specific gating differences at the reverse anion distribution.

Permeation Properties of EAAT3 and EAAT4 Anion Channels—For both isoforms, currents are inwardly rectifying in cells internally dialyzed with the more permeant anion SCN⁻—but outwardly rectifying with SCN⁻ in the external membrane site (Figs. 1 and 2). Reversal potentials enable a calculation of the relative permeabilities using the Goldman-Hodgkin-Katz equation. In the absence of glutamate, the SCN⁻ over Cl⁻ permeability coefficients are identical for EAAT3 and EAAT4. Application of glutamate changes the anion selectivity of EAAT anion channels (5). It causes an increased relative permeability of internal SCN⁻ for EAAT3 and for EAAT4. However, for external SCN⁻, glutamate results in a decreased \( P_{\text{SCN}}/P_{\text{Cl}} \) (\( p < 0.05 \)) of EAAT4 but leaves the reversal potential of EAAT3 currents unaffected (supplemental Fig. S1).

To study unitary anion channel properties, we performed noise analysis (Figs. 3 and 4). Current variances are much larger in cells expressing EAAT3 or EAAT4 (Fig. 3B–E) than in untransfected or uninduced cells (Fig. 3A). Various EAAT anion channels have been recently reported to generate a Lorentzian type of noise (5, 7, 14–16). To demonstrate that the measured current variance in cells expressing EAAT3 or EAAT4 indeed originates from the opening and closing of individual channels, we computed power spectra from macroscopic currents using fast Fourier transformation. Fig. 3 (F and G) shows representative power spectra of EAAT3 and EAAT4 currents that could be well fit with a sum of two Lorentzian components, with cut-off frequencies comparable with those expected from the relaxation time constants (Fig. 3, F and G). We conclude that the measured current variance is Lorentzian noise associated with gating of EAAT-associated channels.

Mean current amplitudes (\( I \)) and current variances (\( \sigma^2 \)) were determined in the last 50 ms of 500-ms test steps to various voltages (Fig. 3B–E). Current variance was analyzed in two ways. First, the ratio of current variance by mean current amplitude was calculated for both anion conditions and in the presence and absence of glutamate. For channel-mediated currents, this ratio is equal to the product of the single channel amplitude (\( I \)) and the fraction of time spent in a closed state (\( 1 - p \), with \( p \) being the absolute open probability) (\( \sigma^2/I = i(1 - p) \) (17)). EAAT3 and EAAT4 did not differ in this value for SCNᵢ/Clᵢ at \(-135 \text{ mV} \), \( 40.5 \pm 16 \text{ fA} \) for EAAT3, \( n = 3 \); and \( 49.8 \pm 11 \text{ fA} \) for EAAT4, \( n = 4 \), \( p = 0.6 \); for Clᵢ/SCNᵢ at \(+55 \text{ mV} \), \( 4.0 \pm 0.8 \text{ fA} \) for EAAT3, \( n = 6 \); and \( 4.6 \pm 0.8 \text{ fA} \) for EAAT4 \( n = 4 \), \( p = 0.1 \). The identity of these values, however, does not prove that single channel amplitudes and absolute open probabilities are the same for both isoforms. It is still possible that smaller unitary conductances of EAAT3 might be masked by smaller absolute open probabilities.

To separate unitary current amplitudes and absolute open probabilities, we performed a variant of stationary noise analysis (11, 12) (Fig. 4). Stationary current variances and mean current amplitudes were measured at various voltages, and variances divided by the product of the mean and the instantaneous current amplitudes were plotted against the ratio of the mean current amplitude by the instantaneous current amplitude (Fig. 4A and D). This transformation of variance-current plots usually used in nonstationary noise analysis (18) allows determination of unitary channel properties by a linear fit to values obtained from measurements of stationary noise. The slope (\( -1/N \)) of the fitted straight gives the number of channels (\( N \)), and the \( y \) axis intercept providing the ratio between unitary current amplitude and instantaneous current amplitude.

We performed such an analysis only at SCNᵢ/Clᵢ in the presence of glutamate. At all other tested conditions, the open
probabilities changed only a little with voltage, precluding a successful analysis. Multiplication of the $y$ axis intercept and the voltage dependence of the instantaneous current provides the voltage dependence of the single channel current amplitudes (Fig. 4, B and E). We obtained comparable results for several cells with varying current amplitudes, indicating that contaminations of the anion current with background currents did not affect the determination of the single channel amplitude.

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This analysis also allowed determination of absolute open probabilities. The $x$ axis intercept defines the value of $I_{ss}/I_{inst}$ that corresponds to an absolute open probability of 1. Dividing $I_{ss}/I_{inst}$ at a given voltage by the $x$ axis intercept gives the absolute open probability. Using this method, we determined the absolute open probabilities at $-155$ mV and normalized the voltage dependences of relative open probabilities to this value (Fig. 4, C and F).

Noise analysis at the same cells after moving to a glutamate-free external solution provides the unitary current amplitude and absolute open probability also under those conditions (Fig. 4, B, C, E, and F). For both isoforms, the single channel current amplitudes are identical in the absence as well as in the presence of glutamate. The sole basis for the glutamate-induced increase of macroscopic current amplitudes is a change of the absolute open probability. We conclude that EAAT3 and EAAT4 anion channels differ neither in the unitary current amplitude nor in the absolute open probability in the absence as well as in the presence of glutamate.

**EAAT4 Glutamate Transport Rates Are Significantly Smaller than Those of EAAT3**—The identity of anion transport rates of EAAT3 and EAAT4 indicates that the well established difference in the contribution of anion current to the total transport current of the two isoforms (2, 3) must be due to different glutamate transport rates. The use of *Xenopus* oocytes provides a simple and direct procedure to determine relative glutamate transport rates (Fig. 5). The oocytes were incubated in glucuronate-based solution, the removal of permeant anions (15) from the external membrane side results in an almost complete efflux of intracellular Cl$^-$ anions. Under this condition, glutamate-induced currents represent isolated glutamate uptake currents (Fig. 5A). Consequently, the external solution was switched from a glucuronate-based to an SCN$^-$-based solution (Fig. 5C). Because of the voltage dependence of the electrogenic glutamate transport (19, 20), currents at positive potentials are isolated anion currents. The specificity of anion currents was tightly monitored by testing their TBOA sensitivity (Fig. 5E).

EAAT3 expresses much better in *Xenopus* oocytes than EAAT4. We adjusted the length of the time period between injection and experiments so that oocytes expressing EAAT3 or EAAT4 exhibited comparable absolute current amplitudes. The amplitudes of uptake currents were $234 \pm 37$ nA for EAAT3 ($n = 5$) and only $86 \pm 17$ nA ($n = 5$) for EAAT4-expressing oocytes at $-120$ mV (Fig. 5, A and B). At $+60$ mV, anion current amplitudes measured $0.8 \pm 0.05$ μA in the absence of glutamate and $1.8 \pm 0.2$ μA in the presence of glutamate for EAAT3 ($n = 5$) and $2.2 \pm 0.09$ μA in the absence and $4.1 \pm 0.1$ μA in the presence of glutamate for EAAT4 ($n = 5$) (Fig. 5, C and D). These two values were used to calculate the uptake current to anion current ratio ($0.13 \pm 0.01$ ($n = 9$) for EAAT3 and $0.02 \pm 0.001$ ($n = 4$) for EAAT4; $p < 0.001$) (Fig. 5F). Because the mean unitary current amplitudes per individual transporter for the two isoforms are identical, these values enable a comparison of glutamate transport rates for the two isoforms. We conclude that EAAT3 glutamate transport rates are at least seven times larger than for EAAT4.
Glutamate Dependence of EAAT3 and EAAT4 Anion Currents—Glutamate increases EAAT3- and EAAT4-associated anion currents with an isoform-specific concentration dependence (Fig. 6). We recently reported that EAAT4 anion currents exhibit a sigmoidal glutamate concentration dependence (8). Apparent dissociation constants \( K_D \) and the Hill coefficient \( n \) depend on the internal and external solutions \( n = 3.7, K_D = 35.0 \pm 0.3 \mu M \) \((n = 5)\) for \( \text{Cl}_{\text{int}}/\text{NO}_3^{\text{ext}} \); \( n = 5.2, K_D = 23.2 \pm 0.4 \mu M \) \((n = 5)\) for \( \text{Cl}_{\text{int}}/\text{SCN}^{\text{ext}} \) (Fig. 6B) (8). For EAAT3, we obtained a perfectly hyperbolic relationship with a Hill coefficient of 1.0 and a \( K_D \) of 20.5 \pm 8 \mu M at \( \text{Cl}_{\text{int}}/\text{SCN}^{\text{ext}} \). EAAT3 and EAAT4 anion channels thus exhibit similar glutamate affinities but differ in the steepness of the concentration dependence of anion currents. The two isoforms also differ in the relative amplitudes of the glutamate-independent anion currents under this anion conditions \( \text{Cl}_{\text{int}}/\text{SCN}^{\text{ext}} \). In the absence of glutamate, TBOA-sensitive anion currents could be measured in cells expressing EAAT3 as well as in cells with EAAT4. For EAAT3, the glutamate-independent current amplitude is \( 67 \pm 3\% \) of the maximum anion current amplitude at a saturating glutamate concentration (Figs. 2B and 6B). For EAAT4 anion channels, relative anion current amplitudes in the absence of glutamate are considerably lower \( (20 \pm 2\% \) \((Figs. 2E and 6B)\).

Two point mutations, G464S and Q467S, were recently found to increase the cooperativity and affect the apparent glutamate affinity of the glutamate activation of EAAT4 anion channels (8). We constructed the homologous point mutations, G410S and Q413S, in EAAT3. EAAT3 expressed much better in oocytes than in cells, and we therefore used the oocyte expression system to study the effects of these mutations on the glutamate dependence of EAAT3 anion currents. G410S and Q413S change the apparent glutamate affinity but leave the steepness of glutamate dependence in EAAT3 basically unaf-



DISCUSSION

EAAT3 and EAAT4 represent two extremes of the apparent specialization of EAAT isoforms into glutamate carriers and glutamate-gated anion channels. EAAT3 is characterized by a high glutamate transport rate (21) and only small associated anion current (2). In contrast, EAAT4 exhibits a predominant anion current and has been generally assumed to function as an anion channel rather than as a glutamate transporter (3). We here demonstrate that the anion conduction pathways of EAAT3 and EAAT4 are functionally very similar, whereas the two isoforms differ greatly in glutamate transport rates (Fig. 5). Unitary current amplitudes and absolute open probabilities are comparable (Fig. 4). The anion conductance of EAAT4 therefore does not dominate total transporter currents because of high anion transport rates but because of low glutamate transport rates (Fig. 5). A large difference in glutamate transport rates was recently also reported for rEAAT3 and hEAAT4. Using a kinetic analysis of reaction steps of the glutamate uptake cycle, Mim et al. (22) determined glutamate transport rates of \( 3 \text{ s}^{-1} \) for hEAAT4 as compared with \( 90 \text{ s}^{-1} \) (both at \(-90 \text{ mV}\) for rEAAT3 (23). A comparison of transport rates (22) and relative anion currents (2, 3) of various EAATs suggest that glutamate transport rates are inversely related to relative anion conductances and that unitary anion channel amplitudes are comparable for all EAATs. Although glutamate transport rates differ greatly among different EAAT isoforms, the anion conduction pathway is conserved. The functional specialization within the EAAT family thus occurs by adjusting the glutamate transport alone.

Single channel amplitudes of EAAT3 and EAAT4 anion channels are too small to be measured directly. We used stationary noise analysis and determined identical unitary current amplitudes for EAAT3 \((\text{at } -155 \text{ mV}, 208 \pm 45 \text{ fA}, n = 4)\) and EAAT4 \((\text{at } -155 \text{ mV}, 164 \pm 39 \text{ fA}, n = 5)\) (Fig. 4) in cells dialyzed with SCN-based solution. Under these conditions, EAAT3 and EAAT4 anion currents are very similar in the voltage dependence of the open probability, the time dependence of current relaxations, and the ratio of current variance and current amplitudes, providing perfect conditions for a comparison of unitary channel properties by noise analysis. The noise analysis presented here provided consistent results, with and without glutamate (Figs. 3 and 4), and the results agree well with our
earlier results on EAAT4, which were obtained by nonstationary noise analysis (5). Earlier publications reported differing single channel amplitudes of EAAT anion channels (5, 14–16). However, because experiments were performed under dissimilar ionic conditions, these earlier results did not allow a direct comparison of anion conduction properties of different isoforms.

Although conduction properties are conserved between EAAT3 and EAAT4 anion channels, there are marked differences in time, voltage, and substrate dependence of anion currents. The isoform specificity of EAAT anion channel gating most likely arises from differences in intersubunit interactions. EAAT transporters are assembled as trimers (9), with each subunit transporting glutamate independently from the adjacent subunits (24–26). We recently reported a cooperative interaction between the subunits in activating the EAAT4 anion channel. No cooperativity was observed in the glutamate dependence of glutamate transport, indicating that intersubunit interaction does not occur in glutamate binding but rather in gating processes coupling glutamate binding and anion channel opening (8). Mutations affecting intersubunit interactions modify gating transitions of EAAT4 anion channels (8), in full agreement with the idea that voltage-dependent gating involves conformational changes of individual subunits relative to their neighbors. In contrast to these results on EAAT4, we did not observe cooperativity in glutamate activation of wild type or mutant EAAT3 anion channels (Fig. 6).

EAAT4 glutamate transporters have been studied by several groups in native cells or heterologous expression systems (3, 22, 25, 27). Rat and human EAAT4 were reported to bind glutamate with apparent dissociation constants between 0.6 (22) and 30 μM (27). We determined apparent glutamate dissociation constants of 10 μM for the glutamate transport (8) and of about 30 μM for activation of the anion current (8) (Fig. 6). In contrast to all other reports, the glutamate concentration dependence of anion currents obtained by our group could not be fit with a Michaelis-Menten relationship but rather with a Hill relation-
ship and a Hill coefficient larger than 3. We measured the glutamate concentration dependence of anion currents by plotting isochronal current amplitudes determined 1 ms after stepping from a holding potential of 0 mV to the test potential. This approach accounts for the voltage-dependent gating of EAAT anion channels that is modified by external substrates (5, 7, 8). All other groups used late current amplitudes, thus lumping the glutamate and voltage dependence of anion channel opening together. The differences between our results and those by other groups are likely because of the different methods used to quantify apparent dissociation constants. We now compared the glutamate dependences of EAAT3 and EAAT4 anion currents, using exactly the same experimental approach for both transporter isoforms. The different steepness of the glutamate dependences of EAAT3 and EAAT4 indicates an isospecific difference, irrespective of the reason for the differences between our results on EAAT4 and those of other groups.

EAAT3 is responsible mainly for neuronal glutamate and cysteine uptake (28) and seems to be the major glutamate carrier in tubular glutamate reabsorption (29). The physiological role of EAAT4 is not clear. It does not play a crucial role in cerebellar glutamate homeostasis (30), in good agreement with its low unitary glutamate transport rates. Mim et al. (22) recently postulated that human EAAT4 mainly serves to bind glutamate with high affinity to prevent spillover to adjacent synapses. Although rat EAAT4 used in our study has a 10-fold larger glutamate dissociation constant than hEAAT4 (8, 22), it also represents a high affinity glutamate transporter well suited for such a task. It has been generally assumed that EAAT4 functions as anion channel and regulates neuronal excitability because of a large unitary conductance of the associated anion channel. Our finding that anion conduction pathways of EAAT are conserved does not argue against a specific role of the EAAT4 anion conductance. In contrast, low glutamate transport rates might even support EAAT4 functioning as a gated channel involved in the regulation of cellular excitability. We recently reported a voltage-dependent gating step that controls anion-to-cation selectivity of EAAT4 anion channels (7). Trains of phasic depolarizations and long lasting depolarizations result in an increased cation permeability and in excitatory inward current through EAAT4 channels (7). EAAT4 anion channels can therefore mediate inhibitory currents in resting cells and excitatory currents in electrically active cells. In dendritic spines of Purkinje cells, where EAAT4 is mainly expressed (31), such excitatory currents might result in substantial depolarizations (7). In such cell compartments, glutamate receptor-mediated Na⁺ signals can cause substantial increases of internal [Na⁺] (32), and dendrites are tightly enclaved by glial cells facilitating extracellular K⁺ accumulation (33). These conditions might cause an inversion of the glutamate transport direction under certain conditions, resulting in glutamate extrusion into the external medium and activation of ionotropic glutamate receptors. One might speculate that the low transport capacity of EAAT4 could minimize transporter-associated glutamate release at such a situation and help regulation of cellular excitability without interfering with extracellular glutamate homeostasis.

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